Bacterial Coral Pathogens of the Genus *Vibrio*

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In loving memory of
Harry M. Ushijima,
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ABSTRACT

Coral reefs play an important role in numerous marine ecosystems, however, their survival is threatened by outbreaks of disease. On their own, reefs have the ability to regenerate after destructive events like natural disasters; however recent threats have pushed coral reefs past the point of recovery and many reefs are now under threat of disappearing forever. Outbreaks of diseases specific to corals have already decimated the reefs of the Florida Keys and the Caribbean. In addition, the baseline levels of disease are increasing, as well as the expansion of disease outbreaks into new regions and the broadening of coral species that are affected. Unfortunately, not all of the characterized diseases have a pathogen positively identified and many of the mechanisms of disease for the known pathogens have yet to be determined. This piece of work describes the isolation, identification, and characterization of three virulent Vibrio strains that infect and cause tissue lysis in Hawaiian corals and species at Palmyra Atoll. First, Vibrio oswensii strain OCN002 causes chronic Montipora white syndrome (cMWS) among the Hawaiian Rice coral (Montipora capitata), a major reef building species, in Kāne‘ohe Bay, Hawai‘i. Second, Vibrio coralliilyticus strain OCN008 causes a comparably faster-spreading disease called acute Montipora white syndrome (aMWS). Third, V. coralliilyticus strain OCN014 is a cause of Acropora white syndrome among the table coral (Acopora cytherea) at Palmyra Atoll. In addition to characterizing infection, common virulence factors between the two V. coralliilyticus were investigated and a direct link between rising global sea surface temperatures and increased virulence of a coral pathogen was established. This work also describes the identification of a novel virulence mechanism utilized by strain OCN008, which may represent the evolution of this pathogenic species in response to the protective properties conferred to coral by the microorganisms normally associated with it.
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CHAPTER 1: INTRODUCTION TO CORAL DISEASE AND CORAL PATHOGENS OF THE GENUS VIBRIO

The declining health of coral reefs

Coral reefs are irreplaceable and one of the most threatened ecosystems on Earth. Corals provide a physical structure that acts as both a nursery to the juvenile stages of numerous organisms and an indispensable dwelling for many coral-associated species (1, 2). Unfortunately, disease, overfishing, pollution, and climate change threaten the existence of the world’s coral reefs (2, 3). Roughly 33% of reef-building coral species currently face an elevated risk of extinction due to climate change (4). The projected increase in greenhouse gasses over the next 50 years will lead to elevated sea surface temperatures (SST) and ocean acidification, which are detrimental to the health of corals (5).

Increased SST is a major cause of coral bleaching, the loss of the photosynthetic dinoflagellates within the coral cells (6). For many coral species, the mutualistic dinoflagellates, Symbiodinium spp., are the major source of fixed carbon. Therefore, bleaching forces the coral to rely solely on heterotrophic feeding, which can result in over a 90% reduction in energy production for some species. Elevated SSTs beyond a tolerable threshold causes a break down in the equilibrium maintained within coral cells and sets off a chain reaction of processes, predominantly involving oxidative stress, which result in the expulsion of the dinoflagellates from the coral tissue (6). However, depending on species, corals can survive for a time on heterotrophic feeding. A sustained increase of 1 to 2 °C above the average summer maxima
temperature for a week is often enough to induce bleaching, while periods longer than several weeks would be catastrophic and could result in mass mortality (7, 5, 2, 4, 8–10).

In addition to elevated SSTs, increased CO₂ emission leads to ocean acidification, which has been shown to negatively affect reef-building organisms (11). An increase in dissolved CO₂ drives a reaction with seawater that produces carbonic acid, lowering the pH. Acidification is problematic for reef-building corals because their skeletons are composed of calcium carbonate, which increases in solubility as pH decreases. In addition, acidification, along with many other environmental stressors, can also induce bleaching. However, it has been suggested that corals are more resistant to acidification than previously thought (12). Although ocean acidification does not pose as dramatic an effect as increased SST, their synergistic influence on coral may prove to be devastating. In addition, the effects of climate change are further exacerbated by the spread of coral diseases, some of which display a positive correlation between virulence and increased SST (8–10, 13–22).

