IDENTIFYING VIRULENCE FACTORS AND REGULATORS CONTRIBUTING TO
PATHOGENESIS BY THE SELECT-AGENT BACTERIUM BURKHOLDERIA
PSEUDOMALLEI

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This doctoral dissertation is dedicated to my loving wife, Malia, without whose support I could not have completed this work; to my son, Henry, for the many years of affection to come; and to my parents for a lifetime of patience.
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ABSTRACT

Many virulence factors contribute to the molecular pathogenesis of Bp, such as type III and VI secretion systems, flagella, toxins, and capsule. A large subset of Bp proteins, particularly those annotated as hypothetical and putative (~35.5% of the genome), are avoided in experiments. Some of these proteins probably contribute to pathogenesis and its’ regulation. Whether for a lack of tools or techniques, many questions about the molecular pathogenesis of Bp remain unanswered.

Engineered K96243, 1026b, and MSHR487 Δasd mutants could not grow without the essential cell-wall component diaminopimelic acid (DAP) and were unable to replicate in mice. All strains were excluded from select-agent regulations, providing the research community with helpful BSL-2 surrogates. Intracellular replication and time-lapse movies of fluorescent two-color cell-fusion assays demonstrated that B0011 behaved similarly to WT. B0011’s utility in virulence factor investigations was demonstrated by testing in the nematode Caenorhabditis elegans. B0011 was also found to kill Tenebrio molitor (yellow mealworm) in a novel feeding assay indicating the biosafe B0011 would be a good surrogate for research of virulence factors. The CDC and Los Alamos have obtained the strains for diagnostics and JCVI for persister cell research.

To identify essential virulence factors, a transposon mutant library was constructed in Bp K96243 Δasd and passaged through macrophages 3 times. gDNA from the passaged pool and the un-passaged pool was isolated and the transposon/genome junctions were enriched using TN-seq. Bacterial genes that were not present in the
samples after passaging through macrophages were considered putative virulence factors. 113 genes underwent greater than 1,000-fold negative selection. Of those, many were deficient in some part of the infectious process. 20 were used to infect mice in an intranasal melioidosis model. 10 mutants demonstrated attenuation and represent new virulence factors.

40 hypothetical regulatory genes were deleted and mutants tested for a decreased plaque forming ability in cell monolayers. Nine regulatory mutants were unable to form plaques, indicating a role in virulence regulation and were complemented with ChIP-seq vectors. Nine regulators were characterized by growth and infection in vitro. Of nine, six regulators were used to infect mice and showed attenuation in vivo.
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Sun, Z., Kang, Y., **Norris, M.H.**, Troyer, R.M., Son, M.S., Schweizer, H.P., Dow, S.W., and Hoang, T.T. Blocking phosphatidylcholine utilization in *Pseudomonas aeruginosa*, via mutagenesis of fatty acid, glycerol and choline degradation pathways, confirms the importance of this nutrient source *in vivo*. *PLoS ONE* 9(7):e103778.....378
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REVIEWED PAPERS PUBLISHED


**Manuscripts in Review:**


**Manuscripts in Preparation** (will submit within a couple of months):


**PATENT**

A provisional patent application (# 61383699; submitted 16 September 2010) has been filed for a single bacterium total transcript amplification method. T.T. Hoang, Y.Kang, and M.H. Norris are named as the inventors on this patent.

**ABSTRACTS PRESENTED at MEETINGS** (presenter underlined):


2. Kang, Y., Norris, M.H., Zarzycki-Siek, J., and Hoang T.T. Lambda red recombineering system for naturally transformable *Burkholderia thailandensis* and *Burkholderia pseudomallei*. Poster
number F-27, presented 05/28/2010 at the Banff Conference on Infectious Diseases, Banff, Alberta, Canada.


4. Norris, M.H., Yun Kang, Katie Propst, Steven Dow, Herbert Schweizer, and Tung T. Hoang. The Burkholderia pseudomallei Δasd mutant exhibits attenuated intracellular infectivity and imparts protection against acute inhalation melioidosis. Poster number F-18, presented 05/28/2010 at the Banff Conference on Infectious Diseases, Banff, Alberta, Canada.


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Chapter 1. Introduction: *Burkholderia pseudomallei*, the etiological agent of melioidosis, a potentially life-threatening tropical infectious disease
The melioid bacillus, *Burkholderia pseudomallei* (*Bp*), is typically found in soil and water environments between the latitudes of 20°N and 20°S and so is primarily a tropical organism [1]. Originally described as causing a glanders-like disease by Alfred Whitmore in 1912, it was named Whitmore’s disease [2]. In 1932, the disease was renamed melioidosis; taken from “melis” Greek for the disease affecting asses (glanders) and “eidos” meaning of the same [3]. The affliction was also called “the Vietnamese time bomb” because soldiers returning to the U.S. from the Vietnam War would become ill many years after exposure [4]. At the beginning of the 19th century, the bacterium itself was known as *Bacillus whitmorii*, but more recently was called *Pseudomonas pseudomallei*. In 1992, based on 16S rRNA sequence homology and cellular profiles it was proposed that many bacteria in the genus *Pseudomonas* be moved to a new genus called *Burkholderia*, changing *Pseudomonas pseudomallei* to *Burkholderia pseudomallei* (*Bp*) [5].

*Bp* can be found in soils at pH 2-9 but optimally at 5-8 [6] preferring to grow in wet environments where survival times greatly increase with increased water content (70 days at 10% wc compared to >726 days at 40% wc) [6,7]. Sample sites in northeastern and central Thailand, near Bangkok, are consistently positive for *Bp* and the soil can contain upwards of 17,000 CFU/mL [8]. In endemic areas such as Thailand very high levels of the bacterium are found in the pooled surface waters of rice paddies [9] coinciding with high disease and exposure rates in among rice farmers [10]. There are currently between 3,000 and 4,000 cases of melioidosis in Thailand each year [11]. The season for greatest infection risk is the beginning of the wet season, May to June, when fields are plowed for rice planting [8]. Typically there is a regional hospital far from the individual’s home and by the time they arrive, the disease has progressed to an acute form leading to a high mortality rate of 40.5% in Northeastern Thailand.
Nearly 80% of individuals diagnosed with melioidosis are farmers and nearly 70% of these patients have poorly controlled or undiagnosed Type 2 diabetes mellitus [13]. Interestingly, children rarely get sick, but in endemic regions ~80% are seropositive by age 4 [14]. It is believed the organism in contaminated waters gains entry into the host through breaks in the skin or via inhalation of aerosolized particulates. Recently, consuming contaminated drinking water has been implicated as a common route of infection [15] and studies have shown *Bp* is able to colonize gastric epithelium [16]. Human-to-human transmission of *Bp* is very rare and has only been documented a handful of times. In two well-documented cases from the 1970’s, prostatic abscesses in male U.S. serviceman were believed to be the infectious source when sexually transmitted to a wife and a neonatal baby [17-19].

Not only does the melioid bacillus pose a public health challenge in Northeastern Thailand but the US Centers for Disease Control has listed *B. pseudomallei* (causing melioidosis) and the closely related *Burkholderia mallei* (causing glanders) as Tier-1 Select Agents. Tier 1 organisms have the potential of posing a severe threat to US public health and safety and the U.S. Congress passed laws to develop vaccines and therapeutics for their associated disease including melioidosis and glanders [20]. Research on these bacteria is a high priority because the historical usage of *B. mallei* in biological warfare by Germany on horses during World War I and on humans by Japan in World War II, and weaponization by the U.S.S.R. [21] and high pathogenicity when aerosolized. An important step in combating a disease is to understand the fundamental basis. In this case, only by understanding the molecular pathogenesis of *Bp* infection will an effective vaccine come to fruition. Additionally, a highly secure Bio-Safety Level 3 (BSL-3) laboratory is required for all live bacteria manipulations.
The disease melioidosis is known as the ‘great mimicker’ due to the wide variety of symptoms and clinical presentations, which resemble other diseases [22]. Melioidosis can present as an acute, chronic, or latent infection. Studies from Northern Australia indicate that 88% of melioidosis cases were acute while 12% were chronic [23]. Overall, bacteremia during melioidosis occurs in ~50% of cases [24]. Pneumonia and respiratory infection is found in half of patients but severity can range from severe septicemia to mild pneumonia with few symptoms [24]. Other presentations include skin and soft-tissue infection, bone and joint infection, visceral abscesses, infection of the parotid gland and urinary tract, and infections of the nervous system, including the brain. \( Bp \) has been known to infect every tissue and organ of the body, with the exception that endocarditis has never been observed [25]. \( Bp \) strains found in Thailand differ genetically to those found in Australia (to be discussed later). In Thailand, melioidosis often presents with parotid gland infections but only a single event has been observed in Australia while prostatic abscesses and neurological melioidosis are more common in Australia than Thailand [24]. When symptoms persist beyond two months, the infection is considered as chronic melioidosis [23]. Chronic melioidosis accounts for ~10% of cases in Thailand and Australia but most cases identified in non-endemic regions are chronic infections. Patients with chronic lung infections typically present with chest pain and bloody sputum. Visible as cavitation and infiltration in X-ray radiography, the lungs appear very similar to tuberculosis-infected lungs. Often times misdiagnosed as tuberculosis, the name great mimicker is certainly appropriate. Unfortunately, drugs used to treat tuberculosis are ineffective for melioidosis, so doctors in endemic regions must be wary about making the correct diagnosis. With education there is less misdiagnosis, resulting in increasing reports of melioidosis cases. Latent \( Bp \) infections are considered initially asymptomatic that are eventually diagnosed as melioidosis.
The longest latent infection described was in a U.S. serviceman taken prisoner during World War II [26], who, presented with an ulcerated wound on his thumb 62 years later. Bacteria isolated from the wound were identified as *Bp*. It can be considered a unique case considering serological studies indicate 3% of the three million military personnel were seropositive for *Bp* exposure after returning from the Vietnam War, compared to the low number of verified cases of melioidosis [27].

Melioidosis is a serious disease that demands immediate treatment. Once *Bp* is verified by culture or if melioidosis is suspected in endemic regions, treatment begins with parenteral therapy. The discovery that ceftazidime, a β-lactam third-generation cephalosporin antibiotic, decreased mortality by ~50% was a milestone in the treatment of melioidosis and is the gold standard for initial parenteral treatment of acute melioidosis in Thailand [28]. The recommended dose for curing melioidosis is 120 mg/kg/day of ceftazidime at 2 g every 8 hours via IV for at least 10-14 days or until improvement. Carbapenems, such as imipenem and meropenem, are considered just as effective during parenteral therapy and are the standard treatment antibiotics in North Australian hospitals. The carbapenems are more effective at inhibiting bacterial growth during *in vitro* testing but the verdict is still out as to their superiority over ceftazidime. Dosages for the carbapenems are in the 70 mg/kg/day range at 1 g every 8 hours [29]. If clinical improvement is observed after 10-14 days, parenteral treatment can be ended and the eradication phase begun.

*Bp* is a facultative intracellular bacterium, similar to *Mycobacterium tuberculosis*, so antibiotics must be taken for a long period to prevent relapse. The prescribed first-line treatment during the eradication phase is trimethoprim-sulfomethoxazole (TMP-SMX) with or without doxycycline for 3-6 months depending on the health of the patient [1]. Relapse of infection is
very common if the therapeutic regimen is not followed. *Bp* has high strain-to-strain genetic variability so *in vitro* antibiotic susceptibility testing is recommended as soon as a strain is isolated because some strains can be resistant to ceftazidime [30] or TMP-SMX [31].

As stated above, much of the population in endemic areas is seropositive but only a small fraction becomes ill. How does the body react and respond to *Bp* infection? Once inhaled into the respiratory tract, *Bp* is exposed to anti-bacterial compounds such as lysozyme, complement, and defensins. *Bp* is known to be resistant to all these molecules allowing for cellular contact between bacteria and cells of the host. The upper airway surfaces can then be colonized, providing for rapid access to the nervous system via the olfactory epithelium and the nerves therein [32]. In recognizing *Bp*, the toll-like receptors (TLRs) and other pathogen recognition receptors (PRR) are the first to detect the pathogen-associated molecular patterns (PAMPs). Many of the TLRs (TLR1-5, 8 and 10) are upregulated in melioidosis patients [33]. When MyD88 (the essential TLR adaptor for NF-κB activation) is knocked out, mice died 50% quicker and pulmonary neutrophil infiltration was repressed while normal phagocytosis was not changed [34]. Initial recognition and activation of innate immunity can have a large effect on disease prognosis. Indeed, certain polymorphisms in TLR4, the canonical TLR for lipopolysaccharide (LPS) detection, have been associated with increased susceptibility to melioidosis [35]. *Bp* is known to invade and replicate within macrophages and polymorphonuclear cells (PMNs) [36]. These cells are believed to be very important for bacterial containment as it has been shown that PMNs from diabetic patients have a reduced ability to migrate and phagocytize *Bp* *in vitro* [37] and that BALB/c mice, which are very sensitive to *Bp* infection, recruit less monocytes to infection sites than resistant mouse strains like C57BL/6 [38]. Additionally, *in vivo* macrophage depletion experiments showed C57BL6 mice infected with *Bp* via the intranasal route were much
more sensitive to \( Bp \) mediated killing and had higher levels of internal \( Bp \) CFUs following infection than untreated controls [39]. It has also been shown that both TNF-\( \alpha \) and IFN-\( \gamma \), which work to activate macrophages, are essential in extending survival during early-stage experimental melioidosis. Administration of IFN-\( \gamma \) directed antibodies into \( Bp \) infected mice lowered the lethal doses by 50\%, emphasizing the essential role for IFN-\( \gamma \) in activating cells of the immune system during initial infection with \( Bp \) [40]. IL-18, probably due to its ability to initiate IFN-\( \gamma \) release that subsequently activates macrophages, also plays a role [41]. Following the initial stages of infection, host-defense is reliant on a T\( _{H1} \) cell response to propagate macrophage activation and bactericidal activities along with clonal expansion of \( Bp \) antigen-specific T cells [42,43]. Dendritic cells have been shown to serve an important role in activating CD4\(^+\) T-cells and to some extent CD8\(^+\) T-cells during melioidosis. Monocytic dendritic cells were pulsed with bulk antigen as well as purified antigens and caused memory T-cell expansion in T cells isolated from seropositive donors, further substantiating the importance of a T\( _{H1} \) response to \( Bp \) infection [44]. One caveat is that HIV has not been implicated as a risk factor in studies, which would be counterintuitive to the hypothesis that cell-mediated immunity is required for host defense [45]. Perhaps HIV patients do not come into contact with the bacterium in its natural environment. Although cell-mediated immunity is the major player in host resistance to \( Bp \) infection, a humoral response is still mounted. Many of the antibodies identified are against the LPS and capsular polysaccharide (CPS) surrounding the bacterium. Virtually all antibodies are IgG of the subclass IgG1 (\(~65\%) that is consistent with a tendency towards a cell-mediated response [46]. Melioidosis survivors usually have very high anti-\( Bp \) antibody titers but they are not protective as relapse is high [47] and seem to be useful only for opsonizing extracellular bacteria. Antibodies have proven useful in positive identification of \( Bp \) where the
latex agglutination test was used for colony identification [48] and in the new dipstick assay for identifying *Bp* in clinical specimens [49] but passive immunizations have failed to protect mice during infection; again lending credence that cell-mediated immunity is paramount to fighting the infection.

Currently there is no vaccine licensed for melioidosis but interest in developing one has increased following the realization that the organism could be weaponized. *Bp* is a facultative intracellular pathogen that will enter host cells and replicate, necessitating a powerful cell-mediated immune response to counter the infection. Many types of auxotrophic strains have been developed and immunization with these strains can create protection from acute melioidosis [50,51]. Live attenuated mutants auxotrophic for purine metabolism and amino acid metabolism have shown the most significant protection from lethal challenge doses but sterile immunity is never achieved and death is merely delayed in animal models [51,52]. Data generated in our lab (delineated in Chapter 5 of this dissertation) found that mice immunized intranasally with a live-attenuated *Bp* prior to intranasal high lethal dose challenge of wildtype were protected from acute melioidosis. Survival was increased from 4 days post-infection to between 20 and 60 days post-infection. Protection was conveyed at the site of initial infection but failed to protect secondary infections in the spleens. Mice then succumbed to a chronic infection at the secondary sites of infection. Survival data like this is typical for live-attenuated studies and future work will address the prevention of chronic infection by boosting the cell-mediated immune response. Heat-killed whole-cell vaccines have been produced with *Bp* and closely related species but the only details gleaned from such a confusing combination of site immunizations and site challenges, is that the heat-killed vaccines convey some protection but that it is dependent on where the immunization is given and what route the challenge dose is given [53]. Species similar
to *Bp*, *B. mallei* and *B. thailandensis*, can produce cross-reactive immunity to *Bp* but in all cases sterile immunity is elusive and more work must be done on optimizing route and dosage of a heat-killed whole-cell vaccine. Several sub-unit vaccines have been tested including the CPS and LPS. LPS has been shown to increase the mean time to death, but again sterile immunity is not occurring [54]. In the previous study the LPS vaccine can only protect against intraperitoneal challenge and not the more relevant respiratory challenge. Subcutaneous challenge was not attempted. A major problem with *Bp* LPS as a subunit vaccine, is that the structure can vary depending on the strain. Three types of LPS have been identified [55] and LPS sub-unit vaccines are type specific. It has not been investigated weather or not cross-protective immunity can be achieved for LPS variant strains without using all LPS types in the vaccine. Mechanisms that could improve the activity of the vaccines include the production of glycoconjugate vaccines. Glycoconjugate vaccines consist of either LPS, CPS, or both conjugated to a known *Bp* immunogenic protein to stimulate both the cell-mediated (protein) and humoral (polysaccharide) immunity. Currently, there have only been conjugations of non-specific immunogens, such as BSA, but still result in higher IgG production against the T-cell independent CPS antigen than either component alone [56]. Mouse survival was not tested. The search for a melioidosis vaccine is ongoing and with increased research on *Bp* virulence factors and how they contribute to host infection, a more effective vaccine will be realized.

When *Bp* cells come into contact with host cells a plethora of host modifications occur in response to bacterial infiltration and virulence factor expression leading to a diseased state. Within-host, *Bp* can infect most tissues and invades, then replicate inside the cytoplasm of many cell types [51,57-61]. To accomplish this feat *Bp* attaches to the host cell via an unknown mechanism, causing actin rearrangement and inducing bacterial phagocytosis [62]. Once,
internalized \( Bp \) utilizes the *Burkholderia secretion apparatus* (Bsa, one of three genomically encoded type 3 secretion systems, T3SS) to secrete the effector BopE through the membrane of the phagocytic vesicle [63,64]. BopE is a guanine nucleotide exchange factor (GEF) for host-cell Rho GTPases that undermines the surrounding cytoskeletal framework, damaging the membrane and allowing vesicular escape before phagosomal degradation of \( Bp \) occurs [65]. The T3SS effector BopA is believed to play a role in host-cell autophagy evasion [66] but data from our lab indicates another protein may have a more significant effect on the process. The action and impact of both effectors to mammalian infections has been demonstrated *in vitro* but BopA and BopE mutants do not make a significant impact on mouse survival [67,68]. Once inside the cytoplasm the well characterized \( Bp \)-intracellular motility protein A, BimA, polymerizes host-cell actin [69]. The polar actin “tail” allows intracellular movement and eventually leads to formation of membrane protrusions [70]. The membrane protrusions allow \( Bp \) to seek out uninfected cells and begin the cycle anew without transitioning into the extracellular milieu. Intracellular motility quickly oscillates between actin-mediated movement and use of flagella, where \( Bp \) swims within the host-cell cytoplasm [71]. During the infectious process an infected cell may fuse with healthy neighboring host cells and is attributed to the main \( Bp \) type 6 secretion system (T6SS-1) of which \( Bp \) has 6. [72]. The formation of multi-nucleated giant cells (MNGCs) has been observed *in vitro* and *in vivo* and is a major hallmark of \( Bp \) infection [70,73]. As discussed earlier, \( Bp \) produces CPS and LPS. Mutants of both are attenuated in mice [74,75]. \( Bp \) encodes genes to make up to seven quorum-sensing (QS) molecules that allow bacterial communication and concerted gene expression. The genes that are activated by quorum sensing in \( Bp \) are still unknown but deletion of the *luxR* homologues (a transcriptional activator of QS) results in decreased virulence in hamsters [76]. Recently, the QS molecules have been shown to
negatively affect macrophage MNCG formation in vitro [77]. The list of virulence factors goes on with flagella and pili contributing to efficient adherence to and invasion of host-cells [78,79]. The bacteria replicate concurrently with the production of several known and unknown virulence factors causing death of the host-cell. However, questions remain; how does Bp sense when to fuse, where to travel, what nutrients to use, how are mediators (e.g. autophagy) of innate immunity avoided, and what causes the observed cytoskeletal rearrangements (Fig. 1)? These questions are indicative of the knowledge gap present in fully understanding the molecular pathogenesis of Bp.

A major hurdle in understanding more about how Bp causes disease is the lack of adequate tools. Tools in the sense of genetic methodologies (Chapters 3 and 4 of this work) and disease models (Chapter 5). Bp is highly resistant to antibiotics and it’s listing as a select agent prohibits the use of many antibiotic markers; making classic genetic analysis difficult. The candidate’s initial projects included the development of non-antibiotic selectable markers for use in Bp and Bm. Two non-antibiotic markers commonly used in plants were adapted and customized for use in Burkholderia sp. The glyphosate acetyl-transferase (gat) and bi-aliphos resistance (bar) genes, conferring resistance to the herbicides glyphosate and glufosinate respectively, were used for various genetic manipulations in Bp strains [80]. In the previously published study, the gat marker was used to delete the aspartate semialdehyde dehydrogenase (asd)-encoding gene in two Bp type strains, 1026b and K96243. Asd is essential for creating diaminopimelic acid (DAP), which is the amino acid cross-linking neighboring strand of peptidoglycan in the gram-negative cell wall. Without Asd or exogenous DAP, bacteria swell and lyse [81]. The publication accomplished two goals: 1) providing the research community with non-antibiotic selectable markers for safe genetic manipulation of select-agent
and 2) creation of two biosafe strains with live-attenuated vaccine potential that can conditionally grow only in the presence of exogenous DAP. Attenuation of both strains has since been verified by high-dose intranasal challenge in BALB/c mice [51]. The data in Chapter 4 of this work was summarized and the CDC approved an application for exclusion of these two strains from the select agent regulations (SAR). These strains can now be used as bio-safe surrogates outside of the SAR. Several other asd mutants have been made in different Bp strain backgrounds and verified for attenuation in BALB/c mice (unpublished data). Current research with this pathogen is highly regulated, requiring SAR, approved personnel, and BSL-3 biocontainment facilities. Time investment and research costs can skyrocket under project requirements. BSL-2-trained personnel and research space far outnumber those available for BSL-3 and represent underutilized resources in research of select agents. Bio-safe strains can be moved to the BSL-2 and utilized as surrogates for many parts of the research project. **The first aim of my dissertation is to demonstrate the utility of our bio-safe strain in infection models for use at BSL-2.** Many models have been used to study Bp infection including in vitro cell infection models. Numerous cell lines have been infected by Bp including epithelial, hepatic, macrophage, and polymorphonuclear leukocyte cell-lines among others [57-59,61]. Monolayers infected with Bp form multi-nucleated giant cells (MNGCs) and eventually plaques. Plaque formation can be used to identify important virulence factors by studying plaque-forming ability of virulence-factor mutants [71,82]. Monolayers can also be used to study invasion efficiency and intracellular replication of Bp [83]. A cell-fusion assay has also been developed utilizing two HEK293T cell lines; one expresses GFP cytoplasmically and the other RFP [71]. When seeded together in one monolayer followed by infection with Bp; intracellular bacteria induce fusion of neighboring cells mixing the cytoplasms creating a yellow color. Live-cell imaging is also a very
important tool in investigating bacteria/host-cell interactions and tracking infectious stages. Besides cell lines, Bp infection models used to investigate virulence include invertebrate animal models such as the nematode, Caenorhabditis elegans [84-86], and wax moth larvae, Galleria mellonella [87,88]. Chapter 5 of this work encompasses the utility testing of our bio-safe Bp strain in various infection models.

While many Bp virulence factors lead to attenuation in mouse models of infections, mutational in vitro cell infection studies indicate a delay of the cellular infectious events rather than abolition [67,72,89] and as described above. Bp has nearly 6,000 genes and an open genome [90,91]. Many genes (approximately 35.5% in Bp strain K96243) are hypothetical or putative with no proven function [90]. It stands to reason that there are Bp-specific virulence factors contributing to disease that remain to be discovered and that mutating these factors may produce a live-attenuated strain that is capable of protecting from inhalation melioidosis. To identify all the Bp genes contributing to infection, known and unknown, we screened a large random transposon library pool through RAW264.7 macrophages. The resulting output transposon pool was compared to the input pool using Tn-seq analysis [92]. Before the advent of Next Generation Sequencing (NGS) microarray technology was used to reduce the amount of screening used in high-throughput signature-tagged mutagenesis (STM) experiments targeting bacterial virulence factors [93]. The technique is based on the principle that if the transposon inactivates a gene important for intracellular survival (i.e., a virulence factor) that particular mutant is unable to survive in the host–cell and the gene will not be detected in the output pool. Tn-seq enriches the genome/transposon junctions then Illumina sequencing is used to sequence the enriched DNA. Tn-seq is a variation on a theme but was used to identify all the genes contributing to antibiotic resistance in Pseudomonas aeruginosa [92]. With all high throughput
screens it is critical to verify contributions by mutational analysis. Several genes were positively identified as aiding in antibiotic resistance. The development of a vaccine remains elusive, placing urgency on elucidating the functional contributions of hypothetical/putative genes to the molecular pathogenesis model. In total, we consider the effort of this work in identification and characterization of novel virulence factors to be innovative and of significance to the field. The details of the Tn-seq experiment are summarized in Chapter 6 of this dissertation.

*Bp* commands an arsenal of virulence factors that are put into use during host-cell infection. To organize and time the use of these virulence factors for full effect, a complex regulatory cascade must be fine tuned and maintained. Identifying the transcriptional regulators that are responsible for such fine-tuning would give insight to the virulence network of *Bp* and provide targets for novel therapeutic development.

Transcriptional regulation of virulence genes has been well studied in *Vibrio cholerae*, as well as in *P. aeruginosa*. In these pathogens external compounds and sensors are known to turn on a suite of transcriptional regulators that then mediate toxin, pili, flagella, phospholipases and proteases production among others. *V. cholerae* responds to temperature, osmolarity, bile, and pH to up regulate cholera toxin synthesis [94]. Moreover, studies have uncovered several regulons of virulence factors that control the ability of *V. cholerae* to attach and adhere to intestinal mucosa (ToxR regulon and the toxin coregulated pilus), but that all need to be coordinated for successful colonization. Secretion of mucinases, lipases, and proteases must also be coordinated to digest the mucous surrounding the membranes. The model pathogen, *P. aeruginosa*, is known to coordinate a complex and intricate web of regulation that allows the bacterium to infect a wide variety of hosts and survive in a wide variety of environments [95].
A few *Bp* transcriptional regulators have been investigated. The AraC-type regulator, HrpB, and its role in regulating the plant-pathogen like T3SS has been elucidated [96]. BsaN has been identified as a direct regulator of the T6SS-5, a major virulence factor [97] and BspR has been identified as a regulator of the major virulence factor T3SS [98]. These regulators are located proximal to these virulence factors and were obvious candidates for direct regulation of these two loci. Our goal was to identify additional regulators including global regulators that affect virulence factor expression during host infection and aide in coordinating the process of host-cell infection. Previous data generated in the lab by Dr. Yun Kang using single-cell transcriptomics [99] of the *Bp* transitome (in preparation) identified the global transcriptional profile of *Bp* as it transits through the host-cell during infection. Many genes were differentially regulated as *Bp* invaded host cells, replicated in the cytoplasm, and formed protrusions. Within this data set, 40 transcriptional regulators were identified that showed differential gene expression during host-cell infectious stages. The targets of these regulators are unknown and we hypothesized that at least some of them would be contributing to the coordinated expression of virulence factors during infection.
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Chapter 2. Non-antibiotic selectable markers and production of the *Burkholderia pseudomallei* Δasd and ΔdapB mutants

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2.1 ABSTRACT

Genetic manipulation in the category B select agents *Burkholderia pseudomallei* and *Burkholderia mallei* has been stifled, due to the lack of compliant selectable-markers. Hence, there is a need for additional select-agent-compliant selectable-markers. We engineered a selectable-marker based upon the *gat* gene (encoding glyphosate-acetyl-transferase), which confers resistance to the common herbicide glyphosate (GS). To show the ability of GS to inhibit bacterial growth, we determined the effective GS concentrations for *Escherichia coli* and several *Burkholderia* species. Plasmids based on *gat*, flanked by unique flip-recombination-target (*FRT*) sequences, were constructed for allelic-replacement. Both allelic-replacement approaches, using i) the counter-selectable marker *pheS* and the *gat-FRT* cassette or ii) the DNA incubation method with the *gat-FRT* cassette, were successfully utilized to create deletions in the *asd* and *dapB* genes of wildtype *B. pseudomallei* strains. The *asd* and *dapB* genes encode for an aspartate-semialdehyde-dehydrogenase (BPSS1704, chromosome 2) and dihydrodipicolinate reductase (BPSL2941, chromosome 1), respectively. Mutants unable to grow on media without diaminopimelate (DAP) and other amino acids of this pathway were PCR verified. These mutants displayed cellular morphology consistent with the inability to cross-link peptidoglycan in the absence of DAP. The *B. pseudomallei* 1026b *Dasd*::*gat-FRT* mutant was complemented with the *asdbp* gene on a site-specific transposon, mini-Tn7-*bar*, by selecting with phosphinothricin for the *bar* gene (encoding bialaphos/phosphinothricin resistance). We conclude that the *gat* gene is one of very few appropriate, effective, and beneficial compliant markers available for *Burkholderia* select-agent species. Together with the *bar* gene, the *gat*-cassette will aid various genetic manipulations in *Burkholderia* select-agent species.
2.2 INTRODUCTION

Members of the genus *Burkholderia*, comprised of over 40 different species, are extremely diverse Gram-negative non-spore-forming facultative bacilli. Many exist as innocuous soil saprophytes or plant pathogens (53), while other *Burkholderia* species cause human and animal diseases. Among these human and animal pathogens are the etiological agents of melioidosis (*Burkholderia pseudomallei*) and glanders (*Burkholderia mallei*) (10, 56, 57). Melioidosis is an emerging infectious disease generally considered endemic to Southeast Asia and Northern Australia (14). Positive diagnoses in many tropical countries around the world have expanded the global awareness of melioidosis (4, 17, 29, 30, 33, 40, 44, 47, 58). In contrast to the ubiquitous nature of *B. pseudomallei*, *B. mallei* is also a highly infectious agent causing glanders, a predominantly equine disease (39, 56). *B. mallei*, a clone derived from genomic downsizing of *B. pseudomallei*, has been used in biowarfare (20). This historical significance, along with the low infectious dose and route of infection, has contributed to the decision by the Centers for Disease Control and Prevention to classify these two microbes as category B select agents (48).

Classification of *B. pseudomallei* as a select agent has stimulated interest and research into the pathogenesis of melioidosis, necessitating the development of appropriate tools for genetic manipulation. In the struggle to elucidate the molecular mechanisms of pathogenesis, selectable markers are indispensable genetic tools (51). Current regulations prohibit the cloning of clinically important antibiotic resistance genes into human, animal, or plant select-agent pathogens, if the transfer could compromise the ability to treat or control the disease. The only antibiotic markers currently approved for use in *B. pseudomallei* are based on aminoglycoside resistance (gentamycin, kanamycin, and zeocin) (51). However, the efficacy of these markers is
limited due to i) high levels of aminoglycoside resistance inherent within the *Burkholderia* genus and ii) spontaneous resistance in *B. pseudomallei* (11, 24, 46). In addition, the use of aminoglycosides for selection (e.g. gentamycin) may require aminoglycoside efflux-pump mutants (11, 38). Utilization of aminoglycoside selection in efflux-pump mutants may be counter-intuitive as aminoglycosides, although not routinely used for treatment, could be used effectively against efflux-pump mutants in case of accidental exposure by aminoglycoside-sensitive strains (22, 50). With the advent of improved selective media containing gentamycin by Ashdown in 1979 (1), isolation of gentamycin sensitive *B. pseudomallei* strains is rare. However, prior to this, many gentamycin and kanamycin sensitive *B. pseudomallei* strains were isolated (19, 23) and successful treatment with aminoglycosides has been documented (13, 62). Another potential drawback is that efflux-pumps play a major role in bacterial physiology and mutating them may change the pathogenic traits under investigation (8, 45). A more logical approach employs alternative non-antibiotic selectable-markers conferring resistance to compounds that are not potentially important in clinical treatment.

There have been very few non-antibiotic resistance markers utilized successfully in *Burkholderia* species. The non-antibiotic selectable-marker, based on Tel', has been successfully developed and used in *Pseudomonas putida, Pseudomonas fluorescens*, and *Burkholderia thailandensis* (3, 32, 49). The engineering of Tel'-FRT cassettes, coupled to flip-recombination-target (*FRT*) sequences, could be used to generate unmarked mutations and allow recycling of the Tel' selectable-marker (3). In addition, utilization of Flp-*FRT* resistant-cassettes to generate mutants allows downstream modification and manipulation, such as fusion integration (34). However, the disadvantage of the Tel'-cassette is the number of genes required (*kilA-telA-telB*)
and the large size (>3 kb), making it less likely to obtain PCR product for allelic-replacement by natural transformation (52). Another potentially useful non-antibiotic selectable marker is based on the *bar* gene encoding for resistance to bialaphos or its degradation product phosphinothricin (PPT) (55). PPT inhibits glutamine synthetase in plants (54), starving the cell for glutamine, and the *bar* gene has been used successfully in Gram-negatives as a selection marker (26). In select-agent *Burkholderia* species, however, the PPT MIC was found to be greater than 1024 mg/ml (M. Frazier, K. Choi, A. Kumar, C. Lopez, R. R. Karkhoff-Schweizer, and H. P. Schweizer, 2007 American Society for Microbiology, Biodefense and Emerging Diseases Research Meeting, Washington, DC). We have found that the effective PPT concentration for *B. pseudomallei* and *B. mallei* to be ~2.5% (25,000 mg/ml; data not shown). The high concentration of PPT required for selection in these species may be costly, considering purified PPT costs ~$380 per gram. Therefore, further development of non-antibiotic makers and search for a more economical source of herbicide to use in restricted select-agent species are required.

Work by Castle *et al.* (6) generated a highly active GAT (glyphosate *N*-acetyltransferase) enzyme for plant engineering, making it possible to utilize the *gat* gene as an effective non-antibiotic marker for bacterial selection with glyphosate. The commonly used herbicide, glyphosate (GS), inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) of plants through competition with phosphoenolpyruvate (PEP) for overlapping binding sites on EPSPS (16), depriving plants of the three aromatic amino acids (Fig. 1). Since humans and animals obtain tryptophan and phenylalanine (giving rise to tyrosine) through dietary intake, GS is relatively non-toxic. Similar to plants, bacteria must make these amino acids, when lacking, from basic precursors. GS has been found to be inhibitory to a variety of different bacteria including
Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, and Bradyrhizobium japonicum (18, 61), while other bacterial strains are able to metabolize low concentrations of GS (31, 36). Although B. pseudomallei has been reported to have two genes (glpA and glpB) for GS degradation and metabolism (43), our searches of all available genomes of Burkholderia species in the GenBank yielded no glpA or glpB genes within this genus. GS resistance by bacteria has been documented through the EPSPS target mutations or GS detoxification mechanisms (41). However, these mechanisms did not give resistance to relatively high GS concentrations. More recently, directed-evolution of the gat-gene, based on various bacterial gat sequences and selection in E. coli, yielded a very active GAT protein sequence with an efficiency increase of nearly four orders of magnitude (6), holding promise as an appropriate non-antibiotic marker for select-agent species.

Here, we engineered and tested a novel non-antibiotic selectable-marker (gat) for use in the select-agent B. pseudomallei. GS is the active ingredient in RoundUp®, which was used for selection (Fig. 1). The effective compound GS is readily available, inexpensive, relatively non-toxic, very soluble, not clinically important, and yields tight selection. The engineered gat marker (~500 bp) was optimized for Burkholderia codon-usage and adapted (with a Burkholderia rpsL promoter) for use in the select agent B. pseudomallei. Effective concentrations of GS in several species of Burkholderia, including the select agents B. pseudomallei and B. mallei, were determined. Using the gat gene, we created deletional mutants of the essential asdBp and dapBBp genes (encoding aspartate-semialdehyde dehydrogenase and dihydrodipicolinate reductase) in two wildtype B. pseudomallei strains. The DasdBp mutant of B. pseudomallei showed a phenotypic defect consistent with the lack of diaminopimelate for cell-
wall cross-linking. Complementation of the *B. pseudomallei* *Dasd*<sub>Bp</sub> mutant with the *asd*<sub>Bp</sub> located on a site-specific transposon, mini-Tn7-*bar*, was successful by using an inexpensive source of phosphinothricin for selection.
2.3 MATERIALS AND METHODS:

**Bacterial strains, media, and culturing conditions.** All strains and plasmids used in this study are located in Tables 1 and 2. All manipulations with *B. pseudomallei* and *B. mallei* were conducted in a CDC/USDA approved and registered BSL3 facility at UHM, and experiments with these select-agents were performed with BSL3 practices by following the recommendations of the *Biosafety in Microbiological and Biomedical Laboratories* (BMBL) 5th edition.

Luria-Bertani (LB) medium (Difco) was used to culture all *E. coli* strains. *Burkholderia* strains (*B. pseudomallei*, *B. mallei*, *B. thailandensis*, *B. cenocepacia*, and *B. dolosa*) were cultured in LB or 1x M9 minimal medium + 20 mM glucose (MG). Diaminopimelate (DAP) was prepared in 1 M NaOH as a 100 mg/ml stock and used when necessary as described previously (3). A ~1 L bottle of the ‘super-concentrated’ herbicide RoundUp® (50% w/v GS) was purchased at a City Mill® hardware store as a source of GS for approximately $50 and used in this study. Purified GS was purchased from Sigma. We also purchased the herbicide Finale (9.5 L with 11.33% w/v phosphinothricin) for $125 at a local farm supply store (Pacific Agricultural Sales and Services), and it was used as a source of phosphinothricin (PPT) in this study. MG medium plus GS or PPT was utilized for *gat* or *bar* selection, respectively. Since GS blocks the biosynthesis of aromatic amino acids (Fig. 1), it is necessary to use minimal media without Phe, Trp, and Tyr. We observed that minimal media provided with any two aromatic amino acids abolished the selective potential of GS. Likewise, minimal media lacking glutamine is required for selection of *bar* with PPT. Antibiotics and non-antibiotic antibacterial compounds in solid media were utilized as follows: For *E. coli*, ampicillin (Ap) 110 µg/ml, GS 0.3%, kanamycin (Km) 35 µg/ml, and PPT 0.3% were used; for *B. mallei*, GS 0.2% effective concentration was
determined; for *B. pseudomallei*, GS 0.3% and PPT 2.5% were used; and for *B. thailandensis*, Km 500 µg/ml, GS 0.04%, and PPT 1.5% were used.

Two derivatives of *E. coli* EPM10B (BioRad), one containing *lacIq* and *pir* (E1869) and the other containing *lacIq*, *pir*, and *leu*<sup>+</sup> (E1889), were routinely used as cloning strains in rich and minimal media, respectively. The *E. coli* conjugal and suicidal strain, EPM10B-*pir116-Dasd-mob-Km-Dtrp::Gm (E1354), was used for plasmid mobilization into *B. pseudomallei* and *B. thailandensis*. Growth of *E. coli* Δasd strains was carried out as previously described (3). *E. coli* strain EPM10B-*pir116-Dasd::Gm (E1345) was used for cloning of *asd*-complementing vectors (e.g. pBAKA; *asd* gene encodes for aspartate-semialdehyde dehydrogenase). Briefly, selection of E1345 complemented with various *asd-* and *gat*-containing constructs (e.g. pBAKA-*DasdBp::gat-FRT* or pBAKA-*DdapBp::gat-FRT*) was performed on MG + GS media supplemented with leucine (Leu). To simplify selection and replace strain E1345, EPM10B-*lacIq/pir/leu*<sup>+</sup>/Δasd::Gm (E1951) was later created to select for *asd*-, *bar*-, and *gat*-containing plasmids on MG + GS or PPT media, so that leucine can be omitted from the minimal media. Selection of *asd*-, *bar*-, and *gat*-containing plasmids in the conjugation proficient strain E1354 was carried out with MG + Leu, Trp, and GS or PPT; in the absence of a complementing *asd* gene (e.g. pBBR1MCS-2-PC<sub>S12-gat</sub>), an additional 1 mM each of lysine (Lys), methionine (Met), threonine (Thr) and 100 µg/ml of DAP were added. For selection against E1354 following conjugation, Leu and Trp were omitted from the growth media. Counter-selection of *pheS* was carried out on MG media containing 0.1% *p*-chlorophenylalanine (cPhe; DL-4-chlorophenylalanine from Acros Organics) as described previously (3). *B. pseudomallei* Δasd<sub>Bp</sub>:*gat-FRT* and Δdap<sub>Bp</sub>:*gat-FRT* mutants were grown on rich LB media + 200 µg/ml DAP. For Δdap<sub>Bp</sub>:*gat-FRT* mutants grown in minimal media, MG medium + 200 µg/ml DAP +
1 mM Lys was used; this minimal medium was also supplemented with 1 mM of both Met and Thr for growing \(\Delta asd_{Bp}::gat-FRT\) mutants.

**Molecular methods and reagents.** Oligonucleotides used in this study are shown in Table 3. All molecular methods and reagents used were as described previously (3).

**Conjugation into Burkholderia spp.** Conjugation between the *E. coli* strain E1354 and *Burkholderia* strains was routinely carried out by growing both donor and recipient to log-phase prior to conjugation. Equal volumes of the donor and recipient (500 ml each) were mixed and centrifuged at 8,000x \(g\) for 1 minute. The supernatant was removed leaving approximately 30 \(\mu l\) of the conjugation mix which was spotted directly onto the surface of an LB plate with 100 \(\mu g/ml\) of DAP and incubated overnight at 37°C. Cells were scraped off using a disposable loop, resuspended and washed twice in 1 ml of 1x M9 buffer (to remove trace amino acids), and then resuspended in a final 1 ml of the same buffer. Aliquots of 100 \(\mu l\) and 200 \(\mu l\) of the cell suspensions were plated on the appropriate media. Conjugation using this method usually resulted in 50-100 colonies for recombination of non-replicating vectors when 100 \(\mu l\) of a conjugation recovery was plated, and 500-700 colonies for replicating plasmids when 100 \(\mu l\) of a 10x dilution was plated.

**Determination of GS minimal inhibitory concentrations (MIC) and effective concentrations.** To determine the liquid MIC for GS, we first grew all strains overnight in LB. One ml of culture was harvested by centrifugation, washed twice in 1x M9 buffer to remove trace amounts of amino acids, resuspended in 1x M9 buffer, and diluted 100x in the same buffer.
GS gradients in MG medium with a starting concentration of 0.8% GS and decreasing by 2x dilutions to 0.00625% GS were inoculated with the 100x dilutions (~10^5 cfu/ml) of each strain listed in Table 4. The liquid MIC was then determined after 2 days of incubation, with shaking, at 37°C. To establish the MIC of GS on solid media, we used MG + GS + 1.5 % agar (w/v). LB cultures of all species were grown to an OD_{600} of ~ 0.8. One ml of each culture was harvested by centrifugation, washed twice in 1x M9 buffer to remove trace amounts of amino acids, and resuspended in 1x M9 buffer. One hundred µl of the high-cell-density cultures were then plated on MG plates containing different concentrations of GS (ranging from 0-0.5%). The concentration where no growth was observed after 2 weeks was identified as the plate MIC. The GS concentration for each species was increased by ~30% above the MIC, and complete growth inhibition after 4 days in liquid and 3 weeks on solid media was taken as the effective concentration ([GS]_{EC}) (Table 4).

**Engineering of pUC57-P_{S12-gat}, pwFRT-PC_{S12-gat}, and pBBR1MCS-2-PC_{S12-gat}**. Driven by the *B. pseudomallei* rpsL promoter (P_{S12}) (60), the *gat* gene sequence was optimized to the codon-usage of *B. pseudomallei*. The *gat*-gene sequence was synthesized by GenScript Corporation, which was cloned into pUC57 as an EcoRV-XhoI fragment yielding pUC57-P_{S12-gat}. pUC57-P_{S12-gat} was digested with EcoRV + XhoI and inserted into the EcoRV + XhoI digested pwFRT-Tp', replacing the Tp'-cassette and yielding pwFRT-P_{S12-gat}. We overlooked a SacI restriction site, which was removed by site-directed mutagenesis using oligonucleotide #894 to yield pwFRT-P_{S12-gat}-SDM. Additionally, the *gat* gene was initially engineered to be driven by the P_{S12}-promoter on pUC57-P_{S12-gat}. However, homologous recombination may occur with the P_{S12}-promoter region at the native locus on the chromosome or an additional P_{S12}-promoter
located on pBAKA. To prevent this possibility, the *gat* gene from pwFRT-P<sub>SI2-gat</sub>-SDM was removed using NcoI + XhoI and ligated into pwFRT-Tel<sup>f</sup> cut with the same enzymes, replacing the Tel<sup>f</sup>-cassette with the *gat* gene, yielding pwFRT-PC<sub>SI2-gat</sub> (Fig. 4A). Unique enzyme sites are present on the *gat* cassette to allow for ease of manipulation (Fig. 4A).

pBBR1MCS-2-PC<sub>SI2-gat</sub> was constructed to first test the effectiveness of GS selection in *E. coli* and *B. thailandensis* by comparing colony numbers on LB + Km and MG + GS media. pBBR1MCS-2 was digested with KpnI, blunt ended, and then digested with XhoI. The resultant fragment was ligated to the 533-bp EcoRV-XhoI PC<sub>SI2-gat</sub> fragment from pwFRT-PC<sub>SI2-gat</sub>, yielding pBBR1MCS-2-PC<sub>SI2-gat</sub>. When introduced into *E. coli* and *B. thailandensis*, this plasmid yielded the same number of colonies on kanamycin-containing media when compared to GS-containing media with RoundUp<sup>®</sup> as a source of GS (data not shown). This indicates that no other ingredients in RoundUp<sup>®</sup> have adverse effects on the selection of *gat*-containing constructs and that this source of GS is appropriate for selective media.

**Testing the inhibitory action of glyphosate on *Burkholderia* select agent species after 24 hours.** We wanted to determine if GS was a bacteriostatic or bactericidal compound by exposing *B. pseudomallei* strains 1026b and K96243 and *B. mallei* ATCC23344 to increasing concentrations of GS for 24 hours. First, *B. mallei* and the two *B. pseudomallei* strains (1026b and K96243) were grown overnight in LB. The cultures were then washed twice in 1 ml of 1x M9 buffer and resuspended in 1 ml of the same buffer, which were inoculated (1:100 dilution) into 3 ml of MG + 0.25%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, or 4.0% GS with 30µl of the washed cultures. Immediately, 100 µl of serial dilutions of each culture were plated to determine initial bacterial cfu for each strain. After 24 hours, 100 µl of serial dilutions from each
culture were plated and bacterial cfu were again calculated. We used the ratio of bacterial cfu at 24 hrs to bacterial cfu at initial exposure to determine the percent of survival after 24 hours of exposure to GS (Fig. 2).

**Determination of the effective concentration of phosphinothricin and construction of pBBR1MCS-2-PC\textsubscript{S12}-bar.** The effective concentration of PPT ([PPT]\textsubscript{EC}) from the herbicide Finale was determined in the same manner as for the GS effective concentration. We first determined whether bar would be an efficient selectable marker in *E. coli* and *B. thailandensis* by constructing pBBR1MCS-2-PC\textsubscript{S12}-bar. The bar gene was amplified from pCAMBIA-1301-bar using oligonucleotides #881 and #882. The product was then used as a template for a second PCR using oligonucleotides #837 and #882 to introduce the PC\textsubscript{S12}-promoter. The 800 bp PCR product was then digested with XhoI and ligated into the EcoRV + XhoI digested pBBR1MCS-2 to yield pBBR1MCS-2-PC\textsubscript{S12}-bar. This construct was then introduced into *E. coli* and, subsequently, into *B. thailandensis* via electroporation. The [PPT]\textsubscript{EC} was determined to be 0.3% and 1.5% for *E. coli* and *B. thailandensis*, respectively. Introduction of pBBR1MCS-2-PC\textsubscript{S12}-bar into *E. coli* and *B. thailandensis* yielded the same number of colonies on kanamycin media as PPT containing media (data not shown), indicating that this source of PPT contains no other ingredients that could adversely affect selection of bar-containing constructs. The [PPT]\textsubscript{EC} for *B. pseudomallei* 1026b, *B. pseudomallei* K96243, and *B. mallei* was found to be 2.5% by the same methods described above for the [GS]\textsubscript{EC}.

**Construction of gat-FRT and bar-FRT vectors.** Using pwFRT-PC\textsubscript{S12}-gat with TCTAGAAA as the wildtype spacer of the flanking FRT sequences, we also constructed four
other plasmids based on four other unique \textit{FRT} sequences: pmFRT-PCS\textsubscript{12}-\textit{gat} with flanking \textit{FRT}-spacer TGTAGATA; pFRT1-PCS\textsubscript{12}-\textit{gat} with TCTTGAAA spacer; pFRT2-PCS\textsubscript{12}-\textit{gat} with TCTAGGAA spacer; and pFRT3-PCS\textsubscript{12}-\textit{gat} with TCTCGAAA spacer. The differences in the spacer sequence yield unique \textit{FRT}s. The unique \textit{FRT}s in these 5 plasmids (pwFRT-PCS\textsubscript{12}-\textit{gat}, pmFRT-PCS\textsubscript{12}-\textit{gat}, pFRT1-PCS\textsubscript{12}-\textit{gat}, pFRT2-PCS\textsubscript{12}-\textit{gat}, pFRT3-PCS\textsubscript{12}-\textit{gat}) allow for multiple rounds of allelic-replacement by recycling the same marker with Flp-\textit{FRT} excision, reducing the risk of chromosomal deletions and rearrangements as observed previously (2). To construct these four plasmids, laboratory vectors pmFRT-Gm\textsuperscript{r}, pFRT1-Gm\textsuperscript{r}, pFRT2-Gm\textsuperscript{r}, and pFRT3-Gm\textsuperscript{r} were PCR amplified with oligos #715 and #716 to obtain plasmid backbones without the Gm\textsuperscript{r}-marker. Each plasmid backbone was digested with EcoRV + XhoI and ligated with the PCS\textsubscript{12}-\textit{gat} fragment obtained from pwFRT-PCS\textsubscript{12}-\textit{gat} by EcoRV + XhoI digestion. Essentially, the sequences of all four new pFRT-PCS\textsubscript{12}-\textit{gat}-\textit{FRT} plasmids are the same as pwFRT-PCS\textsubscript{12}-\textit{gat}, with the exception of the \textit{FRT}-spacer sequences flanking the \textit{gat} cassette. The \textit{FRT}-flanked \textit{bar} cassette on pwFRT-PCS\textsubscript{12}-\textit{bar} was constructed by first amplifying the \textit{bar} gene from pCAMBIA-1301-\textit{bar}, via a two-step PCR, as described above for the construction of pBBRMCS1-2-PCS\textsubscript{12}-\textit{bar}. The 800 bp \textit{bar} fragment was digested with XhoI and ligated into the EcoRV + XhoI digested pwFRT-PCS\textsubscript{12}-Tel\textsuperscript{r}, replacing the Tel\textsuperscript{r}-cassette with the \textit{bar} gene to produce pwFRT-PCS\textsubscript{12}-\textit{bar}.

**Construction of pBAKA-\textit{\Delta asd\textsubscript{Bp}}::\textit{gat-FRT} and pBAKA-\textit{\Delta dap\textsubscript{Bp}}::\textit{gat-FRT}**. The \textit{B. pseudomallei} K96243 asd\textsubscript{Bp} gene was amplified from chromosomal DNA using oligonucleotides #892 and #893. This asd\textsubscript{Bp} gene sequence is essentially identical for both K96243 and 1026b. The 1.4 kb fragment was digested with EcoRI + HindIII and cloned into pUC18 digested with
the same enzymes. After cloning, the purified plasmid, pUC18-\(a_{sdBp}\), was then electroporated into the \(dam^{-}\) strain GM33. Plasmids were isolated, digested with BclI (\(dam\) methylation sensitive) + EcoRV, and blunt ended. The plasmid backbone was then ligated to the 0.7 kb fragment from SmaI digested \(pwFRT-PC_{S12-gat}\), resulting in a 250 bp deletion in the \(asdBp\) gene. pUC18-\(\Delta asdBp::gat-FRT\) was then digested with EcoRI + HindIII and the 1.9 kb fragment was cloned into pBAKA, cut with the same enzymes, to produce pBAKA-\(\Delta asdBp::gat-FRT\). The \(gat\) gene is in the same orientation as the \(asd_{Bp}\) gene (Fig. 5A).

To construct pBAKA-\(\Delta dapB_{Bp}::gat-FRT\), the \(B. \ pseudomallei\) K96243 \(dapB\) gene was amplified from chromosomal DNA using oligonucleotides #1048 and #1050. The 1.9 kb fragment was digested with HindIII + XbaI and ligated into pBAKA cut with the same enzymes. pBAKA-\(dapB_{Bp}\) was then digested with SalI and ligated with the 0.7 kb fragment from the SalI digestion of \(pwFRT-PC_{S12-gat}\), producing pBAKA-\(\Delta dapB_{Bp}::gat-FRT\). The \(gat\) gene is in the opposite orientation as the \(dapB_{Bp}\) gene.

**Engineering of \(B. \ pseudomallei\) \(\Delta asdBp::gat-FRT\) and \(\Delta dapB_{Bp}::gat-FRT\) mutants.** E1354 was utilized as the conjugal donor to introduce the allelic-replacement vectors, pBAKA-\(\Delta asdBp::FRT-gat\) and pBAKA-\(\Delta dapB_{Bp}::gat-FRT\), into \(B. \ pseudomallei\) strain K96243. Conjugations were carried out as described above, and 100 \(\mu l\) and 200 \(\mu l\) of the conjugation mixtures were plated on MG medium + 200 \(\mu g/ml\) DAP + 0.3\% GS + 1 mM each of Lys, Met, and Thr; these last 3 amino acids (3AA) are required for the specific \(Dasd\) mutation (Fig. 5B). Colonies appearing after 3 to 4 days were streaked-out on the same medium supplemented with 0.1\% cPhe to counter-select against \(pheS\). It is critical that the media, in the presence of cPhe, contain no competing phenylalanine for clean counter-selection as previously described (3). GS
resistant mutants were screened by patching with toothpicks onto plates with and without DAP (MG + 0.3% GS + 0.1% cPhe + 1 mM 3AA ± 200 mg/ml DAP). Mutants unable to grow without DAP were purified once on LB + DAP, and patched again on MG + 0.3% GS + 0.1% cPhe + 1 mM 3AA ± 200 mg/ml DAP to confirm. Purification from potential background on LB + DAP is recommended and is very important because GS is static, rather than cidal, at this effective concentration. Further screening and confirmation of DAP requiring mutants were performed by PCR, using oligonucleotides #1062 and #1063 which annealed to the chromosome outside of the region cloned for allelic-replacement (Fig. 5A and 5C).

To engineer the *B. pseudomallei* K96243 ΔdapB<sub>bp</sub>::gat-FRT mutant, methodologies were essentially the same as for the engineering of ΔasdB<sub>bp</sub>::gat-FRT, except only 1 mM Lys was supplemented in the media with DAP rather than all 3AA (Fig. 5B). DAP requiring colonies were further purified as above on LB + DAP because GS is a static agent. Screening of DdapB<sub>bp</sub> mutants was carried out by PCR using oligonucleotides #1070 and #1071, which anneal outside of the oligonucleotides used for cloning (Fig. 5C).

For *B. pseudomallei* strain 1026b, we engineered and confirmed the DasdB<sub>bp</sub> mutant essentially as above for strain K96243 via counter selection with cPhe/pheS. To demonstrate that the DNA incubation approach also works using gat for selection with GS in strain 1026b, we used the published DNA incubation and natural transformation approach to delete the dapB<sub>bp</sub> gene in strain 1026b (52). pBAKA-ΔdapB<sub>bp</sub>::gat-FRT was used as a template along with oligos #1049 + #1051 in a PCR to obtain a linear 2.7 kb ΔdapB<sub>bp</sub>::gat-FRT fragment. Allelic-replacement was performed as previously published (3), but selection was carried-out on MG medium + 0.3% GS + 200 µg/ml DAP + 1 mM Lys. GS resistant colonies that were DAP requiring were purified and further confirmed by PCR with oligos #1070 and #1071 as above.
Phenotypic lysis of Δasd and ΔdapB mutants without DAP. The *B. pseudomallei* wildtype K96243, K96243-Dasd<sub>Bp</sub>::gat-FRT mutant, and K96243-DDapB<sub>Bp</sub>::gat-FRT mutant were first grown overnight in LB (wildtype strain) or LB + DAP (Dasd<sub>Bp</sub>::gat-FRT and DdapB<sub>Bp</sub>::gat-FRT strains). One ml of each culture was centrifuged, the cell pellets were washed twice with LS (LB no salt) medium, and resuspended in 20 ml of LS. Ten ml of each concentrated cell resuspension was spotted on LS and LS + DAP plates and incubated at 37°C. After 18 hours, cells were resuspended in sterile saline (0.85% NaCl) and smeared on glass slides. The slides were then air dried and fixed with 1% paraformaldehyde (in PBS) for 1 hour. This fixing method was initially tested on wildtype K96243 and the slide was incubated in rich LB media for 3 weeks to ensure no growth was observed, indicating complete killing. Finally, the cells were stained with safranin for 10 minutes, gently rinsed with water, and examined under 100x oil immersion (Fig. 6).

Construction of mini-Tn7-bar and mini-Tn7-bar-asdBp. To construct the site-specific mini-Tn7-bar transposon, mini-Tn7-Tel' was digested with XbaI (cut in the flanking FRT-spacer regions) and the bar-cassette from pwFRT-PC<sub>S12</sub>-bar (also digested with XbaI in the FRT-spacer regions) was ligated to replace the Tel' cassette. Recovery of the FRT sequences was verified by confirming the orientation of the cloned PC<sub>S12</sub>-bar fragment and recovery of the XbaI sites via restriction enzyme digestions. To construct mini-Tn7-bar-asdBp, the asdBp gene with 600 bp of upstream sequence was amplified from the *B. pseudomallei* K96243 chromosome to include the putative promoter. The 1.8 kb asdBp gene was PCR amplified from K96243 chromosomal DNA using oligos #893 and #1117, and the product was digestd with EcoRI + HindIII. mini-Tn7-bar was digested with the same enzymes and ligated to this 1.8 kb asdBp gene resulting in mini-Tn7-bar-asdBp. The asdBp gene was cloned in the same orientation as the bar-cassette. Functionality
of the \( asd_{Bp} \) was verified by transformation into a \( Dasd E.coli \) strain (E1345) and growth was observed on LB in the absence of DAP.

**Complementation of the \( B. pseudomallei \Delta asd_{Bp}::gat-FRT \) mutant.** The \( B. pseudomallei \) 1026b \( \Delta asd_{Bp}::gat-FRT \) mutant was complemented using the mini-Tn7-\( bar-asd_{Bp} \) vector. The mini-Tn7-\( bar-asd_{Bp} \) vector and its helper plasmid (pTNS3-\( asd_{Ec} \)) were transformed individually into \( E. coli \) E1354 (a conjugation proficient tryptophan auxotroph) and conjugated into \( B. pseudomallei \Delta asd_{Bp}::gat-FRT \) in a tri-parental mating experiment. Conjugation mixtures were scraped from the plate using a disposable loop and resuspended in 1 ml of 1x M9 minimal media. Then 100 \( \mu l \) of a 1:10 dilution was plated on MG medium + 200 \( \mu g/ml \) DAP + 2.5% PPT + 1 mM of each Lys, Met, and Thr. This medium prevents the \( E. coli \) donor (Trp auxotroph) from growing and selects for PPT resistant \( B. pseudomallei \). Of seventy colonies initially patched onto 1x M9 + 1% casamino acids ± DAP, forty-five separate isolates grew in the absence of DAP, indicating a complementation frequency of \( \sim 64\% \). Ten isolates were screened for positive integration using oligonucleotides #876, which anneals in the Tn7L region of the mini-Tn7-\( bar \) site-specific transposon, and one of the oligos #1079, #1080, or #1081, specific for each of the three possible integration sites on the chromosome (11). Of the ten isolates screened, two had Tn7-\( bar-asd_{Bp} \) integrated downstream of \( glmS1 \), while the remaining 8 isolates had Tn7-\( bar-asd_{Bp} \) integrated downstream of \( glmS2 \). Neither integration at \( glmS3 \) nor multiple integrations at different \( glmS \) integration sites were observed. Two positive isolates with insertion at \( glmS1 \) and three isolates with insertion at \( glmS2 \) were chosen for further characterization below.
Growth of wildtype *B. pseudomallei* strain 1026b, its ∆\textit{asd}\textsubscript{\textit{Bp}}::\textit{gat-FRT} mutant, and ∆\textit{asd}\textsubscript{\textit{Bp}}::\textit{gat-FRT/attTn7-bar-asd}\textsubscript{\textit{Bp}} complements. To further characterize the \textit{Dasd} mutation, we first grew strain 1026b, the \textit{Dasd}\textsubscript{\textit{Bp}} mutant strain, and several complements overnight in LB + DAP, at 37°C with shaking at 250 rpm. One ml of each culture was harvested by centrifugation at 8,000x \textit{g} for 2 minutes. The pellet was washed twice with 1x M9 buffer to remove any residual nutrients and resuspended in 1 ml of 1x M9 buffer. To determine the amino acid auxotrophic properties of these strains, the cell suspensions were diluted 20x in 1x M9 buffer. Five µl of each culture was spotted onto plates with MG medium + 200 µg/ml DAP + 1 mM each of Ile + Lys + Met + Thr; the same amount of the diluted cultures was spotted onto 5 other plates, each missing one of the four amino acids or DAP. Growth on the plates was observed after 24 hrs and 7 days (Fig. 8).

**GenBank Accession Numbers.** The sequences for all constructs in figure 4 were submitted to GenBank. The accession numbers are: pwFRT-PC\textsubscript{\textit{S12-gat}}, FJ384986; pwFRT-PC\textsubscript{\textit{S12-bar}}, FJ858786; mini-Tn7-\textit{gat}, FJ858785; and mini-Tn7-\textit{bar}, FJ826509.
2.4 RESULTS AND DISCUSSION

Effectiveness of glyphosate (GS) against *Burkholderia* species. Although studies have measured the GS inhibitory concentrations for *P. aeruginosa*, *E. coli*, *B. subtilis*, and *B. japonicum* (18, 61), no studies have determined the GS inhibitory concentration for *Burkholderia* species. It was previously shown that growth inhibition of *B. japonicum* was observed at lower GS concentrations (5 mM or 0.085%), and rapid death occurred at a higher GS concentration (10 mM or 0.17%) (61). Thus, GS could be bacteriostatic depending on the concentration used. We initially determined the inhibitory or killing action of GS for three *Burkholderia* select-agent strains (Fig. 2). When exposed to different concentrations of GS for 24 hours, *B. mallei* was found to be more sensitive to GS than *B. pseudomallei* and over 60% death was observed when exposed to a concentration as low as 0.25%. Both *B. pseudomallei* strains replicated slightly less than double at 0.25% GS and were killed at higher concentrations of GS (Fig. 2). Clearly, no significant replication was observed at concentrations as low as 0.25% GS. Although to be determined in future studies, the mechanism of GS inhibition on *Burkholderia* species is likely similar to the mechanism of EPSPS inhibition in plants, having been confirmed for *P. aeruginosa*, *E. coli*, *B. subtilis*, and *B. japonicum* (18, 61).

We next wanted to determine the minimal inhibitory concentration of GS for members of the *Burkholderia* genus, then identify the effective GS concentration ([GS]_{EC}) in liquid and on solid media (see Materials and Methods). Significantly high cell densities typical in genetic manipulations (e.g. 10^5 cfu in liquid media and 100 ml of ~10^9 cfu/ml was plated on solid media containing different concentrations of GS) were inoculated to determine the [GS]_{MIC} where no spontaneous resistance to GS was observed after 2 days (liquid media) or 2 weeks (solid media). The [GS]_{EC} above the [GS]_{MIC} was defined and utilized to ensure no growth of high inocula in
liquid media after 4 days or no growth for 3 weeks on solid media (Table 4). We determined the 
[GS]EC within this time frame for liquid and solid media, because this time period is sufficient to 
observe most mutants that will arise during allelic-replacement and also allows most 
*Burkholderia* species to grow on minimal media during selection. The [GS]EC for *E. coli* and *B.
pseudomallei, B. mallei,* and *B. cenocepacia* K56-2 are higher than other *Burkholderia* (Table 4). We have utilized the [GS]EC on Table 4 to select for the gat gene (below) successfully in *E. coli,* *B. thailandensis,* and the two wildtype *B. pseudomallei* strains. Thus, we are confident that the 
[GS]EC in Table 4 for other species are appropriate.

Roundup® is an appropriate source of GS for selection. We have not encountered any 
problem with the solubility of GS at high concentration (10% was the highest concentration 
tested using purified GS). Indeed, the ‘super-concentrated’ Roundup® purchased contained 50% 
GS in aqueous solution. In addition to this advantage, GS is readily available, inexpensive, 
relatively non-toxic, not in clinical use, and gives tight selection (below). Although purified GS 
could be purchased from Sigma and other distributors, we do not have concerns with using 
Roundup® for selection, since purified GS gave the same [GS]EC. One bottle of ‘super-
concentrated’ Roundup® purchased from a local garden supply store has lasted through the 
duration of this study. Roundup® with lower concentrations of GS are also available, although we 
recommend the ‘super-concentrated’ Roundup® as it is potent enough for >150 liters of cultures 
when used at 0.3% final concentration. The other evidence that Roundup® is appropriate for 
selection, with no observed adverse affects on bacteria from other ingredients, is that selection of 
pBBR1MCS-2-PC_{S12}-gat (kanamycin and GS resistant) in *E. coli* and *B. thailandensis* on 
kanamycin or GS from Roundup® yielded the same number of colonies (data not shown).
**Engineering of a gat-cassette and effective selection with glyphosate.** We engineered the gat-gene through GenScript Corporation, based on the previously described GAT protein sequences (6), using an approach similar to the synthesis of the pheS gene (3). We utilized this approach to optimize the codon-usage for efficient expression in *Burkholderia* species, while eliminating many restrictions sites within the gene and strategically placing others at certain locations for future manipulation. The engineered gat-cassette, including the *B. cenocepacia* PC$_{S12}$ promoter of the *rpsL* gene, is only 533 bp (Fig. 3). The small size of the gat-cassette makes it easy to manipulate, clone, and perform PCR for use in the DNA incubation method of allelic-replacement in naturally competent *Burkholderia* species (52). As proof-of-concept, we utilized this cassette in the DNA incubation approach to delete the asdB$_{t}$ gene of *B. thailandensis* at its [GS]$_{EC}$ (data not shown). This confirmed that the [GS]$_{EC}$ on Table 4 are sufficient for selection.

**Deletional mutagenesis of the essential *B. pseudomallei* asd$_{Bp}$ and dapB$_{Bp}$ genes using GS and gat.** The reliability of any marker for mutagenesis would best be demonstrated by the successful mutagenesis of essential genes. Therefore, we chose two essential genes, *asd* and *dapB*, that are absolutely required for diaminopimelate (DAP) synthesis and cell-wall cross-linking in most Gram-negative bacteria (Fig. 5B) (9, 12, 25, 27, 42). Mutation of the *asd* gene makes Gram-negative bacteria auxothrophic for three amino acids (Thr, Met, DAP), while *dapB* mutants only require DAP. Although Lys and Ile are also made from the same pathway, the provided DAP and Thr should act as precursors for Lys and Ile biosynthesis, respectively (Fig. 5B). The *asd$_{Bp}$* and *dapB$_{Bp}$* genes encode for aspartate-semialdehyde-dehydrogenase (BPSS1704 on chromosome 2) and a dihydrodipicolinate reductase (BPSL2941 on chromosome 1), respectively.
To knock-out the \( \text{asd}_{\text{Bp}} \) and \( \text{dapB}_{\text{Bp}} \) genes in \( B. \text{pseudomallei} \), we engineered \( \text{gat-FRT} \) cassettes for allelic-replacement (Fig. 4A). As mentioned above, utilization of a \( \text{gat-FRT} \) cassette was successfully tested in \( B. \text{thailandensis} \) to delete the \( \text{asd}_{\text{Bt}} \) gene using the DNA incubation and natural transformation method (52), where the selection with 0.04% GS yielded \( \sim 80\% \) \( B. \text{thailandensis} \) \( \text{Dasd}_{\text{Bt}} \) mutation frequencies (data not shown). We then utilized pBAKA and the \( \text{pheS} \) counter-selection approach as previously described (3), with the \( \text{gat-FRT} \) cassette from \( \text{pwFRT-PC}_{S12}-\text{gat} \), to inactivate the \( \text{asd}_{\text{Bp}} \) and \( \text{dapB}_{\text{Bp}} \) genes in \( B. \text{pseudomallei} \) (Fig. 4A and 5A). Independent merodiploids resulting from the first recombination in strain K96243 were obtained with 0.3% GS after three days of growth (see Materials and Methods). Streaking of merodiploids on media containing 0.1% cPhe and 0.3% GS for counter-selection to resolve the mutations yielded DAP-requiring colonies at frequencies of \( \sim 25\% \) and \( \sim 80\% \) for the \( B. \text{pseudomallei} \) K96243 \( \text{Dasd}_{\text{Bp}} \) and \( \text{DdapB}_{\text{Bp}} \) mutants, respectively. To demonstrate this principle of allelic-replacement in another \( B. \text{pseudomallei} \) strain, we utilized this same approach with cPhe/\( \text{pheS} \) counter-selection to create a 1026b \( \text{Dasd}_{\text{Bp}} \) mutant, which yielded a lower mutation frequency of \( \sim 10\% \) in this essential gene. Since strain 1026b is also naturally competent, we wanted to utilize the published DNA incubation method for allelic-replacement (52) by engineering a \( \text{DdapB}_{\text{Bp}} \) mutant, yielding 1026b \( \text{DdapB}_{\text{Bp}} \) mutants at a frequency of \( \sim 25\% \). We confirmed these mutations by PCR with oligos annealing to chromosomal regions outside of the initial oligos used for cloning (Fig. 5A and 5C). Because GS is bacteriostatic at the concentration used (Table 4 and Fig. 2), it is critical to purify all mutants from the potential background contamination before reconfirming the phenotype, PCR confirmation, and growth for long-term storage at -80°C. Phenotypically, \( B. \text{pseudomallei} \) \( \text{DdapB}_{\text{Bp}} \) mutants required DAP for growth (data not shown), while \( \text{Dasd}_{\text{Bp}} \) mutants required DAP, Thr, and Met (Fig. 8). Using wildtype and mutants of strain
K96243 as examples, we further characterized the phenotype of DasdBp and DdapBp mutants. In the presence of DAP, both DasdBp and DdapBp mutants displayed normal rod-shape cellular morphology (Fig. 6). However, in the absence of DAP, these two mutants lack DAP for cell-wall biosynthesis and cross-linking, demonstrating ‘cell-rounding’ characteristics and evidence of lysis (Fig. 6).

**B. pseudomallei DasdBp mutant complementation with a site-specific mini-Tn7-bar-asdBp transposon.** We engineered a site-specific transposon based on mini-Tn7, which has previously been demonstrated to integrate at three possible glmS sites in the *B. pseudomallei* chromosome (11) (Fig. 4D and 7A). Our construct, mini-Tn7-bar, is based on the non-antibiotic bar gene that encodes for resistance to bialaphos and phosphinothricin (a bialaphos degradation product also known as glufosinate). Since bialaphos can be very expensive, a cheaper alternative, phosphinothricin (PPT), can be used. We determined the effective concentration of PPT ([PPT]EC) for *B. mallei* and *B. pseudomallei* to be ~2.5%. Many herbicide brands (e.g. Basta, Buster, Dash, Finale, Hayabusa, Ignite, Conquest, Liberty, Rely, Shield, Harvest, Sweep, Arise, and others) contain PPT as the active ingredient. Since the [PPT]EC was quite high, we picked the herbicide Finale because it contains the highest PPT concentration (11.33% w/v) we could find, although other brands not available on our island (e.g. Liberty and Ignite) can contain 20-25% PPT. In this study, we utilized the 11.33% PPT in Finale as the working stock to make media at the 2.5% [PPT]EC. As proof-of-concept, we introduced the constructed mini-Tn7-bar-asdBp into the *B. pseudomallei* 1026b DasdBp strain to complement the DasdBp mutation. The suicidal helper plasmid pTNS3-asdEc, harboring the *E. coli asdEc* gene for maintenance in an *E. coli* DasdEc strain, aids the transposition of the Tn7-bar-asdBp transposon to one of three possible
glmS chromosomal targets (Fig. 7A). We selected PPT resistant colonies in the presence of DAP, Lys, Met, and Thr to prevent bias in immediately selecting for complements. After patching on media ± DAP, it was found that ~64% of PPT resistant colonies tested (45 out of 70) were complements of the DasdBp mutation and did not require DAP. It was further confirmed that the majority of the complements (8 out of 10 tested) had the mini-Tn7-bar-asdBp transposed to the region downstream of the glmS2 target, while 2 out of 10 recombined at the glmS1 target (Fig. 7B). No transposition at the glmS3 target was observed. These data indicated that PPT in Finale® was appropriate for selection of the bar gene and yielded a fairly high frequency of transposition.

To further characterize five complements along with the wildtype 1026b and the DasdBp mutant, we spotted these strains on various media lacking one of the five amino acids (DAP, Lys, Met, Thr, and Ile) in the aspartate family of amino acid biosynthetic pathways. Media lacking Met, Thr, or DAP yielded no growth of the DasdBp mutant compared to the wildtype 1026b and the five complements, confirming that the Asd reaction gives rise to these amino acids (Fig. 5B and 8). Ile and Lys were not required in the DasdBp mutant, as Thr and DAP will yield Ile and Lys, respectively (Fig 8E and 8H). In summary, the DasdBp mutant of B. pseudomallei displayed a similar phenotype to asd mutants of other Gram-negatives, and the successful complementation of this mutant suggests that our allelic-replacement approach did not introduce any undesirable mutations during selection with Roundup® and Finale®.

**Conclusions.** (i) We engineered and successfully demonstrated the use of a novel non-antibiotic gat-marker, based on resistance to glyphosate in Burkholderia species. This cassette was demonstrated to be useful for allelic-replacement of essential genes in B. pseudomallei, adding valuably to the limited number of select-agent approved markers. The advantages of
using GS-containing herbicides to select for the gat-cassette in recombinant work include cost effectiveness, availability, low toxicity, non-clinical use, high solubility, relatively tight selection, and the small size of the gat-marker. The gat-cassette was used successfully in more than one allelic-replacement strategy to delete two essential genes confirming its value, the usefulness of pheS as a counter-selectable marker, and the compatibility with the DNA incubation method for naturally competent Burkholderia species (52). (ii) We initiated the successful utilization of a second non-antibiotic resistance marker, based on the better characterized bar gene (55), encoding for bialaphos and phosphinothricin (glufosinate) resistance. This will hopefully also expand its future use in select-agent species. One minor disadvantage of using gat and bar is the requirement for minimal media lacking two of the three aromatic amino acids (Phe, Tyr, or Trp) and glutamine, respectively. However, for most mutations, using 1x M9 + 20 mM glucose should suffice for B. pseudomallei, B. mallei, and B. thailandensis. Note that we added DAP, Lys, Met, and Thr to the media in this study because of the mutant-specific amino acid requirements (e.g. DasdBp and DdapBp). (iii) We created two mutants in two wildtype B. pseudomallei strains, which may be promising as future attenuated vaccine candidates, since DAP is a bacterial specific product not available in mammalian hosts. (iv) Finally, it is important to point out that all genetic tools used in this study are completely devoid of antibiotic resistance during introduction and selection. The potential use of gat and bar may be expanded to other select agent species (e.g. Brucella and Francisella), since minimal media lacking Phe, Tyr, Trp, and Gln has been defined for some of these species (7, 21). These compounds (GS and PPT), originally designed to kill plant weeds, may prove quite useful for the future selection of recombinants in bacterial select-agent species.
2.5 ACKNOWLEDGEMENTS

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Graduate stipend to M.H.N. was supported by an NSF IGERT Award 0549514 to B.A.W.
2.6 FIGURE LEGENDS

FIG. 1. (A) A 946 ml bottle of the ‘super concentrated’ herbicide Roundup® used in this study, available for ~$50 from most local hardware stores and garden or farm supply centers. Indicated are the 50% glyphosate active ingredient and the chemical structure of glyphosate. The glyphosate N-acetyl-transferase (Gat), encoded by the gat gene, catalyzes the inactivation of glyphosate via N-acetylation. (B) Pathways of aromatic amino acids biosyntheses. Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) required for aromatic amino acid biosyntheses, starving bacteria for tyrosine, phenylalanine, and tryptophan.

FIG. 2. Bacterial survival after incubation with different concentrations of GS for 24 hours. B. mallei was more sensitive to GS than both B. pseudomallei strains and killing of B. mallei by GS was observed at 0.25% GS. B. pseudomallei strain 1026b is significantly more resistant to GS than strain K96243. Insignificant replication of both B. pseudomallei strains (less than double) after 24 hours was observed at 0.25% GS. Killing was observed at 2% GS for strain K96243 and 3% GS for strain 1026b.