*Examples of coral diseases responsive to temperature and anthropogenic stressors*

Diseases specific to coral have devastated a number of reefs around the world, and the incidence of coral disease is increasing (23–29). The first report of coral disease was the observation of black band disease in the Caribbean, which was reported in the 1970s (30). Subsequent outbreaks of several diseases (14, 15, 31, 32) have reduced coral cover in the Caribbean by approximately 80% since the first reports of disease (33). The history of coral disease in the Caribbean represents an extreme scenario; areas of high coral cover that have been decimated by the spread of disease in the last few decades.
An outbreak of the tissue loss disease white plague type II in 1995 was particularly destructive for reefs in the Caribbean. Initially, white plague type II predominantly affected the coral *Dichocoenia stokes*, killing up to 38% of colonies in some areas (14, 34). Four months after the discovery of the outbreak, the disease spread over an area of 200 kilometers and 16 other coral species started displaying similar disease signs over the course of the outbreak (14). The bacterium *Aurantimonas coralicida* was identified as the responsible etiological agent (14, 34). Since the 1995 outbreak, *A. coralicida* was implicated in subsequent outbreaks in 1996 and 1997. Interestingly, all three outbreaks coincided with the warmer summer months, and warmer SSTs, suggesting a positive correlation between SST and virulence.

In addition to white plague type II, another tissue loss disease originally called white pox, but now known as acroporid serratisosis, has spread throughout the Caribbean and infected the coral species *Acropora palmata* (15). *A. palmata* was once one of the most predominant corals in the Caribbean. However, due to the effects of acroporid serratisosis, it was listed under the United States Endangered Species Act in 2006 (35). Mortality among the *A. palmate* populations caused by acroporid serratisosis exceeded 50% in some areas. The causative agent of acroporid serratisosis was identified as a strain of the human pathogen, *Serratia marcescens*, which is often found in human feces. The incidents of the disease were directly linked to sewage outflow; the virulent strain, PDR60, originated from human wastewater (36, 37). White plague type II and acroporid serratisosis represent coral diseases that are influenced by elevated SSTs and anthropogenic influence. They also highlight the potential damage that can result from disease outbreaks.
The global coral pathogen *Vibrio coralliilyticus*

The coral pathogens described above are exclusively isolated from coral infections in the Caribbean; in contrast, the bacterium *Vibrio coralliilyticus* has been proposed as a disease agent for multiple coral genera on reefs in the Indian Ocean, Red Sea, the Great Barrier Reef, Micronesia, Polynesia, and the Mediterranean (38–44). The host range of *V. coralliilyticus* includes *Pachyseris speciosa* and *Pocillopora damicornis*, the latter of which is threatened by increased sea surface temperatures, harvesting for the aquarium trade, and harmful algal blooms (45–47). Other susceptible species include *Acropora cytherea* and *Oculina patagonica*, which display a high sensitivity to bleaching conditions (44, 45), and *Montipora aequituberculata*, which was one of the more frequently infected coral species in two major disease outbreaks on the Great Barrier Reef (39, 48). Furthermore, *V. coralliilyticus* is responsible for disease in economically important bivalve crops, including the Greenshell mussel *Perna canaliculus*, the Pacific oyster *Crassostrea gigas*, the flat oyster *Ostrea edulis*, and the scallop *Pecten maximus* (49–51), which are all part of the billion-dollar shellfish industry. Some of these susceptible hosts are also threatened by elevated SSTs, which has been linked to increased virulence and higher host mortality associated with some strains *V. coralliilyticus*. (17, 52).

*Temperature-dependent infection by V. coralliilyticus*

The link between elevated temperature and virulence is exemplified by *V. coralliilyticus* strain BAA-450 (hereafter BAA-450) (17, 38, 53). BAA-450 was isolated from a bleached colony of the coral *Pocillopora damicornis*, (38). Fragments of *P. damicornis* were observed to bleach or lose tissue after inoculation with BAA-450, and infection was subsequently concluded
to be affected by water temperature. During infections at 24 °C, the intracellular dinoflagellates were degraded and the coral bleached (17). At 27 °C, infections resulted in tissue lysis and increased host mortality. At temperatures below 23 °C, BAA-450 was incapable of infecting *P. damicornis*.

When water temperatures are elevated from 23 to 27 °C, both behavioral and physiological changes occur in BAA-450. This pathogen displays chemotaxis towards mucus secreted by *P. damicornis*, rather than just opportunistically adhering, and elicits a chemotactic response towards the molecule dimethylsulfoniopropionate (DMSP) in the coral mucus (54). During periods of elevated water temperature, the mucus concentration of DMSP increases, as well as the overall chemotactic response of BAA-450 and its chemotactic response towards DMSP (55). This was supported by the inability of BAA-450 to infect *P. damicornis* when functionality of its polar flagellum was disrupted by transposon mutagenesis (56). In addition to chemotaxis, electron microscopy studies suggest that tissue invasion of *P. damicornis* by BAA-450 occurs only when water temperatures reach 27 °C (52). During invasion, BAA-450 proliferates in the *P. damicornis* ectodermal cells and then penetrates into deeper tissue layers causing tissue lysis. The mechanism BAA-450 uses to induce tissue lysis after it penetrates into the coral tissue is still under speculation.

Increased production of an extracellular metalloprotease at elevated growth temperatures may contribute to the disparity between BAA-450 infections at different temperatures (17). This metalloprotease displays homology to the *V. cholera* hemagglutinin involved in attachment to mammalian hosts, and in a purified form is capable of lysing *P. damicornis* tissue, consistent with a role in infection (17, 57). However, deletion of the gene encoding the BAA-450
metalloprotease homolog in another pathogenic strain of *V. coralliilyticus*, strain P1, did not result in reduced virulence, leaving the role of metalloproteases in this association open (58).

It has been hypothesized that periods of elevated SST result in the upregulation of multiple genes associated with pathogenesis in BAA-450. Proteomic analysis of BAA-450 identified 136 gene products with significant upregulation at 27 °C, which included hemolysins, an RTX toxin, pili for adhesion, and secretion systems thought to contribute to virulence (59). Though shown to be upregulated, these putative virulence factors were not demonstrated to be involved in pathogenesis.

The coral immune system

Research on the immune responses coral have to pathogens, such as *V. coralliilyticus*, have been mainly descriptive studies. However, they have identified a number of conserved components of an innate immune system (60–70). Comparative studies of predicted protein sequences and structures have identified potential pattern recognition receptors in coral that bind to conserved microbe-associated molecular patterns (MAMPs), including bacterial structural molecules like flagellin, lipopolysaccharide, and teichoic acid. Potential pattern recognition receptors in coral have been speculated to activate an innate immune response upon recognition of MAMPs (60, 62, 71–73). In addition, enzymatic assays and sequence analysis have uncovered constituents of a humoral response in coral, including upregulation of the complement molecule C3, antibacterial peptides, and phenoloxidase activity with melanization (74, 63, 75, 68). The first antibacterial peptide discovered to be synthesized by a hard coral, damicornin, is produced and stored in the ectodermal granular cells of *P. damicornis* and displays inhibitory activity
against Gram-positive bacteria (63). However, damicornin does not have significant activity against BAA-450, and this pathogen seemed to inhibit expression of this peptide according to transcriptomic studies. In other studies with the coral *Acropora millipora* and *V. coralliilyticus* strain P1, it was revealed that after exposure to this pathogen there was a significant upregulation of some presumed immune and stress-related proteins (68). In all, bacterial pathogens do trigger a response in corals, however, it is unclear how analogous the reaction is to the immune response described in other established invertebrate systems.

The only immune-related gene in coral demonstrated to serve a similar function as its homolog in more defined systems is TNFα, a cytokine involved with triggering apoptosis (69). A homolog of TNFα was identified in the coral *Acropora digitifera*, which encoded a gene product that triggered apoptosis and bleaching when coral cells were exposed to the purified molecule. The purified TNFα was also able to trigger apoptosis in human T lymphocytes, suggesting conservation of function. In addition, purified human TNFα elicited a similar response in coral cells upon exposure. Although TNFα is conserved among a range of different organisms, both vertebrates and invertebrates, these results do suggest that some gene homologs identified in coral may serve a similar function, but more experimental data is required before any conclusion can be drawn from these descriptive studies.

The extracellular mucus layer is considered the first line of defense corals have against the environment, which serves as physical barrier against pathogenic microorganisms (76–78). Coral mucocytes excrete mucin and a range of different sugars, lipids, and proteins to create the mucus layer that, along with ciliary action, physically removes sediment, marine debris, and microorganisms from the coral surface (78, 79). This mucus layer is colonized by a suite of bacteria, archaea, fungi, viral particles, and protozoans (76, 80–82). The coral *P. damicornis* was
demonstrated to be able to maintain the mucus microflora at a consistent level of $10^5$ cells/ml of mucus through recurrent sloughing (78). The bacteria that colonize the mucus of healthy coral, part of the normal microflora, must sustain a replication rate greater than the mucus sloughing rate to prevent removal from the coral (76, 77, 80). Though the normal microflora is distinct from the surrounding seawater, the mechanisms involved in maintaining this specific community structure are not fully understood (76, 80, 83). However, the coral mucus layer can be overwhelmed when bacteria such as fast-growing *Vibrio* spp., which have some of the shortest generation times of any bacteria, establish a population (39, 84).