FIG. 3. A schematic diagram of the engineered 533-bp gat-gene on pwFRT-PC_{S12-gat}. The B. cenocepacia rpsL promoter (PC_{S12}) and ribosomal-binding-site (rbs) are shown in relation to the gat gene. Below the schematic are the corresponding nucleotide and protein sequences. Codons were optimized according to codon preference within the B. pseudomallei K96243 asd gene. Also indicated are the -35 and -10 regions of the PC_{S12}-promoter. Restriction sites were positioned strategically (in bold) for subsequent cloning and manipulation.
FIG. 4. Maps of the pwFRT-PC$_{S12}$-gat (A), pwFRT-PC$_{S12}$-bar (B), mini-Tn7-gat (C), and mini-Tn7-bar (D). (A) pwFRT-PC$_{S12}$-gat is flanked with symmetrical restriction-sites (HindIII to SacI) that will cut to remove the gat-cassette flanked with identical wildtype FRT sequences. Not shown are four other FRT-gat cassettes with flanking unique FRT-sequences (pmFRT-gat, pFRT1-gat, pFRT2-gat, and pFRT3-gat), where the gat marker is flanked by identical FRTs with unique spacer sequences. The DNA sequences and restriction sites for all five gat-FRT are identical with the exception of the spacers. (B) pwFRT-PC$_{S12}$-bar with bar flanked by wildtype FRT sequences and symmetrical restriction enzyme sites. (C) mini-Tn7-gat and (D) mini-Tn7-bar were engineered to allow site-specific integration of cloned gene(s), using the non-antibiotic bar or gat selectable marker, with the assistance of a helper-plasmid (pTNS3-asd$_{Ec}$).

Abbreviations: bar, gene encoding bialaphos/phosphinothricin resistance; bla, beta-lactamase encoding gene; ori, ColE1 origin of replication; FRT, flippase recognition target sequences; oriT, conjugal origin of transfer; PC$_{S12}$, promoter of the B. cenocepacia rpsL gene; R6K$_{γ}$ori, π protein dependent R6K origin of replication; Tn7L/Tn7R, left and right transposase recognition sequences; T$_{1}$T$_{0}$, transcriptional terminator.

FIG. 5. (A) Gene replacement strategy using a gat-FRT cassette to inactivate the B. pseudomallei strain K96243 and 1026b asdBp gene. Oligoss #892 and #893 were used in the initial cloning of the asdBp gene into the allelic-replacement vector pBAKA, and the asdBp gene was inactivated with the gat-FRT cassette. Deletion of the chromosomal asdBp gene with pBAKA-Dasd$_{Bp}$::gat was performed as shown. PCR verification of the Δasd$_{Bp}$ mutant was done using outside oligoss #1062 and #1063. The asdBp gene of both K96243 and 1026b strains were inactivated using pBAKA and pheS for counter-selection. Similarly, the dapB$_{Bp}$ gene of strain
K96243 was also inactivated using pBAKA and pheS for counter-selection (not shown, see Materials and Methods). Oligos #1049 and #1051 were used to amplify the DdapBp::gat cassette from plasmid pBAKA-DdapBp::gat to inactivate the dapBp gene from strain 1026b using the DNA incubation method (52) (Materials and Methods). (B) Bacterial amino acid biosynthetic pathway of the aspartate family, where asparate is used to synthesize DAP, Lys, Met, Thr, and Ile. The indicated reactions catalyzed by Asd and DapB are central to this pathway and mutants of these genes cannot cross-link their cell-walls due to the lack of DAP. (C) PCR verification of the DasdBp and DdapBp mutants. In each case as expected, the PCR products indicated that the chromosomal fragment of the mutant is larger than the wt, and the no template negative control (nc) showed no PCR product. Abbreviations: asdBp, B. pseudomallei asd gene encoding aspartate-semialdehyde-dehydrogenase; asdPa, P. aeruginosa asd gene; dapBp, B. pseudomallei gene encoding dihydrodipicolinate reductase; gat, glyphosate resistance gene encoding glyphosate acetyl transferase; GS, glyphosate; M, 1-kb ladder (New England Biolabs); Plac, lac-promoter; pheS, mutant B. pseudomallei gene encoding the a-subunit of phenylalanyl tRNA synthase.

**FIG. 6. Phenotypic characterization of B. pseudomallei K96243 DasdBp and DdapBp mutants.** Wildtype K96243 was rod-shape when grown in the absence or presence of DAP (left top and bottom panels). The mutant DasdBp (middle panels) and DdapBp (right panels) strains grow, but ‘pop-and-die’ without the ability to cross-link their cell-walls in the absence of DAP. The majority of the bacteria are in the process of forming protoplasts. Some protoplast cells could be observed (black arrows) as well as cell debris (white arrows) due to bacterial lysis, which were absent when these mutants were grown in the presence of DAP (bottom panels).
FIG. 7. Single-copy complementation of the *B. pseudomallei* 1026b DasdBp mutant using mini-Tn7-bar-asdBp. (A) The suicidal plasmid mini-Tn7-bar-asdBp and its suicidal helper plasmid, pTNS3-asdEc, were introduced into *B. pseudomallei* 1026b DasdBp mutant by conjugation. Tn7 has three possible integration sites on different chromosomes (denoted by red triangles), which can result in complementation of the DasdBp mutation from three different chromosomal loci as depicted according to the annotation of *B. pseudomallei* K96243. Screening of ten random complements was done using oligonucleotide Tn7L (#876) and an oligonucleotide specific for each potential integration site (#1079, #1080, or #1081) as indicated by arrows. (B) For each isolate, PCR verification of ten random complements was performed for all three glmS sites (lanes 1, 2, and 3). Insertion downstream of glmS1 would result in a 218 bp PCR product, downstream of glmS2 would result in a 263 bp fragment, and downstream of glmS3 would result in a 309 bp PCR product. Isolates #1, 2, 3, 4, 5, 6, 8, and 10 had Tn7 inserted downstream of glmS2. Isolates #7 and #9 showed a PCR product near 200 bp indicating Tn7 integration downstream of glmS1. Abbreviations: asdEc, *E. coli* aspartate-semialdehyde-dehydrogenase gene; M, 100-bp ladder (New England Biolabs); P1, P1 integron promoter; glmS1, glmS2, and glmS3 encode for three different *B. pseudomallei* glucosamine 6-phosphate synthetases; tnsABCD, Tn7-transposase genes.

FIG. 8. Growth characteristics of the *B. pseudomallei* K96243 DasdBp mutant and five complements, relative to wildtype (wt), on media lacking amino acids (AA) of the aspartate family. (A) On 1x M9 minimal glucose (MG) medium, the DasdBp mutant did not grow compared to wt, whereas five complements (numbered 1-5) using mini-Tn7-bar-asdBp transposon
all grew as well as wt. Spots 1 and 2 are Tn7-bar-asd<sub>Bp</sub> complements transposed at the glmS<sub>1</sub> site, while spots 3-5 are complements transposed at the glmS<sub>2</sub> site. (B) The Dasd<sub>Bp</sub> mutant grew similarly to the wt on MG media when provided with all five AA of the aspartate family (DAP, Lys, Met, Thr, and Ile). The Dasd<sub>Bp</sub> mutant cannot grow when provided with four of the five AA when one aa such as Met (C), Thr (D), or DAP (F) was omitted from MG medium; whereas the wt and all complements grew well on these media. (E) The Dasd<sub>Bp</sub> mutant still grew when Ile was omitted from MG medium containing the other 4 AA, because Thr in the medium could be converted to Ile in this pathway. (G) Surprisingly, no growth was observed when Lys was omitted from the MG medium + 4 AA (Met, Thr, DAP, and Ile). We suspect that the amount of DAP provided was shuffled for use in cell-wall biosynthesis and very little gets converted to Lys for growth and the Dasd<sub>Bp</sub> mutant grows slowly on this media. When the plate in panel (G) was incubated for another six days, growth was observed for the Dasd<sub>Bp</sub> mutant (H), indicating that some DAP does get converted to Lys. All other plates, where the Dasd<sub>Bp</sub> mutant did not grow after one day, did not show growth of this mutant after seven days (data not shown).
## TABLE 1. Bacterial strains used in this study

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<td>Coli Genetic Stock Center</td>
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**Abbreviations:** bar, gene encoding bialaphos (phosphinotricin) resistance; gat, gene encoding glyphosate acetyltransferase; Gm⁺, gentamicin resistant; GS⁺, glyphosate resistant; Km⁺, kanamycin resistant; PPT⁺, phosphinotricin resistant.

**Please use laboratory identification number (Lab ID) when requesting strains.**

**Details on the engineering of these strains are to be published elsewhere.**
## Table 2. Plasmids used in this study

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<td>E1763</td>
<td>Ap', GS'; cloning vector pUC57 containing <em>B. pseudomallei</em> codon optimized gat</td>
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<td>pwFRT-P&lt;sub&gt;S12&lt;/sub&gt;-gat</td>
<td>E1798</td>
<td>Ap', GS'; gat-cassette flanked by wild-type FRT</td>
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<tr>
<td>pwFRT-P&lt;sub&gt;S12&lt;/sub&gt;-gat-SDM</td>
<td>E1812</td>
<td>Ap', GS'; gat-cassette flanked by wild-type FRT after SDM removing internal SacI site</td>
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<tr>
<td>pwFRT-PC&lt;sub&gt;S12&lt;/sub&gt;-gat&lt;sup&gt;b&lt;/sup&gt;</td>
<td>E1929</td>
<td>Ap', GS'; gat-cassette flanked by wild-type FRT with the P&lt;sub&gt;S12&lt;/sub&gt; replaced by the PC&lt;sub&gt;S12&lt;/sub&gt;</td>
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<tr>
<td>pwFRT-PC&lt;sub&gt;S12&lt;/sub&gt;-bar</td>
<td>E2209</td>
<td>Ap', PPT'; bar-cassette flanked by wild-type FRT</td>
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<td>pwFRT-Tp'</td>
<td>E1659</td>
<td>Tp'; Tp'-cassette flanked by wild-type FRT</td>
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<td>pwFRT-Tel'</td>
<td>E1584</td>
<td>Tel; Tel'-cassette flanked by wild-type FRT</td>
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<sup>a</sup>Other abbreviations: Ap', ampicillin resistance; P<sub>S12</sub>, rpsL promoter from *B. pseudomallei*; PC<sub>S12</sub>, rpsL promoter of *B. cenocepacia*; SDM, site-directed mutagenesis; Tel', tellurite resistant.

<sup>b</sup>Four other *FRT* mutants exist for this plasmid, where the only sequence difference in the mutated plasmids is within the spacer sequence of each *FRT* (see Materials and Methods for detail).

<sup>c</sup>Please use laboratory identification number (Lab ID) when requesting plasmids.
### TABLE 3. Oligonucleotide primers used in this study

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<tr>
<th>Oligos number and name</th>
<th>Sequences*</th>
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<tr>
<td>557; M13-RP………………….5'-AGCGGATAACAATTTCACACAGGA-3'</td>
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<td>558; M13-FP………………….5'-CGCCAGGGTTTCCCAGTCACGAC-3'</td>
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<td>715; pPS854-XhoI………...5'-AAGCTCGAGCTAATTCC-3'</td>
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<td>716; pPS854-Cla-EcoRV………..5'-CAATATCGATATCCATTGCTTGACTGACAAAG-3'</td>
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<td>837; PS12(cenocepacia)…………5'-ATCAGCGCCGACTTAATGGTTTTCCGGGAATATCATGCTGTGTTTC CGAATAATTGGTTTAACTTTAAGGAGATATACC-3'</td>
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<td>876; Tn7L……………………5'-CTTTAAGAAGAGGATATCCATGAGCCCGGAACGACGCC-3'</td>
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<td>881; bar-start………………...5'-CTTTAAGAAGGAGGATATACCATGAGCCCGGAACGACGCC-3'</td>
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<td>882; bar-XhoI………………...5'-GAAACTCGAGTCAAATCTCGGTGCCGGGCA-3'</td>
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<td>892; Bpasdup-HindIII…………5'-CGTCAAGCTTTCGCGCCGCTTG-3'</td>
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<td>893; Bpasddown-EcoRI…………5'-GGTGTGAATTGCTCGTAATCGCGTAG-3'</td>
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<td>894; gat-SacSDM……………….5'-GCAAATCGGAGCTAATTCCGCGACAGC-3'</td>
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<td>1048; dapB-up-XbaI……………..5'-CGCTCTAGAGCCATGCAGGCGG-3'</td>
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*Restriction enzyme sites used in this study are underlined*
TABLE 4. Glyphosate effective concentrations ([GS]EC) for *Burkholderia* species and *E. coli*

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<tr>
<th>Strain</th>
<th>Effective Concentrations (EC)</th>
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<td></td>
<td>[GS] (%) Liquid(^a)</td>
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<td><em>B. cenocepacia</em> K56-2</td>
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<td><em>B. cenocepacia</em> Bc7</td>
<td>&lt;0.005%</td>
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<td><em>B. dolosa</em> AUO158</td>
<td>&lt;0.005%</td>
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<tr>
<td><em>B. mallei</em> ATCC23344</td>
<td>0.1%</td>
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<tr>
<td><em>B. pseudomallei</em> K96243</td>
<td>0.1%</td>
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<tr>
<td><em>B. pseudomallei</em> 1026b</td>
<td>0.1%</td>
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<tr>
<td><em>B. thailandensis</em> E264</td>
<td>0.01%</td>
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<tr>
<td><em>E. coli</em> K12</td>
<td>0.1%</td>
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</table>

\(^a\) Effective concentration in liquid media determined by absence of growth after 4 days.

\(^b\) Effective concentration on solid media by absence of growth or spontaneous resistant colonies after 3 weeks.
Figure 2

Effect of glyphosate on *Burkholderia* select-agent spp. after 24 hours exposure

Survivors (%) vs Glyphosate Concentration (%)

- B. pseudomallei 1026b
- B. pseudomallei K96243
- B. mallei
Figure 3
Figure 4
Figure 6
Figure 7

(B) Complement Isolate Number

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</tbody>
</table>

PCR Product Sizes: Lane 1 = glmS1 = 218 bp  Lane 2 = glmS2 = 263 bp  Lane 3 = glmS3 = 309 bp
**Figure 8**

(A) MG 24 hrs

(B) +5 aa 24 hrs

(C) +4 aa / -Met 24 hrs

(D) +4 aa / -Thr 24 hrs

(E) +4 aa / -Ile 24 hrs

(F) +4 aa / -DAP 24 hrs

(G) +4 aa / -Lys 24 hrs

(H) +4 aa / -Lys 7 days
2.7 REFERENCES


Chapter 3. Stable site-specific fluorescent tagging constructs optimized for *Burkholderia* species

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Stable site-specific fluorescent tagging constructs optimized for *Burkholderia* species in Applied and Environmental Microbiology 2010 Nov;76(22):7635-7640.

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3.1 ABSTRACT

Several vectors were constructed that facilitate stable fluorescent labeling of *Burkholderia pseudomallei* and *Burkholderia thailandensis*. These vectors combined the effectiveness of the miniTn7 site-specific transposition system with fluorescent proteins optimized for *Burkholderia* spp., enabling bacterial tracking during cellular infection.
3.2 INTRODUCTION

*Burkholderia pseudomallei* is a highly infectious Gram-negative bacterium and is a facultative intracellular pathogen. The ability to observe infectious processes of this bacterium at various stages is critical in understanding pathogenesis. Fluorescent proteins facilitate bacterial tagging and have been powerful investigative tools in deciphering biological processes [100-105]. However, the lack of optimized fluorescent constructs used to label *B. pseudomallei* for visualization necessitate further development. Although there are many fluorescent tools available besides the green fluorescent protein, they are not optimized for use in *B. pseudomallei* and the less pathogenic model species, *B. thailandensis*. Commercially available fluorescent proteins are optimized for eukaryotic expression or, at the very best, for bacteria with low G/C genomes and hence codon preference may cause problems [106,107] during protein expression in *Burkholderia* spp. Also, there is usually an ineffective promoter driving transcription in *Burkholderia* spp., and available constructs are usually replicating plasmids that require selective maintenance. The restricted use of antibiotic markers in select agents (e.g. *B. pseudomallei*) adds another level of complexity to the genetic manipulation of these species [108]. Hence, these obstacles have limited the applications of fluorescent proteins in pathogenesis studies of *Burkholderia* spp.
3.3 RESULTS and DISCUSSION

The well-established miniTn7 system [109-111] inserts itself at a unique neutral site(s) in the bacterial genome with the aid of a non-replicating helper plasmid encoding the transposase [112]. The *B. pseudomallei* chromosome contains three insertion sites downstream of three *glmS* genes, whereas *B. thailandensis* contains two insertion sites downstream of two *glmS* genes [113]. Insertion of miniTn7 is quite stable in the bacterial genome [110,114] without the need for selective maintenance. Here, we constructed and demonstrated the use of fluorescent proteins encoded on miniTn7-based site-specific transposition vectors for fluorescent tagging of *B. pseudomallei* and *B. thailandensis*. We optimized the fluorescent protein genes (cyan, red, and yellow) for *B. pseudomallei*, which were synthesized through Genscript Corporation based on the amino acid sequences from Evrogen (http://www.evrogen.com/), by driving their transcription with a P$_{S_{12}}$ promoter [115] and changing the codons to those preferred by *B. pseudomallei*. Since the eGFP is sufficiently bright in *Burkholderia spp.*, we utilized the gfp gene driven by the P$_{S_{12}}$ promoter (Table 1). The four new fluorescent proteins were combined with two miniTn7 backbones to produce eight fluorescent tagging vectors (Fig. 1 and Table 1). The first series of four vectors encode the non-antibiotic selectable marker *gat* (resistance to glyphosate) [80,116] and the other series of vectors encode kanamycin resistance (Kan') (Fig. 1). Both are currently approved selectable markers in *B. pseudomallei* [80,113]. Nevertheless, prior CDC/USDA approval for each laboratory to use these markers is necessary and must be sought. All manipulations of *B. pseudomallei* were carried out in a CDC/USDA approved biosafety level 3 laboratory, following the guidelines presented in the Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th edition [117].
The four vectors based on *gat* were used to tag *B. pseudomallei* 1026b (Fig. 1). To introduce the fluorescent tags, tri-parental matings were conducted using an *E. coli* donor (E1354, Table 1) carrying a helper plasmid (pTNS3-asmEc), *B. pseudomallei* 1026b, and one of the four fluorescent vectors shown in Fig. 1, as previously described [80]. *B. pseudomallei* containing the inserted transposon were selected for on 1x M9 minimal glucose media containing 0.3% (v/v) glyphosate, as previously described [80]. Colonies appeared ~2 days later and were purified on the same media. Insertion at one of the three *glmS* sites in the chromosome was verified by PCR as described previously [80,113,118]. *B. pseudomallei* could be labeled and all four colors could be observed as shown in Fig. 2. To visualize fluorescently labeled bacteria, all samples were fixed in 1% paraformaldehyde based on previously published protocols for 30 min [37,119]. Fluorescent microscopy was carried out using the suggested filter cube sets as shown in Table 2. As our laboratory has not applied for approval to introduce Kanr genes into *B. pseudomallei*, we used the *gat*-based constructs to tag *B. pseudomallei* for the rest of our experiments. Regardless, the other four fluorescent vectors based on Kanr (Fig.1) were constructed for use in *B. pseudomallei* by those laboratories with appropriate USDA/CDC approval, and we have validated proper transposition and fluorescence in *B. thailandensis*, as well as in *Burkholderia cenocepacia* strain K56-2 and *Pseudomonas aeruginosa* strain PAO1 (data not shown).

The ability to tag *Burkholderia* spp. with different colors could facilitate studies where one or more strains expressing different colors could be located or tracked. To demonstrate this, we took advantage of the intracellular replication of *B. pseudomallei* by using two of the fluorescent strains engineered above (e.g. green and red) to infect the murine macrophage cell-line, RAW264.7, in a modified aminoglycoside protection assay [120]. Briefly, the respective *B.
*pseudomallei* cultures were grown overnight and used to infect the RAW 264.7 monolayers at an MOI of 10:1 for 1 h [89,121-124]. Afterwards, the extracellular bacteria were removed and the monolayers were washed twice with 1x PBS. Fresh DMEM containing 750 µg/ml amikacin and 750 µg/ml kanamycin was then added to inhibit extracellular bacterial replication. At 7 h post-infection, the monolayers were fixed with 1% (w/v) paraformaldehyde for 30 min and visualized using fluorescent microscopy [37,119]. As Figure 3 indicates, *B. pseudomallei* tagged with different colors were easily distinguishable within the murine macrophage cell-monolayers and can be seen inside host cells as the bacteria replicate. When both green and red fluorescent *B. pseudomallei* are mixed together and used to infect a murine macrophage cell monolayer at a total MOI of 10:1, differently colored bacteria can be distinguished and neighboring bacteria of either color can be differentiated from one another (Fig. 4). To observe the different infectious stages with fluorescently tagged *B. pseudomallei*, RAW 264.7 macrophages were infected at an MOI of 1:5 (1 bacteria per 5 host cells). In Figure 5A and 5B, the host-cells were infected with RFP-tagged *B. pseudomallei*, fixed, permeabilized, then stained with the far-red lipophilic styryl dye FM 4-64-FX (Molecular Probes). This stains all lipid bilayers far-red, including vacuoles, leaving the slightly orange color of the RFP-tagged *B. pseudomallei* visible i) in the macrophage vesicle (Fig. 5A) and ii) during vesicular escape (Fig. 5B). Alternatively, *B. pseudomallei* could be labeled with GFP for visualization (Fig. 5C and 5D). By infecting the macrophages at an MOI of 1:5 with GFP-tagged *B. pseudomallei*, then staining host-cell actin far-red, one can visualize bacterial replication in the cytoplasm (Fig. 5C) and the formation of actin tails during protrusion from the host cell (Fig. 5D).

In summary, we have constructed and demonstrated the use of transposon vectors for site-specific stable fluorescent tagging of *B. pseudomallei* with four unique colors. These tools
will be beneficial for microbiological studies involving the tracking or microscopy of *B. pseudomallei* during cellular infection. There are real needs for these vectors in the field and several applications can be envisioned. Infection studies that require tracking more than one strain through the infectious process would benefit from these tagging vectors, which do not require plasmid maintenance. Although bioluminescent tools have been of value in *in vivo* and non-invasive imaging of *B. pseudomallei* animal infections [125], our fluorescent constructs are of similar value [126,127]. Fluorescence activated cell sorting could also be used to enumerate host cells infected with a particular strain or strains of fluorescent *B. pseudomallei* or to monitor gene expression when using engineered constructs [102,128]. We believe these constructs will be beneficial to colleagues in this field and can be obtained upon request (Table 1).

This work was supported by National Institutes of Health grant R21-AI074608 to T.T.H. A graduate stipend for M.H.N. was provided by an NSF IGERT award (0549514) to Bruce A. Wilcox.
3.4 FIGURE LEGENDS

FIG. 1. Maps of miniTn7-gat-cfp and miniTn7-kan-cfp (A), miniTn7-gat-gfp and miniTn7-kan-gfp (B), miniTn7-gat-rfp and miniTn7-kan-rfp (C), and miniTn7-gat-yfp and miniTn7-kan-yfp (D). These constructs allow for site-specific transposition of fluorescent protein genes (cfp, gfp, rfp, and yfp), using the non-antibiotic resistance marker gat or the kanamycin resistance marker kan assisted by the helper plasmid pTNS3-asdEc. Differences in plasmid size are denoted in parenthesis. The P_{S12} promoter drives all fluorescent proteins. The gat or kan cassette is driven by the PC_{S12} on all constructs. These selectable markers are flanked by FRT sequences for Flp protein excision. Abbreviations: oriT, RP4 conjugal origin of transfer; PC_{S12}, rpsL promoter of B. cenocepacia; P_{S12}, rpsL promoter of B. pseudomallei; R6K_{γ}, π protein-dependent R6K origin of replication; Tn7L and Tn7R, left and right transposase recognition sequences; T_{0}T_{1}, transcriptional terminator.

FIG. 2. Fluorescent microscopy of B. pseudomallei labeled at the attTn7 site with gat-cfp (A), gat-gfp (B), gat-rfp (C), and gat-yfp (D). Fluorescent signals were obtained using the respective filter cube sets on a Zeiss AxioObserver D1 microscope and AxioCam MRc 5 monochrome camera. Pseudo-color was applied to the signal intensity at the time of capture using Zeiss AxioVision software. The middle row is comprised of differential interference contrast (DIC) images from the respective samples. At the time of capture, Zeiss AxioVision software was used to superimpose the fluorescent signal and DIC images displayed in the bottom row. When comparing the overlay in the bottom row to the DIC image in the middle, it can be seen that almost all bacteria are fluorescing at one exposure time or at a set fluorescent intensity. Differing expression of fluorescent protein genes and extended fixation can result in slightly
different fluorescent intensities among a population of bacteria, since extended paraformaldehyde fixation could damage the fluorescent proteins [129]. Total magnification is 630x and scale bars equal 10µm.

**FIG. 3.** Fluorescent microscopy of *B. pseudomallei* labeled at the *att*Tn7 site with *gat-cfp* (A), *gat-gfp* (B), *gat-rfp* (C), and *gat-yfp* (D) to infect murine macrophage-like cell line RAW264.7. Cell monolayers were seeded overnight onto poly-L-lysine coated coverslips at the bottom of a 6-well plate. The respective *B. pseudomallei* cultures were grown overnight and used to infect the RAW 264.7 monolayers at an MOI of 10:1 for 1 h. Afterwards, the extracellular bacteria were removed and the monolayers were washed twice with 1x PBS. Fresh DMEM containing 750µg/ml amikacin and 750 µg/ml kanamycin was then added to inhibit extracellular bacterial replication. The infection was allowed to proceed for 6 hrs after which the DMEM was removed; the monolayers were washed with 1x PBS and fixed with fresh 1% (w/v) paraformaldehyde for 30 min. Images were obtained as in Fig. 2. Total magnification is 630x and scale bars equal 10µm.

**FIG. 4.** Dual infection of RAW264.7 macrophages by differentially labeled (green and red) *B. pseudomallei*. Infections were carried out identically to those in Fig. 3. (A) The green fluorescent signal was obtained indicating where *gfp*-tagged *B. pseudomallei* are replicating inside macrophages. (B) The red fluorescent signal was obtained from the same field indicating where *rfp*-tagged *B. pseudomallei* are replicating within macrophages. A DIC image was then captured and is presented in (C). Overlay of images captured sequentially in (A), (B), and (C). Images were superimposed at the time of capture using Zeiss AxioVision software. (E) and (F)
are close-ups of the two macrophages indicated by arrows in (D), where the two differently fluorescing *B. pseudomallei* strains are clearly visible and distinguishable within the macrophages and even within the same host cell. Total magnification in (A), (B), (C), and (D) is 630x and all scale bars equal 10µm.

**FIG. 5. Tracking of *B. pseudomallei* infectious stages.** Infections were carried out as in Fig. 3 except *B. pseudomallei* were used to infect macrophages at an MOI of 1:5 to enable isolated bacterial infection. (A) RAW 264.7 monolayers were infected with RFP-tagged *B. pseudomallei*. The infection was allowed to progress for 1 h and then vesicles were stained far-red with the lipophilic styryl dye, FM-4-64-FX (Molecular Probes). Phase contrast microscopy in the red fluorescent channel captured an image of two RFP-tagged *B. pseudomallei* in a phagocytic vesicle. The image in (B) was obtained similarly except that a single RFP-tagged *B. pseudomallei* is possibly escaping the far-red stained phagocytic vesicle. (C) RAW 264.7 macrophages were infected with GFP-tagged *B. pseudomallei* for 2 hrs after which the monolayers were then fixed, permeabilized, and host-cell actin was stained far-red with phalloidin. GFP-tagged *B. pseudomallei* can be seen polymerizing host-cell actin enabling observation of actin-based intracellular motility. (D) GFP-tagged *B. pseudomallei* were used to infect RAW 264.7 monolayers for 6 hrs. The bacteria are polymerizing host-cell actin to infect neighboring host cells via membrane protrusions. The arrows indicate GFP-tagged *B. pseudomallei* at the tip of polymerized actin tails. Total magnification in (A), (B), (C), and (D) is 1,000x.
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Lab ID(^c), GenBank ID No.</th>
<th>Relevant properties</th>
<th>Sources</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>EPMa10B-pir116/Δasd/Δtrp::Gm(^f)/mob-Kan(^f)</td>
<td>E1354</td>
<td>Gm(^f), Kan(^f); F(^d) ΔmcrAΔ(mrr-hsdRMS-mcrBC) ΔlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara, leu)7697 galU galK rpsL nupG Tn-pir116-FRT2 Δasd::wFRT Δtrp::Gm(^f)-FRT5 mob[recA::RP4-2 Tc::Mu-Kan(^f)]</td>
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</tr>
<tr>
<td>EPMa10B-lacP/pir</td>
<td>E1869</td>
<td>F(^d) ΔmcrAΔ(mrr-hsdRMS-mcrBC) ΔlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara, leu)7697 galU galK rpsL nupG lacP(^f)-FRT8 pir-FRT4</td>
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</tr>
<tr>
<td><strong>Burkholderia spp.</strong></td>
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<td>B. pseudomallei</td>
<td></td>
<td></td>
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<td>1026b</td>
<td>B0004</td>
<td>Type-strain; clinical melioidosis isolate</td>
<td>[130]</td>
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<td>1026b/attTn7-gat-cfp</td>
<td>B0036</td>
<td>GS(^f), 1026b with miniTn7-gat-cfp inserted</td>
<td>This study</td>
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<td>B. thailandensis</td>
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<td>E264</td>
<td>E1298</td>
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<td>[131]</td>
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<td>GS(^f), miniTn7 integration vector based on gat</td>
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<td>pRK2013</td>
<td>E1358</td>
<td>Kan(^f); Helper plasmid encoding conjugative proteins.</td>
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<td>pTNS3-asd(_{Ec})</td>
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<td>Suicidal helper plasmid containing asd(_{Ec}) and transposase for the Tn7 site-specific transposition system</td>
<td>[118]</td>
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<tr>
<td>pUC7-P(_{S12})-cfp</td>
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<td>Ap(^f); plasmid harboring B. pseudomallei codon optimized cfp driven by P(_{S12})</td>
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</table>

\(^a\) Abbreviations: gat, gene encoding glyphosate acetyltransferase; Gm\(^f\), gentamycin resistant; GS\(^f\), glyphosate resistant; kan, gene encoding kanamycin resistance; Kan\(^f\), kanamycin resistant; P\(_{S12}\), rpsL promoter from B. pseudomallei.

\(^b\) Please use laboratory identification number (Lab ID) when requesting strains and plasmids.

\(^c\) Plasmids denoted with an Addgene number should be obtained from Addgene.

\(^d\) Details on the engineering of these strains are to be published elsewhere.
<table>
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<th>Fluorescent protein (original source)</th>
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<th>Emission max. (nm)</th>
<th>Recommended fluorescence microscopy filter set</th>
<th>Maturation at 37°C</th>
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<td>Tag CFP <em>(Aequorea macrodactyla)</em></td>
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<td>Omega Optical sets XF114-2 or XF130-2</td>
<td>fast</td>
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<td>eGFP <em>(Aequorea victoria)</em></td>
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<td>Omega Optical set <strong>XF116-2</strong> or Chroma Technology set 41017</td>
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<td>Turbo YFP <em>(Phialidium sp.)</em></td>
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<td>538</td>
<td>Omega Optica set XF104-3 or Chroma Technology set 42003</td>
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<td>Turbo RFP <em>(Entacmaea quadricolor)</em></td>
<td>553</td>
<td>574</td>
<td>Omega Optical sets QMAX-Yellow, XF108-2, XF101-2, and XF111-2</td>
<td>superfast</td>
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</table>

These characteristics were found on the Evrogen website (http://www.evrogen.com/).
Figure 1
Figure 2
Figure 4
3.5 REFERENCES


Chapter 4. The *Burkholderia pseudomallei* Δasd mutant exhibits attenuated intracellular infectivity and imparts protection against acute inhalation melioidosis in mice

Published as:

The *Burkholderia pseudomallei* Δasd mutant exhibits attenuated intracellular infectivity and imparts protection against acute inhalation melioidosis in mice


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4.1 ABSTRACT

*Burkholderia pseudomallei*, the cause of serious and life threatening diseases in humans, is of national biodefense concern because of its potential use as a bioterrorism agent. This microbe is listed as a select agent by the CDC, therefore, development of vaccines is of significant importance. Here, we further investigated the growth characteristics of a recently created *B. pseudomallei* 1026b Δasd mutant *in vitro*, in a cell model, and in an animal model of infection. The mutant was typified by an inability to grow in the absence of exogenous diaminopimelate (DAP) and, upon single-copy complementation with a wildtype copy of the asd gene, growth was restored to wildtype levels. Further characterization of the *B. pseudomallei* Δasd mutant revealed a marked decrease in RAW264.7 murine macrophage cytotoxicity when compared to the wildtype and the complemented Δasd mutant. RAW264.7 cells infected by the Δasd mutant did not exhibit signs of cytopathology or multi-nucleated giant cell (MNGC) formation observed in wildtype *B. pseudomallei* cell infections. The Δasd mutant was found to be avirulent in BALB/c mice and mice vaccinated with the mutant were protected against acute inhalation melioidosis. Thus, the *B. pseudomallei* Δasd mutant may be a promising live-attenuated vaccine strain and a biosafe strain to be considered for exclusion from the select agent list.
4.2 INTRODUCTION

*Burkholderia pseudomallei*, a gram-negative saprophyte and facultative intracellular pathogen, is a common cause of environmentally acquired septicemia in Southeast Asia and Northern Australia [1,133,134]. It is the etiological agent of the disease, melioidosis, and is listed as a category B select agent by the U.S. Centers for Disease Control and Prevention. Bacterial select agent research is currently focused on basic research into virulence and pathogenesis to fulfill the five main points of the United States Public Health Security and Bioterrorism Preparedness and Response Act of 2002 [20]. One of the goals is to develop and maintain medical countermeasures (such as drugs, vaccines and other biological products, medical devices, and other supplies) against biological agents and toxins in a bioterrorism event. To combat potential foul play associated with intentional release of select agents, a focus on vaccine development for first responders, such as military and health service professionals, is of the utmost importance [135]. Currently, there are no vaccines against *B. pseudomallei* and treatment entails prolonged regimens of intravenous and orally administered antibiotic therapy [136].

In a recent publication, we engineered a *B. pseudomallei* strain with a deletional mutation in the aspartate-β-semialdehyde dehydrogenase (*asd*) gene that is auxotrophic for diaminopimelate (DAP) in rich media and auxotrophic for DAP, lysine, methionine, and threonine in minimal media [80], which is consistent with similar mutations in many other bacterial species [81,137-139]. DAP is a diamin acid that crosslinks to D-alanine in neighboring peptidoglycan strands, and the ∆*asd* mutant exhibited the "pop-and-die" phenotype associated with an inability to synthesize DAP for cell-wall biosynthesis. Previous works have created *asd* mutants in *Salmonella typhimurium* [81] and *Legionella pneumophila* [140] and demonstrated a growth requirement for DAP. In addition, the *S. typhimurium* ∆*asd* strain has been extensively
used in clinical studies with human subjects as a vaccine delivery strain [141,142]. The pathway for synthesizing DAP from aspartate is absent in mammals, therefore no DAP is present in mammalian hosts, including humans [81,143]. The other amino acids (lysine, methionine, and threonine) made from aspartate via Asd are essential amino acids in humans, affording another possible level of nutrient limitation in vivo. Without a considerable exogenous concentration of DAP, the Δasd mutant is unable to crosslink its cell wall and cannot replicate. Even when supplied with high levels of DAP, intracellularly replicating L. pneumophila Δasd could not recover to wildtype levels of pathogenicity in both macrophage and protozoan infection models [140].

Live-attenuated vaccines are particularly effective vaccines because live bacteria may replicate modestly in the host, similar to situations encountered during an actual infection. In addition, live-attenuated vaccines contain complex epitopes not found in subunit or heat-inactivated vaccines, thus stimulating parts of the immune system that could otherwise be neglected (e.g. a strong Th1 response) [144]. Previous studies testing the efficacy of auxotrophic B. pseudomallei and B. mallei strains as live-attenuated vaccines have resulted in varying degrees of success [145,146]. In order to determine if the Δasd strain may be appropriate as a future vaccine candidate, we evaluated the growth and attenuation of the B. pseudomallei 1026b Δasd mutant in vitro and in cell culture. Animal studies were carried out to determine virulence levels and attenuation of the Δasd strain. Efficacy of the B. pseudomallei 1026b Δasd strain as a live-attenuated vaccine against inhalation melioidosis was then ascertained in a BALB/c mouse model.
4.3 MATERIALS and METHODS

**Bacterial strains, media, and culture conditions.** All manipulations of *B. pseudomallei* were conducted in CDC/USDA approved and registered BSL3 facilities at the University of Hawaii at Manoa and Colorado State University, and experiments with select agents were performed in accordance with the recommended BSL3 practices [147]. Derivatives of *Escherichia coli* strain EPMax10B (BioRad), E1345, E1354, E1869, and E1889 (Table 1) were routinely used for cloning or plasmid mobilization into *B. pseudomallei* as described previously [80,118]. Luria-Bertani (LB) medium (Difco) was used to culture *E. coli* strains. *B. pseudomallei* strains were cultured in LB or 1x M9 minimal medium supplemented with 20 mM glucose (MG). Antibiotics and non-antibiotic anitbacterials in solid media were utilized as follows: for *E. coli*, glyphosate (GS) 0.3% (w/v) and phosphinothricin (PPT) 0.3% (w/v) were used; for *B. pseudomallei*, GS 0.3% (w/v) and PPT 2.5% (w/v) were used. Growth of *E. coli* Δ*asd* strains and preparation of DAP were carried out as previously described [148]. Selection for *bar* or *gat* genes in *E. coli* and *B. pseudomallei* strains was performed as previously described [80]. *B. pseudomallei* Δ*asd::gat* strains were grown on LB containing 200 mg/ml DAP or on MG containing, 1 mM Lys, 1 mM Met, 1 mM Thr, and 200 mg/ml meso-DAP, as described previously [80].

**Molecular methods and reagents.** Molecular methods, PCR conditions, and conjugation into select agents were conducted as described previously [80,148,149].

**Engineering of *B. pseudomallei* Δ*asd*::*gat*-FRT.** *B. pseudomallei* Δ*asd*::*gat*-FRT was engineered as described previously [80] but briefly, the allelic-replacement vector, pBAKA-
\( \Delta asdBp::FRT-gat \) was conjugally introduced into \( B. \) \( pseudomallei \) strain 1026b and selection of the mutation was carried out on MG medium + 200 \( \mu g/ml \) DAP + 0.3\% GS + 1 mM each of Lys, Met, and Thr; these last 3 amino acids (3AA) are required for the specific \( \Delta asd \) mutation. Colonies were streaked-out on the same medium supplemented with 0.1\% cPhe to counter-select against \( pheS \). GS resistant mutants were DAP were purified once on LB + DAP, and patched again on MG + 0.3\% GS + 0.1\% cPhe + 1 mM 3AA ± 200 mg/ml DAP to confirm.

**Construction of single-copy \( rfp \)-containing vectors.** The red fluorescent protein gene (\( rfp \)) was optimized for the codon preference of \( B. \) \( pseudomallei \) and the constitutive \( B. \) \( pseudomallei \) \( rpsL \) promoter (\( P_{S12} \)) was incorporated upstream of the gene [150]. Constructed as previously described [150], \( rfp \) was cloned from pUC57-\( P_{S12}-rfp \) into mini-Tn7-\( PC_{S12}-bar \) to yield mini-Tn7-\( bar-rfp \) (Fig. 1). The mini-Tn7-\( PC_{S12}-bar \) [80] construct was digested with PstI + SpeI and ligated to the \( rfp \) fragment obtained from pUC57-\( P_{S12}-rfp \) digested with PstI + XbaI, producing mini-Tn7-\( bar-rfp \). Next, the complementation and fluorescent tagging transposon was constructed by digesting mini-Tn7-\( bar-asdBp \) [80] with PstI + SpeI and ligating it to the \( rfp \) fragment from PstI + XbaI digested pUC57-\( P_{S12}-rfp \), yielding the single copy complementation/fluorescent tagging vector, mini-Tn7-\( bar-asdBp-rfp \). In addition to the \( bar \) based vector, the fluorescent tagging vector mini-Tn7-\( gat-rfp \) based on \( gat \), constructed as previously described [150], was also utilized below.

**Engineering of \( rfp \)-tagged \( B. \) \( pseudomallei \) strains and complemented mutants.** E1354 was utilized as the conjugal donor to introduce the single copy vector mini-Tn7-\( bar-rfp \) into the \( B. \) \( pseudomallei \) \( \Delta asd \) mutant for fluorescent tagging, producing \( B. \) \( pseudomallei \)
Δasd:gat-FRT/attTn7-bar-rfp (Δasd/rfp). The mini-Tn7-bar-asd<sub>Bp</sub>-rfp construct was introduced into the Δasd mutant for complementation and fluorescent tagging, yielding *B. pseudomallei* Δasd:gat-FRT/attTn7-bar-asd<sub>Bp</sub>-rfp (Δasd/complement/rfp). The mini-Tn7-gat-rfp construct was introduced into wildtype *B. pseudomallei* strain 1026b for fluorescent tagging of the wildtype, resulting in *B. pseudomallei* attTn7-gat-rfp (wt/rfp). These strains were obtained from a tri-parental mating experiment using the pTNS3-asd<sub>Ec</sub> helper plasmid and bacteria containing the integrated transposon were selected and screened via PCR as described previously [80,118,151]. The mini-Tn7 system allows site-specific insertion of the transposon at a neutral site in the chromosome, downstream of any *glmS* gene, of which *B. pseudomallei* has three [113]. In all cases the transposon had inserted at the highly favored *glmS*<sub>2</sub> site. Fluorescence was verified by fixing the bacteria with fresh 1% paraformaldehyde in phosphate buffered saline (PBS) for 30 min followed by imaging with a Zeiss Axio Observer D.1 fluorescent microscope and accompanying AxioVision release 4.7 software.

Construction of fluorescent strains, including selection for glyphosate or phosphinothricin resistant colonies, and transposon integration and screening were performed as previously described [80,113,118,150]. Sample preparation and fluorescent imaging were also carried out as previously described [150].

**Growth analysis of the rfp-tagged *B. pseudomallei* Δasd mutant, complemented Δasd mutant, and wildtype strain.** Growth curve experiments were performed on the three RFP-labeled *B. pseudomallei* strains engineered above (wt/rfp, Δasd/rfp, and Δasd/complement/rfp). These strains were grown overnight at 37°C in LB medium, where the Δasd mutant was supplemented with 200 mg/ml of DAP. Overnight cultures were then washed twice with 1x M9 medium to remove trace amounts of DAP and resuspended in an equal volume of 1x M9
medium. Resuspended cultures were diluted 100-fold into fresh LB medium, without DAP, and
shaken at 225 rpm at 37°C. At each time point, 300 µl aliquots were removed, diluted two-fold
in LB medium, and their optical densities were measured at 600 nm using an Eppendorf
Biophotometer.

**DAP dependency of *B. pseudomallei Δasd/rfp***. A growth curve experiment was
performed on the *B. pseudomallei Δasd/rfp* and *B. pseudomallei wt/rfp* strains. The strains were
grown overnight and washed with 1x M9 and inoculated into LB media as described above,
supplemented with different concentrations of DAP (0, 50, 100, 200, and 500 mg/ml). At each
indicated time point, 300 µl aliquots were removed and the optical densities at 600nm were
determined.

**Intracellular replication assays.** Both murine macrophage RAW264.7 and human
cervical carcinoma HeLa cell lines were grown in a 5% CO₂ environment at 37°C in Dulbecco’s
Modified Eagles Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). Gibco 100x anti-
biotic/anti-mycotic was added at a 1x working concentration (containing 100 U/ml of penicillin,
100 mg/ml of streptomycin, and 250 ng/ml of amphotericin B) to the cell culture media during
cell culture growth but was omitted during the infection assay. Intracellular replication assays
were performed using a modified kanamycin protection assay as previously described [120].
Briefly, cells (HeLa and RAW264.7 lines) were cultured in DMEM to confluence, scraped from
cell-culture flasks, and seeded at 1x10⁵ cells per well into 24-well Corning CellBIND culture
plates. To prepare cells for infection study, cells were allowed to attach overnight and were
washed twice with 1x PBS in the morning.
The three bacterial strains used in this experiment were the same as used above for the complementation growth study. To investigate whether exogenous DAP allowed intracellular infection, two series of infection were carried out with the Δasd/rfp strain. One series was allowed to infect during the entire course of the study in the presence of DAP and the other had DAP omitted from the media after 1 h of infection (T=1). During the first hour of infection, both series of the *B. pseudomallei* Δasd/rfp strain were supplemented with 200 mg/ml of DAP in the cell culture media, so as not to bias invasion ability during attachment and internalization. Assays with the wildtype and complemented Δasd/rfp mutant strains were carried out essentially the same as those with the Δasd/rfp mutant, except that no DAP was added. Briefly, *B. pseudomallei* strains were grown to high-cell-density, washed twice with 1x PBS, then diluted to ~1x10⁶ CFU/ml. At time zero, 1 ml of DMEM containing diluted bacteria was added to the macrophage monolayers (MOI 10:1). After allowing the infection to progress for one hour, the media was removed and the monolayers were washed twice with 1x PBS to remove any unattached bacteria. Next, fresh DMEM with 700 mg/ml each of amikacin and kanamycin was added to the monolayers to kill any non-internalized bacteria and inhibit extracellular bacterial replication. During the assay, medium was removed from the wells at three time points (2, 6, and 24 h post-infection) and the infected cell monolayers were washed twice with 1x PBS, and then lysed with 0.1% Triton X-100. Serial dilutions of the lysates were plated on Brucella agar (Difco) plus 4% (v/v) glycerol (BAG) media at 37°C, as described previously [89,152]. BAG medium was supplemented with 200 mg/ml of DAP when enumerating *B. pseudomallei* Δasd/rfp colonies. Colonies were counted within 48 h. Experiments with both HeLa and RAW264.7 cell lines, in combination with all bacterial strains, were performed in triplicate and the standard error of the mean (SEM) was calculated for each.
RAW264.7 macrophage cytotoxicity assay. Macrophages were cultured as described above and seeded into a 96-well CellBIND plate at ~5x10^4 cells per well. A kanamycin protection and infection assay was carried out, as described above for the intracellular replication assay, with bacteria infected at an MOI of 10:1. At 2, 6, 12, and 24 h post-infection, the cellular supernatant was removed and lactate dehydrogenase (LDH) levels were determined using the CytoTox 96 Non-radioactive Cytotoxicity Assay (Promega). LDH levels of infected monolayers were compared and normalized to maximal LDH levels (after complete monolayer lysis using 0.1% Triton X-100) to determine percent cytotoxicity. The cytotoxicity assay was carried out in triplicate and the standard error of the mean (SEM) was calculated.

Light microscopy and time course of *B. pseudomallei wt/rrp*, Δasd/rrp, and Δasd/complement/rrp infection of RAW264.7 murine macrophages. Light microscopy of infected cell monolayers was carried out as described [150], except for a few modifications. Glass coverslips were sterilized in 70% (v/v) ethanol and then treated for 4 h with 150 mg/ml poly-L-lysine in sterile double distilled water (ddH2O). The glass coverslips were washed twice with ddH2O and allowed to air-dry within a sterile petri dish overnight. In our experience, glass coverslips treated with poly-L-lysine provided the best surface for cell attachment and microscopic imaging. The 22 x 22 mm coverslips were placed at the bottom of the wells in a 6-well Corning CellBIND plate prior to seeding. RAW264.7 macrophages were seeded at ~8x10^5 cells per well, allowed to attach overnight, and the infection was initiated by adding different bacterial strains at an MOI of 10:1. At 1 h post-infection, the coverslips were washed twice with 1x PBS and then fresh DMEM media containing 700 mg/ml of kanamycin was added to inhibit
extracellular bacterial replication. At 2, 6, 12, and 24 h post-infection the media was removed and the cell monolayers were washed twice with 1x PBS and fixed with 1% paraformaldehyde for 30 min. After 30 min, the paraformaldehyde was removed and the coverslips were washed twice with 1x PBS. For safe removal of fixed samples from the BSL-3 for imaging, this method must be initially tested by incubating fixed cover slips for 5 d in LB to confirm the absence of growth and viable bacteria. Coverslips were mounted with a slide-mounting buffer containing 50% glycerol in 1x PBS. Images were obtained as previously described [150].

**Animal studies.** BALB/c mice between 4 and 6 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in micro-isolator cages under pathogen-free conditions. The Institutional Animal Care and Use Committee at Colorado State University approved the animal experiments conducted for these studies. *B. pseudomallei* infections were done using intranasal (i.n.) inoculation [52]. Animals were anesthetized with 100 mg/kg ketamine plus 10 mg/kg xylazine. The desired challenge dose of *B. pseudomallei* was suspended in PBS and 20 µl was delivered i.n., alternating nostrils. For the challenge studies, groups of 5 mice were challenged with the wildtype and mutant strain. For the vaccination studies, mice (n=10) were administered 1x10⁷ CFU Δasd mutant *B. pseudomallei* intranasally, and then boosted in the same manner 3 weeks later. Two weeks following the boost, mice were challenged intranasally with 4x10³ CFU wild type *B. pseudomallei* 1026b. For all *B. pseudomallei* challenge and survival studies, animals were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints. Lungs, liver, and spleen were removed and homogenized using a tissue stomacher (Teledyne Tekmar, Mason OH) and homogenates were plated in serial dilutions to determine bacterial counts in the *B.
*pseudomallei* challenged mice 75 d post-infection. Statistical differences in survival times were determined by Kaplan-Meier curves followed by the log-rank test (Prism5 software, GraphPad, LaJolla, CA).
4.4 RESULTS

Construction and growth analysis of the rfp-tagged *B. pseudomallei* Δasd, Δasd/complement, and wildtype strains. In the previous work [80], we developed two non-antibiotic markers, *bar* and *gat*, which are effective for the genetic manipulation of *B. pseudomallei*. In this study it became apparent that another marker besides *gat* was needed for fluorescent tagging of *B. pseudomallei* during infection studies. Therefore, we constructed a new non-antibiotic based single-copy transposon vector (mini-Tn7-bar-rfp; Fig. 1) for stable site-specific insertion of red fluorescent protein genes (*rfp*) without the need for plasmid maintenance. We used the *gat* select agent compliant non-antibiotic marker to fluorescently tag wildtype bacteria as previously described [150]. However, mini-Tn7-bar-rfp based constructs were used to fluorescently tag the *B. pseudomallei* Δasd::gat-FRT strain, as well as to tag and complement this mutant strain.

The amino acid requirements of the *B. pseudomallei* Δasd/rfp mutant were previously demonstrated by showing that the mutant could not grow in the absence of methionine, threonine, and DAP on minimal media plates [80]. In this study, we wanted to show that this mutant is unable to grow in rich liquid media in the absence of DAP. A growth-curve experiment was initiated to allow the comparison of growth between *B. pseudomallei* wt/rfp, *B. pseudomallei* Δasd/rfp, and the *B. pseudomallei* Δasd/complement/rfp strains in a rich nutrient source (LB medium). The *B. pseudomallei* Δasd mutant displayed an inability to grow compared to wildtype strain (Fig 2A). This was expected, as in previously published work the *B. pseudomallei* 1026b Δasd mutant began to lyse after 6 h without DAP [80]. When the *B. pseudomallei* Δasd/rfp strain was complemented using a transposon containing a single copy of the *B. pseudomallei* asd gene, the growth defect of the mutant was abolished and normal growth
was restored. This indicated that the growth defect exhibited by the mutant was solely caused by deletion of the \textit{asd} gene.

We next investigated the effect of different concentrations of DAP on growth of the \(\Delta\text{asd}/\text{rfp}\) mutant in rich media (Fig. 2B). In the presence of DAP, all curves showed a significant growth lag, however, when compared to the wildtype, the OD\textsubscript{600} eventually reached wildtype levels. This demonstrated that the \(\Delta\text{asd}\) mutant can grow well when DAP is added to the media and not at all in the absence of DAP.

\textbf{The \textit{B. pseudomallei} \(\Delta\text{asd}\) mutant is highly attenuated in intracellular replication.} Assessment of the \(\Delta\text{asd}\) mutant attenuation in HeLa and RAW264.7 cell infection models were necessary before animal vaccination. In agreement with previous work [120], our experience suggested that an MOI of 10:1 would initiate an infection that would maximally affect the cell-monolayer within 24 h. Internalization was very inefficient in HeLa cells, with only \(~500\) CFU out of \(~1\times10^6\) CFU internalized by the monolayers (Fig. 3A). As shown in Figure 3A, the \textit{B. pseudomallei} \(\Delta\text{asd}/\text{rfp}\) strain is unable to replicate in HeLa cells. It was able to attach and become internalized as well as the wildtype indicated by similar intracellular CFUs obtained at 2 h post-infection. However, by 6 h post-infection (\(T=6\)), the \(\Delta\text{asd}/\text{rfp}\) mutant alone (without DAP) was only able to replicate modestly. By 24 h post-infection, intracellular mutant bacteria were undetectable. However, when complemented with a single wildtype copy of the \textit{asd} gene the \(\Delta\text{asd}\) mutant strain behaved exactly as wildtype, reaching a maximum of \(~1\times10^6\) CFU. Interestingly, the \(\Delta\text{asd}/\text{rfp}\) strain could infect HeLa cell monolayers when the growth medium was supplemented with 200 \(\mu\text{g/ml}\) of DAP and only slowed down the rate of decline in RAW
264.7 macrophages. This indicates that a sufficient amount of DAP was transported into HeLa cells.

*B. pseudomallei* was internalized efficiently and replicated much more significantly within RAW264.7 cells (Fig. 3B). The *B. pseudomallei Δasd/rfp* strain is not internalized efficiently or is killed more efficiently by macrophages than the wildtype, denoted by one log difference in CFU at 2 h post-infection. The Δasd mutant could not sustain a wildtype level of replication even in the presence of 200 µg/ml of DAP, unlike HeLa cell infection. By the end of the assay (24 h), the Δasd/complement/rfp and the wt/rfp strains replicated to a similar level (~1x10^6 CFU) within the RAW264.7 macrophage monolayer. This indicates that even a single copy of the *asd_Bp* gene can restore the mutant’s abilities to grow within cells.

**Cytotoxicity and light/fluorescent microscopy time course of the *B. pseudomallei* 1026b Δasd mutant infection of RAW264.7 murine macrophages.** Although the Δasd mutant does not replicate to high cell numbers like the wildtype, it was of interest whether or not the Δasd mutant damages the cell monolayers comparably to the wildtype. LDH assays of all strains infecting RAW264.7 monolayers at 2 and 6 h post-infection revealed little differences in cytotoxicity when compared to the non-infected control (Fig. 4A) while LDH levels of the wildtype and complement began to rise at 12 h post-infection. The wildtype and complemented Δasd mutant infected monolayers had surpassed the maximum cytotoxicity at 24 h (determined by lysing the initially seeded macrophages), reaching ~100% (Fig. 4A). Independently of DAP, the Δasd mutant did not damage the monolayers to the same level as the wildtype and was still comparable to the non-infected control. LDH levels of the non-infected macrophage monolayer rose at 24 h, indicating the spontaneous lysis of macrophages at high confluency, a usual
occurrence where rapid division leads to low nutrient availability, macrophage death, and LDH release.

Intracellular replication and host-cell cytotoxicity were then placed in a visual context by tracking the \textit{rfp}-tagged bacteria via fluorescent microscopy. Visible in representative images from 24 h post-infection was a pervasive red fluorescence indicative of high numbers of intracellular bacteria (Fig. 4B). The majority of macrophages are joined together in multinucleated-giant-cells (MNGCs). Upon closer inspection (Fig. 5), the MNGCs were observed teeming with bacteria in both the \textit{wt/rfp}- and \textit{Δasd/complement/rfp}-infected monolayers. The bacteria-containing protrusions are clearly visible extending from the surface of the macrophages (Fig. 5). The \textit{Δasd} mutant infected monolayers neither contain high numbers of replicating bacteria nor do they show any sign of MNGC formation in the monolayer at 24 h and in fact appear as healthy as the non-infected control (Fig. 4B). Although not unexpected, the data reaffirms that the mutant was internalized by the macrophages but was unable to produce cytopathology (or MNGCs), with or without DAP, consistent with the wildtype infection.

**Attenuation, vaccination, and acute protection of the \textit{Δasd} mutant in mice.** We first tested the \textit{Δasd} mutant for attenuation \textit{in vivo}. The LD\textsubscript{50} of \textit{B. pseudomallei} 1026b in BALB/c mice has been determined to be approximately 900 CFU via the inhalation route [152]. An intranasal (i.n.) dose of 4,500 CFU has been experimentally determined to produce 100% mortality in BALB/c mice after 3 d [52,153]. Intranasal inoculation mimics inhalation melioidosis and produces a characteristic acute pneumonic infection to which BALB/c mice succumb within a few days. Five BALB/c mice (n=5) were challenged i.n. with 4,500 CFU of \textit{B. pseudomallei} 1026b and another five BALB/c mice were challenged i.n. with $1\times10^7$ CFU (5 logs
x LD$_{50}$). Survival of the mice was then monitored. After 3 d, mice challenged with wildtype *B. pseudomallei* had all been euthanized due to progressive infection (Fig. 6A). In contrast, mice infected with asd mutant *B. pseudomallei* showed no outward signs of infection and were observed for 75 d post-challenge and all remained healthy during this period (Fig. 6A). Thus, the Δasd mutant was highly attenuated when compared to wildtype strain of *B. pseudomallei*. To assess possible bacterial persistence *in vivo*, mice challenged with mutant *B. pseudomallei* were euthanized on day 75 and the lungs, livers, and spleens were homogenized, diluted, and plated on LB agar. Bacteria were not detected in any organ, using assays with limits of detection of approximately 50 CFU/organ, indicating the mutant bacteria did not persist in organs typically infected during the chronic phase of infection with virulent *B. pseudomallei*. The numbers of mice used in these studies were judged to be adequate [52] to assure that the mutant bacterium was avirulent in immune competent mice.

We then considered if the Δasd mutant could be used as a vaccine against inhalation melioidosis in BALB/c mice. Numerous publications support the fact that single vaccinations with attenuated live *B. pseudomallei* vaccines are generally unable to protect mice from developing chronic melioidosis [145,154,155]. Therefore, we investigated whether an i.n. prime-boost vaccination strategy could extend protection against development of chronic melioidosis. The i.n. route of infection and vaccination emulates aerosol exposure/vaccination and typically vaccination at the route of pathogen entry generally leads to more effective disease prevention [156]. Ten BALB/c mice were primed with an i.n. vaccination of 1 x 10$^7$ CFU of the *B. pseudomallei* 1026b Δasd mutant. Three weeks later, the same mice were boosted with another i.n. vaccination of 1x10$^7$ CFU of the Δasd mutant. The time period between the initial exposure and the boost would presumably allow for an adaptive cellular and humoral immune response to
occur. Two-weeks post-boost, the mice were challenged with $4 \times 10^3$ CFU of wildtype strain 1026b and survival was compared to unvaccinated mice challenged with the same amount of the wildtype. The boost was administered 2 weeks before the infection to further enhance the immune response presumably allowing time for enhanced adaptive immunity [157,158]. The data show that vaccinated mice survived significantly longer than the unvaccinated control mice (Fig. 6B). While the prime-boost strategy used in this study protected mice from acute infection, it failed to protect mice from development of chronic *B. pseudomallei* infection, as nearly all of the vaccinated and challenged mice developed infection of organs at secondary sites, particularly the spleen (data not shown).
4.5 DISCUSSION

The essentiality of the *asd* gene in *E. coli* has been known for some time, but its requirement for growth and infectivity of select agent species has not been thoroughly investigated. This study evaluated the growth and pathogenicity of the *B. pseudomallei Δasd* mutant produced in the previous work [80] and its potential for use as a live-attenuated vaccine. By performing growth experiments, it was found that without DAP the mutant is unable to replicate and complementation, with a single copy of wildtype *asd*/*bp*, is sufficient for *in vitro* and intracellular replication compared to the wildtype bacterium in both HeLa and RAW264.7 cells. These studies demonstrated that by adding DAP, the Δasd strain can be easily propagated within a laboratory setting and, by complementing the *asd* gene, a markerless balanced lethal system could be used for various *B. pseudomallei* studies [159]. It is important to note that while adding DAP during *asd* mutant infection can re-establish wildtype growth levels in some cell lines (i.e. HeLa cells), it is not homologous to replication of the mutant after single copy complementation, where high levels of replication are seen within both cell lines. This may have important implications in future subcutaneous vaccine experiments due to the epithelial nature of the HeLa cell line.

In the absence of DAP, the mutant was unable to replicate in both the HeLa and RAW 264.7 cell infection models. Cytotoxicity data showed that the mutant did not cause increased death or distress to the macrophages, indicating that the mutant is unable to replicate within or cause significant damage to host cell macrophages via endotoxin or exoenzyme release. The link between cytotoxicity and inflammation has been known for some time and can be partially attributed to free radical release during cellular damage both *in vitro* and *in vivo* [160]. Although inflammatory modulators were not measured in the cytotoxicity assay, a tentative hypothesis
would place the corresponding inflammatory modulator levels in the same trend as LDH. By tagging the *B. pseudomallei* strains with RFP and tracking them *in vitro* during intracellular replication, we were able to confirm the intracellular location of the mutant and further demonstrate the utility of non-antibiotic selectable markers in pathogenesis research.

*B. pseudomallei Δasd* mutants should be considered biosafe strains suitable for laboratory use and exclusion from the USDA/CDC select agent lists. First, the Δasd mutant was constructed by deleting several hundred bases in the middle of the asd gene [80], producing a stable mutant unable to revert. Additionally, as DAP is not present within mammals, there is no source of exogenous DAP, affording another level of safety for this strain. On the other hand, it can be seen that the *B. pseudomallei Δasd* mutant invades host-cells although, like the *B. pseudomallei purM* mutant [52], the Δasd mutant is unable to replicate in the host and bacterial persistence cannot occur. These data, together with the use of Δasd mutants of other species as vaccine delivery strains in humans [81,141] provides strong evidence supporting the removal of this strain from select agent lists, as was previously achieved with the 1026b DpurM strain Bp82 (33).

Previous vaccination studies utilizing live-attenuated strains and a single vaccination were unable to prevent death from chronic infection [65,74,145,161,162]. However, we had reason to believe that the Δasd mutant would be more effective than previous live attenuated strains. It has been shown that a more protective immune response can be achieved by increasing short-term vaccine persistence, which we attempted with the booster vaccine [163-165]. Unfortunately, while vaccination with the Δasd mutant did indeed protect against acute melioidosis, the vaccine failed to protect against chronic melioidosis. This failure might have been because because the Δasd mutant vaccine was unable to persist long enough or disseminate
and proliferate enough, even after the boost, to induce systemic protection. The route of vaccination can be important because of increased protection at the site of challenge (e.g. mucosal surfaces), however, this may not generate systemic protection [156]. This is a possible reason for why mice were protected from the initial lung infection but eventually succumbed to systemic infection at secondary sites. Even so, protection from acute pneumonic melioidosis may provide a vital increase in survival time that could allow for the administration of antibiotic therapeutics.

Future investigations should be carried out to address whether short-term persistence, proliferation, and dissemination of the Δasd mutant, achieved by adding DAP to the vaccine, would provide systemic protection against chronic melioidosis. Within-host persistence of the Δasd mutant would then be contingent on the amount of DAP administered with the vaccination. Addition of DAP to mutant infected HeLa cells, an epithelial cell line, did allow some intracellular replication therefore it may be highly beneficial to incorporate a subcutaneous vaccine containing DAP. Longer exposure to the cutaneous and subcutaneous dendritic cells could prolong T-cell activation at draining lymph nodes [157] and create the powerful cell-mediated immune response hypothetically necessary for sterile immunity [166]. Other means of producing systemic dissemination and, perhaps, protection would be to incorporate an intramuscular or subcutaneous vaccination along with the inhaled vaccination. This two-pronged approach may give rise to longer protection from chronic or latent infection, which is proving more difficult to combat than acute melioidosis. Seemingly, the greatest prospect for an effective vaccine against melioidosis is a live-attenuated strain. In conclusion, this initial work suggests the utility of the B. pseudomallei Δasd mutant as a live-attenuated vaccine against acute melioidosis and further justifies its potential removal from the select agent list.
4.6 ACKNOWLEDGEMENTS

This project was supported by Award Number AI065359 from the National Institute of Allergy and Infectious Diseases and by the Center of Biomedical Research Excellence grant P20RR018727 from the National Center for Research Resources of the National Institutes of Health to TTH. SWD and HPS were supported by NIH NIAID grant AI065357. The content is solely the responsibility of the authors and does not necessarily represent the official view of the funding agencies.
4.7 FIGURE LEGENDS

FIG 1. Mini-Tn7-bar-rfp, single-copy tagging vector based on phosphinothricin resistance, harboring rfp driven by the P$_{S12}$ promoter. After insertion aided by pTNS3-asd$_{Ec}$ [150], the non-antibiotic resistance marker, which is flanked by identical FRTs, can be removed by Flp-mediated excision. Abbreviations: bar, gene encoding bialaphos/phosphinothricin resistance; FRT, Flp recombination target sequences; oriT, RP4 conjugal origin of transfer; PC$_{S12}$, promoter of the B. cenocepacia rpsL gene; P$_{S12}$, promoter of the B. pseudomallei rpsL gene; R6K$_{γ}$ori, π protein dependent R6K origin of replication; rfp, red fluorescent protein encoding gene; Tn7L/Tn7R, left and right transposase recognition sequences; $T_o T_1$, transcriptional terminator.

FIG 2. Growth curve experiments performed with B. pseudomallei strains. (A) B. pseudomallei strains were grown in the absence of DAP. The wildtype strain and the Δasd strain complemented with a single copy of the asd gene on a site-specific transposon exhibit the same growth rates and final optical densities, while the Δasd mutant exhibited a typical DAP-dependent phenotype. (B) The B. pseudomallei Δasd mutant was tested in different concentrations of DAP, ranging from 0 µg/ml to 500 µg/ml. When compared to wildtype, the Δasd mutant exhibits absence of growth without DAP. All other concentrations of DAP afforded a partial growth rate recovery and final optical density, albeit only after a lag in growth.

FIG 3. Infection of HeLa and RAW264.7 cells by B. pseudomallei and the Δasd strain. HeLa (A) and RAW264.7 (B) cell monolayers were infected at an MOI of 10:1. The complemented Δasd strain shows no decrease in its ability to invade and replicate within either cell line.
However, the $\Delta asd$ mutant in the absence of DAP cannot sustain an infection in either cell line, denoted by an overall drop in bacterial numbers.

**FIG 4.** (A) Cytotoxicity of *B. pseudomallei* strains to the RAW264.7 murine macrophage cell line. RAW264.7 cells were infected with the $\Delta asd$ mutant (in the presence and absence of DAP), the complemented mutant strain, and wildtype strain. Between 2 h and 6 h post-infection there was a slight increase in cytotoxicity associated with infection by the complemented and wildtype strains when compared to the $\Delta asd$ mutant infected monolayers. By 12 h post-infection, cytotoxicities of the complement and wildtype infected monolayers were more obvious, while cytotoxicity caused by the $\Delta asd$ mutant remained similar to the non-infected control. At 24 h post-infection, the complement and wildtype infected monolayers exhibited maximal cytotoxicity. (B) Microscopy and time course of the cytopathic effects of *B. pseudomallei* $\Delta asd$ infection. Monolayers were infected at an MOI of 10:1 then analyzed for red fluorescence at 2 h and 24 h post-infection. Differential interference contrast (DIC) images were overlaid with the red fluorescent channel. Red fluorescence indicates the presence of *B. pseudomallei*. Note the high bacterial levels in the complement and wildtype infected monolayers at 24 h and the confluent MNGC formation. This coincides with high levels of cytotoxicity at 24 h post-infection. Abbreviations: CT, non-infected control; $\Delta asd$, *B. pseudomallei* $\Delta asd/rfp$; $\Delta asd$ + DAP, *B. pseudomallei* $\Delta asd/rfp$ in the presence of 200 µg/ml of DAP; $\Delta asd$ + complement, *B. pseudomallei* $\Delta asd/complement/rfp$; WT, *B. pseudomallei* wt/rfp. Error bars represent the SEM of three experiments. Statistical significance was determined by the two-tailed unpaired T-test (***, $P < 0.0005$).
FIG 5. Intracellular replication of *B. pseudomallei*. RAW264.7 murine macrophage monolayers were visualized using a combination of differential interference contrast and red fluorescence microscopy 24 h post-infection with the *B. pseudomallei Δasd/complement/rfp*, (A) and (B), and with *B. pseudomallei wt/rfp*, (C) and (D), strains. All macrophages in the field of view are involved in MNGCs and are filled with intracellular bacteria about to burst into the extracellular milieu. (A) and (C) were captured at 600x magnification while (B) and (D) are zoomed in images of the regions denoted in (A) and (C), respectively. Note the large number of bacteria projecting out of the remaining macrophages in (C) and (D). There were no bacteria and an absence of protrusions as well as MNGCs at all time points in monolayers infected with the Δasd mutant (data not shown).

FIG 6. (A) *B. pseudomallei 1026b Δasd* mutant is avirulent to mice. Mice (n=5 animals per group) were challenged i.n. with either 4,500 CFU *B. pseudomallei 1026b* (wildtype) or 1x10⁷ CFU *B. pseudomallei 1026b Δasd* mutant, and survival was monitored. Statistical differences in survival times were determined by Kaplan-Meier curves followed by log-rank test (**, *P* < 0.01 for *B. pseudomallei 1026b wt* vs. *B. pseudomallei 1026b Δasd* mutant). (B) Intranasal vaccination with *B. pseudomallei 1026b Δasd* mutant protects mice from lethal *B. pseudomallei* challenge. Mice (n=10 animals per group) were primed i.n. with 1x10⁷ CFU *B. pseudomallei 1026b Δasd* and boosted in the same manner 3 weeks later. Two weeks post-boost, mice were challenged i.n. with 4x10³ CFU wildtype *B. pseudomallei 1026b*. Survival was monitored, and statistical differences in survival times were determined by Kaplan-Meier curves followed by log-rank test (***, *P* < 0.0001 for vaccinated vs. non-vaccinated mice). Data represents two individual pooled experiments.
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\textsuperscript{a} E. coli strains are available lab strains. Please use Lab ID number when requesting.
Figure 2

Figure 3
Figure 4

(A) Cytotoxicity Time Course

(B) Microscopic images showing the effect of different treatments on cell morphology.

**Figure 4**
Figure 5
Figure 6
4.8 REFERENCES


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Chapter 5. Select-Agent excluded *Burkholderia pseudomallei* strains and cell infection model systems for research in BSL2 laboratories

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5.1 ABSTRACT

*Burkholderia pseudomallei* (*Bp*), causes a serious and life threatening disease in humans called melioidosis. Since this microbe is a potential bioterrorism agent, it is listed as a select-agent (SA) by the CDC, therefore requiring strict regulations and costly BSL3 laboratories to perform research, which can hamper research progress. Additionally, some experiments are nearly impossible to carry out under BSL3 practices and procedures, and the closely related BSL2 *Burkholderia thailandensis* species is not an appropriate model for *Bp* infection. To alleviate these problems for current researchers and to attract new investigators into this field, we developed SA exempt *Bp* strains and eukaryotic cell infection models that could be manipulated in BSL2 laboratories. The SA exempt *Bp* strain B0011 was also tested in a nematode infection model.

Recently, the CDC excluded the *Bp* 1026b Δasd (B0011) strain from the select-agent list. Here, we investigated the cellular infection characteristics of strain B0011 in cellular models of infection, including epithelial, macrophage, and kidney cell lines, among others. The mutants could be grown in the BSL2 lab with the addition of exogenous diaminopimelate (DAP) in rich media. We investigated different concentrations of exogenous DAP added to eukaryotic cell growth media during cell infection experiments using B0011. Invasion and intracellular replication assays were then carried out in addition to capturing time-lapse microscopy of mutant bacteria during cell infection of the various cell lines. Hallmarks of a chronic infection model have also been observed in other cell lines such as human microglial and neuroblastoma cell lines. Utilizing this model, it was discovered that the mutant strain could chronically infect and grow in the nucleus of host cells, which was then verified in wildtype acute infections using electron microscopy. Here, we showed that with supplemental DAP certain eukaryotic cells and
*C. elegans* could be infected by strain B0011 as well as the wildtype *Bp* strain, and could serve as an appropriate model for BSL2 studies. We also demonstrated that the mealworm *Tenebrio molitor* could serve as BSL2 model for our *asd* strains. Animal studies with two newly excluded *Bp* strains K96243 Δ*asd* and MSHR487 Δ*asd* demonstrated avirulence thus providing two new strain backgrounds for BSL2 work.

In conclusion, cell infection and animal models are indispensable tools in pathogenesis studies of this intracellular pathogen, *Bp*. Our *Bp* mutants and cell infection models provide valuable, safe, and cost effective alternative resources for scientists to study *Bp* pathogenesis, which will help expedite progress in this field.
5.2 INTRODUCTION

*Burkholderia pseudomallei* (*Bp*), a gram-negative soil bacterium and facultative intracellular pathogen, is a common cause of environmentally acquired septicemia in Southeast Asia and Northern Australia although global prevalence is on the rise [1,133,134]. It causes the disease melioidosis in humans and animals and is listed as a Tier-1 select-agent by the Centers for Disease Control and Prevention (CDC). Research priorities regarding SA bacteria are currently centered on basic research into virulence and pathogenesis mechanisms. Fulfilling the five main goals of the Public Health Security and Bioterrorism Preparedness and Response Act of 2002 [20], including development of medical countermeasures (such as vaccines) against biological agents and toxins that may be involved in such emergencies, is the driving force behind much of the research taking place in the United States. BSL2 laboratories across the nation far outnumber BSL3 laboratories and represent significantly underutilized assets. More resources could be applied to solving these goals if basic research into the pathogenesis of *Bp* could be elucidated without the restrictive accessibility and costs of running a BSL3 laboratory.

Previously, we engineered a *Bp* 1026b strain with a deletional mutation in the aspartate-beta-semialdehyde dehydrogenase (*asd*) gene that is auxotrophic for diaminopimelate (DAP) in rich media [80] and could not crosslink the cell wall. In our more recent paper [51], we showed that the *Bp* 1026b Δasd strain (B0011) is attenuated in cell infection and BALB/c mouse infection models, and furthermore, that the strain is an effective vaccine for acute melioidosis. We also determined that with the addition of exogenous DAP, strain B0011 is able to continue infecting host-cells *in vitro*. Pending select-agent exemption, this would then allow observation of the non-infectious strain in various BSL2 experiments, where adding DAP to the media would allow controlled bacterial growth yet maintain the attenuation of the strain. It was also shown
that our strain is very amiable to downstream genetic manipulation by tagging it with multiple fluorescent proteins and producing secondary chromosomal mutations.

*B. thailandensis* is a popular BSL1 alternative to *Bp* but the reality remains that it is a BSL1 organism and does not cause disease in healthy individuals, indicating significant differences in pathogenesis. Presently, exempt *Bp* strains have not demonstrated effectiveness in infection models with a simple chemical additive, such as DAP. Cell infection assays, including intracellular replication and invasion assays, are indispensable for investigating infectivity of *Bp*. With this in mind, CDC exemption of strain B0011 from SA regulations was requested and approved. Many labs are interested in studying pathogenesis of *Bp* but the inaccessibility of BSL3 labs may be hampering research progress. Here, we demonstrated in several models that the Δ*asd* strain may be used to investigate the pathogenesis of *Bp* at BSL2. We also demonstrated a BSL2 model using the mealworm *Tenebrio molitor*. BALB/c animal studies were carried out on two *Bp* Δ*asd* strains from the K96243 and MSHR487 backgrounds. We now have 3 *Bp* SA excluded strains for use in a variety of BSL2 assays.
5.3 MATERIALS and METHODS

**Bacterial strains, media, and culture conditions.** All manipulations of wildtype *Bp* were conducted in CDC/USDA approved and registered BSL3 facilities at the University of Hawaii at Manoa John A. Burns School of Medicine (JABSOM) and experiments with select agents were performed in accordance with the recommended BSL3 practices [7]. *Escherichia coli* strain EOP50 and its rifampicin resistant derivative, EOP50-rif, were used as a nutrient source for *Caenorhabditis elegans* maintenance. Luria-Bertani (LB) medium (Difco) was used to culture *E. coli* strains. Wildtype *Bp* strains were cultured in LB while Δ*asd* strains were cultured in no salt LB with 200 µg/ml diaminopimelic acid (LS+DAP200) or in minimal salts media containing 1 mM lysine, 1 mM, methionine, 1 mM threonine, and 200 µg/ml DAP (3AA+DAP). Antibiotics in solid media were utilized as follows: for *E. coli*, rifampicin (Rif) 200 µg/ml and for *Bp Δasd* kanamycin (Km) 1000 µg/ml and trimethoprim (Tp) 100 µg/ml; non-antibiotics 0.3% glyphosate (GS) in minimal media. Growth on and preparation of DAP were carried out as previously described [8]. All bacterial growth was carried out at 37°C and shaken cultures were maintained at 225 rpm.

**Molecular methods and reagents.** Molecular methods and reagents were carried out as described previously [5,6,9,10].

**Engineering of the *Bp* 1026b virulence factor mutants.** The *Bp* hcp-I (BPSS1498) and was knocked out in B0011 Δ*asd::gat* background using lambda RED recombineering as previously described [9] but with modifications as follows. Two primers whose 3’ regions anneal to the upstream or downstream M13 regions surrounding the *FRT-dhfr-pheS* cassette and
designed to contain 45 bp of homology to the upstream or downstream regions of the targeted
gene, respectively, were used in the PCR to create an *FRT-dhfr-pheS* cassette bounded upstream
by 45 bp homologous to the upstream region of the knock out target and downstream by 45 bp
homologous to the downstream region of the knock out target (~2.3 kb). Primers were then used
in amplification by PCR of the lambda RED region from pKaKa1, to include the *beta, gam, and
exo* genes to create a second DNA fragment (~3 kb). Strain B0011 was grown in 4 ml of M9
media + 20 mM glucose + 3AA+DAP (MG+3AA+DAP) overnight to an optical density at 600
nm (O.D.600) of 1-1.4 then all 4 ml were pelleted by centrifugation and all supernatant removed.
1 µg of each of the two DNA fragments was combined in a total volume of 5 µl and resuspended
with the pelleted bacteria. The suspension was incubated at room temperature for 30 min then
allowed to outgrow in 3-4 ml of the same media at 37°C with shaking. The bacteria were then
pelleted and plated on MG+3AA+DAP+Tp60. Colonies appeared 5-6 d later and were screened
for correct insertion size using primers outside of the region used to create the knockout cassette.
After Flp mediated excision of the *gat* cassette from B0011 Δasd::gat [8], lambda RED was used
to delete BPSS1818 and BPSS1860 with the *gat-pheS-FRT* cassette (instead of *dhfr-pheS*) in the
GS<sup>S</sup> strain B0011 Δasd::FRT. The *wbiI::gat-sacB* insertional mutant was made in B0011
Δasd::FRT by conventional allelic replacement methods [5,8]. All mutants were verified using
PCR.