*The coral probiotic hypothesis*

The relatively slow growth and lack of an adaptive immune system means that corals are unable to adapt to rapidly evolving threats. However, it has been hypothesized that the normal microflora plays a role in the defense of their coral hosts (40, 76, 80, 83, 85–87). According to the coral probiotic hypothesis, the normal microflora, which is distinct from the surrounding seawater, can shift by changing its constituents or evolution of existing microorganisms (via mutation or gene transfer) to respond to changing environmental conditions (85). The coral microflora is thought to release antibacterial compounds, produce toxic metabolites, sequester nutrients, and fill niches to form a network of antagonistic interactions that prevents domination by any single group of bacteria, such as a pathogen (76, 87, 80, 88, 40, 89–91, 81). Furthermore, bacterial strains isolated from healthy corals inhibited the growth of the pathogens *V. coralliilyticus* and *V. shiloi* during *in vitro* studies. Many of these inhibitory strains belong to the genus *Pseudoalteromonas*, which includes multiple species that are known to produce a wide variety of antimicrobial compounds (76, 92, 87–89, 91). In contrast, BAA-450 can seemingly
overcome the antibacterial activity of different isolates from healthy coral when challenged at elevated water temperatures (40, 86–88).

The coral microflora protects against the pathogen Vibrio shiloi

The protective properties of the normal microflora are believed to be responsible for the acquired immunity of the coral Oculina patagonica to V. shiloi infection (93). In 1997, V. shiloi was discovered to infect and induce bleaching in O. patagonica and, at the time, was the most elucidated coral infection system. V. shiloi was determined to bind to a β-D-galactopyranoside-containing receptor in the coral mucus released by the endosymbiotic dinoflagellates, followed by penetration into the O. patagonica cells (94, 95). Within the coral cells, V. shiloi would enter a viable-but-not-culturable (VBNC) state, multiple intracellularly, and release an extracellular peptide that bound and inhibited photosynthesis in the dinoflagellates (96, 94, 97, 98). The extracellular peptide, toxin P, binds to the intracellular dinoflagellates and facilitated transport of NH₃ into the cells, which lead to their death and bleaching of the coral host (98). It was hypothesized that toxin P caused an uptake of NH₃ more rapidly than NH₄⁺ into the dinoflagellate cells and caused a disassociation of the available NH₄⁺ into NH₃ and H⁺, which lowered the pH of the cytosol and damaged the cell. The observed drop in cytosolic pH and photosynthetic quantum yield after dinoflagellates were incubated with toxin P and NH₄Cl corroborated this. However, in 2004, stocks of V. shiloi no longer infected O. patagonica specimens and could not be detected in inoculated specimens or bleached corals from the field (93). However, when corals were treated with the broad-spectrum antibiotic nalidixic acid beforehand, V. shiloi infected O. patagonica, induced bleaching, and could be detected in bleached specimens. Therefore, it was suggested that the coral animal did not develop resistance
to *V. shiloi*, but the normal microflora evolved to prevent infection from this pathogen. Like *V. shiloi*, the coral microflora is hypothesized to affect infection by *V. coralliilyticus*, but no *in vivo* or functional studies have demonstrated this.

**Current work**

This dissertation includes research on infection models using two species of coral. First, *Montipora capitata*, a major reef-building coral in Kāne‘ohe Bay, Hawai‘i, is affected by a progressive infection called chronic *Montipora* white syndrome (cMWS) and is one of coral diseases observed in Kāne‘ohe Bay. Lesions of cMWS usually have sediment and algal growth directly adjacent to the disease front, due to the relatively slow rate of tissue loss. Levels of cMWS are not seasonally dependent and mean prevalence in Kāne‘ohe Bay ranges from 0.02 to 0.87%. In surveys, the case fatality rate for cMWS was 28% over two years, however, 32% of afflicted colonies showed some regrowth of tissue and a cessation of disease progression over the survey period.

A second, comparatively faster disease called acute *Montipora* white syndrome (aMWS) also affects *M. capitata* colonies. Colonies displaying aMWS are sporadically observed in Kāne‘ohe Bay, but are distinguished from cMWS by comparatively faster tissue loss that results in the exposure of large areas of white coral skeleton. During aMWS outbreaks, afflicted colonies can die within weeks of the first signs of disease. In 2010 and 2011, outbreaks of aMWS in Kāne‘ohe Bay affected 388 and 969 observed colonies, respectively (Aeby unpublished data).
The second coral species, *Acropora cytherea* is one of the major reef-building corals at Palmyra Atoll, which experienced a bleaching event following the 2009 El Niño event that caused a 2 °C increase in SST (99). Following the bleaching event, an outbreak of the coral disease *Acropora* white syndrome (AWS) spread throughout Palmyra Atoll’s *Acropora* population affecting 25% of the coral colonies at some study sites (99, 100). Similar to aMWS, AWS is a tissue loss disease that destroys healthy coral tissue and can lead to complete colony mortality (100, 101). The two coral species described above represent major constituents of reefs at Palmyra Atoll and Kāne‘ohe Bay, which require investigation of the pathogens that threaten them to prevent their loss.

The number of defined etiological agents is meager compared to the total number of described coral diseases, while, in comparison to the pathogens of eukaryotic organisms, the mechanisms of infection for coral pathogens are not well understood. With the gradual decline of the environment and the spread of infection to non-coral hosts, the identification of coral disease-causing agents and an understanding of their mechanisms of infection needs to be improved upon because effective management of any disease requires an understanding of what is causing it. This dissertation describes the identification of three etiological agents of coral disease, *Vibrio owensii* strain OCN002, *V. coralliilyticus* strain OCN008, and *V. coralliilyticus* strain OCN014, which can induce the coral diseases chronic *Montipora* white syndrome, acute *Montipora* white syndrome, and *Acropora* white syndrome, respectively. In addition, virulence factors were identified that are shared between the geographically distant *V. coralliilyticus* strains OCN014 and OCN008, providing evidence for a conserved set of virulence factors for this species. Lastly, a novel virulence mechanism is described in strain OCN008, which produces the broad-spectrum
antibiotic andrimid to disrupt the protective coral microflora on *M. capitata* to enable colonization and infection.
CHAPTER 2: VIBRIO OWENSHII INDUCES THE TISSUE LOSS DISEASE MONTIPORA WHITE SYNDROME IN THE HAWAIIAN REEF CORAL MONTIPORA CAPITATA

INTRODUCTION

Coral disease is a progressing threat to many reefs around the world. Reefs in the Florida Keys and Caribbean have been devastated since the first accounts of coral disease were documented in the early 1970s (30, 31, 102–104), and reports of disease in the Indo-Pacific are increasing (24, 26–29, 105, 106). Major disease outbreaks can lead to breakdown of reef structure, disruption of local ecosystems, and threaten tourism-dependent economies of areas like Hawaii and the Caribbean (2, 106–108). Understanding coral disease is critical in developing management and conservation strategies to protect these valuable resources. However, disease processes are complex and our knowledge of causal factors of disease is extremely limited [5, 16].

Bacteria have been implicated as the etiological agent for several coral diseases [17-26]. The coliform bacterium Serratia marcescens, an opportunistic human pathogen commonly found in sewage, causes acroporid serratiosis in the Elkhorn coral Acropora palmata. [19, 27, 28]. Vibrio shiloi, an intracellular coral pathogen that attacks zooxanthellae following penetration of coral cells, causes bleaching (94, 95, 119). Vibrio coralliilyticus causes cell lysis of Pocillopora damicornis at elevated temperatures or bleaching at lower temperatures (17, 53). Vibrio coralliilyticus has also been implicated in outbreaks of tissue loss diseases (white syndromes) in corals in Palua, Marshall Islands, and the Great Barrier Reef (39, 120).

1Ushijima et al. 2012. PLoS ONE 7:e46717
In Hawaii, coral disease is an emerging problem. Twelve diseases have been described from across the archipelago and the frequency of disease outbreaks has increased [9, 34, 35]. *Montipora* white syndrome (MWS) is a disease of concern that has been reported across the archipelago [34, 35]. MWS is a chronic and progressive disease that produces a subacute tissue-loss pattern (Figure 1). There is a positive association between levels of MWS and *M. capitata* coverage, following the standard host-pathogen relationship [34]. MWS is transferred by direct contact between coral fragments, and there is no apparent link between MWS and time of year, suggesting that water temperature does not play a major role in the infection process (121). Recently, the reefs of Kaneohe Bay experienced outbreaks of an acute tissue loss disease in *M. capitata* (Aeby, unpublished data). Therefore, we will refer to the slower moving MWS as chronic *Montipora* white syndrome (cMWS) to differentiate between the two types of lesions.