**Cell lines and culture conditions.** Human embryonic kidney cells (293T), human
neuroblastoma cells (HTB-11 a.k.a. SK-N-SH cells [11-13]), human epithelial cells (HeLa), and
human microglial cells (CHME [13,14]) were cultured in Dulbecco’s modified Eagle’s medium
(DMEM; HyClone) containing 4500 mg/l glucose with 4.0 mM L-glutamine. Murine microglial
cell line BV-2 ([13,15]) and murine macrophage cell line RAW264.7 were also cultured in DMEM containing 4500 mg/l glucose with 4.0 mM L-glutamine. All cultures were supplemented with 10% (v/v) heat-inactivated standard fetal bovine serum (FBS; HyClone) and antibiotic/antimycotic solution (100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B; HyClone). Cells were maintained at 37°C and 5% CO₂ in a humidified incubator. 293T, HeLa, and RAW264.7 cell lines were maintained at 50-80% confluency and were passaged by scraping the cells from the flasks using a cell scraper. BV-2, CHME, and HTB-11 cell lines were passaged by trypsinization with 0.25% trypsin (Gibco) and 1 mM EDTA in pre-warmed phosphate buffered saline (PBS) while being maintained at 50-80% confluency. Cell lines were maintained in Corning™ flasks and plates with CellBIND™ surfaces. Cell concentrations were verified with the Scepter handheld automated cell counter (Millipore).

**C. elegans strains, media, and culture conditions.** The two *C. elegans* strains used in this study (N2 and DH26) were obtained from the *C. elegans* Genetics Center maintained at the University of Minnesota. Both strains are hermaphroditic. N2 is a wildtype strain [16] and DH26 is a temperature sensitive mutant that cannot reproduce at temperatures above 25°C [17,18]. To reduce risk of contamination during prolonged [19] *C. elegans* culturing, *E. coli* EOP50 was plated on LB+Rif 200 to obtain spontaneous rifampicin resistant mutants (EOP50-rif). EOP50-rif were then used as nematode food by spotting onto Nematode Growth Media (NGM; [18]) supplemented with 200 µg/ml rifampicin and allowed to grow at 37°C overnight. The plates were removed and allowed to cool in a 20°C incubator for several hours. Nematode cultures were passaged to fresh bacterial lawns by “chunking” whereby a cube of agar containing worms
is cut out of the plate and moved to another. Cultures were grown at 20°C and passaged every 4-6 days onto fresh *E. coli* EOP50-rif lawns.

**Growth curves of B0011.** Growth curves of the mutants were initiated by growing them overnight in 96-deepwell plates then diluting them 200 x into the indicated media. The 96-well plate was incubated at 37°C with shaking in the BioTek ELx808IU and measuring the O.D. 600 nm every 30 min for 48 h. Each growth curve was done in duplicate with the average and the SEM shown (Fig. 1).

**Determination of ideal exogenous DAP concentrations.** Previously, we found that a DAP concentration of 200 µg/ml would allow for growth of B0011 in HeLa cells and for slow killing in RAW264.7 cells after 24 h. Several different concentrations of DAP were added to the infections to see if higher DAP concentrations would allow better or prolonged growth. Briefly, cell lines (BV-2, CHME, HeLa, HTB-11, and RAW264.7) were harvested and seeded into 24 well plates at 70% maximum confluency as determined by cell counting using the Scepter and allowed to attach overnight. After attaching, the monolayers were washed twice in pre-warmed 1x PBS. Wildtype *Bp* 1026b and B0011 were grown overnight in LS+DAP200 then diluted to a multiplicity of infection (MOI) of ten bacteria per host cell (10:1) in pre-warmed DMEM containing 10% FBS and 250 µg/ml of DAP to allow cell wall cross-linking during the initial infection. The DMEM containing B0011 bacteria was added to the washed monolayers and the infection progressed for 1 h. After 1 h, the monolayers were again washed twice with pre-warmed PBS. Different concentrations of DAP were added (0 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, 2000 µg/ml) to separate aliquots of DMEM with 10% FBS containing 750 µg/ml
amikacin and 750 µg/ml of kanamycin (to kill extracellular bacteria). The infection was allowed to progress for 24 h. At 24 hours post infection (hpi) the media was removed and the monolayers were washed twice with pre-warmed PBS. 1 ml of 0.2% (v/v) Triton-X100 non-ionic detergent in PBS was added to the wells and incubated for 15 min. The mixture was pipetted repeatedly to disrupt the cell monolayers then serially diluted in PBS. 100 µl aliquots were plated on LS-DAP200 and incubated at 37°C for 48 h or until colonies were visible. Colonies were counted and CFU/well determined. Numbers shown are the averages of two independent experiments (Fig. S1).

**Infection characteristics of various Bp strains and their cognate Δasd mutants.** Bp strains isolated from various sources were obtained from Drs. Paul Keim and Apichai Tuanyok at Northern Arizona State University, while the Δasd mutants of those strains were produced in our previous work using lambda RED recombineering [9]. Wildtype Bp strains were grown overnight in LS while the Δasd strains were grown overnight in LS+DAP200. When the optical densities of the cultures were equal, wildtype strains were diluted in DMEM + 10% FBS and Δasd strains were diluted in the same media with 250 µg/ml DAP. HeLa and RAW264.7 cell monolayers were treated as in the determination of optimal exogenous DAP concentration above. The infection was allowed to progress for 1 h before the addition of antibiotics to kill the extracellular bacteria and maintain intracellular infection. Monolayers were lysed at 24 h, diluted, and plated for CFU/well as described above. The numbers indicated are the average of two independent experiments graphed with the standard error of the mean (SEM; Fig. S2).
Invasion and intracellular replication assays of *Bp* 1026b WT and the strain B0011 with and without DAP. Invasion and intracellular replication assays were carried out using a slightly modified kanamycin protection assay as previously described [6,20]. The cell lines utilized were: 293T, BV-2, CHME, HTB-11, and RAW264.7. 293T cells do not attach well so to stabilize the monolayers during washing, Corning™ 24-well plates with CellBIND™ surface were prepared by incubating the wells with a 1:40 dilution of phenol-Red free BD Matrigel Matrix with reduced growth factors in PBS for 30 min prior to seeding. Invasion assays were initiated as described above in DMEM + 10% FBS for *Bp* 1026b and with 250 µg/ml DAP for strain B0011. 1 hpi the medium was removed and the monolayers were washed twice with pre-warmed PBS and fresh DMEM containing 750 µg/ml amikacin and 750 µg/ml of kanamycin was added to kill extracellular bacteria for another hour. At this point the medium was removed again and washed twice with pre-warmed PBS. Monolayers were lysed with 0.2% Triton-X100 in PBS, diluted, plated onto LS+DAP200, and incubated at 37°C for 48 h. Colonies were enumerated and invasion efficiency was determined by dividing the number of intracellular bacteria at 2 hpi by the total number of infecting bacteria. The experiment was carried out in triplicate and the numbers are the average of all three replicates with the error bars representing the SEM (Fig. 2A). The student t-test was used to determine the significance between invasion efficiencies of the wildtype and strain B0011.

The intracellular replication assays were carried out by using the same cell lines and also set up as described above. Each cell line was infected singly with wildtype *Bp* 1026b, strain B0011 with 250 µg/ml of DAP for the whole infection, or strain B0011 without DAP. The monolayers were infected at an MOI of 10:1 for 1 h then washed and the medium was replaced with DMEM containing 750 µg/ml amikacin and 750 µg/ml kanamycin. At the indicated time...
points, from 2 h until 4 days post infection (dpi), monolayers were again lysed, diluted, and intracellular bacteria enumerated after 48 h incubation on LS+DAP200. The numbers shown are the average of three experiments; SEMs are shown but were so low that they are not visible in the graphs (Fig. 2B-F).

**Plaque assay of HEK 293T cells infected with strain B0011 in the presence or absence of DAP.** Plaque assays were carried out as previously described [21] but with some modifications. Briefly, 293T cells were seeded in CellBIND™ coated Corning™ 6-well plates with a 35 mm diameter. The plates were incubated with a 1:40 dilution of phenol-red free BD Matrigel Matrix with reduced growth factors in PBS for 30 min prior to seeding. After a short period of drying within a bio-safety cabinet, cells were seeded at 8x10^5 cells/well and allowed to attach overnight. In the morning the media was removed and the monolayers were washed twice with PBS. An overnight culture of B0011 was resuspended in DMEM with 250 µg/ml of DAP. The culture was diluted down in the same media to reach an MOI of 100:1 and 10:1. The suspension of bacteria was used to infect two monolayers at an MOI of 100:1 and a third at 10:1 all for 1 h. After 1 h, the bacteria-containing media was removed and the monolayers were gently washed twice with PBS to remove any extracellular bacteria. DMEM containing melted 0.5% agarose, 750 µg/ml of amikacin, and 750 µg/ml of kanamycin was cooled to near solidity then used to overlay one of the 293T monolayers infected at an MOI of 100:1. The other two monolayers were overlaid with the same media but supplemented with 250 µg/ml of DAP. At 24 hpi, 400 µl of DMEM with 0.1% (w/v) neutral red solution (Invitrogen) was added to the agarose overlay and incubated for 2 h to allow staining. Plates were inverted on a light box and images
were captured and the negative black and white image created using imageJ version 1.440 (Fig. 4C-E).

**Microscopy and live-cell time lapse imaging of acute and persistently infected cell lines.** Light microscopy of infected cell monolayers was carried out as described [10], except for a few modifications. Glass bottom 12-well plates were obtained from MatTek Corporation (Ashland, MA). The plates were sterile, uncoated, and contained bottoms of No. 1.5 covers lips. Glass coverslip bottoms were treated for four hours with 150 µg/ml poly-L-lysine in ddH2O. The plates were washed twice with ddH2O and allowed to air-dry within a biosafety cabinet overnight. For 293T cells, the wells were treated as indicated above with the BD Matrigel Matrix. Monolayers were seeded at nearly 3x10^8 cells/well verified by the Scepter handheld automated cell counter. Cells were allowed to attach and grow for 24 h prior to initiating the infection. Cell lines were infected with an MOI of 10:1 bacteria in 200 µl of DMEM + 250 µg/ml DAP to increase the contact between the bacteria in the DMEM and the monolayers. After 1 h the medium containing bacteria was removed, the monolayers were washed 2X with PBS, and DMEM containing 250 µg/ml DAP, 750 µg/ml amikacin, and 750 µg/ml kanamycin was added for the remainder of the experiment. For fluorescent microscopy, the DMEM was removed and washed from the monolayers with PBS then replaced by Medium 199 (with Earle’s Salts, L-glutamine, 2.2 g/l sodium bicarbonate, and without phenol red; gibco) + 250 µg/ml DAP. DMEM is somewhat auto-fluorescent in the green channel due to the thiamine in the media. M199 contains significantly less thiamine so the auto-fluorescence is greatly reduced.

Live-cell imaging consisted of 10 min movies taken in a pre-warmed room after the ambient equipment temperature stabilized for an hour. A Zeiss Axio-observer D1 fluorescent
microscope was utilized to capture images. The Axiovision software with the Smart Experiment Time-Lapse Module was used to capture live-cell infection by B0011. Images were captured at 1 frame/s for 10 min total. Images were compiled into videos using imageJ version 1.440.

Long term live-cell two color fluorescent time-lapse fusion assay. GFP and RFP tagged HEK293 cells were obtained from Dr. Jeff Miller’s lab at UCLA [22]. These cell lines were maintained in DMEM + 10% FBS as described above with the addition of 3 µg/ml of puromycin and passaged by gently tapping the side of the culture flask. At ~80% confluence both cell lines were treated with 0.25% trypsin (Gibco) and 1 mM EDTA in pre-warmed phosphate buffered saline (PBS) for 5 minutes to break up aggregations of cells. The Millipore Scepter was used to enumerate the cell concentration and equal numbers of each cell line were mixed, resuspended thoroughly, then seeded into 12-well cover glass bottom plates that had been pre-treated with BD Matrigel as described above. After allowing attachment overnight, an infection at an MOI of 1:1 was carried out as described above, where after 1 h of infection media containing DAP + aminoglycosides was added. The plate was then placed in a stage mounted incubator chamber custom fabricated by Bioscience Tools where a 5% CO₂ atmosphere was maintained at 37°C. During the next 2 hours the monolayers were observed closely to identify infected cells. Once an infected cell was identified by visualizing intracellular bacteria or protrusions, the multi-channel live-cell time-lapse fluorescent imaging using a custom capture sequence was initiated and images in the green, red, and brightfield were captured every 2 min for 24 h. At the conclusion of the experiment, images were exported to imageJ for intensity normalization and movie production.
Transmission electron microscopy of wildtype *Bp* 1026b infected RAW264.7 macrophages. 60 mm tissue culture dishes were treated for four hours with 150 µg/ml poly-L-lysine in sterile double distilled water (ddH₂O). The dishes were washed twice with ddH₂O and allowed to air-dry within a biosafety cabinet overnight. RAW264.7 murine macrophages were scraped from flasks and allowed to attach in DMEM overnight. The next day wildtype *Bp* 1026b were resuspended in 200 µl of DMEM at a concentration adequate for an MOI of 10:1 and used to infect the monolayer for 1 h. After 1 h, the media was removed and monolayers were washed twice with PBS. Fresh DMEM containing 750 µg/ml amikacin and 750 µg/ml kanamycin was added for the remainder of the experiment to kill all uninternalized bacteria. At 18 hpi, the media was removed and the monolayers were fixed for 2 h with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4. The dishes were washed twice with 0.1 M cacodylate buffer for 20 min each. Samples were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h then dehydrated in a graded ethanol series. Epoxy resin was used to infiltrate samples and allowed to polymerize at 60°C for 2 d. Samples were visualized using a 120 kV Hitachi HT7700 digital transmission electron microscope. Images were captured using an AMT XR-41 2048 x 2048 pixel bottom-mount high-resolution camera (Fig. S3).

**Determination of endonuclear B0011.** 293T cells were infected with strain B0011 as above for the intracellular replication assays but in 6-well CellBIND™ coated Corning™ 6-well plates with a 35 mm diameter. At 20 and 24 hpi, the culture medium was removed and the monolayers were washed with PBS then scraped off the well bottom using a cell scraper. The cells were then washed in cold PBS by centrifugation after which the supernatant was discarded. The cells were resuspended in chilled hypotonic buffer solution (20 mM Tris-HCl pH 7.4, 10
mM NaCl, and 3 mM MgCl₂) by pipetting and incubated on ice for 15 min. 25 µl of NP40 detergent (10% w/v) was added and the tube vortexed for 10 s at the highest setting. The homogenate was then centrifuged at 4°C and 1,850 x g for 10 min. The supernatant containing the cytoplasmic fraction was removed and saved. The pellet containing the nuclei was resuspended in 3 ml of buffer S1 (0.25 M sucrose and 10 mM MgCl₂) then layered carefully over a 3 ml cushion of buffer S3 (0.88 M sucrose and 0.5 mM MgCl₂) and centrifuged at 4°C and 2,800 x g for 10 min. This step is done twice to provide a cleaner nuclear pellet that was then resuspended in nuclear lysis buffer (0.2% Triton-X100 in PBS) and vortexed at the highest setting for 10s. The cytoplasmic and nuclear lysates were serially diluted in PBS and plated on LS+DAP200 to determine cytoplasmic and nuclear bacterial load. This experiment was performed in triplicate and the error bars represent the SEM of the CFU obtained from the different fractions.

**C. elegans survival, prolonged killing, and intraorganism replication assay.** Survival and prolonged killing assays were carried out as previously described [23,24], but with a few modifications. We used *C. elegans* strain DH26, a temperature sensitive mutant, in both the survival and prolonged killing assays. Survival assay experiments were initiated by transferring 30 worms in the L2 stage to B0011 lawns on NGM supplemented with 3AA+DAP (when growing the B0011 strains). Animals were left on the lawns for the duration of the survival assay. The assay plates were moved to the non-permissive temperature of 28°C to prevent reproduction of the nematodes. Progeny nematodes may confound the data, so eliminating them prevents accidental counting during the assay. At the indicated time points, the number of dead worms was observed using a Zeiss SteREO Discovery V.8 stereomicroscope and prodded gently
with a platinum wire to verify death. The survival assay experiment was carried out in triplicate, where the survival curve represents the average % surviving and the error bars are the SEM (Fig. 5A). For the prolonged killing assay, the nematodes were exposed to B0011 for 30 min then removed from the lawn of bacteria, washed 3X with PBS, then transferred to a new NGM plate containing a lawn of EOP50. The prolonged killing assay is shown as survival % of three trials of independent experiments compared to a control of EOP50 (Fig. 5B).

To enumerate intracellular bacteria within *C. elegans*, an assay was adapted from the previously described method [25] with a few modifications. Nematodes were transferred to a plate containing a lawn of B0011 for 1 h, after which they were picked using a “worm hook” made from a platinum loop, and were placed ten per microcentrifuge tube for each of the three indicated time points. The nematodes were washed 3X with PBS in their separate tubes by centrifugation in a tabletop centrifuge for 5 min at 540 x g. The worms were then resuspended in M9 minimal salts media containing the 3AA, and either with or without 400 µg/ml of DAP. This media also contained 750 µg/ml of amikacin and 750 µg/ml of kanamycin to promote invasion by strain B0011 and kill off other extracellular bacteria. At the indicated time points, the nematodes were again centrifuged and washed 3X in PBS. 1 ml of modified worm lysis buffer (this buffer contains 10X Pfu buffer, 60 µg/ml Proteinase-K, and 0.2% Triton X-100 non-ionic detergent in PBS) [26] was added to each tube and incubated for up to an hour at 42°C with intermediate vortexing until the worms looked mostly digested under a stereomicroscope. The solution was serially diluted in PBS and CFU/tube determined. Time points were done in triplicate and the numbers shown are the average with the SEM (Fig. 5C).
**C. elegans live-animal time-lapse imaging.** 200 µl of $Bp\Deltaasd::gat-FRT/attTn7-kan-gfp$ grown in LS+DAP200 overnight was plated on NGM plates supplemented with 3AA+DAP using a disposable spreader. The bacteria were allowed to grow for 48 h at 37°C until a thin lawn was visible. The plates were then transferred to 28°C for 2 h prior to transferring *C. elegans* nematodes from the EOP50 lawn to the lawn of *gfp*-tagged B0011 for 1 h. Live *C. elegans* were visualized with the Zeiss SteREO Discovery V.8 stereomicroscope and the KLD cold halogen light source. The attached green fluorescent LED excitation unit and emission filters were used to visualize intra-organism green fluorescence. A Retiga 2000R high precision camera was attached via binocular node and time-lapse images were captured by QCapture Pro7 software. ImageJ was used to process the images into movies and for production of montages.

**Challenge of *C. elegans* with live cells, heat-killed cells, and cell-free supernatant.** Strain B0011 was grown over night in LS+DAP200 media and frozen at -80°C in aliquots of 30% glycerol. A single frozen aliquot was thawed the following morning and serially diluted in S-basal media [27]. The dilutions were plated onto LS+DAP200 plates to determine the CFU/ml of the frozen aliquots thus taking into account bacterial survival percentage during freezing. For the experiments the aliquots were thawed and diluted to the desired CFU in S-basal media. *Bp* were heat inactivated by heating the diluted aliquots to 80°C for 1 h. 100 ul of the least dilute samples were spotted on LS+DAP200 plates to confirm heat inactivation of all bacteria. To confirm bacteria were intact and had not lysed, samples were visualized under 1,000X magnification. Cell-free supernatants were obtained by centrifugation of undiluted samples at 20,000 X g followed by filtration of the supernatant through a Millipore PES 0.22 µm filter. Aliquots were plated on LS+DAP200 to ensure supernatant were cell-free.
20 age matched L2 *C. elegans* strain DH26 were collected and placed 1.5 ml centrifuge tubes containing 10 ul of S-basal media. The nematodes were then exposed to the indicated CFU of strain B0011, heat-killed B0011, or corresponding dilutions of cell-free supernatant in a total of 50 µl of S-basal media containing 3AA+DAP for 30 min. The nematodes were then spun down and resuspended in S-basal media containing 3AA+DAP and heat-killed EOP50 as a food source in the wells of a 12-well plate to make sure the food source did not overgrow them. At the indicated time points survival percentages were determined by observation under a Zeiss SteREO Discovery V.8 stereomicroscope, survival was verified in unmoving worms by gentle prodding with a titanium loop. The numbers presented are the averages of two independent experiments carried out in triplicate and the error bars are the SEM of the survival percentages (Fig. 5D-F). Modified prolonged killing assays using B0011 virulence factor mutants were carried out in the same manner but exposed to only 1x10^6 CFU of the indicated strains.

*Tenebrio molitor* (yellow mealworm) survival assay. Fresh giant mealworms were obtained from PETCO. Worms were removed from the food source and starved for 24 h at room temperature. It is essential to purchase twice as many worms than what is needed due to mealworm die-off during starvation. 100 mm by 15 mm petri dishes were used as the feeding chambers for each replicate and were humidified by placing a 35 mm petri dish containing a folded paper towel soaked with double distilled water inside. A piece of the rim of the 35 mm dish was shaved off with scissors to allow for humidification, ensuring the opening was small enough to prevent the worms from entering the humidifier. 20 starved worms were added to each feeding chamber.
Bacterial cultures were grown to an OD\textsubscript{600} of 5 in 6 ml of LS broth+200 µg/ml DAP by heavy inoculation 24 h prior to initiating the experiment. For each replicate, 1 g of oats was measured into borosilicate glass tubes and autoclaved to sterilize. 2 ml of each strain were concentrated via centrifugation and were then resuspended in 1 ml LS broth+1,000 µg/ml DAP. 1 ml of each culture was used to soak 1 g of oats for every replicate and strain. Once the oats absorbed all liquid they were added to the inside of the 100 mm petri plate (outside of the 35 mm plate humidifier), this is taken as time zero (T=0). Survival assays were carried out in triplicate with 20 worms per replicate for a total of 60 worms per strain tested.

The lids of the 100 mm petri dishes were taped shut to prevent mealworm escape and placed in a ziploc bag to maintain humidity then incubated at room temperature. Animals were checked every 12-24 h for movement, if the animals did not move they were picked up gently with forceps and observed for any movements. If the animals did not respond or showed melanization they were considered dead and marked as such. Survival curves were plotted by pooling the replicates in GraphPad Prism 6.

**Engineering of Bp \textit{\textDelta asd::gat–FRT/attTn7-kan-gfp}**. The \textit{Bp} 1026b \textit{\Delta asd::gat –FRT} strain (B0011) was engineered as previously described [5] and was tagged with pTn7-\textit{kan-P\textsubscript{S12}-gfp} as previously described [10]. B0011 was grown for 36 h in 4 ml of LS+DAP200 then washed 4 times in room temperature sterile double distilled water (ddH\textsubscript{2}O). The pellet was resuspended in 40 µl of ddH\textsubscript{2}O and added to 2 mm electroporation cuvettes along with 250 ng of both pTn7-\textit{kan-P\textsubscript{S12}-gfp} and pTNS3-\textit{asdEc}. Following a pulse of 250 kV, the bacteria were resuspended in 1 ml of LS+DAP200 and recovered for 1 h at 37°C with shaking. After 1 h, bacteria were pelleted and plated onto LS+DAP200+Km1000. Colonies appeared after 3-4 days and 4 isolates were
streaked for purity on the same media. Two colonies from each were PCR confirmed to contain the Tn7 element at the \textit{glmS3} site [28] and verified via fluorescent microscopy as being green fluorescent.
5.4 RESULTS

Determination of ideal exogenous DAP concentrations for intracellular infection models. Previously, we had established the ideal concentration of DAP for log growth of strain B0011 in rich media at 250 µg/ml of DAP [6]. Since DAP allows growth of the attenuated mutant in liquid media, we wanted to investigate whether DAP present in cell monolayer growth media would allow for intracellular replication of the attenuated B0011 strain. In addition to intracellular replication, we wanted to determine the concentration of DAP that would give us optimal infection of various cell lines. Many different cell lines were chosen, some that have been extensively utilized in cell infection models (HeLa [6,20,21] and RAW264.7 [6,29,30]) and others that may prove useful for future Bp pathogenesis experiments. Wildtype Bp 1026b was able to infect all the cell lines tested including the newly introduced murine (BV-2) and human microglial (CHME) cell lines as well as the human neuroblastoma cell line (HTB-11). The wildtype was also able to produce plaques in these cell lines (data not shown). When the culture media was not supplemented with DAP post-infection there were no B0011 bacteria detectable at 18 hpi in any of the cell lines, as expected. To determine the appropriate concentration of DAP, the amount supplemented was titrated up from 250 µg/ml of DAP, the optimal concentration used in shaken culture, to 2,000 µg/ml of DAP. There is a step-down in infection efficiency as the amount of DAP increases. Fewer bacteria are recovered at higher supplemental DAP concentrations (Fig. S1). DAP is an amino acid specific to bacterial peptidoglycan which at higher concentrations begins to activate the innate immunity of the cell lines via the NOD1 pathway [31] resulting in more efficient host-cell mediated killing. The numbers of recovered B0011 bacteria were a log or two lower than those of the wildtype. At 24 hpi, under bright-field microscopy, it was observed that mutant bacteria were infecting the cell lines with a similar
phenotype as the wildtype (Fig. 1). At 18 h the mutant can be seen polymerizing actin at a velocity of ~17.4 µm/min measured by the average distance traveled in the supplementary time-lapse videos (compared to 4.8 µm/min for *Rickettsia rickettsii* [32] and ~10 µm/min reported for *Listeria monocytogenes* [33]) and protruding from the various cell lines, indicating key infectious stages of phagosome escape, actin polymerization, and protrusion occur similarly between the wildtype *Bp* 1026b and the biosafe Δ*asd* strain, B0011.

**Infection characteristics of various *Bp* strains and their cognate Δ*asd* mutants.** Many experiments with *Bp* have focused on the type strains 1026b and K96243. While the importance of these virulent type strains cannot be underscored, it may be important to understand that they are isolates from acute human infections and that within-host adaptation is known to occur frequently [34]. *Bp* isolated from human chronic cases, from the soil environment, and from different geographic locations can all infect humans but subtle genomic differences may yield diverse pathogenic outcomes. To this end we tested nine naturally competent *Bp* in the two commonly used cell line models, HeLa human epithelial cells and RAW264.7 murine macrophages. All wildtype strains tested besides the chronic case isolates were able to infect intracellularly to around 10⁶ CFU/well (Fig. S2) and produce plaques (data not shown). Strain Bp4141, Bp4144, and Bp6340 are clinical isolates from chronic cases occurring in Australia isolated over the course of a decade (Table 1). The number of intracellular bacteria was consistently 10 and 100 times lower than the acute and environmental isolates in the two independent experiments for one or both cell lines (Fig. S2) although there were some disparities between HeLa and RAW264.7 cell lines. Whether chronic *Bp* isolates have adapted to a slower infectious course within-host remains to be seen but it is certainly plausible given
adaptations in other chronically infecting pathogens such as those of *Pseudomonas aeruginosa* (22) and *Burkholderia dolosa* [35].

That aside, we thought it would be of use if strains from alternate sources, such as those above, could be accessible to BSL2 researchers and assist in answering some of these interesting questions. The $\Delta asd$ mutants of all the strains were created previously [9] and used to infect the monolayers above and the CFU/well recovered were compared to those of the wildtype. Some of the $\Delta asd$ strains were unable to infect while others could. Numbers were generally higher for the RAW264.7 monolayers, compared to HeLa cells, possibly because of the phagocytic nature of the cell line. Fine-tuning of the DAP concentration may be necessary for the different strains as their growth behavior in supplemental DAP has not been investigated. Interestingly, the $\Delta asd$ mutant of the chronic Australian isolate had nearly the same replication numbers as its cognate wildtype strain in RAW264.7 macrophages after 24 h.

**B0011 growth rate in different media.** Strain B0011 was grown in several different growth media to find the best one for future experiments (Fig. 1A). Low salt Lennox LB broth, no salt LB broth, and M9 minimal media supplemented with 200 $\mu$g/ml DAP were chosen along with the unsupplemented media. Growth curves in triplicate are shown for all media. Strain B0011 was unable to grow without DAP supplementation regardless of media. LB broth supplemented with DAP gave the best growth rate and eliminating the salt allowed an increased rate of exponential phase growth. We then wanted to compare the growth rate of B0011 to the wildtype 1026b strain (Fig. 1B). Again, without DAP strain B0011 could not grow. In LB broth without salt (LS) strain B0011 had the same growth rate as wildtype 1026b.
Invasion and intracellular replication assays of *Bp* 1026b WT and the B0011 Δasd mutant with and without DAP. Alternate strains would be useful in studying some aspects of *Bp* pathogenesis such as innate pathogenesis or differences between acute, chronic, and latent forms of infection in BSL2 cell models. However, the type strain 1026b is still the major workhorse for investigating the molecular pathogenesis of *Bp*, so demonstrating the usefulness of strain B0011 in invasion and intracellular replication assays is paramount. A wide variety of cell lines were chosen to determine the invasion efficiencies; including HEK293T, BV-2, CHME, HTB-11, and RAW264.7 (Fig. 2A). As it can be seen, the invasion efficiencies between the wildtype 1026b and B0011 were very similar in all the cell lines and the efficiencies were found to be insignificantly different by the student t-test. This indicates that attachment and invasion of the Δasd mutant B0011 occurs virtually identically as the widltype regardless of the cell type. Whereas *B. thailandensis* may exhibit certain cell line tropism and decreased invasion efficiencies [36], the B0011 exhibits wide tropism and wildtype invasion efficiencies irrespective of cell type.

The intracellular replication assays were carried out in the same cell lines as the invasion assays. To properly illustrate intracellular growth characteristics, the assays were carried out to 4 d (96 h; Fig. 2B-F). In all cell lines tested, the wildtype reached a peak of 1x10⁶ CFU/well at 18 hpi and rapidly declined to very low numbers by day four of the assay. Without supplemental DAP after the initial 1 h infection, B0011 was unable to replicate within any of the cell lines and no CFU were recoverable at 18 hpi. When DAP was supplemented in the cell culture media at 250 µg/ml throughout the experiment, strain B0011 was able to infect HEK293T cells the same as the wildtype, reaching 1x10⁶ CFU/well after 18 hpi then declining in numbers for the remainder of the experiment (Fig. 2B). Contrastingly, the Δasd mutant B0011 was unable to
“acutely” infect all other cell lines (Fig. 3C-3F). Instead the detected CFUs drop to between 1x10^{4}-1x10^{5} CFU/well then plateau to “persist” within the monolayers for the remaining 4 d of the experiment. Strain B0011 infected the cell monolayers well beyond the normal 24 h wildtype infection and we predicted that it would continue to infect past the 4 d window of the initial intracellular replication assay.

**Intracellular replication of B0011 in HEK293T and HeLa cell lines.** A finer scale infection assay showed that with DAP, B0011 can infect 293T (Fig. 3A) cells and Hela cells (Fig. 3B) similar to wildtype 1026b. Without DAP, B0011 cannot grow intracellulary and eventually dies. For comparison the B0011 ΔhcpI mutant was used and showed that in this particular model the major T6SS is contributing to intracellular replication more so in HeLa epithelia cell than the HEK293T kidney cells. In both cell lines the B0011 ΔhcpI mutant numbers increase like the wildtype and B0011 strains then in HeLa cells intracellular CFU drop by 12 and 24 hpi. Bright-field phase images represent well-wide MNGCs full of B0011 bacteria in the process of fusing with healthy HEK 293T cells that then completely lyse by 48 hpi (Fig. 3C, 3D, and V1). To this end, the plaque assay was attempted with B0011 in HEK293T cells. Without DAP the cell monolayers appeared healthy at 24 hpi (Fig. 3E), whereas when infected with DAP at an MOI of 10:1 and 1:1 the plaques can clearly be seen (dark areas; Fig. 3F, and 3G).

**Cell lines remain persistently infected by the *Bp* 1026 Δasd mutant, B0011, for up to 3 weeks.** Cell monolayers were infected with B0011 at an MOI of 10:1 with DAP and monitored via light microscopy and live-cell time-lapse imaging at various dpi (Fig. S3). At five and nine
dpi Δasd mutant bacteria can be seen infecting BV-2 murine microglial cells at high density (Fig. S3A). Carets indicate multiple bacteria undergoing actin-mediated protrusion and flagella-based intracellular movement. In Figure S3B, CHME human microglial cells are observed at 16 dpi where carets indicate a few of the many protruding bacteria infecting the host-cells. 48 h later at 18 dpi compact spheres packed with moving bacteria are observed. Carets at 18 dpi indicate two of the spheres, one small and one large. The phenotypic difference between these perfectly round bacteria-filled spheres and the very rough, bacteria free apoptotic cells lying between and around them indicate a unique origin. The source of these spheres was initially a mystery that will be addressed later. Infected HTB-11 human neuroblastoma cells can be seen in Figure S3C at 4 dpi. Multiple bacteria are swimming in the cell and around the nucleus, while others protrude, searching for neighboring cells to infect. Two weeks later (14 d), bacteria are still infecting some of the cells. Mutant bacteria are tightly packed at high cell density within the live cell and are observed in motion. In Figure S3D, RAW cells stay infected for many days similarly to the other cell lines. All of the images are single frames of 5-10 min time-lapse videos located online in the supplemental materials (V6-8).

*Burkholderia pseudomallei* can undergo endonucleobiosis during the course of infection. Our new model of persistently infected cells allowed easy observation of the infectious process at BSL2. Rather than having only 24 h to observe infected monolayers, there was an average of 3 weeks available. Frequent observation during this period allowed surveillance of infection events that would normally be difficult to detect in “acute” infection models. Time-lapse movies of HTB-11 human neuroblastoma cells infected with strain B0011 revealed bacteria swimming within the nucleus of the host cell and running into the nucleolus.
Another host-cell was found containing two daughter nuclei, one of which was highly infected with over 50 gfp-tagged B0011 bacteria (Fig. S4A, S4B, and S4C). Time-lapse video shows the bacteria moving around in the nucleus and possibly exiting the nuclear membrane through an unknown mechanism (V9). The presence of endonuclear Bp has never been described and, to the authors’ knowledge, never been observed. After seeing the way the bacteria fill the nucleus, it became clear that the perfect spheres enclosing moving bacteria in other cell lines described above were actually free-floating intact nuclei of lysed cells containing bacteria at high density. The larger the nuclei the more swollen and tightly packed the bacteria, consistent with observations of endonucleobiosis in Euglena hemichromata [37]. It has also been observed that the nucleus remains intact after cell membrane destruction by wildtype Bp 1026b (Fig. S4G); whether nuclear located bacteria play a role in pathogenesis remains to be investigated.

In case this was an aberration observed solely with B0011 and to verify if wildtype Bp infects the nucleus, transmission electron microscopy (TEM) was carried out on fixed RAW264.7 macrophage monolayers infected with wildtype at 24 hpi. In Figure S4D-F, TEM images captured many wildtype Bp 1026b in the nuclei of host-cells. Figure S4D shows two nuclei of a five nuclei multi nucleated giant cell (MNGC) infected with bacteria. Figure S4D is a close up of one nucleus from a different MNGC infected with 6 bacteria in the same plane and Figure S4F is a giant free-floating nucleus full of bacteria reminiscent of the bacteria filled spheres seen earlier. This heavily infected nucleus is almost indistinguishable from nuclear Rickettsia infection [38] implying there are plenty of nutrients to sustain high cell density growth while remaining secluded from the host immune system components. Observation of free-
floating nuclei after destruction of the cell membrane was frequently observed in wildtype infected monolayers (Fig. S4G).

It was observed that during infections endonuclear bacteria were present more so during the later stages. At two time points, 20 and 24 hpi, cytoplasmic and endonuclear CFU of B0011 were determined. Endonuclear \( Bp \) increased 10 fold between the two time points and accounted for 0.4% of the total bacteria infecting the monolayers. This may not seem substantial but in an intracellular population of millions of bacteria quite a few bacteria are entering the nucleus. Endonucleobiosis of \( Bp \) does not seem to be an isolated event but a previously unknown intracellular niche exploited by this organism during pathogenesis, the mechanism of which remains to be elucidated.

**B0011 is useful for live-cell imaging at BSL2.** Utilizing the previously published GFP/RFP HEK293 cell lines [22] at BSL2 in monolayer infection studies with B0011 can be a very valuable tool. For this experiment it was possible to use time-lapse fluorescent microscopy to track strain B0011 ability to infect monolayers with monolayer fusion detectable as MNGCs at around 12 hpi (Fig. 4A and V2). By 20 hpi the entire monolayer appears fused into MNGCs and by 24 hpi the monolayer is destroyed by cascading lysis. B0011 can also be used to visualize more detailed processes of intracellular infection. In figure 4B, fusion of two HEK293T cells is visualized. B0011 bacteria can be seen producing actin tails and protruding from the surface of the host-cell as the two host-cells fuse (Fig. 4B top panel and V3). Bacteria can also be tracked as they transit through the host-cell (Fig. 4B bottom panel). The blue line tracks one bacterium through one host-cell as it transits into the next. The green line tracks a second bacterium as it alternates from protruding to cytoplasmic locations and back again.
Use of B0011 in the *C. elegans* challenge model. Another model useful for study of *Bp* pathogenesis is the *C. elegans* nematode model [23,39,40]. It is an animal model easily reproducible in the BSL2 lab and does not require IACUC oversight since *C. elegans* are invertebrates. Our *Bp Δasd* mutant strain, B0011, can be used in this model at BSL2 with the addition of DAP to the NGM plates. After the worms are placed on the media they begin ingesting B0011 bacteria while moving through the thin lawn. Figure 5A shows the survival of *C. elegans* over time after they are transferred to the various bacterial lawns. Strain B0011 kills the nematodes at the same rate as *B. thailandensis* E264, which has been previously shown to kill the same as wildtype *Bp*, thus clearly agreeing with previously published observations of wildtype kill-curves [23]. A few *C. elegans* do not survive until the first time point in all replicates observed and survival percentages drop exponentially thereafter. All nematodes stopped moving by 30-40 h after assay initiation and death was verified by gentle prodding. Worms placed on negative control plates containing *E. coli* had high survival rates the entire length of the experiment.

Another effect *Bp* has on *C. elegans* survival is prolonged killing [39,40]. After a short exposure (30 min in this study) to B0011, nematodes were transferred to a fresh plate of *E. coli* and monitored for survival. Nematode survival was monitored for 4 days and Figure 5B shows the Kaplan Meier survival curves for three separate trials. The data agrees with previously published results regarding prolonged *C. elegans* killing by wildtype *Bp*. In this study, we exposed the nematodes for a shorter amount of time and observed them longer than the previous studies. By day 4 the survival rate was reduced to ~ 65%. It is apparent that even a short
exposure to \textit{Bp} can significantly reduce the lifespan of \textit{C. elegans} compared to the negative control that was never exposed to strain B0011 and only \textit{E. coli}.

A modified whole organism infection model was developed with the potential to identify and characterize the effects of virulence factors required for intracellular replication at the whole-animal level. At the indicated time points post-infection, worms were enzymatically digested and intracellular bacteria were counted. In the presence of DAP, strain B0011 was able to replicate and maintain bacterial levels at \(\sim 1 \times 10^4\) CFU/tube and \(\sim 1 \times 10^3\) CFU/worm (Fig. 5C). When DAP was not present, internalized CFU plummeted by 24 hpi. The CFUs recovered without DAP were 1,000 fold less than when DAP was provided during infection. The negative \textit{E. coli} control showed no presence of bacteria by the first time point. Survival of nematodes throughout the experiment was verified by observing continuous nematode movement throughout the assay (data not shown) indicating the intracellular bacteria levels observed were not lethal.

Time-lapse bright field imaging of \textit{C. elegans} mock infected with \textit{E.coli} compared to \textit{C. elegans} fed with B0011 reveals clear phenotypic changes in the nematode (Fig. V4). They became larger and the bodies appeared distended as the intestinal and motor muscles become paralyzed during infection. The typical sinusoidal movement associated with healthy worms was replaced with disjointed spasmodic movement with no forward progress in infected worms. Visualizing internal bacteria would also be of use, so we fed the \textit{gfp}-tagged B0011 strain to \textit{C. elegans} and were able to visualize the fluorescent bacteria lining the intestinal lumen. This could be useful in competition experiments with two different color strains of B0011 (e.g. \textit{gfp}- and \textit{rfp}-tags, [10]). Tagging B0011 with \textit{gfp} also allows time-lapse live organism stereomicroscopy. Video 5 shows the green fluorescent bacteria filling the intestinal lumen during nematode
locomotion. A pronounced intestinal bulge of bacteria seems to be giving the nematode considerable digestive difficulty.

**Challenge of *C. elegans* with live cells, heat-killed cells, and cell-free supernatant.** To further fine-tune the model of *C. elegans* we wanted to titer the number of bacteria the nematodes were exposed to. Expectedly, the virulent effect was dose-dependent but when the *C. elegans* were exposed to $10^6$ bacteria in small liquid volumes, the prolonged killing effect was greater than when placed directly on a lawn of *Bp* (Fig. 5D). As the CFU used for the challenge was titrated down the killing effect was still present but the killing rate decreased, ranging from an exponential decline at $10^6$ CFU to more of a linear decline at $10^2$ CFU. This trend was also observed when using heat-killed (Fig. 5E) and cell-free supernatant (Fig. 5F). For all CFU used, the live cells killed the best followed by heat-killed bacteria, then cell-free supernatant. Secreted components present in the cell-free supernatant, i.e. proteases and heat labile and stable toxins contribute the least. Intrinsic components, such as endotoxin and heat-stable toxins, are the next greatest contributors to *C. elegans* killing. This can be analyzed by comparing the cell-free supernatant (just secreted components) to the heat-killed (secreted and intrinsic components) (Fig. 5G). Live cells presumably have secreted, intrinsic, and also cell contact dependent active virulence components (toxin secretion directly into host organisms). Even 30 min exposures of 100 bacteria are potent enough to kill all worms ~8 days post-exposure. Strain B0011 is capable of killing *C. elegans* with all the processes tested.

**Challenge of *C. elegans* with B0011 virulence factor mutants.** To demonstrate that *C. elegans* and B0011 can be used to screen virulence factors we used the fast-kill challenge model
and the modified prolonged killing model. In the fast-kill model the *C. elegans* were placed on lawns of the indicated strains for the duration of the experiment. Two virulence factors of known function, *hcp1* a component of the T6SS-5 [41] and *wbiI* the last gene in O-antigen biosynthesis, were mutated and tested for attenuation in the two *C. elegans* models [42,43]. In the fast-kill model the nematodes challenged with the ∆*hcpI* mutant compared to B0011 had an almost tripled median time to death from 6 h to 19 h. The survival curves were highly significantly different. The *wbiI* mutant also had a doubling of median survival time from 6 h to 12 h and a significantly different survival curve when compared to B0011 challenged *C. elegans*. In this model, *C. elegans* exposed to the B0011 putative virulence factor mutants ∆BPSS1818 and ∆BPSS1860 survived the same as those exposed to B0011 (Fig. 4H). The same mutants were used to infect *C. elegans* in the slow-killing model (Fig5I). In the slow killing model the *wbiI* mutant did not survive significantly longer than B0011 exposed worms while the ∆*hcpI*-exposed worms had a significantly different survival curve with no increase in median survival time. In contrast the nematodes exposed to ∆BPSS1818 mutant bacteria had a highly significant difference in survival compared to B0011 exposed nematodes with a median increase in survival time from 40 to 68 hpi. Nematodes exposed to the putative attachment factor mutants ∆BPSS1860 showed the largest difference in the slow killing model with survival curves similar to the EOP50 exposed control.

**Studying *Bp* virulence at BSL2 with the mealworm survival assay.** Mealworms have a more complicated immune system than the nematode *C. elegans*. The ability to study the virulence of *Bp* in a higher-level organism at BSL2 is an invaluable model system. Similar to *G. mellonella*, the mealworm *T. molitor* begins to melanize during an infection until completely
moribund where the entire worm turns dark in color. Of particular note, these worms appear to be paralyzed from the mid-section outwards. The paralysis first manifests itself as twitching then spreads to encompass the entire animal, leaving only the legs with the ability to move before mealworm death. Observations indicate that mealworm paralysis is followed eventually by a spreading melanization and appear to have the same focus in the animal’s anatomy. Strain B0011 was fed to the worms, the region of melanization begins at the junction of the crop and the proventriculous (mid-gut). The

The practicality of the mealworm model in studying Bp virulence with B0011 at BSL2 was demonstrated by a survival assay with B0011 and several virulence factor mutants (fig. 6A). Feeding on B0011 spiked oats kills the mealworms much faster than the EOP50 control indicating the mealworm can be a new, cheap, fast, and effective model for studying Bp pathogenesis at BSL2. Survival curves of mealworms infected with wbiI and hcpI mutants were both significantly different than the B0011 with many worms surviving on average double those infected with B0011 but without an increase in median survival time (Fig. 6A). ΔBPSS1860 infected mealworms had increased median survival times from 48 h to 60 h but only slightly longer average survival times and was significant compared to the B0011 control curve (Fig. 6A). In the T. molitor model, bacterial dissemination can be followed from the digestive track by feeding the worm B0011 tagged with stable GFP constructs [10]. Figure 6B shows a picture of the worm and a diagram of its digestive tract anatomy. After brief feeding of mealworms with GFP-tagged B0011, the entire tract can be removed by severing the head and carefully pulling the final few segments until the tract comes free. The tract can be mounted on a microscope slide for viewing. The anatomical diagram can be compared to the DIC images of the mealworm (Fig. 6B compared to 6C top panels). The fluorescent bacteria are visible in the digestive tract and you
can see permeation of the surrounding tissues with fluorescent bacteria (Fig. 6C middle panels). Paralyzed worms that still reacted to touch but could not move were dissected. A large portion of the crop and anterior end of the midgut were liquefied and were unable to be removed intact. Under magnification the mealworm digestive tract cells appeared to be fused into the characteristic MNGCs of monolayer infections (Fig. 6C bottom panels). MNGC type formations are termed nodules in insects and are hallmarks of innate cell-mediated. Within the nodule green-fluorescent bacteria can be seen.

**BALB/c attenuation studies of two new *Bp* strains.** The previously constructed *Bp* K96243 Δ*asd* mutant and the newly made MSHR487 Δ*asd* mutant were tested for avirulence in the BALB/c inhalation melioidosis animal model. The LD$_{100}$ dose for both strains is ~4,500 CFU with all animals succumbing to infection by 4 dpi. 1x10$^7$ CFU were used to challenge the mice (~2,000 time the LD$_{100}$) and survival was followed. All mice challenged with the Δ*asd* mutants survived 60 days until the end of the experiment. Organs loads were determined to be 0 CFU in all organs tested (lungs, liver, spleen) indicating the bacteria were unable to infect the mice and were completely attenuated. The CDC has since excluded these two strains from the SA regulations.
5.5 DISCUSSION

*Burkholderia pseudomallei* research has been restricted to BSL3 laboratories in the United States. This limits much of the pathogenesis research to entities and institutions that have access to and can afford to run a BSL3 lab. It is no surprise that BSL2 labs far outnumber BSL3 labs in the United States, representing underutilized assets in the search for therapeutics and vaccines. Recently a bio safe vaccine strain developed by this laboratory has been excluded from the select agent list and can be used at BSL2 pending IBC approval and entity specific exclusion.

Previously, the utility of *Bp* 1026b Δ*asd* strain B0011 as a vaccine was demonstrated. In this paper, experiments were undertaken to expand the utility of this strain into standard assays used by researchers for *Bp* pathogenesis investigation. After finding that 250 µg/ml of DAP was an ideal concentration for allowing infection of multiple cell types of different lineages, images were captured of strain B0011 protruding from host cells while polymerizing actin. This shows that the mutation in the *asd* gene has no effect on the molecular pathogenesis of the mutant and that cell-type tropism seen with other model bacteria does not occur.

Several other wildtype *Bp* strains isolated from different sources (environmental and clinical) and different disease presentations (acute and chronic) were tested along with their Δ*asd* counterparts in two common cell infection models, HeLa and RAW264.7. First, this demonstrated that regardless of the source, all the strains were able to infect the cell lines and second, that there may be subtle changes in chronic isolates that predisposes them to slower growth rates during infection. That aside, the Δ*asd* mutants of the strains were still able to invade and infect the two cell lines but grew at a slower rate, similarly to what was seen with strain B0011. Future work will focus on getting the *asd* mutants of these other strains excluded from
the SA list for BSL 2 use. This would allow researchers to study the effects of source and disease presentation on pathogenesis in a BSL 2 laboratory.

Efficacy of strain B0011 in the invasion assay was tested and compared to wildtype 1026b. The mutation in the asd gene does not affect the ability of the mutant to enter any of the five cell lines tested, where approximately 1 in 50 cells become infected. Since there was no defect in invasion, the assumption that there is no defect in attachment is probably true. The mutant attaches, invades, and escapes the vacuole the same as the wildtype and would therefore be a good BSL2 model for studying these Bp infection stages in the cell types tested. Beyond invasion, the intracellular replication assay was carried out in several cell lines and found that in 293T and HeLa cells, when DAP was present, strain B0011 grew intracellularly at the same rate as the wildtype. Strain B0011 would be very useful for intracellular replication assays in 293T cells at BSL2. Microscopy revealed the presence of MNGCs followed by plaque formation. When a plaque assay was performed in 293T cells with DAP, plaques were the same size as wildtype at 24 hpi, indicating strain B0011 would be excellent for plaque assays at BSL2.

However, intracellular replication assays turned out quite different in the other cell lines tested. An initial drop then plateau of intracellular CFU and microscopic observation indicated long-term persistent infections atypical of rapid infections caused by wildtype bacteria. Previous studies of chronic animal melioidosis distinguish acute infection from chronic infections as those with lethality occurring before 7 days as acute and after 7 days as chronic [44,45]. In agreement with the previous studies, strain B0011 infects cell lines, besides 293T, chronically and up to the lifespan of the monolayer (23 days in this study). Until now there has not been a practical in vitro chronic cell infection model at any safety level, let alone for use at BSL2. Observation of these chronic cell infections revealed a novel intracellular niche previously unknown to be exploited.
by *Bp*. Endonucleobiosis was verified in wildtype *Bp*, necessitating modification of the infection model to reflect *Bp* exploitation of the intracellular niche. The nuclear niche is exploited by pathogens of the genus *Rickettsia* as a means to escape host-cell defenses and more recently intracellular bacteria have been found to produce proteins called nucleomodulins that directly modify host cell gene expression [46,47]. Once *Bp* enters the nucleus, it escapes the defenses and enters a high nutrient environment where it can potentially modify host-cell behavior. Furthermore, interesting implications arise when direct contact between host cell genetic material and naturally competent *Bp* strains capable of DNA uptake as nutrient source occurs. As an example, *Plasmodium falciparum* has been shown to take host cell DNA into its own nucleus [48] and genomic analysis of *Rickettsia bellii* indicates gene exchange between different bacteria while inside host amoeba nuclei [38].

Live-cell imaging is a technique that can tell us many things about bacteria host-cell interactions. B0011 can fuse neighbor cells, cause plaques, and be tracked through a host-cell during infection. When coupled with fluorescent microscopy a great many experiments can be envision such as live-cell co-localization experiments and membrane dynamics, all at BSL2 with a bio safe strain. Timing of the events can also be compared between strains in these types of experiments.

Expanding the *Bp C. elegans* infection model into BSL2 labs would allow for greater access to an animal model and allow further verification of data beyond cell infection models. We demonstrated that our *Bp* 1026b Δasd mutant, B0011, can replace wildtype 1026b in the typical survival and prolonged killing assays. A whole organism intracellular replication model was also introduced so, if desired, a virulence factor’s effect on intracellular replication at the animal level could be investigated. Bright-field videography of our B0011 infecting *C.elegans* in
virulence screens could be used in concert with high-throughput image analysis applications such as the recently published WormToolbox [49]. Fluorescent protein tagged B0011 can be visualized in the worm gut allowing studies such as in vivo observation of competition. Several virulence factor mutants were compared in their ability to kill C. elegans. The nature of the virulence factor under investigation will determine which C. elegans assay will demonstrate attenuation. It must be taken into account that during the fast-killing assay C. elegans are in constant contact with the bacteria that are also constantly ingested. In the slow-killing assay we saw the largest impact with a putative attachment factor mutant that our lab has been investigating. The brief exposure of the nematodes to the bacteria would necessitate efficient attachment for virulence factor-mediated damage to occur. This work has also introduced a new animal feeding model with the mealworm Tenebrio molitor. The mealworm-feeding model is very easy to use with mealworms being freely available at your neighborhood pet shop, such as PETCO®. The model also allows for tissue dissemination studies that can be difficult at the size of the nematode and the ease of removing the entire digestive tract quickly provides another benefit to this model. The role of LPS in invertebrate pathogenesis was verified in the various models. Previous works found no effect on pathogenesis but experimental design flaws of the initial study are believed to be the reason [23]. The T6SS-5 also contributed to invertebrate killing in the models tested, verifying its important role in animal pathogenesis.

We already demonstrated the usefulness of these models by discovering a novel intracellular niche occupied by Bp during acute and chronic infections that had not been observed in available models. Besides the innate costs of running a BSL3, time-lapse bright field and fluorescent microscopy equipment can be costly as well as cumbersome. Movement of these animal/cell infection models and assays to the BSL2 allows for much simpler observation and a
decrease in redundant, sometimes expensive, equipment. The ability to study \textit{Bp} pathogenesis in BSL2 model systems should accelerate the already burgeoning field and help reduce research costs. Lastly, we also developed and tested two new $\Delta$asd \textit{Bp} strains. \textit{Bp} K96243 is a very popular type strain in \textit{Bp} infection investigations. \textit{Bp} MSHR487 is an Australian strain from a chronic type infection. Both strains were found to be avirulent in BALB/c mice with no bacteria recovered from inside the animals. These strains have been excluded from the select agent regulations by the CDC and are available after entity specific exclusion is obtained. There are now 4 SA excluded strains of \textit{Bp} available, 3 produced in our lab, and with the diversification of strain background could be used in various investigations of \textit{Bp} pathogenesis.
5.6 ACKNOWLEDGEMENTS

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5.7 FIGURE LEGENDS

Figure 1. Growth curve of B0011 on various media and compared to wildtype. Growth of B0011 on LB agar was slower than on media with lower salt concentrations and a comprehensive growth curve using a Biotek plate reader was used to compare growth rates in different salted media. (A) Salt does affect the growth of B0011 and can be seen in comparing LB-Lennox broth (green line, 0.5% NaCl) to no salt LB broth (blue line, 0.0% NaCl). LB miller broth (1% NaCl) was not tested because of a long growth lag in this media. (B) When 200 µg/ml DAP is added to the media B0011 grows the same as wildtype 1026b and without DAP, B0011 cannot grow in no salt LB.

Figure 2. Invasion and replication assays of B0011 in several cell types. A) Invasion assays were carried out by infecting the indicated cell lines with B0011 and wildtype 1026b. DMEM was supplemented with 250 ug/ml DAP for the mutant. There was some variability between cell lines, but the variability in invasiveness between the WT and the Δasd mutant was not significant as determined by the student t-test. Replication assays were also performed on B) HEK 293T, C) BV2 murine microglial, D) CHME, human microglial, E) HTB-11, human neuroblastoma, and F) RAW264.7 macrophage cells. Infection of 293T cells by the mutant was similar to the WT. The remaining cell lines showed a decrease at the initial time-points. Bacterial numbers plateaued and persisted for up to 2 weeks pi. Each time point is done in triplicate with the error bars showing the SEM (not visible at this scale).

Figure 3. B0011 infects 293T and HeLa cells and causes plaques similar to wildtype. B0011 was tested in many cell lines. Intracellular replication of B0011 was most comparable to
wildtype 1026b in 293T cells (A) and HeLa (B). Phase contrast microscopy indicated cell fusion, bacterial replication, and host-cell lysis were occurring similarly to virulent \( Bp \) 1026b in the infection model at 24 and 48 hpi (C,D). Without DAP, B0011 was unable to create plaques in 293T cell monolayers. Plaques formed in the same time frame as wildtype infections when infecting 293T cells with B0011 in the presence of DAP within agarose overlays (F,G).

Figure 4. B0011 live-cell fusion and infection events using time-lapse microscopy. B0011 was used to infect monolayers consisting of two strains of HEK293 cells, each expressing either cytoplasmic GFP or RFP. When neighboring cells fuse and cytoplasm mixes, a yellow color is produced [22]. (A) Using live-cell time-lapse fluorescent microscopy (every 5 min for 30 h, above) of cell fusion all the way to plaque formation. (B) At higher magnification the moment of cell fusion and infectious processes such as actin polymerization are observed (top), and tracking software can track individual bacteria in live cells at BSL2 (blue and green lines; every 1 min for 4 h, bottom).

Figure 5. B0011 used with the \( C. \ elegans \) infection model at BSL2. In A), 30 \( C. \ elegans \) nematodes grown on \( E. \ coli \) OP50 to the L2 stage were transferred to a plate containing either \( B. \ thailandensis \) E264, B0011, or \( E. \ coli \) OP50. Nematodes were checked for movement at the indicated time-points and verified by gentle prodding. Survival % was determined to be the number of live animals/total number of animals and is the SEM of three experiments. B) 30 animals were exposed to the 1026b \( \Delta asd \) mutant or \( E. \ coli \) food source for 30 m then transferred onto \( E. \ coli \) lawns. Survival percentages are presented as Kaplan Meier survival curves in 3 independent experiments. C) Whole organism intracellular replication assay. Nematodes were
used in a modified intracellular invasion assay then lysed to determine intracellular bacteria. D, E, F, G, H) B0011 kills *C. elegans* in a dose dependent manner with the major contributor being live-cells and mainly the T6SS-1 surface component HcpI. I) Fast-killing assay on *Bp* mutants of known and putative virulence factor. 20 *C. elegans* were placed on plates containing the indicated *Bp* strain and survival % measured. Performed in triplicate with the SEM shown. J) Slow-killing assay on *Bp* mutants of known and putative virulence factors. 20 *C. elegans* were exposed to 1x10⁶ CFU of the indicated *Bp* strain for 30 m. Survival % is shown from an experiment done in triplicate with the SEM shown.

**Figure 6. B0011 in the Tenebrio molitor mealworm-feeding model.** B0011 is able to kill the mealworm *T. molitor* through feeding, (A). We found that the T6SS-1 *hcpI* is not important for killing of the worm in this particular feeding model. Combining numbers form three independent experiments generated the Kaplan-meir curve. B) Diagram of *T. molitor* anatomy (external, top; internal digestive tract anatomy, bottom). C) Bright field image of the *gfp*-tagged B0011 infected excised *T. molitor* digestive tract at 20X magnification (top). Green fluorescent channel of the same images (middle). Magnified images of the indicated insets at 63X in the green channel (bottom).

**Figure 7. BALB/c attenuation studies using two newly excluded *Bp ∆asd* mutants.** A) Survival curve of five BALB/c mice infected intranasally with 1x10⁷ CFU of the *Bp K96243 ∆asd* mutant. B) Survival curve of five BALB/c mice infected intranasally with 1x10⁷ CFU of the *Bp MSHR487 ∆asd* mutant. 3,000 CFU of wildtype killed all five mice by 3 dpi. All mice infected with ∆asd mutants survived the 60-day study. C and D) Bacterial organ loads of lung,
liver, and spleens of all Δasd mutant infected mice. No bacteria were detected in either *Bp* K96243 Δasd mutant or MSHR487 Δasd mutant infected mice, respectively.

**Figure 8. Proposed *Bp* cellular pathogenesis model including endonuclear niche.** *Bp* (purple) attach and are internalized using unknown bacterial gene products and host-cell surface receptors (yellow). Once internalized the bacteria escape the endosome utilizing the T3SS$_{Bsa}$ and move within the host cell by using flagella or by polymerizing host cell actin utilizing BimA. *Bp* can then enter the host cell nucleus (green) via active penetration of the nuclear envelope or become trapped after reformation of the envelope following mitosis. Bacteria can then be passed to daughter cells during replication, or form MNGCs with neighboring cells by actin-based membrane protrusion or direct membrane fusion using T6SS.

**Figure S1. Exogenous DAP can allow B0011 to infect many cell types.** The indicated cell lines were first grown in various concentrations of DAP alone to control for possible DAP toxicity. Cell lines appeared healthy up to 2,000 µg/ml of DAP (data not shown). The adequate concentration was first determined in all cell lines by supplementing DMEM/10% FBS with 0 µg/ml to 2,000 µg/ml of DAP at the indicated increments. As expected, bacteria were not recoverable from any of the cell lines 18 hpi without DAP present. Interestingly, a trend appeared that as the exogenous DAP concentration was increased the CFU recovered at 18 hpi decreased in most cell lines. A) BV-2, murine microglial cell line. B), CHME, human microglial cell line. C), HeLa, human epithelial cell line. D), HTB-11, human neuroblastoma cell line. E) RAW264.7, murine macrophage cell line. The bottom row are phase contrast microscopy
pictures taken 24 hpi at 600X. Carets indicate B0011 bacteria protruding from host-cells at 18 hpi.

**Figure S2. Differential infection by various Bp strains.** A) HeLa and B) RAW264.7 cells were infected with 9 strains of *Bp* isolated from various sources and their cognate ∆asd mutants produced previously (Table 1 and [9]) by lambda RED recombination. The data shown is the average of two independent 24 h intracellular replication experiments at an MOI of 1:1 with the SEM. Strains cause differential infection, particularly WT Bp6340 from a chronic case grows less than all the other strains tested in both cell lines. Adaptation to a chronic lifestyle could allow the bacteria to reach a homeostasis with the host cell and might explain why levels of the Bp6340 ∆asd are similar to wildtype *Bp* 1026b in the infected RAW monolayers after 24 h.

**Figure S3. Bp 1026b ∆asd persistently infects some cell lines in vitro.** Besides 293T cells, the replication assay and initial microscopy indicated that the cell lines remain infected with B0011 far beyond the normal *in vitro* “acute” infection time of ~24 h. Monolayers were infected at an MOI of 10:1 and media containing 750 µg/ml amikacin, 750 µg/ml of kanamycin, and 250 µg/ml DAP. Every 3 days, fresh media was added to the monolayers. Pictures are representative images at different time points taken from video clips during the course of infection. Carets indicate infecting bacteria. A) BV2, murine microglial cell line at 5 d.p.i. (100X) and 9 d.p.i. (40X), B) CHME, human microglial cell line at 16 d.p.i (100X) and 18 d.p.i (100X). Spheres containing tightly packed, motile bacteria are seen predominantly at later stages in many cell types. C) HTB-11, human neuroblastoma cells at 4 d.p.i. (60X) and at 22 d.p.i. (100X) a cell full of
bacteria (boxed). D) RAW264.7, murine macrophages at 7 d.p.i. (20X) and MNGC bounding a plaque at 10 d.p.i. (20X).

**Figure S4. During *in vitro* infection, both *B. pseudomallei* 1026b Δasd strain (B0011) and wildtype 1026b replicate in the host-cell nucleus.** The presence of near-perfect spheres containing tightly packed, motile bacteria in Fig. 5 was identified as intra-nuclear bacteria close observation of live cell time-lapse video. HTB-11 cells 8 d.p.i. containing *gfp*-tagged B0011 within a daughter nuclei of an undivided neuroblastoma cell. A) Green fluorescent channel. B) DIC bright field channel. C) Green fluorescent channel and brightfield overlay. D) TEM image of a RAW264.7 macrophage MNGC containing two nuclei with carets indicating endonuclear WT *Bp* 1026b at 1.2 kX magnification. E) TEM close-up image of a RAW264.7 nucleus containing six endonuclear bacteria at 4.0 kX magnification, two of which are indicated by carets. F) TEM image of a large nucleus without a cell membrane containing hundreds of endonuclear bacteria. G) RAW264.7 monolayer infected by WT *Bp* 1026b at 24 h. Nuclei are dyed blue with DAPI and the cell membranes are dyed red with FM 4-64X lipophilic dye. The blue, red, and DIC brightfield images are overlaid. The caret indicates two intact nuclei that have lost the cell membrane and remain floating in the media.

**Figure S5. The *Bp* 1026b Δasd mutant (B0011) can be used in the *C. elegans* infection model.** Nematodes were used in a modified intracellular invasion assay then lysed to determine intracellular bacteria counts. D) Green fluorescent image of *gfp*-tagged B0011 within the lumen of *C. elegans*. E) Time-lapse imaging of healthy worms compared to those fed strain B0011 as nutrient source. F) *gfp*-tagged B0011 are visible in the *C. elegans* gut. G) Fluorescent time-lapse
imaging of live infected *C. elegans*. The *gfp*-tagged bacteria are visible in the gut of the moving animal.

**Supplemental videos:**

**Video 1**: 293T cells 48 hpi a large amount of protruding and intracellular B0011 bacteria can be seen in this 5 m time-lapse video of Figure 3D.

**Video 2**: Red and green fluorescent 293 cells being infected with B0011. Time-lapse fluorescent microscopy of Figure 4A. Bright field is on the left and fluorescent channels on the right. Each frame is 5 minutes taken for 24 hours.

**Video 3**: DIC time-lapse microscopy of Figure 4B. B0011 was used to infect 293T cells. A picture was captured every minute for 5 hours. The image on the left is unmodified. The image on the right tracks two bacteria through the fusion of the neighboring cells.

**Video 4**: A short video of a healthy *C. elegans* nematode beside a B0011 infected nematode for comparison.

**Video 5**: A short fluorescent video taken of *C. elegans* after being fed *gfp*-tagged B0011.

**Video 6**: A short time-lapse video of B0011 infected BV-2 cells in Figure 5A 9 dpi.
**Video 7:** A short time-lapse video of B0011 infected CHME cells 16 dpi from Figure 5B. Spheres containing packed moving bacteria are visible.

**Video 8:** A short time-lapse video from Figure 5C of B0011 infected HTB-11 cells 22 dpi. The host-cell is still alive and is tightly packed with bacteria.

**Video 9:** A short time-lapse video from Figure 6B of B0011 infecting the nucleus of HTB-11 cells 22 dpi.
### TABLE 1. Bacterial strains and plasmids used in this study

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<td>EOP50</td>
<td>Rif'; E. coli B strain uracil auxotroph spontaneous rifampicin resistant mutant for <em>C. elegans</em> maintenance</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>EOP50-rif</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. pseudomallei</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1026b</td>
<td>B0004</td>
<td>Type-strain; clinical melioidosis isolate</td>
<td>[51]</td>
</tr>
<tr>
<td>1026b-(\Delta)asd::gat-FRT</td>
<td>B0011</td>
<td>GS'; 1026b with gat cassette inserted in the (\text{as}d) (_B) gene</td>
<td>[5]</td>
</tr>
<tr>
<td>1026b-(\Delta)asd::gat-FRT/attTn7-kan-gfp</td>
<td>X0004</td>
<td>GS', Km'; 1026b (\Delta)asd::gat-FRT mutant with mini-Tn7-kan-gfp integrated</td>
<td>This study</td>
</tr>
<tr>
<td>Bp0085</td>
<td>B0040</td>
<td>WT strain; clinical strain from a sepsis case (fatal) in Thailand, 2006</td>
<td>[9]</td>
</tr>
<tr>
<td>Bp0085-(\Delta)asd::pheS-gat</td>
<td>B0079</td>
<td>GS'; Bp0085 with pheS-gat-FRT fragment replacing the asd gene</td>
<td>[9]</td>
</tr>
<tr>
<td>Bp0091</td>
<td>B0042</td>
<td>WT strain; clinical strain from a sepsis case (fatal) in Thailand, 2006</td>
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<tr>
<td>Bp0091-(\Delta)asd::pheS-gat</td>
<td>B0080</td>
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<tr>
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<tr>
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<td>Bp4001</td>
<td>B0054</td>
<td>WT strain; environmental isolate from soil in Australia, 1997</td>
<td>[9]</td>
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<tr>
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<td>B0082</td>
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<td>WT strain; clinical strain from a sepsis case (fatal) in Australia, 1999</td>
<td>[9]</td>
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<tr>
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<td>B0083</td>
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<td>[9]</td>
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<tr>
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<td>B0064</td>
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<tr>
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<td>B0066</td>
<td>WT strain; clinical isolate from chronic case (survived) in Australia, 1991</td>
<td>[9]</td>
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<tr>
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<td>GS'; Bp4141 with pheS-gat-FRT fragment replacing the asd gene</td>
<td>[9]</td>
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<td>Bp4144</td>
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<td>WT strain; clinical isolate from chronic case (survived) in Australia, 1995</td>
<td>[9]</td>
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<tr>
<td>Bp4144-(\Delta)asd::pheS-gat</td>
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<td>GS'; Bp4144 with pheS-gat-FRT fragment replacing the asd gene</td>
<td>[9]</td>
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<tr>
<td>Bp6340</td>
<td>B0078</td>
<td>WT strain; clinical isolate from chronic case (survived) in Australia, 2003</td>
<td>[9]</td>
</tr>
<tr>
<td>Bp6340-(\Delta)asd::pheS-gat</td>
<td>B0087</td>
<td>GS'; Bp6340 with pheS-gat-FRT fragment replacing the asd gene</td>
<td>[9]</td>
</tr>
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</table>

\(^{a}\)Abbreviations: asd, *B. pseudomallei* aspartate semi-aldehyde dehydrogenase encoding gene; FRT- yeast 2 \(\mu\)m plasmid recombination target; gat, gene encoding glyphosate acetyltransferase; GS', glyphosate resistant; gfp, green fluorescent protein encoding gene; Km', kanamycin resistant; pheS- engineered mutant version of the \(\alpha\)-subunit of phenylalanyl tRNA synthase; Rif', rifampicin resistant.

\(^{b}\)Please use laboratory identification number (Lab ID) when requesting strains and plasmids. Request of select agent bacteria requires entity specific approval and requests may not be granted.
Figure 1
Figure 2
Figure 3 and Video1
Figure 4 and Videos 2 and 3
Figure 5  and Video 4
Figure 6
Figure 7
Figure 8
Figure S1
Figure S2
Figure S4
5.8 REFERENCES


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Chapter 6. Utilization of Tn-seq to analyze the intramacrophagic virulome of *Burkholderia pseudomallei*

(To be submitted to PLoS Pathogens)

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6.1 ABSTRACT

The select-agent bacterium *Burkholderia pseudomallei* possesses a large repertoire of virulence factors that culminate in the infection known as melioidosis. The objective of the research is to identify unknown virulence genes that contribute to the cellular infectious process through a genome-wide screen. A comprehensive transposon mutant library was constructed from an engineered strain of *B. pseudomallei* and used to infect RAW264.7 murine macrophages. Tn-seq was used to obtain between 6 and 10 million reads per sample. HT-Seq python scripts were used to count the reads per gene, which were visualized using IGV (Integrative Genome Viewer 2.3) and analyzed using MeV (Multi-Experiment Viewer). Bacterial genes that had lower reads after serial passaging through the macrophages were considered putative virulence genes. 901 genes were identified as being 10-fold negatively selected against. 333 of those genes were 100-fold negatively selected against and 113 were 1,000-fold negatively selected against. Several loci already associated with virulence were identified, including genes of the T6SS-5, Bsa (*Burkholderia* secretion apparatus T3SS), flagella, and LPS biosynthesis; lending confidence that the data are reliable. DAVID gene ontology analysis of the gene list indicated enrichment for the term pathogenesis. Numerous loci encoding secondary metabolite biosynthetic genes and transcriptional regulators were also enriched for. Cytotoxicity assays were then used to screen 134 mutants for virulence levels. 22 mutants that showed decreased cytotoxicity towards macrophages were further characterized in intracellular replication, attachment, and invasion assays. 5 genes were complemented for data verification and neighboring genes were investigated. 20 mutants were assessed in the BALB/c mouse inhalation melioidosis model with 10 demonstrating various levels of attenuation. Tn-seq is a powerful tool that allowed the identification of previously unknown virulence factors that contribute to *B.*
*pseudomallei* cell infection. Refinement of the intramacrophagic virulome provides novel targets for therapeutics and increases our understanding of the arsenal that *B. pseudomallei* utilizes to overcome host defenses.
6.2 AUTHOR SUMMARY

When the Public Health Security and Bioterrorism Preparedness and Response Act was passed by the 107th US congress in 2002, research on treatment and prevention of illnesses caused by the newly termed select agent bacteria (such as *B. pseudomallei*) became a public health priority. Several major virulence factors have been identified but full understanding of *B. pseudomallei* cellular infection is still lacking in depth and detail. Here we use a genome wide screen and a relatively new method of Tn-seq to identify the intramacrophagic virulome and novel virulence genes of *B. pseudomallei*. Initial analysis showed a large enrichment in putative and hypothetical genes indicative of the dearth of knowledge of the intracellular pathogenesis of *B. pseudomallei*. We found several novel secondary metabolite, putative, and hypothetical genes that play a role during macrophage infection and cell-cell spread. Screening of mutants allowed identification of virulence factors that can be putatively assigned roles in attachment, invasion, intracellular replication, cell-cell spread as well as *in vivo* infection.
6.3 INTRODUCTION

The melioid bacillus, *Burkholderia pseudomallei* (*Bp*), is typically found in soil and water environments between the latitudes of 20°N and 20°S and so to is melioidosis primarily a tropical disease [1]. In Thailand, very high levels of the bacterium are found in the pooled surface waters of rice paddies [9] coinciding with high disease and seroprositivity rates in rural rice farmers. Typically there is a regional hospital at great distance from the individuals home and by the time they arrive at the hospitals, the disease has progressed acutely leading to a high mortality rate of 40.5% [12]. 79% of individuals diagnosed as having melioidosis are farmers and nearly 70% of those are patients that have type 2 diabetes mellitus that is poorly controlled or undiagnosed [13]. Not only does the melioid bacillus pose a public health challenge in Northeastern Thailand but the US Centers for Disease Control have listed *Bp* as a Tier-1 select agent. Tier 1 organisms have the potential to pose a severe threat to US public health and safety and the US congress passed laws to develop vaccines and therapeutics for them, including *Bp* [20]. An important step in combating a disease, is understanding the fundamentals at the base. In this case, only by understanding the molecular pathogenesis of *Bp* will an effective vaccine come to fruition.

When *Bp* cells come into contact with host cells, a plethora of host modifications occur in response to bacterial infiltration and virulence factor expression leading to the diseased state. Within-host, *Bp* can infect most tissues and invades, then replicates inside the cytoplasm of many cell types [51,57-61]. To accomplish this feat *Bp* attaches to the host cell via an unknown mechanism, causing actin rearrangement and inducing bacterial phagocytosis [62]. Once, internalized *Bp* utilizes the *Burkholderia secretion apparatus* (Bsa, one of three genomically encoded type 3 secretion systems, T3SS) to secrete the effector BopE through the membrane of
the phagocytic vesicle [63,64]. BopE is a guanine-nucleotide-exchange factor that interacts with the Rho GTPase Cdc42 undermining the surrounding cytoskeletal framework, damaging the membrane and allowing vesicular escape before phagosomal degradation of \( Bp \) occurs [65,197]. Once inside the cytoplasm the well characterized \( Bp \)-intracellular motility protein A, BimA, polymerizes host-cell actin [69]. The polar actin “tail” allows intracellular movement and eventually leads to formation of bacteria containing membrane protrusions [70]. The membrane protrusions allow \( Bp \) to seek out uninfected cells and begin the cycle anew without transitioning into the extracellular milieu. Intracellular motility can quickly oscillate between actin-mediated movement and use of flagella, where \( Bp \) swims within the host-cell cytoplasm [71]. During the infectious process an infected cell may fuse with healthy neighboring host cells and is at least partially attributed to the main \( Bp \) type 6-secretion system (T6SS-5; nomenclature according to Shalom et. al.) [72,180]. The formation of multi-nucleated giant cells (MNGCs) has been observed \textit{in vitro} and \textit{in vivo} and is a major hallmark of \( Bp \) infection [70,73]. The bacteria replicate concurrently with the production of several known and unknown virulence factors causing death of the host-cell. However questions remain, how does \( Bp \) sense when to fuse, where to travel, what nutrients to use, how are mediators (e.g. autophagy) of innate immunity avoided, and what causes the observed cytoskeletal rearrangements? These questions are indicative of the knowledge gap present in fully understanding the molecular pathogenesis of \( Bp \).

Mutating many of these virulence factors lead to attenuation in mouse models of infections but \textit{in vitro} cell infection mutational studies indicate a delay of the cellular infectious events rather than abolishment [67,72,89]. \( Bp \) has nearly 6,000 genes and an open genome [90,91]. Many genes (approximately 35.5% in \( Bp \) strain K96243) are hypothetical or putative with no proven function [90]. It stands to reason that there are \( Bp \)-specific virulence factors
contributing to disease that remain to be discovered and that mutating these factors may produce a live-attenuated strain that is capable of protecting from inhalation melioidosis. Our data indicate this to be true. The development of a vaccine remains elusive, placing urgency on elucidating the functional contributions of hypothetical/putative genes to the molecular pathogenesis model. In total, we consider the effort of this work in identification of novel virulence factors to be of significance to the field.