In this study a pathogen responsible for cMWS was isolated and classified as *Vibrio owensii* strain OCN002 based on *rrsH* gene sequence, multi-locus sequence analysis, colony morphology, and metabolic capabilities. Isolation of OCN002 in pure culture, the demonstration of virulence by exposure of healthy *M. capitata* fragments with an axenic culture, and the re-isolation of the introduced bacterium from coral showing signs of disease, fulfilled Henle-Koch’s 2nd, 3rd, and 4th postulates (123, 124). OCN002 expands the range of bacteria known to cause disease in corals and is the first bacterial coral pathogen identified in Hawaii.
RESULTS

Initial screen for a potential cMWS pathogen

Contact-dependent transmissibility of MWS from diseased to healthy coral fragments suggested that the causative agent of MWS could be an infectious agent [34]. To isolate a potential pathogen, bacteria from a diseased coral fragment in Kāne'ohe Bay were plated on a complete medium, purified by streaking, and tested for their ability recreate signs of disease with healthy coral under laboratory conditions. A preliminary screen was conducted using corals inoculated with groups of five bacteria to rapidly assess the potential virulence of a large number of bacteria. Tissue loss only occurred in one of the 10 groups of isolates tested. All three of the coral fragments showed tissue loss after 21 days. The diffuse tissue loss, typical of cMWS, was characterized as the regression of coral tissue from the skeleton, resulting in exposure of white skeletal projections (Figure 2) and resembled corals with MWS observed in the field (Figure 1).
None of the fragments in control aquaria or those exposed to other groups of bacteria showed signs of tissue loss.

To determine which bacterium in the group of five isolates was responsible for tissue loss, the five bacteria were tested individually for their ability to induce signs of disease. Corals exposed to four of the strains and those in control aquaria remained healthy. In contrast, the fifth strain caused tissue loss on all three coral fragments after 24 days. This strain was designated OCN002 and pursued further based on its potential as the etiologic agent of cMWS.

**Figure 2. Progression of tissue loss in experimental coral fragment.** (A) Coral pre-inoculation. (B) The same fragment exhibiting disease signs of cMWS 26 days post-inoculation as evidenced by exposed white skeletal elements. Fragments were processed for microbial analysis at this stage of tissue loss. (C) Experimental fragments with late stage cMWS. The majority of tissue has been lost exposing the white skeleton.

*OCN002 induces signs of cMWS*

To test if strain OCN002 was responsible for the signs of disease in *M. capitata* observed above, healthy fragments of *M. capitata* were exposed to the putative disease-causing bacterial strain at a concentration of 10⁷ cells/ml, filtered seawater (FSW) as a control, or bacterial strain OCN004 as a second control. Strain OCN004 was isolated from extracellular mucus of healthy *M. capitata*. Seven of the 13 fragments exposed to OCN002 showed signs of disease after an average of 28 days subsequent to the addition of the bacteria (Figure 2B) (McNemar’s test,
P=0.014, n=13/treatment). In contrast, none of the 13 fragments in control aquaria showed signs of tissue loss. The infection trials showed that inoculation of healthy coral with OCN002 causes tissue loss under laboratory conditions.

*Increased bacterial abundance associated with disease signs in infection trials is similar to that in field samples*

One of the hallmarks of coral disease is rapid over-growth of coral with bacteria not normally associated with healthy coral, with bacteria of the genus *Vibrio* well represented (39, 113). A similar pattern of increased bacterial abundance and proportion of *Vibrio* spp. relative to those of healthy control fragments was found in diseased coral fragments in infection trials (Figure 3). Coral fragments in tanks inoculated with OCN002 that showed signs of disease had an average increase of 2.5 X 10^3 (SE±9.55) CFU/ml, and a 78% (SE±26.35%) increase in *Vibrio* spp. (Table 3). In contrast, no significant increases in either bacterial abundance or proportion of *Vibrio* spp. were found in either group of control fragments (seawater or OCN004) nor from coral fragments exposed to the test strain (OCN002) that did not develop disease signs (Figure 3,
Table 2. Coral fragments to which sterile seawater had been added had an average decrease of $1.0 \times 10^1$ (SE±3.79) CFU/ml, and a 14% (SE±8.07%) decrease in *Vibrio* spp. proportions. Coral to which strain OCN004 had been added had an average decrease of $1.1 \times 10^1$ (SE±4.52) CFU/ml, as well as a 16% (SE±7.72%) decrease in *Vibrio* spp. proportions (Table 2).

Furthermore, the fragments in tanks inoculated with strain OCN002 that remained disease-free had an average increase of $1.3 \times 10^1$ (SE±466.98) CFU/ml, but a 3% (SE±0.36%) decrease in the *Vibrio* spp. proportions (Table 2).
Similar to the laboratory results described above, mucus collected from healthy *M. capitata* directly from the field had a significantly lower abundance of cultivable bacteria as compared to field collected samples of cMWS coral (Mann-Whitney U test, W=15, p = 0.012, n = 10). Healthy coral mucus had an average of $4.8 \times 10^2$ (SE±176.83) CFU/ml, whereas diseased fragments had an average of $1.3 \times 10^4$ (SE±4122.13) CFU/ml. Healthy coral also had a significantly lower proportion of *Vibrio* spp. as compared to diseased coral (Mann-Whitney U test, W= 15, p= 0.012, n= 10). Healthy coral had an average of 2.4\% (SE±1.19\%) *Vibrio* spp. compared to diseased coral, which had an average proportion of 28\% (SE±5.35\%). Both the abundance of bacteria and the percentage of cells that were *Vibrio* spp. were significantly higher in cMWS samples, suggesting similar responses of bacterial communities after the onset of disease on coral in the field and in infection trials.

*Re-isolation of strain OCN002 from diseased coral in infection trials*

If strain OCN002 was the cause of cMWS in the infection trials described above, it should be possible to re-isolate the strain from fragments of coral that showed signs of disease; re-isolation of a putative pathogen from subjects of an infection trial is one of Henle-Koch’s postulates for determination of disease causation [36, 37]. To facilitate identification of strain OCN002, PCR primers were designed to amplify a 1.2 kb region of DNA that includes a 594 bp open reading frame similar in sequence to the *moxR* genes of several bacteria and two flanking non-coding intergenic regions. The *moxR* gene is predicted to encode a AAA+ ATPase. This region was chosen based on the presence of two intergenic regions, which are not well conserved, and therefore served as a reasonable identifier of strain OCN002. All 70 of the bacterial isolates tested from the mucus of the seven coral fragments (ten from each coral) that
showed signs of MWS after exposure to OCN002 yielded 1.2 kb PCR products with sequences identical to that from OCN002, suggesting that the recovered isolates were the same as, or very similar to, strain OCN002.

Sequence analysis suggests, but does not confirm, that the bacteria isolated from diseased coral were part of the clonal population added to the aquaria in the infection trials. To determine if direct descendants of OCN002 were recovered from diseased coral, a replicating plasmid was introduced to strain OCN002 to genetically tag the bacteria, and the resulting strain was used to infect fragments of *M. capitata*. All 30 bacterial isolates tested after recovery from laboratory infected corals contained the plasmid, pRL1383a, based on plasmid recovery and sequence analysis. In addition, each of the 30 isolates produced a PCR product with the same sequence as that of the *moxR* region of strain OCN002. Taken together, these results indicate that the specific strain of OCN002 containing plasmid pRL1383a, added during the infection trials, was recovered from the coral fragments that developed lesions. Conversely, no CFUs were recovered from coral exposed to OCN002 that remained healthy throughout the course of the infection trial on medium selective for the plasmid, suggesting that strain OCN002 does not persist on corals if it does not promote the disease state.

*OCN002 is a strain of Vibrio owensii*

To classify OCN002 taxonomically, the sequence of its 16S ribosomal RNA (*rrsH*) gene was determined and analyzed. Using the universal primers 8F and 1513R, 1505 bp of the 1542 bp *rrsH* gene of strain OCN002 was amplified by PCR (125) and sequenced directly using the primers used for amplification. Nucleotide designations at each position were unambiguous,
indicating that if multiple copies of the \textit{rrsH} gene are present in this organism, they are likely exact copies of one another. BLAST analysis of the resulting 1324 bp sequence indicated that strain OCN002 is a member of the genus \textit{Vibrio}, consistent with its ability to grow on TCBS medium. A bifurcating phylogenetic tree was generated using a region of the 16S rRNA genes from strain OCN002 and 17 other bacteria representing 9 species of \textit{Vibrio} (Figure 4). Strain OCN002 appears to be closely related to multiple strains of \textit{V. communis}, \textit{V. owensii}, and \textit{V.}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{phylogenetic_tree.png}
\caption{Phylogenetic tree showing relatedness among members of the Harveyi clade constructed using sequences of \textit{Vibrio rrsH} genes, which encodes the 16S rRNA. The evolutionary history was inferred using the Maximum Likelihood method. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to each branch.}
\end{figure}
*harveyi*, exhibiting greater than 99% identity in the sequence of the *rrsH* genes, indicating that OCN002 is in the monophyletic Harveyi clade (126).