Here, a genome wide screen and the relatively new method of Tn-seq [92] were used to identify the intramacrophagic virulome and novel virulence genes. Initial analysis showed a large enrichment in putative and hypothetical genes indicative of the dearth of knowledge of the intracellular pathogenesis of Bp. Cytotoxicity assays were used to characterize the virulence of 134 mutant Bp strains of the initial 901 putative virulence candidates identified. Intracellular replication assays were used to measure reduced replication inside RAW264.7 macrophages. Growth curves were carried out to make sure there were no in vitro growth defects accounting for reduced intracellular replication. Mutants showing no in vitro growth defect and reduced intracellular replication were submitted to invasion and attachment assays. Fluorescent microscopy was used to characterize cell fusion, actin polymerization, and plaque formation when compared to wildtype and a T6SS-5 mutant (tssC-5::T24) identified in the Tn-seq data. Two genes were chosen for mini-Tn7 based complementation and verification in intracellular replication assays. Neighboring genes of these 2 were investigated in their cytotoxicity and intracellular replication characteristics. Several novel secondary metabolite, putative, and hypothetical genes that play a role during macrophage infection and cell-cell spread were identified and verified to be essential for full virulence of Bp. Twenty candidate mutants were used to challenge BALB/c mice in an intranasal infection model. Ten of those mutants
demonstrated some level of attenuation and have now been added to the arsenal that is the *Bp* intramacrophagic virulome.
6.4 RESULTS

Tn-seq analysis of the intramacrophagic virulome of Bp. To identify any and all genes of the ‘intramacrophagic virulome’ a saturating transposon library was created in Bp K96243 Δasd strain [28] using a pBT20 transposon based on non-antibiotic complementation (Fig. 1). Briefly, the transposon mutant library was pooled and used to infect the murine macrophage cell-line, RAW264.7. The infection was allowed to proceed for 12 h, for a total of three passages. The genomic DNA from passaged and unpassaged pools were isolated and transposon/genome junctions identified by Tn-seq on the Illumina system [27]. Infection replicates at multiplicity of infections (MOIs) of 10:1 and 1:1 were carried out and data compared to the unpassaged pool. Genes with reads before but not after passaging were implicated as the ‘intramacrophagic virulome’. Following analysis we found many genes in both replicates and grouped them into those ≥10, ≥100, or ≥1,000 times negatively selected against. Presumably, the higher the negative selection the more essential the genes are to the intracellular infectious process. A majority of genes were shared between replicates (Fig. 2A). 901 genes showing ≥10 negative selection were shared between both replicates. 333 of those genes (roughly one third) underwent ≥100 negative selection in both replicates and 113 genes ≥ 1,000 (Table S1). Each time the stringency of selection was increased 10 times, one third of the genes dropped out. The genes shared between replicates, located in the overlapping regions of the Venn diagrams (Fig. 2A), were further analyzed for functionality and a large amount were ascribed a hypothetical or putative function. When looking at the classification for all genes in the K96243 genome ~35.5% are hypothetical or putative. Among the genes with ≥10-times negative selection from the Tn-seq data that number increases to 51.3% and among genes with ≥1,000-times negative selection, hypothetical and putative genes comprise 76% of the group
Fig. 2B). At higher stringency of selection there is an increasing majority of genes considered hypothetical or putative. When analyzed in the context of intracellular infection, it becomes clear that there remains a lack of knowledge surrounding \( Bp \) activity during host-cell infection.

**Functional Analysis of Genes Identified from Tn-seq.** Functionally annotated genes were clustered with DAVID [29] to compare functional enrichment in our data to levels in the \( Bp \) K96243 genome. Many known virulence factors were present in the gene lists as well as some metabolic processes that \( Bp \) conducts during host cell infection (Fig. 3A). The ≥100-fold selection showed the greatest enrichment in virulence associated terms including, “pathogenesis, LPS, flagellum and cilia” while increasing the stringency to ≥1,000-fold left “flagella and cilia” and metabolic processes. The processes “actin cytoskeletal rearrangement” and “activation of Rho GTPase activity” are known to be important for \( Bp \) cellular pathogenesis as described above. “Trehalose biosynthesis” is also known to be important for infection and is enriched 10 times in all groups beyond the number present in the K96243 genome. The “chemotaxis” functional group was enriched by 5 times and those genes may be responsible for \( Bp \) detection of chemical gradients in the cell. Functional ontology analysis was accompanied by looking at the individual genes of known virulence factors. If the screen was successful, many of the well-characterized virulence factors would have ≥10 times negative selection. Flagellar motor, hook, and filament genes as well as genes involved in flagella expression were identified (Fig. S1A). Genes involved in LPS biosynthesis (Fig. S1B) and structural genes of the T2SS, T3SS, T6SS, and twin arginine targeting systems also experienced ≥10 times negative selection (Fig. S1C). The well-characterized Bsa effectors BopA, BopE, and Cif\(_{bp}\) were enriched between 10 and 100 times underscoring the relevance of the dataset to \( Bp \) pathogenesis. A quick perusal of read
saturation across samples and chromosomes gives an immediate indication of the rich data obtained by the method (Fig. 3B). The horizontal axis represents each of the \textit{Bp} chromosome and samples with log_{10} reads as the vertical lines. Virulence loci are visible by looking at areas in the 10:1 and 1:1 that have less read saturation than the initial pool, represented in Figure 3B by emphasizing the known virulence locus of the T6SS-5 and Bsa. The virulence loci (areas of decreased read saturation) include two loci of branched amino acid transport and utilization, two genomic islands (GI7 and GI16), four unknown and two known (malleilactone and syringolin) secondary metabolite operons, two well characterized (T6SS-5 and Bsa) and three uncharacterized (BPSS0958-0963, BPSS1250-1265, and BPSS1614-1630) \textit{Bp} secretion apparatuses (Fig. S2A chromosome 1 and Fig. S2B chromosome 2). Genes that received zero reads from either replicate after passage were analyzed and divided into groups based on the number of reads in the initial pool. Two flagella genes are located on the list. FliL is at the top of the list with several thousand reads from the initial pool compared to none after the passaging. The \textit{Salmonella enterica} FliL mutant is impaired in swimming and completely deficient in swarming where the torsional stress causes rod breakage and ejection [30]. Other genes with over 1,000 reads are stress-induced morphogen \textit{bolA}, histidine utilization gene \textit{hutf}, and several housekeeping genes. Genes of known functional roles in pathogenesis (i.e., Bsa and T6SS) validate that this method can be used to identify other genes of unknown function in the \textit{Bp} intramacrophagic virulome.

**Characterization of Hypothetical and Putative Virulence Factor Candidates.**

For this study the primary interest was in the hypothetical, putative, and uncharacterized genes and whether they could be described as virulence factors. All genes from the \(\geq 100\) and
1,000 list with hypothetical function annotations were chosen and combined with all genes with putative functions from the ≥1,000 list resulting in a 134 gene list of putative virulence factors for further characterization. 134 insertional mutants, representing the 134 genes, made in the Bp strain 1026b background were obtained for a high-throughput cytotoxicity screen in a modified aminoglycoside protection assay to measure damage to RAW264.7 macrophage monolayers (Table S2). The *tssC*-5 gene (BP1026B_II1588), forming part of the contractile sheath along with *tssB*-5 in the major T6SS-5 and confirmed with great detail in *P. aeruginosa* [31], was identified in the ≥100 list, and its insertional mutant was used as a negative control for this experiment along with wildtype 1026b. Figure 4A shows the data obtained from the cytotoxicity experiment carried out in triplicate for each mutant represented as a percentage of total monolayer lysis (refer to table S2 for identification of mutants). Many mutants exhibited lower cytotoxicity than wildtype at 24 hours post-infection (hpi) and 32 of them exhibited statistically significant lower levels of lactate dehydrogenase (LDH) release (Table 1). Growth curves on all 32 mutants were carried out to ensure the low cytotoxicity readings were not because of *in vitro* growth defects (Fig. 4B). Our experimental setup was designed to limit this effect but was essential before moving forward with characterization. Two mutants showed significantly different growth characteristics when compared to wildtype (Fig. 4B, orange line). Mutant 84 (Fig. 4B, blue line, BP1026B_I2891, oxidoreductase, FAD-binding protein) and mutant 96 (Fig. 4B, red line, BP1026B_I0173, addiction module antidote protein) had interesting growth characteristics but were excluded from further characterization. Mutant 60 (Fig. 4B, green line, BPS1026B_I1231, hypothetical protein) was slightly lower than wildtype but was left in the pool for further characterization.
**Intracellular Replication Assays of 21 Putative Virulence Factors.**

Modified aminoglycoside protection assays performed in triplicate were used to determine defects in intracellular replication of 21 mutants from the list while using internal positive (mutant 39, BP1026B_I1079) and negative (mutant 126, tssC-5) controls as determined from the cytotoxicity assay in comparison to wildtype. Average intracellular CFU as a percentage of wildtype was compared to average cytotoxicity as a percentage of wildtype at 24 hpi (Fig. 4C). For some of the mutants (mutants 21, 25, 27, 60, 78, 95) cytotoxicity and intracellular replication agree, suggesting a linkage between cell injury and replication for these virulence factors. Others (mutants 2, 4, 5, 7, 8, 16, 20, 108) show an inverse relationship between cytotoxicity and intracellular replication with low cytotoxicity levels and wildtype levels of intracellular replication. Only three of the 21 mutants (mutants 110, 113, 119) have higher cytotoxicity percentages than CFU percentages but both being lower than wildtype.

Additional time points of the aminoglycoside protection assays were carried out to look at the overall intracellular growth trend of all 21 mutants (Fig. 4D). Two strains had extremely different replication assay curves. Mutants 60 and 95 had no deficiencies in *in vitro* growth but showed a severe defect at 2, 12, and 24 hpi. Mutant 60 (BP1026B_I1231) encodes a hypothetical protein, which shows high similarity to the *E. coli* ZipA protein. In *E. coli* the ZipA protein is a membrane-anchored protein that recruits the Fts complex during cell division [32]. Mutant 95 (BP1026B_I0717) encodes the transmembrane cell division/chromosome segregation protein FtsK [33,34]. This mutant also showed no defect in *in vitro* growth but substantial defect in intracellular replication. Mutants 4 (BP1026B_I1575), 20 ((BP1026B_I1256), 25 (BP1026B_I2174), 108 (BP1026B_I1664), and 113 (BP1026B_I10522) were deficient in intracellular replication at 12 hpi compared to wildtype (Fig. 4D, red line and bar). Mutants 21
27, and the two cell division mutants had replication levels comparable to or less than the tssC-5 mutant (Fig. 4D, orange line and bar) at 12 hpi. The remaining mutants had less replication than wildtype but not significantly so. By 24 hours the two cell division mutants had the highest defect in replication compared to wildtype and 7 mutants (20, 21, 25, 27, 110, 113, 119) had significantly lower intracellular CFUs with the mean CFU, $2.42 \times 10^5$, being 48% of the wildtype and positive control levels. All 22 mutants were then submitted to an invasion assay to determine if the defect of these mutants was at the vacuolar escape stage of the intracellular infection. \textit{Bp} were allowed to infect the cell monolayers for 1 h to allow internalized bacteria to escape the phagocytic vesicle into the host-cell cytoplasm. Following an hour of killing, the host cells were lysed and intracellular CFU were counted then divided by the amount used for the infection, resulting in the invasion efficiency (Fig. 5A). Those mutants that showed an invasion frequency significantly lower than the wildtype (mutants 2, 4, 5, 16, 20, 21, 25, 27, 60, 78, 80, 95, 109, 110) were submitted to an attachment assay to help define which stage of infection the mutants were deficient in (Fig. 5B). Both cell division mutants, FtsK and ZipA, were deficient in invasion but only the FtsK mutant was deficient in attachment.

At least some of the mutants were believed to have a deficiency in cell-cell spread. To understand the mutants’ ability to spread from cell to cell, plaque assays were carried out for 14 of the putative virulence factor mutants and plaque diameters were measured and compared to both the tssC-5 mutant, the internal positive control, and wildtype 1026b (Fig. 5C). Nine of the mutants produced significantly smaller plaques when compared to the wildtype but were not as small as the tssC-5 mutant. These nine mutants are hypothesized to be deficient in cell-cell spread to some degree. Insertional mutants in genes BP1026B_I1969 (a LivH-like branched
chain amino acid permease) and BP1026B_I1463 (putative glutathione-S-transferase) produced plaques with a diameter slightly smaller than wildtype. Insertional mutants of seven genes (BP1026B_I1231, BP1026B_I1764, BP1026B_I2174, BP1026B_I2220, BP1026B_I1256, BP1026B_I1463 and BP1026B_I1694) formed plaques significantly smaller than wildtype but slightly larger than the \textit{tssC-5} insertion mutant.

**Microscopy and Cell Infection Characteristics of Select Putative Virulence Factors.**

Plaque diameters were measured but plaque phenotype was investigated to note any differences to wildtype. Figure 6A shows representative plaque images from RAW264.7 macrophages infected with the indicated mutants for 24 h. Mutants of BP1026B_I1969 and BP1026B_I1463 were verified to create plaques phenotypically similar to wildtype but of smaller size. Mutants of BP1026B_I1764, BP1026B_I2174, BP1026B_I2220, and BP1026B_I1256 were unable to form plaques similar to the wildtype. The specified mutants appeared unable to cause cell fusion as efficiently as wildtype resulting in globular MNGCs that never fuse with neighboring uninfected cells. Plaques formed by these mutants resemble larger versions of MNGCs formed by the \textit{tssC-5} mutant. Gene BP1026B_I1231 (\textit{zipA}) created plaques but were neither circular nor as extensive as the plaques of wildtype. All 32 mutants that were analyzed in the growth curve experiment were used to infect RAW264.7 murine macrophages. The infected monolayers were fixed and the monolayers were stained as described in the materials and methods to visualize nuclei (blue), actin (green), cell membranes (red), and bright field for monolayers. Several mutants did not form plaques as well as the wildtype. Mutant 39 and the \textit{tssC-5} were used as positive and negative controls for the data in addition to the uninfected and wildtype controls for plaque formation. The insertion mutants within the \textit{ftsK},
zipA, and antidote protein (mutant 96, BP1026B_I0173) had major decreases in MNGC formation and plaque formation. Insertions in ftsK and the antidote protein (mutant 96, BP1026B_I0173) greatly affected in vitro growth so an inability to infect was expected. However, the zipA (BP1026B_I1231) insertion mutant formed long strands of bacteria that are stuck at various cell division stages in vitro (Fig. 6B, left) similar in phenotype to an effective Yersinia pestis vaccine strain mutated in the nlpD gene [35]. The Bp zipA mutant showed decreased invasion efficiencies but was still able to infect RAW264.7 macrophages. Long strands of Bp are seen in the phase contrast image inside the MNGC and when overlaid with the fluorescence you can see red tendrils of infecting bacteria (Fig. 6B, right). Actin polymerization appears to have a slight defect most likely because of difficulty localizing to the poles, translating to a defect in cell-cell spread.

Other mutants did not have such an obvious bacterial phenotype but the infection characteristics were much different for some of the mutants tested compared to our controls. Several mutants demonstrated reduced plaque formation (Fig. 6C). Bacteria in red have spread to many cells but fusion and plaque formation appears to lag behind that of the wildtype infected monolayers.

**Virulence Phenotype Summation.**

A visual summation of all the virulence data is presented in a “mutant virulence phenotype” matrix or MVP matrix (Fig. 7). Each mutant was assigned a number based on the significance of difference to wildtype in the various assays then clustered on similar trends. Red tiles mean similarity to wildtype, the brighter the green the more lacking the phenotype of the mutant compared to wildtype. All mutants have lower cytotoxicity levels compared to wildtype
whether they are significant or not. Mutants were clustered into 7 groups based on performance in all the assays. Group 1 consists of only the BP1026B_I1256 mutant and performed consistently worse than wildtype in all the assays suggesting a deficiency in all aspects of infection. Group 2 includes BP1026B_I10927 (79), BP1026B_I1764 (8), and BP1026B_I1463 (7). These 3 genes behave the same as wildtype except for in the plaque formation category, where they form significantly smaller plaques indicative of a cell-cell spread deficiency. Group 3 genes have deficiencies after attachment including invasion, replication, and plaque formation. The \textit{tssC-5} insertion mutant is part of this group that also includes BP1026B_I1231, BP1026B_I2220, and BP1026B_I2174. The outlier is the \textit{ftsK} mutant, which was unable to progress through any of the infectious stages. The three mutants in Group 4 (BP1026B_I2818, BP1026B_I0522, and BP1026B_I3714) show no difference in plaque formation but show inabilities in invasion or replication. Group 5 mutants (BP1026B_I2523 and BP1026B_I1664) show behavior similar to wildtype in all assays except cytotoxicity and may represent genes that cause release of a cytotoxic compound. Eight group 6 mutants (BP1026B_I1747, BP1026B_I1969, BP1026B_I1522, BP1026B_I1575, BP1026B_I0034, BP1026B_I0207, BP1026B_I1508, and BP1026B_I1870) show no difference in intracellular replication or plaque formation but show deficiencies in the beginning stages of infection resulting in lower cytotoxicity. Group 7 includes wildtype 1026b and the internal positive control mutant, BP1026B_I1079, both with no deficiencies in any of the assays.

\textbf{Analysis and Complementation of Two Virulence Candidates and Neighboring Genes.}
The *Bp* *phoU* gene is BP1026B_I2174, and the *E. coli* *phoU* has been well characterized as a negative regulator of the phosphate regulon (knocking out results in constitutive expression of the pho regulon). *PhoU* lies at the center of the phosphate transport operon with phosphate permease subunit genes (BP1026B_I2175-BP1026B_I2178, *ptsB, A, C, S*) and phosphate sensing (BP1026B_I2172, *phoB*) and regulatory genes (BP1026B_I2173, *phoR*); (Fig. 8C). The *phoU* gene has been shown to mediate adherence of entero-pathogenic *E. coli* to epithelial cell cultures and the murine urinary tract [36,37] that upon mutation results in attenuation of septicemia-inducing *E. coli* in pigs [38]. The *Bp* phosphate regulon sensor protein, *phoR* (BP1026B_I2172), also underwent >1,000 times negative selection during the initial screen. However, the BP1026B_I2174 mutant was not defective in attachment to RAW264.7 cells. This may be because phosphate-limiting conditions do not predominate until *Bp* is in the phagocytic vesicle, therefore deregulation of virulence expression does not occur until the invasion stage.

The second gene is BP1026B_II1256. It is a hypothetical protein that is Sec secreted with an alpha-beta hydrolase fold. BP1026B_II1256 is predicted to be in an operon with a non-ribosomal peptide synthase (NRPS) (BP1026B_II1250) which underwent >10 times negative selection and an N-acylhomoserine lactone synthase (BP1026B_II1251) which underwent >1,000 times negative selection. These genes form a secondary metabolite biosynthetic cluster with several polyketide synthases (PKS) and the entire region was analyzed with antiSMASH [39]. The region from BP1026B_II1224 to BP1026B_II1284, was identified as a 95.312 kb secondary metabolite cluster with BP1026B_II1256 near the middle (Fig. S4A). The product is an NRPS-typeI PKS-homoserine lactone hybrid molecule with the predicted scaffold indicated in figure S4B. This region is conserved among many *Bp* strains (Fig. S4C) but a homologue of BP1026B_II1256 is conspicuously absent from *Burkholderia thailandensis* E264. Additionally, a
~15kb region containing secondary metabolite genes near a mobile element, BP1026B_II1278 through BP1026B_II1286, is 1.4 Mbp away on the *B. thailandensis* E264 chromosome II. The region contains transposase genes, hypothetical genes, and a T6SS Rhs element VgrG gene. Gene clusters as compared by antiSMASH and webACT show the missing gene and regions (Fig. S4C and S4D). Movement of the genes may result in changing of the core scaffold, tailoring reactions, or expression of the secondary metabolite. The regions are conserved between *Bp* strains 1026b and K96243 (data not shown).

Mini-Tn7-*gat* was used to complement the 2 insertion mutants [28]. Complementation was verified with an intracellular replication assay in RAW264.7 macrophages (Fig. 8A and B) with insets showing growth defects in the linear scale. Complemented strains had intracellular replication similar to the wildtype. Plaque assays also verified complementation with both mutants showing significant defects in comparison to the complemented strains and wildtype but not as low as the *tssC*-5 mutant (Fig. 8C). Neighboring insertional mutants to these two target genes were used to infect RAW264.7 macrophages and their cytotoxicities as a percentage of total monolayers lysis were measured and compared to the cytotoxicity levels of the gene of interest, the *tssC*-5 mutant, mutant 39, and wildtype (Fig. 8D and E). Mutants in genes *pstA* (BP1026B_I2176) and *phoR* (BP1026B_I2172), which neighbor BP1026B_I2174, were found to have cytotoxicity levels lower than wildtype, *phoR* had significantly lower levels. The NRPS/PKS containing BP1026B_II1256 is predicted to span 60 genes from BP1026B_II1224 to BP1026B_II1284. In this work we only looked at the immediate operon from BP1026B_II1245 to BP1026B_II1256 that are in the same operon as BP1026B_II1256 (Fig. 8E). Only our original target gene showed very low cytotoxicity levels. BP1026B_II1256 may be a tailoring gene that modifies a major functional portion of the NRP.
BALB/c attenuation study.

Twenty candidate virulence factors that showed decreased cytotoxicity were tested in the BALB/c intranasal melioidosis mouse model (Fig. 9A). 10 mutants had no attenuation in vivo (Fig. 9B). 8 mutants (BP1026B_I1522, BP1026B_I1664, BP1026B_I1764, BP1026B_I2174, BP1026B_II0207, BP1026B_II1256, BP1026B_II1463, and BP1026B_II1508) showed various levels of attenuation but only one mutant (BP1026B_I1231) was completely avirulent with all mice looking healthy and only one had low levels of bacteria in the lung at 60 dpi (Fig. 9I and J). Mice infected with 10 times the LD$_{50}$ (4,500 CFU) of wildtype $Bp$ 1026b succumb to the infection by day 3 (red line, all curves). Five mice were infected with each insertion mutant. From most attenuated to least attenuated: mutants of BP1026B_II1463, and BP1026B_II1508 had nearly all mice survive to 4 dpi (days post infection) with 60% survival the rest of the study (Fig. 9G). Two surviving mice from each had bacteria in the lungs and high levels of bacteria in the spleens (Fig. 9H). Splenic abscesses were observed in some survivors. Mutants of BP1026B_I1522, BP1026B_I1764, and BP1026B_I2174 had a 40% survival rate (Fig. 9D). There were no bacteria detected in surviving mice after infection with mutant BP1026B_I1522 but survivors from the other two groups had between 10 and 1,000 CFU in the lungs (Fig. 9E). Mutants of BP1026B_I1664, BP1026B_II0207, and BP1026B_II1256 had a 20% survival rate (Fig. 9C) and all surviving mice had bacteria in the lungs but not in the liver or spleen (Fig. 9D). Generally the higher bacterial load in the spleen the more pronounced the splenomegaly.
6.5 DISCUSSION.

Nutrient acquisition: A major component of understanding intracellular infection is identifying where *Bp* obtains nutrients for such robust growth. The branched chain amino acid transporter *livH* experienced almost 4,000 time negative selection. The *liv* ABC transporter is essential for maximal *Streptococcus pneumoniae* infection in both pneumonic and septicemic models [40]. *Francisella tularensis* has also been shown to utilize free amino acids derived from autophagic degradation of host-cell proteins as an energy source during intracellular infection [41]. The *livH* *Bp* mutant did not show a growth defect *in vitro* indicating branched chain amino acids are a nutrient source in the host cell. A cystine binding transport gene (BPSL1867, BP1026B_I1828) was selected for >3000 times in both replicates. Cystine is essentially two cysteine molecules covalently linked via a disulfide bond and is released upon degradation of host proteins. A histidine degradation gene (BPSL2339, BP1026B_I0987) was also identified. Genes involved in phosphate sensing (BPSL1365, BP1026B_I2172, *phoR*) and regulation of the phosphate regulon (BPSL1363, BP1026B_I2174, *phoU*) had very high levels of negative selection, whose effects on infection by *Bp* were characterized in this work. Taken together *Bp* transports and degrades amino acids while replicating intracellularly and requires the ability to respond to nutrient limitations present inside the host cell.

Regulators: Twelve regulatory genes were identified in the Tn-seq data with >1,000 times negative selection in both replicates. Several of the regulators have been characterized by our lab independently of this work and have been shown to be of importance in the inhalation BALB/c mouse infection model (in preparation).
Secretion systems: The Bsa (T3SS-3) effector genes *bopC, bopA, and bopE* were negatively selected for through the passage in addition to the Bsa sorting platform gene BPSS1538 (BP1026B_II1634, *bsaV*) and the secretion apparatus gene BPSS1543 (BP1026B_II1639, *bsaQ*). Interestingly, potential effector genes BPSS1386 (BP1026B_II1478, a hypothetical protein with a GTP binding domain and an actinin-type actin binding domain; effective T3 score of 0.76299 [42]) and BPSS1416 (BP1026B_II1508, a hypothetical protein 73% homologous at the amino acid level to the *Pseudomonas syringae* effector HopAN1) of the T3SS-1 apparatus were also identified as having >100 and >1,000-times negative selection respectively. Host actinin is regulated by phosphorylation to mediate association of F-actin bundles with cell adhesion points and between actin filaments [43]. The actin-binding domain of BPSS1386 would target the protein to the ends of actin bundles where the GTP binding domain would serve as a GTPase-Activating Protein (GAP) and terminate downstream adhesion or rearrangement events. The *P. syringae* HopAN1 protein has been observed to inhibit the innate immune response in plants [44] and may perform similar functions in *Bp*. BPSS1394 (BP1026B_II1486, *sctN*) and BPSS1407 (BP1026B_II1499, *sctD*), genes encoding basal body proteins of the T3SS-1 were identified in the Tn-seq data but barely made the >10-fold cutoff. In agreement with previous studies of apparatus knockouts, weak selection of the structural genes indicates they are not contributing greatly to pathogenesis. Strong selection of the effectors of T3SS-1 indicates that they play an important role and may be secreted via other secretion systems affecting pathogenesis.

Structural genes of the major T6SS-5 were identified with the *tssC-5* gene undergoing the highest negative selection. Gene *tssL-1* (BPSL3111, BP1026B_I0182), a *dotU/icmH* homologue, of the T6SS-1 had very high levels of negative selection in both replicates of the Tn-seq data but cytotoxicity levels did not show any difference from wildtype. Previously published data show
that Syrian hamsters vaccinated against components of T6SS-1 can be protected from infection and mutating this T6SS resulted in slight attenuation over the wildtype [20]. The tssC-2 gene (mutant 40, BPSS0098, BP1026B_I0106) of the T6SS-2 showed a high level of negative selection and cytotoxicity levels lower but not significantly lower than wildtype. Two genes of the T6SS-3 (BPSS0173, BP1026B_I10194 and BPSS0175, BP1026B_I0174) underwent low levels of negative selection. The ATPase and tagAB-4 genes of T6SS-4 were both negatively selected against with tagAB-4 experiencing nearly 500 times negative selection. The protein contains pentapeptide repeat regions and a partial C-terminal PipB2 domain of the Salmonella secreted effector PipB2. PipB2 is secreted through both T3SSs of Salmonella and interacts with the host-cell kinesin-1 motor complex while in the bacteria containing vacuole to modulate vesicle trafficking [45,46].

Other known virulence factors: The Tn-seq data had many genes involved in capsular polysaccharide synthesis. Genes wcbB, wcbD, wcbG experienced >100 times negative selection while wcbP, wcbS, and wcbT experienced >10 times negative selection. The importance of the capsular polysaccharide to Bp replication within macrophages has bee previously reported [47]. A putative undecaprenyl phosphate N-acetylglucosaminyl transferase (BPSL2671, BP1026B_I0648) involved in LPS biosynthesis experienced very high levels of negative selection at >4,000 times negative selection in both replicates. A hypothetical protein involved in LPS biosynthesis (BPSL0537, BP1026B_I2958) experienced >100 times negative selection after passaging. A Bp LPS mutant has been shown to have the same replication levels inside RAW264.7 macrophages [48] which would explain why more genes involved in LPS synthesis were not identified with our screen. The YadA-like autotransporter adhesin, bpaC (BPSL1631, BP1026B_I1575) showed negative selection and our data agreed with the previous publications
that this protein effects attachment in B. mallei and Bp but has no impact on mouse survival [49,50].

**Uncharacterized virulence factors:** Secondary metabolite operons encode for a diverse array of molecules; from the siderophore malleobactin [51] to the proteasome inhibitor syringolin [52]. There are a predicted 16 clusters on the first chromosome and 24 clusters on the second chromosome spanning a total of ~1.3 Mb of DNA or ~18% of the genome. Dedicating such a large region of the genome to these clusters underscores the importance of the produced molecules to Bp survival in diverse environments. Several NRPS/PKS secondary metabolite operons were indicated as having importance during the infection (BPSL1710-1727/BP1026B_I1663-1681, BPSL2214-2229/BP1026B_I1161-1176, BPSS0482-0489/BP1026B_I10536-0544, BPSS2023-BPSS2037/BP1026B_I12177-BP1026B_I12192). A few genes from several of the NRPS operons experienced >1,000 fold negative selection. One such secondary metabolite is investigated in this work.

Other uncharacterized genes that show a high amount of negative selection are GI7 and GI16, which include transporter, regulatory, and hemagglutinin genes with varying similarities to Yersinia, Vibrio, and E. coli genes. Genes in GI8, BPSL1660-1665/BP1026B_I1605-1610, encoding hemagglutinin, hemolysin, and toxin secretion genes with homology to Vibrio were also identified. An unidentified VgrG T6SS in the BPSS0958-0963/ BP1026B_I1049-1055 region made it on the list.

**Melioidosis BALB/c mouse experiment**

The previously unknown virulence factors screened in this study include genes annotated as hypothetical and putative whose role in infection remains unclear. Out of the initial 134-
unknown/hypothetical virulence factor mutants, the pool was narrowed down to 20 virulence factors from the various screens. Of the 20 screened in animal studies, 10 demonstrated some level of attenuation in vivo. These 10 genes produce virulence factors that directly affect the mammalian infection. One of the genes BP1026B_I2174 has high homology to *phoU* (as described above), a phosphate regulon transcriptional regulator, which has been shown to co-activate T3SS and T6SS proteins under phosphate limiting conditions in *Edwardsiella tarda* [53]. Bacteria were only found in the lungs of surviving mice so *phoU* may be important for chronic disease or dissemination. Gene BP1026B_II1508 shows 65%ID 75%H at the amino acid level to the *Pseudomonas syringae* HopAN1 T3SS effector (as described above). The exact function of the HopAN1 effector during plant infections is unknown but it reduces the hypersensitivity response (analogous to the animal innate immune response to PAMPs) to *P. syringae* in plants [54]. Previous works have shown that the T3SS-2 is only important for plant infections by deleting genes BPSS1386-1411 (according to the K96243 gene ID) [55]. In this work we targeted two effectors known as BPSS1386 and BPSS1416. The BPSS1386 (BP1026B_II1478) mutant was just as infectious as wildtype and killed all 5 mice by 3 dpi in agreement with the previous study. However, the BPSS1416 mutant (HopAN1; BP1026B_II1508), was attenuated with all mice surviving to 4 dpi and 60% surviving thereafter. However, high levels of bacteria were isolated form 2 out of 3 surviving mice indicating establishment of a chronic infection. Synthesis of the data indicates BP1026B_II1508 may be important during acute infection to avoid the host immune system. This *Bp* effector was not targeted previously and may be secreted via one of the other T3SS during host-cell infection.

The novel virulence candidate BP1026B_II1463 is a sigma class glutathione-S-transferase family protein. These proteins are essential in the scavenging of oxidative compounds
that can damage DNA and proteins that are a known component of innate immunity. During initial inflammation, activated macrophages and neutrophils release large amounts of reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS). ROS has been shown to be important for controlling *Bp* intracellular replication in mouse monocytes and in C57Bl/6 mice [56,57]. RNI has been show to be unimportant in controlling bacterial replication in the resistant C57Bl/6 [57] but plays a very important role in controlling bacterial replication during early infection in the sensitive BALB/c mouse model [58]. Glutathione-S-transferases play pivotal roles in detoxification during oxidative stress [59] including to ROS and RNI. Without a way to prevent macrophage induced oxidative damage, many bacteria would be killed by DNA and protein damage resulting in the survival rates observed in BALB/c mice challenged with GST mutant *Bp*.

BP1026B_I1522 is a hypothetical protein with BLAST matches to a polyhydroxybutyrate (PHB) depolymerase. PHB depolymerase enzymes act on the biopolymer PHB. PHB is stored as a granule inside the cell and used as an alternative source of acetyl-CoA under conditions of oxygen and nutrient limitation [60]. PHB cycle enzymes of *Legionella pneumophila* have been shown to be important during bacterial infection [61]. Without the ability to metabolize PHB *L. pneumophila* was unable to replicate within U937 macrophages and amoebae. Enzymes of the PBH provide an alternative source of acetyl CoA to the glyoxylate shunt, the importance of which has been demonstrated in *Bp* infection models [62]. Recently, enzymes of the PHB have been implicated as drug targets for bacterial pathogens including *M. tuberculosis* [63,64]. Neither of the two surviving mice infected with the BP1026B_I1522 mutant had any detectable bacteria in the organs tested implying that PHB may be an important nutrient source during host
infection. The *Bp* PHB mutant is attenuated; consequently targeting these enzymes in *Bp* may be a viable co-treatment to the current regimen of therapy.

Gene BP1026B_I1764 encodes a metalloendopeptidase with an NlpD/M23 peptidase domain. Annotations from 1026b predict a start site that would produce a peptide with an extra 60 AA on the N-terminal of the protein compared to the K96243 annotation. All secretion signal predictions are very weak for the 1026b protein BP1026B_I1764 (Sec 0 and T3SS 0.08904) but the shorter K96243 input protein BPSL1806 gives a high probability that it is secreted by the T3SS (0.8084) [42]. All similar proteins only match to the ~200 C-terminal AA containing the Gly-Gly endopeptidase NlpD domain, with the 200 N-terminal AA belonging to the PRK06132 domain of unknown function. The *Bp nlpD* gene is encoded by BPSL1504 and is predicted to be Sec secreted with a very low T3SS prediction score. The NlpD product is believed to contribute to cell division and results in an inability to divide properly, similar to what was observed with the *zipA* mutant. Our microscopy indicates the BP1026B_I1764 insertion mutant divides the same as wildtype during host-cell infection (data not shown) so the target of the peptidase during host-cell infection is not *Bp* peptidoglycan. Mice surviving infection with the BP1026B_I1764 mutant have low levels of the bacteria in the lung indicating the gene may play a role in dissemination. The domain structure is similar to the well-characterized protease LasA of *P. aeruginosa* that affects many aspects of cell invasion and the T3SS [65] and may help unmask the protein’s true function.

The least attenuated strains are the mutants of BP1026B_I1664, BP1026B_I10207, and BP1026B_I11256. The gene region of T6SS-4 (according to Burtick et. al.)/T6SS-3 (according to Shalom et. al.) was found to have lower read density than the surrounding area. The last gene of the secretion system BP1026B_I10207 (BPS185) is a small gene known as T6SS associated
gene D-3 \((\text{tagD-3})\). No insertions were detected in the initial pool, but this mutant piqued our interest. I-TASSER analysis \([66,67]\) used ten threading templates to predict the structure and function. All ten templates indicate the hypothetical protein of unknown function was a PAAR repeat protein homologous to T4 phage gp5 and other bacterial PAAR motif T6SS proteins. Recently, PAAR repeat proteins have been shown to sit atop the VgrG proteins at the tip of the T6SS needle, sharpening it and diversifying effector functions during injection \([68]\). The function of T6SS-4/3 in \(Bp\) infection is unknown but Syrian hamsters challenged with an \(hcp4\) mutant died just as wildtype \([20]\). In our study we used BALB/c mice, where 20% survival was observed, perhaps allowing for observation of nuanced virulence. Interestingly, this T6SS is only present in the select agents \(B. pseudomallei\) and \(B. mallei\) but absent in \(B. thailandensis\).

BP1026B_I1664, and BP1026B_II1256 are genes in two different NRPS/PKS operons. The unmodified peptide scaffolds were predicted and are in the supplementary information (Fig. S4 and S5). The BP1026B_II1256 mutant had a low level of attenuation and, while one mouse survived, was not significantly different than wildtype. Mutant BP1026B_I1664 had a significant increase in time to death of infected mice compared to wildtype. The carbamoyl transferase activity of this gene likely tailors the NRP to its fully functional final form. A transporter in this gene cluster also experienced >1,000 fold negative selection and the mutant was subjected to the cytotoxicity screen but the results of this assay were not significantly different than wildtype, potentially highlighting the weakness of such an assay in identifying some virulence factors. However, this assay, although not perfect, was a good high throughput method for us to screen many mutants at once. Secondary metabolites have a wide range of mammalian cell modifying properties. The effect of these \(Bp\) secondary metabolites on the host-cell during infection will require further analysis. Of the 20% survival group each surviving mouse had low levels of
bacteria in only the lungs, indicative of minor roles in bacterial spread. Future investigations of this T6SS and secondary metabolites will help to determine roles in infection.

Taken together, this data has determined 10 novel virulence factors that contribute to RAW macrophage and murine melioidosis infection. They make up a diverse group of proteins, which collectively with other characterized virulence factors, allows Bp to infect the host efficiently. The arsenal that is the intramacrophagic virulome is fluid and future work will tackle the task of characterizing the function of these newly described factors.
6.6 MATERIALS and METHODS

**Bacterial strains, media, and culture conditions.** All manipulations of wildtype *B. pseudomallei* were conducted in CDC/USDA approved and registered BSL-3 facilities at the University of Hawaii at Manoa John A. Burns School of Medicine (JABSOM) and experiments with select agents were performed in accordance with the recommended BSL-3 practices [147]. *Escherichia coli* strain E2188 (ΔdapA::pir Δasd::mob-Kan) was used for cloning and culturing of the R6K-ori based pBT20-asd<sub>pΔa</sub>-T7 plasmid. The strain was grown in 1xM9 minimal media with 20mM glucose (MG), 1mM lysine and 100 µg/ml diaminopimelic acid (DAP) with the plasmid maintained through complementation of methionine and threonine auxotrophy. Wildtype *B. pseudomallei* strains 1026b were cultured in LB while the K96243 Δasd strain [80] was cultured in LB with 400 µg/ml diaminopimelic acid (LB+DAP400) or in minimal salts media containing 1 mM lysine, 1 mM, methionine, 1 mM threonine, and 400 µg/ml DAP (3AA+DAP). *B. pseudomallei* 1026b::T24 insertion mutants were kindly provided by Dr. Herbert Schweizer at Colorado State University. Antibiotics were not used for selection but LB + 1,000µg/ml kanamycin was used for purifying the 1026b::T24 insertion mutants. Growth on and preparation of DAP were carried out as previously described [148]. All bacterial growth was carried out at 37°C and shaken cultures were maintained at 225 rpm.

**Molecular methods and reagents.** Molecular methods and reagents were carried out as described previously [51,80,150,167]

**Cell line and culture conditions.** Murine macrophage cell line RAW264.7 was cultured in DMEM containing 4500 mg/l glucose with 4.0 mM L-glutamine. All cultures were
supplemented with 10% (v/v) heat-inactivated standard fetal bovine serum (FBS; HyClone) and antibiotic/antimycotic solution (100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B; HyClone). Cells were maintained at 37°C and 5% CO₂ in a humidified incubator. RAW264.7 cell lines were maintained at 50-80% confluency and were passaged by scraping the cells from the flasks using a cell scraper. Cell lines were maintained in Corning™ flasks and plates with CellBIND™ surfaces. Cell concentrations were verified with the Scepter handheld automated cell counter (Millipore).

**Transposon library creation in the B. pseudomallei K96243 Δasd strain.** Three mls each of *E. coli* strain E2188 harboring the pBT20-asd₉₋₁₇ and the *B. pseudomallei* K96243 Δasd strain were grown to log phase and the cells were harvested by centrifugation in a biosafety cabinet within the BSL3. The pellets were combined and gently resuspended by pipetting in 50 µl of LB. The mixture was spotted onto an LB plate containing 200 µg/ml of DAP and incubated for 12 h at 37°C. After 12 h, the conjugation mixture was scraped off the plate with a plastic inoculation loop and resuspended in 1x M9 salts buffer. The mixture was plated onto 3-150 mm petri dishes containing MG media without any additional amino acids. Without additional amino acids neither donor nor recipient can grow unless the K96243 Δasd strain has the BT20-asd₉₋₁₇ transposon inserted in the genome. Minimal media was used to remove a good portion of the biosynthetic/metabolic insertion mutants from the pool as virulence factors were the major target. There were approximately 20,000-30,000 colonies per plate. All colonies were scraped from the plates and pooled into 6 ml of MG and then 500 µl aliquots were frozen in glycerol at -80°C.
Passaging the K96243 transposon mutant pool through RAW264.7 macrophages. RAW264.7 macrophages were grown to near confluency in T75 cell culture flasks. The K96243 pool was grown to log phase in MG and diluted down to an MOI of 10:1 and 1:1. The different MOI dilutions were each used to infect a flask of RAW264.7 macrophages for 1 h. Bacteria were washed from the monolayer and DMEM containing 750 µg/ml amikacin and 750 µg/ml kanamycin was added. The infection was allowed to progress for 12 h before the DMEM media was removed and the monolayers were washed 3 times with pre-warmed PBS. The monolayers were incubated with 2 ml of 0.2% Triton X-100 in PBS and gently rocked back and forth for 5 minutes to lyse the monolayers and liberate the intracellular B. pseudomallei. The 2 ml were harvested and 500 µl of each MOI were inoculated into 5 ml of MG broth and grown 12 h at 37°C with shaking. The respective cultures were diluted down to their respective MOIs (10:1 or 1:1) and used to infect another two T75 flasks of RAW264.7 macrophages in the same manner as above. This was repeated 3 times total. At the end of each passage the liberated bacteria were isolated and grown in MG broth then frozen at -80°C.

**gDNA isolation and Tn-seq.** 500 µl of the transposon library samples were inoculated into 4.5 ml of MG media and grown overnight. Genomic DNA (gDNA) of the passaged and unpassaged pools was isolated with the Promega Wizard Genomic DNA Purification Kit following the protocol for gram-negative bacteria. The kit protocol was checked for sample sterility prior to removal from the BSL3 laboratory. The samples were prepared by combining steps from the TUFTS University Core Facility protocol and as described previously [92]. 10 µg of gDNA samples (5-200 µl) were resuspended in shearing buffer (40% glycerol+10 mM Tris pH 8 + 1mM EDTA) to a total volume of 800 µl. The samples were nebulized separately for 4-5
minutes at 35 p.s.i. of N₂. 30 mg/ml of dextran sulfate in water was added to each sample to a final concentration of 0.2 µg/ml. Add 1/10th the volume of 3M potassium acetate + 6M acetic acid and vortex. Precipitate with an equal volume of isopropanol at -80°C for 10 min. The samples were centrifuged and washed with 70% ethanol and the pellets were resuspended in 16 µl Qiagen EB buffer for quantification via Nanodrop. The sheared DNA was end repaired with the NEB Next End Repair Kit according to protocol then purified with the Qiagen QIAquick PCR Purification Kit according to protocol. Each sample was divided into 4 samples and 3’ A-tailing was carried out with in-house Taq polymerase at 70°C for 20 min. Design of adapters was as previously described with modification for sequence specific to our transposon [92] while preparation of adapters and ligation was according to the TUFTS sequencing core recommendations. Samples were purified with the QIAquick PCR kit then digested with XbaI overnight at 37°C to separate the two genome-transposon junctions. The DNA samples were run on a 2% TAE gel and the DNA smears between 200 and 400 bp were excised and purified through the QIAquick Gel Extraction Kit according to protocol and eluted with 30 µl of EB buffer. Samples were at a concentration of between 27 ng/µl and 54 ng/µl of DNA in water. Circularization of adaptor ligated gDNA and exonuclease digestion were carried out as previously described using Ampligase heat stable ligase [92]. The transposon ends were amplified with PCR and eluted with the MinElute PCR Purification Kit in 10 µl EB. The molar amount of DNA was calculated and the three samples were multiplexed for Illumina sequencing at the TUFTS sequencing core.

**Tn-seq data analysis.** After demultiplexing, each sample received between 5 and 8 million reads. Reads were mapped with Bowtie [231] using the Galaxy suite [232-234]. The
SAM files were visualized with Integrative Genomics Viewer (IGV) [235]. Expression data was not being produced so to identify read differences between genes not operons, the HTSeq python script htseq-count was used to count reads per feature (gene) of each sample [236]. Reads of the passaged samples were compared to the unpassaged to determine fold negative selection. Heat maps were generated by importing read files into MeV [237]. Gene lists were manipulated by sorting with Excell into >10, >100, >1000 fold negative selection and 0 reads post-passage groups. Functional analyses of these groups were done by broadcasting groups of genes via Gaggle and Firegoose [238] to KEGG. Functional ontology enrichment was analyzed using Comparative GO [239] and DAVID [198]. Gene clusters with lower read densities after passaging were identified by visual analysis of the read maps in IGV. Prism 6 was used to create all graphs.

**Cytotoxicity assay.** The cytotoxicity assay was preformed as previously described [51]. *B. pseudomallei* strain 1026b::T24 insertion mutants were obtained from Colorado State University. RAW264.7 macrophages were inoculated in 96-well CellBIND plates then spun at 350 x g to assist cellular attachment. *B.p.* strains were grown overnight with LB in 96-deepwell plates sealed with a breathable microporous film. The next day the bacteria were serially diluted and used to infect the RAW264.7 macrophages in triplicate for each mutant at an MOI of 1:1 in 30 µl. After 1 h of infection unattached bacteria were washed off with 1xPBS followed by addition of DMEM containing 1,500 µg/ml of amikacin was added to kill any uninternalized bacteria. Initially cytotoxicity levels at 12, 24 and 48 hpi were measured but the ideal time point for measurement of lactate dehydrogenase (LDH) release was at 24 hpi. LDH released into the supernatant was measured at 24 hpi in triplicate for each mutant with the Promega CytoTox 96
Non-Radioactive Cytotoxicity Assay kit. A BioTek ELx808 plate reader was used to measure the absorbance at 490 nm after 30 min incubation at room temperature in the dark according to the protocol. The average cytotoxicity levels are presented with the standard error of the mean (SEM).

**Growth curves of B. pseudomallei 1026b insertion mutants.** Growth curves of the mutants were initiated by growing them overnight in 96-deepwell plates then diluting them 200 x into fresh LB. The 96-well plate was incubated at 37°C with shaking in the BioTek ELx808IU and measuring the O.D. 600 nm every 30 min for 48 h. Each growth curve was done in duplicate with the average and the SEM shown.

**Intracellular replication assays of B. pseudomallei 1026b insertion mutants.** Intracellular replication assays were carried out using a modified aminoglycoside protection assay as previously described [51,120] and as above. RAW264.7 macrophages were infected singly with wildtype B. pseudomallei and 21 insertion mutants with the tssC-5::T24 and BP1026B_I1079::T24 mutants as negative and positive controls, respectively. The monolayers were infected at an MOI of 1:1 for 1 h then washed and the medium was replaced with DMEM containing 1,500 µg/ml amikacin. At 2, 12, and 24 hpi monolayers were lysed with 0.2% Triton-X100 in PBS, lysates were diluted in PBS, plated onto LB, and incubated at 37°C for 48 h. Colonies were enumerated and bacterial numbers were determined. The experiment was carried out in triplicate and the numbers represent the average of three replicates with the error bars representing the SEM. The student t-test was used to determine the significance between replication of the wildtype and mutant strains. Calculating the average measurements from the
cytotoxicity assay and the intracellular assay and presenting it as a percentage of wildtype allowed comparison of the two assays.

**Invasion assays.** Invasion assays were initiated by diluting *B. pseudomallei* 1026b strains in PBS to an MOI of 1:1 in DMEM. The dilutions were plated on LB and colonies were counted 48 h later to accurately determine the number of CFU used to initiate the infections. The dilutions were used to infect the RAW264.7 macrophages in 96-well CellBIND plates at an MOI of 1:1. At 1 hpi the bacteria-containing medium was removed and the monolayers were washed twice with pre-warmed PBS and fresh DMEM containing 1,500 µg/ml amikacin was added to kill extracellular bacteria for another hour. At this point (2 hpi) the medium was removed and monolayers washed twice with pre-warmed PBS. Monolayers were lysed with 0.2% Triton-X100 in PBS, diluted, plated onto LB and incubated at 37°C for 48 h. Colonies were enumerated and invasion efficiency was determined by dividing the counted colonies by the total number of infecting bacteria. The experiment was carried out in triplicate and the numbers represent the average of all three replicates with the error bars representing the SEM. The student t-test was used to determine the significance between invasion efficiencies of the wildtype and insertional mutants.

**Attachment assays.** The attachment assay was carried out by diluting *B. pseudomallei* 1026b strains in PBS to an MOI of 1:1 in DMEM. The dilutions were plated on LB and colonies were counted 48 h later to accurately determine the number of CFU used to initiate the infection. The dilutions were used to infect the RAW264.7 macrophages in 96-well CellBIND plates at an MOI of 1:1. At 1 hpi the bacteria-containing medium was removed and the monolayers were...
washed 3 times with pre-warmed PBS. Monolayers were lysed with 0.2% Triton-X100 in PBS, diluted, plated onto LB and incubated at 37°C for 48 h. Colonies were enumerated and attachment efficiency was determined by dividing the attached number by the initial number of infecting bacteria. The experiment was carried out in triplicate and the numbers represent the average of all three replicates with the error bars representing the SEM. The student t-test was used to determine the significance between attachment efficiencies of the wildtype and insertional mutants.

**Plaque assays.** *B. pseudomallei* 1026b strains were grown overnight and diluted and used to infect RAW264.7 monolayers at an MOI of 1:1 in 24-well CellBIND plates the same as the intracellular replication assay. After 1 h of infection the bacteria containing media was washed off the monolayers and further washed once more with PBS. 1.2% low-melt SeaPlaque agarose (Lonza) in DMEM was heated to 60°C cooled to ~37°C then amikacin was add to 1,500 µg/ml. 500 µl was used to overlay each monolayer and they were incubated for 24 h at 37°C in 5% CO₂. The monolayers were fixed with 4% paraformaldehyde (PFA) in PBS for 45 min and the agarose plugs were removed. Monolayers were then stained with a 1% crystal violet solution for ease of viewing. Plaques were viewed with a Zeiss AxioObserver D1 and the accompanying AxioVision 64 bit 4.9.1 software was used to measure plaque diameter. 10 to 20 plaques per well were measured for comparison. A multiple one-way ANNOVA tested significance against the wildtype plaque diameter. Numbers presented are the average with the SEM.
Complementation and plaque assay of 5 gene candidates with mini-Tn7-kan. The 5 genes, BP1026B_I2174 (mutant 25), BP1026B_I2220 (mutant 27), BP1026B_I2818 (mutant 21), BP1026B_I10034 (mutant 110) and BP1026B_I1256 (mutant 20), were amplified by PCR and cloned into mini-Tn7-gat [80]. The resultant plasmids were maintained in an E. coli pir+ mob+ strain and mated into the respective insertion mutants as previously described [118] then selected on MG+ 0.3% glyphosate media where Tn7 insertion for each mutant was verified by PCR as previously described [80,113]. Plaque assays for the 5 mutants and the respective complemented strains was carried out as described above for the other plaque assays.

Differential interference contrast and fluorescence microscopy. Glass bottom 12-well plates were obtained from MatTek Corporation (Ashland, MA). The plates were sterile, uncoated, and contained bottoms of No. 1.5 covers lips. Glass coverslip bottoms were treated for four hours with 150 µg/ml poly-L-lysine in ddH2O. The plates were washed twice with ddH2O and allowed to air-dry within a biosafety cabinet overnight. Monolayers were seeded at nearly 3x10^8 cells/well verified by the Scepter handheld automated cell counter. Cells were allowed to attach and grow for 24 h prior to initiating the infection. Cell lines were infected with an MOI of 1:1 bacteria and the rest of the infection was carried out as above for the plaque assay. After 24 h of infection the monolayers were fixed with 4% PFA for 45 min before removing the agarose overlay. Fixed monolayers were permeabilized with 0.2% Triton X-100 in PBS for 5 minutes. Monolayers were washed with PBS then incubated with 1% BSA in PBS for 30 min to remove background. The actin was stained with AlexaFluor 488 phalloidin (Invitrogen) according to the manufacturers protocol. After washing, the cell membranes and nuclei were stained by mixing FM 4-64FX (a lipophilic styryl dye that dyes membranes red, Invitrogen) and ProLong Gold
antifade with DAPI (to stabilize dyes for long term storage and DAPI to dye nuclei blue, Invitrogen). Plates were sealed, protected from light, and stored at 4°C for up to 2 months. Images were captured with an AxioObserver D1 and accompanying Axiovision 4.9.1 software. Multi-color fluorescent images were captured with the multichannel fluorescence acquisition module of the Axiovision software. Images were deconvolved using the imageJ plugin Iterative Deconvolve 3D.

**Animal studies.** BALB/c mice between 4 and 6 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in micro-isolator cages under pathogen-free conditions. The Institutional Animal Care and Use Committee at Colorado State University approved all animal studies (CSU protocol 12-3586A). Every infection with *B. pseudomallei* was done using intranasal (i.n.) inoculation [82]. Animals were anesthetized with 100 mg/kg ketamine plus 10 mg/kg xylazine. A challenge dose of 4,500 CFU (10 times the LD$_{50}$) of the various *B. pseudomallei* were suspended in PBS and 20 µl was delivered i.n., alternating nostrils. For all *B. pseudomallei* survival studies, animals were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints. Lungs, liver, and spleen were removed and plated to determine bacterial counts in surviving infected mice 60 d post-infection. Statistical differences in survival times were determined by Kaplan-Meier curves followed by the log-rank test. At 60 dpi all surviving animals were humanely sacrificed and the lungs, liver, and spleen were removed and homogenized in 1 ml saline. Aliquots were plated and observed for organ load CFU.
6.7 FIGURE LEGENDS

FIG 1. Tn-seq experimental setup. The *P. aeruginosa* PAO1 *asd* gene was cloned into the mariner transposon pBT20. It was mated into *B. pseudomallei* K96243 Δ*asd*. Only the *B.p.* that had the wildtype copy of the *asd* gene inserted in the genome would have a return of the wildtype phenotype and grow on minimal glucose media without DAP. All colonies were pooled and used to infect RAW264.7 murine macrophages for 12 h and a total of 3 passages. gDNA from the unpasaged and passaged pools was isolated and sequenced with Illumina sequencing. Data was analyzed using IGV. 134 insertion mutants of hypothetical and putative genes that demonstrated high levels of negative selection were obtained and used to infect RAW264.7 macrophages to verify importance of the genes during infection.

FIG 2. Genes shared between replicates and hypothetical enrichment. A, Venn diagrams of the genes shared between replicates. The number of genes present in only the MOI of 10:1 replicate (blue) and the number of genes present in only the MOI 1:1 replicates (red) are indicated and those shared are in the overlapping region. Each Venn diagram has an increasing negative stringency increasing from ≥10 to ≥1,000. A majority of genes are shared between the replicates. B, Pie charts indicating the percentage representation of hypothetical/putative genes in the K96243 genome, and genes shared between replicates of the ≥10 and the ≥1000 data sets. Hypothetical/putative genes undergo higher negative selection and are overrepresented amongst genes the more stringent the cutoff.
FIG 3. Gene functional enrichment of low read saturation regions and genes. A, Gene ontology enrichment of genes with known or predicted functions. The X-axis is the gene function. The Y-axis is the fold enrichment of the function in our data compared to that present in the genome. The genes in the ≥10 (blue bar), ≥100 (green bar), and ≥1,000 (red bar) are the same genes in the overlapping Venn diagrams of Figure 2A representing the genes shared between replicates. B, Visual comparison of the mapped reads from the unpassaged pool compared to the two passaged replicates in the IGV program. Highlighted as an example is the well-characterized region of *B. p.* chromosome two. This region contains the T6SS-5 and the T3SS-3 *Burkholderia* secretion apparatus (*Bsa*). Vertical grey bars represent mapped reads in log-scale. Lower read saturation is visible in this region of the passaged replicates (10:1 and 1:1; bottom two rows) compared to the unpassaged (POOL; top row) sample, reiterating the importance of this region to bacterial replication in host cells.

FIG 4. Cytotoxicity and intracellular replication screening of novel putative virulence factors. A, 134 gene candidates were tested for reduced cytotoxicity. The y-axis is the LDH released as a % of total possible LDH release. Vertical bars represent the average of 3 biological replicates with error bars representing the standard error of the mean (SEM); * means p<0.05 as determined by the student t-test. For comparison the red line indicates the average level of cytotoxicity attributed to infection by wildtype. B, Growth rates of the insertion mutants were carried out to make sure there were no in vitro growth defects by measuring the OD every 30 minutes for 48 h. Two mutants had growth defects and were not investigated further (blue and red line). The remaining mutants (green and black lines) were all similar to wildtype (orange line) indicating there were no other in vitro growth defects and that cytotoxicity readings were
not due to an inability to grow. Growth curves were done in duplicate and the average is shown with the SEM. C, Cytotoxicity of *Bp* insertion mutants with lower cytotoxicity than the wildtype was calculated as a % of wildtype (black bar) and compared to intracellular bacteria CFU of % of wildtype (grey bar) at 24 hpi to observe trends in the two measurements. D, Intracellular replication assays for the insertion mutants were carried out and presented in linear scale where the groups of mutants were significantly different at the 12 or 24 hpi time points. The 12 and 24 hpi time points are presented as bar graphs to allow for ease of comparison between wildtype (WT; red line and bar), the internal positive control insertion mutant BP1026B_I1079 (39; light blue line and bar), the internal negative control insertion mutant BP1026B_II1588 (*tssC*-5::T24, 126; orange line and bar) and the remaining insertion mutants. *=*\( p<0.05 \), **\( p<0.005 \), ***\( p<0.0005 \), ****\( p<0.00005 \) by the student t-test.

**FIG 5. Virulence assays characterize the putative virulence factors.** A, Invasion assays were carried out on all mutants that had intracellular replication deficiencies and compared to the *tssC*-5, BP1026B_I1079 insertion mutants, and wildtype. Numbers shown are the number of intracellular bacteria recovered after 2 h infection divided by the total number of bacteria used to initiate the infection. The average of three experiments and the SEM is shown. B, Attachment assays were carried out for all mutants that demonstrated a deficiency in the invasion assay and compared to the BP1026B_I1079 insertion mutants and wildtype. The numbers shown are the number of bacteria recovered after 1 h of attachment divided by the total number of bacteria used to initiate the infection. All numbers are the average of three replicate experiments with the SEM. C, Plaque assays were carried out on genes of interest and compared to the *tssC*-5 and the
wildtype. At least 10 plaques were measured with the bars representing the average and the SEM. *\(=p<0.05\), **\(=p<0.005\), ***\(=p<0.0005\), ****\(=p<0.00005\) by the student t-test.

**FIG 6. Plaque phenotypes of putative virulence factor mutants.** A, Plaque phenotypes of the mutants most deficient in plaque formation from Fig. 5C were observed via DIC microscopy at 24 hpi after removing the agarose overlay. The mutants were compared to the internal negative control \(tssC-5\) and positive control BP1026B_I1079 mutants and wildtype. Carats indicate the MNGC/plaque formation in the representative images. For ease of viewing a light red border has been drawn around the regions of MNGC formation for clarity.

**FIG 7. Mutant Virulence Phenotype “MVP” Matrix.** A summation of all virulence assays of 23 mutants including the negative internal control \(tssC-5\) and positive internal control mutant 39 compared to wildtype 1026b. Columns represent the different assays and rows represent the different mutants. If a mutant behaved like wildtype in the assays the heat map tile is red. The more deficient in the assays than wildtype the more green the tile. The heat map was clustered using Pearson correlative hierarchical clustering to place mutants that behave similarly near each other. They are divided into 7 different groups based on their behaviors in the assays and more simply those that have deficiencies in the beginning and later stages of the cellular infection model.
FIG 8. Complementation of two novel virulence factors and contributions of neighboring genes. Intracellular replication assay of BP1026B_I2174 (A) and BP1026B_I11256 (B) mutants along with their Tn7-complemented strains compared to wildtype and the tssC-5 mutant, measured in triplicate with the SEM shown. The x-axis is log scale internal CFU with y-axis as time post-infection. Insets show details in linear scale. C, Plaque assay of the same strains with at least ten plaques measured 24 hpi. Significance determined by one-way ANOVA compared to WT as control. **** =p<0.00005. D and E are LDH assays performed in triplicate presented as % total cytotoxicity of wildtype. Yellow bar is gene of interest, grey/black are neighboring genes as labeled on x-axis, orange is the negative control, blue is the positive control, and red is the wildtype control. *=p<0.05, **=p<0.005, ***=p<0.0005, ****=p<0.00005 by one-way ANOVA compared to WT as control.

FIG 9. BALB/c virulence mutant attenuation study. Twenty mutants from the original 134 were selected for attenuation studies in the BALB/c inhalation melioidosis model. Five mice were infected with 10x the LD50 or 4,500 CFU of each of the transposon mutants or wildtype 1026b and survival was observed. Mice infected with wildtype become moribund 3 dpi and the survival curve is red in all the figures in order of increasing attenuation. A, all survival curves. B, Survival curve of mutants that killed all mice. C, Survival curve of mutants with 20% survival. D, Survival curve of mutants with 40% survival. E, Survival curve of mutants with 60% survival. F, Survival curve of the mutant with 100% survival. All curves compared to wildtype (red line) using Log-rank (Mantel-Cox) test with *=p<0.05 and **=p<0.005. D,F,H, and J, Bacterial organ loads of each surviving mouse from the survival curves to the immediate left of each organ load graph.
Table 1. Genes showing greater than 10, 100, and 1,000 fold negative selection in both replicates compared to the unpassaged pool. Gene ID for these lists is I the K96243 gene ID. Functional annotation is shown for select genes in the >10 group and all genes in the >1,000 group. Genes involved in secondary metabolite biosynthesis, flagellar assembly, secretion, and regulation are indicated by the color, see legend.

Table 2. List of genes targeted in this study. A list of 134 insertion mutants targeted in this study. This table indicates the gene mutant number in this study (1-134), the K96243 and the 1026b gene IDs, the annotated function in each genome, additional data obtained through bioinformatics, and virulence data (where applicable).


FIG S2. Potential virulence loci identified in the Tn-seq data. For each column, mapped reads for each sample are on the left with each gene on the right and a heat map representation in the middle. These are regions of low read saturation in both the “10_1” and “1_1” passaged pools when compared to the unpassaged “POOL” sample. Black means 10 times more reads and yellow means greater than 100 times more reads than blue for a given gene row. A summary of
functions for selected gene regions are below the heat maps. A, *Bp* chromosome I. B, Chromosome II.

**FIG S3. Gene receiving 0 reads after passage.** A, Genes that had 0 reads in both replicates after passaging (y-axis) and the number of reads received from the unpassaged pool (x-axis), red circles. B, Heat maps comparing the reads from the unpassaged pool (left) and the average reads from both passaged replicates.

**FIG S4 and S5. anti-SMASH analysis of secondary metabolite operons.** A, secondary metabolite clusters and predicted function of genes in the cluster. B, anti-SMASH predicted peptide scaffold without tailoring modifications by nearby genes resulting in potentially different “isotope” modifications. C, Cluster homology among the Burkholderia and conservation of genes by color. S4D, only webACT alignment of the 1026b region and the *B. thailandensis* E264 region showing absence of a BP1026B_II1256 homologue in the E264 genome. The deletion and relocation of BP1026B_II1278 through BP1026B_II1286 homologues in the E264 genome is also depicted in C.
Pool

Intracellular infection of RAW 264.7 Mφ cell-line

Negative Selection
3X

3X

70,000 colonies
M9 + glucose
no DAP medium

Transposition into Bp K96243 Δasd mutant

Pool

Intracellular infection of RAW 264.7 Mφ cell-line

Bacterial chromosomal DNA isolation
TN-Seq multiplex NG Illumina sequencing
TUFTS or in house microarray

Genes essential for Intracellular infection

Figure 1

Figure 2
Figure 3
Figure 4
Figure 6
Figure 7

Figure 8
Figure 9
Figure S1
### Figures S2

#### Figure S2A

<table>
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<tr>
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### Gene IDs and Descriptions

- **BPSL0194-0202**: TonB receptor/heme transporter
- **BPSL0194-0202**: GI7BPSL1661-1664 hemaglutinin/hemolysin, toxin secretion proteins
- **BPSL2214-2229**: NRPS/PKS operon similar to syringomycin
- **BPSS0290-0312**: malleilactone operon
- **BPSS0802-0805**: extracellular branched chain amino acid binding and transport
- **BPSS0958-0963**: A VgrG T6SS
- **BPSS0482-0489**: extracellular hemaglutinins
- **BPSS1495-1528**: T6SS-1
- **BPSS1614-1630**: T3SS-2
- **BPSS2023-2037**: secondary metabolite operon, unknown
- **BPSS2057-2071**: GI16- proteins similar to *Y. pestis*

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**Note**: Figures S2A and S2B illustrate the reads heat maps for different gene IDs across various chromosomes. The gene IDs are associated with specific functions and activities as described above.
Figure S3
Figure S4
Figure S5
6.8 REFERENCES


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Chapter 7. Identifying *Burkholderia pseudomallei* transcriptional regulators that contribute to virulence.

To be published in future works.
7.1 INTRODUCTION

*Burkholderia pseudomallei* (*Bp*) commands an arsenal of virulence factors that are put into use during host-cell infection. To organize and time the use of these virulence factors for full effect, a complex regulatory cascade must be fine tuned and maintained. Identifying the transcriptional regulators that are responsible for such fine-tuning would give insight to the virulence network of *Bp* and provide targets for novel therapeutic development.

Transcriptional regulation of virulence genes has been well studied in *Vibrio cholerae* as well as in *Pseudomonas aeruginosa*. In these pathogens external compounds and sensors are known to turn on a suite of transcriptional regulators that then mediate toxin, pili, flagella, phospholipases and proteases production among others. *V. cholerae* responds to temperature, osmolarity, bile, and pH to up regulate cholera toxin synthesis [1]. Indeed, studies have uncovered several regulons of virulence factors that control the ability of *V. cholerae* to attach and adhere to intestinal mucosa (ToxR regulon and the toxin coregulated pilus), but that all need to be coordinated for successful colonization. Secretion of mucinases, lipases, and proteases must also be coordinated to digest the mucous surrounding the membranes. The model pathogen, *P. aeruginosa*, is known to coordinate a complex and intricate web of regulation that allows the bacterium to infect a wide variety of hosts and survive in a wide variety of environments [2].

A few *Bp* transcriptional regulators have been investigated. The AraC-type regulator, HrpB, and its role in regulating the plant-pathogen like T3SS has been elucidated [3]. BsaN has been identified as a direct regulator of the T6SS-5, a major virulence factor [4] and BspR has been identified as a regulator of the major virulence factor T3SS [5]. These regulators are located proximal to these virulence factors and were obvious candidates for direct regulation of these two loci. Our goal was to identify additional regulators including global regulators that affect
virulence factor expression during host infection and aide in coordinating the process of host-cell infection (Fig. 1). Previous data generated in the lab by Dr. Yun Kang using single-cell transcriptomics [6] of the Bp transitome (in preparation) identified the global transcriptional profile of Bp as it transits through the host-cell during infection. Many genes were differentially regulated as Bp invaded host cells, replicated in the cytoplasm, and formed protrusions. Within this data set, 40 transcriptional regulators were identified that showed differential gene expression during host-cell infectious stages (Fig. 2). The targets of these regulators are unknown and we hypothesized that at least some of them would be contributing to the coordinated expression of virulence factors during infection.

In this work, I utilized lambda RED recombineering to produce knockout mutants of all 40 transcriptional regulators in the naturally competent BSL3 select agent Bp strain 1026b [7]. The 40 transcriptional knockout mutants were then tested for deficiencies in plaque formation within HEK293T cells, a human embryonic kidney cell line. The mutants that showed reduced ability to form plaques compared to wildtype were submitted to a growth curve to make sure the inability to form plaques was not due to an inability to grow in vitro. After demonstrating the mutants were important for monolayer plaque formation and not affected in in vitro growth they were complemented with ChIP-seq vectors to ensure functionality of the vector encoded transcriptional regulators. Six of the regulators were chosen for animal studies in BALB/c mice. Survival of the mice was observed over 60 days post-infection. Bacterial organ loads in the lung, liver, and spleen were determined in all surviving mice.
7.2 MATERIALS and METHODS

**Bacterial strains, media, and culture conditions.** All manipulations of *B. pseudomallei* were conducted in CDC/USDA approved and registered BSL3 facilities at the University of Hawaii at Manoa, and experiments with select agents were performed in accordance with the recommended BSL3 practices [8]. Derivatives of *Escherichia coli* strain EPMaX10B (BioRad), E1345, E1354, E1869, and E1889 (Table 1) were routinely used for cloning or plasmid mobilization into *B. pseudomallei* as described previously [9,10]. Luria-Bertani (LB) medium (Difco) was used to culture *E. coli* strains. *B. pseudomallei* strains were cultured in LB or 1x M9 minimal medium supplemented with 20 mM glucose (MG). Antibiotics and non-antibiotic anitbacterials in solid media were utilized as follows: for *E. coli*, glyphosate (GS) 0.3% (w/v); for *B. pseudomallei*, GS 0.3% (w/v) was used. Growth of *E. coli* Δasd strains and preparation of DAP were carried out as previously described [11]. Selection for gat genes in *E. coli* and *B. pseudomallei* strains was performed as previously described [10].

**Generation of 40 regulatory mutants in Bp 1026b using lambda RED recombineering.** The 40 *Bp* transcriptional regulatory genes were knocked out in strain 1026b using lambda RED recombineering as previously described [7] but with modifications as follows. For each gene, two primers whose 3’ regions anneal to the upstream or downstream M13 regions surrounding the *FRT-gat-pheS* cassette and designed to contain 45 bp of homology to the upstream or downstream regions of the targeted gene, respectively, were used in the PCR to create an *FRT-gat-pheS* cassette bounded upstream by 45 bp homologous to the upstream region of the knock out target and downstream by 45 bp homologous to the downstream region of the knock out target (~2.3 kb). Primers were then used in amplification by PCR of the lambda
RED region from pKaKa1, to include the beta, gam, and exo genes to create a second DNA fragment (~3 kb). Strain 1026b was grown in 4 ml of M9 media + 20 mM glucose overnight to an O.D. of 1-1.4 then all 4 ml were pelleted by centrifugation at 14,000 g and all supernatant removed. 1 µg of each of the two DNA fragments was combined in a total volume of 5 µl and resuspended with the pelleted bacteria. The suspension was incubated at room temperature for 30 min then allowed to outgrow in 3-4 ml of the same media at 37°C with shaking for 2 h. The bacteria were then pelleted and plated on MG+0.3% GS. Colonies appeared 2-3 d later and were screened for correct insertion size using primers outside of the region used to create the knockout cassette.

**HEK293T plaque assay using 40 Bp regulatory mutants.** Plaque assays were carried out as previously described [12] but with some modifications. Briefly, 293T cells were seeded in CellBIND™ coated Corning™ 24-well plates with an ~ 16 mm diameter. The plates were incubated with a 1:40 dilution of phenol-red free BD Matrigel Matrix with reduced growth factors in PBS for 30 min prior to seeding. After a short period of drying within a bio-safety cabinet, cells were seeded at 7.5x10⁴ cells/well and allowed to attach overnight. In the morning the media was removed and the monolayers were washed twice with PBS. Overnight cultures of Bp regulatory mutants were resuspended in DMEM. The culture was diluted down in the same media to reach an MOI of 1:1. The suspension of bacteria was used to infect monolayers at an MOI of 1:1 all for 1 h. After 1 h, the bacteria-containing media was removed and the monolayers were gently washed twice with PBS to remove any extracellular bacteria. DMEM containing 1.2% low melt agarose, 750 µg/ml of amikacin, and 750 µg/ml of kanamycin was cooled to near solidity then used to overlay the 293T monolayers. At 24 hours post-infection (hpi), 400 µl of
DMEM with 0.1% (w/v) neutral red solution (Invitrogen) was added to the agarose overlay and incubated for 2 h to allow staining. Plaques were imaged on an Olympus IX82 inverted microscope and captured with QCapture Pro 7 software. At least 10 plaques per mutant were measured (where available) with the QCapture Pro 7 software.

**Growth curves of plaque deficient mutants.** Growth curves of the regulatory mutants were initiated by growing them overnight in 96-deepwell plates then diluting them 200 times into LB. The 96-well plate was incubated at 37°C with shaking in the BioTek ELx808IU and the O.D. 600 nm was measured every 30 min for 48 h. All growth curves were done in quadruplicate with the average and the SEM shown.

**Complementation of the regulatory mutants of interest.** Mutant strains had the chromosomally located gat resistance marker removed by Flp-FRT excision [13] and were verified by PCR and phenotypically as GS\(^S\). The ChIP-seq vector pAM3GIQ-3xTY1 was constructed in our lab and is a GS\(^R\) plasmid that encodes lacI to repress the \(P_{lac}\) promoter in \(Bp\). The lac promoter is induced by IPTG (removing repression by LacI) and drives expression of cloned genes. The multiple cloning site (MCS) is designed to allow N-terminal translational fusions to contain the ChIP-seq tag TY1 repeated three times (3xTY1). Genes BPSL0849, BPSL1938, BPSL2496, BPSS0012, BPSS1134, BPSS1569, BPSS1889, and BPSS2146 were cloned by NdeI and HindIII double digests as translational fusions with the 3xTY1 tag. Gene BPSS1471 was blunt end cloned as a translational fusion. The plasmids were then electroporated into their cognate GS\(^S\) mutant strains that had the chromosomally located gat resistance marker removed by Flp-FRT excision. Transformants were selected for on MG+0.3% GS plates and
verified by PCR. Complementation was verified by plaque assay as described above but by adding various amounts of IPTG in the agarose overlay for ideal induction of the cloned genes (0.5, 2.5, 5, and 10 mM IPTG). Plaques were measured in the same manner as above.

**BALB/c acute inhalation melioidosis animal model and bacterial organ load determination study.** Three tubes of each mutant to be tested were frozen at -80°C for 48 h. One tube was thawed and diluted to determine the CFU/ml of the remaining tubes. After determining the concentration and on the day of the challenge experiment, a second tube was thawed and diluted as required in PBS. Each group of five BALB/c male mice between 4 and 6 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in micro-isolator cages under pathogen-free conditions at ABSL3 in the JABSOM Bio-containment Facility (JBF). The Institutional Animal Care and Use Committee at the University of Hawaii at Manoa approved all the animal studies (Protocol # 10-1073-4). Animals were anesthetized with 100 mg/kg ketamine plus 10 mg/kg xylazine until unresponsive. The mice were inoculated with 10x the LD50, or 4,500 CFU of each regulator mutant in 20 µl of PBS by pipette into the nose alternating nostrils, 5 mice per group. For all *Bp* survival studies, animals were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints. Mice surviving 60 d were euthanized with CO2 and the lungs, livers, and spleens were removed. Statistical differences in survival times were determined by Kaplan-Meier curves followed by the log-rank test. Each organ was separately resuspended in 5 ml PBS and homogenized in a Seward Stomacher® 80 Biomaster for 4 minutes. Aliquots were diluted and bacterial organ loads determined.
7.3 RESULTS and DISCUSSION

The mutants that were differentially regulated include domains from many families of regulators including GntR, AraC, Lrp, LysR, TetR, LuxR, PadR, DeoR, AcrR, MerR, RocR, and RpiR. Regulators within each domain have a wide range of gene targets with little besides protein domain organization gleaned from that information. These 40 regulators also demonstrated differential gene expression during transit through the host-cell infection, implying that they are induced or repressed in response to specific signals during the infection that direct them to up or down-regulate their target genes. Although all three data sets are from inside the host cell, the different intracellular niches are diverse enough to induce changes in transcriptional regulator profiles that account for the several thousand differentially expressed genes detected.

The regulatory mutants were made using co-incubation lambda RED recombineering. A few were unable to be made and deemed essential for growth. The 35 transcriptional mutants made in \textit{Bp} 1026b and verified by PCR were tested in multiple plaque assays for deficiencies in plaque formation. Nine of the 35 regulator mutants had severe inabilities to create plaques on HEK293T monolayers (Fig. 3) and are indicated in figure 2 with red dots. The results were highly significant for these nine candidates with significance at $p<0.0001$ from the unpaired student t-test. The nine regulators encompass many of the transcriptional regulatory families: AcrR (BPSS1471), AraC (BPSS1889), DmlR (BPSS0012), Lrp (BPSL0849 and BPSL2496), LysR (BPSS2146), PadR (BPSS1134), TetR (BPSS1569), and putative regulator BPSL1938. Most of the regulators had marked up-regulation while \textit{Bp} was in the vacuolar stage (BPSL1938, BPSS1889 and BPSS2146) or cytoplasm (BPSL0849 and BPSL2496). Two other regulators demonstrated significant down-regulation in either the vacuole (BPSL1569) or cytoplasm (BPSS1134). BPSS1471 showed up-regulation in the cytoplasm and during protrusion. These
nine regulators contribute to intricate regulation of virulence factors during infection at the predicted stages of infection; turning on genes essential for progression of the infection through the vacuole, cytoplasm, and protrusion stages eventually resulting in plaque formation.

Growth curves on the mutants of interest were done to make sure plaque-forming deficiencies were not due to an inability to grow in vitro. Figure 4 shows the growth rates of the mutants compared to wildtype Bp 1026b in the rich media LB broth. Mutants in BPSL1938 and BPSL2496 showed considerable growth defects in in vitro growth indicating these two mutants have general growth defects not related to infection. The other mutants had growth rates similar to wildtype Bp 1026b. Mutants in BPSS1471 and BPSS2146 had a lag-phase a little longer than wildtype but eventually reached the same optical density. These two mutants plus the remaining regulatory mutants were hypothesized to have a defect in virulence regulation and not in in vitro growth.

After Flp-FRT excision of the gat cassette from the chromosome, the mutants were complemented with ChIP–seq vectors. ChIP-seq vectors were chosen so that we could identify the direct regulon of our newly identified virulence regulators in the future. For the purpose of this study, I looked at the ability of the complemented strains to form plaques on HEK293T cell monolayers in comparison to wildtype 1026b and their respective mutants (Fig. 5). Complemented BPSL1889 and BPSL2496 mutants were unable to form plaques as wildtype (WT). Inefficient complementation may be because we used an inducible promoter that causes constant transcription. Figure 2 shows that expression of the regulators fluctuates as the bacteria infect host-cells thus requiring subtle changes in expression to successfully form plaques. Attempts were made at varying the induction levels of IPTG for BPSL1889 and BPSL2496 in the media to no avail. These two vectors were also cloned with native promoters and some
degree of plaque formation was obtained. Complemented BPSL1569, BPSL1938, BPSS1134, BPSS1471, and BPSS2146 all created plaques on HEK293T monolayers similar to the wildtype and much higher than the mutant strains indicating complementation was successful. The complemented strains were saved and are undergoing the ChIP-seq protocol to identify the direct regulon of each virulence regulator.

Once initial characterization of the regulator mutant infection phenotypes was complete, the mutants were tested for attenuation in the inhalation melioidosis BALB/c mouse model. Verification of a role in pathogenesis needs to be done with an *in vivo* animal infection model. Groups of five mice were infected with each transcriptional regulator mutant (knockout mutants of BPSL0849, BPSL1569, BPSS0012, BPSS1134, BPSS1471, BPSS2146 and wildtype). The intranasal infection model where lethal inoculum is pipetted into the nares of anesthetized mice, simulating inhalation melioidosis, was used. Five groups had all mice survive the length of the 60-day experiment (Fig. 6A, 6C-F, column I). In our experience this timeframe is appropriate to allow for chronic infections deaths to occur. Wildtype *Bp* infected mice are succumb to the infection around 3-4 days whereas the BPSL0849, BPSS0012, BPSS1134, BPSS1471, BPSS2146 mutants infected mice survived the length of the study. BPSL1569 mutant infected mice began succumbing to chronic infection at ~40 dpi (Fig. 6B, I). Mice that survived the 60 days of the study were humanely euthanized and their lung, liver, and spleen removed and homogenized to determine bacterial organ load (Fig. 6A-F column II). Of all groups, only BPSL0849 mutant infect mice survived the length of the study and whose organs were sterile and free of *Bp* (Fig. 6A). Three of the five mice infected with the BPSS0012 mutant had bacteria in the lungs while the spleen and liver were clear, indicating that in the host the DmlR regulator BPSS0012 is responsible for regulating processes needed for dissemination beyond the lungs.
Mice infected with the BPSS1471 mutant also demonstrated similar survival and bacterial dissemination characteristics but only one mouse had *Bp* in the lungs, suggesting a role for BPSS1471 in survival of *Bp* in all tissues including the lungs (Fig. 6E). Three mice infected with the BPSL1569 mutant died of chronic infections and one that survived had very high levels of *Bp* in all the organs tested and gross splenic abnormality (Fig. 6B). The last mouse appeared to have no *Bp* in its organs. The regulator encoded by BPSL1569 is suggested to have an intermediate role in pathogenesis and may control bacterial behavior associated with acute infectious processes such as growth at primary infection sites. All mice infected with *Bp* mutant BPSS1134 survived the 60-day infection but there were very high levels of bacteria in the organs following homogenization and plating (Fig. 6D). Bacteria were found in the lungs of four mice, in the spleens of three, and in the liver of one. The BPSS1134 mutant was able to disseminate but was unable to cause acute disease. Mice infected with the last regulator mutant, BPSS2146, survived the length of the experiment and one mouse had bacteria in the lungs and spleen indicating an intermediate level of attenuation.

Taken together, I created 40 transcriptional regulator mutants. Through screening we narrowed down to nine regulators that were involved in plaque formation in cell-monolayers. Complementation of the regulator knockouts verified essentiality in the cell monolayer infection model while growth curves verified little effect on *in vitro* growth kinetics. BALB/c mouse infections showed attenuation of six regulators of interest. One regulator still infected mice with a mortal chronic infection while the remaining five presented with various levels of attenuation and inability to disseminate. Based on this work, we have identified six new virulence regulators that play a vital role in coordinating virulence factor expression. Ongoing projects include next generation illumina sequencing using the ChIP-seq vectors produced in this work. ChIP-seq will
allow us to identify the promoters that the regulators physically interact with, also known as the direct regulon. We are also working on RNA-seq experiments for each of the regulator mutants versus the wildtype \( Bp \). After analyzing the data from both of these experiments we will know the direct and indirect regulon of each regulator and be able to round out a clearer picture of virulence regulation in \( Bp \).
7.4 FIGURE LEGENDS:

FIG 1. Transcriptional regulators affect virulence processes in *Bp*. As *Bp* infects a host-cell a highly intricate and coordinated process occurs whereby the bacterial transcriptional regulators activate genes for phagosome escape, cytoplasmic replication, and cell-cell spread. Activation of virulence genes in a coordinated manner results in bacterial internalization A), invasion B), actin polymerization C), and membrane protrusion/spread D).

FIG 2. 40 transcriptional regulators differentially regulated inside host-cells. Each row lists a transcriptional regulator gene ID along with its putative function. The heat maps indicate the gene expression in the vacuole (V), cytoplasm (C), and during protrusion (P) in comparison to control bacteria grown in cell culture media. Replicates and average of those replicates are shown for each region of the cell where green indicates higher expression compared the control, red lower expression, black same as control, and grey not detected. Red dots highlight the transcriptional regulators of particular interest in this work.

FIG 3. HEK293T plaque assay of 40 transcriptional regulators. Plaque diameters were measured during monolayers infections of HEK293T cells. Plaque size on the Y-axis is the average diameter of 10 to 20 plaques with the standard error of the mean shown. Gene IDs are listed on the X-axis. Significant diameter differences were determined by the unpaired student t-test for each regulator compared to wildtype *Bp* 1026b. ****= p<0.0001.

FIG 4. Growth curve of *Bp* transcriptional regulator mutants. Starter cultures were diluted 100 times in LB broth. The optical density (OD) at 600 nm was read every 30 min for 48 h.
Black lines indicate wildtype Bp1026b and mutants that grew the same as wildtype. Red and green lines represent mutants that had a longer lag but final OD similar to wildtype. Blue and purple lines represent the two mutants that were unable to grow to the same OD as wildtype. Each line is the average of a quadruplicate experiment with standard error of the mean shown.

**FIG 5. Plaque assay of complemented Bp transcriptional regulator mutants.** Regulator mutants complemented with ChIP-seq vectors induced with IPTG. Plaque diameter in µm is shown on the Y-axis and strain ID is shown on the X-axis. 10-20 plaques were measured and the standard error of the mean is shown compared to wildtype.

**FIG 6. Bp transcriptional regulators are attenuated in the inhalation melioidosis BALB/c mouse infection model.** For all regulators, five mice were infected with 10x the LD$_{50}$ and survival was monitored in I. In each survival curve mice infected with wildtype 1026b were used as control. For each regulator, bacterial organ loads were determined in the indicated organ for each surviving mouse in II. A) BPSL0849, B) BPSL1569, C) BPSS0012, D) BPSS1134, E) BPSS1471, and F) BPSS2146
Figure 1
Figure 2
HEK293T plaque assay

Transcriptional regulator mutant

Plaque size (µm)

WT

BPSL0849
BPSL1569
BPSL1938
BPSL2496
BPSS0012
BPSS1134
BPSS1471
BPSS2146

Time (hours)

OD 630

Figure 3

Figure 4
Figure 5
Figure 6

A

B

C

D

E

F

Lung
Spleen
Liver

CFU/organ

BPSL0849 BPSL1569
BPSS0012 BPSS1134
BPSS1471 BPSS2146

Days

Percent survival
7.5 REFERENCES


APPENDICES
Engineering of Tellurite-Resistant Genetic Tools for Single-Copy Chromosomal Analysis of *Burkholderia* spp. and Characterization of the *Burkholderia thailandensis* betBA Operon

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There are few appropriate single-copy genetic tools for most Burkholderia species, and the high level of antibiotic resistance in this genus further complicates the development of genetic tools. In addition, the utilization of resistance genes for clinically important antibiotics is prohibited for the bioterrorism agents Burkholderia pseudomallei and Burkholderia mallei, necessitating the development of additional nonantibiotic-based genetic tools. Three single-copy systems devoid of antibiotic selection based on two nonantibiotic selectable markers, tellurite resistance (Tel') and Escherichia coli aspartate-semialdehyde dehydrogenase (asd)_, were developed to facilitate genetic manipulation in Burkholderia species. These systems include one mariner transposon, a mini-Tn7-derived site-specific transposon, and six FRT reporter fusion vectors based on the lacZ, gfp, and luxCDABE reporter genes. Initially, we showed that the random mariner transposon pBT20-Δhla-Tel'-FRT efficiently transposed within Burkholderia cenocepa, Burkholderia thailandensis, B. pseudomallei, and B. mallei. We then utilized the mini-Tn7 Tel'-based transposon vector (mini-Tn7-Tel'-betBA) and a transposase-containing helper plasmid (pTNS3-asd+) to complement the B. thailandensis ΔbetBA mutation. Next, one of the FRT-lacZ fusion vectors (pFRT1-lacZ-Tel') was integrated by Flp (encoded on a helper plasmid, pCD135K-Flp-oriT-asd+) to construct the B. thailandensis ΔbetBA::FRT-lacZ-Tel' reporter fusion strain. The betBA operon was shown to be induced in the presence of choline and under osmotic stress conditions by performing β-galactosidase assays on the B. thailandensis ΔbetBA::FRT-lacZ-Tel' fusion strain. Finally, we engineered B. thailandensis ΔbetBA::FRT-gfp-Tel' and ΔbetBA::FRT-lux-Tel' fusion strains by utilizing fusion vectors pFRT1-gfp-Tel' and pFRT1-lux-Tel', respectively. The induction of the betBA operon by choline and osmotic stress was confirmed by performing fluorescent microscopy and bioluminescence imaging analyses.

The genus Burkholderia, consisting of more than 40 different species, occupies diverse ecological niches ranging from the soil rhizosphere to the human respiratory tract (39). Within this genus, members exhibit considerable genetic diversity and broad metabolic capabilities (26, 39), facilitating their adaptation to a variety of environmental conditions including nutrient limitation, the presence of antibiotics and toxic compounds, and pH fluctuations. Many Burkholderia species are known plant pathogens, including Burkholderia caryophylli, B. plantarii, and B. glumae, while others (e.g., B. cepacia complex) cause opportunistic infections (39). In addition, Burkholderia pseudomallei and B. mallei are primary pathogens for humans and animals and are listed as category B select agents in the United States.

To best exploit the genomic information available for several Burkholderia species, a wide array of tools is required for molecular genetic and pathogenesis studies of these bacteria. For Burkholderia species not classified as select agents, antibiotic-resistance-based tools could be used for genetic manipulation. However, the Centers for Disease Control and Prevention restricts the introduction of markers conferring resistance against clinically important antibiotics into the two select agents B. mallei and B. pseudomallei. At present, only gentamicin, kanamycin, and zeocin resistance markers are approved for limited use for B. pseudomallei, while only the kanamycin and zeocin resistance markers are approved for B. mallei (35). However, most wild-type strains of B. mallei and B. pseudomallei have high levels of resistance to all three antibiotics (7, 29, 36), and even at high concentrations, the selection is not tight, and spontaneous resistance still arises (10, 15, 32). Consequently, there is still a need to expand universal genetic tools based on nonantibiotic selectable markers, allowing broader applications in various Burkholderia species.

Several nonantibiotic selection schemes have been used in bacteria including, but not limited to, resistance to various compounds (e.g., arsenate; bialaphos or its degradation product, phosphinothricin; mercury; and tellurite [Tel]) and metabolic markers (e.g., lactose utilization and purine and amino acid biosynthesis). Potential drawbacks to using arsenate and mercury are high toxicity levels and narrow selective concentration ranges (4, 16). Bialaphos and its degradation product, phosphinothricin, have been shown to be ineffective for Burkholderia select agents, requiring concentrations greater than 1,000 µg/mL, whereas these bacteria have been shown to be
sensitive to Tel concentrations of less than 1 μg/ml (M. Frazier, K. Choi, A. Kumar, C. Lopez, R. R. Karkhoff-Schweizer, and H. P. Schweizer, presented at the American Society for Microbiology Biodefense and Emerging Diseases Research Meeting, Washington, DC, 2007). Therefore, the nonantibiotic selectable marker based on Tel resistance (Telf) could be useful for genetic manipulation in various Burkholderia species, particularly \textit{B. mallei} and \textit{B. pseudomallei}. The Telf marker, consisting of three genes (kilA, telA, and telB) (38), has been successfully employed as a nonantibiotic selectable marker originally in \textit{Pseudomonas putida} (34), in several other gram-negative bacteria (25), and, more recently, in \textit{B. thailandensis} (2). Additionally, the \textit{asd} gene (a metabolic marker encoding aspartate-semialdehyde dehydrogenase for amino acid biosynthesis) has been used as a nonantibiotic selectable marker in Δasd backgrounds (2, 30). Combining the Telf marker and the \textit{asd} gene may expand the repertoire of genetic tools available for \textit{Burkholderia} species.

Strategies and tools for the manipulation of genetic elements as a single copy on the chromosome have been developed, such as \textit{Himar1}-based mariner transposons (22, 32), the mini-Tn7 site-specific transposition system (1, 9), and FRT-lacZ fusion vectors (12, 37). The random \textit{Himar1}-based mariner transposon plasmid pBT20 was successfully used for mutant library construction in \textit{Pseudomonas aeruginosa} (6, 19, 22) and has also been proven useful for transposition in a broad range of gram-negative bacteria (20). Similarly, the \textit{Himar1}-based transposons carrying the Km\textsuperscript{r} cassette were proven to be useful in \textit{B. pseudomallei} (32). The second single-copy system based on the mini-Tn7 site-specific transposon, when used in conjunction with the transposase-encoding helper plasmid, has broad applications for the introduction of single-copy chromosomal elements into gram-negative bacteria (9) and the select agent \textit{B. mallei} (8). Lastly, after mutant construction with an FRT-flanked selectable marker and Flp excision, the introduction of an Flp-containing helper plasmid and an FRT-lacZ fusion vector allows for simple Flp-catalyzed recombination to the “FRT scar” at the target gene downstream of the native promoter, facilitating regulation studies without prior knowledge of the promoter sequence (12, 37). Nevertheless, there are disadvantages to these existing systems when used in \textit{Burkholderia} species, particularly in the select agents \textit{B. pseudomallei} and \textit{B. mallei}, due to the antibiotic resistance markers used (e.g., gentamicin, kanamycin, ampicillin, and streptomycin) and the occurrence of spontaneously resistant mutants (10, 15, 32). Moreover, to our knowledge, no FRT-reporter fusion vectors based on reporter genes other than lacZ have been developed.

In this study, genetic tools using the Telf marker for selection were developed for single-copy analyses of chromosomally targeted genetic elements. These include a \textit{Himar1}-based random mariner transposon plasmid and a mini-Tn7 site-specific transposon vector. We also engineered FRT-reporter fusion vectors based on three common reporters, lacZ, gfp, and the \textit{luxCDABE} operon, allowing for Flp-catalyzed recombination. These systems expand upon our previously published nonantibiotic selectable marker approach for allelic replacement (2) and will aid in routine genetic manipulations including transposon mutagenesis, complementation studies, and promoter regulation studies of \textit{Burkholderia} species. Most importantly, all genetic tools presented here are completely devoid of antibiotic resistance selection and are in compliance with select-agent regulations. We utilized these tools to characterize the \textit{B. thailandensis} betBA operon, encoding betaine aldehyde dehydrogenase (BetB) and choline dehydrogenase (BetA).

\section*{MATERIALS AND METHODS}

\textbf{Bacterial strains, media, and culturing conditions.} All the strains and plasmids involved in this study are listed in Tables 1 and 2. \textit{Escherichia coli} strain EPMa10B-pplT16 was routinely used as a cloning strain. \textit{E. coli} strain DH5α-pir was used for the cloning of pBT20-Δbla-Telf-FRT. \textit{E. coli} strain E1345 was used to clone \textit{E. coli} \textit{asd} (\textit{asdEc})-containing vectors. The \textit{E. coli} conjugal and suicidal strain E1354 was routinely used for introducing plasmids into \textit{Burkholderia} species through conjugation. An alternative \textit{E. coli} conjugal donor, E463, was used for the conjugal transfer of transposon plasmid pBT20-Δbla-Telf-FRT. \textit{Luria-Bertani} (LB) medium (Difco) was used to culture all \textit{E. coli}, \textit{Burkholderia cenocepacia}, \textit{B. pseudomallei}, and \textit{B. mallei} strains. \textit{B. thailandensis} wild-type strain E264 and its derivatives were cultured in LB medium or 1/10 M9 minimal medium supplemented with 20 μM glucose (MG). For the single-copy complementation study (see Fig. 3), \textit{B. thailandensis} strains were grown in 1/10 M9 minimal medium plus 1% Brij 58 (Sigma) and 20 μM glucose or 30 μM choline chloride (Sigma). One percent Brij 58 was added to prevent bacterial clumping during growth. To study betB regulation, \textit{B. thailandensis} strains were grown in M9 minimal medium (M9) and Brij 58 alone with different concentrations of Tel (see Fig. 5) or in no-salt LB medium (LS; Teknova) supplemented with different NaCl concentrations (see Fig. 6). Antibiotics and nonantibiotic bacte- ricidial compounds were added to the media utilized for both selection and plasmid maintenance as follows: 110 μg/ml ampicillin (Ap), 25 μg/ml chloramphenicol (Cm), 15 μg/ml gentamicin (Gm), 35 μg/ml kanamycin (Km), 25 μg/ml streptomycin (Sp), and 20 μg/ml potassium Tel (TEKNOVA) for \textit{E. coli}; 125 μg/ml Tel for \textit{B. cenocepacia} strain K562 and \textit{B. thailandensis}; 200 μg/ml Tel for \textit{B. cenocepacia} strain J2315; and 25 μg/ml Tel for \textit{B. pseudomallei} strains K92643 and 1026b and \textit{B. mallei} strain ATCC 23344. For the growth of \textit{E. coli} Δasd strains E463, E1345, and E1354, without \textit{asdEc}-containing plasmids, 100 μg/ml of diaminopimelic acid (Sigma) was supplied. All manipulations of \textit{B. pseudomallei} and \textit{B. mallei} were conducted in a CDC/USDA-approved and registered bio-safety level 3 facility at the University of Hawaii at Manoa. All experiments with these two select agents were performed with biosafety level 3 practices according to recommendations described previously (32a).

\textbf{Molecular methods and reagents.} All restriction enzymes, deoxynucleoside triphosphates, T4 DNA polymerase, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs and used as recommended by the supplier. Plasmids and DNA gel bands were isolated using the ZypReactor gel plasmid maintenance as follows: 110 μg/ml ampicillin (Ap), 25 μg/ml chloramphenicol (Cm), 15 μg/ml gentamicin (Gm), 35 μg/ml kanamycin (Km), 25 μg/ml streptomycin (Sp), and 20 μg/ml potassium Tel (TEKNOVA) for \textit{E. coli}; 125 μg/ml Tel for \textit{B. cenocepacia} strain K562 and \textit{B. thailandensis}; 200 μg/ml Tel for \textit{B. cenocepacia} strain J2315; and 25 μg/ml Tel for \textit{B. pseudomallei} strains K92643 and 1026b and \textit{B. mallei} strain ATCC 23344. For the growth of \textit{E. coli} Δasd strains E463, E1345, and E1354, without \textit{asdEc}-containing plasmids, 100 μg/ml of diaminopimelic acid (Sigma) was supplied. All manipulations of \textit{B. pseudomallei} and \textit{B. mallei} were conducted in a CDC/USDA-approved and registered bio-safety level 3 facility at the University of Hawaii at Manoa. All experiments with these two select agents were performed with biosafety level 3 practices according to recommendations described previously (32a).

\textbf{Conjugal transfer of vectors into \textit{Burkholderia} species.} \textit{E. coli} strain E463 was used as the conjugal donor to introduce transposon plasmid pBT20-Δbla-Telf-FRT into all \textit{Burkholderia} species. Another \textit{E. coli} conjugal strain, E1354, was used to introduce the mini-Tn7 and FRT-lacZ vectors and their respective helper plasmids into \textit{B. thailandensis} strains. Conjugation of non-select-agent \textit{Burkholderia} species was carried out as follows. The donor and recipients were grown to log phase for conjugation. One milliliter of each culture was harvested separately by centrifugation at 9,000 × g for 1 min at room temperature and washed twice with 1 ml of LB medium. The cell pellets of the donor and recipients were then resuspended together in 30 μl of LB medium. The 30-μl cell suspension was spotted onto cellulose acetate filters (Sartorius) on LB agar plates and incubated at 37°C for 8 h. Filters were then vortexed in 1 ml of 1× M9 minimal medium, and 100 μl of this cell suspension and 100 μl of 10× dilutions were plated onto LB or MG plates with appropriate concentrations of Tel. Conjugations into \textit{B. pseudomallei} or \textit{B. mallei} cells were performed directly on LB plates without filters. Bacteria were gently scraped off the LB plates with disposable inoculation loops and resuspended in 1× M9 medium, and plating was done similarly as described above. Plates were usually incubated for 2 to 3 days at 37°C until single Telf colonies were observed.
TABLE 1. Bacterial strains utilized in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lab ID*</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>E1249</td>
<td>F− λ− merA Δ(mrr-hsdRMS-mcrBC) ΔlacX74 deor recA1 endA1 araD139 Δ(ara leu) galU galK rpsL mupG Tn-pir116-FRT2</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>DH5α-pir</td>
<td>E0175</td>
<td>Te+ λ+ φ80lacZΔM15 (lacZYA-argF)U169 deor recA1 endA1 hsdR17(ri-mcrBK_2) phoA supE44 thi-1 gyrA96 relA1 supF7697 zygD23::Tn10</td>
<td>31</td>
</tr>
<tr>
<td>HPS1-mob-Δasd-pir</td>
<td>E0463</td>
<td>Te+ Km2 Cm1; e14 (merA) recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(lac-proAB) rif zxx::mini-Tn5-Lac4 (lacF1+ lacZ M15) Δasd::FRT uidA::pir zygD232::Tn10 recA::RP4-2 Te::Mu Km2</td>
<td>—b</td>
</tr>
<tr>
<td>EPMAX10B-pir116-Δasd::Gm′</td>
<td>E1345</td>
<td>Gm2; F− λ− merA Δ(mrr-hsdRMS-mcrBC) Δ80lacZΔM15 ΔlacX74 deor recA1 endA1 araD139 Δ(ara leu) galU galK rpsL mupG Tn-pir116-FRT2 Δasd::Gm2</td>
<td>—b</td>
</tr>
<tr>
<td>EPMAX10B-pir116-Δasd-mob-Km′-Δrps::Gm′</td>
<td>E1354</td>
<td>Km2 Gm2; F− λ− merA Δ(mrr-hsdRMS-mcrBC) Δ80lacZΔM15 ΔlacX74 deor recA1 endA1 araD139 Δ(ara leu) galU galK rpsL mupG Tn-pir116-FRT2 Δasd::FRT rpsA::RP4-2 Te::Mu Km2</td>
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</tr>
<tr>
<td>DH5α-attB::pCD13SK-Flp</td>
<td>E0982</td>
<td>Sp4; F− φ80lacZΔM15 (lacZYA-argF)U169 deor recA1 endA1 hsdR17(ri-mcrB) phoA supE44 thi-1 gyrA96 relA1 attB::pCD13SK-Flp</td>
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B. thailandensis

<table>
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<th>Source or reference</th>
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<td>E264</td>
<td>E1298</td>
<td>Prototroph</td>
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<tr>
<td>E264-ΔbetBA::FRT</td>
<td>E1671</td>
<td>B. thailandensis ΔbetBA::FRT mutant</td>
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<tr>
<td>E264-ΔbetBA::FRT/attTn7::Tel′</td>
<td>E1709</td>
<td>Tel′; B. thailandensis ΔbetBA::FRT mutant with empty vector mini-Tn7-Tel′ integrated at the attTn7 site</td>
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<td>E264-ΔbetBA::FRT/attTn7::Tel′-betBA</td>
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<td>E264-ΔbetBA::FRT-lacZ-Tel′</td>
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<td>E264-ΔbetBA::FRT-gfp-Tel′</td>
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<td>E264-ΔbetBA::FRT-lacZ-Tel′/attTn7::betBA</td>
<td>E1849</td>
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<td>E264-ΔbetBA::FRT-gfp-Tel′/attTn7::betBA</td>
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<td>E264-ΔbetBA::FRT-lacZ-Tel′/attTn7::betBA</td>
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B. cenocepacia

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<td>K56-2</td>
<td>E1554</td>
<td>Prototroph; cystic fibrosis isolate</td>
<td>P. Sokol</td>
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<td>J2315</td>
<td>E1553</td>
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<td>J. Goldberg</td>
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B. pseudomallei

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<td>K96243</td>
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<td>1026b</td>
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B. mallei ATCC 23344

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<td>B0001</td>
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<td>40</td>
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* For strains constructed in this study, please see the text for further details. Please use the laboratory identification (Lab ID) number to request strains.

b — details on the engineering of these strains will be published elsewhere.

Construction and testing of pBT20-Δbla-Tel′-FRT. A new mariner transposon vector was constructed based on the Tel′ marker (Fig. 1). pwFRT-PC17::Tel′ was digested with SmaI, and the 3.2-kb PC17::Tel′ fragment was cloned into the pBT20-Δbla backbone (∼4.3 kb) following Bsal digestion and blunt ending. This replaced the Gm′ cassette with the Tel′ marker, resulting in transposon vector pBT20-Δbla-Tel′-FRT. The transposition frequencies of pBT20-Δbla-Tel′-FRT were determined by conjugation into several different Burkholderia species and strains: B. cenocepacia strains K56-2 and J2315, B. thailandensis strain E264, B. pseudomallei strains K96243 and 1026b, and B. mallei strain ATCC 23344. Following conjugation as described above, the mating mixtures were diluted and plated onto LB plates and LB plates supplemented with the appropriate concentration of Tel. The transposition frequencies for individual conjugation experiments were calculated based on the ratio of the number of colonies counted that were grown on LB medium plus Tel to the number of colonies that were grown on LB medium. Three independent conjugation experiments were carried out to obtain the average transposition frequency and standard error of the mean for each strain. Similar control conjugation experiments, omitting the E. coli conjugal donor harboring pBT20-Δbla-Tel′-FRT, were performed on all recipient strains to ensure that no spontaneous mutants arose from Tel selection. For B. thailandensis and B. cenocepacia, 15 random Tel′ colonies were purified on LB plates with Tel and PCR screened using telB-specific oligonucleotides 834 and 854.
B. pseudomallei previously (17), for the pwFRT-PC

pwFRT-P

pPS856-pFRT2-pFRT1-
lacZ

E0790 Gm r;

-Telr E2053 Tel r; pFRT1-
lacZ

purified on LB plates with Tel and PCR screened using /H9004

bla

pBT20-

/H9004

/H11032

/E1707 Tel r; pFRT1-lacZ with Gm' cassette replaced by Tel' cassette

/E1708 Tel' pFRT2-lacZ with Gm' cassette replaced by Tel' cassette

/E1044 Gm' Ap'; lacZ' cassette flanked by wild-type FRT sequences

/E0042 Ap'; gfp-containing vector

/A-Pilx2

/E1863 Ap'; luxCDABE bioluminescence operon-containing vector

/FRT1-Gmr-

lacZ

/E1827 pCD13SK-Flp-

laFRT1-lacZ-Tel' replace the

FRT1-Gmr-FRT1 fragment

/FRT2-Gm'-lacZ-Tel'

/E2050 Gm' Tel'; FlpFRT1-laZ-Tel' replaced by the

FRT2-Gm'-FRT2 fragment

/FRT1-Gmr-lacZ-Tel'-FRT2-lacZ-Tel'

/E2051 Tel'; FlpFRT1-lacZ-Tel' with Flp-excised FRT1-Gm' Tel'; FlpFRT2-lacZ-Tel' with Flp-excised FRT2-Gm' Tel'; FlpFRT2-gfp-Tel'

/E2053 Tel'; FlpFRT1-lacZ-Tel'-FRT2-lacZ-Tel' with gfp replacing lacZ

/E2055 Tel'; FlpFRT2-lacZ-Tel' with gfp replacing lacZ

/E2064 Tel'; FlpFRT2-lacZ-Tel'-FRT2-lacZ-Tel' with luxCDABE replacing lacZ

/E2065 Tel'; FlpFRT2-lacZ-Tel' with luxCDABE replacing lacZ

/E0803 Sp'; Flp-containing suicidal vector

/E1827 pCD13SK-Flp-orT-asd E0

/E1862 pFipAB-5

/E1662 Tp'; broad-host-range Flp-containing vector

TABLE 2. Bacterial plasmids utilized in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Lab ID</th>
<th>Relevant properties</th>
<th>Reference or source</th>
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<td>pBT20-Δbla</td>
<td>E1029</td>
<td>Gm'; mariner transposon plasmid</td>
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<tr>
<td>pwFRT-P&lt;sub&gt;S22&lt;/sub&gt;-Tel'</td>
<td>E1584</td>
<td>Tel'; P&lt;sub&gt;S22&lt;/sub&gt;-Tel' cassette flanked by wild-type FRT sequences</td>
<td>2</td>
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<tr>
<td>pBT20-Δbla-pTel'-FRT</td>
<td>E1727</td>
<td>Tel'; mariner transposon plasmid based on Tel'</td>
<td>This study</td>
</tr>
<tr>
<td>pCD111-Gm'&lt;sub&gt;p116&lt;/sub&gt;-orT</td>
<td>E1254</td>
<td>Gmt Gm'; conjugation vector</td>
<td>Laboratory collection</td>
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<tr>
<td>pUC18R6KT-miniTn7</td>
<td>E1190</td>
<td>Ap'; Tn7-based broad-host-range transposon vector</td>
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<tr>
<td>mini-Tn&lt;sub&gt;7&lt;/sub&gt;-Tel-bla</td>
<td>E1645</td>
<td>Ap'; Tel'; Tel' cassette cloned into pUC18R6KT-miniTn7</td>
<td>This study</td>
</tr>
<tr>
<td>mini-Tn&lt;sub&gt;7&lt;/sub&gt;-Tel</td>
<td>E1825</td>
<td>Tel'; mini-Tn&lt;sub&gt;7&lt;/sub&gt;-bla-Tel' with bla gene deleted</td>
<td>This study</td>
</tr>
<tr>
<td>mini-Tn&lt;sub&gt;7&lt;/sub&gt;-Tel'betBA</td>
<td>E1829</td>
<td>Tel'; mini-Tn&lt;sub&gt;7&lt;/sub&gt;-Tel' with betBA operon cloned</td>
<td>This study</td>
</tr>
<tr>
<td>pTNS3</td>
<td>E1189</td>
<td>Ap'; helper plasmid for Tn7 transposition system</td>
<td>9</td>
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<tr>
<td>pTNS3-asd E0</td>
<td>E1831</td>
<td>pTNS3 with bla replaced by the E. coli asd gene</td>
<td>This study</td>
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<tr>
<td>pFRT1-lacZ</td>
<td>E0790</td>
<td>Gm'; FRT1-lacZ fusion containing suicidal vector</td>
<td>37</td>
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<tr>
<td>pFRT1-lacZ-Tel'</td>
<td>E1707</td>
<td>Tel'; pFRT1-lacZ with Gm' cassette replaced by Tel' cassette</td>
<td>This study</td>
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<td>pFRT2-lacZ</td>
<td>E0787</td>
<td>Gm'; FRT2-lacZ fusion containing suicidal vector</td>
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<td>pFRT2-lacZ-Tel'</td>
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<td>Tel'; pFRT2-lacZ with Gm' cassette replaced by Tel' cassette</td>
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<tr>
<td>pPS856-Δxbas</td>
<td>E1044</td>
<td>Gm' Ap'; lacZ' cassette flanked by wild-type FRT sequences</td>
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<td>pPS747</td>
<td>E0042</td>
<td>Ap'; gfp-containing vector</td>
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<td>pA-Pilx2</td>
<td>E1863</td>
<td>Ap'; luxCDABE bioluminescence operon-containing vector</td>
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<td>pFRT1-Gm'-lacZ-Tel'</td>
<td>E2049</td>
<td>Gm' Tel'; pFRT1-lacZ-Tel' with FRT1 replaced by the</td>
<td>This study</td>
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<tr>
<td>pFRT2-Gm'-lacZ-Tel'</td>
<td>E2050</td>
<td>Gm' Tel'; pFRT2-Gm'-FRT2 fragment</td>
<td>This study</td>
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<tr>
<td>pFRT1-lacZ-Tel'-FRT2-lacZ-Tel'</td>
<td>E2051</td>
<td>Tel'; pFRT1-lacZ-Tel' with Flp-excised FRT1-Gm' Tel'; FlpFRT2-lacZ-Tel' with Flp-excised FRT2-Gm' Tel'; FlpFRT2-gfp-Tel'</td>
<td>This study</td>
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<tr>
<td>pFRT1-gfp-Tel'</td>
<td>E2053</td>
<td>Tel'; pFRT1-lacZ-Tel'-FRT2-lacZ-Tel' with gfp replacing lacZ</td>
<td>This study</td>
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<tr>
<td>pFRT1-gfp-Tel'</td>
<td>E2055</td>
<td>Tel'; pFRT2-lacZ-Tel' with gfp replacing lacZ</td>
<td>This study</td>
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<td>pFRT1-lacZ-Tel';</td>
<td>E2064</td>
<td>Tel'; pFRT1-lacZ-Tel'-FRT2-lacZ-Tel' with luxCDABE replacing lacZ</td>
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<td>pFRT1-lacZ-Tel';</td>
<td>E2065</td>
<td>Tel'; pFRT2-lacZ-Tel' with luxCDABE replacing lacZ</td>
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<tr>
<td>pCD13SK-Flp-orT</td>
<td>E0803</td>
<td>Sp'; Flp-containing suicidal vector</td>
<td>37</td>
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<tr>
<td>pCD13SK-Flp-orT-asd E0</td>
<td>E1827</td>
<td>pCD13SK-Flp-orT with asd E0 replaced by the Sp' cassette</td>
<td>21</td>
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<td>pFipAB-5</td>
<td>E1662</td>
<td>Tp'; broad-host-range Flp-containing vector</td>
<td>2</td>
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</tbody>
</table>

<sup>a</sup> For plasmids constructed in this study, please see the text for further details. Please use the laboratory identification (Lab ID) number when requesting plasmids.

<sup>b</sup> —, details on the engineering of this plasmid are to be published elsewhere.

For B. pseudomallei and B. mallei, five random Tel' colonies were purified on LB plates with Tel and PCR screened using kilΔ oligonucleotides 831 and 1066, telΔ oligonucleotides 827 and 1067, and telΔ oligonucleotides 834 and 854 (Table 3). Southern hybridization analysis was also performed, as described previously (17). For B. pseudomallei and B. mallei Tel' colonies using a telA-specific probe after the digestion of chromosomal DNA with XhoI.

Construction of mini-Tn<sub>7</sub> site-specific transposon and their helper plasmid. The mini-Tn<sub>7</sub>-Tel' site-specific transposon, based on Tel', was constructed as described below. pwFRT-PC<sub>S22</sub>-Tel' was digested with SacI and blunt ended, and the p<sub>PC</sub><sub>S22</sub>-Tel' fragment (3.2 kb) was cloned into pUC18R6KT-miniTn7 (EcoRV digested), resulting in mini-Tn<sub>7</sub>-Tel'-bla. The R6K<sub>ori</sub>-ori<sub>T</sub> region, amplified from pCD111-Gm'<sub>p116</sub>-orT using oligonucleotides 696 and 702, was ligated with the mini-Tn<sub>7</sub>-Tel'-bla backbone (including the Tel' cassette) following AflII and NarI digestion and blunt ending. This resulted in a mini-Tn<sub>7</sub>-Tel' vector (Fig. 2A).

TABLE 3. Oligonucleotide primers utilized in this study

<table>
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<td>89 (asd&lt;sub&gt;Δ&lt;/sub&gt;-np)</td>
<td>5'-CGGTGTTACTTACTCCGGTGCGGAATAGGC-3'</td>
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<tr>
<td>91 (asd&lt;sub&gt;Δ&lt;/sub&gt;-down)</td>
<td>5'-TACTGGATCCGGCAAAATGGGCTGCAATTAG-3'</td>
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<tr>
<td>696 (orT-Clai-1)</td>
<td>5'-TGGTATCTGATTTACCTAAAGGTATACTTT-3'</td>
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<tr>
<td>702 (R6K)</td>
<td>5'-TGTCACGCTTGAATGTTTC-3'</td>
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<td>713 (lacZ)</td>
<td>5'-TTGGTGGAGAGGGCGGATC-3'</td>
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<tr>
<td>827 (telA-Smal)</td>
<td>5'-GGGAAGACCCCTGCCGAGCGCA-3'</td>
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<tr>
<td>831 (tel-kilA)</td>
<td>5'-AGCTAAATGAGGAAAGAACA-3'</td>
</tr>
<tr>
<td>834 (tel-BXhol)</td>
<td>5'-CCCTCTGAGCAAGAAATCAGGACCT-3'</td>
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<tr>
<td>854 (telB-down)</td>
<td>5'-TACCAAGAAGATGGACAC-3'</td>
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<tr>
<td>861 (Bet-betBA-HindIII)</td>
<td>5'-CCCGCAAGCTTCCGGCGCA-3'</td>
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<tr>
<td>862 (Bet-betBA-KpnI)</td>
<td>5'-AACGGGTATCCGGCCCAGGCGGAT-3'</td>
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<tr>
<td>873 (gntS1-DN)</td>
<td>5'-GTCCTGCTGCACCGCAAACTA-3'</td>
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<tr>
<td>875 (glnS1-DN)</td>
<td>5'-AGATCGGGATGATATTGTGCAGG-3'</td>
</tr>
<tr>
<td>876 (Tn&lt;sub&gt;7&lt;/sub&gt;)</td>
<td>5'-ATTAGCTTACGACGTACCAGCA-3'</td>
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<tr>
<td>885 (gfp-BspHI-down)</td>
<td>5'-CAGAGTACATGACACCTCTCTTTATGTA-3'</td>
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<sup>a</sup> Oligonucleotides were synthesized as previously described (9).
FIG. 2. The mini-Tn7-Tel’ integration vector and its pTNS3-asdEc helper plasmid. (A) mini-Tn7-Tel’, a Tn7-based suicidal vector with the Tel’ marker. (B) pTNS3-asdEc, suicidal helper plasmid encoding the transposable, which catalyzes Tn7 transposition. (C) Chromosomally inserted mini-Tn7-Tel’ elements at two different B. thailandensis attTn7 sites as previously described (6). Oligonucleotides 874, 875, and 876, indicated by arrows, were used to screen for the location of transposable. glmS1 and glmS2 encode glucosamine-6-phosphate synthetases; MCS, multiple-cloning site; P1, P1 integrase promoter; Tn7L, Tn7 transposase left recognition sequence; Tn7R, Tn7 transposase right recognition sequence; T0, transcriptional terminators; insABCD, Tn7 transposases.

Helper plasmid pTNS3-asdEc (Fig. 2B), containing the Tn7 transposase genes, was also constructed based on asdEc. The asdEc fragment was amplified from E. coli K-12 chromosomal DNA using oligonucleotides 874 and 91. The 2.5-kb PCR product was digested with EcoRI and PstI and blunt ended, and the 1.6-kb asdEc gene was ligated into the pTNS3 backbone (digested with BglI and blunt ended), yielding pTNS3-asdEc.

Single-copy complementation of E264-ΔbetBA::FRT. The mini-Tn7-Tel’ and the betBA operon fragment (amplified from E264 with oligonucleotides 861 and 862) were digested with HindIII and blunt ended, and the 1.6-kb asdEc gene was ligated into the pTNS3 backbone (digested with BglI and blunt ended), yielding pTNS3-asdEc.

Characterization of growth of E264-ΔbetBA::FRT and the complemented strain. Growth curve experiments were performed on three B. thailandensis strains: wild-type E264, the E264-ΔbetBA::FRT containing the mini-Tn7-Tel’-betBA control, and the E264-ΔbetBA::FRTattTn7::Tel’-betBA complement. These strains were grown overnight at 37°C in LB medium. Cultures grown overnight were washed twice with M9 medium, 1% Brij 58, and 20 mM glucose or 30 mM choline chloride, and growth was initiated by shaking at 250 rpm and 37°C. At all time points, aliquots of each culture were taken, and optical densities were measured at 600 nm.

Construction of FRT-lacZ, FRT-gfp, and FRT-lux fusion vectors. An flp-carrying helper plasmid containing a nonantibiotic resistance marker was first created for the recombination of the various reporter fusion vectors. pCD138K-Flp-orT was digested with Ndel and SalI, blunt ended, and ligated with the above-mentioned 1.6-kb asdEc fragment. The resulting helper plasmid, pCD138K-Flp-orT-asdEc (see Fig. 4A), contains the asdEc marker in place of the Sp’ cassette. Two pFRT-lacZ-Tel’ vectors, pFRT1-lacZ-Tel’ and pFRT2-lacZ-Tel’ (Fig. 4B), were constructed in this study by replacing the Gm’ cassette with the Tel’ cassette. To create pFRT1-lacZ-Tel’, pwFRT-PC12-Tel’ was digested with EcoRV and XhoI and blunt ended, and the resulting PC12-Tel’ fragment was cloned into pFRT1-lacZ (digested with BglI and SalI and blunt ended to remove the Gm’ cassette). Similarly, pFRT2-lacZ-Tel’ was constructed by cloning the PC12-Tel’ fragment into the pFRT2-lacZ backbone.

Four different fusion vectors were constructed based on the gfp reporter gene and the luxCDABE operon. In order to replace the lux gene with the promoterless gfp reporter gene or the lac operon, several cloning steps were carried out to eliminate one of the BamHI sites flanking the FRT sequence, leaving a unique BamHI site downstream of the FRT sequence. First, pPS856-ΔXba5 was digested with SmaI to recover the FRT-Gm’-FRT cassette, which was cloned into the pFRT1-lacZ-Tel’ backbone following digestion with the same enzyme. This cloning step resulted in the creation of pFRT1-Gm’-lacZ-Tel’ (with the Gm’ cassette in the same orientation as the lacZ gene) and pFRT2-Gm’-lacZ-Tel’ (with the Gm’ cassette in the opposite orientation of the lacZ gene). To excise the Gm’-FRT fragment, both pFRT1-Gm’-lacZ-Tel’ and pFRT2-Gm’-lacZ-Tel’ were introduced into Flp-containing strain DH5α-attB::pCD138K-Flp. The resulting constructs, pFRT1-lacZ-Tel’-ΔBam and pFRT2-lacZ-Tel’-ΔBam, were digested with Ndel, blunt ended, and then digested with BamHI. These Tel’ cassette-containing vectors were ligated with the gfp gene from pPS747 (digested with HindIII, blunt ended, and then digested with BamHI), yielding pFRT1-gfp-Tel’ and pFRT2-gfp-Tel’, respectively (Fig. 4C). Finally, pFRT1-lacZ-Tel’-ΔBam and pFRT2-lacZ-Tel’-ΔBam were digested with BamHI and Ndel and blunt ended, and the luxCDABE operon, obtained from pAKlux2 (Addgene plasmid 14080) following EcoRI digestion and blunt ending, was cloned to yield pFRT1-lacZ-Tel’ and pFRT2-lacZ-Tel’ (Fig. 4D).
Engineering of B. thailandensis E264-BetBA::FRT reporter fusion strains. To construct the E264-BetBA::FRT-lacZ-Tel' reporter strain for the betBA promoter study, pFR1-T-lacZ-Tel and the helper plasmid (pCD135K F-oriT-ascEc) were conjugated from E1354 into E264-BetBA::FRT in a triparental mating experiment. Colonies on MG plates with 125 μg/ml Tel were screened by PCR using oligonucleotides 713 and 861 to confirm the orientation of the lacZ gene relative to the betBA promoter region. This strain was then used in the choline induction study (see Fig. 5B and C). The E264-BetBA::FRT-lacZ-Tel' fusion strain was complemented by engineering the E264-BetBA::FRT-lacZ-Tel'/attTn7::betBA strain in several steps (see Fig. 6A). First, the Tel' cassette in strain E264-BetBA::FRT-lacZ-Tel'/attBET-Ba (described above) was FAB excised using pFLP-ABS according to a previously described procedure (2). The resulting strain, E264-BetBA::FRT-lacZ-Tel'/attBET-Ba, was conjugated with two E1354 donor strains harboring either pFR1-lacZ-Tel or pCD135K F-oriT-ascEc, in a triparental mating experiment. This triparental mating mixture was then plated onto MG plates with 125 μg/ml Tel to select for fusion strain E264-BetBA::FRT-lacZ-Tel'/attBET-Ba, harboring a lacZ reporter driven by the native betBA promoter. Colonies were screened by PCR using oligonucleotides 713 and 861 (Fig. 6A). Isolates with lacZ integrated at the betBA locus were purified once on LB medium and used in the osmotic regulation study (see Fig. 8B and C). Fusion vector pFR1-gfp-Tel was also integrated into the ΔbetBA::FRT mutant and complemented strains as described above. The resulting fusion strains, E264-ΔbetBA::FRT-gfp-Tel and E264-ΔbetBA::FRT-gfp-Tel'/attTn7::betBA, were screened by PCR using oligonucleotides 861 and 885 (Fig. 6A). Similarly, vector pFR1-lac-Z-Tel was used to construct fusion strains E264-ΔbetBA::FRT-lac-Z-Tel' and E264-ΔbetBA::FRT-FRT-lac-Z-Tel'/attBET-Ba, which were screened by PCR using oligonucleotides 861 and 1030 (see Fig. 6A).

Choline and osmotic regulation studies of the betBA operon. β-Galactosidase activity of the integrated betBA::FRT-lacZ-Tel' fusion was measured under various growth conditions. To study choline induction of the betBA operon, fusion strain E264-ΔbetBA::FRT-lacZ-Tel' was grown overnight in LB medium. Cultures grown overnight were washed twice with 1 volume of 1X M9 medium and resuspended in an equal volume of the same medium. Resuspended cultures were then diluted 100-fold into a solution containing fresh 1X M9 medium, 1% Brij 58, 20 mM glucose, and 0, 1, 2, 4, or 8 mM choline chloride. Growth curve experiments were performed on each culture by diluting sample aliquots twice in Brij 58, 20 mM glucose, and 0, 1, 2, 4, or 8 mM choline chloride. Growth curve experiments were performed on each culture by diluting sample aliquots twice in Brij 58 and measuring the optical density at 600 nm (see Fig. 5B). Additional 1 ml cell culture aliquots were taken at each time point during the growth curve experiments to assay for β-galactosidase activity. These assays were done in triplicate and are displayed as average Miller units (28), with standard errors of the mean (28).

RESULTS

Engineering and utilization of a random Tel' transposon in Burkholderia species. A Himar1-based mariner transposon carrying a Km' marker has been used successfully in B. pseudomallei although with some reported leakiness (32). To further develop and test a mariner transposon based on the alternative nonantibiotic Tel' marker for a broader range of Burkholderia spp., we replaced the Km' marker on mariner transposon plasmid pBT20. pBT20, originally based on the Km' marker with a bla gene in its plasmid backbone, is not appropriate for selection in Burkholderia species due to their high level of Km' and may be inappropriate for use in the select-agent species B. pseudomallei and B. mallei. In this study, we constructed a mariner transposon, pBT20-Δbla-Tel'-FRT, based on the nonantibiotic Tel' marker (Fig. 1) previously shown to be effective in B. thailandensis (2). We eliminated the bla gene from pBT20 and replaced the Km' cassette on the transposon with the Tel' marker for selection in both E. coli and Burkholderia. To demonstrate the effectiveness of this transposon, we conjugated pBT20-Δbla-Tel'-FRT from a suicidal Δasf E. coli strain into four different Burkholderia species: B. cenocepacia (two strains), B. thailandensis, B. pseudomallei (two strains), and B. mallei. For each species, three independent mating experiments were conducted, and the average transposition frequencies were determined and are shown in Table 4. We determined the effective Tel concentrations for the four Burkholderia species, and no spontaneous Tel' mutants were detected when 105 CFU were plated alone on LB medium with Tel as controls (see Materials and Methods). On average, conjugation mixtures were resuspended in 1 ml of LB medium and diluted 10-, where 100-μl volumes were plated onto LB medium with Tel, yielding 50 to 200 colonies depending on the species. Fifteen random Tel' colonies from B. cenocepacia (J2315 and K56-2) and B. thailandensis were screened by PCR using telB-specific oligonucleotides, and five random colonies from B. pseudomallei (K96243 and 1026b) and B. mallei were positively screened by PCR using kilA-, telA-, and telB-specific oligonucleotides (see Fig. S1A to S1F in the supplemental material). Southern blot analysis was also performed on the 15 Tel' isolates of B. pseudomallei and B. mallei using a telB-specific probe (see Fig. S1G in the supplemental material). Single bands with different sizes were obtained in all isolates, suggesting random transposition into the B. pseudomallei and B. mallei genes.
genomes (see Fig. S1G in the supplemental material). All Burkholderia species tested displayed similar transposition frequencies, ranging from $2.08 \times 10^{-6} \pm 0.16 \times 10^{-6}$ to $1.51 \times 10^{-5} \pm 0.23 \times 10^{-5}$ (Table 4), which are comparable to the frequencies obtained when using a Km$^r$-based transposon in B. pseudomallei (32). However, analyses of 5 to 15 Tel$^r$ colonies showed that 100% of the Tel$^r$ colonies contained the transposon, demonstrating the effectiveness of Tel$^r$ selection (see Fig. S1 in the supplemental material). No spontaneous resistance was observed when 10$^9$ CFU were plated, indicating that the spontaneous resistance frequency is $<10^{-9}$. If required, the transposon insertion sites could easily be determined by sequencing the flanking region of the transposon using semirandom PCR methods as previously described (19, 24). The Tel$^r$ cassette in our transposon, flanked by FRT sequences (17), could then be excised by Flp recombinase for subsequent recycling of the Tel$^r$ cassette or integration of FRT-reporter fusions (below) at the transposed loci for immediate gene regulation studies.

**Engineering and testing of the single-copy mini-Tn7-Tel$^r$ site-specific transposon by complementing the B. thailandensis ΔbetBA mutant.** The mini-Tn7 site-specific transposon and helper plasmid (carrying the Tn7 transposase genes) were utilized in various species (1, 3, 8, 23). This system could be used for single-copy complementation studies, promoter-reporter fusion integration, and reporter gene (e.g., fluorescence and bioluminescence proteins) tagging in Burkholderia species (1, 3, 8, 23). However, all these systems contain antibiotic resistance markers for selection, requiring the need for reengineering with nonantibiotic selectable markers. In this study, we developed a mini-Tn7-Tel$^r$ site-specific transposon and a helper plasmid for Burkholderia species based on two nonantibiotic selectable markers, the Tel$^r$ cassette and the asd$_{Ec}$ gene, respectively (Fig. 2A and B). This mini-Tn7-Tel$^r$ vector contains a multiple-cloning site for conveniently cloning genes of interest for subsequent site-specific transposition, which is catalyzed by the transposase encoded on the pTNS3-asd$_{Ec}$ helper plasmid. The location of the chromosomally inserted transposon could be determined by PCR with site-specific and transposon-specific oligonucleotides (9) (Fig. 2C). The FRT-flanked Tel$^r$ cassette allows Flp-catalyzed excision of the Tel$^r$ marker while maintaining the introduced gene of interest at the specific transposition site.

As a proof of concept, the mini-Tn7-Tel$^r$ system was used to complement the betBA mutation in B. thailandensis. Previously, we engineered a B. thailandensis ΔbetBA mutant that exhibits a growth defect when grown in choline as a sole carbon source (2). A wild-type copy of the betBA operon was cloned into the multiple-cloning site of mini-Tn7-Tel$^r$ (Fig. 2A), resulting in the mini-Tn7-Tel$^r$-betBA vector. The helper plasmid pTNS3-asd$_{Ec}$ and the mini-Tn7-Tel$^r$-betBA vector were simultaneously conjugated by triparental mating into strain E264-ΔbetBA::FRT. Site-specific transposition of the betBA-Tel$^r$ complement was confirmed by PCR as previously described (9). In the majority of Tel$^r$ isolates (8 out of 10 screened), the mini-Tn7 transposon was inserted downstream of the glmS2 gene on the second chromosome, while two transpositions occurred downstream of the glmS1 gene on the first chromosome (9). None of the isolates displayed integration on both chromosomes (see Fig. S2 in the supplemental material).

To show the complementation of the ΔbetBA mutant, the constructed strain E264-ΔbetBA::FRT/tel7::Tel$^r$-betBA was tested for its ability to grow on choline as a sole carbon source. As shown in Fig. 3A, this chromosomally integrated copy of the betBA operon recovered the growth ability of the ΔbetBA mutant strain on choline as a sole carbon source, displaying a growth rate and an overall cell density comparable to those of wild-type strain E264. The transposition of the empty mini-Tn7-Tel$^r$ control into the ΔbetBA mutant yielded no complementation of the ΔbetBA mutation, and it was unable to grow with choline as a sole carbon source (Fig. 3A). Growth curve studies for these three strains on glucose as a sole carbon source were also conducted (Fig. 3B) to show that the integrated mini-Tn7 system did not alter any other growth phenotypes of mutant strain E264-ΔbetBA::FRT.

**Engineering of reporter gene constructs and regulation studies of the betBA operon.** Recombination of the FRT-lacZ reporter fusion with the single chromosomally located “FRT scar”...
aided by the Flp-encoding helper plasmid, following mutant construction with an FRT-flanked antibiotic resistance cassette and Flp excision, has facilitated regulation studies of target genes at their native chromosomal loci (12, 37). In our experience, when coupled with FRT-based resistant-marker approaches for chromosomal mutagenesis (e.g., allelic replacement or FRT-based transposon) (32), these fusion vectors were found to be simple and powerful tools for studying gene regulation without promoter mapping or prior knowledge of promoter sequence or location. The disadvantages of previously reported fusion vectors and helper plasmids (12, 37) are the use of antibiotic resistance markers (Apr, Cmr, Gmr, and Spr) and the limitation of a single reporter gene (lacZ). Here, the Telr cassette replaced the Gmr cassette in the previously reported vectors pFRT1-lacZ and pFRT2-lacZ (37), resulting in pFRT1-lacZ-Telr and pFRT2-lacZ-Telr (Fig. 4B). The difference between these two new vectors is the orientation of the FRT sequence relative to that of the reporter gene, accounting for the selection of the appropriate fusion vector relative to the orientation of the “FRT scar” on the chromosome, thus aligning the reporter gene in the same direction as the promoter of interest. Abbreviations: cI857, temperature-sensitive repressor; gfp, green fluorescent protein gene; lacZ, β-galactosidase gene; luxCDABE, genes encoding the bacterial bioluminescent operon; T1T2, transcriptional terminators.

**FIG. 4.** Plasmid maps of the FRT-reporter fusion vectors and their helper plasmids. (A) pCD13SK-Flp-oriT-asdEc is a flp-containing helper plasmid for the recombination of all FRT-reporter fusions. (B to D) Various fusion vectors were constructed based on the reporters lacZ (B), gfp (C), and the luxCDABE operon (D). With the exception of the FRT oriented relative to the reporter genes, all of the paired pFRT1-Telr and pFRT2-Telr vectors have the same sequence. Depending on the orientation of FRT on the chromosome, either pFRT1-Telr or pFRT2-Telr would be used to orient the promoterless reporter fusion in the same direction as the promoter of interest. Abbreviations: cI857, temperature-sensitive repressor; gfp, green fluorescent protein gene; lacZ, β-galactosidase gene; luxCDABE, genes encoding the bacterial bioluminescent operon; T1T2, transcriptional terminators.
ture, oxygen, choline, and glycine betaine. However, little is known about the regulation of the \textit{betBA} operon in \textit{B. thailandensis}. As a proof of concept, we used one of the reporter fusion vectors to determine the regulatory mechanism of the \textit{betBA} operon in \textit{B. thailandensis} by choline and osmotic stress. \textit{B. thailandensis} strain E264-\textDelta betBA::\textit{FRT}-lacZ was engineered using fusion vector pFRT1-lacZ-Tel' and helper plasmid pCD13SK-\textit{oriT-asd}_{Ec} (Fig. 4A and B and 5A). Flp-catalyzed recombination and the orientation of the \textit{FRT}-lacZ fusion at the “\textit{FRT scar}” within the chromosome of the \textDelta betBA mutant, a location at which the \textit{lacZ} reporter gene is controlled by an unknown \textit{betBA} promoter, were verified by PCR using oligonucleotides 713 and 861 (Fig. 5A). Choline induction of the \textit{betBA} operon was studied by growing fusion strain E264-\textDelta betBA::\textit{FRT}-lacZ-Tel' in 1× M9 medium with glucose and supplemented with different concentrations of choline. These conditions resulted in similar growth rates and overall cell densities (Fig. 5B).  

\textit{β}-Galactosidase assays were performed in triplicate for all of the growth cultures shown above (B) at various time points, indicating that \textit{betBA} is inducible by choline. Two alternative fusion strains, E264-\textDelta betBA::\textit{FRT}-\textit{gfp}-Tel' (D) and E264-\textDelta betBA::\textit{FRT}-\textit{lux}-Tel' (E), were grown in MG medium or MG medium plus 8 mM choline. (D and E) The expressions of GFP (D) and bioluminescent proteins (E) were significantly induced in the presence of choline. Images in D are representative of multiple fields for the same samples. OD\textsubscript{600}, optical density at 600 nm.
FIG. 6. Induction of the betBA operon by osmotic stress. (A) Strategy for constructing fusion strain E264::ΔbetBA::FRT-LacZ-Tel'/attTn7::betBA. Oligonucleotide 861 was used along with oligonucleotide 713, 885, or 1030 for screening, and they are indicated by arrows. Parallel diagonal lines indicate a large distance of separation on the chromosome. (B) The resulting strain E264::ΔbetBA::FRT-LacZ-Tel'/attTn7::betBA, and wild-type (WT) strain E264 were grown in LS (0 M NaCl) medium supplemented with 0.3 and 0.4 M NaCl. Both the wild-type and fusion strains produced identical growth characteristics in LS medium and LS medium plus 0.3 M NaCl yet displayed slightly decreased levels of growth in 0.4 M NaCl after mid-log phase. (C) At
to compare the expression levels of the betBA operon in different choline concentrations (Fig. 5C). As choline concentrations increased, corresponding increases in β-galactosidase activities were observed, indicating that the betBA operon was responsive to choline. Utilizing two alternative gene fusion vectors (pFRT1-gfp-Tel and pFRT1-lacZ-Tel), fusion strains E264-ΔbetBA::FRT-gfp-Tel and E264-ΔbetBA::FRT-lacZ-Tel were also constructed. By comparing the fluorescent and luminescent intensities, the induction of the betBA operon by choline was observed using the gfp or lux reporter, respectively (Fig. 5D and E).

A previously reported study has shown that the E. coli betBA operon was involved in osmotic regulation and was induced by osmotic stress (13). Here, we demonstrated that osmotic stress operon was involved in osmotic regulation and was induced by 0.3 and 0.4 M NaCl. Consequently, fusion strain E264-ΔbetBA::FRT-lacZ-Tel/attTn7::betBA was constructed in the E264-ΔbetBA::FRT/attTn7::betBA background (Fig. 6A) because we found that the growth of the ΔbetBA mutant was significantly affected by NaCl without complementing the ΔbetBA mutation. The constructed strain, E264-ΔbetBA::FRT-lacZ-Tel/attTn7::betBA, and wild-type strain E264 were grown in LS (0 M NaCl) medium supplemented with 0.3 and 0.4 M NaCl. These strains produced identical growth characteristics in LS medium and LS medium with 0.3 M NaCl, but both strains displayed slightly decreased growth rates in 0.4 M NaCl after the mid-log growth phase (Fig. 6B). The presence of 0.3 M NaCl significantly induced the betBA operon as determined by β-galactosidase assays (Fig. 6C). Despite the increased induction of the betBA operon in the presence of 0.4 M NaCl relative to that in the absence of NaCl, this comparison was unreliable due to differing growth rates. These data indicated that the betBA operon in B. thailandensis was induced by osmotic stress. Similarly, two fusion strains (E264-ΔbetBA::FRT-gfp-Tel/attTn7::betBA and E264-ΔbetBA::FRT-lacZ-Tel/attTn7::betBA) were constructed to show the induction of the betBA operon by observing the increased fluorescence and bioluminescence levels under conditions of osmotic stress (Fig. 6D and E).

**DISCUSSION**

In this study, the nonantibiotic Tel cassette was utilized to construct three genetic systems: a random mariner transposon, a mini-Tn7 site-specific transposon vector, and six FRT-reporter fusion vectors based on three different reporters. A constitutive promoter, PC512 (B. cenocepacia rpsL gene) (41), was included upstream of the Tel' cassette in all these tools to ensure the efficient expression of this resistance marker. First, the constructed Himar1-based random mariner transposon was successfully tested in four different Burkholderia spp.: B. cenocepacia, B. thailandensis, B. pseudomallei, and B. mallei. PCR screening with Tel-specific oligonucleotides revealed that 100% of Tel' colonies harbored the Tel' cassette. Next, the mini-Tn7 system was utilized successfully to complement the ΔbetBA mutant. Finally, three different FRT-reporter fusion vectors were used to study the regulation of the B. thailandensis betBA operon. Results showed that the betBA operon, which is essential for B. thailandensis choline degradation, was induced significantly by choline and osmotic stress (NaCl).

There are several advantages to including FRT-flanked resistance cassettes in random transposon mutagenesis and allelic replacement (2, 32). First, unmarked mutations can be obtained subsequent to allelic replacement or transposon mutagenesis by Flp excision of FRT-flanked resistance selection cassettes. The use of FRT-flanked resistance cassettes in allelic replacement allows for easier selection, resulting in higher mutation frequencies than that reported for a recently published approach to obtain unmarked mutations where there was a lack of positive selection for the second homologous recombination (15). Furthermore, the lack of positive selection requires laborious screening, and mutating essential genes may not be possible. In addition, the remaining “FRT scar” adds flexibility to subsequent fusion integrations, aiding in the construction of fusion strains for regulation studies of nonessential genes without prior knowledge of the identity and location of promoter sequences. For essential genes, mutant fusion strains can be complemented with the mini-Tn7-Tel' system presented here, and gene regulation studies can be performed. Because single copies are more representative of the natural genetic regulation mechanism, as opposed to multicopy plasmids, single-copy tools could ameliorate the difficulties of complementation and promoter studies. The six FRT-reporter fusion vectors, based on three different reporters (lacZ, gfp, and the luxCDABE operon), add further flexibility and provide simplified visualization and quantification of gene expression during regulation studies. For example, by fusing the gfp reporter downstream of a target gene with pFRT-gfp-Tel' vectors, it is possible to measure gene expression via detecting the bacterial green fluorescent protein (GFP) signal under different growth conditions or during eukaryotic cell infections. Similarly, by utilizing the pFRT-lux-Tel' vectors, the regulation of target genes during animal model infections can be studied by measuring the bacterial bioluminescence intensity.

The genetic tools described in this paper will aid in elucidating the physiology, environmental behavior, and pathogenic mechanisms of Burkholderia species. Although the model or...
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ganism B. thailandensis was utilized primarily to demonstrate
the efficacy of these tools, Telr selection was successfully tested
in the select agents B. pseudomallei and B. mallei by using the
transposon pBT20-⌬bla-Telr-FRT. Thus, we believe that these
tools could be particularly useful for various studies of B.
pseudomallei and B. mallei. The alternative nonantibiotic asdEc
selectable marker in helper plasmids pTNS3-asdEc and
pCD13SK-Flp-oriT-asdEc may allow their use in other selectagent species. The genetic tools presented here could be further developed by substituting the Telr cassette with other
nonantibiotic selectable markers. Finally, because these tools
are completely devoid of any antibiotic resistance markers,
they are in full compliance with CDC select-agent regulations.
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Multiple FadD Acyl-CoA Synthetases Contribute to Differential Fatty Acid Degradation and Virulence in Pseudomonas aeruginosa

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Abstract

A close interconnection between nutrient metabolism and virulence factor expression contributes to the pathophysiology of Pseudomonas aeruginosa as a successful pathogen. P. aeruginosa fatty acid (FA) degradation is complicated with multiple acyl-CoA synthetase homologs (FadDs) expressed in vivo in lung tissue during cystic fibrosis infections. The promoters of two genetically linked P. aeruginosa fadD genes (fadD1 and fadD2) were mapped and northern blot analysis indicated they could exist on two different transcripts. These FadDs contain ATP/AMP signature and FA-binding motifs highly homologous to those of the Escherichia coli FadD. Upon introduction into an E. coli fadD/fadR double mutant, both P. aeruginosa fadDs functionally complemented the E. coli fadD/fadR mutant, allowing degradation of different chain-length FAs. Chromosomal transgenesis, growth analysis, induction studies, and determination of kinetic parameters suggested that FadD1 has a substrate preference for long-chain FAs while FadD2 prefers shorter-chain FAs. When compared to the wild type strain, the fadD2 mutant exhibited decreased production of lipase, protease, rhamnolipid and phospholipase, and retardation of both swimming and swarming motilities. Interestingly, fadD1 mutant showed only increased swimming motility. Growth analysis of the fadD mutants showed noticeable deficiencies in utilizing FAs and phosphatidylcholine (major components of lung surfactant) as the sole carbon source. This defect translated into decreased in vivo fitness of P. aeruginosa in a BALB/c mouse lung infection model, supporting the role of lipids as a significant nutrient source for this bacterium in vivo.

Introduction

To occupy a diverse range of ecological niches, Pseudomonas aeruginosa must evolve and maintain a wide array of metabolic pathways for nutrient uptake and utilization. This adaptive flexibility allows P. aeruginosa, a ubiquitous Gram-negative saprophyte, to occupy environmental niches in both soil and water and to transition into a potentially pathogenic lifestyle with humans, plants, animals, and other microbes [1–4]. This bacterium has been responsible for a myriad of infections including serious bacteremia and nosocomial pneumonia [5–8], and it has been shown to be the major cause of morbidity and mortality among cystic fibrosis (CF) patients aged 18–24 years [9,10]. P. aeruginosa thrives both environmentally and within a human host because of its extensive repertoire of virulence factors (e.g., LasA/LasB and alkaline proteases, phospholipases, lipases, exotoxin A, type III secretion exoenzymes S/T/U/Y, rhamnolipid, alginate biofilm, hydrogen cyanide synthesis, and others) and its capacity to metabolize 70–80 different organic substrates as sole carbon sources, notably different chain-length fatty acids (FA, C4–C10) [11]. Our previous work suggested that P. aeruginosa expresses phospholipases and lipases in vivo that degrades phosphatidylcholine (PC; Fig. 1A) as a nutrient source for bacterial replication in the lungs of CF patients [12]. In support of these results, Miller et al. [13] have shown that P. aeruginosa utilizes type IV pili to twitch towards phospholipids (i.e. phosphatidylethanolamine and PC) and long-chain FA (LCFAs).

Fatty acid degradation (Fad) in the model microbe, Escherichia coli, employs enzymes of the Fad pathway encoded by the fad regulon [14,15]. E. coli possesses a single FadD, a 62-kDa fatty acyl-CoA synthetase (FACS or AMP-forming fatty acid:CoA ligase), encoded by the fadD gene [16,17]. The FadD protein possesses two highly conserved sequence elements corresponding to a proposed ATP/AMP signature motif [17,18], as well as a signature motif involved in FA substrate binding and specificity [19] (Fig. 1C). Following FadL-mediated importation of exogenous FAs through the outer membrane [20–22] and an unknown transportation process through the inner membrane, FadD appears to employ these two motifs to activate FAs in a two-step process [18,19]. In the first step of activation, an acyl bond between the ω-phosphoryl group of an ATP and the carboxyl group of a FA is formed creating a fatty acyl-adenylate intermediate and releasing pyrophosphate. In the second step, the release of AMP occurs concomitantly during thioester bond
Figure 1. The proposed FA degradation pathway in *P. aeruginosa* based on *E. coli* β-oxidation. (A) Phosphatidylcholine (PC) is the major component of lung surfactant. PC can be cleaved by *P. aeruginosa* phospholipase C and lipases producing free fatty acids that are degraded via the β-oxidation pathway by this bacterium. (B) FAs are transported through the outer membrane aided by an unidentified *P. aeruginosa* FadL [13,67]. In *E. coli*, FA may be transported through the inner membrane via an unknown mechanism coupled to a single peripheral membrane FadD protein [18]. However, *P. aeruginosa* contains at least two FadDs (FadD1 and FadD2). Although there are over a dozen potential FadE homologues in the *P. aeruginosa* genome, the specific enzyme(s) that catalyzes this reaction has not been identified. FadB catalyzes the next two steps followed by cleavage of the 3-keto-acyl-CoA by FadA. Two fadBA operons (fadBA1 and fadBA5) have been identified in *P. aeruginosa* [29,30]. (C) Alignment of the *P. aeruginosa* FadD1 and FadD2 ATP/AMP-signature and FA-binding motifs with the FadD motifs of *E. coli* [18,19]. Abbreviation for Fad-proteins:
formation between the fatty acyl group and the sulfhydryl group of coenzyme A in the second step [23]. This FadD-catalyzed reaction produces fatty acyl-CoA, a molecule capable of degradation by the β-oxidation cycle or exerting transcriptional control on the *E. coli* *fad*-regulon by interacting with the FadR regulator to derepress *fad*-genes [24–27]. However, it seems currently that some *fad*-genes in *P. aeruginosa* are induced, not by fatty acyl-CoA, but by LCFAs [24–28]. While the biochemistry and physiology of FadD have been well characterized in *E. coli*, relatively little is known about FadD in *P. aeruginosa*. The *P. aeruginosa* β-oxidation cycle in FadD has only been partially characterized with respect to FadBAs (Fig. 1B) [29,30]. Fad enzymes, including the broad substrate specificity of the FACS, have also been characterized in *Pseudomonas fragi* [31–33]. A study on *Pseudomonas putida* originally isolated and characterized one FACS with a broad substrate range [34]. Additional work further characterized the role of this *P. putida* enzyme and identified a second FACS, naming them FadD1 and FadD2, respectively [35,36]. In this dual FadD system, it was shown that FadD1 played a dominant role in FA metabolism while FadD2 was activated only when FadD1 was inactivated [36]. Comparison of the significantly larger size of the *Pseudomonas* genome relative to that of *E. coli*, such genetic redundancies are not unexpected. However, the importance of the redundancy and functions of these enzymes in Fad are uncertain.

Studies on other species have indicated a link between FACS, nutrient metabolism, and the expression of virulence factors [37–43]. In *Mycobacterium tuberculosis*, 36 *fad*D homologues were identified [39]. A null mutation in the *M. tuberculosis* *fadD28* gene showed significant replication restriction in mouse lungs, as a result of defects in cell-wall biosynthesis and the production of complex lipids [40]. In addition, *fadD33* in the *M. tuberculosis* H37Ra strain was shown to play a role in supporting growth in mouse livers [42]. Similar to these *Mycobacterium* studies, the use of random transposon mutagenesis has led to the isolation of a *fadD* mutant in *Salmonella enterica* serovar Typhimurium which was shown to reduce the expression of *hle* (a proposed transcriptional activator of genes in the type III secretion system [38]) and invasion genes three- to five-fold [41]. A *Xanthomonas campestris* *fadD* homolog *rpfB* mutant has decreased production of protease, endoglucanase, and polygalacturonate lyase due to the inability to generate a diffusible extracellular factor containing a FA moiety [37]. A Tn5 insertion in the *fadD* gene of *Sinorhizobium meliloti* displayed an increased swimming phenotype compared to wild-type, resulting in an observed decrease in alfalfa root nodulation [43]. Many of these studies correlated *fadD* mutations with decreased virulence, but did not confirm or elucidate its enzymatic role in FA metabolism. We have previously shown that *P. aeruginosa* expresses *fadD1* and *fadD2* (PA3299 and PA3300) during lung infections in CF patients, suggesting the importance of Fad in lipid nutrient acquisition in vivo [12]. However, the role of *fadD* on virulence and growth of the bacteria in vivo has not been characterized.

Here, we characterized the FadD1 and FadD2 (PA3299 and PA3300) and the respective genes with relevance to their biochemistry and the effect on *P. aeruginosa* pathophysiology. The results of genetic analyses and biochemical characterization provided insight into reasons why redundancies in *fadD* are beneficial to this pathogen. Interestingly, *fadD* mutants displayed alterations in swimming and swarming motility and the production of lipases, phospholipases, rhamnolipids, and proteases. The *fadD* mutants with reduced ability to grow on phosphatidylcholine as a sole carbon source showed decreased fitness in a mouse lung infection model. These results provide the initial characterization of *P. aeruginosa* *fadD* genes and suggest a pathophysiological link between Fad and virulence.

## Results

### Comparison of two *fadDs* in *P. aeruginosa*

Our previous work showed that two *P. aeruginosa* *fadDs* (*fadD1* and *fadD2*) were expressed in *vivo* during lung infection in CF patients [12]. FadD1 (PA3299) and FadD2 (PA3300) are 72% similar (54% identical) and 72% similar (53% identical) to the *E. coli* *fad*D genes, respectively, while *P. aeruginosa* FadD1 and FadD2 are 76% similar (60% identical) to each other. In addition, *fadD1* and *fadD2* are adjacent genes, separated by 234-bp and a possible Rho-independent transcriptional terminator (Fig. 2C). Convincingly, the ATP/AMP signature and FA-binding motifs described for the *E. coli* *fadD*s are highly conserved in both *P. aeruginosa* FadD1 and FadD2 (Fig. 1C). This preliminary analysis suggests that *fadD1* and *fadD2* are both involved in Fad.

To confirm that *fadD1* and *fadD2* are important for Fad, we complemented the *E. coli* *fadD1*/*fadD2* strain (E2011) with these *P. aeruginosa* genes. This *E. coli* strain contains a mutation in the *fadD*-gene (*fadD*), the main repressor of the *fad*-regulon in *E. coli*, allowing the constitutive expression of other *fad*-genes of the aerobic Fad-pathway [24–27]. Both *fadD1* and *fadD2* of *P. aeruginosa* were able to functionally complement the *E. coli* *fadD* mutant (Table 1). The *E. coli* K12 wildtype strain was able to metabolize C<sub>12</sub>:0 to C<sub>18</sub>:1<sup>ω9</sup> as expected, because long-chain acyl-CoA (≥C<sub>12</sub>) binds efficiently to FadR to induce the *fad*-regulon. The results of supplementation analysis with *E. coli* *fadD* gene (fadD<sub>E</sub>) on plasmid pET15b resulted in growth similar to that of *E. coli* K12 on C<sub>12</sub>:0 to C<sub>18</sub>:1<sup>ω9</sup> as well as on C<sub>10</sub>:0 because of the deregulated *fad*-regulon as previously observed [44]. Both *P. aeruginosa* *fadDs* were able to complement the *E. coli* *fadD1*/*fadD2* strain to a similar level as the *fadD<sub>EC</sub>* complement (Table 1), suggesting that both FadD1 and FadD2 could activate LCFAs and medium-chain FAs (MCFAs). The various complements did not grow on short-chain FAs (SCFAs), suggesting that other *E. coli* Fad-enzymes may not support growth on SCFAs [45] and not necessarily that the *Pseudomonas* FadD proteins are incapable of producing short-chain fatty acyl-CoAs. The E2011 control strains, either with or without the pET15b empty vector, showed no growth on any FA (Table 1).

### *fadD2* and *fadD1* exist on two transcripts and are induced by FA of different lengths

To gain information on the regulatory regions of the *P. aeruginosa* *fadDs*, we mapped their transcriptional start sites to assign putative promoter sequences, and then determined transcription levels of each gene on various carbon sources (Figs. 2 and 3). Promoter mapping experiments indicated that each *fadD* had an independent transcriptional start site, suggesting that they were independently transcribed; however, northern blot analyses indicated that *fadD2* and *fadD1* can be co-transcribed on a single larger transcript or as smaller independent transcripts (Fig. 3A and 3B). Both *fadD2* and *fadD1* can exist as two different transcripts, suggesting some level of regulation by the predicted transcriptional terminator or attenuator sequence within the intergenic region (Fig. 2C).
Figure 2. SMART mapping of the transcriptional start sites for fadD2 and fadD1. (A) One SMART product was observed after PCR amplification of the cDNA with SMART and fadD2 primers (oligonucleotides #798 and #373). Sequencing of the single band with a nested fadD2 primer (oligonucleotide #374) displayed a reverse-complement sequence chromatogram, showing the fadD2 transcriptional start site (indicated by +1 at the CTTGC sequence) and the underlined SMART primer sequence. (B) Likewise, the downstream fadD1 transcriptional start site was mapped (at the G of the sequence GCCTA) by sequencing a single PCR product. (C) fadD2 and fadD1 coding sequences and the predicted promoter regions are indicated (boxed). The intergenic region between fadD2 and fadD1 contains a potential transcriptional terminator or attenuator sequence (inverted arrows). For each gene, three black arrows indicate primers 1, 2 and 3 (#372/#375, #373/376, and #374/377) used for mapping fadD2 and fadD1. Dashed lines indicate missing protein sequences and dots indicate stop codons.

doi:10.1371/journal.pone.0013557.g002

Table 1. Complementation of the E. coli fadD mutant with P. aeruginosa fadD homologues.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Growth on different FAs and casamino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C₆:₀</td>
</tr>
<tr>
<td>K12 (wildtype)</td>
<td>none</td>
<td>-</td>
</tr>
<tr>
<td>E2011 (fadD/fadR)</td>
<td>none</td>
<td>-</td>
</tr>
<tr>
<td>E2011 (fadD/fadR)</td>
<td>pET15b</td>
<td>-</td>
</tr>
<tr>
<td>E2011 (fadD/fadR)</td>
<td>pET15b-fadD₁</td>
<td>-</td>
</tr>
<tr>
<td>E2011 (fadD/fadR)</td>
<td>pET15b-fadD₁</td>
<td>-</td>
</tr>
<tr>
<td>E2011 (fadD/fadR)</td>
<td>pET15b-fadD₂</td>
<td>-</td>
</tr>
</tbody>
</table>

*(-) denotes no growth on a patch; (+) denotes growth: (+1) is very little growth and (+6) is heavy growth after 3 days.

doi:10.1371/journal.pone.0013557.t001
Figure 3, we hypothesize that the promoter upstream of *fadD2* drives the expression of both genes, and the intergenic terminator attenuates the larger *fadD1* transcript. The *fadD1* promoter immediately downstream of this regulatory element was induced by LCFA (e.g. C18:1<sup>D9</sup>), and initiated the expression of the smaller *fadD1* transcript (Fig. 2C, 3A and 3B). Presumably, when there was no termination of transcription from the *fadD2* promoter, *fadD2* and *fadD1* were transcribed together on the larger transcript of the same size observed on both blots (Fig. 3A and 3B). Based on the determination that these *fadD* genes could be independently transcribed or co-transcribed, it was necessary to determine which chain-length FA induced *fadD1* and *fadD2*. The observed levels of induction showed that *fadD1* was mainly induced by LCFA, particularly C18:1<sup>D9</sup>, while *fadD2* was specifically induced by short- to medium-chain FA (C8:0, C10:0) (D). Both *fadD* genes showed some level of expression under all conditions tested, indicating low levels of constitutive expression.

**fadD** mutants showed reduced ability to grow on various FAs

To further confirm the involvement of each *fadD* in Fad, we generated single and double mutants for growth analysis on various FAs as sole carbon sources (Fig. 4). As previously observed for *fadBA* mutants [30], growth defects were exemplified by slower growth and lower overall final cell densities in various FA media, presumably, due to reduced rates of Fad and growth inhibiting intermediates. Both *fadD1* and *fadD2* single mutants had various levels of defects when grown on all FAs (Fig. 4). However, the *fadD1* mutant displayed a greater growth defect on all FAs than the single *fadD2* mutant, with the exception of C8:0 and C10:0 where *FadD2* seems to be equally as important as *FadD1* (Fig. 4D and 4E). The Δ*fadD2D1* mutant showed more dramatic growth defects on all FAs than the individual single mutants, indicating that both proteins were involved in the metabolism of all chain-length FAs tested. The lack of a complete defect in Fad of this double mutant suggests that other *fadDs* exist in *P. aeruginosa*. The complemented single and double mutants fully restored growth on all FAs (Fig. 4), while empty vector miniCTX2 controls did not complement growth on the FAs (data not shown). No apparent defects were observed for any mutant grown with casamino acids (CAA) as a sole carbon source (Fig. 4A). Based on the physiological data (Fig. 3 and 4), *fadD1* was found to be important for the metabolism of all FAs, particularly the unsaturated LCFA oleate, while *fadD2* was also important in Fad but more so for MCFA (C8:0 and C10:0) degradation.
Kinetic properties of purified FadDs support differential FA chain-length preferences

To determine the substrate specificities of each FadD and further clarify the reason why P. aeruginosa possesses multiple fadD homologues, both FadD proteins were purified to near homogeneity from an E. coli fadD strain to ensure that all acyl-CoA synthetase activities were derived only from purified recombinant FadD1 or FadD2 (Fig. 5A). FadD1 of P. aeruginosa coupled CoASH to LCFA better than to SCFA or MCFA, as exemplified by larger $V_{\text{max}}$ and lower $K_m$ values for C18:1Δ9 and C16:0 than FAs of other chain-lengths (Fig. 5B and Table 2). The reverse was true for FadD2, where this enzyme had higher $V_{\text{max}}$ and lower $K_m$ for SCFA and MCFA than LCFA (Fig. 5C and Table 2). The catalytic efficiency ($k_{\text{cat}}/K_m$) of FadD1 was significantly higher for LCFA (C18:1Δ9, C16:0, and C14:0) than MCFA (C12:0 to C8:0) or SCFA (C6:0 and C4:0), while the catalytic efficiency of FadD2 was higher for MCFA and SCFA than LCFA (Table 2). The kinetic parameters for ATP and catalytic efficiency of both enzymes were comparable when ATP was limited in the reaction, with FadD1 being a slightly better catalytic enzyme for ATP than FadD2 (Table 2). Clearly, multiple FadDs in P. aeruginosa, with broad substrate conversion capabilities and overlapping chain-length preferences, afford this bacterium the ability to optimally metabolize FAs of various chain-lengths.

fadD mutants influence virulence behavior of P. aeruginosa

Based on work in other bacteria that showed an interconnection between fadD genes and expression of virulence factors [37–43], we sought to determine if a similar connection existed in P. aeruginosa. Increased swarming motility of S. meliloti, leading to altered virulence, was previously attributed to hyperflagellation observed by transmission electron microscopy (TEM) [43]. While no apparent differences in structure or numbers of flagella were observed for the fadD mutants compared to wildtype PAO1 using TEM in the current study (data not shown), we showed that fadD mutations could still significantly influence swarming and swimming motility in P. aeruginosa (Fig. 6). The fadD2 mutant was severely defective in swimming and swarming motility relative to the wildtype PAO1 strain (Fig. 6). Although the fadD1 single mutant showed no apparent difference in swimming motility, it displayed increased swimming migration compared to PAO1. Swarming was most pronounced in the ΔfadD2D1 mutant. In the ΔfadD2D1 mutant, it was very interesting to observe that the fadD1
mutation suppressed the swarming and swimming defects of the fadD2 mutation. Each complemented strain showed that swimming and swarming motility could be restored to wildtype levels, indicating no unforeseen secondary or polar mutations affected these behaviors.

The production of other virulence factors was also monitored for the fadD mutants. Interestingly, the fadD2 mutant showed significantly decreased production of total hemolysins, proteases, lipases, and rhamnolipids (Fig. 7). No other mutant or complement showed noticeable decreases in the production of these virulence factors compared to the wildtype PAO1 strain. The suppression of the fadD2 mutation by the fadD1 mutation, in the DfadD2D1 mutant, reversed the reduction in virulence factor expression seen in the single fadD1 mutation alone (Fig. 7). Similar suppression was observed in swimming and swarming motilities (Fig. 6). The altered virulence behaviors and suppression were not due to differences in growth-rates or overall final cell densities, as all seven strains (i.e. wildtype, mutants, and complements) grew identically in LB media prior to testing for these virulence traits (Fig. 7E). Mechanisms governing these differences remain to be elucidated.

Compromised ability of fadD mutants to degrade FA and PC leads to reduced P. aeruginosa fitness in mice

Since fadD1 and fadD2 are expressed in vivo during CF lung infections [12] and are potentially important for PC degradation (Fig. 1A), it was necessary to determine whether these mutants are deficient in growth on PC. Growth analysis on PC showed only slight decreases in the maximum cell density of the individual single fadD mutants, while the fadD2 mutant exhibited a delayed log phase (Fig. 8A). The DfadD2D1 mutant exhibited the greatest growth defect, while the single and double fadD complements restored growth to wildtype levels. Since PC is the major component (70%) of the essential lung surfactant [46] and is a potential nutrient source in vivo [12], it was important to assess whether the growth defects of these mutants on PC would result in decreased fitness in vivo.

### Table 2. Kinetic properties of FadD1 and FadD2 with various substrates.

<table>
<thead>
<tr>
<th>Substrate varied</th>
<th>FadD1 Kinetic Parameter</th>
<th>FadD2 Kinetic Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vmax, b (nmol/min/mg)</td>
<td>kcat, c (s⁻¹)</td>
</tr>
<tr>
<td>ATP</td>
<td>213</td>
<td>0.219</td>
</tr>
<tr>
<td>C4:0</td>
<td>137</td>
<td>0.141</td>
</tr>
<tr>
<td>C6:0</td>
<td>133</td>
<td>0.137</td>
</tr>
<tr>
<td>C8:0</td>
<td>125</td>
<td>0.128</td>
</tr>
<tr>
<td>C10:0</td>
<td>116</td>
<td>0.119</td>
</tr>
<tr>
<td>C12:0</td>
<td>130</td>
<td>0.134</td>
</tr>
<tr>
<td>C14:0</td>
<td>130</td>
<td>0.134</td>
</tr>
<tr>
<td>C16:0</td>
<td>154</td>
<td>0.158</td>
</tr>
<tr>
<td>C18:1, D9</td>
<td>217</td>
<td>0.223</td>
</tr>
</tbody>
</table>

*Kinetic constants (Vmax and Km) determined using Hanes-Woolf plot.

1nmol of acyl-CoA formed/min/mg of protein.

2Determined using MW of FadD1 (61,655) and FadD2 (61,373).

3mM of ATP or FA.

4mM⁻¹ s⁻¹; represents enzyme catalytic efficiency.

5doi:10.1371/journal.pone.0013557.t002
In vitro competition between the various fadD mutants and their complements were first tested to determine if the defect reduced their ability to utilize various sole carbon sources. Each mutant and complement pair was inoculated into seven growth media with different sole carbon sources including LB, CAA, glucose, PC, C_{18:1}^{cis9}, choline, and glycerol, and bacterial CFU were determined after 24–48 h growth (Fig. 8B). As expected, all three single or double fadD mutants were less competitive than their corresponding complements but only in media containing PC or C_{18:1}^{cis9} as sole carbon sources. Next, to evaluate the fitness of the fadD mutants within the lung environment, in vivo competition between the mutants and their complements was analyzed. Following intratracheal inoculation of equal ratios of each mutant and its complement pair into BALB/c mice (6×10^6 CFU/animal), bacterial CFU recovered from the lungs were determined 24 h and 48 h postinfection and the competitive index (CI) was calculated (Fig. 8C and 8D). The CI is defined as the ratio of mutant CFU relative to CFU of the respective complement [47].

In all of these competition experiments, with the exception of the fadD2 mutant after 48 h, the average total CFU/mouse recovered was greater than the initial inoculum showing that these strains maintained the ability to replicate within the mouse lung. Although the fadD1 single mutant showed decreased competitive fitness within the lung compared to the complement, the CI of the fadD1 mutant remained relatively unchanged between the two time points analyzed. At 24 h postinfection, all of the mutants exhibited decreased competition levels relative to their respective complements and the fadD2 and ΔfadD2D1 mutant strains showed greater reduced fitness than the fadD1 mutant. By allowing the infections to persist for 48 h, the reduced CI of the fadD2 mutant showed a significantly higher defect in competitive fitness. By 48 h, the CI of the ΔfadD2D1 mutant was half of that observed at 24 h. Clearly, the ΔfadD2D1 mutant with significantly reduced ability to degrade PC (Fig. 8A), while showing no altered virulence factor secretion (Fig. 7), had its competitive fitness reduced by three-fold. This strongly suggests that the ability to degrade PC as a nutrient source allows P. aeruginosa to replicate within the lung environment.

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Figure 7. Analysis of protease, hemolysin, lipase, and rhamnolipid production by *P. aeruginosa* fadD mutants. The fadD2 mutant displayed significantly decreased production of proteases (A), hemolysins (B), lipases (C), and rhamnolipids (D), while no growth defects in LB were observed (E). These assays were conducted in triplicate and are expressed as a percentage of the mean value of the wildtype PAO1 ± s.e.m. doi:10.1371/journal.pone.0013557.g007

**Discussion**

This study focused on characterizing two *P. aeruginosa* acyl-CoA synthetases (FadD1 and FadD2), which are expressed during lung infection in CF patients suggesting their importance in lipid degradation for bacterial replication [12]. The transcriptional profile and substrate preferences of FadD1 and FadD2 were determined to initially shed light onto why *P. aeruginosa* has this genetic redundancy. The *fadD1* and *fadD2* of *P. aeruginosa* were differentially regulated in response to the type of available FA (Fig. 3), suggesting that each FadD has a different FA substrate preference. While both *fadD1* and *fadD2* were controlled by their common and independent promoters, the expression of *fadD1* downstream could be partially attenuated by some FAs due to a putative intergenic Rho-independent transcriptional-terminator (Fig. 2). Based on the growth defects, both fadD genes are individually important for the degradation of all chain-length FAs tested. These results are further supported by the observation that the ∆*fadD2D1* mutant had the greatest growth defect on all chain-length FAs (Fig. 4). These growth defects may be due to a bottleneck in the conversion of exogenous FA to acyl-CoA created by the inactivation of two genes that facilitate this process. The complementation study in *E. coli* was initially inconclusive with respect to metabolism of SCFA and some MCFAs by *P. aeruginosa* FadDs (Table 1), as other *E. coli* Fad-proteins (e.g. FadE, FadA and FadD) do not allow the metabolism of shorter-chain FAs [45]. However, the kinetic parameters, especially the catalytic efficiencies (Table 2), provided more precise biochemical evidence for the differences in substrate preferences. By comparing the kinetic measurements of these enzymes, it appears that FadD1 preferred LCFA for degradation, while FadD2 was more suited to the degradation of SCFAs and MCFAs (Fig. 5 and Table 2). Together, the enzyme kinetics, gene-fusion, and growth analyses data all support the importance of FadD2 for degradation of SCFA and MCFa and FadD1 is more suited for LCFA degradation (Fig. 3, 4 and 5). Therefore, we can conclude that these proteins have different substrate preferences and are not functionally equivalent.

We showed here that mutations in *fadD3* genes, important for FA β-oxidation in *P. aeruginosa*, also influenced two modes of motility and virulence factor expression. Our *fadD2* mutant had reduced swimming and swarming motilities and decreased virulence factor expression (proteases, phospholipases, rhamnolipids, and lipases), while the *fadD1* mutant and the ∆*fadD2D1* double mutant only showed an increased swimming phenotype. In the double mutant background, the presence of the *fadD1* mutation suppresses the phenotype of the *fadD2* mutation (Figs. 6 and 7). It will be interesting to determine the exact cause of the phenotypic suppression in future investigations. At this point, we speculate that the reduced swimming phenotype of the *fadD2* mutant is due to decreased production of rhamnolipids (Fig. 6 and 7), as rhamnolipids were previously shown to be necessary for *P. aeruginosa* swarming motility [48]. These phenotypic differences in virulence factor expression further support the observation that FadD1 and FadD2 are not functionally equivalent. Although we did not exhaust the large list of virulence determinants, nor were able to show the exact method by which FadD2 influences their expression, this characterization of several virulence factors links Fad and virulence factor expression in *P. aeruginosa*.

Additionally, the expression of genes in *in vivo* that encode proteins with β-oxidative activity, along with several other PC degradation genes, strongly support the hypothesis that lipids within the lung may be important nutrient sources for *P. aeruginosa* [12] and serve as signals to control virulence factor expression [13]. Phospholipase- and lipase-derived components of PC (LCFA, glycerol, and phosphorylcholine) could individually serve as sole carbon sources (Fig. 1A) and provide nitrogen and phosphorous contributing to virulence [49,50]. Of these three PC components, the two LCFA from each PC molecule yield the most carbon and energy. Therefore, the determination that both FadD1 and FadD2 were important for LCFA degradation was pivotal as PC, the major component of lung surfactant, is primarily composed of LCFA (C<sub>16:0</sub>, 50-60%; C<sub>14:0</sub>, C<sub>12:0</sub>, C<sub>11:0</sub>, and C<sub>10:0</sub>, each at 10-20%) [46]. To that end, we analyzed the growth of these mutants on PC as a sole carbon source. The delayed log-phase of the *fadD2D1* mutant is likely due to the decreased expression of lipase and phospholipase, thereby reducing the cleavage rate of exogenous PC into its usable components and thus slowing growth. Since it was shown that the ∆*fadD1D2* mutant had no apparent deficiencies in lipase or phospholipase expression, yet exhibited the greatest decrease in growth on all FAs tested, we believe that its reduced growth on PC is attributed to a reduced ability to degrade FAs, as this double mutant degrades phosphorylcholine and glycerol as well as the complement. Since the *fadD* mutants fully retained the ability to degrade choline and glycerol and only had reduced levels of FA degradation, it was not surprising that the ∆*fadD1D2* double mutant could still degrade PC.

Because PC is a major lung surfactant common in all mammals, including mice [51], a mouse lung infection model [52] was utilized to analyze the competitive growth *in vivo* between the *P. aeruginosa* fadD mutants and their complements. Replication of the *fadD2* mutant was observed after 24 h, while all other strains showed an increase after 48 h, indicating all strains were able to replicate *in vivo*. This could be explained by the fact that our ∆*fadD1D2* double mutant still had significant ability to degrade PC and its components, and that *P. aeruginosa* expresses genes in the lung for both amino acid and PC degradation [12] and possibly DNA [53]. After 24 h and 48 h, all mutants showed lower *in vivo* fitness than their complements, which means that lipids are significant nutrient sources in the mouse lung. The CIs of the *fadD2* mutant were consistently lower than those of the *fadD1* mutant after both 24 h and 48 h, attributed to the *fadD2* mutant’s decreased production of virulence factors. Although the ∆*fadD2D1* mutant only showed partial defect in PC degradation and no effect in virulence factor expression or the ability to grow with amino acids, this partial defect in PC degradation translated into significantly reduced *in vivo* lung fitness. This is further supported by the *in vitro* competition results where all single and double *fadD* mutants exhibited competition defect only on PC and C<sub>10:0</sub>. Therefore, the significantly lowered CI after 48 h for the double ∆*fadD1D2* mutant can only be due to a reduced ability to degrade PC as a nutrient source for replication in the mouse lung.

In this study, a pathophysiological link between the acquisition of lipid nutrients and virulence *in vivo* was established by i) characterizing these two *P. aeruginosa* *fadD* genes expressed during lung infection in CF patients, ii) determining that there may be some connection between *fad*-genes and the expression of certain virulence traits, and iii) showing that mutations in these genes
Figure 8. Growth analysis on phosphatidylcholine and competition studies. (A) The ΔfadD2D1 mutant exhibited a growth defect when grown on PC as a sole carbon source, while the fadD2 mutant had a delayed log phase compared to the wildtype PAO1 strain. The growth defects were fully recovered in complemented strains, as they had identical growth rates compared to the wildtype PAO1 strain. (B) In vitro competition studies of the various fadD mutants and their complemented strains in different growth media (n = the number of independent in vitro competition experiments performed with each carbon source). In vivo lung competition of the various fadD mutants and their complemented strains after 24 h (C) and 48 h (D), n equals the number of mice in each group that were inoculated with a total of 6 x 10^6 CFU/mouse. The solid red line indicates the geometric mean of the competitive indices (CI) in each competition group. CI<1 indicates the fadD2 mutant was less competitive than its complemented strain in various growth media (B) or within the lungs (C and D) (*, P<0.05 based on one sample t test) [47]. Numbers above the red line represent the average total recovered CFU/mouse for each competition group.

doi:10.1371/journal.pone.0013557.g008

Bacterial strains and growth media

Strains and plasmids used in this study are shown in Tables 3 and 4. E. coli EPMa10B was routinely used as a strain for cloning and was cultured in Luria-Bertani (LB) medium (Difco). P. aeruginosa strain PAO1 and derivatives were cultured on Pseudomonas Isolation Agar or Broth (PIA or PIB; Difco) or LB medium. PAO1::fadD1::FRT, PAO1::fadD2::FRT, PAO1::fadD2D1::FRT, and their complements were cultured in 1x M9 minimal medium supplemented with 10 mM choline (PC, Sigma; Fig. 8A) for growth analyses. The fusion strains PAO1::fadD1::FRT::lacZ/attB::miniCTX2-fadD1 and PAO1::fadD2::FRT::lacZ/attB::miniCTX2-fadD2 were cultured in 1x M9 +1% Brij-58 (+1% CAA, 1x M9 +1% Brij-58 +0.2% phosphatidylcholine (PC, Sigma; Fig. 8A) for growth analyses. The fusion strains PAO1::fadD1::FRT::lacZ/attB::miniCTX2-fadD1 and PAO1::fadD2::FRT::lacZ/attB::miniCTX2-fadD2 were cultured in 1x M9 +1% Brij-58 +1% CAA±0.1% (w/v) C14:0, C16:0, or C18:1 #341 for induction studies (Fig. 3C and 3D). For in vitro competition studies (Fig. 8B), each mutant/complement mixture of equal cell density was grown on PC as a sole carbon source, while the fadD mutants, completely blocked in PC utilization, will have significantly lower in vivo fitness, further supporting PC as a significant nutrient source for this important pathogen in mamlan lung.

Materials and Methods

Ethics statement

All animal experiments were conducted in compliance with the NIH (National Institutes of Health) Guide for the Care and Use of Laboratory Animals and were approved by the University of Hawaii Institutional Animal Care and Use Committee (protocol No. 06-023-04).

Construction of PAO1 fadD mutant and complementation strains

Three fadD mutant strains (PAO1::fadD1::FRT, PAO1::fadD2::FRT, and PAO1::fadD2D1::FRT) were engineered, respectively, using three allelic-replacement plasmids (pEX18T-fadD1::Gm-FRT, pEX18T-fadD2::Gm-FRT, and pEX18T-fadD2D1::Gm-FRT) as previously described [55]. These gene-replacement vectors were constructed by inserting the SmaI Gmr::FRT cassette at the EcoRV site to inactivate fadD1 gene, the SmaI site to inactivate fadD2 gene, or at the deleted fadD2D1 SmaI-EcoRV locus. These newly engineered mutant strains, PAO1::fadD mutants were confirmed by PCR (data not shown).

These newly engineered mutant strains, PAO1::fadD1::FRT, PAO1::fadD2::FRT, and PAO1::fadD2D1::FRT, were complemented using the relevant gene(s) on the miniCTX2 single copy integration vector as described previously [56]. The resulting strains, PAO1::fadD1::FRT/attB::miniCTX2-fadD1, PAO1::fadD2::FRT/attB::miniCTX2-fadD2, and PAO1::fadD2D1::FRT/attB::miniCTX2-fadD2, were used in the growth curve experiments (Fig. 4 and 8A). Controls were also performed with the empty miniCTX2 integrated into each mutant strain.

Growth characterization of fadD mutant and complemented strains

The fadD mutants, their corresponding complemented strains, and the PAO1 wildtype strain were initially grown overnight in PIB medium. The overnight cultures were centrifuged and the cell pellets were washed twice with 1x M9 minimal media and resuspended with equal volumes of the same 1x M9 media. The cell resuspensions were then diluted 100-fold in 1x M9 +1% Brij-58 +1% CAA or 0.2% of the individual FAs (C14:0 to C16:0, or C18:1 #340, #342 and #343; Fig. 4) or 0.2% PC (Fig. 8A), and growth was then initiated. At each time point, aliquots of individual cultures were diluted 4-fold in 4% Brij-58 (pre-incubated at 42°C) to clarify any insoluble FA and OD540 measurements were taken.

General molecular methods

All molecular methods and their components were employed as previously described [34]. Oligonucleotides utilized in this study were ordered through Integrated DNA Technology (IDT, Table 5).

Complementation of E. coli fadD mutant

The E. coli fadD (fadC::Km) mutant (E2011) was engineered by transferring the fadC::Km mutation from JWC285 into the K27326 (fadD) strain via P1 transduction. The resulting double mutant strain was then used for the complementation study. To construct the E. coli complementation vectors, coding regions of P. aeruginosa fadD1 and fadD2 were amplified from PAO1 chromosomal DNA using oligos #341 and #342, and #339 and #340, respectively. PCR products were then digested with NdeI + BamHI and ligated individually into PET15b, digested with the same enzymes, yielding pET15b-fadD1 and pET15b-fadD2. As a control for the complementation study, the E. coli fadD gene (fadDEc) was also amplified from strain K12 chromosomal DNA using oligos #1092 and #1093. The 1.8-kb PCR product was digested with AseI + BamHI, and ligated with pET15b digested with NdeI + BamHI, yielding pET15b-fadDeC. These three vectors, pET15b-fadD1, pET15b-fadD2, and pET15b-fadDeC, were introduced into E. coli strain E2011 for complementation. E2011, harboring each complementation vector, was patched on 1x M9 minimal medium +1% Brij-58 +1% CAA or 0.2% various fatty acids, and growth was determined after 3 days incubation at 37°C (Table 1).

correspond to a deficiency in the ability to replicate in mouse lungs. These data support results from a previous in vivo gene expression study showing that P. aeruginosa expresses gfp, hbl, and especially, fad-genes to degrade PC as one of the nutrient sources in the lungs [12]. We predict that P. aeruginosa mutants, completely blocked in PC utilization, will have significantly lower in vivo fitness, further supporting PC as a significant nutrient source for this important pathogen in mamlan lungs.
Construction of $fadD1$-$lacZ$ and $fadD2$-$lacZ$ fusion strains and induction by FAs

To take advantage of the native $fadD1$-promoter and create a transcriptional fusion of $P_{fadD1}$-$lacZ$ $pFRT1-lacZ$ was integrated at the $FRT$ locus in strain PAO1-$fadD1::FRT$ as previously described [29]. The resulting fusion strain, PAO1-$fadD1::FRT-lacZ$ was PCR confirmed using oligos #341 + #713, which are specific for the $fadD1$ and $lacZ$ genes, respectively. Similarly, PAO1-$fadD2::FRT-lacZ$ was constructed and PCR confirmed using oligos #377 + #501 (data not shown). Complementation vectors miniCTX2-327 and miniCTX2-fadD2 were then integrated into these newly developed $lacZ$-fusion strains, to yield two complemented fusion strains, PAO1-$fadD1::FRT-lacZ/attB::miniCTX2-fadD1$ and PAO1-$fadD2::FRT-lacZ/attB::miniCTX2-fadD2$, respectively.

$\beta$-Galactosidase activities were measured for these two complemented fusion strains under various growth conditions. Cells were first grown overnight in PIB medium, washed twice with one volume of 1x M9, and resuspended in an equal volume of the same medium. Cell resuspensions were then diluted 100-fold into fresh 1x M9 +1% Brij-58 +1% CAA +6% lysate, and growth curve experiments were performed. Cell cultures were taken at mid-log phase (OD$_{540}$ $\approx$ 0.1%) and induction by FAs $\frac{C_{16}:0}{C_{16}:1\text{n}-7}$ was used to induce the fusion strains.

Table 3. Bacterial strains used in this study.

<table>
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$\text{P. aeruginosa}$

<table>
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<th>Strains</th>
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$\text{For strains constructed in this study, please see text for further details.}$

$\text{Please use lab ID for requesting strains.}$

doi:10.1371/journal.pone.0013557.t003
Table 4. Plasmids used in this studya.

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*aFor plasmids constructed in this study, please see text for further details.

bPlease use lab ID for requesting plasmids.

doi:10.1371/journal.pone.0013557.t004

~2.0) and β-galactosidase assays were performed in triplicate and Miller Units (mean±s.e.m.) were determined [57] (Fig. 3).

FadD1 and FadD2 purification

Histidine-tagged FadD1 and FadD2 were expressed on the pET15b vector and purified using a Ni2+NTA column (Qiagen, Valencia, CA) as described elsewhere [58]. The E. coli K27-T7 (fadD) strain was used for protein expression to prevent any possible E. coli FadD contamination in protein preparations (Fig. 5A).

Measurement of fatty acyl-CoA synthetase (FadD1 and FadD2) activity

Fatty acyl-CoA synthetase activity was monitored using Ellman's reagent, as previously described in several studies, to detect the amount of free thiol (i.e. CoASH used in the reaction) [23,59–61]. Reactions (450 μl total) were prepared with 20 μg of purified FadD1 (or FadD2) in a reaction buffer containing final concentrations of 150 mM Tris-HCl (pH 7.2), 10 mM MgCl2, 2 mM EDTA, 0.1% Triton X-100, 5 mM ATP, 0.5 mM coenzyme A (CoASH), and an individual FA (30 to 300 μM) in thin-walled glass tubes. Briefly, to perform the reaction, each mixture was assembled containing all components above (excluding CoASH) and the 405 μl mixture was pre-incubated at 37°C for 3 min. The reaction was then initiated with the addition of 45 μl of CoASH (5 mM stock in 20 mM Tris-HCl, pH 6.7, diluted to a final concentration of 0.5 mM) that was pre-incubated at 37°C for 3 min, quickly mixed, and incubated at 37°C during the course of the reaction. Immediately after mixing, a time zero point was taken by removing 75 μl from the 450 μl reaction mix and adding it to 600 μl of 0.4 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, dissolved in 0.1 M potassium phosphate at pH 8.0) and the ΔA12 were measured. Subsequent 75 μl aliquots of the reaction were taken at 20-sec intervals and mixed with DTNB for additional measurements. Additionally, control experiments without FadD enzymes were performed exactly as above to show no change in absorbance at 412 nm and verify the stability of CoASH and DTNB under these conditions. Reactions with FadD were repeated to obtain triplicate data for each FA at each concentration. For each FA substrate, decreases in ΔA412 values (loss of CoASH) over time were used to calculate the initial velocity (V0) for each FA concentration (Fig. 5B and 5C). The maximum velocity (Vmax) of the enzymes and affinity for the different substrates (Michaelis constant, Km) were then determined using the Hanes-Woolf plot, rather than the Lineweaver-Burk plot, for increased accuracy [62]. To determine the Vmax and Km for the substrate ATP, the same procedure was followed, except that the concentration of C18:1 was constant (1 mM) and varying concentrations of ATP (0.05 to 2.5 mM) were used (Table 2).

Motility assays

Strains for swarming and swimming were grown overnight in LB medium. Cell pellets of 500 μl culture aliquots were washed twice with equivalent volumes of 1x M9 medium and resuspended.
in equal volumes of the same medium. Swimming motility was assayed by spotting 5 µl of the resuspended cultures onto BM2-glucose swarm agar plates, made as described previously [63]. Swimming motility was assayed by pin-stabbing 0.3% LB agar plates with the overnight liquid cultures grown in LB. All inoculated plates were allowed to dry at room temperature for 10 min, incubated at 37°C for 16 to 18 h, and motility zones were compared (Fig. 6).

**Protease, phospholipase, lipase, and rhamnolipid detection**

Strains were grown in LB medium and cultures were used for OD$_{600}$ measurements at various time points (Fig. 7E). For protease, phospholipase, and lipase assays, clarified supernatants were inoculated plates were allowed to dry at room temperature for glucose swarm agar plates, made as described previously [63]. Plate-based assays were conducted in triplicate and the clearance incubation at 37°C [64], and the plates were imaged using a UV transilluminator after incubation at 37°C for 3 days to visualize lipase activity. These plate-based assays were conducted in triplicate and the clearance zone diameters for skim milk and blood agar plates or the fluorescent halo diameters for the rhodamine B plates were measured and compared by percentage conversion relative to the wildtype PA01 value and were expressed as an average ± s.e.m (Fig. 7A, 7B and 7C).

**Promoter mapping**

The transcriptional start sites of the fadD1 and fadD2 genes were determined as previously described [60]. Briefly, PA01 was grown in 1x M9 minimal media supplemented with 0.2% C$_{16}$,0 to mid-log phase. This FA was chosen prior to the gene induction studies and, in retrospect, it was as appropriate as any other FA to map the fadD promoters. Cells were harvested and total RNA was isolated for subsequent cDNA synthesis and quantitative real-time PCR (qRT-PCR) analysis to determine fadD expression under different growth conditions [64]. The cDNA was subsequently used as the template in PCR, using oligos #798 + #376 and #798 + #373 for fadD1 and fadD2, respectively. The PCR product was sequenced using a second nested oligo #377 for fadD1, or #374 for fadD2 (Fig. 7). The PCR product was sequenced using a second nested oligo #377 for fadD1, or #374 for fadD2 (Fig. 7).

**Northern blot analysis**

Wildtype strain, PA01, was grown in 1x M9 minimal medium supplemented with 0.2% C$_{16}$,0, C$_{14}$,0, or C$_{12}$,0 as sole carbon sources. After reaching mid-log phase (OD$_{600}$=1.0), cells were harvested at 4°C and total RNA was isolated. Thirty µg of each RNA sample was used for northern analysis as described previously [66]. The fadD1 and fadD2 genes were PCR amplified from pET15b-fadD1 and pET15b-fadD2 using oligos #341 + #342, and #339 + #340, respectively, and used individually as probes (Fig. 3A and 3B).

**In vitro and in vivo competition studies**

Various fadD mutant strains (fadD1, fadD2 and fadD2 fadD1 mutants) and their corresponding complemented strains were utilized for the in vitro and in vivo competition studies (Table 3). A mucA insertional mutation was introduced into all strains to overproduce alginate, as we used a mouse model to allow these mucoid strains to survive and replicate in the lung as described previously [52]. Briefly, a 450-bp internal region of the mucA gene was PCR amplified from PA01 chromosomal DNA using oligos #973 and #974 and cloned into the PvuI site of pUC18. The resultant vector pUC18-mucA was electroporated into the various fadD mutant/complemented strains and the mucoid transformants were selected on PIA plates supplemented with 500 µg/ml carbenicillin (Cb500). One mucoid colony of each mutant/complemented strain was then inoculated separately in 3 ml of PIB + Cb500. After 24 h of incubation in a shaking incubator at 37°C, these cultures were diluted 100 times into 5 ml of fresh PIB + Cb500 and grown overnight. Three ml of each overnight culture was centrifuged (20,000 x g) for 10 min at 4°C and clarified supernatants were collected. The cell density of each culture was calculated by plating 10-fold serial dilutions on LB plates. Each culture was then adjusted to 2x10$^6$ CFU/ml in its own clarified supernatant, obtained above. At this point, each diluted fadD mutant strain (fadD1, fadD2 and fadD2 fadD1 mutants) and its

<table>
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<th>Table 5. Primers used in this study.</th>
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<td><strong>Number and Name</strong></td>
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</tr>
<tr>
<td>fadD cloning</td>
</tr>
<tr>
<td>302; fadD-up-Hind</td>
</tr>
<tr>
<td>303; fadD-down</td>
</tr>
<tr>
<td>339; fadD2-Ndel</td>
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<tr>
<td>340; fadD2-BamHI</td>
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<tr>
<td>341; fadD1-Ndel</td>
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<tr>
<td>342; fadD1-BamHI</td>
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<tr>
<td>501; Gm-up-reverse</td>
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<tr>
<td>713; lacZs</td>
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<tr>
<td>1092; EcfadD-up-AseI</td>
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<tr>
<td>1093; EcfadD-down-BamHI</td>
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<th>mucA cloning</th>
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<tr>
<td>937 – mucA-up</td>
<td>5’-GAGGGCTAGAAGCTTCGAG-3’</td>
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<tr>
<td>938 – mucD-down</td>
<td>5’-AAGGCCTGATTCAACCCAGGAGT-3’</td>
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*Restriction enzyme sites utilized in this study are underlined. 
Primers synthesized RNase free and HPLC purified.

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corresponding complemented strain were mixed at a 1:1 CFU ratio and the resulting mixtures (*fadD1/complement, *fadD2/ complement, and *fadD2D1/complement*) were used for inoculation into various growth media (*in vitro* competition) or mouse lungs (*in vivo* competition).

For *in vitro* competition, each mutant/complement mixture of equal cell density was diluted 100x into various growth media with LB, glucose, CAA, PC, C18:1, glycerol, or choline as sole carbon sources. All cultures were grown at 37°C with shaking for 1–2 days until the total cell densities reached ~1x10⁷ CFU/mL. Bacteria were then quantified by plating dilutions onto LB plates with and without tetracycline to determine the total number of bacteria (growth with no tetracycline) and the number of complemented bacteria (growth with tetracycline). These numbers were used to determine the *in vitro* CI (CFUmutant/CFUcomplement) when grown in media [47]. All experiments were performed in triplicate and statistical analysis was performed using Graphpad Prism 5.0 software (Fig. 8B).

Male BALB/c mice, 6–8 weeks old, were purchased from Charles River Laboratories and used in this *in vivo* competition study [52]. Before challenge, the mice were anesthetized by the intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. Thirty μl of the mutant/complemented strain mixture (3x10⁸ CFU of each) was inoculated intratracheally into BALB/c mice using the BioLITE Intubation System (Braintree Scientific). After 24 or 48 h, mice were humanely euthanized and lungs were harvested and homogenized. Bacteria were quantified by plating dilutions onto growth media with and without tetracycline to determine the total number of bacteria (growth with no tetracycline) and the number of complemented bacteria (growth with tetracycline). These numbers were used to determine the *in vivo* CI (CFUmutant/CFUcomplement) when grown in mouse lungs [47]. A control condition was included using PAO1-mucA::pUC10/lPAO1-mucA::pUC10-miniCTX2 to show that no competitive advantage or disadvantage was conferred by the presence of the Tet’ marker during in vivo growth (data not shown). Statistical analysis was performed using Graphpad Prism 5.0 software (Fig. 8C and 8D).

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**Author Contributions**

Conceived and designed the experiments: YK CBW TTH. Performed the experiments: YK JZS MHN. Analyzed the data: YK TTH. Contributed reagents/materials/analysis tools: JZS CBW MHN. Wrote the paper: YK TTH.

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Transcript amplification from single bacterium for transcriptome analysis

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**Supplemental Material**

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Method

Transcript amplification from single bacterium for transcriptome analysis

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Total transcript amplification (TTA) from single eukaryotic cells for transcriptome analysis is established, but TTA from a single prokaryotic cell presents additional challenges with much less starting material, the lack of poly(A)-tails, and the fact that the messages can be polycistronic. Here, we describe a novel method for single-bacterium TTA using a model organism, Burkholderia thailandensis, exposed to a subinhibitory concentration of the antibacterial agent, glyphosate. Utilizing a B. thailandensis microarray to assess the TTA method showed low fold-change bias (less than twofold difference and Pearson correlation coefficient R = 0.87–0.89) and drop-outs (4%–6% of 2842 detectable genes), compared with data obtained from the larger-scale nonamplified RNA samples. Further analysis of the microarray data suggests that B. thailandensis, when exposed to the aromatic amino acid biosynthesis inhibitor glyphosate, induces (or represses) genes to possibly recuperate and balance the intracellular amino acid pool. We validated our single-cell microarray data at the multi-cell and single-cell levels with lacZ and gfp reporter-gene fusions, respectively. Sanger sequencing of 192 clones generated from the TTA product of a single cell, with and without enrichment by elimination of rRNA and tRNA, detected only B. thailandensis sequences with no contamination. These data indicate that RNA-seq of TTA from a single cell is possible using this novel method.

Innovative methods in single-cell technology are needed to enhance the investigations of Bacteria and Archaea genomic material, particularly if we are to develop deeper insights into the functional and metafunctional genomics of these “prokaryotes.” Functional-genomics or transcriptomics of a single-cell can produce a wealth of information at resolutions that cannot be attained by analysis of multi-cell populations or communities. Such advances hinge on the development of innovative methods for single-cell isolation (Podar et al. 2009) and transcript amplification from a minute amount of starting material with low gene expression bias. Single eukaryotic cell mRNA amplification methods for transcriptome analysis, via microarray (Kurimoto et al. 2007; Scanlon et al. 2009) and mRNA sequencing (Tang et al. 2009), have recently been described. These existing methods of transcript amplification, pioneered for eukaryotic transcript amplification, involve multiple rounds of exponential (Kurimoto et al. 2006) and/or linear (Scanlon et al. 2009) amplification of cDNA. However, no study has described total transcript amplification (TTA) from a single bacterium, possibly due to the major challenges one may encounter when working with single-bacterium TTA. These challenges include (1) the low amount of RNA (~0.1–2 pg/prokaryotic cell vs. ~10–50 pg/eukaryotic cell); (2) the lack of poly(A)-tails for ease of tagging and mRNA amplification; and (3) the fact that the messages can be polycistronic, and full-length amplification is critical for detecting expression of all genes in an operon. Due to these characteristics of prokaryotic transcripts, our experience with TTA using existing linear and exponential amplification methods for single bacterial cells shows the methods are labor intensive and yield unusable data with extensive gene expression bias and low reproducibility. If these challenges could be resolved, one could envisage numerous applications that may provide a wealth of functional-genomic information that was not previously possible (Supplemental Fig. S1).

Here, we describe a novel method for TTA from a single prokaryotic cell. Using Burkholderia thailandensis as a model bacterium exposed to a subinhibitory concentration of the antibacterial agent glyphosate (GS) (Norris et al. 2009), we developed a novel method for TTA using φ29 polymerase multiple displacement amplification (MDA) of circularized cDNA. We used microarray to assess the reproducibility, level of gene expression bias, and gene presence that resulted from this novel method. This low bias and simple single-tube method is reproducible and is not labor intensive. The data yielded a less than twofold difference in fold-changes compared with the nonamplified samples. In a typical experiment, we could amplify and detect ~94%–96% of the detectable transcripts (2842 genes) from a single cell by microarray. Exposure to GS up- or down-regulates many genes, resulting from GS inhibition of aromatic amino acid biosynthesis, to possibly compensate for amino acid imbalance. From the microarray data obtained through TTA of single cells exposed to GS versus no GS, we randomly picked five up-regulated genes, three down-regulated genes, and two control genes that showed no fold-change to validate our microarray data by reporter-gene fusions. We propose...
that this novel method can be applied to RNA-seq and will stim-
ulate various important prokaryotic research areas that require
single-cell level transcriptome analysis (Supplemental Fig. S1).

Results

Single-cell isolation and amplification method

We utilized laser capture microdissection (Emmert-Buck et al. 1996)
to isolate \(B.\) \textit{thailandensis} single cells, followed by \(B.\) \textit{thailandensis}
microarray analysis to assess our single-cell TTA method. Although
various single-cell isolation techniques have been described (Podar
et al. 2009), we chose to use the Zeiss Laser Capture Microdissection
(LCM) MicroBeam IV system (hereafter referred to as the Zeiss LCM)
to isolate single \(B.\) \textit{thailandensis} cells grown in 1× M9 minimal
glucose media (MG) ± GS (Fig. 1). We have recently discovered that
\(B.\) \textit{thailandensis} is very sensitive to the herbicide GS (Norris et al.
2009) because bacteria, in the presence of GS, cannot synthesize
aromatic amino acids (Fischer et al. 1986). At a subinhibitory GS
concentration of 0.01% compared with no GS, there is no apparent
difference in growth rate or final cell density, which renders GS
exposure appropriate as a model for gene-expression analysis be-
tween these two growth conditions (Fig. 1B). Our approach (Fig.
1A) was to perform large-scale RNA preparation from each of the
two cultures (nonamplified samples). Single cells were then iso-
lated from both cultures (Fig. 1C–E) and subjected to our single-cell
TTA method (amplified samples). Microarray experiments were
then performed to obtain gene expression fold-change (between
the two growth conditions, i.e., ± GS) for both the amplified and
the nonamplified samples, which were then compared to assess
the suitability of the TTA method (Fig. 1A). We expected that genes
differentially expressed between the two conditions from the
amplified RNA samples would correlate, with minimal fold-
change bias, to data from the large-scale nonamplified RNA
preparations from more than \(10^9\) bacteria.

We devised a novel method for single-cell TTA, using \(q29\)
polymerase for MDA of circularized cDNA (Fig. 2). Full details of
the method can be found in the Methods section. Briefly, single

Figure 1. Single \(B.\) \textit{thailandensis} cell isolation. (A) Experimental design for evaluating the single-cell transcript amplification method. \(B.\) \textit{thailandensis}
grown in two different conditions were used in large-scale (nonamplified) and single-cell level (amplified) microarray analysis. Fold-changes (between
condition 1 and 2) of all genes detected from the nonamplified and amplified samples were then compared by correlation analysis. (B) Comparable growth
curves of \(B.\) \textit{thailandensis} in MG medium ± 0.01% GS (w/v) added at mid-log phase (red arrow) and harvested 30 min post-exposure (black arrow). (C)
Fluorescent \(B.\) \textit{thailandensis} cells were observed under 1000× magnification. The section of the membrane containing a single bacterium was drawn and
cut by the focused laser (green line) and catapulted at a distance from the cell with unfocused low-intensity laser beam (blue spot), which aseptically
catapulted and isolated the single cell into the lid of a 0.2-mL PCR tube containing the cell lysis buffer. (D) Bright-field mode showing the section of the
membrane where the single bacterium had been. (E) Fluorescence mode confirming that the bacterium of interest has been transferred from the
membrane slide to the PCR tube lid.
B. thailandensis cells were isolated (Fig. 1C–E) and subjected to a lysis step with the nonionic detergent Triton X-100, EDTA, and lysozyme (Fig. 2). At this point, an optimal 5’-phosphate-dependent exonuclease step can be incorporated to remove tRNA and rRNA to enrich for mRNA, if the end product of the TTA method is to be used for deep sequencing (see Methods and below). Otherwise, cDNA synthesis was performed thereafter with DNA random hexamers and MMLV reverse transcriptase. McrBC and DpnI were added to remove bacterial chromosomal DNA and any potentially contaminating DNA, which are methylated and will be digested by these enzymes. The newly synthesized single-stranded (ss) cDNA (ss-cDNA) was 5’-end phosphorylated and ligated intramolecularly with CircLigase ssDNA ligase, which will not ligate ss-DNA <15 bp (e.g., excess random hexamers). The circularized ss-cDNA was then randomly primed with RNA hexamers and subjected to MDA with φ29 DNA polymerase. The use of thiophosphate-linked RNA random hexamers is critical to reduce falsely primed DNA by-product and primer dimers, while not interfering with the DNA synthesis (Takahashi et al. 2009). The thiophosphate-linked RNA random hexamers are also stable in the presence of φ29 DNA polymerase RNase activity (Lagunavicius et al. 2008). The φ29 DNA polymerase amplification was split into two steps (steps 5 and 6, Fig. 2), where the initial step was performed in a small total volume (~10 μL) to increase the template and substrate concentrations. The second MDA step was performed by adding another 90 μL of a φ29 DNA polymerase and substrate mixture to increase the cDNA yield. As a precaution, we included McrBC and DpnI endonucleases again to prevent amplification of potentially contaminating DNA during the MDA steps of circularized cDNA. The highly polymerized double-stranded (ds) cDNA (ds-cDNA), labeled with 5-(3-aminoallyl)-2’-deoxyuridine-5’-triphosphate (aa-dUTP), was fragmented to ~1–4 kb and incorporated with Cy dyes for microarray analysis (Fig. 2).

There are several advantages of this novel single-cell TTA method. In this study, the φ29 DNA polymerase provides rapid and efficient amplification from starting materials of a single cell with minimal fold-change bias (below). Several strategies were employed to eliminate template-independent amplification, including the use of thiophosphate-linked RNA random hexamers.
hexamers as previously described (Takahashi et al. 2009), elimination of exogenous DNA contamination with endonucleases (McrBC and DpnI), and minimization of the reaction volume (10 μL for initial amplification and 100 μL total). All manipulations were performed from beginning to end in a single tube in a PCR chamber. In addition, this simple method is not labor intensive and requires very few manipulation steps, thereby reducing potential manipulation-derived contaminants. The typical yield of ds-cDNA from a single cell is ~25–30 μg, sufficient for microarray analysis (Fig. 3). If more ds-cDNA is required and further amplification is necessary for the technical microarray replicates below, additional ε29 DNA polymerase and substrate mixture can be added to the original amplification tube containing ~25–30 μg of ds-cDNA. This will increase ds-cDNA yield to ~75–90 μg.

**Microarray analysis of genes expressed at the single-cell level**

Our final TTA method amplifies and detects 94%–96% of the total transcripts detectable by microarray (2842 genes), using from five cells down to the single-cell level. Although ~10 μg of labeled ss-cDNA is sufficient for nonamplified samples in a typical microarray experiment, our amplified product is ds-cDNA. Therefore, to optimize single-cell microarray experiments for our amplified samples, we initially performed TTA on groups of five *B. thailandensis* cells and used different amounts of the amplified cDNA for each microarray slide (Fig. 3). When 10–14 μg of amplified cDNA from TTA of the five cells was used, ~10.8% of the transcripts were not amplified to an amount detectable by microarray, while expression of these genes was detectable in the non-amplified samples (Fig. 3A). Increasing the amount of amplified cDNA used in microarray experiments to 20–25 μg reduced "drop-outs" to ~4.1% (Fig. 3B). Further increasing the amount of amplified cDNA used to 30–35 μg did not significantly reduce the percentage of genes that was missing from the amplified samples (~4.4%) (Fig. 3C). We next attempted amplification of total transcript from a single cell for microarray analysis, and the results were once again significantly improved at the higher concentration of cDNA used (Fig. 3E–G). To determine the reason for these missing genes (drop-outs) from the microarray data in the amplified samples, we examined their GC content, operon size, RNA secondary structure, or gene expression variation among the individual cells of the population (Supplementary Analysis). These analyses revealed these missing genes resulted from their low expression levels, which suggests the low abundance of their transcripts causes "drop-out" of these genes in the amplified samples (Fig. 3D,H). Although there were good Pearson correlations between the fold-change of the amplified and nonamplified samples in all these experiments (Fig. 3A–C, E–G), we recommend using 30 μg of amplified cDNA in a typical microarray experiment, as this amount of cDNA used yielded a minimal number of genes absent from the microarray data (~4%–5% drop-outs) (Fig. 3C,G).

Having optimized the amount of amplified cDNA (30 μg) to use in microarray experiments, we performed biological (Fig. 4A–C) and technical replicates (Fig. 4D) to obtain fold-change data from 2 pg of diluted total RNA to an actual single cell. Initially, microarray experiments were performed in triplicate using three independent amplifications of 2 pg of purified and diluted RNA. We correlated the fold-change of the amplified to nonamplified

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**Figure 3.** Fold-change scatter plots of expressed genes obtained from nonamplified versus amplified samples. 10–14 μg (A), 20–25 μg (B), or 30–35 μg (C) of DNA amplified from five-cell samples were hybridized to different slides, and the fold-changes of detected genes were plotted against those obtained from the nonamplified sample. The number located at the right bottom corner of each plot indicates the percentage of missing genes (drop-outs) from each amplified sample compared with the nonamplified sample (2842 genes total). (D) Gene expression levels from the nonamplified sample (black dots) were compared between two growth conditions (MG ~ 0.01% GS). Expression levels of genes that were missing in the five-cell amplified samples are colored green (as a result of using 10–14 μg of cDNA), red (using 20–25 μg of cDNA), or purple (using 30–35 μg of cDNA), and are overlaid on the same graph in D. Similar comparisons were conducted with different amounts of cDNA amplified from one-cell samples: 10–14 μg (E), 20–25 μg (F), or 30–35 μg (G). Missing genes or drop-outs from each sample were color-coded similarly and overlaid with the total number of genes detected in the nonamplified samples (H). The 8 value in the upper left corner of each plot represents the Pearson correlation coefficient. All microarray experiments in this figure were conducted without the optional mRNA enrichment step.
Each amplified replicate produces 3.2%–3.9% gene drop-outs, and the Venn diagram for the missing genes among these replicates indicated significant overlap among these low abundance transcripts (Fig. 4A). The averaged data from the triplicates showed excellent Pearson correlation of the fold-change to those of the nonamplified samples ($R = 0.98$) (Fig. 4A). A total of 4.7% drop-out genes in the averaged data indicated that we successfully amplified and detected ~95% of the total transcript expressed relative to the nonamplified sample when starting with 2 pg of purified total RNA. Similar results were observed for three groups of five cells (Fig. 4B). When microarray data were obtained from three individual single cells as biological replicates, comparable fold-change correlations and percentages of missing genes were observed (first three graphs of Fig. 4C). The averaged data

**Figure 4.** Microarray data fold-change comparison of nonamplified and amplified samples starting from 2 pg of diluted RNA (A), five cells (B), or one cell (C) as biological replicates; or a single cell hybridized to three different slides as technical replicates (D). The first three plots of each item are biological replicates (A–C) or technical replicates (D). The number in the bottom right corner of each plot indicates the percentage of genes that were missing in the amplified samples compared to the nonamplified samples. The Pearson correlation coefficient between the amplified and nonamplified fold-change data is shown at the upper left corner of each plot. The high correlation coefficient values ($P < 0.0001$) and the tight grouping of the dots within the twofold difference boundaries suggest a relatively low bias. The percentages of overlap among missing genes from each group are displayed as area-proportional Venn diagrams of three independent biological (A–C) or technical replicates (D). The color for each circle in the Venn diagram corresponds to the colored boxes in each scatter plot. The last plot of each item shows averaged data from the three biological (A–C) or technical replicates (D). All microarray experiments in this figure were performed without the optional mRNA enrichment step.
showed excellent correlations ($R = 0.96$), with 93.3% of the transcripts from single cells amplified and detected compared with the nonamplified sample (last graph of Fig. 4C). Technical replicates, where single-cell transcripts were amplified to sufficient level for three microarray slides, were relatively reproducible, confirming the consistency among different microarray experiments (Fig. 4D). An important point to note is that, in the averaged data, there was very little fold-change bias or skewing as a result of the amplification of transcripts from a single cell, and fold-change variations of all detectable genes were significantly less than a two-fold difference (i.e., all dots lie well within the $2 \times$ difference lines in Fig. 4). The averaged data set from three TTA replicates of a single cell (Fig. 4C) indicated that reliable data could be generated by using this TTA method, and $>93\%$ of the transcripts from a single cell could be amplified and detected in microarray experiments.

Correlation of the gene expression levels between amplified single cells versus nonamplified control and independent single cell amplifications was also analyzed (Supplemental Fig. S2). Expression levels from single cell amplified and nonamplified samples were poorly correlated (Supplemental Fig. S2A,B), indicating significant amplification bias, probably due to different amplification efficiencies among individual genes. However, correlations among transcripts amplified from independent single cells were significantly higher (Supplemental Fig. S2C,D), indicating more consistent amplification efficiencies for any particular gene in the two cells. Thus, any amplification bias of different genes due to differences in amplification efficiencies would “cancel out,” when comparing gene expression levels in single cells of two conditions to obtain fold-change. Much like real-time RT-PCR comparison to obtain reliable gene expression fold-change, amplification efficiencies of a particular gene in two different conditions must be consistent, while amplification efficiency variations between different genes are acceptable. Thus, single cell gene expression profiling using the method described here should be performed and compared between single cells in two different conditions (e.g., GS and no GS). However, this method is not recommended for obtaining gene fold-changes by comparing between amplified and nonamplified samples.

**GS-dependent gene expression by single-cell microarray analysis and validation via reporter gene fusions**

With the averaged microarray data generated from the TTA of a single bacterium performed in technical triplicates (Fig. 4D), we assembled a list of genes that were induced or repressed in the presence of GS (Supplemental Tables S1, S2). We chose five genes up-regulated in the presence of GS, three genes down-regulated by GS, and two control genes with no fold-change (Supplemental Table S3) to perform reporter-gene fusions with lacZ and GFP for microarray data confirmation. The genes selected for microarray validation are involved in amino acid metabolism (Fig. 5A). After 2 and 4 h exposure to GS, increased green fluorescence signals and β-galactosidase activities indicated that the expression of the five genes up-regulated in our microarray data did increase as a result of GS (Fig. 5B,C). As indicated by the fluorescence signals and β-galactosidase activities, expression levels of the three genes down-regulated in the microarray data also decreased as a result of GS, while expression levels of the two control genes showed only insignificant changes (Fig. 5B,C). Overall, gene fusion experiments and the fold-change from the nonamplified sample microarray data showed strong agreement to the single-cell amplified samples microarray data (Fig. 5). Only a few genes in the single-cell data with a fold-change of two or more were less than two in the nonamplified data (potential false positives) (Supplemental Tables S1 and S2). However, the majority of the genes expressed comparably in the amplified and nonamplified samples, suggesting a strong and reliable correlation between the fold-change data generated from single cells and large-scale nonamplified samples (Fig. 4D; Supplemental Tables S1, S2).

Among the up-regulated genes in particular, three genes targeted for validation were responsible for aromatic amino acid (AAA) biosynthesis (BTH_I1295, BTH_I2909, and BTH_I3337) and two genes were responsible for shuffling benzoate and alanine to possibly replenish central pathway intermediates (BTH_I0506 and BTH_I0922) (Fig. 5A). Of the three genes down-regulated due to GS, two targeted genes convert pyruvate in the synthesis of other amino acids, potentially balancing other amino acid species (e.g., Met, Ile, and Val), and one gene was involved in conversion of TCA cycle intermediates (Fig. 5A). It seems that GS not only affects the EPSPS target (Fig. 5A) and reduces AAA synthesis but also has an overall influence on the amino acid pool. Reducing AAA signals a concurrent reduction in other amino acids (Met, Ile, Val, and Ala) to balance the cellular amino acid population.

**Potential application to next-generation sequencing**

We next evaluated if this TTA method can be used for transcriptome analysis by deep sequencing. RNA-seq methods for prokaryotes require mRNA enrichment to eliminate rRNA and tRNA, which can be upward of 90%–95% of the total RNA in the cell, to reduce the number of reads and costs. Additionally, transcriptome profiling by deep sequencing is less tolerant of potential contaminants or nonspecific amplification. Therefore, we analyzed our TTA products using real-time RT-PCR and Sanger sequencing to assess the potential use of this method for transcriptome analysis by deep sequencing. An mRNA enrichment step was added to our amplification method to amplify transcripts from single cells (Fig. 2). By real-time RT-PCR, we then analyzed amounts of rRNAs (23S and 16S) and a tRNA (Ala) relative to the mRNA amount of one gene (BTH_I2028) before and after enrichment in amplified single cell samples grown in MG medium (Fig. 6). The amounts of rRNAs and one tRNA species were significantly reduced after enrichment, relative to the mRNA transcript of BTH_I2028 (Fig. 6). A microarray analysis was then performed on the enriched or unenriched samples amplified from single cells grown in MG medium (same as the real-time experiment above) and MG + GS medium. Data from the enriched sample showed low fold-change bias resulting from the enrichment, with an optimized amount of 5'-phosphate–dependent exonuclease ($1 \times 1 \times 10^{-5}$ units of enzymes). However, a higher level of amplification bias was observed when more exonuclease ($10 \times 1 \times 10^{-4}$ units of enzymes) was used, possibly due to nonspecific digestion of mRNA (Fig. 6).

Single-cell cDNA libraries were constructed to analyze the purity of our enriched and amplified ds-cDNA to determine the potential for RNA-seq application (Supplemental Fig. S3). Sanger sequencing reads of 96 independent clones from each of the unenriched and enriched cDNA libraries were analyzed, and ~96% (188 out of 192) matched to *B. thailandensis* sequences (Supplemental Table S4). Approximately 4% did not match to any sequences in GenBank. This is typical in RNA-seq experiments, where ~5% of sequences cannot be assigned (i.e., no match) (Yoder-Himes et al. 2009). This demonstrated the effectiveness of the strategies to...
Figure 5.

Legend on next page.
eliminate template-independent amplification and exogenous DNA contamination. The significant reduction of rRNA sequences in the enriched sample was confirmed by both real-time RT-PCR and sequencing data, suggesting successful depletion of rRNAs in the enrichment step. Taken together, these results showed promise to apply our TTA method for transcriptome profiling by deep sequencing, which may allow detection of low abundance transcripts that were drop-outs in our less sensitive microarray analysis approach.

Discussion

There are currently no published methods for single prokaryotic cell TTA. However such a method is desperately needed if we are to expand multiple areas in prokaryote functional genomics (Supplemental Fig. S1). The model used in this study, B. thailandensis exposed to GS, was appropriate and sufficient for assessment of our TTA method because we could compare the transcriptomic fold-change data obtained from single cells to data obtained from the

Figure 6. Evaluation of mRNA enrichment in amplified single cell samples. Top panel presents mRNA amount of a gene (BTH_I2028) relative to genes of 16S rRNA, 23S rRNA, and a tRNA-Ala detected by real-time RT-PCR. Unenriched represents amplified sample without the mRNA enrichment step. Enriched (1× or 10×) means treated with 1 × 10⁻⁸ U (as described in Methods) or 1 × 10⁻⁴ U of the Terminator 5'-Phosphate-Dependent Exonuclease, respectively. The relative transcriptional levels of rRNAs and tRNA are significantly higher than the BTH_I2028 gene (mRNA) in the unenriched sample but are greatly reduced in the enriched samples. Microarray analysis was performed for amplified samples (enriched and unenriched); fold-changes were compared to nonamplified samples as shown in the bottom plots. Fold-change correlation for the BTH_I2028 gene is indicated by the red dots. The number at the bottom right corner represents the percentage of transcripts that were missing in the single cell. Enrichment with 10× the amount of 5'-phosphate–dependent exonuclease resulted in a slightly higher fold-change bias as indicated by the Pearson correlation coefficient shown at the upper left corner for each plot.

Figure 5. Validation of microarray data via reporter-gene fusions. (A) Proposed amino acid metabolism pathways influenced by glyphosate (GS) in B. thailandensis. Two connecting arrows indicate two or more reaction steps. The target for GS is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Fischer et al. 1986). In A, B, and C, green and orange boxes indicate genes induced and repressed, respectively, by GS. (B,C) gfp and lacZ reporters were fused to five GS-induced genes, three GS-repressed genes, and two GS-insensitive control genes (housekeeping gene controls in black boxes). (B) Cells were examined under 630× magnification at 2 and 4 h post-exposure to GS. Differential interference contrast (DIC) and green fluorescence images were merged, and the representative fields are shown. (C) At the same time points, β-galactosidase activities for these fusion strains were determined in triplicate, and the Miller units were plotted with the SEM. The numbers above the bars in the histogram in C indicate fold-induction or fold-repression differences by GS, as measured by β-galactosidase assays. For comparison, the microarray data fold-change of the corresponding genes from the amplified sample and the nonamplified sample are also displayed below the graph. As a general trend, these gene-fusion data agree with the microarray data at both 2 and 4 h post–GS-exposure.
larger-scale nonamplified RNA samples. Since there were excellent microarray data correlations of fold-change between the amplified and nonamplified samples in this study and the differences were significantly less than twofold when three replicates were averaged (Fig. 4), we are confident that the fold-changes generated from TTA of single cells between two different conditions are reliable (Supplemental Tables S1, S2). In summary, we have developed a single-tube TTA method that is simple, requiring very few steps. This method yielded reproducible data, low fold-change bias, and a high number of genes efficiently amplified from a single prokaryotic cell (94%–96% presence or low transcript drop-out as detectable by microarray).

We envisage that the method described will be used for transcriptome analysis by deep sequencing (Croucher et al. 2009; Passalacqua et al. 2009) of the amplified ds-cDNA, with an optional step to eliminate rRNAs and tRNAs for enrichment of mRNA (Figs. 2, 6). Deep sequencing may increase the dynamic range of detectable genes expressed to include low or highly expressed genes that may not be detectable or differentiated by microarray (Wang et al. 2009), and this may decrease or eliminate drop-outs. The limitation of this method is that antisense transcripts are not detectable, because the final product of our TTA method is ds-cDNA, and thus it is not strand specific (Vivancos et al. 2010). Our method yielded ds-cDNA, which could potentially be used in tiling arrays, possibly identifying untranslated regions and novel operons (Toledo-Arana et al. 2009). Hence, this method, beyond microarray, has the potential to detect global gene expression of a single cell, novel operons, and novel coding and noncoding regions (Sorek and Cossart 2010) in both cultured and noncultured prokaryotes (Supplemental Fig. S1).

Finally, we envisage that our method can be applied to single eukaryotic cell transcript amplification. This could be performed by substituting the DNA random hexamers to a poly(T) oligo during cDNA synthesis, and an mRNA enrichment step is not necessary. The remaining steps of the protocol are otherwise identical.

Methods

Bacterial strains, media, and culturing conditions

Escherichia coli EPMax10B-lacF-pir (Nortis et al. 2010) was routinely used as a cloning strain. The B. thailandensis wild-type strain, E264 (Brett et al. 1998), and its derivatives were cultured in LB or 1× M9 minimal medium (Sambrook and Russell 2001) supplemented with 20 mM glucose (MG). For the B. thailandensis microarray and reporter-gene fusion studies (Fig. 5B,C), B. thailandensis wild-type strain E264 and derivatives were grown in MG medium + 1% (w/v) Brij-58 and exposed to a final concentration of 0.01% (w/v) GS. Brij-58 (1% w/v) was added to all cultures to prevent bacterial clumping during growth.

Molecular reagents

T4 polynucleotide kinase, dNTPs, MMLV (Moloney murine leukemia virus) reverse transcriptase, and endonucleases (McrBC and DpnI) were purchased from New England Biolabs. Ready-Lyse lysozyme, Terminator 5’-Phosphate-Dependent Exonuclease, CircLigase ssDNA ligase, and q29 DNA polymerase were purchased from Epicentre Biotechnologies. Inorganic pyrophosphatase was purchased from Roche Applied Science. TRIzol reagent, RNaseOut reagent, RNase-free DNA random hexamers, and Live/Dead BacLight cell stain were purchased from Invitrogen. The RNeasy MiniKit and RNaseOut reagent were purchased from Qiagen. DNA oligonucleotide primers and random RNA hexamers with five thio-phosphate-linkages (6R5S) (Takahashi et al. 2009) were synthesized through Integrated DNA Technology (IDT). DNase I, aminooaryl-dUTP (aa-dUTP), and all the other chemicals used in this study were purchased from Sigma. Cy3 and Cy5 dyes were purchased from Amersham Biosciences.

Single bacterium total transcript amplification

Single bacterium level transcript preparation

Large-scale total RNA was extracted from B. thailandensis wild-type cells (Fig. 1A) using TRIzol reagent and purified with the RNeasy Mini Kit by following the manufacturer’s total RNA cleanup protocol. Two picograms of RNA, representing the single bacterium level of RNA, was prepared by serially diluting the purified total RNA to a final concentration of 10 pg/μL in DEPC water containing 1 U/μL of RNaseOut. Then, 0.2 μL (2 pg) of the final dilution was directly added to 2 μL of the lysis buffer (100 mM Tris-Cl at pH 8.0, 200 mM KCl, 0.2 mM EDTA, 0.1% Triton X-100, 2 mM DTT, 0.04 U/μL RNaseOut, 2 × 10⁻⁷ M Ready-Lyse lysozyme).

Single bacterium isolation

For single cell isolation, B. thailandensis cells were treated with RNaseOut reagent, and stained with Live/Dead BacLight fluorescent dyes. Stained cells were then smeared onto a PALM membrane (Carl Zeiss), and observed under 1000× magnification on the Zeiss LCM system. Sections of membrane containing one or five fluorescent bacteria were cut by the focused laser and catapulted with unfocused low-intensity laser beam into 2 μL of lysis buffer contained within a 0.2-mL PCR tube lid. The cDNA synthesis and amplification of the single cell total transcript were then performed as below.

Single bacterium transcript amplification

The B. thailandensis cells or diluted 2 pg total RNA was incubated in the lysis buffer for 5 min at 37°C, then heated for 2 min to 80°C. When necessary (e.g., RNA-seq is used rather than microarray), an optional mRNA enrichment step should be performed here. A 0.5 μL of the enrichment mixture (1 × 10⁻⁵ U Terminator 5’-Phosphate-Dependent Exonuclease and 5 nmol MgCl2) is added and incubated for 10 min at 37°C. A 2 μL aliquot of RT mixture was then added, consisting of 4 U RNaseOut, dNTPs (0.25 nmol each), preheated DNA random hexamers (0.2 pg for one cell, 1 pg for five cells, or 2 pg for 2 pg diluted total RNA), 10 nmol MgCl2, and 2 U MMLV. The reverse transcription was carried out for 2 h at 48°C. A 0.24 μL aliquot of endonucleases mixture (4 nmol GTP, 1 U McrBC, and 2 U DpnI) was then added to each tube, followed by incubation for 15 min at 37°C to degrade the chromosomal DNA. ss-DNA phosphorylation and ligation were accomplished by adding a 0.7 μL aliquot of the ss-DNA ligation mixture (0.25 nmol ATP, 12.5 nmol MnCl2, 1 U T4 polynucleotide kinase, 10 U CircLigase ss-DNA Ligase) to each tube followed by incubation for 1 h at 37°C and then for 8 h at 60°C for ligation. The circularized ss-DNA (~5 μL) was then used as template in a two-step multiple primed rolling circle amplification (MPRCA) reaction as follows. First, a 25 mM dNTP/aa-dUTP stock was prepared following an established protocol, where a 1:2 dTTP:aa-dUTP ratio was used to efficiently label DNA with high GC% (http://pfgrc.jcvi.org/index.php/microarray/protocols.html). A 4.5 μL mixture (1 × 029 reaction buffer, 20 nmol DTT, 0.5 μL dNTPs/aa-dUTP stock, 15 pmol of RNA random hexamers 6R5S, and 4 nmol GTP) was then added, followed by heating for 2 min to 90°C then immediate cooling on ice. McrBC (2 U, 0.2 μL) was then added to each tube and incubated

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for 5 min at 37°C. Fifty units of α29 polymerase (0.5 μL) was added, followed by incubation for 2 h at 30°C to initiate the MPRCA reaction in a small total volume (~10 μL). After the initial MPRCA, a larger volume (90 μL) of the MPRCA mixture (1× α29 reaction buffer, 360 nmol DTT, 4.5 μL dNTPs/aa-dUTP, 150 pmol RNA random hexamers, 100 U α29 polymerase, 90 nmol GTP, 5 U McrBC, 10 U DpnII, and 20 U pyrophosphatase) was added, and the reaction was carried out for another 32 h at 30°C. After this step, the cDNA yield is typically 25–30 μg. For the higher yields (75–90 μg) required to perform microarray technical replicates, another 200 μL of the MPRCA mixture (1× α29 reaction buffer, 800 nmol DTT, 10 μL dNTPs/aa-dUTP, 300 pmol RNA random hexamers, 200 U α29 polymerase, 200 nmol GTP, 10 U McrBC, 20 U DpnII, and 40 U pyrophosphatase) was added to the reaction. The reaction mixture (300 μL total) was then aliquoted into three 0.2-ml PCR tubes and incubated for another 16 h at 30°C. After amplification, the reaction was stopped by heat-inactivation at 65°C for 10 min. The newly synthesized double-stranded cDNA was then randomly fragmented to 1–4 kb in length with Dnase I and extracted with phenol/chloroform (1:1). Fragmented cDNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 3 volumes of isopropanol/chloroform (1:1). Fragmented cDNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 3 volumes of isopropanol and then incubated at ~80°C for 1 h. The DNA pellet was then collected by centrifugation at 20,000g for 10 min, washed with 70% ethanol, and vacuum-dried.

Two-color microarray and data analysis

For microarray analysis of the nonamplified samples, cDNA was synthesized from 10 μg of total RNA following established protocols (http://pfgrc.jcvi.org/index.php/microarray/protocols.html). Typically, 8–12 μg of nonamplified cDNA or 25–30 μg of amplified cDNA was used for each condition in microarray hybridization. Both the nonamplified and amplified cDNA were labeled with Cy3 (no-GS condition) and Cy5 (GS condition) dyes and then hybridized to the B. thailandensis 70-mer replicate arrays (geo accession number GPL7113) following the established protocols (http://pfgrc.jcvi.org/index.php/microarray/protocols.html). Microarray slides were scanned with a GenePix 4000B microarray scanner using GenePix Pro software 5.1. Individual TIFF images from each channel were processed with SpotFinder software 3.2.1 (available at http://www.tm4.org) to quantify the gene expression levels. Raw microarray data were processed and normalized by low intensity filtering, total intensity normalization, LOWESS normalization, standard deviation regularization, and in-slide replicate analysis using MIDAS software 2.21 (available at http://www.tm4.org). Finally, normalized gene expression data were used to generate data tables using MEV software 4.5.1 (available at http://www.tm4.org).

Fold-change data for all detected genes were obtained from triplicates of the nonamplified or amplified samples. GS-induced genes (fold-change ≥ 2, P ≤ 0.05) are summarized in Supplemental Table S1, and GS-repressed genes (fold-change ≥ 2, P ≤ 0.05) are summarized in Supplemental Table S2.

Scatter plots and Venn diagrams

GraphPad Prism 5 software was used generate all scatter plots and calculate the correlation coefficients in Figures 3, 4, and 6 and Supplemental Figure S2. The area-proportional Venn diagrams were drawn based on images generated using a free online software (http://bioinforx.com/free/bxarrays/venndiagram.php).

Gene assignment and pathway designation

Gene description assignment for some genes was assisted by reference to the Burkholderia Genome database (http://www.burkholderia.com). Genes involved in the GS pathway (Fig. 5A) were assigned according to the Kyoto Encyclopedia of Genes and Genomes (KEGG).

Microarray data validation via gene fusion studies

Eight genes predicted to be involved in the GS pathway (Fig. 5A) and two controls genes were chosen for validation of the microarray data. Two promoter-less reporter-genes, gfp and lacZ, were integrated downstream from each target gene via two-step λ red protein-mediated recombineering (data to be published elsewhere). Briefly, the gfp-pherS-gat fragment was amplified from pUCP28T-gfp-pherS-gat (laboratory plasmid) using oligos containing 40–45 bp homologous sequences to the downstream region of the target gene. The PCR product was then integrated downstream from the target gene after the stop codon to make a transcriptional fusion in the B. thailandensis chromosome via λ red protein-mediated recombineering. Positive integration was selected on 1× M9 minimal medium supplemented with 0.04% (w/v) GS and screened by PCR. Next, the second λ red protein-mediated recombineering was accomplished by introducing the lacZ fragment (with the 5’-end homologous to the downstream region of the gfp gene, and the 3’-end homologous to the downstream region of the gat gene) to replace the pherS-gat fragment. The final gfp-lacZ fusion strains were obtained via pherS counter-selection on cPhe-containing media, as previously described (Barrett et al. 2008), and confirmed by PCR using oligos which anneal to the target genes and the lacZ gene.

These newly engineered fusion strains were first grown in LB medium overnight. Cells were harvested by centrifugation, washed twice with 1× M9 minimal medium, and resuspended in the same medium. Resuspended cells were diluted 100× into MG medium + 1% (w/v) Brij, and two identical cultures of each fusion strain were grown to mid-log phase, at which point GS was added to one of the cultures to a final concentration of 0.01% (w/v). At 2 and 4 h post-exposure to GS, samples of both cultures (with and without GS) were taken for fluorescence microscopy and β-galactosidase assay. To immobilize bacteria for fluorescence microscopy, cells were mixed with warm (42°C) molten agarose to a final concentration of 0.1% (w/v) and were immediately mounted between glass slides and coverslips to solidify the agar. Fluorescence was observed under the 38HE filter set on a Zeiss Axio Observer D1 microscope, and images were recorded with an AxioCam MRc 5 camera. Differential interference contrast (DIC) and green fluorescence images were merged at the time of capture using Zeiss AxioVision software. Multiple images were captured for each sample, and representative fields are shown in Figure 5B. β-Galactosidase assay (Sambrook and Russell 2001) was performed in triplicate on these fusion strains, and average activities are shown in Figure 5C with SEM. For comparison of the fold-change data from the microarray and β-galactosidase assay, the GS-induced gene fold-changes were calculated as the gene expression levels in the presence of GS divided by those in the absence of GS; the GS-repressed or GS-insensitive gene fold-changes were calculated based on gene expression levels in the absence of GS, divided by gene expression levels in the presence of GS.

Microarray data accession number

Microarray data are available in the NCBI GEO repository under accession number GSE23419.

Real-time RT-PCR

Primers and probes for each target were designed using Integrated DNA Technologies Primer Quest software (http://www.idtdna.com) and are shown in Supplemental Table S5. Amplified ds-cDNA
from isolated single cells grown in MG medium were used as template for real-time RT-PCR, which was performed as previously described (Son et al. 2007). Real-time PCR was conducted in eight replicates for each target. To control for variations between runs, all PCRs were performed at the same time in one 96-well plate.

We followed the previously reported data analysis method (Peirson et al. 2003) to provide more accurate quantitative real-time PCR data. Real-time RT-PCR data were averaged over eight replicates for each target, and fold-changes were calculated using DART-PCR (Peirson et al. 2003). Accordingly, the average efficiencies of each target are within 4.1% differences (<5%) and permit accurate analysis. The expression level of mRNA BTH_I2028 was taken as 1 for each amplified sample tested, and the amounts of rRNA and rRNAs were normalized relative to this value.

cDNA library construction for sequencing

For construction of the single-cell cDNA library, aliquots of the same preparation of amplified ds-cDNA samples for real-time RT-PCR above were used. Amplified ds-DNA (10 μg) from unenriched or enriched (1 × 10^{-8} U exonuclease) samples were randomly fragmented with DNase I, blunt-ended with T4 DNA polymerase and 1 mM dNTPs, and ran on a 1% agarose gel. Fragments in two size ranges (0.1–1.5 kb and 1.5–4.0 kb) were extracted from the agarose gel and cloned into the Smal site of pUC19 vector for library construction (Supplemental Fig. S3). Sanger sequencing reads of the inserts were generated at a local sequencing facility and identified using a homology search with the BLASTn algorithm (http://www.ncbi.nlm.nih.gov).

Competing interest statement

A provisional patent application (no. 61383699, submitted September 16, 2010) has been filed for the single bacterium TTA method described in this paper. T.T.H., Y.K., and M.H.N. are named as the inventors on this patent.

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Authors’ contributions: Y.K. performed the single-cell TTA, microarray analysis, real-time RT-PCR study, and cDNA library construction. M.H.N. performed the fluorescence microcopy experiments and contributed to data analysis. J.Z-S. contributed to data analysis. W.C.N. generated the B. thailandensis microarray slides, provided technical advice and performed critical reading of the manuscript. S.P.D. secured funding for the Carl Zeiss LSM system used in this study, assisted with the single cell isolation training, and critical editing of this manuscript. T.T.H. conceived of the method and supervised the experiments. T.T.H. and Y.K. wrote the manuscript.

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Son MS, Matthews WJJ, Kang Y, Nguyen DT, Hoang TT. 2007. In vivo critical editing of this manuscript. T.T.H. conceived of the method and supervised the experiments. T.T.H. and Y.K. wrote the manuscript.

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Knockout and pullout recombineering for naturally transformable *Burkholderia thailandensis* and *Burkholderia pseudomallei*

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Phage λ-Red proteins are powerful tools for pulling and knocking out chromosomal fragments but have been limited to the γ-proteobacteria. Procedures are described here to easily knock out (KO) and pull out (PO) chromosomal DNA fragments from naturally transformable *Burkholderia thailandensis* and *Burkholderia pseudomallei*. This system takes advantage of published γ-proteobacteriophages (e.g., λ-Red–mediated recombination of a suicidal plasmid at recA loci), natural transformation, screening for KOs with primers annealing to chromosomal homologous regions (800–1,000 bp) and overlapping PCR can be difficult for larger DNA fragments with high G + C content) is not required, thus reducing the number of oligos used; (vi) recombination aided by λ-Red proteins effectively reduces the length of homologous regions required (40–45 bp), whereas no recombination occurs without the induction of λ-Red proteins (i.e., DNA incubation alone); (vii) possible secondary chromosomal mutation(s) in the 800–1,000 bp of homology resulting from overlap-extension PCR are avoided in λ-Red recombineering, which requires only 40–45 bp of homology.

There are other advantages specific to λ-Red–mediated in vivo cloning (Fig. 2). PCR amplification of the insert, which can generate mutations in the fragment to be cloned, is not required. In addition, large PCR products can be difficult to amplify, especially from the high-G + C DNA of *Burkholderia* species. Using this protocol to PO large chromosomal fragments from *Burkholderia* spp. is much faster than, for example, recA-mediated recombination of a suicidal plasmid at

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**INTRODUCTION**

The ability to manipulate the bacterial chromosome for molecular genetics, pathogenesis and bacteria-host interaction studies is crucial for the discovery of novel vaccine, therapeutic and diagnostic targets. The Gam, Exo and Beta proteins of coliphage λ aid in the RecA-independent homologous recombination process to pull out (PO) or knock out (KO) regions from bacterial chromosomes. These λ-Red proteins facilitate high-frequency recombination between the chromosome and small homologous sequences (approximately 40–45 bp) flanking a selectable marker. However, PO and KO manipulation of the bacterial genome using the λ-Red system has been limited to the γ-proteobacteria class⁶. Therefore, protocols to expand the use of λ-Red recombineering beyond the γ-proteobacteria are needed, particularly for two closely related naturally transformable β-proteobacterial species: *Burkholderia thailandensis* and *Burkholderia pseudomallei*.

*B. thailandensis* is a relatively nonpathogenic bacterium often used as a model microbe to study various aspects of the potential bioterrorism agent *B. pseudomallei*. *B. pseudomallei* is the etiological agent of melioidosis, a globally emerging and often fatal infectious disease⁷. Work by Thongdee et al.⁷ has demonstrated the KO of chromosomal fragments in both naturally transformable *Burkholderia* species, using PCR fragments generated by three-fragment overlap-extension PCR. The requirement for large homologous regions (800–1,000 bp) and overlapping PCR can be hampered by the size and G + C content of the target DNA. In addition, screening for KOs with primers annealing to chromosomal regions outside the 800–1,000 bp of homology can be difficult because of increased ampiclon size. Because of these limitations and the lack of PO protocols, we developed protocols to extend the λ-Red recombineering potential in these two species. Here we present protocols for λ-Red recombineering to capture or delete, for example, large chromosomal DNA fragments from two β-proteobacteria species: *B. thailandensis* and *B. pseudomallei*.
Figure 1 | Genetic constructs for KO and PO recombineering in *B. thailandensis* and *B. pseudomallei*. (a) Plasmid pKaKa1 contains \(\lambda\)-Red genes (*gam*-bet-exo)\(^{11}\) driven by the arabinose-inducible promoter (**P**\(_{\text{araB}}\)). (b) Plasmid pKaKa2 is maintained in ara- *E. coli* and *B. pseudomallei* strains by the *B. thailandensis* arabinose-utilization operon and growth on arabinose minimal medium, in which \(\lambda\)-Red genes are driven by the rhamnose-inducible promoter. (c) Broad-host-range (bhr) replicating plasmid used for PO recombineering (in vivo cloning). Target-PO1 and Target-PO2 indicate primers (blue arrows) with 40–45 bp homology to the targeted chromosomal region, used to obtain PCR products (oriT-ColE1-ori-gat-ori1600-rep) to PO targeted sequences. (d) The pheS-gat and sacB-gat FRT cassettes used for KO recombineering experiments. Target-KO1 and Target-KO2 indicate primers (blue arrows), with 40–45 bp homology to chromosomal regions, used to amplify this cassette in KO experiments. Abbreviations: **araC**, of pKaKa1, activator of the arabinose-inducible promoter (P\(_{\text{araB}}\)) from *E. coli*; **araBCDEFGHI** of pKaKa2, *B. thailandensis* arabinose-utilization operon\(^{12}\); **bla**, encodes \(\beta\)-lactamase; **ColE1-ori**, **ColE1** origin of replication obtained from high-copy-number pUC vectors\(^{12}\); **FRT**, yeast 2 \(\mu\)m plasmid Flp recombination target; **gat**, glyophosphate (GS) acetyltransferase for GS resistance\(^{13}\); **Km**, kanamycin resistance, encoded by nptII\(^{12}\); **lacZ**, encodes Lacz \(\alpha\)-peptide; **mob-oriT**, RP4-dependent conjugal origin of transfer of *B. bronchiseptica* cryptic plasmid pBBR1 (ref. 16); **oriT-rep**, Pseudomonas and *Burkholderia* bhr origin of replication\(^{12}\); **ori-**-rep, bhr replicon of *B. bronchiseptica* pBBR1 plasmid\(^{16}\); **oriT**, plasmid RP4 origin of transfer for conjugation; **P**\(_{\text{araH}}\), arabinose-inducible promoter; **P**\(_{\text{gal}}\), *E. coli* lactose operon promoter; **P**\(_{\text{intact}}\), rhamnose-inducible promoter; **P**\(_{\text{araD}}\) and **P**\(_{\text{araE}}\), constitutive promoters of *B. pseudomallei* and *B. cenocepacia* rpsL genes\(^{12}\); **pheS**, engineered gene encoding a mutant version of the \(\alpha\)-subunit of phenylalanyl tRNA synthase\(^{12}\); **rhaR** and **rhaS**, regulators of the rhamnose-inducible promoter\(^{12}\); **sacB**, encoding for a modified levanasucrase counterselectable marker\(^{14}\).

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**Figure 1** Genetic constructs for KO and PO recombineering in *B. thailandensis* and *B. pseudomallei*. (a) Plasmid pKaKa1 contains \(\lambda\)-Red genes (*gam*-bet-exo)\(^{11}\) driven by the arabinose-inducible promoter (**P**\(_{\text{araB}}\)). (b) Plasmid pKaKa2 is maintained in ara- *E. coli* and *B. pseudomallei* strains by the *B. thailandensis* arabinose-utilization operon and growth on arabinose minimal medium, in which \(\lambda\)-Red genes are driven by the rhamnose-inducible promoter. (c) Broad-host-range (bhr) replicating plasmid used for PO recombineering (in vivo cloning). Target-PO1 and Target-PO2 indicate primers (blue arrows) with 40–45 bp homology to the targeted chromosomal region, used to obtain PCR products (oriT-ColE1-ori-gat-ori1600-rep) to PO targeted sequences. (d) The pheS-gat and sacB-gat FRT cassettes used for KO recombineering experiments. Target-KO1 and Target-KO2 indicate primers (blue arrows), with 40–45 bp homology to chromosomal regions, used to amplify this cassette in KO experiments. Abbreviations: **araC**, of pKaKa1, activator of the arabinose-inducible promoter (P\(_{\text{araB}}\)) from *E. coli*; **araBCDEFGHI** of pKaKa2, *B. thailandensis* arabinose-utilization operon\(^{12}\); **bla**, encodes \(\beta\)-lactamase; **ColE1-ori**, **ColE1** origin of replication obtained from high-copy-number pUC vectors\(^{12}\); **FRT**, yeast 2 \(\mu\)m plasmid Flp recombination target; **gat**, glyophosphate (GS) acetyltransferase for GS resistance\(^{13}\); **Km**, kanamycin resistance, encoded by nptII\(^{12}\); **lacZ**, encodes Lacz \(\alpha\)-peptide; **mob-oriT**, RP4-dependent conjugal origin of transfer of *B. bronchiseptica* cryptic plasmid pBBR1 (ref. 16); **oriT-rep**, Pseudomonas and *Burkholderia* bhr origin of replication\(^{12}\); **ori-**-rep, bhr replicon of *B. bronchiseptica* pBBR1 plasmid\(^{16}\); **oriT**, plasmid RP4 origin of transfer for conjugation; **P**\(_{\text{araH}}\), arabinose-inducible promoter; **P**\(_{\text{gal}}\), *E. coli* lactose operon promoter; **P**\(_{\text{intact}}\), rhamnose-inducible promoter; **P**\(_{\text{araD}}\) and **P**\(_{\text{araE}}\), constitutive promoters of *B. pseudomallei* and *B. cenocepacia* rpsL genes\(^{12}\); **pheS**, engineered gene encoding a mutant version of the \(\alpha\)-subunit of phenylalanyl tRNA synthase\(^{12}\); **rhaR** and **rhaS**, regulators of the rhamnose-inducible promoter\(^{12}\); **sacB**, encoding for a modified levanasucrase counterselectable marker\(^{14}\).

Limitations of the present protocol

The limitations of the current protocol are that (i) there is a requirement for minimal medium (typically 1× M9 glucose (MG)) supplemented with glyophosphate (GS) or chlorinated phenylalanine (cPhe) during gat selection or pheS counterselection; (ii) when introduced into *E. coli* at high copy number, certain PO gene products may be toxic to the cell, potentially limiting the nature of downstream applications; and (iii) this protocol has been performed successfully in only the naturally transformable strains of *B. thailandensis* and *B. pseudomallei* (~50% of the strains tested in this study). We have not been able to extend its use to the non-naturally transformable strains of *B. thailandensis* and *B. pseudomallei* (K96243 and *Burkholderia mallei* strain ATCC23344) because of potentially inefficient electrotransformation and/or restriction barriers against double-stranded DNA. Various published electroproporation techniques\(^{11-13}\) were tested in non-naturally transformable strains (e.g., *B. pseudomallei* K96243 and *B. mallei* ATCC23344), but no colonies or recombinants were obtained. In these non-naturally transformable species and strains, mutation could be created with other established mutagenesis procedures as previously described\(^{8,12,14}\). However, \(\lambda\)-Red recombineering has a very broad host range (bhr), and we strongly believe that these

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<th>Accession numberb</th>
<th>Relevant features and use</th>
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<td>GS; Bp6340 with pheS-gat-FRT fragment replacing the asd gene</td>
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(continued)
tools will work in many other natural transformable strains. For example, we have extended the use of this technology successfully in nine other naturally transformable *B. pseudomallei* environmental and clinical strains (Table 1).

### Experimental design

**Genetic constructs for recombineering.** This protocol takes advantage of our recently published *pheS* counterselectable and *gat*-selectable markers, along with the arabinose-utilization operon (*araBCDEFGHI*) and the more established *sacB* counterselectable marker. Two new *bhr* replicating plasmids containing λ-Red genes (gam, bet and exo) that are inducible with arabinose and rhamnose, pKa1 and pKa2, respectively, were engineered for this protocol (Fig. 1a,b). Pka1 is typically used for *B. thailandensis*, using the kanamycin-resistance selectable marker and the *pheS* gene as a counterselectable marker in the presence of cPhe, as described previously. As kanamycin resistance has limited use in wild-type *B. pseudomallei* strains, the alternative *B. thailandensis* arabinose-utilization operon (*araBCDEFGHI*) was used as a metabolic marker for selection of pKa2 in *B. pseudomallei*, whereas the *sacB* gene was chosen for counterselection, using sucrose. The pKa1 and pKa2 plasmids contain the *Bordetella bronchiseptica* bhr origin for replication in Gram-negative bacteria. These plasmids can be electrotransformed into *B. thailandensis* and *B. pseudomallei* or conjugated from RP4-harbouring *E. coli* (e.g., E1299, Table 1), in which they can eventually be cured using cPhe or sucrose after recombineering. The λ-Red proteins Gam, Redβ and Redα (encoded by gam, bet and exo) are

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**Table 1** Continued.

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<td>EU626138</td>
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</table>

<sup>a</sup>Lab ID when requesting *E. coli* strains and plasmids. <sup>b</sup>GenBank accession number. <sup>c</sup>Details on the construction of these strains and plasmids will be published elsewhere. <sup>d</sup>Acquisition, possession and manipulation of these strains in the United States are limited to FBI-screened and cleared personnel, and experiments must be performed in a CDC/USDA-approved and registered BSL-3 select agent laboratory.

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**Figure 2** Overview of an example of PO recombineering in *B. pseudomallei*. The 31.5-kb *mba* cluster was pulled out of the *B. pseudomallei* chromosome, using a PCR-amplified fragment of the pAM3G backbone (Fig. 1c), with correctly oriented 40- to 45-bp homologous sequences toward the *mbaf* and *mbaS* genes. Positive clones can be immediately obtained in one step by glyphosate-resistance selection. The pAM3G-*mba* bhr replicating plasmid could be screened (with the primers indicated by arrows) and isolated from glyphosate-resistant *B. pseudomallei* colonies and transformed into *E. coli* for further characterization. Red crosses indicate λ-Red-mediated homologous recombination.
Based on optimized expression of Gam and mutated versions of Redβ and Redα17. Upon induction of the λ-Red proteins, linear PCR products are naturally transformed into \textit{B. thailandensis} or \textit{B. pseudomallei} to generate POs or KOs. The relevant strains, plasmids and oligonucleotides used in this protocol are shown in Tables 1 and 2.

**In vivo PO strategy.** The \textit{in vivo} cloning or PO protocol involves the amplification of a PCR product from the engineered plasmid pAM3G, containing a GS-resistance selectable marker\(^9\), a bhr origin of replication and an origin of transfer for conjugation (Figs. 1c and 2). The PCR-amplified fragment (~2.8 kb), flanked by sequences homologous to the chromosomal PO region, is incubated with cells expressing the \(\lambda\)-Red proteins. As examples of the PO protocol, we will individually describe the \textit{in vivo} cloning of the \textit{B. thailandensis} arabinose-utilization operon (\textit{araABCDEFGHI}, 11.6 kb)\(^10\) and the \textit{B. pseudomallei} siderophore malleobactin biosynthetic cluster (\textit{mba}-cluster, 31.5 kb)\(^18\) into the

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<tr>
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<td>5’-GCCTGCGCGCGACGAGCGGCGCGCTCGTATGGTGGGAAATTGTTG-3’</td>
</tr>
<tr>
<td>#1486; asd-K03</td>
<td>5’-GCCTGCGCGCGACGAGCGGCGCGCTCGTATGGTGGGAAATTGTTG-3’</td>
</tr>
<tr>
<td>#1487; asd-K04</td>
<td>5’-GCCTGCGCGCGACGAGCGGCGCGCTCGTATGGTGGGAAATTGTTG-3’</td>
</tr>
<tr>
<td>#1488; asd-up</td>
<td>5’-ATCTGATCGAGCGGTG-3’</td>
</tr>
<tr>
<td>#1489; asd-down</td>
<td>5’-GTAAATGCCGACGAGGT-3’</td>
</tr>
</tbody>
</table>

*Underlined are homologous sequences to targets on the chromosome. Boldface sequences in the long oligos are identical to the shorter oligos used in the second PCR (Step 5 of the protocol). In italics are reverse complementary sequences of mbaF-K01 and mbaS-K05, where these primers are used to generate ~100 bp PCR product to create the unmarked mutation (Step 13A).
pAM3G replicating plasmid backbone. The PO strategy for the mba cluster is depicted in Figure 2. Positive PO frequencies of GS-resistant colonies with araABCDEFGHI clones ranged from 88 to 93% in B. thailandensis (Table 3), whereas cloning frequencies for the mba cluster ranged from 90 to 100% in B. pseudomallei (Table 4).

**Overview of KO strategy.** The KO procedure uses a PCR-amplified pheS-gat FRT cassette flanked by 40–45 bp of sequence homologous to the targeted chromosomal regions (Fig. 1d). Upon introduction of the pKaKa2 plasmid and induction of the expression of λ-Red proteins, KOs can be achieved in one step by transformation of the PCR-amplified pheS-gat cassette and selection on GS. The helper plasmid pKaKa2 could be cured by counterselection with sacB on sucrose-containing medium (Fig. 1b). Figure 3 depicts an example of chromosomal KO using pKaKa2 in B. pseudomallei at high frequencies (88–92%, Table 4). We foresee that for those laboratories approved to use kanamycin-resistance selection in B. pseudomallei, pKaKa2 could be used to generate KO in B. pseudomallei (Fig. 1a,d). In this case, pKaKa1 could be cured by counterselection with pheS on cPhe-containing medium.

**Recycling of useful markers.** There are very few selectable or counterselectable markers for genetic manipulation in most bacteria, and the ability to reuse these precious markers in the same strain is essential. This protocol describes two strategies for recycling the pheS-gat or sacB-gat FRT cassettes (Fig. 3). A second round of λ-Red protein induction followed by incubation of cells with a short PCR fragment flanked by 40–45 bp homologous to the targeted region on the chromosome (Fig. 3) will generate an unmarked KO mutant in B. thailandensis or B. pseudomallei. This strategy can be achieved quickly because of the presence of pKaKa1 or pKaKa2 from the previous KO procedure. In an alternative strategy that does not require λ-Red proteins, the unmarked KO mutants can be generated by incubating cells with a PCR fragment containing flp (Fig. 3).

**Overview of the protocol.** This PO/KO protocol is summarized in Figure 4. Steps 1–12 demonstrate the generation of PO/KO in B. pseudomallei and B. thailandensis (left section). Following KO with the pheS/sacB-gat FRT cassette, unmarked mutations can be created with overlapping oligos (middle; Step 13A) or Flp-mediated excision (right; Step 13B). When desired, the helper plasmid can be cured by pheS/sacB counterselection (Step 14). The step-by-step protocol is described below.

---

### Table 3 | Pullout recombineering efficiencies of the ara operon in B. thailandensis.

<table>
<thead>
<tr>
<th>DNA amount</th>
<th>First PCR product%</th>
<th>Second PCR product%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 µg</td>
<td>41 ± 9 (92)</td>
<td>245 ± 40 (89)</td>
</tr>
<tr>
<td>0.5 µg</td>
<td>3 ± 1 (92)</td>
<td>19 ± 3 (90)</td>
</tr>
<tr>
<td>0.1 µg</td>
<td>1 ± 1 (100)</td>
<td>4 ± 0.3 (93)</td>
</tr>
</tbody>
</table>

Each experiment was performed in triplicate, and average number of GS-resistant colonies is shown with standard error of the mean. On average, 12 GS-resistant colonies were PCR screened for positive pullout and the percentage is shown in parentheses.

---

### Table 4 | Pullout/knockout recombineering efficiencies of the mba cluster in B. pseudomallei.

<table>
<thead>
<tr>
<th>DNA amount</th>
<th>Pullout %</th>
<th>Knockout %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninduced</td>
<td>Induced</td>
</tr>
<tr>
<td>2 µg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 µg</td>
<td>0</td>
<td>55 ± 11 (90)</td>
</tr>
<tr>
<td>0.1 µg</td>
<td>ND</td>
<td>19 ± 5 (100)</td>
</tr>
</tbody>
</table>

Each experiment was performed in triplicate, and average number of GS-resistant colonies is shown with standard error of the mean. On average, 12 GS-resistant colonies were PCR screened for positive PO/KO and the percentage is shown in parentheses. ND, not determined.
**Figure 4** | λ-Red recombineering scheme for PO/KO in *B. thailandensis* and *B. pseudomallei*. Strategy for PO/KO recombineering is shown on the left (blue); the middle (purple) and right (green) sections present the strategies for creating unmarked mutants by λ-Red recombination or Flp-mediated excision, respectively. Detailed descriptions for each step are provided in the procedure. Numbers in red correspond to the steps in the protocol.
**MATERIALS**

**REAGENTS**

- Amino acids: 2.6-diaminopimelic acid (DAP; 100 mg ml⁻¹ in 1 M NaOH, Acros Organics, cat. no. 235540010); l-lysine (Lys; 1 M; filter sterilized, Acros Organics, cat. no. 125121001); l-threonine (Thr; 1 M; filter sterilized, Acros Organics, cat. no. 303540050); l-methionine (Met; 1 M; filter sterilized, Acros Organics, cat. no. 166160250); dl-threonine (Thr; 1 M; filter sterilized, Acros Organics, cat. no. 138930050) and (S)-tryptophan (Trypt; 1 M; filter sterilized, Acros Organics, cat. no. 140591000).

- Bacterial strains

  - *Burkholderia strains* (Table 1)
  - *Burkholderia thailandensis* E264 (available from BEI Resources)
  - *Burkholderia pseudomallei* 1026b, Bp0085, Bp0091, Bp0094, Bp4001, Bp4003, Bp4122, Bp4141, Bp4144 and Bp6340! CAUTION Acquisition, possession and manipulation of *B. pseudomallei* strains in the United States are limited to FBI-screened and -cleared personnel, and experiments must be performed in CDC- and US Department of Agriculture (USDA)-approved and registered BSL-3 select agent facilities.

  - E. coli strains (Table 1; available from T.T.H.)

- Plasmids (Table 1; available from T.T.H.)

  - pKaKα1 or pKaKα2, replicating plasmids containing inducible λ-Red genes (Fig. 1)

- pAM3G, bhr plasmid encoding gat (GS acetyltransferase), source of PCR fragment for PO recombinering

- pwFRT-PC, the-gat or pwFRT-PC-sucB-gat plasmid DNA used as template for KO recombinering

- pCD135K-flp-orF-rasid, plasmid DNA used as a template to amplify flp gene-containing fragment

- Oligonucleotides (see Table 2; Integrated DNA Technologies)

- Target-PO1 (5'-GATCCCTTAAATGGATCCTTATATGCGTCTATTTATG-3'); N₁₆ refers to 40 bases of sequence homologous to the target gene. Underlined bases can be common to the target and also anneal to the PCR template to increase efficiency and reduce costs.

- Target-PO2 (5'-GATCCCTTAAATGGATCCTTATATGCGTCTATTTATG-3'); use with oligo orF-rep-internal to confirm PO by PCR

- Target-KO1 (5'-GCGAGATTTGACAGATGCTGAG-3'); use with oligo Target-rev to confirm PO by PCR

- Target-KO2 (5'-CAGCTCTTAAATGGATCCTTATATGCGTCTATTTATG-3'); use with oligo Target-rev to confirm PO by PCR

- Target-KO3 (N₁₆-identical to the 5'-end of Target-KO1)

- Target-KO4 (N₁₆-identical to the 5'-end of Target-KO2)

- Target-KO5 (5'-TACCACCAAATTAACGCTTGTGCTA-3'); this oligo has N₁₆ as Target-KO2)

- Target-up-out (N₁₆-anneals outside of the homologous region; use with Target-down-out to confirm KO by PCR)

- Target-down-out (N₁₆-anneals outside of the homologous region; use with Target-up-out to confirm KO by PCR)

- Plac-up (5'-GGCAATACGCGGAAAGCGCGTCTC-3')

- Plac-down (5'-TAAATGATCTTATAGCTGCTATTTATG-3')

- DNA ladder (100 bp; range 100–1,500 bp, New England Biolabs, cat. no. N3231L)

- DNA ladder (1 kb; range 0.5–10 kb, New England Biolabs, cat. no. N3231L)

- dNTPs (2 mM; New England Biolabs, cat. no. N0447L)

- dNTPs (200–400 mM; Sigma-Aldrich, cat. no. 600153)

- DNA ladder (100 bp; range 100–1,500 bp, New England Biolabs, cat. no. N3231L)

- dNTPs (2 mM; New England Biolabs, cat. no. N0447L)

- l-threonine (Thr; 1 M; filter sterilized, Acros Organics, cat. no. A20065-100.0)

- l-methionine (Met; 1 M; filter sterilized, Acros Organics, cat. no. A20065-100.0)

- l-lysine (Lys; 1 M; filter sterilized, Acros Organics, cat. no. 125121001)

- M9 minimal salts solution, 20 ml of 2 M arabinose, 500 ml of 1 M glucose or 20 ml of 1 M MgSO₄ and 25 ml of CaCl₂. The liquid media can be prepared and stored at 4 °C. The sugar and salt solutions can be stored at room temperature for several months. Use LB agar and LB broth Lennox (Teknova, cat. nos. L9330 and L9310) as solid medium preparation

- l-lysine (l-lys-gat; Sigma-Aldrich, cat. no. K4378)

- Glycine: super-concentrated RoundUp (50% (vol/vol) GS from Home Depot or other farm or garden supply stores)

- Kanamycin sulfate salt (Sigma-Aldrich, cat. no. K4378)

- Liquid medium preparation

  - Use LB broth Lennox to prepare rich media. To prepare 1 liter of MG medium, add 200 ml 5× M9 minimal salts solution, 20 ml of 1 M glucose, 500 μl of 1 M MgSO₄, 25 μl of CaCl₂ and 780 ml sterile DDW. Final concentrations are listed as follows: 1× M9 minimal salts, 20 mM glucose, 500 μM MgSO₄ and 25 μM CaCl₂. A volume of 1 liter of M9 arabinose (MA) medium is prepared by adding 200 ml 5× M9 minimal salts solution, 20 ml of 2 M arabinose, 500 μl of 1 M MgSO₄, 25 μl of CaCl₂ and 780 ml sterile DDW. Final concentrations are listed as follows: 1× M9 minimal salts, 40 mM arabinose, 500 μM MgSO₄ and 25 μM CaCl₂. The liquid media can be prepared and stored at room temperature for several months.

- Solid medium preparation

  - All solid media could be prepared fresh or stored at 4 °C for several months. Use LB or LS agar to prepare rich plate media. One liter of MG or MA with agar is prepared by first mixing 15 g agar in 780 ml DDW and sterilizing. After autoclaving, add 200 ml 5× M9 minimal salts solution, 20 ml of 1 M glucose or 20 ml of 2 M arabinose. Let it cool to ~50 °C before adding 500 μl of 1 M MgSO₄ and 25 μl of CaCl₂.
The final concentrations are 1.5% (wt/vol) agar, 1× M9 minimal salts, 20 mM glucose or 40 mM arabinose, 500 μM MgSO4, and 25 μM CaCl2. Approximately 50 plates can be prepared from 1 liter of medium. When growing B. pseudomallei asd-specific mutant in minimal medium (Table 1), Met, Lys and Thr are added at a final concentration of 1 mM, and DAP was added at 200 μg ml⁻¹. Sucrose is added at a concentration of 15% (wt/vol). cPhe is added at a concentration of 0.1% (wt/vol), as previously described.

PROCEDURE

Preparing PO/KO DNA fragment ● TIMING 1 d

1. Design oligonucleotide primers such that the last 45 bases on the 5’ end are homologous to two corresponding regions on the chromosome for the PO/KO method, and order oligos prior to initiating the protocol. When designing the pair of PO or KO oligos, avoid having five consecutive matching base pairs between them within their 5’ ends to prevent intrafragment recombination. All oligos do not require PAGE purification; however, the majority of the long oligos (60–65 bp) produced will be truncated or will have incorrect 5’ ends as the coupling or synthesis efficiency is not 100% (coupling efficiencies are available from oligo manufacturer), resulting in reduced recombineering efficiency (e.g., ~29% of the 60-bp oligos have truncated or incorrect ends when the coupling efficiency is 99%, or up to 95% will have truncated or incorrect ends if coupling efficiency is as low as 95%). To remedy this problem and increase recombineering efficiency at reduced cost, a two-step PCR approach is described below. The first PCR product is obtained using the long oligos, which is used as the template for subsequent amplification by shorter oligos (16–20 bp) annealing to the 5’ ends of the first PCR product. The shorter oligos required for the second PCR should have sequences identical to the 5’ end of the long oligos from the first PCR, which will thus repair them and yield more accurate 5’ ends (Fig. 1 and Table 2).

2. Set up one PCR reaction using the long oligos and the following components in a thin-walled PCR tube.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (μl)</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH2O</td>
<td>35.0</td>
<td>—</td>
</tr>
<tr>
<td>Pfu buffer (10×)</td>
<td>5.0</td>
<td>1×</td>
</tr>
<tr>
<td>dNTPs (2 mM)</td>
<td>5.0</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>pAM3G or pwFRT-pheS/sacB-gat DNA (~20 ng μl⁻¹)</td>
<td>1.0</td>
<td>~20 ng</td>
</tr>
<tr>
<td>Target-PO1 or Target-KO1 (30 μM)</td>
<td>1.0</td>
<td>30 pmol</td>
</tr>
<tr>
<td>Target-PO2 or Target-KO2 (30 μM)</td>
<td>1.0</td>
<td>30 pmol</td>
</tr>
<tr>
<td>Pfu polymerase (2.5 U μl⁻¹)</td>
<td>2.0</td>
<td>5 U</td>
</tr>
</tbody>
</table>

△ CRITICAL STEP It is essential to use Pfu polymerase or other polymerases that generate blunt ends in order to avoid the addition of unwanted bases (e.g., A at the 3’ end with Taq). The proofreading capability of Pfu polymerase will also significantly increase the fidelity of the PCR products.

3. Carry out the PCR as described below. Set the lid temperature to 98 °C to prevent condensation of the sample during the reaction.

<table>
<thead>
<tr>
<th>Cycle number</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Termination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94 °C, 2 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2–34</td>
<td>94 °C, 30 s</td>
<td>58 °C, 30 s</td>
<td>72 °C, 3 min (pAM3G) or 2 min (pwFRT-pheS/sacB-gat)</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td></td>
<td></td>
<td>72 °C, 5 min</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td></td>
<td></td>
<td></td>
<td>4 °C, hold</td>
</tr>
</tbody>
</table>

■ PAUSE POINT PCR samples can be retained in the PCR machine or stored at 4 °C for a short period of time. For extended storage, it is recommended that the reaction be placed in a −20 °C freezer.

4. Visualize all PCR reactions by agarose gel electrophoresis using a 1.0% agarose gel submerged in TAE buffer (see REAGENT SETUP). Run alongside DNA ladder (e.g., 1 kb DNA ladder) for estimating PCR product size. If oligos are
designed as recommended, PCR products of ~2.7 kb (oriT-ColE1ori-gat-ori1600-rep from pAM3G template) or ~1.8 or 2.3 kb (pheS/sacB-gat from pwFRT-pheS/sacB-gat template) can be expected. Purify DNA from the gel with desired kit or protocol and quantify using a spectrophotometer at 260 nm. This first PCR product could be used directly for recombineering in *B. thailandensis*, with lower frequencies compared with the second PCR product (Table 3); however, the first PCR product should not be used directly for *B. pseudomallei* because of the lower frequencies obtained (Table 4).

PAUSE POINT If not used for transformation immediately, the DNA can be stored at 4 °C for a short period of time. For extended storage, it is recommended that the reaction be placed in a −20 °C freezer.

5] Set up the second PCR using the product from Step 4 as template. Typically, set up four PCR reactions to obtain sufficient amounts of DNA, using the shorter oligos as below. Multiply all of the following components by four and combine in a master mix. Pipette 50 μl of the master mix into four thin-walled PCR tubes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (μl)</th>
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</thead>
<tbody>
<tr>
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<td>1×</td>
</tr>
<tr>
<td>dNTPs (2 mM)</td>
<td>5.0</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>oriT-ColE1ori-gat-ori1600-rep or pheS/sacB-gat DNA (~20 ng μl⁻¹)</td>
<td>1.0</td>
<td>~20 ng</td>
</tr>
<tr>
<td>Target-PO3 or Target-KO3 (30 μM)</td>
<td>1.0</td>
<td>30 pmol</td>
</tr>
<tr>
<td>Target-PO4 or Target-KO4 (30 μM)</td>
<td>1.0</td>
<td>30 pmol</td>
</tr>
<tr>
<td><em>Pfu</em> polymerase (2.5 U μl⁻¹)</td>
<td>2.0</td>
<td>5 U</td>
</tr>
</tbody>
</table>

▲ CRITICAL STEP It is essential to use *Pfu* polymerase or other polymerases that generate blunt ends in order to avoid the addition of unwanted bases. The proofreading capability of *Pfu* polymerase will significantly increase the fidelity of the PCR products.

6] Carry out the PCR as described below. Set the lid temperature to 98 °C to prevent condensation of the sample during the reaction.

<table>
<thead>
<tr>
<th>Cycle number</th>
<th>Denaturation</th>
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<td>2–34</td>
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<td>58 °C, 30 s</td>
<td>72 °C, 3 min (pAM3G) or 2 min (pwFRT-pheS/sacB-gat)</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td></td>
<td></td>
<td>72 °C, 5 min</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td></td>
<td></td>
<td>4 °C, hold</td>
<td></td>
</tr>
</tbody>
</table>

PAUSE POINT PCR samples can be retained in the PCR machine or stored at 4 °C for a short period of time. For extended storage, it is recommended that the reaction be placed in a −20 °C freezer.

7] Visualize all PCR reactions by agarose gel electrophoresis, using a 1.0% agarose gel submerged in TAE buffer (see REAGENT SETUP). Run alongside DNA ladder (e.g., 1-kb DNA ladder) to estimate PCR product size. If oligonucleotides are designed as recommended, PCR products of ~2.7 kb (oriT-ColE1ori-gat-ori1600-rep fragment) or ~1.8 or 2.3 kb (pheS/sacB-gat FRT cassette) can be expected. Purify DNA from all bands produced with desired kit or protocol and quantify using a spectrophotometer at 260 nm.

Alternative strategy: If cost is not an issue, PAGE-purified long oligos could be ordered and the first PCR product could be used directly for recombineering without the need for a second PCR. The PCR with PAGE-purified long oligos should be set up exactly as the first PCR.

PAUSE POINT If not used for transformation immediately, the DNA can be stored at 4 °C for a short period of time. For extended storage, it is recommended that the reaction be placed in a −20 °C freezer.

? TROUBLESHOOTING
Introduction of pKaKa1 or pKaKa2 into *Burkholderia* species

8] The plasmids encoding λ-Red proteins are pKaKa1 (for *B. pseudomallei* and *B. thailandensis*) and pKaKa2 (for *B. pseudomallei*). One of these plasmids should be introduced into bacteria prior to PO/KO experiments, by electroporation or conjugation. Option A describes delivery of pKaKa1 by electroporation. pKaKa1 contains the kanamycin-resistance marker and selection is performed on LB + 500 μg ml⁻¹ Km for *B. thailandensis* or on LB + 1,000 μg ml⁻¹ Km for *B. pseudomallei*₂¹. Delivery of pKaKa2 by electroporation or conjugation is described in options B and C, respectively. pKaKa2 confers arabinose-utilization ability to *B. pseudomallei* and is selected for on MA medium. The conjugation method (option C) is particularly useful for delivering plasmid into the select agent *B. pseudomallei*, as it reduces the risk of generating aerosols during electroporation. Although we only describe the mating protocol for pKaKa2 into *B. pseudomallei*, conjugation of pKaKa1 into *B. pseudomallei* could be done similarly. For KO in *B. pseudomallei*, the pKaKa1 helper plasmid should be used in conjunction with the sacB-gat fragment, whereas pKaKa2 should be used with pheS-gat fragment.

**(A) Electroporation of pKaKa1 into *B. thailandensis* or *B. pseudomallei* ● TIMING 4 d

(i) Grow 5 ml of *B. thailandensis* or *B. pseudomallei* in LB broth from a single colony overnight at 37 °C in a shaking incubator set to 225 r.p.m. (This is the end of day 1.)

(ii) After the culture has reached a sufficient density (OD₆₀₀ 0.8–1.5), spin down all 5 ml in a minicentrifuge at 10,000 g for 1 min. Wash the cell pellet with 1 ml of cold, sterile ddH₂O. Repeat this four more times, adhering to the same centrifugation conditions.

! CAUTION Pipette and wash gently to minimize aerosolization of *B. pseudomallei* culture. Filtered pipette tips should be used for *B. pseudomallei* to avoid contamination of the pipette. Instead of using cold ddH₂O, 300 mM sucrose maintained at room temperature may be substituted when washing cells as previously described.₁⁵ Comparable electroporation efficiencies can be obtained by using either method.

(iii) Remove all water from the washed pellet and resuspend the cell pellet with 40 μl of cold, sterile ddH₂O. Add 1 μl of purified pKaKa1 plasmid DNA (0.2–0.5 μg) to the competent cells and transfer the mixture into an electroporation cuvette. Place the electroporation cuvette in the electroporator shock chamber and apply a shock of 2.5 kV, 25 μF and 200 Ω. Ensure an exponential decay of the applied shock and the absence of an arc.

? TROUBLESHOOTING

(iv) Immediately add 1 ml of rich broth medium (e.g., LB broth) to the cuvette and transfer to a round-bottom culture tube. Incubate the culture for 1 h at 37 °C with shaking at 225 r.p.m. in order to allow for kanamycin-resistance gene expression (Fig. 1a).

(v) Pipette 100 μl of the recovery mixture onto an LB + 500 μg ml⁻¹ Km ( *B. thailandensis* ) or LB + 1,000 μg ml⁻¹ Km ( *B. pseudomallei* ) plate while centrifuging the rest of the recovered culture at 16,000g for 1 min prior to plating. Remove all but 100 μl of medium, then spread the remaining culture on another LB + Km plate. Incubate both plates in a 37 °C incubator until colonies appear, ~2 d later.

**(B) Electroporation of pKaKa2 into *B. pseudomallei* ● TIMING 4 d

(i) In a 15-ml Falcon tube, grow *B. pseudomallei* in 5 ml of LB broth overnight at 37 °C in a shaking incubator set to 225 r.p.m. (This is the end of day 1.)

(ii) After the culture has reached a sufficient density (OD₆₀₀ 0.8–1.5), spin down all 5 ml in a minicentrifuge at 10,000g for 1 min. Wash the cell pellet with 1 ml of cold, sterile ddH₂O. Repeat this four more times, adhering to the same centrifugation conditions. Alternatively, instead of using cold ddH₂O, 300 mM sucrose maintained at room temperature may be substituted when washing cells as previously described.₁⁵ Comparable electroporation efficiencies can be obtained by using either method.

(iii) Remove all water from the washed pellet and resuspend the cell pellet with 40 μl of cold, sterile ddH₂O. Add 1 μl of purified pKaKa2 plasmid (~0.2–0.5 μg) DNA to the competent cells and transfer the mixture into an electroporation cuvette. Place the electroporation cuvette into the electroporator shock chamber and apply a shock of 2.5 kV, 25 μF and 200 Ω. Ensure an exponential decay of the applied shock and the absence of an arc.

? TROUBLESHOOTING

(iv) Immediately add 1 ml of rich broth medium (e.g., LB broth) to the cuvette and transfer to a 15-ml culture tube with a screw cap. Incubate the culture for 1 h at 37 °C with shaking at 225 r.p.m. for recovery.

(v) Transfer the recovery mixture into a 1.5-ml microcentrifuge tube and spin in a minicentrifuge at 10,000g for 1 min. Discard the supernatant, wash the cell pellet twice with 1 ml of 1× M9 buffer and resuspend the cell pellet in the same volume of 1× M9 buffer. Plate 100 μl of the resuspended cells onto an MA agar plate, while centrifuging the rest at 16,000g for 1 min. Remove all but 100 μl of buffer, then spread the remaining culture on another MA agar plate. Incubate both plates in a 37 °C incubator until colonies appear, ~2 d later.

**(C) Conjugation of pKaKa2 into *B. pseudomallei* ● TIMING 3 d

(i) Grow a single colony of *B. pseudomallei* in 2 ml of LB broth, and grow the *E. coli* donor harboring pKaKa2 (E1354/pKaKa2) in 2 ml of MA, 1 mM Leu, 1 mM Lys, 1 mM Met, 1 mM Thr, 1 mM Trp and 100 μg ml⁻¹ DAP, overnight at 37 °C in a shaking incubator set to 225 r.p.m. (This is the end of day 1.)
(ii) After both cultures have reached a sufficient density (OD$_{600}$ 0.8–1.5), spin down 1 ml of each culture separately in a minicentrifuge at 10,000g for 1 min. Remove all medium from the E. coli donor and all but 40 μl LB from the B. pseudomallei tube, and resuspend both pellets in this 40 μl of LB. Spot this 40-μl mixture on the surface of a dried and prewarmed (37 °C) LB agar plate, and incubate at 37 °C for 4 h.

(iii) Gently scrape the cells off the plate with sterile inoculation loop and resuspend in 1 ml of 1 M9 in a 1.5-ml microcentrifuge tube; spin in a minicentrifuge at 10,000g for 1 min. Discard the supernatant, wash the cell pellet once more with 1 ml of 1× M9 buffer and resuspend the cell pellet in the same volume of 1× M9 buffer. Plate 100 μl of the resuspended cells onto an MA agar plate and incubate in a 37 °C incubator until colonies appear, ~2 d later.

**Induction of λ-Red protein expression ● TIMING ~18 h**

9| The λ-Red proteins encoded on pKaKa1 (Step 8A, and pKaKa2 (Step 8B) require different induction conditions, as detailed below. Option A describes the induction of the λ-Red proteins encoded on pKaKa1 with arabinose. Option B describes the induction of the λ-Red proteins encoded on pKaKa2 with rhamnose.

(A) Induction of the λ-Red proteins encoded on pKaKa1

(i) Grow the colonies of B. thailandensis or B. pseudomallei harboring pKaKa1 in 4 ml of LB broth containing 300 or 1,000 μg ml$^{-1}$ Km, respectively.

(ii) When the culture grows to an OD$_{600}$ of ~1.4 (B. thailandensis) or ~0.8 (B. pseudomallei), add arabinose to a final concentration of 10 mM to induce the λ-Red system on pKaKa1. After ~4 h of induction, harvest the culture by centrifugation. Concentrate the culture ~200 times by resuspending all cell pellets in 20 μl of LB broth. Proceed immediately to Step 10.

▲ CRITICAL STEP Induction at the proper OD is crucial for efficient recombineering. If the OD is too low, toxicity of λ-Red proteins can cause the cells to stop growing. If the OD is too high, cells tend to be ‘unhealthy’ after induction and recombineering efficiencies decrease.

(B) Induction of the λ-Red proteins encoded on pKaKa2

(i) Grow the colonies of B. pseudomallei harboring pKaKa2 in 4 ml of MA at 37 °C with shaking at 225 r.p.m.

(ii) When the culture grows to an OD$_{600}$ of ~0.8, add rhamnose to a final concentration of 0.2% to induce the λ-Red system on pKaKa2. After ~4 h of induction, harvest the culture by centrifugation. Concentrate the culture ~200 times by resuspending all cell pellets in a 20-μl volume of LB broth. Proceed immediately to Step 10.

▲ CRITICAL STEP Induction at the proper OD is crucial for efficient recombineering. If the OD is too low, toxicity of λ-Red proteins can cause the cells to stop growing. If the OD is too high, cells tend to be ‘unhealthy’ after induction and recombineering efficiencies decrease.

**DNA incubation ● TIMING ~4 d**

10| Add 0.5–2.0 μg of the PO/KO DNA obtained in Step 7 of the protocol, depending on the desired frequency (Tables 3 and 4), to a 20-μl aliquot of induced and concentrated cells from Step 9A or 9B. Incubate the cultures for 30 min at room temperature without shaking. After 30 min, add 2 ml of LB and incubate at 37 °C with shaking at 225 r.p.m. for 1 h.

▲ CRITICAL STEP The DNA sample should be added to the concentrated cells in a small volume (≤10 μl), as close contact between the cells and DNA is critical for efficient DNA uptake. If the volume of the DNA sample exceeds 10 μl, dry it down in a vacuum concentrator.

11| Pipette the recovery mix into a 1.5-ml microcentrifuge tube and centrifuge at 16,000g for 1 min. Wash the pellet twice with 1 ml of 1× M9. Remove 800 μl of the 1× M9 and resuspend the cell pellet in the remainder of the M9. Pipette and spread 50 and 150 μl of the recovery mix onto two different MG plates containing 0.04% GS for B. thailandensis or 0.3% GS for various strains of B. pseudomallei (with the exception of strain Bp0091, for which 0.1% GS was used). Colonies should be visible in ~3 d.

**PCR screening for successful PO/KO**

12| The screening for correct clones for PO (A) or KO (B) is done differently, as detailed below.

(A) PO screen in B. thailandensis and B. pseudomallei ● TIMING ~3 d

(i) Single colonies, containing the PO fragment as a replicating plasmid, should appear with a frequency similar to those shown in Table 3 or 4 (typically, when 0.5–2 μg PO DNA was used, 20–200 colonies should be expected for B. thailandensis or 20–60 colonies for B. pseudomallei). To verify a positive PO, a Target-rev gene-specific oligonucleotide (e.g., araI-rev or mbaS-rev) and a pAM3G-specific oligonucleotide (e.g., ori1600-rep-internal) should be used for PCR verification (Table 2 and Fig. 5). PCR verification should be set up exactly as described above in Steps 2 and 3 with extension time ~1 min kb$^{-1}$.

? TROUBLESHOOTING
Figure 5 | PCR confirmation for pullout and knockout in *B. thailandensis* and *B. pseudomallei*. (a) As an example, the arabinose-utilization operon was pulled out from the *B. thailandensis* chromosome using the pAM3G backbone with 45-bp homologous sequences to the araA and araI genes. The genetic map of the resulting plasmid is shown with the oligos in blue used for PCR screening. Using these oligos, ten independent POs were screened by PCR. As indicated by the arrow, a PCR product with the correct size was obtained in 89% (eight out of nine) of the GS-resistant colonies. Abbreviations: NC, negative control using wild-type *B. thailandensis* as template; L, 1-kb DNA ladder from New England Biolabs. (b) Similarly, the mbo cluster was pulled out from the *B. pseudomallei* strain 1026b chromosome, and the genetic map of the resulting plasmid, pAM3G-mbo, was shown with the oligos in blue used for PCR screening. Using these oligos, 10 independent POs were screened by PCR. As indicated by the arrow, PCR products with the correct size were obtained in 90% (nine out of ten) of the GS-resistant colonies.

(ii) Once a successful PO of the desired fragment is verified, inoculate the positive colonies into liquid MG + 0.01% GS (*B. thailandensis*) or MG + 0.1% GS (*B. pseudomallei*). Isolate the plasmid with a plasmid isolation kit and electroporate into an *E. coli* cloning strain (e.g., EPMAX-10B or E1889 in Table 1) for maintenance and downstream manipulation. Electrotransformation of *E. coli* is described elsewhere. Alternatively, instead of purifying plasmids from liquid culture, pipette 100 μl of the culture into a 1.5-ml microcentrifuge tube and boil the tube for 8 min. Centrifuge the tube at 16,000g for 1 min to spin down the cell debris. Electroporate 5 μl of the boiled supernatant into an electrocompetent *E. coli* cloning strain (e.g., EPMAX-10B or E1889 in Table 1) for further downstream applications.

**TROUBLESHOOTING**

(B) KO screen in *B. pseudomallei* ● TIMING ~5 h

(i) Single colonies, with the *pheS/sacB-gat* fragment recombined into the chromosome deleting the target gene, should appear with a similar frequency as shown in Tables 4 and 5 (typically, when 0.5–2 μg KO DNA is used, 20–50 colonies should be expected). To verify a successful KO, a set of Target-up-out and Target-down-out oligos annealing to chromosomal regions outside the targeted KO primers are used (e.g., mbaF-out and mbaS-out, Table 2). PCR verification should be set up exactly as described above in Steps 2 and 3 with extension time ~1 min kb⁻¹.

**TROUBLESHOOTING**

(ii) After verification of the KO, it is critical to purify the positive colonies once before growth for storage. To purify, transfer positive colonies onto an MG + GS plate and streak out for single colonies.

Recycling of the *pheS/sacB-gat* markers for KO in *B. pseudomallei* ● TIMING 3 d

13] If desired, the *pheS/sacB-gat* fragment can be removed from the mutant obtained in Step 12 by two different strategies. Option A describes replacement of the *pheS-gat* or *sacB-gat* cassette by a small PCR fragment homologous to the target gene,

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Knockout recombineering efficiencies of the essential <em>asd</em> gene in various naturally transformable clinical and environmental <em>B. pseudomallei</em> isolates.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninduced</td>
<td>0</td>
</tr>
<tr>
<td>Induced</td>
<td>144 (100%)</td>
</tr>
</tbody>
</table>

Two micrograms of DNA (*pheS-gat* cassette flanked by id homologous regions) was used for each incubation. Mutants were selected on MG + GS plates supplemented with 1 mM each of Met, Thr and Lys and 200 μg ml⁻¹ DAP specific for this mutation. GS was used for selection at final concentration of 0.3% (wt/vol) in all strains, with the exception of 0.1% GS used for Bp0091. Twenty GS-resistant colonies were tested phenotypically and by PCR confirmation with external chromosomal primers, and the frequencies of true mutants are shown in parentheses.

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by λ-Red recombination, generating a markerless mutant. Option B describes Flp-mediated excision of the pheS-gat or sacB-gat fragment, generating an unmarked mutant with a single FRT scar remaining in the target gene.

(A) Replacement of the pheS-gat or sacB-gat cassette with overlapping oligos

(i) Typically, set up two PCR reactions to obtain sufficient amounts of DNA, using the overlapping primers. Multiply all of the following components by two and combine in a master mix. Pipette 50 μl of the master mix into two thin-walled PCR tubes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (μl)</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>30.0</td>
<td>—</td>
</tr>
<tr>
<td>Pfu buffer (10×)</td>
<td>5.0</td>
<td>1×</td>
</tr>
<tr>
<td>dNTPs (2 mM)</td>
<td>5.0</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>aTarget-KO1 (1 μM)</td>
<td>1.0</td>
<td>1 pmol</td>
</tr>
<tr>
<td>aTarget-KO5 (1 μM)</td>
<td>1.0</td>
<td>1 pmol</td>
</tr>
<tr>
<td>Target-KO3 (30 μM)</td>
<td>3.0</td>
<td>30 pmol</td>
</tr>
<tr>
<td>Target-KO4 (30 μM)</td>
<td>3.0</td>
<td>30 pmol</td>
</tr>
<tr>
<td>Pfu polymerase (2.5 U μl⁻¹)</td>
<td>2.0</td>
<td>5 U</td>
</tr>
</tbody>
</table>

*Target-KO1 and Target-KO5 anneal to each other and serve as template.

▲ CRITICAL STEP It is essential to use Pfu polymerase or other polymerases that generate blunt ends in order to avoid the addition of unwanted bases. The proofreading capability of Pfu polymerase will significantly increase the fidelity of the PCR products.

(ii) Carry out the PCR as described below. Set the lid temperature to 98 °C to prevent condensation of the sample during the reaction.

<table>
<thead>
<tr>
<th>Cycle number</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Termination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94 °C, 2 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2–34</td>
<td>94 °C, 30 s</td>
<td>58 °C, 30 s</td>
<td>72 °C, 30 s</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>72 °C, 5 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td></td>
<td>4 °C, hold</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

■ PAUSE POINT At this time, the DNA can be stored at 4 °C for a short period of time. For extended storage, it is recommended that the reaction be placed in a −20 °C freezer.

(iii) Visualize PCR reactions by agarose gel electrophoresis, using a 2.0% agarose gel submerged in TAE buffer (see REAGENT SETUP). Run alongside DNA ladder (e.g., 100-bp DNA ladder) to estimate PCR product size. A single PCR product of ~100 bp should be observed. Recover and purify DNA from the gel with desired kit or protocol and quantitate using a spectrophotometer.

■ PAUSE POINT The DNA can be stored at 4 °C for a short period of time. For extended storage, it is recommended that the reaction be placed in a −20 °C freezer.

(iv) Inoculate the purified colony obtained in Step 12B(ii) into 4 ml of MA broth (B. pseudomallei with pKAk2 and the pheS-gat cassette) or 4 ml of LB + 1000 μg ml⁻¹ Km broth (B. pseudomallei with pKAk1 and the sacB-gat cassette) and incubate at 37 °C with shaking at 225 r.p.m. When the culture grows to an OD₆₀₀ of ~0.8, add rhamnose to a final concentration of 0.2% (B. pseudomallei with pKAk2 and the pheS-gat cassette) or arabinose to a final concentration of 10 mM (B. pseudomallei with pKAk1 and the sacB-gat cassette) in order to induce the λ-Red system. After 4 h of induction, harvest all 4 ml of culture by centrifuging at 16,000g for 1 min. Discard the supernatant and resuspend the cell pellet in 20 μl of LB. Incubate the resuspended cells with DNA obtained in Step 13A(iii) below immediately.

▲ CRITICAL STEP Induction at the proper OD is crucial for efficient recombineering. If the OD is too low, toxicity of λ-Red proteins can cause the cells to stop growing. If the OD is too high, cells tend to be ‘unhealthy’ after induction and recombineering efficiencies decrease.
(v) Add 0.5–1.0 μg of PCR DNA (~10 μl) obtained in Step 13A(iv) to the 20 μl of resuspended cells from Step 13A(i). Incubate the mixture for 30 min at room temperature without shaking. After 30 min, add 2 ml of LB and incubate at 37 °C with shaking at 225 r.p.m. for 2 h.

▲ CRITICAL STEP A small volume of the DNA sample (≤10 μl) should be added to the concentrated cells, as close contact between the cells and DNA is critical for efficient uptake of DNA. If the volume of the DNA sample exceeds 10 μl, dry it down in a vacuum concentrator.

(vi) Aliquot the recovery mix into two 1.5-ml microcentrifuge tubes and centrifuge at 16,000g for 1 min. Combine the pellet and wash it twice with 1 ml of 1× M9. Remove 800 μl of the 1× M9 and resuspend the cell pellet in the remainder of the 1× M9. Spread 50 and 150 μl of the cell suspension onto two MG plates containing 0.1% cPhe (B. pseudomallei with pKaKa2 and the pheS-gat cassette) or two LS + 15% sucrose plates (B. pseudomallei with pKaKa1 and the sacB-gat cassette; see REAGENT SETUP). Colonies should be visible in ~2 d.

(vii) To verify successful recombination and loss of the pheS-gat or sacB-gat fragment, colonies can be patched onto MG ± 0.3% GS plates to confirm GS sensitivity. The same set of oligonucleotides used in Step 12B(i) (Target-up-out and Target-down-out), which anneal outside the homologous regions, should be used for PCR verification. PCR verification should be set up exactly as described in Steps 2 and 3 with extension time ~1 min kb−1.

? TROUBLESHOOTING

(B) Flp-mediated excision of the pheS-gat or sacB-gat fragment

(i) Typically, set up four PCR reactions to obtain sufficient amounts of DNA. Multiply all of the following components by four and combine in a master mix. Pipette 50 μl of the master mix into four thin-walled PCR tubes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (μl)</th>
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</tr>
<tr>
<td>dNTPs (2 mM)</td>
<td>5.0</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>pCD135K-Flp-oriT-asdEc (~20 ng μl⁻¹)</td>
<td>1.0</td>
<td>~20 ng</td>
</tr>
<tr>
<td>Plac-up (30 μM)</td>
<td>1.0</td>
<td>30 pmol</td>
</tr>
<tr>
<td>Flp-down (30 μM)</td>
<td>1.0</td>
<td>30 pmol</td>
</tr>
<tr>
<td>Pfu polymerase (2.5 U μl⁻¹)</td>
<td>2.0</td>
<td>5 U</td>
</tr>
</tbody>
</table>

▲ CRITICAL STEP It is essential to use Pfu polymerase or other polymerases that generate blunt ends in order to avoid the addition of unwanted bases. The proofreading capability of Pfu polymerase will significantly increase the fidelity of the PCR products.

(ii) Carry out the PCR explained as below:

Set the lid temperature to 98 °C to prevent condensation of the sample during the reaction.

<table>
<thead>
<tr>
<th>Cycle number</th>
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</tr>
<tr>
<td>36</td>
<td>4 °C, hold</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

■ PAUSE POINT At this time, the DNA can be stored at 4 °C for a short period of time. For extended storage, it is recommended that the reaction be placed in a −20 °C freezer.

(iii) Visualize PCR reactions by agarose gel electrophoresis, using a 1.0% agarose gel submerged in TAE buffer (see REAGENT SETUP). Run alongside DNA ladder (e.g., 1-kb DNA ladder) to estimate PCR product size. A single PCR product of ~2.6 kb should be observed. Recover and purify DNA from the gel with desired kit or protocol and quantitate using a spectrophotometer.

■ PAUSE POINT The DNA can be stored at 4 °C for a short period of time. For extended storage, it is recommended that the reaction be placed in a −20 °C freezer.

(iv) Inoculate the purified colony obtained in Step 12B(ii) into 2 ml of LB and incubate at 37 °C with shaking at 225 r.p.m. Once the OD 600 reaches 0.8–1.5, harvest the cells by centrifuging at 16,000g for 1 min. Discard the supernatant and resuspend the cell pellet in 20 μl of LB. Incubate the resuspended cells with DNA obtained in Step 13B(iii) below immediately.
Add 0.5–1.0 μg of PCR DNA (~10 μl) obtained in Step 13B(iv) to the 20 μl of resuspended cells from Step 13B(i). Incubate the mixture for 30 min at room temperature without shaking. After 30 min, add 2 ml of LB and incubate at 37 °C with shaking at 225 r.p.m. for 2 h.

**CRITICAL STEP** A small volume of the DNA sample (<10 μl) should be added to the concentrated cells, as close contact between the cells and DNA is critical for efficient uptake of DNA. If the volume of the DNA sample exceeds 10 μl, dry it down in a vacuum concentrator.

Aliquot the recovery mix into two 1.5-ml microcentrifuge tubes and centrifuge at 16,000 g for 1 min. Wash the pellet twice with 1 ml of 1× M9. Remove 800 μl of the 1× M9 and resuspend the cell pellet in the remainder of the 1× M9. Spread 50 and 150 μl of the cell suspension onto two MG plates containing 0.1% cPhe (*B. pseudomallei* with pKaKa2 and the pheS-gat cassette) or two LS + 15% sucrose plates (*B. pseudomallei* with pKaKa1 and the sacB-gat cassette). Colonies should be visible in ~2 d.

To verify successful FRT recombination and loss of the pheS-gat or sacB-gat fragment, colonies can be patched onto MG ± 0.3% GS plates to confirm GS sensitivity. The same set of oligonucleotides as in Step 12B(i) (Target-up-out and Target-down-out), which anneal outside of the homologous regions, should be used for PCR verification. PCR verification should be set up exactly as described in Steps 2 and 3 with extension time ~1 min kb⁻¹.

**TROUBLESHOOTING**

Curing of pKaKa1 or pKaKa2

**TIMING ~5 d**

14| The pKaKa1 or pKaKa2 helper plasmid can be cured from *B. thailandensis* or *B. pseudomallei* in one step if required.

Option A describes the curing of pKaKa1 by pheS counterselection and is achieved on cPhe-containing medium. Option B describes the curing of pKaKa2 by sacB counterselection and is achieved on sucrose-containing medium.

**(A) Curing of pKaKa1 via pheS counterselection**

(i) Pick 1–2 isolates of *B. thailandensis* or *B. pseudomallei* harboring pKaKa1 using a sterile inoculation loop and streak the cells out on MG + 0.1% cPhe plates. Incubate the plates at 37 °C for 1–2 d until single colonies appear.

(ii) To confirm the loss of pKaKa1 plasmid, patch 10–20 single colonies from MG + cPhe plate onto LB + 500 μg ml⁻¹ Km (*B. thailandensis*) or 500 μg ml⁻¹ Km (*B. pseudomallei*) plate and incubate at 37 °C. Be sure to include *B. thailandensis* or *B. pseudomallei* harboring pKaKa1 and wild-type *B. thailandensis* or *B. pseudomallei* on the plate as positive and negative controls, respectively.

**(B) Curing of pKaKa2 by sacB counterselection**

(i) Pick 1–2 isolates of *B. pseudomallei* harboring pKaKa2 using a sterile inoculation loop and streak the cells out on LS + 15% sucrose plates. Incubate the plates at 37 °C for 1–2 d until single colonies appear.

(ii) To confirm the loss of pKaKa2 plasmid, patch 10–20 single colonies from LS + sucrose plate onto MA plate and incubate at 37 °C. Be sure to include *B. pseudomallei* harboring pKaKa2 and wild-type *B. pseudomallei* on the plate as positive and negative controls, respectively.

**TROUBLESHOOTING**

Troubleshooting advice can be found in Table 6.

**TABLE 6 | Troubleshooting table.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Possible solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>No second PCR product or high level of non-specific products is obtained</td>
<td>High-GC fragment causes inefficient denaturation and non-specific annealing.</td>
<td>Try a gradient PCR with annealing temperature range 50–70 °C, include 5–10% DMSO in the PCR reaction to aid amplification of high-GC fragments</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Majority of the long primers are truncated (not PAGE purified) in this batch, resulting in first PCR products with truncated ends</td>
<td>Inform primer synthesis company and resynthesize the non–PAGE purified long primers free of charge (from IDT and so on), and retry the PCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poor design of primers</td>
<td>Redesign the primers so that their GC% is 50–60% with no significant primer-dimer and hairpin structure formation, and the amplicons are as small as possible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>359</td>
<td></td>
</tr>
</tbody>
</table>

(continued)
TIMING
Steps 1–7, Preparing KO/PO DNA: 1 d
Step 8, Introduction of pKaKa1 or pKaKa2: 3–4 d
Step 9, Induction: 18 h
Steps 10 and 11, DNA incubation: 4 d
Step 12, PCR screening: 1–3 d
Step 13, Recycling of markers: 3 d
Step 14, Curing of pKaKa1 or pKaKa2: 5 d

ANTICIPATED RESULTS
PO recombineering efficiency of chromosomal fragments from *B. thailandensis*
Using the protocol provided will typically produce 40–200 colonies depending on the amount of DNA and type of PCR product used (Table 3). As an example, the *B. thailandensis* ara operon was pulled out of the chromosome with a PCR fragment containing a bhr origin of replication. Successful removal of the ara operon was verified using an ara-specific primer and a plasmid-origin-specific primer (Fig. 5a). A high percentage (~90%) of the colonies contained the pulled-out fragment (Table 3 and Fig. 5a). Such a percentage should be common as these numbers were obtained from experiments that were done in triplicate, and bacteria that do not contain gat should not grow on medium containing GS.

PO recombineering in *B. pseudomallei*
Using the helper plasmid pKaKa2 (constructed with the ara operon pulled out from *B. thailandensis*), the efficiencies of PO recombineering in *B. pseudomallei* are shown in Table 4; these are relatively lower than those of PO in *B. thailandensis* (Table 3). Introduction of pKaKa2 into *B. pseudomallei* can be achieved through electroporation or conjugation. High efficiency can be expected from both methods. Selection and maintenance of pKaKa2 in *B. pseudomallei* is simple and tight, as cells without the plasmid will not be able to metabolize arabinose as a nutrient source. A higher concentration of GS (0.3% instead of 0.04% for *B. thailandensis*) should be used for selection of the PO plasmid. Successful PO of chromosomal fragments should be confirmed by PCR using two oligos, of which one anneals to the plasmid backbone and the other to the PO fragment (e.g., mba cluster in Fig. 5b). High frequencies (90–100%) of successful PO can be expected (Fig. 5b and Table 4). No GS-resistant colonies were observed when the λ-Red system was not induced, indicating that recA-mediated recombination did not recombine 40–45 bp of homology at any detectable frequencies (Table 4).

KO recombineering in *B. pseudomallei* strains
We first demonstrated the KO recombineering in *B. pseudomallei* by knocking out the mba cluster in strain 1026b. The efficiencies of KO recombineering in the *B. pseudomallei* mba cluster are shown in Table 4: 20–50 colonies are usually

---

**Troubleshooting table (continued).**

<table>
<thead>
<tr>
<th>Step Problem</th>
<th>Possible reasons</th>
<th>Possible solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>8A(iii) and 8B(iii) Arcing is observed when applying electopulse</td>
<td>Inefficient washing results in high-salt concentration, or too much DNA is used</td>
<td>Perform additional washes to the cell pellet, and/or use reduced amount of DNA (e.g., 0.5–1 μg instead of 2 μg)</td>
</tr>
<tr>
<td>12A(i) and 12B(i); 13A(vii) and 13B(vii) No PCR product or high level of non-specific products is obtained</td>
<td>High-GC fragment causes inefficient denaturation and non-specific annealing</td>
<td>Try a gradient PCR with annealing temperature range 50–70 °C, include 5–10% DMSO in the PCR reaction to aid amplification of high-GC fragments</td>
</tr>
<tr>
<td>12A(ii) No colonies are obtained</td>
<td>Size of the PO fragments and toxicity of their gene products may be an issue</td>
<td>Use a fragment containing a low-copy-number origin for PO (e.g., pBBR-based origin)</td>
</tr>
</tbody>
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obtained when 0.5–2 μg KO DNA fragment is used. Independent GS-resistant isolates were screened by phenotypic and PCR confirmation (Fig. 6a,b), showing 100% frequency of successful KO. Generally, high frequencies (90–100%) are expected for the successful KO of chromosomal fragments when the gat marker is used for selection (Fig. 6a,b). Two options, via a second λ-Red-mediated recombination or Flp-catalyzed excision, were provided for the removal of the pheS-gat or sacB-gat FRT cassette from the mutant chromosomes. The λ-Red-mediated recombination results in a clean deletion of the chromosome fragment, whereas the Flp-catalyzed (λ-Red independent) excision leaves a single ‘FRT-scar’ inside the target fragment. Alternatively, instead of flp-containing PCR product incubation, we have expressed Flp transiently by conjugating the suicidal vector, pCD135K-Flp-oriT-asd<sup>Δ</sup>g<sup>+</sup>, from E. coli Δasd strain (E1354 in Table 1) and immediately counterselected the conjugation mix on cPhe- or sucrose-containing medium, yielding GS-sensitive clones. Use of the pheS-gat or sacB-gat FRT cassette for KO makes the creation of unmarked mutations easy, as little to no resistance to cPhe or sucrose was observed. As an example, the removal of the pheS-gat cassette was close to 100% efficient (Fig. 6c,d with the mba cluster as an example). The confirmation of unmarked mutations is simple, as the PCR products amplified using outside primers are small (approximately 200–500 bp; Fig. 6c,d). After introduction of the pKaKa2 plasmid, unmarked mutant could be obtained in ~10 d. pKaKa2 could be easily cured or maintained for the subsequent manipulation, such as creating multiple mutations in the same strain or introducing reporter-gene fusions. If required, the curing of pKaKa2 on sucrose medium could be confirmed by the inability to grow on arabinose as a sole carbon source. The curing efficiency was observed to be close to 100% (Fig. 6). If pKaKa1 in conjunction with the sacB-gat fragment was used for B. pseudomallei KO, then the efficiencies for KO and curing of helper plasmid pKaKa1 are expected to be similar to those when pKaKa2 and pheS-gat are used. Since the first submission of this protocol, we have used this system to PO and KO genes in both species with relative ease.

To demonstrate and extend the use of λ-Red recombineering in other naturally transformable B. pseudomallei strains, we tested 20 additional B. pseudomallei strains and found that 9 were naturally transformable (Table 1). We chose these nine strains encompassing clinical and environmental isolates (Table 1). The pheS-gat cassette was used to successfully KO the asd gene in these nine B. pseudomallei strains, and results are shown in Table 5 and Figure 7. The frequencies

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**Figure 6** Phenotypic and PCR confirmation for the mba-cluster knockouts in the B. pseudomallei. As an example, the B. pseudomallei strain 1026b mba cluster was knocked out, using the pheS-gat fragment with 45-bp homologous sequences to the mbaF and mbaS genes. A total of 25 independent knockout mutants were spotted onto a CAS (chromo azurol S) plate<sup>22</sup>, along with the wild-type 1026b strain (+) and the enterobactin-negative E. coli mutant (−, JW0586-1 (ref. 28)) as the positive and negative control, respectively. As the mba cluster is involved in malleobactin synthesis and secretion, knockout mutants display the malleobactin-negative phenotype as an absence of orange halo. The knockout frequency is 100% in these 25 isolates. (b) Oligos annealing outside the homologous region were used to screen ten isolates from a for successful KOs (oligos mbaF-out and mbaS-out as in Fig. 3). A total of 100% (ten out of ten) of the GS-resistant colonies were shown to have the pheS-gat fragment inserted into the mba cluster, as indicated by the arrow. NC indicates negative control using wild-type 1026b as the PCR template. L indicates the 1-kb DNA ladder from New England Biolabs. (c,d) Following the second λ-Red-mediated recombination with overlapping oligos (c) or Flp-mediated excision (d), unmarked mutations were generated. PCR confirmation was done for both methods, using the same outside oligos as in b, yielding smaller PCR products lacking pheS-gat as indicated by arrows in c and d. In both c and d, L indicates the 100-bp DNA ladder from New England Biolabs. (e) After mutant construction, the pKaKa2 helper plasmid was cured from the 1026b-Δmba::pheS-gat by sacB counterselection on sucrose-containing medium. Total nucleic acids were purified from strain 1026b-Δmba::pheS-gat/pKaKa2 (lane 1) and four isolates of cured strain 1026b-Δmba::pheS-gat without pKaKa2 (lanes 2–5) for Southern hybridization analysis, using the entire pKaKa2 plasmid as a probe. As indicated by arrow, the helper plasmid pKaKa2 exists only in strain 1026b-Δmba::pheS-gat/pKaKa2, whereas the entire pKaKa2 sequence is completely lost in 1026b-Δmba::pheS-gat after curing. L, prebiotinylated DNA ladder from New England Biolabs (mixture of HindIII-digested λ DNA and HaeIII-digested φ×174 DNA). (f) To demonstrate the integrity of the PO fragment, boiling prep of two 1026b/pAM3G-mba isolates were electroporated directly into 1026b-mba::FRT mutant for the complementation study (B1 and B2). In addition, the same boiling prep of 1026b/pAM3G-mba were transformed into an E. coli restriction-minus strain; plasmids were then reisolated from E. coli and reintroduced to complement the 1026b-mba::FRT mutant (E1 and E2). These four complemented strains were spotted on a CAS plate along with positive (+, wild-type 1026b) and negative (−, 1026b-mba::FRT) controls, and all four isolates regained the ability to produce malleobactin. (g) Boiling prep from five different isolates of 1026b/pAM3G-mba were electroporated into E. coli JW0586-1 strain, and the resulted strains were spotted onto CAS plates with positive (+, E. coli K-12 strain) and negative (−, E. coli JW0586-1 strain) controls. As shown, the B. pseudomallei 1026b mba operon complemented the enterobactin-deficient phenotype of E. coli JW0586-1 strain.
of true mutants were high (>50%, Table 5), even for KO of the essential asd gene, and screening two colonies should yield mutants using this system.

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AUTHOR CONTRIBUTIONS Y.K. created the constructs and performed the experiments in B. pseudomallei. M.H.N. performed the experiments in B. thailandensis. B.A.W. provided guidance for M.H.N. in this project. A.T. and T.T.H. wrote this manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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Elucidating the *Pseudomonas aeruginosa* Fatty Acid Degradation Pathway: Identification of Additional Fatty Acyl-CoA Synthetase Homologues

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Abstract

The fatty acid (FA) degradation pathway of *Pseudomonas aeruginosa*, an opportunistic pathogen, was recently shown to be involved in nutrient acquisition during BALB/c mouse lung infection model. The source of FA in the lung is believed to be phosphatidylcholine, the major component of lung surfactant. Previous research indicated that *P. aeruginosa* has more than two fatty acyl-CoA synthetase genes (*fadD*; PA3299 and PA3300), which are responsible for activation of FAs using ATP and coenzyme A. Through a bioinformatics approach, 11 candidate genes were identified by their homology to the *Escherichia coli* FadD in the present study. Four new homologues of *fadD* (PA1617, PA2893, PA3860, and PA3924) were functionally confirmed by their ability to complement the *E. coli* *fadD* mutant on FA-containing media. Growth phenotypes of 17 mutants on different FAs, as sole carbon sources, indicated that the four new *fadD* homologues are involved in FA degradation, bringing the total number of *P. aeruginosa* *fadD* genes to six. Of the four new homologues, *fadD*::PA1617 contributed the most to the degradation of different chain length FAs. Growth patterns of various *fadD* mutants on plant-based perfumery substances, citronellic and geranic acids, as sole carbon and energy sources indicated that *fadD* is also involved in the degradation of these plant-derived compounds. A decrease in fitness of the sextuple *fadD* mutant, relative to the Δ*fadD1D2* mutant, was only observed during BALB/c mouse lung infection at 24 h.


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Introduction

*Pseudomonas aeruginosa* is an important human pathogen [1], [2] responsible for myriad of infections of the human body [3–11]. This ubiquitous bacterium is also a leading cause of mortality and morbidity in patients with cystic fibrosis (CF) [1], [2].

Phosphatidylcholine (PC), the major component of lung surfactant [12], was suggested as a potential nutrient source for pathogenesis during *P. aeruginosa* infection of the CF lung [13]. The major carbon source within the PC molecule comes from the two highly reduced long-chain fatty acids (LCFA). Many fatty acid degradation (β-oxidation) genes are expressed by *P. aeruginosa* during CF lung infection (e.g. *fadD*; PA3299, *fadD2*; PA3300, *fadA5*; PA3013, and *fadB5*; PA3014) [13] and mutants defective in the fatty acid (FA) degradation pathway were reported to have decreased fitness during mouse lung infection [14]. A link between FA degradation genes and virulence was also observed [14] and *P. aeruginosa* can chemotax towards FA [15]. Furthermore, FA was shown to modulate type three-secretion system expression in this bacterium [16].

Despite the connection between virulence and FA degradation during infections, not all genes involved in *P. aeruginosa* FA degradation are characterized (Fig. 1A). In contrast, genes needed by *Escherichia coli* for aerobic β-oxidation (*fadL*, *fadD*, *fadE*, and *fadA* [17–20]), anaerobic FA degradation (*fadK* and *fadIF*) [21], and auxiliary genes (*fadH* [22] and *fadM* [23]) are well characterized. For an exogenous FA to be degraded by this pathway, it must first be transported by the membrane transporter (FadL) into the cell [24]. FA is then activated with the use of adenosine triphosphate (ATP) and coenzyme A (CoASH) by FadD (fatty acyl-CoA synthetase, FACS) [19], [25]. The activated FA molecule can then proceed through the β-oxidation pathway (Fig. 1A). In *E. coli*, genes encoding enzymes needed for β-oxidation (*fadL*, *fadD*, *fadE*, and *fadA*) are expressed in the absence of FAs by the transcriptional regulator FadR. Acyl-CoA of chain length ≥ C12:0 can bind to FadR to induce FA degradation [18], [26], [27] resulting in growth on FA (≥ C10:0). Cyclic AMP and receptor protein complex levels [28], presence of oxygen [29], and osmotic pressure [30] also affect expression of FA degradation genes in *E. coli*. However, the existence of a central regulator, such as *fadR*, is unknown in *P. aeruginosa*, and only a few *fad*-genes have been found to be regulated by a FA sensor, PsrA [31].

*P. aeruginosa* exhibits greater metabolic capabilities for FA degradation than *E. coli* by growing aerobically on short, medium, and long-chain FAs as sole carbon and energy sources [31]. With a
The genome of 6.3 Mb, *P. aeruginosa* could potentially have more FA degradation genes than *E. coli* [32], suggesting possible redundancies and a higher level of complexity in this pathway. Three potential *fadL* genes have been investigated thus far in *P. aeruginosa* and their exact role in FA transport still remains unclear [15]. Two *fadBA* operon homologues (*fadAB1* and *fadBA5*) have been studied so far. The *fadAB1* (PA1736–PA1737) operon was shown to be strongly induced by medium-chain fatty acids (MCFA, C10:0 and C12:0) and, to a lesser extent, LCFA (C14:0–C18:1 Δ9) [33]. The *fadBA5* (PA3013–PA3014) operon was determined to be...
involved in LCFA metabolism and to be induced by LCFA, especially oleate (C_{18:1}) [31]. We have recently identified two FACS homologues of *P. aeruginosa*, fadD1 (PA3299) and fadD2 (PA3900) [14]. The FadD1 and FadD2 of *P. aeruginosa* were determined to have broad specificity for FA of different chain lengths. FadD1 has preference for LCFA whereas FadD2 has higher activities for shorter chain FAs. *fadD1, fadD2, and fadD2D1* mutants showed growth defects when grown on minimal media with different length FAs as sole carbon sources. fadD1 was determined to be induced by LCFA and to be more important for growth on LCFA while *fadD2* was important for growth on short-chain fatty acids (SCFA) and was induced by MCF. The double mutant *fadD2D1* displayed an impaired ability to grow on PC as a sole carbon source. This growth defect translated into decreased in vivo fitness during mouse lung infection, indicating that FadD1 and FadD2 may mediate *P. aeruginosa* replication in the CF lung [14]. However, the double mutant *fadD2D1* was still able to grow on FA, suggesting the involvement of other *fadD* homologues in FA degradation [14].

We surveyed the *P. aeruginosa* genome for additional *fadD* homologues to gain more insight into the degradation of FAs in this bacterium. Four new *fadD* homologues PA1617, PA2993, PA3860, and PA3924 were identified out of 11 potential candidates. Through genetic analyses, their contribution to FA degradation was assessed. The final four candidates were determined to be FACS homologues, but PA1617 (*fadD4*) was found to be the major contributor to FA degradation. Involvement of the newly discovered *fadD4* in catalysis of plant-derived acyclic terpenes suggests that the function of multiple FACS in *P. aeruginosa* is the degradation of compounds closely related to FAs. Growth defect on PC and decreased fitness in mouse lung of the sextuple *fadD* mutant supports the role of FA as a nutrient in *in vivo*.

**Results**

**Identification of *P. aeruginosa* Fatty acyl-CoA Synthetase Homologues**

To identify *fadD* homologues of *P. aeruginosa*, *E. coli* FadD amino acid sequence was compared to *P. aeruginosa* PAO1 ORFs via BLAST [34]. The amino acid sequence of genes obtained in the search were further analyzed for the presence of ATP/AMP [19], [35–37] and fatty acid binding motifs [38]. Genes that encode eleven proteins containing amino acid sequences with high degree of similarity to the motifs found in *E. coli* FadD (Fig. S1) were chosen for complementation tests. Identity and similarity of the proteins range from 22% to 31% and from 37% to 52%, respectively (Table S1). When cloned into a high copy number pUC19 vector, only genes encoding PA3860, PA1617, PA2993, and PA3924 were found to complement the *E. coli* fadD<sup>−</sup>/*fadR<sup>−</sup> (E2011) strain on minimal medium containing oleate (C_{18:1}) and decanoate (C_{10:0}) (Table S1) and were designated *fadD3, fadD4, fadD5*, and *fadD6*, respectively. Their ATP/AMP and FA binding motifs show high degree of similarity to those of *E. coli* FadD (Fig. 1B).

All four *P. aeruginosa* fadD genes (*fadD3, fadD4, fadD5*, and *fadD6*) were tested further for their ability to support growth of *E. coli* fadD<sup>−</sup>/*fadR<sup>−</sup> (E2011) on various FAs as a single copy on the *E. coli* chromosome. The *E. coli* fadD<sup>−</sup>/*fadR<sup>−</sup> double mutant was used to ensure that FadR does not inhibit expression of other *E. coli* β-oxidation enzymes. Mini-Tn7 based complementation vectors were constructed and integrated into the E2011 chromosome at the *attTn7* site and resulting strains were tested for growth on FAs (Table 1). As expected, wildtype *E. coli* control strain K-12 showed growth on longer FAs (C_{12:0}–C_{18:1}) but not on the MCF, C_{10:0}, or SCFAs (C_{4:0}–C_{6:0}). The E2011 and the integrated empty-vector control strain were not able to grow on any of the FAs. E2011 complemented with *E. coli* fadD (*fadD<sub>W</sub>*) grew on C_{12:0}, C_{14:0}, and C_{16:0} comparably to K-12. *P. aeruginosa* fadD3, fadD4, fadD5, and fadD6 genes individually allowed E2011 to grow on C_{14:0}, C_{16:0}, and C_{18:0} to similar levels as K-12. fadD3 and fadD6 complemented E2011 to a lesser degree than *fadD4* and *fadD5* on C_{12:0}, and four *fadD* genes supported minimal growth of E2011 on C_{8:0} to the same level as *fadD<sub>W</sub>*. E2011 complemented with *fadD<sub>W</sub>, fadD3, fadD4, fadD5, or fadD6 did not grow on C_{4:0}–C_{8:0}, which was in agreement with previous observations that other *E. coli* FA degradation enzymes do not support metabolism of shorter FAs [39].

**Contribution of *fadD3, fadD4, fadD5*, and *fadD6* to FA Degradation**

To determine the role of the *fadD* homologues (*fadD3, fadD4, fadD5, and fadD6*) in *P. aeruginosa* FA degradation, strains with various combinations of *fadD* mutations were created. To prevent potential masking of phenotypes by *fadD1* and *fadD2*, 15 mutants were constructed in the *P. aeruginosa* PAO1 *fadD1D2* background. Four triple, seven quadruple, four quintuple mutants and one sextuple mutant (Table 2) were tested for growth on C_{4:0}–C_{18:1} along with wildtype PAO1 and the *fadD1D2* mutant.

As expected, all 17 mutant strains grew the same as PAO1 on glucose at 24 h and 96 h (Tables 3 and 4). On C_{4:0}, growth of all mutants was not observed, which was the same as PAO1 indicating that none of the *fadD* homologues contribute to the degradation of this FA or the differences were too small to be detected via plate-based growth assays. Throughout the study, the Δ*fadD3D4D5D6* strain had the same growth as PAO1 on C_{4:0}–C_{18:1} indicating that FadD1 and FadD2 are most likely providing a majority of FACS activity in *P. aeruginosa* (Tables 3 and 4). No difference in growth was observed between Δ*fadD1D2* strain and Δ*fadD1D2D3, Δ*fadD1D2D5, Δ*fadD1D2D6, Δ*fadD1D2D5D6*, Δ*fadD1D2D5D6*, or Δ*fadD1D2D6D5D6* on C_{6:0}–C_{18:1}. There was significantly less growth for Δ*fadD1D2D4* on C_{6:0}–C_{18:1} at 24 h in comparison to Δ*fadD1D2*, suggesting that *fadD4* is important for degradation of all FAs from C_{6:0} to C_{18:1}.

Addition of *fadD3, fadD5*, or fadD6 mutation to Δ*fadD1D2D4* strain in a quadruple mutant combination resulted in larger deficiencies in growth on FAs in comparison to the triple Δ*fadD1D2D4* mutant (Tables 3 and 4), indicating that *fadD3, fadD5*, and *fadD6* also take part in FA degradation and suggesting the dominance of FadD4 over these homologues. The Δ*fadD1D2D3D4, Δ*fadD1D2D4D5*, and Δ*fadD1D2D4D6* strains showed no growth on C_{9:0} and C_{8:0} even after four days, in contrast to the Δ*fadD1D2D4* mutant (Table 4), indicating that *fadD3, fadD5*, and *fadD6* are involved in the degradation of these FAs.

All quintuple mutants exhibited some level of growth on several FAs after 96 h (Table 4), whereas no growth was present for the sextuple mutant combination (Δ*fadD1D2D3D4D5D6*), indicating that all four new *fadD* homologues contribute to FA degradation and that only six aerobic FACS genes are likely present in *P. aeruginosa*. Quintuple mutants with both *fadD4* and *fadD5* mutations (Δ*fadD1D2D3D4D5D5D6* and Δ*fadD1D2D4D5D6D5D6*) were more deficient in FA degradation (Table 3). Growth patterns of the four quintuple mutants after 96 h (Table 4) suggest that *fadD4*, besides *fadD1* and *fadD2*, is much more important for FA degradation than *fadD3, fadD5*, and *fadD6* combined, and *fadD5* contributes to FA degradation more than *fadD3* and *fadD6*. Furthermore, by comparing the phenotypes of double, triple, and quadruple mutants at two time points (Tables 3 and 4) a hierarchy of contributions of *fadD* homologues to the degradation of different
Chain-length FAs can be assigned as follows: i) FadD4 degrades C₆₀–C₁₄:₀ (ΔfadD1ΔfadD2 in Table 3); ii) FadD5 degrades C₆₀–C₁₄:₀ (ΔfadD1ΔfadD2ΔfadD5 versus ΔfadD1ΔfadD2ΔfadD4 in Tables 3 and 4); iii) FadD3 degrades C₆₀–C₁₂:₀ (ΔfadD1ΔfadD2ΔfadD3ΔfadD4 versus ΔfadD1ΔfadD2ΔfadD4 in Tables 3 and 4); and iv) FadD6 degrades C₆₀–C₁₂:₀ (ΔfadD1ΔfadD2ΔfadD6ΔfadD4 versus ΔfadD1ΔfadD2ΔfadD6 in Tables 3 and 4).

fadD1 and fadD2 in Comparison to fadD3, fadD4, fadD5, and fadD6

The growth phenotypes of various combinatory mutants on FAs indicated that out of the newly discovered FACS genes (fadD3, fadD4, fadD5, and fadD6), fadD4 is most important for FA degradation (Tables 3 and 4), in addition to fadD1 and fadD2 [14]. To investigate further the contribution of fadD4 to FA degradation in comparison to fadD1 and fadD2, growth curve experiments were performed on SCFA, MCFA, and LCFAs with ΔfadD1ΔfadD2ΔfadD4, ΔfadD3ΔfadD4ΔfadD5, ΔfadD1ΔfadD2ΔfadD3ΔfadD5, and ΔfadD1ΔfadD2ΔfadD3ΔfadD4ΔfadD5ΔfadD6 mutants along with PAO1 and ΔfadD1ΔfadD2 strains (Fig. 2). The growth experiments on FAs were conducted up to 30 h, which was sufficient to distinguish differences in growth patterns between various strains. The growth rates calculated from growth curves in Fig. 2 are presented in Table S3. The ΔfadD1ΔfadD2 mutant strain had impaired growth in comparison to PAO1 on FAs (Fig. 2B–2E). The phenotype of ΔfadD1ΔfadD2ΔfadD3ΔfadD4ΔfadD5ΔfadD6 in C₆₀–C₁₂:₀ Av (Fig. 2B–2E) was characterized by lower final optical density (OD) and/or longer lag phase than ΔfadD1ΔfadD2ΔfadD4, indicating that fadD3, fadD5, and fadD6 also contribute to FA degradation. In comparison to ΔfadD1ΔfadD2 and ΔfadD1ΔfadD2ΔfadD3ΔfadD4ΔfadD5ΔfadD6, ΔfadD1ΔfadD2ΔfadD3ΔfadD4ΔfadD5ΔfadD6 exhibited very small amounts of growth, and no increase in turbidity was observed for ΔfadD1ΔfadD2ΔfadD3ΔfadD4ΔfadD5ΔfadD6 on FAs (Fig. 2B–2E). The ΔfadD3ΔfadD4ΔfadD5ΔfadD6 mutant had almost identical growth in comparison to PAO1 in C₆₀ and C₁₂:₀ Av (Fig. 2B and 2E). In C₆₀ and C₁₄:₀ ΔfadD3ΔfadD4ΔfadD5ΔfadD6 showed a similar final OD as PAO1 but longer lag phase (Fig. 2C and 2D). These data indicate that, although the activity of FadD4 is masked by the dominance of FadD1 and FadD2, the FadD4 plays a significant role in the degradation of FAs in P. aeruginosa.

Role of fadD Homologues in the Utilization of Plant-derived Acyclic Terpenes

One of the P. aeruginosa fadD homologues, fadD5 (PA2893; attD), was proposed to be part of the acyclic terpenes utilization (ATU) pathway and to contribute to degradation of citronellol and geraniol (perfumery compounds found in plants) by activating citronellic acid (CA) and geranic acid (GA) through addition of CoASH [40]. However, mutation of PA2893 alone did not abolish growth on acyclic terpenes possibly suggesting the involvement of other homologue(s) [40]. To determine the role of fadD5 and other fadD homologues in degradation of acyclic terpenes as plant-derived nutrient sources, we grew PAO1 along with 17 combinatory fadD mutants in 1x M9 minimal media +1% (w/v) Brij-58 supplemented with 0.2% (w/v) fatty acids or 20 mM glucose (Glu) +0.25 mM IPTG for three days at 37°C. – indicates no growth on a patch and + indicates growth.

+1 is very little growth whereas +6 is very heavy growth comparable to K12 on glucose at day 3.

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Table 1. Single copy complementation of the E. coli fadD mutant with P. aeruginosa fadD homologues.

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<td>+5</td>
<td>+5</td>
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Strains were grown on 1x M9 medium +1% (w/v) Brij-58 supplemented with 0.2% (w/v) fatty acids or 20 mM glucose (Glu) +0.25 mM IPTG for three days at 37°C. – indicates no growth on a patch and + indicates growth.

To determine if newly discovered homologues modulate virulence, single unmarked mutants ΔfadD3, ΔfadD4, ΔfadD5, and ΔfadD6, along with ΔfadD1ΔfadD2ΔfadD3ΔfadD4ΔfadD5ΔfadD6 strain and its complement were tested for production of proteases, lipases, phospholipases, and rhamnolipids. No difference in production of these virulence determinates was observed between PAO1 and all strains tested (data not shown).
Table 2. Strains utilized in this study.

<table>
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<td>Km−; fadD− (olcD88) fadR:Km−</td>
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</tr>
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</tr>
<tr>
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<td>This study</td>
</tr>
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</table>

Abbreviations:
Cd′; carbencillin resistance; Ec; E. coli; fadD; gene encoding fatty acyl-CoA synthetase; Flp; Saccharomyces cerevisiae recombinase; FRT; Flp recognition target; Gm′, gentamicin resistance; Km′, kanamycin resistance; mucA, anti-sigma factor, repressor of alginate biosynthesis in P. aeruginosa; Pa, P. aeruginosa; pheS, gene encoding a mutated z-subunit of phenylalanyl tRNA synthase; Tc′, tetracycline resistance.

doi:10.1371/journal.pone.0064554.t002

Involvement of New fadD Homologues in PC Degradation and in vivo Growth

Our previous study indicated that the ∆fadD1D2 mutant had a decreased ability to degrade PC and was less fit in BALB/c mice lungs [14]. We hypothesized that the sextuple fadD mutant, which does not grow on FAs, would exhibit impaired growth on PC and have significantly decreased in vivo fitness. We first investigated the role of the four newly discovered FACS genes in PC degradation (Fig. 4A). Before death phase, ∆fadD1D2 exhibited slower growth rate and lower final turbidity than PAO1. ∆fadD1D2D4 had a longer lag phase in comparison to ∆fadD1D2 before reaching a similar OD, implying that fadD4 contributes to degradation of PC. The ∆fadD1D2D3D4D5D6 mutant further exhibited a significant growth defect on PC. The large differences in growth rate and final OD between the sextuple mutant and ∆fadD1D2D4 suggest that not only fadD4 but also fadD3, fadD5, and fadD6 are required for growth on PC, which contains a mixture of FA chain lengths.

When in vitro competition studies were conducted on the sextuple fadD mutant and its competitor the complemented sextuple fadD mutation, mutation of all six FACS genes did not affect fitness when the bacteria were grown in rich Luria Bertani (LB) medium, and minimal medium supplemented with casamino acids, glucose, glycerol, and choline (Fig. 4B). In contrast, the in vitro competitive index (CI) in oleate (C18:1(ω)7) and PC were low (~0.15 and ~0.3, respectively) indicating that ∆fadD1D2D3D4D5D6 has a growth disadvantage on these carbon sources.
sources. The *in vivo* competition study showed that the sextuple \( fadD \) mutant was out numbered by its complement (Fig. 4C). An almost 10-fold increase in CFU per lung above inoculum (6 x10⁶) was observed for both time points indicating bacterial replication *in vivo*. At 24 h, the amount of the sextuple \( fadD \) mutant was half of its complement, which is lower than the reported CI for the \( ΔfadD1ΔD2 \) mutant at 24 h [14]. Even at 48 h the CI was significantly lower than 1, indicating that deletion of \( fadD \) genes decreases *in vivo* fitness of sextuple \( fadD \) mutant.

Discussion

Previous research on \( fadD1 \) and \( fadD2 \) indicated that more than two FACS genes are present in *P. aeruginosa* [14]. In this study, we focused on identification of additional \( fadD \) homologues. Four genes, \( fadD3, fadD4, fadD5, \) and \( fadD6 \) (PA3860, PA1617, PA2893, and PA3924, respectively) were found to encode FACS (Tables S1 and 1). Each of these genes contributes at a varying degree to FA degradation (Tables 3 and 4). Surprisingly, none of the new \( fadD \)s were involved in degradation of butyrate (\( C_{4:0} \); Table 3). It is possible that other unidentified genes with acyl-CoA synthetase functions are responsible for growth on \( C_{4:0} \). Butyrate could also be processed through the acetoacetate degradation pathway (\( ato \)), an alternative pathway for degradation of SCFA [41]. This could be possible since two homologues of both of *E. coli* acetoacetyl-CoA transferase complex proteins, AtoA and AtoD, are present in *P. aeruginosa*. PA2000 (identity 45% and similarity 62%), PA0227 (identity 28% and similarity 62%), PA1999 (identity 40% and similarity 64%), and PA5445 (identity 33% and similarity 55%), respectively.

Growth studies with various mutants using FAs as sole carbon and energy sources indicated that FACS homologues are not of equal physiological significance and that there are disparities in importance and FA preference between them. \( fadD1 \) and \( fadD2 \), along with \( fadD4 \), are responsible for almost all FA degradation and dominate over other homologues. When \( fadD1 \) and \( fadD2 \) are inactivated, the majority of growth on SCFAs, MCFAs and LCFA is due to \( fadD4 \) (Tables 3 and 4, Fig. 2). In comparison, \( fadD3, fadD5, \) and \( fadD6 \) have small contributions to overall growth on FAs and their individual involvement can be only observed when \( fadD1, fadD2 \) and \( fadD4 \) are absent (Table 4). This is not unprecedented, since *Pseudomonas putida* FadD2 is only active when FadD1 is not present [42]. It could be possible that gene(s) ruled out by screening in *E. coli* for growth on LCFA (Table S1), might be involved in SCFA and/or MCFA degradation. However, lack of growth for the sextuple \( fadD \) mutant on \( C_{6:0}–C_{18:1}\text{unsat} \) (Table 4) strongly indicates that *P. aeruginosa* has a total of six aerobic FACS genes.

*P. aeruginosa* is commonly found in soil, water, and on plant surfaces [43–45] and it is known to degrade over 70 different organic substances such as aromatic compounds, organic acids (e.g. isovalerate), alcohols, and acyclic terpenes (e.g., citronellol and geraniol) [44]. Sources of nutrients for pseudomonads on plant surfaces have not been determined. Citronellol and geraniol (perfumery compounds and possible bacterial nutrient sources found in plants) are degraded through the acyclic terpene utilization (ATU) pathway, \( β \)-oxidation pathway, and leucine/isovalerate utilization pathway [40,46]. The \( fadD5 \) (PA2893 or *atuH*) was proposed to be part of ATU and to be involved in activation of the CA and GA intermediates of the

---

### Table 3. Growth of various *P. aeruginosa fadD* mutants on FAs after 24 h.

<table>
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<th>( C_{10:0} )</th>
<th>( C_{12:0} )</th>
<th>( C_{14:0} )</th>
<th>( C_{16:0} )</th>
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<th>Glu</th>
</tr>
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<td>+3</td>
<td>+3</td>
<td>+2</td>
<td>+3</td>
<td>+2</td>
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<td>+4</td>
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<tr>
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<td>+2</td>
<td>+3</td>
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</tr>
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<td>+3</td>
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<td>+2</td>
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</table>

Strains were grown on 1x M9 medium +1% (w/v) Brij-58 supplemented with 0.2% (w/v) fatty acids or 20 mM glucose (Glu).

- \( +1 \) indicates no growth on a patch and \( + \) denotes growth.

\( +4 \) is a heavy growth comparable to PAO1 on glucose at 24 h.

\( +6 \) is a very heavy growth comparable to PAO1 on glucose at 96 h.

doi:10.1371/journal.pone.0064554.t003
pathway. However, fadD5 was confirmed experimentally not to be part of ATU, and other homologues were thought to be also involved and to 'mask' the phenotype [40]. We investigated the possible role of fadD homologues in the degradation of acyclic terpenes, and we reasoned that combination of various fadD mutations would allow involvement of FACS homologues in ATU to be assessed. Surprisingly, fadD5 along with fadD1, fadD2, fadD3, and fadD6 had minimal if any contributions to the degradation of CA and GA (Fig. 3). Interestingly, fadD5 is located right next to genes known to be involved in ATU and seems to be the last gene in atuABCDEFGH cluster [40]. On the other hand, fadD4 is not only involved in ATU but it is almost solely responsible for degradation of these compounds as can be observed from growth phenotypes of the single fadD4 mutant and its complement (Fig. 3D and 3F). Notably, homologues of fadD4 with high similarity are present in Pseudomonas fluorescens (e.g., Pit10_4205 in Pit-1, 72% identity and 84% similarity), Pseudomonas protegens (e.g., PFL_1744 in strain Pf-5, 71% identity and 82% similarity), and Pseudomonas mendocina (e.g., MDS_2302 in strain NK-01, 75% identity and 87% similarity) and some strains of these pseudomonads are known to degrade acyclic terpenes [40], [47].

The ability of P. aeruginosa to degrade lipids and FAs, especially the main component of lung surfactant PC, has been linked to replication of this opportunistic pathogen during infection of CF patients’ lungs [13]. Previously, we determined that ΔfadD1, ΔfadD2, and double ΔfadD1ΔD2 mutants have decreased fitness in BALB/c mice due to their deficiencies in degradation of FAs and PC [14]. We hypothesized that P. aeruginosa strains with greater defects in utilization of FAs and PC in vitro will have larger disadvantages during in vivo growth. ΔfadD1ΔD2ΔD3ΔD6 mutant exhibited the most significant growth defect in FAs and PC (Fig. 2, 4A and 4B), and similar level of virulence factors (i.e. proteases, hemolysins, lipases) production was observed between sextuple fadD mutant, its complement, and PAO1 (data not shown). The ΔfadD1ΔD2ΔD3ΔD6 mutant had some decrease of in vivo fitness in comparison to the ΔfadD1ΔD2 at 24 h (Fig. 4C and [14]); but at 48 h, ΔfadD1ΔD2ΔD3ΔD4ΔD6 mutant was not less fit in mice lungs than ΔfadD1ΔD2 mutant. This latter result was surprising, as the impaired ability to utilize PC did not result in a more dramatic phenotype in vivo at 48 h (Fig. 4C). There are several possibilities, which could account for this unexpected phenotype. The sextuple mutant could utilize in vivo other constituents of PC such as choline and glycerol later in the infection. Additionally, pulmonary surfactants are composed of 10% proteins [48] and amino acids were suggested to be used by P. aeruginosa during lung infection [49] and could serve as an alternative nutrient source for sextuple fadD mutant. Other FACS genes (i.e. anaerobic which we could not identify because of limitations of our aerobic in vitro screening method) could be important for in vivo growth.

In summary, we have identified four additional FACS homologues of P. aeruginosa and determined their involvement in degradation of different FAs. The dual catabolic function of fadD4 (PA1617) for FAs and acyclic terpenes exemplifies the interconnection of metabolic pathways and multiple roles that FACS homologues play in this ubiquitous bacterium. Our in vivo data show that nutrient acquisition during lung infection is a complicated process, involving alternative pathways that require

Table 4. Growth of various P. aeruginosa fadD mutants on FAs after 96 h.

<table>
<thead>
<tr>
<th>Strains</th>
<th>C₄:0</th>
<th>C₆:0</th>
<th>C₈:0</th>
<th>C₁₀:0</th>
<th>C₁₂:0</th>
<th>C₁₄:0</th>
<th>C₁₆:0</th>
<th>C₁₈:1&lt;sup&gt;α&lt;/sup&gt;</th>
<th>Glu</th>
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<td>ΔfadD1ΔD2ΔD4ΔD5</td>
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<td>ΔfadD1ΔD2ΔD4ΔD6</td>
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<td>ΔfadD1ΔD2ΔD3ΔD4ΔD5</td>
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<td>ΔfadD1ΔD2ΔD3ΔD4ΔD6</td>
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<td>+</td>
</tr>
</tbody>
</table>

+ indicates no growth on a patch and + denotes growth.

<sup>α</sup> is a very heavy growth comparable to PAO1 on glucose at 24 h.

Strains were grown on 1x M9 medium +1% (w/v) Brij-58 supplemented with 0.2% (w/v) fatty acids or 20 mM glucose (Glu).

doi:10.1371/journal.pone.0064554.t004

Fatty Acyl-CoA Synthetases in P. aeruginosa
further investigation. Knowledge of all fadD genes needed for FA degradation significantly increases our understanding of the FA degradation pathway and its importance for in vivo replication of *P. aeruginosa*.

**Materials and Methods**

**Ethics Statement**

All animal experiments were approved by University of Hawaii at Manoa Institutional Animal Care and Use Committee (protocol no. 06-023-6) and were conducted in compliance with the NIH guidelines.
Strains and plasmids utilized in this study are listed in Tables 2, S2, and 5, respectively. All *P. aeruginosa* mutants constructed and utilized in this study are derived from strain PAO1. *E. coli* E1869 strain (Table S2) was routinely used for cloning and *E. coli Δasd* or *ΔdapA* strains (E464, E1353, and E2072, Table S2) were used for mobilization of plasmids as described previously [50]. *E. coli* and *P. aeruginosa* strains were cultured in rich and minimal media as described in Materials and Methods.

**Figure 3. Growth phenotypes of various fadD homologues mutants on acyclic terpenes.** Strains were grown in liquid 1x M9 medium +1% (w/v) Brij-58 supplemented with 20 mM glucose, 0.1% (w/v) of citronelllic acid, or 0.1% (w/v) geranic acid at 30°C. Optical densities (ODs) of cultures were measured and compared to PAO1 at day one (A, C, and E). Growth of ΔfadD4 mutant and ΔfadD4/attB::fadD4 complement strain in different carbon source were compared to PAO1 and ODs from day six are presented (B, D, and F). Results shown are from representative experiments that were performed twice by measuring triplicate cultures. doi:10.1371/journal.pone.0064554.g003

(National Institutes of Health) Guide for the Care and Use of Laboratory Animals.

**Bacterial Strains and Growth Media**

Strains and plasmids utilized in this study are listed in Tables 2, S2, and 5, respectively. All *P. aeruginosa* mutants constructed and
described by Kang et al. [14] unless indicated otherwise. Fatty acids stocks were prepared as previously described [31].

General Molecular Techniques

Molecular techniques were performed as previously described [50]. Oligonucleotides (Table 6) were synthesized through Integrated DNA Technologies.

Identification of \textit{P. aeruginosa} Fatty acyl-CoA Synthetase Homologues

Potential \textit{P. aeruginosa} fadD homologues were identified through BLAST [34] utilizing \textit{E. coli} FadD sequence and alignment of \textit{E. coli} FadD ATP/AMP [19], [35–37] and fatty acid binding motifs [38] with the FadD motifs of \textit{P. aeruginosa} fadD homologues. Prediction of function of genes was obtained from Pseudomonas Genome Database (www.pseudomonas.com) [51]. PA2557, PA3860, and PA4198 were PCR amplified and cloned into pUC19 as BamHI fragments. The \textit{fadD} homologues PA1617, PA1997, PA2555, PA3568, PA2893, and PA3924, were PCR amplified and cloned into pUC19 as HindIII/EcoRI, BamHI/SmaI, HindIII/KpnI, HindIII/SalI, and XbaI/BamHI fragments, respectively. For functional complementation testing, pUC19 vectors containing PAO1 \textit{fadD} homologues were transformed into \textit{E. coli} \textit{fadD}2/fadR2 strain (E2011) and the resulting transformants were patched onto 1x M9 +1% (w/v) Brij-58 + ampicillin 100 \textmu g/ml supplemented with 20 mM glucose, 0.2% (w/v) oleate (C18:1 \textit{D}9), or decanoate (C10:0).

Single Copy Complementation of the \textit{E. coli} \textit{fadD}--/\textit{fadR}-- Mutant

To construct \textit{fadD}3, \textit{fadD}5, and \textit{fadD}6 single copy complementation vectors, first \textit{fadD}3, \textit{fadD}5, and \textit{fadD}6 PCR product were cloned into pET15b as NdeI/BamHI fragments. Next, the pUC19 vectors containing PAO1 \textit{fadD} homologues were transformed into \textit{E. coli} \textit{fadD}2/fadR2 strain (E2011) and the resulting transformants were patched onto 1x M9+1% (w/v) Brij-58+ ampicillin 100 \textmu g/ml supplemented with 20 mM glucose, 0.2% (w/v) oleate (C18:1 \textit{D}9), or decanoate (C10:0).
with XbaI. To construct miniTn7-fadD, the fadD PCR product was cloned as BamHI/blunt-end fragment into miniTn7-Gm’-FRT cassette inserted into fadD3

Various miniTn7 vectors were integrated into E2011 using pTNS2 [52]. For the complementation study, two colonies of K-12, E2011, E2011/attTn7::miniTn7-Gm’, E2011/attTn7::fadD, E2011/attTn7::fadD3, E2011/attTn7::fadD4, E2011/attTn7::fadD5, and E2011/attTn7::fadD6 were patched onto 1x M9 medium +1% (w/v) Brij-58+0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) supplemented with 0.2% (w/v) FAs or 20 mM glucose. Plates were incubated for three days at 37°C and bacterial growth was scored from +1 to +6. Very little growth was marked as +1 and very heavy growth on a patch comparable to 374 K12 on glucose at day three was marked as +6.

Construction of Mutant Strains of PAO1

The fadD3, fadD4, fadD5, and fadD6 gene replacement vectors were obtained as follows. pEX18T-fadD3-Gm’-pheSpa was constructed by digesting pUC19-PA3860 with MscI and SgrAI, blunt-ending, and ligation it with Gm’-pheSpa-FRT cassette that was Smal excised from pwFRT-Gm’-pheSpa. The PA3860-Gm’-pheSpa fragment was excised from the resulting vector using BamHI and cloned into pEX18T. Similarly, pEX18T-fadD3-Gm’-pheSpa was obtained by first sub-cloning fadD4 gene as a HindIII/EcoRI fragment from pUC19-PA1617 into pEX18T, and fadD4 was deactivated at the Xhol site by inserting the Gm’-pheSpa-FRT cassette Sall excised from pmFRT-Gm’-pheSpa. pEX18T-fadD5-Gm’ was constructed by cloning fadD5 PCR product (oligos #437 and #438) as BamHI/blunt-end fragment into pEX18T that was

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**Table 5. Plasmids used in this study.**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Lab ID</th>
<th>Relevant Properties</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miniCTX2</td>
<td>E0076</td>
<td>Tc’; P. aeruginosa site specific integration vector</td>
<td>[58]</td>
</tr>
<tr>
<td>miniCTX2-fadD2D1</td>
<td>E2143</td>
<td>Tc’; fadD2D1 cloned into miniCTX2</td>
<td>[14]</td>
</tr>
<tr>
<td>miniCTX2-fadD2D1D4</td>
<td>E2811</td>
<td>Tc’; fadD4 gene cloned into miniCTX2-fadD2D1</td>
<td>This study</td>
</tr>
<tr>
<td>miniCTX2-fadD4</td>
<td>E2589</td>
<td>Tc’; fadD4 gene cloned into miniCTX2</td>
<td>This study</td>
</tr>
<tr>
<td>miniTn7-Gm’</td>
<td>E2643</td>
<td>Ap’, Gm’, pUC18R6Kmini-Tn7 [52] with FRT-Gm’ cassette and lac promoter cloned</td>
<td>Laboratory collection</td>
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<tr>
<td>miniTn7-fadD3</td>
<td>E2645</td>
<td>Ap’, Gm’, fadD3 cloned into miniTn7-Gm’</td>
<td>This study</td>
</tr>
<tr>
<td>miniTn7-fadD4</td>
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<tr>
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<td>This study</td>
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<tr>
<td>miniTn7-PA3860</td>
<td>E2377</td>
<td>Ap’, Gm’, fadD3 with native rbs cloned into miniTn7-Gm’</td>
<td>This study</td>
</tr>
<tr>
<td>miniTn7-PA3924</td>
<td>E2854</td>
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<td>This study</td>
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<tr>
<td>miniTn7-fadD3-fadD5-fadD6</td>
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<td>pCD135K-flp-onIT</td>
<td>E0783</td>
<td>Sp’; suicidal Flp-expressing plasmid</td>
<td>[33]</td>
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<td>pET15b</td>
<td>E0047</td>
<td>Ap’, T7 expression vector</td>
<td>Novagen</td>
</tr>
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<td>Ap’, pET15b with fadD3 gene</td>
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<tr>
<td>pEX18T</td>
<td>E0055</td>
<td>Ap’, gene replacement vector</td>
<td>[53]</td>
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<td>pEX18T-fadD3-Gm’-pheSpa</td>
<td>E2438</td>
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<td>pEX18T-fadD6-Gm’</td>
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<td>pFLP2</td>
<td>E0067</td>
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<td>pPS65</td>
<td>E0050</td>
<td>Ap’, Gm’, plasmid with Gm’-FRT cassette</td>
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<td>pTN52</td>
<td>E1189</td>
<td>Ap’, helper plasmid for Tn7 transposition system</td>
<td>[52]</td>
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<td>pUC18-mucA’</td>
<td>E1907</td>
<td>Ap’, pUC18 with internal fragment of mucA cloned</td>
<td>[14]</td>
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<td>pUC19</td>
<td>E0014</td>
<td>Ap’, cloning vector</td>
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<td>Ap’, PAO1 PA3860 gene cloned into pUC19</td>
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<td>E2380</td>
<td>Ap’, Gm’, plasmid with Gm’-pheSpa-mFRT cassette</td>
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Abbreviations:
Ap’, ampicillin resistance; lac, E. coli lactose operon; rbs, ribosomal binding site; Sp’, streptomycin resistance.
doi:10.1371/journal.pone.0064554.005

Fatty Acyl-CoA Synthetases in P. aeruginosa

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digested with BamHI and Smal, and *fadD5* was deactivated at the blunt-ended XhoI site by inserting the Gm r- cassette SmaI excised cassette from pPS856. To construct pEX18T-*fadD5*-Gmr, PCR product (oligos #1093 and #512) was cloned as BamHI/ SmaI fragment into pEX18T that was digested with BamHI and BamHI-Smal digestion, various strains (PAO1, double, triple, quadruple, quintuple, and sextuple *fadD* mutants) were purified on LB. After 24 h incubation at 37 °C, two colonies of each strain were patched onto 1x M9 solid medium + 1% (w/v) Brij-58 supplemented with 0.2% (w/v) FAs or 20 mM glucose. Plates were incubated at 37 °C. 

**Table 6. Oligonucleotides primers utilized in this study.**

<table>
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<th>Primer number and name</th>
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<td>438; PA2893-BamHib, c</td>
<td>5'-CAGTATGCCACCGTGCTAGACGGGCTG-3'</td>
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<tr>
<td>512; PA3924-BamHib, c</td>
<td>5'-TGTCTGGAATCGGGTCGTTTACCTTACC3'-3'</td>
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<tr>
<td>1093; EcfadD-down-BamHib</td>
<td>5'-AACGGGATCTAGAGGCTGGTTCATG-3'</td>
</tr>
<tr>
<td>1109; PA3924-NDleib</td>
<td>5'-GTGACCCATATGCCGCGTGTGCTG-3'</td>
</tr>
<tr>
<td>1151; PA1221 BamHl-upib</td>
<td>5'-ACCGTGGATCCTCTGAGTTCGTTTACCTTACC3'-3'</td>
</tr>
<tr>
<td>1152; PA1221 BamHl-downid</td>
<td>5'-AGGCCGATCGTGCAGGATCGCATG-3'</td>
</tr>
<tr>
<td>1153; PA2557 BamHl-upib</td>
<td>5'-TGGGCGATCGCCTGCTGTTTACTC-3'</td>
</tr>
<tr>
<td>1154; PA2557 BamHl-downid</td>
<td>5'-GAAAGCGAAGGCCCACTCCTGAGGATCGCATG-3'</td>
</tr>
<tr>
<td>1155; PA3860 BamHl-upid</td>
<td>5'-GAACGGGATCTACTATATGTTGAGACTGATG-3'</td>
</tr>
<tr>
<td>1156; PA3860 BamHl-downid</td>
<td>5'-CTGAGGAAATCGACGCTGTTTACCTTACC3'-3'</td>
</tr>
<tr>
<td>1157; PA4198 BamHl-upid</td>
<td>5'-CCAGAAGGTGCGTTTACCTTACC3'-3'</td>
</tr>
<tr>
<td>1158; PA4198 BamHl-downid</td>
<td>5'-CGACACAGTCTGCTGATCGGATCGCATG-3'</td>
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<tr>
<td>1218; fadD-EC-HindIII-upid</td>
<td>5'-TCTATAAGTCTGGGGTGATTGAAACTG-3'</td>
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<td>1251; fadD3-NDleib</td>
<td>5'-AACCACATGTAATCGTCCCATTCAC3'-3'</td>
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<td>1252; PA3568-Up-HindIIIb</td>
<td>5'-ACTCCAAGCTCCATCTACGCTACTTAC-3'</td>
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<td>1253; PA3568-Down-Salib</td>
<td>5'-GGCTGCGTACGCGTGTGCTG-3'</td>
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<td>1254; PA1997-Up-HindIIIb</td>
<td>5'-CGCTGCTCCAGACGAGTACGGAGA-3'</td>
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<td>1255; PA1997-Down-Snal</td>
<td>5'-CTGAGGATGCGGATCTGTCG-3'</td>
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<td>1256; PA0996-Up-BamHib</td>
<td>5'-CTCTCGTGGTTGACCG-3'</td>
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<td>1257; PA0996-Down-BamHibid</td>
<td>5'-CCACCGGTATCTCCAGACACACGATTAGA-3'</td>
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<td>1258; PA2555-Up-HindIIib</td>
<td>5'-GCGTGAGCTTCCGCTACTCATA-3'</td>
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<td>1259; PA2555-Down-Kpig</td>
<td>5'-CCGCGGATCCGACGACCTCGTACCTTCTTAC-3'</td>
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<td>1260; PA1617-Up-HindIIid</td>
<td>5'-CTTGAAGCTCCTGGCCGCAGACTACA-3'</td>
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<td>1261; PA1617-Down-EcoRIb, c, d</td>
<td>5'-GTCTAGACTGTCGCGTCG-3'</td>
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<td>1441; PA1614-HindIIIc</td>
<td>5'-GAGCTCTGTAAGGAGGACGACCAAC-3'</td>
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<td>1444; PA1617-NDleib</td>
<td>5'-ATGCCATATGCTGACGTCAATGACACGACTACA-3'</td>
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<td>2109; PA2893-Up, d</td>
<td>5'-GGCTATTTGCCGAAGTGCACTGAACTGATGAT-3'</td>
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<tr>
<td>2110; PA3294-Up, d</td>
<td>5'-GGGATCCATGTAATCGTCCCATTCAC3'-3'</td>
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</table>

*Restriction enzyme sequences are underlined.

1Single copy complementation in *E. coli.*

2Single copy complementation in *P. aeruginosa.*

3fadD homologues cloning.

4doi:10.1371/journal.pone.0064554.t006

Growth Phenotypes of Multiple *fadD* Mutants on Fatty Acids

To assess involvement of *P. aeruginosa fadD* homologues in FAs degradation, various strains (PAO1, double, triple, quadruple, quintuple, and sextuple *fadD* mutants) were purified on LB. After 24 h incubation at 37 °C, two colonies of each strain were patched onto 1x M9 solid medium +1% (w/v) Brij-58 supplemented with 0.2% (w/v) FAs or 20 mM glucose. Plates were incubated at 37 °C for four days. Growth of each strain was scored from +1 (little growth) to +6 (very heavy growth comparable to PAO1 on glucose at 96 h).
Growth Curves Experiments

To further characterize various fadD mutants of *P. aeruginosa*, growth curve studies were performed using FAs as sole carbon source as described previously [14]. Doubling time of various strains in log-phase (Table S3) was calculated as follows: doubling time \(= \frac{0.301(t_2-t_1)}{\log OD_2 - \log OD_1} \) [57].

Growth of fadD Mutants on Acyclic Terpenes

The ΔfadD4ΔattB::fadD4 strain was constructed using a single copy complementation vector miniCTX2::fadD4, which was obtained by cloning the fadD4 PCR product (oligos #1443 and #1261) as HindIII and EcoRI fragment into miniCTX2 and integrated into ΔfadD4 mutant chromosome as described previously [56]. Stocks of citronellic (Sigma) and geranic acid (Sigma) were prepared by neutralizing the compounds with 3% (w/v) were prepared by neutralizing the compounds with equal molar sodium hydroxide and dissolving in 1% (w/v) Brij-58. PAO1 and various fadD mutants were grown overnight (14–16 h), starter culture were prepared as described by Kang et al. [14] and inoculated at 200-fold dilution into 1x M9 minimal medium +1% (w/v) Brij-58 supplemented with 0.1% (w/v) of citronellic acid, 0.1% (w/v) geranic acid or 20 mM glucose. Triplicate cultures were shaken at 30°C and optical densities were measured at day one and day six.

Virulence Factors Production

Lipase, protease, phospholipase, and rhamnolipid productions by fadD mutants were tested as previously described [14].

In vitro and in vivo Competition Studies

For in vitro and in vivo competition studies, the ΔfadD1D2D3D4D5D6 strain was complemented with fadD2D1 and fadD4 cloned into miniCTX2 and fadD4, fadD5, and fadD6 cloned into miniTn7-Gm'. MiniCTX2-fadD2D1D4 complementation vector, was constructed by cloning fadD4 gene PCR product (oligos #1443 and #1261) as HindIII/blunt-end fragment into miniCTX2-fadD2D1 digested with XhoI, blunt-ended and digested with HindIII. To construct miniTn7-fadD3-fadD5-fadD6 vector, first fadD3 was sub-cloned as BamHI fragment from pUC19-PA3890 into miniTn7-Gm', resulting in miniTn7-PA3860. The fadD6 was amplified with oligos #512 and #2210 and cloned as a BamHI/XbaI fragment into miniTn7-PA3860, resulting in miniTn7-PA3924. The fadD5 was amplified with oligos #438 and #2109 and digested with BamHI and XbaI, and cloned as BamHI/XbaI fragment into miniTn7-PA3924, resulting in miniTn7-PA3924. The fadD4 was amplified with oligos #1443 and #2210 and cloned as a BamHI/XbaI fragment into miniTn7-Gm', resulting in miniTn7-PA3860. The fadD6 was amplified with oligos #512 and #2210 and cloned as a BamHI/XbaI fragment into miniTn7-Gm', resulting in miniTn7-PA3860. The fadD5 was amplified with oligos #438 and #2109 and digested with BamHI, blunt-ended and digested with XbaI. To construct the final vector, the miniTn7-PA3924 was digested with XbaI, blunt-ended and digested with NdeI and the 2.5 kb fragment (containing fadD6) was cloned simultaneously along with fadD5 fragment into miniTn7-PA3860 digested with NdeI and SpeI. Integration of these plasmids into the *P. aeruginosa* chromosome was performed as described previously [58] and [52].

The in vitro competition between ΔfadD1D2D3D4D5D6 and its complement (strain P1021) on LB, or casamino acids (CAA), choline, glucose, glycerol, oleate (C18:1 Δ9) or PC was performed as described previously [14].

The in vivo competition study was performed as previously described [14]. Briefly, mucA was inactivated in the PAO1-ΔfadD1D2D3D4D5D6 and its complement strains utilizing pUC18-mucA'. Equal amounts of alginate overproducing sxtPE mutants and its complement were resuspended in their own supernatants and mixed. Fourteen BALB/c mice were inoculated intratracheally with 6 x 10^9 colony forming units (CFU) of mixture of mutant (strain P973) and complement (strain P1020) as described previously [14]. At each time point (24 h and 48 h) seven mice were humanly euthanized, lungs were homogenized in 0.85% (w/v) saline and serial dilutions were plated on LB and LB-tetracycline 100 μg/ml to determine the total CFU and the complemented strain CFU. The competitive index (CI) was calculated as described [14].

Supporting Information

Figure S1 Alignment of motifs of potential fatty acyl-CoA synthetase homologues. Amino acids with similar properties are assigned the same colors using CLC Sequence Viewer 6 software (www.clcbio.com).

Figure S2 Growth phenotypes of various fadD homologues mutants on acyclic terpenes at day six. Strains were grown in liquid 1x M9 medium +1% (w/v) Brij-58 supplemented with 0.1% (w/v) of citronellic acid or 0.1% (w/v) geranic acid at 30°C. Optical densities (ODs) of cultures were measured and compared to PAO1.

Table S1 Potential FadD homologues of *P. aeruginosa* identified through BLAST and tested for complementation in *E. coli fadD"fadR"* (E2011).

Table S2 Additional strains utilized in this study.

Table S3 Doubling time in minutes (min) of various strains in log-phase were calculated from growth curves in Fig. 2.

Acknowledgments

We thank Patrick Videau for cloning and screening four of the eleven potential fadD homologues. We also wish to thank Mike Son and Geraldine Cadaline for their assistant in creation of three mutant strains. We are grateful to Chad B. Walton for his assistance with the animal study.

Author Contributions

Conceived and designed the experiments: JZS MHN YK TTH. Performed the experiments: JZS MHN. Analyzed the data: JZS MHN YK TTH. Contributed reagents/materials/analysis tools: JZS MHN YK ZS APB IM. Wrote the paper: JZS TTH. Edited manuscript: JZS MHN YK ZS APB IM.

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Blocking Phosphatidylcholine Utilization in *Pseudomonas aeruginosa*, via Mutagenesis of Fatty Acid, Glycerol and Choline Degradation Pathways, Confirms the Importance of This Nutrient Source *In Vivo*

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Abstract

*Pseudomonas aeruginosa* can grow to very high-cell-density (HCD) during infection of the cystic fibrosis (CF) lung. Phosphatidylcholine (PC), the major component of lung surfactant, has been hypothesized to support HCD growth of *P. aeruginosa in vivo*. The phosphorylcholine headgroup, a glycerol molecule, and two long-chain fatty acids (FAs) are released by enzymatic cleavage of PC by bacterial phospholipase C and lipases. Three different bacterial pathways, the choline, glycerol, and fatty acid degradation pathways, are then involved in the degradation of these PC components. Here, we identified five potential FA degradation (Fad) related *fadBA*-operons (*fadBA-1*–*5*, each encoding 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA thiolase). Through mutagenesis and growth analyses, we showed that three (*fadBA145*) of the five *fadBA*-operons are dominant in medium-chain and long-chain Fad. The triple *fadBA145* mutant also showed reduced ability to degrade PC in vitro. We have previously shown that by partially blocking Fad, via mutagenesis of *fadBAS* and *fadD*, we could significantly reduce the ability of *P. aeruginosa* to replicate on FA and PC in vitro, as well as in the mouse lung. However, no studies have assessed the ability of mutants, defective in choline and/or glycerol degradation in conjunction with Fad, to grow on PC or in vivo. Hence, we constructed additional mutants (Δ*fadBA145*Δ*glpD*, Δ*fadBA145*Δ*betAB*, and Δ*fadBA145*Δ*betABΔglpD*) significantly defective in the ability to degrade FA, choline, and glycerol and, therefore, PC. The analysis of these mutants in the BALB/c mouse lung infection model showed significant inability to utilize PC in vitro, resulted in decreased replication fitness and competitiveness in vivo compared to the complement strain, although there was little to no variation in typical virulence factor production (e.g., hemolysin, lipase, and protease levels). This further supports the hypothesis that lung surfactant PC serves as an important nutrient for *P. aeruginosa* during CF lung infection.

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Introduction

*Pseudomonas aeruginosa* is widespread in nature, inhabiting soil, water, plants and animals. In hospitals, it can be found in sinks, respirators, humidifiers and occasionally on the hands of medical personnel [1,2]. The ubiquitous nature of this bacterium has allowed it to adapt to a broad range of hosts in which it can cause diseases. The role of *P. aeruginosa* as an opportunistic human pathogen is of particular concern, especially because it is a frequent cause of nosocomial infections such as pneumonia, urinary tract infections, and bacteremia [1,3,4]. *P. aeruginosa* infection in the respiratory tract of cystic fibrosis (CF) patients causes a rapid deterioration in lung function and thus patient survival [5,6]. The pathogenicity of *P. aeruginosa* infection in CF patients has been extensively studied in terms of biofilm production [7–9] and quorum sensing (QS) controlled virulence [10–12]. However, little effort has been placed towards the contribution of *P. aeruginosa* nutrient acquisition aids high-cell-density (HCD) replication during lung infection.

Previous studies have shown that *P. aeruginosa* can undergo HCD replication in the lung of CF patients reaching >10^9 CFU/ml [13–15]. HCD replication is highly energy demanding,
Phosphatidylcholine Utilization in P. aeruginosa

Results and Discussion

P. aeruginosa has five fadBA-operators potentially involved in fatty acid degradation

The well-established aerobic fatty acid degradation (Fad) pathway in E. coli was used as a model to characterize P. aeruginosa Fad. E. coli possesses only a single copy of each fad gene for aerobic Fad [28,29], and the cyclic degradation of fatty acids by two carbons per cycle is primarily catalyzed by an acyl-CoA dehydrogenase coded by fadE, and the products of the fadB-operators, 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA thiolase, respectively. Up to five potential fadBA-operators were identified in P. aeruginosa by BLAST analysis of the E. coli fadBA sequence against the P. aeruginosa genome, including fadBA1 (PA1737, PA1736), fadBA2 (PA3590, PA3589), fadBA3 (PA2554, PA2553), fadBA4 (PA1786, PA4785), and fadBA5 (PA3014, PA3013) (Fig. S1). Of these five FadBAs, FadBA5 showed the greatest homology to the E. coli FadBA with FadB5 having 72% similar (34% identical) and FadA3 having 76% similar (61% identical) to the E. coli FadBA, respectively [30,31].

Considering the larger size of the P. aeruginosa genome (6.29 Mb) [32] and its wide range of environmental niches and metabolic capabilities, it is not surprising that P. aeruginosa has up to five fadBA-1 operon homologues. Therefore, it is important next to narrow down which of these five operons are important in Fad, using a mutagenesis approach.

P. aeruginosa fadBA145-operators are important for degrading PC and medium- and long-chain fatty acids

Our previous work showed that the ΔfadBA5 mutant has a reduced ability to utilize LCFA as a sole carbon source, but this ΔfadBA5 mutant can still grow on LCFA as a carbon source, indicating the existence of other potential fadBA-operators in P. aeruginosa for LCFA degradation [31]. Further supporting this idea, the fadBA1-1 operon was shown to be induced by medium-chain fatty acids (MCFA) and to a lesser extent by LCFA [33]. The fadBA5-1 operon plays the most significant role in LCFA degradation, because neither the single ΔfadBA1 mutant, nor the single ΔfadBA4 mutant, showed much defects in their ability to utilize MCFA and LCFA as sole carbon sources (Fig. S2). It is possible that the FadBA1 and/or other FadBAs might have overlapping functions with FadBA5 in the metabolism of different chain length FAs, but these activities are masked by the more dominant FadBA5. Evidence for the involvement of other FadBAs is lacking, and needs to be addressed.

Because it is too overwhelming to test all possible double, triple, and quadruple fadBA-mutant combinations and the complicated dominance of fadBA5, we demonstrated the involvement of each fadBA-1 operon by testing different triple mutant combinations. In this study, we generated several triple mutants (ΔfadBA125, ΔfadBA135, ΔfadBA145, ΔfadBA225, ΔfadBA245, and ΔfadBA345) and a quintuple mutant ΔfadBA1-5 (Table 1) for growth analysis on MCFA and LCFA as sole carbon sources, to further characterize the function of these fadBA-1 operons. The growth defects were previously defined by the slower growth rate and lower overall final cell densities compared to wildtype strain PAO1, which suggest a reduced ability to metabolize these FAs, presumably due to the accumulation of growth inhibiting intermediates [17,31]. Significant growth defects were observed for any combinations of triple mutants in which both the fadBA5-1 and fadBA1-1 operons were deleted (i.e., ΔfadBA125, ΔfadBA135 and ΔfadBA145 mutants), revealing the importance of fadBA1 and fadBA5 contributing to growth on MCFA and LCFA (Fig. 2A–D). Although the level of defects is different for each type of FA used, the trend is consistent for these mutants in all FAs (Fig. 2A–D). Only the ΔfadBA145 triple mutant showed the same growth defect as the ΔfadBA1-5 quintuple mutant on all fatty acids tested, suggesting the importance of all three fadBA1, fadBA4, and fadBA5 operons and the minor role of fadBA2 and fadBA3-operators in metabolizing MCFA and LCFA. The importance of fadBA4 was further confirmed by the fact that the ΔfadBA245 and ΔfadBA345 triple mutants showed an additional growth defect on all FAs compared to the ΔfadBA2345 mutant (Fig. 2A–D). However, the fadBA4-1 operon displays less of an involvement in metabolizing all FAs tested compared to both fadBA1 and fadBA5-1 operons (i.e., comparing ΔfadBA245 to ΔfadBA125 or ΔfadBA345 to ΔfadBA135). Knowing that only fadBA1, fadBA4, and fadBA5 are important for degrading...
Figure 1. Phosphatidylcholine (PC) degradation pathways in *Pseudomonas aeruginosa*. (A) PC is the main component of lung surfactant and can be cleaved by phospholipase C and lipases, producing free fatty acids, glycerol, and phosphorylcholine. Three different pathways then further metabolize each component: the bet pathway for choline head group metabolism, the glp pathway for glycerol metabolism, and the β-

...
oxidation pathway for the degradation of the FAs. (B) The proposed \textit{P. aeruginosa} FA $\beta$-oxidation pathway. Abbreviations: FadA, 3-ketoacyl-CoA thiolase; FadB, cis-$\Delta^2$-trans-$\Delta^2$-enoyl-CoA isomerase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA epimerase, and 3-hydroxyacyl-CoA dehydrogenase; FadD, fatty acyl-CoA synthetase; FadE, acyl-CoA dehydrogenase; FadL, outer membrane long-chain fatty acid translocase; OM, out membrane; IN, inner membrane.

doi:10.1371/journal.pone.0103778.g001

MCFa and LCFa of PC, we continued with further experiments from this point forward in our study using the $\Delta$fadBA145 mutant background, rather than the $\Delta$fadBA1-5 quintuple mutant.

We next tested the $\Delta$fadBA145 mutant for its ability to grow on PC. The $\Delta$fadBA145 triple mutant displayed a reduced growth rate and lower final cell density as compared to wildtype PAO1 when grown with PC as a sole carbon source (Fig. 3A). There was no growth defect observed for this mutant when grown in control LB medium (Fig. 3B). The $\Delta$fadBA145 mutant strain is competitively less fit than the complement in the mutant background, rather than the $\Delta$fadBA145 mutant strain by integrating miniCTX2-$\Delta$fadBA5 mutant strain on PC observed in figure 3A is due to a decreased ability to degrade LCFs of PC and not glycerol or choline. All the evidence we have here strongly suggests the involvement of the three $\Delta$fadBA,4,5-operons in Fad and PC degradation. We complemented $\Delta$fadBA145 mutant strain by integrating miniCTX2-fadBA45 (i.e., the dominant fadBA operon as explained above) as a single copy into the $\Delta$fadBA145 mutant background (Table 1). The complemented strain was fully restored to wildtype growth on PC (Fig. 3A) and FAs (not shown). Hence, all complementation experiments in this study for any $\Delta$fadBA145 mutant background were performed with only the dominant fadBA5-operon.

Mutants blocked in FA, glycerol, and choline degradation displayed dramatically reduced ability to utilize PC in vitro

The enzymatic activity of phospholipase C on PC releases the phosphorylcholine headgroup and the diacylglycerol (DAG) molecule (Fig. 1A). The phospholipase headgroup is first transported across the cell membrane and dephosphorylated by a phosphatase [23,34,35] to yield choline, which has previously been shown to be sufficient for \textit{P. aeruginosa} to grow on a sole carbon, nitrogen, and energy source [36]. \textit{P. aeruginosa} BetAB (encoding choline dehydrogenase and a glycine betaine aldehyde dehydrogenase) catalyzes the conversion of choline to glycine betaine [23]. Glycine betaine is successively demethylated to form dimethylglycine (DMG), sarcosine (monomethylglycine), and finally glycine [24,37]. The DAG molecule is cleaved by the \textit{P. aeruginosa} lipase, liberating a glycerol molecule and two LCFS. Glycerol metabolism has been well characterized in \textit{P. aeruginosa}. The operon primarily consists of \textit{glpD} (a sn-glycerol-3-phosphate dehydrogenase [38]), \textit{glpF} (a membrane-associated glycerol diffusion facilitator [27,39]), \textit{glpK} (a glycerol kinase [27,39]), \textit{glpM} (a membrane protein affecting alginic acid synthesis [26]), and \textit{glpR} (a regulator of the \textit{glp} operon [25]).

Since our previous data showed that \textit{betAB} and \textit{glpD} were expressed \textit{in vivo} [15], they may potentially be involved in PC degradation during lung infection. However, before we could address the \textit{in vivo} aspect of PC degradation, further experiments are needed to characterize \textit{P. aeruginosa} PC degradation \textit{in vitro}. We engineered double pathway mutants $\Delta$fadBA145$\Delta$betAB (blocked in FA and choline degradation) and $\Delta$fadBA145$\Delta$glpD (blocked in FA and glycerol degradation) and a triple pathway mutant $\Delta$fadBA145$\Delta$betAB$\Delta$glpD (blocked in FA, choline, and glycerol degradation) (Table 1), to further determine whether these mutants are deficient in growth on PC. As expected, all three mutants experienced various growth defects with decreased cell density and delayed log-phase when grown on PC (Fig. 3A). The triple pathway mutant $\Delta$fadBA145$\Delta$betAB$\Delta$glpD exhibited the most significant reduced ability to utilize PC, reaching to only about one-third of the wildtype final cell density. We complemented these mutants (i.e., $\Delta$fadBA145$\Delta$betAB, $\Delta$fadBA145$\Delta$glpD, and $\Delta$fadBA145$\Delta$betAB$\Delta$glpD) by integrating the respective miniCTX2-fadBA5/betAB, miniCTX2-fadBA5/glpD or miniCTX2-fadBA5/betAB/glpD as a single copy on the \textit{P. aeruginosa} chromosome and the complemented strains fully recovered these mutants’ ability to grow on PC as compared to wildtype PAO1 (Fig. 3A). No mutants or complement showed any growth defects on control LB medium (Fig. 3B).

We performed an \textit{in vitro} competition study between pathway mutants ($\Delta$fadBA145$\Delta$betAB, $\Delta$fadBA145$\Delta$glpD, $\Delta$fadBA145$\Delta$betAB$\Delta$glpD) and their complements to examine whether the mutation reduced their ability to metabolize various carbon sources. As expected, all the pathway mutants were less competitive than their respective complements in media containing PC and other sole carbon sources involved in the respective pathways (Fig. 4A panels ii-iv). For example, the $\Delta$fadBA145$\Delta$betAB mutant was less competitive than its complement when grown using PC, FA, or choline, as sole carbon source (Fig. 4A panel ii), which is not the case in other control media (e.g., LB, glucose, CAA or glycerol). Likewise, the $\Delta$fadBA145$\Delta$glpD mutant was less competitive than its complement only if PC, FA, or glycerol was used as sole carbon source (Fig. 4A panel iii). The triple pathway mutant was almost completely outcompeted by its complemented strain with the competitive indices (CI) dramatically reduced to ~0.1 when growing in the media containing PC, choline, glycerol, or oleate FA (Fig. 4A panel iv). Overall, these \textit{in vitro} data showed that we have three mutants ($\Delta$fadBA145$\Delta$betAB, $\Delta$fadBA145$\Delta$glpD, $\Delta$fadBA145$\Delta$betAB$\Delta$glpD) and their complement that could be used to assess the utilization of PC \textit{in vivo}, through competitive index experiments within the mouse lung.

Blocking FA, glycerol, and choline degradation significantly reduces replication fitness of \textit{P. aeruginosa} \textit{in vivo}

Since PC is the major component of lung surfactant in mammals, including mice [21], a mouse lung infection model [40] was utilized for \textit{in vivo} competition study to evaluate the fitness of the PC degradation pathway mutants within the lung environment. The mucoid, exopolysaccharide alginate-overproducing phenotype is a distinguishing feature of \textit{P. aeruginosa} isolated from CF patients [40,41]. An alginate-overproducing strain carrying a mucA insertion mutant, which allows the mucoid strain to survive and replicate in the lung, has been successfully used in BALB/c mouse model to establish the connection between nutrient acquisition and \textit{in vivo} lung fitness for \textit{P. aeruginosa} [17]. Therefore, we constructed various mucA$^{-}$ alginate-overproducing strains, such as $\Delta$fadBA145-mucA$^{-}$, $\Delta$fadBA145$\Delta$glpD-mucA$^{-}$, $\Delta$fadBA145$\Delta$betAB-mucA$^{-}$, $\Delta$fadBA145$\Delta$betAB$\Delta$glpD-mucA$^{-}$ and their complemented strains for...
this study. Prior to the animal study, the phenotypes of all mucA strains were confirmed by patching on minimal media plates with FA, choline, or glycerol as sole carbon sources along with all appropriate controls (all mucA wild-type strains and complemented strains). As expected, the mucA mutation did not affect the metabolism of any of these carbon sources (Fig. S3). As previously described [17,40], BALB/c mice were inoculated via intratracheal intubation with equal ratios of each mutant and its complement pair (6×10⁶ CFU/animal). At 24 h post-infection, bacterial CFU recovered from the lungs were determined, followed by CI calculations. For all the strains, the average total CFU per mouse lung recovered at 24 hours post-infection was greater than the
Table 1. Bacterial strains used in this studya.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lab IDb</th>
<th>Genotype/Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli</td>
<td>E1231</td>
<td>F− λ− mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80dlacZ ΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δara, leu7697 galU galK rpsL supG</td>
<td>BioRad</td>
</tr>
<tr>
<td>SM10</td>
<td>E006</td>
<td>thi thr leu tonA lacY supE recA:RP4-2Tc:Mu KmA</td>
<td>[48]</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA01</td>
<td>P007</td>
<td>Prototroph</td>
<td>[49]</td>
</tr>
<tr>
<td>PA01- mucA^b</td>
<td>P447</td>
<td>Cb^*, PA01 with pUC18 inserted in mucA gene</td>
<td>This study</td>
</tr>
<tr>
<td>ΔfadBA125</td>
<td>P122</td>
<td>Gm^*, PA01-ΔfadBA1::FRT, ΔfadBA2::FRT, ΔfadBA5::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>ΔfadBA135</td>
<td>P124</td>
<td>Gm^*, PA01-ΔfadBA1::FRT, ΔfadBA3::FRT, ΔfadBA5::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>ΔfadBA145</td>
<td>P319</td>
<td>Gm^*, PA01-ΔfadBA1::FRT, ΔfadBA4::FRT, ΔfadBA5::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>ΔfadBA235</td>
<td>P317</td>
<td>Gm^*, PA01-ΔfadBA2::FRT, ΔfadBA3::FRT, ΔfadBA5::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>ΔfadBA245</td>
<td>P130</td>
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<td>P555</td>
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<td>This study</td>
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<td>P561</td>
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<td>This study</td>
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<td>This study</td>
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<td>Gm^<em>, Tet^</em>, ΔfadBA145 ΔglpD complemented with miniCTX2-fadBA5/ΔglpD</td>
<td>This study</td>
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<td>This study</td>
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<td>This study</td>
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<td>This study</td>
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<td>P570</td>
<td>Gm^<em>, Cb^</em>, ΔfadBA145 ΔglpD with pUC18 inserted in mucA gene</td>
<td>This study</td>
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<td>P572</td>
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<td>P574</td>
<td>Gm^<em>, Cb^</em>, ΔfadBA145 ΔbetAB ΔglpD with pUC18 inserted in mucA gene</td>
<td>This study</td>
</tr>
<tr>
<td>ΔfadBA145- mucA^b complement</td>
<td>P584</td>
<td>Gm^<em>, Cb^</em>, Tet^*, ΔfadBA145-mucA^b complemented with miniCTX2-fadBA5</td>
<td>This study</td>
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<tr>
<td>ΔfadBA145 ΔglpD- mucA^b complement</td>
<td>P578</td>
<td>Gm^<em>, Cb^</em>, Tet^*, ΔfadBA145 ΔglpD-mucA^b complemented with miniCTX2-fadBA5/ΔglpD</td>
<td>This study</td>
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<tr>
<td>ΔfadBA145 ΔbetAB- mucA^b complement</td>
<td>P580</td>
<td>Gm^<em>, Cb^</em>, Tet^*, ΔfadBA145 ΔbetAB-mucA^b complemented with miniCTX2-fadBA5/ΔbetAB</td>
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<tr>
<td>ΔfadBA145 ΔbetAB ΔglpD- mucA^b complement</td>
<td>P582</td>
<td>Gm^<em>, Cb^</em>, Tet^*, ΔfadBA145 ΔbetAB ΔglpD-mucA^b complemented with miniCTX2-fadBA5/ΔbetAB/ΔglpD</td>
<td>This study</td>
</tr>
</tbody>
</table>

^aFor strains constructed in this study, please see text for further details.

^bPlease use Lab ID for requesting strains.

doi:10.1371/journal.pone.0103778.t001

initial inoculum (Fig. 4B), indicating that all these P. aeruginosa strains maintained the ability to replicate within the mouse lung. The ΔfadBA145 mutant still replicated significantly in vivo compared to its complement. Surprisingly, the ΔfadBA145 CI is quite high compared to other FAD mutants (i.e., fadD mutants) we have previously published where CI is approximately 0.5 [17,18]. The ΔfadBA145 ΔbetAB, ΔfadBA145 ΔglpD in vivo CI is lower than the ΔfadBA145 when compared to their respective complements (Fig. 4B), showing the importance of glycerol and choline degradation as potential nutrient sources in vivo. Most significantly, the mean CI value for the triple pathway mutant (i.e., ΔfadBA145 ΔbetABΔglpD) showed that the triple pathway mutant had a significantly reduced ability to survive and multiply in the lungs of mice compared to its complement.

We monitored all strains tested in vivo for different virulence expression, including proteases, rhamnolipid, hemolysins and lipases (Fig. S4). With similar level of these common secreted virulence factors observed between strains (Fig. S2), the low CI is most likely due to its inability to metabolize PC and the three components of PC (LCFAs, glycerol, and phosphorylcholine) as a nutrient source, rather than resulting from altered virulence expression. Overall, the altered ability for the pathway mutants to metabolize PC as nutrient in vivo was clearly mirrored by their competitive fitness within the lung.
In summary, *P. aeruginosa* possesses an impressive repertoire of virulence factors, and the expression of most of these only occurs during the HCD replication and their timely expression is regulated by QS [42], which occurs at HCD. *P. aeruginosa* requires large amount of readily available energy to reach and maintain HCD and produce the high-energy dependent virulence structure like biofilm. Thus, exploration of the nutrient sources supporting such an energy intensive processes is of importance, especially for chronic *P. aeruginosa* lung infections in CF patients. 

In addition, the identification of the genes and pathways for *P. aeruginosa* HCD replication in CF lungs provides fundamental knowledge for possibly developing new therapeutic strategies targeting bacterial nutrient metabolism in the lung, thereby preventing bacterial HCD. The expression of genes involved in *P. aeruginosa* PC degradation within the lungs of CF patients has been previously demonstrated [15]. Our study focused on providing further evidence to determine whether PC serves as a significant nutrient source during *P. aeruginosa* lung infection. In order to decipher the role of PC in vivo, we first characterized PC degradation pathways in vitro. Of the three components released by the enzymatic cleavage of PC by bacterial phospholipase C and lipases (phosphorylcholine, LCFAs, and glycerol), LCFAs are highly reduced and yield the most carbon and energy. In our study, five potential *fadBA*-operons were investigated and three of them (i.e., *fadBA*1,4,5-operons) proved to be significantly involved in Fad. The *in vitro* growth analysis of different pathway mutants (*fadBA*145, *fadBA*145ΔbetAB, *fadBA*145ΔglpD, *fadBA*145ΔbetABΔglpD) on PC provided direct evidence to support that *P. aeruginosa* utilizes the FA, glycerol and choline degradation pathways to degrade individual components of PC.

**Figure 3. Growth analysis on phosphatidylcholine.** (A) Some mutants exhibited growth defects on PC as a sole carbon source. The growth defects were fully recovered in complemented strains, as they had identical growth rates compared to the wildtype PAO1 strain. (B) No growth defects in control LB medium were observed.

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Our in vivo competition study was performed utilizing a mouse lung infection model [40] to evaluate the fitness of the pathway mutants within the lung environment. The triple pathway mutant ΔfadBA145ΔbetABΔglpD exhibited the greatest growth defect on relevant carbon sources in vitro and was outcompeted by its complement in vivo. Since no altered expression of virulence factors was observed for all the pathway mutants and their complement pairs compared to wildtype PAO1, it is highly likely that the decreased ability to utilize PC resulted in lower replication fitness in the lung environment. This study strongly supports the hypothesis that P. aeruginosa utilizes lung surfactant PC as one of the nutrient sources for chronic lung infection.
Materials and Methods

Ethic Statement
All animal experiments were performed in compliance with the NIH (National Institutes of Health) Guide for the Care and Use of Laboratory Animals and were approved by the University of Hawaii Institutional Animal Care and Use Committee (protocol no. 06-023-04).

Bacterial strains and growth conditions
Bacterial strains and plasmids utilized in this study are listed in Table 1 and 2. E. coli EP-Max10B was used as cloning strains and cultured in Luria-Bertani (LB) medium (Difco), Pseudomonas Isolation Agar or Broth (PIA or PIB; Difco) or LB medium were used to culture P. aeruginosa strain PAO1 and derivatives. All fatty acids (FAs) stocks were made as previously described [17]. Strains for growth analyses were cultured in 1 x M9 minimal medium supplemented with each of C12:0 (medium-chain fatty acid), C16:0, and C18:1 (LCFAs) as a sole carbon source. The single copy integration vector, miniCTX2, was used to engineer the complemented strains for each triple-pathway mutant.

General molecular methods
Oligonucleotides were synthesized through Integrated DNA Technology and are listed in Table 3. All molecular methods and their components utilized were employed as previously described [44].

Construction of mutants and complementation strains
All mutants were constructed as described previously [45]. Briefly, the fadBA (fadBA1, fadBA2, fadBA3, fadBA4, fadBA5) operons, betAB operon, and gldP gene were amplified by PCR using respective upstream and downstream primer pair listed in Table 3. The PCR products were purified from the gel, digested with appropriate restriction enzymes, and cloned into the gene replacement vector pEX18T, digested with the same restriction enzymes, to yield each of the pEX18T-target gene constructs. After deletions were made on plasmid in each of the fadBA-operons, the gldP gene, and the betAB-operon through restriction digestion [fadBA1: PstI, BamHI; fadBA2: StuI, BamHI; fadBA3: NolI, SmaI; fadBA4: EcoRV; fadBA5: SphI, PstI] and blunted (except for gldP, which was blunt-ended using SmaI), the 1.1 kb FRT-Gm-’FRT cassette obtained from pPS856 digested with SmaI was inserted into each gene. These newly constructed pEX18T vectors were transformed into E.coli SM10 or ER2566-mob, and conjugated into PAO1 to engineer the unmarked mutations as previously described [45]. To obtain all triple mutants, we invested an enormous amount of work to first create all single and double mutants with the proper confirmed Fip/FRT-excision of the gentamicin antibiotic resistance cassette to recycle this resistance marker for subsequent mutagenesis (data not shown).

The single copy integration vector, miniCTX2, was used to engineer the complemented strains for each triple-pathway mutant

**Table 2. Plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Lab ID</th>
<th>Relevant properties</th>
<th>Reference</th>
</tr>
</thead>
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<td>pFlp2</td>
<td>E0067</td>
<td>Apr, sacB; Flp-containing plasmid</td>
<td>[45]</td>
</tr>
<tr>
<td>pPS856</td>
<td>E0050</td>
<td>Apr; Gm; plasmid with Gm'-FRT-cassette</td>
<td>[45]</td>
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<tr>
<td>pUC18</td>
<td>E0135</td>
<td>Apr; cloning vector</td>
<td>[50]</td>
</tr>
<tr>
<td>pUC18-'mucA'</td>
<td>E1907</td>
<td>Apr; mucA internal region cloned into pUC18</td>
<td>This study</td>
</tr>
<tr>
<td>pUC19</td>
<td>E0014</td>
<td>Apr; cloning vector with Ppa</td>
<td>[50]</td>
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<tr>
<td>pUC19-gldP</td>
<td>E1843</td>
<td>Apr; pUC19 with gldP gene cloned in downstream of Ppa</td>
<td>This study</td>
</tr>
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<td>pEX18T</td>
<td>E0055</td>
<td>Apr, ornT, sacB; gene replacement vector</td>
<td>[45]</td>
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<tr>
<td>pEX18TfadBA1::Gm</td>
<td>E0202</td>
<td>Apr, Gm; pEX18T with fadBA1 operon with Gm'-FRT-cassette insertion</td>
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</tr>
<tr>
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<tr>
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<tr>
<td>pEX18TfadBA5::Gm</td>
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<td>pEX18TgldP::Gm</td>
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<td>E1070</td>
<td>Apr, Gm; pEX18T with betAB operon with Gm'-FRT-cassette insertion</td>
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<td>miniCTX2</td>
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<td>Tet; site-specific integration vector</td>
<td>[46]</td>
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<td>miniCTX2-fadBA5::gldP</td>
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<td>Tet; miniCTX2 with cloned fadBA5::gldP</td>
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<td>Tet; miniCTX2 with cloned fadBA5::betAB::gldP</td>
<td>This study</td>
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</table>

*For plasmids constructed in this study, please see text for further details.

[Please use Lab ID for requesting plasmids. doi:10.1371/journal.pone.0103778.t002]
as previously described [46]. Briefly, *fadBA5* and *betAB* were PCR amplified with primers 888/889 and 522/895, respectively. The miniCTX2-*fadBA5* fragment was inserted into miniCTX2, both digested with *SacI* and *BamHI*, yielding miniCTX2-*fadBA5*-betAB-glpD, which was cloned into miniCTX2, respectively.

**Table 3. Primers used in this study.**

<table>
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<tr>
<th>Primer number and name</th>
<th>Sequence*</th>
</tr>
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<td>186; <em>fadBA1</em>-upstream</td>
<td>5’-CGAAGCTTGAAGGTGCTATCTTCC-3’</td>
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<td>187; <em>fadBA1</em>-downstream</td>
<td>5’-GGCGGATTCTCCTACACG-3’</td>
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<tr>
<td>218; <em>fadBA2</em>-upstream</td>
<td>5’-GGGCGAGCTTCG-3’</td>
</tr>
<tr>
<td>219; <em>fadBA2</em>-downstream</td>
<td>5’-GGGCGAGCTTCG-3’</td>
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<td>220; <em>fadBA3</em>-upstream</td>
<td>5’-GGGCGAGCTTCG-3’</td>
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<td>221; <em>fadBA3</em>-downstream</td>
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<td>221; <em>fadBA4</em>-upstream</td>
<td>5’-GGGCGAGCTTCG-3’</td>
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<tr>
<td>222; <em>fadBA4</em>-downstream</td>
<td>5’-GGGCGAGCTTCG-3’</td>
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<td>272; <em>fadBA5</em>-HindIII</td>
<td>5’-AGTCTAGATCTCATTAGCC-3’</td>
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<td>273; <em>fadBA5</em>-EcoRI</td>
<td>5’-GGGCGAGCTTCG-3’</td>
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<td>518; <em>glpK</em>-BamHI</td>
<td>5’-AGTCTAGATCTCATTAGCC-3’</td>
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<tr>
<td>519; <em>glpK</em>-SacI</td>
<td>5’-CTGGCGGAGCTTCG-3’</td>
</tr>
<tr>
<td>522; <em>betA</em>-SacI</td>
<td>5’-CAACGAGCTTCG-3’</td>
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<tr>
<td>523; <em>betAB</em>-HindIII</td>
<td>5’-GGGCGAGCTTCG-3’</td>
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<tr>
<td>888; Xho-<em>fadBA5</em></td>
<td>5’-CTGGCGGAGCTTCG-3’</td>
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<tr>
<td>889; <em>fadBA5</em>-Bam</td>
<td>5’-GGGCGAGCTTCG-3’</td>
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<tr>
<td>895; Spe-<em>betAB</em></td>
<td>5’-CTGGCGGAGCTTCG-3’</td>
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<td>896; Hind-<em>glpD</em></td>
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<td>927; <em>SacI</em>-Puc-<em>glpD</em></td>
<td>5’-CTGGCGGAGCTTCG-3’</td>
</tr>
</tbody>
</table>

*Restriction enzyme sites utilized in this study are underlined.

Growth characterization of mutants and complementation strains

Growth curve analyses have been described previously [17]. Briefly, all strains utilized were initially grown overnight in *Pseudomonas* Isolation Broth (PIB). The overnight cultures were centrifuged and the cell pellets were washed twice with 1× M9 minimal medium, and then a 1:100 dilution was made into respective media (described above) for different growth curves. 125 µl aliquots of the diluted cultures were transferred to a sterile, polystyrene 96-well assay plate (Falcon Microtest flat bottom plate, catalog no. 35–1172; Becton-Dickinson Labware). Growth was recorded using an ELx808 Absorbance Microplate Reader (BioTek Instruments, Winooski, VT) under the following conditions: temperature 37°C, and shaking at a low speed. The plate was read at 630 nm every 30 min for 40 h. All of the data was transferred and plotted using Prism.

Virulence factors detection

Strains used for virulence factors detection were grown in LB medium. At each time point, aliquots of individual culture were used for OD540 measurement (Fig. 3B). The detection of proteases, hemolysins, lipases, and rhamnolipid was performed as described elsewhere [17]. All assays were conducted in triplicate, and the data were analyzed as previously described [17].

Growth Phenotype Confirmation of Mucoid and Non-mucoid Strains

To confirm that mutations in *mucA* do not have additional effects on nutrient metabolism of the pathway mutant strains, all of the pathway mutants and complement strains were purified on LB plates and then replica plated on M9 plates supplemented with 250 µl/ml carbenicillin (Cb250) for *mucA* strains. After 24 h incubation at 37°C, single colony of each strain was patched on LB plate, which served as a control. Growth pattern was observed after 24–36 h incubation at 37°C (Fig. S3).
In vitro and in vivo competition studies

In vitro and in vivo competition studies were performed as previously described [17]. Briefly, seven growth media with different carbon sources, including Luria-Bertani (LB) medium, casamino acids (CAA), glucose, PC, C11:0, choline, and glycerol, were used in this study. The bacterial CFU were determined after inoculation into each of the medium for 24–48 h. The CI was calculated as the CFU ratio of mutant/wildtype recovered at each time point divided by the CFU ratio of mutant/wildtype in the input inoculum [47]. The smaller the CI value, the more significant the reduction in fitness of the mutant.

Various aligned-overproducing strains, \( \Delta \text{fadBA145-mucA}^+ \), \( \Delta \text{fadBA145-mucA} \), \( \Delta \text{fadBA145betA-mucA} \), \( \Delta \text{fadBA145betA} \), and the complement strains for each mutant utilized in this study are listed in Table 1. The use of the mucA \(^+\) mutation is essential in this animal model as previously described [40].

Supporting Information

Figure S1 Five potential \( \text{fadBA} \)-operon homologues of \( \text{P. aeruginosa} \). (A) Genes of operons (GenBank accession numbers in parentheses) are shown in light purple with percent of identity and similarity to the \( \text{E. coli} \) FadBA. \( \text{fadBA1} \) is 3.363 kb; \( \text{fadBA2} \) is 2.760 kb; \( \text{fadBA3} \) is 2.346 kb; \( \text{fadBA4} \) is 2.887 kb; and \( \text{fadBA5} \) is 3.353 kb. (B) Alignment of \( \text{P. aeruginosa} \) FadAs and FadBs with \( \text{E. coli} \) FadA and FadB motifs. Amino acids with similar properties are assigned the same colors using CLC Sequence Viewer 6. (TIF)

Figure S2 Growth analysis of different single \( \text{fadBA} \) mutants on medium (C12:0) and long chain-length fatty acid (C14:0, C16:0 and C18:1). Along with the wildtype PAO1 strain, mutants \( \Delta \text{fadBA1} \), \( \Delta \text{fadBA2} \), \( \Delta \text{fadBA3} \), \( \Delta \text{fadBA4} \) and \( \Delta \text{fadBA5} \) were grown in 1% M9 medium supplemented with 0.5% different test FAs (A to D) and 1% Brij-58 or LB broth as a control (E). Only the \( \Delta \text{fadBA5} \) mutant showed various defects when grown with FAs of different chain-lengths, no significant growth defects were observed for the rest of single \( \text{fadBA} \) mutants. All of the mutants grew to the same level as wildtype when grown in LB. (TIF)

Figure S3 Growth Phenotype Confirmation of Mucoid and Non-mucoid Strains. Along with the wildtype PAO1 and PAO1-mucA \(^-\) strains, all of the pathway mutants and their corresponding complement strains were patched on 1×M9 solid medium +1% (w/v) Brij-58 supplemented with 0.2% (w/v) C11:0 (B), 40 mM glycerol (C), or 30 mM choline (D). (A) Growth on LB was performed as a control. Alginate over-producing strains show a light sheen surface indicated by white arrow in panel A. Similar growth defects were shown between mucoid and non-mucoid strains on different plates. A detailed plate layout is shown in panel E with strains identification of Table 1 in parentheses. (TIF)

Figure S4 Analyses of proteases, hemolysins, lipases, and rhamnolipid productions by \( \text{P. aeruginosa} \) various pathway mutant. No mutants displayed significant \((P \leq 0.05, \text{based on student } t\text{-test})\) decrease in productions of proteases (A), rhamnolipid (B), hemolysins (C), and lipases (D). (TIF)

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We would like to thank previous (Asha S. Nayar, and Joon Kim) and current (Jan Zarzycki-Siek) lab members for their assistance in some mutagenesis experiments, as well as current lab members (Andrew Bluham and Ian McMillan) for their critical reading of this manuscript.

Author Contributions

Conceived and designed the experiments: ZS YK MNH RMT HPS SWD TTH. Performed the experiments: ZS YK MNH RMT MSS. Analyzed the data: ZS YK MNH TTH. Contributed reagents/materials/analysis tools: TTH HPS SWD YK MHN RMT. Contributed to the writing of the manuscript: ZS TTH.

References

Phosphatidylcholine Utilization in P. aeruginosa