Members of the Harveyi clade can be differentiated from one another by metabolic capabilities, a list of which is compiled in Table 3. Strain OCN002 is positive for both DNAse and extracellular protease activity and has γ-hemolytic activity. Characteristic swimming and swarming motility were observed, and the strain grew in liquid LB medium amended to NaCl concentrations of 0.1% to 6%. No growth was observed in LB at 10% NaCl or in the absence of added salt, but viable cells could be isolated after 4 days of incubation under the former conditions. Characteristic of bacteria in the Harveyi clade, strain OCN002 had an exceptionally fast growth rate. At 23°C, the average winter sea surface temperature of Kaneohe Bay (127), and 28°C, the average summer sea surface temperature of Kaneohe Bay (127), generation times during log phase growth were 9.9 and 8.0 min, respectively. OCN002 is unable to utilize trisodium citrate as a carbon source, a characteristic that separates *V. owensii* and *V. communis* from other members of the harveyi clade [41, 42]. In addition, the colony morphology of OCN002 was more similar to that of *V. owensii* than that of *V. communis*. On TCBS agar, OCN002 forms smooth, round colonies like *V. owensii* and *V. communis*. On other media types OCN002 forms opaque colonies like *V. owensii*, which differs from the translucent colonies of *V. communis* (128, 129). Collectively, the physiological and morphological characteristics of OCN002 examined in this study are consistent with those of strains classified as *V. owensii*.

The genetic and metabolic similarities of strains within the Harveyi clade can complicate species designation of isolates. Recently, multilocus sequence analysis (MLSA) has been used to distinguish similar bacterial strains and categorize them phylogenetically (130, 131). Six housekeeping genes, *pyrH*, *gapA*, *mreB*, *ftsZ*, *gyrB*, and *topA*, were amplified by colony PCR
from strain OCN002, sequenced, and concatamerized. The resulting 3098 bp sequence was aligned with corresponding sequences from 17 other strains representing 11 species of the genus *Vibrio*, and a maximum likelihood phylogenetic tree was generated (Figure 5). The concatemer alignment indicated that OCN002 is most closely related to the recently described species *V. owensii* (128). Thus, from the combined characterization and MLSA of OCN002, we conclude that this bacterium is a strain of *V. owensii*.

![Figure 5. Phylogenetic tree showing relatedness of OCN002 constructed using Vibrio MLSA results. The evolutionary history was inferred by using the Maximum Likelihood method. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to each branch.](image)

DISCUSSION

Chronic *Montipora* white syndrome (cMWS) is a coral disease that has been killing the common reef coral, *Montipora capitata*, in Kāne‘ohe Bay for the past several years [34]. We used a rapid screening method to initially identify a potential coral pathogen responsible for this disease and then subsequently isolated, identified and demonstrated that the bacterium, *V. owensii* strain OCN002, is a pathogen capable of causing cMWS in *M. capitata*. Identification of the etiological agent for this disease is an important first step towards future management and the development of conservation strategies for our dwindling reef resources.

The pathogen, *V. owensii*, is a recently described species of the Harveyi clade isolated from diseased crustaceans in Australia (128, 129). The Harveyi clade currently consists of 11 species: *V. harveyi*, *V. rotiferianus*, *V. alginolyticus*, *V. campbellii*, *V. parahaemolyticus*, *V. mytili*, *V. natriegens*, *V. azureus* *V. communis*, *V. jasicida* and *V. owensii*, many of which are associated with disease (115, 132–135). The first three of these species along with *V. proteolyticus* have been implicated in causing Yellow Band Disease in corals from the Indo-Pacific and Caribbean (115). YBD is caused by a consortium of bacteria; in infection trials, disease signs were only observed when coral were infected with multiple species concurrently (115). In contrast, cMWS resulted from infection by strain OCN002 alone, suggesting that OCN002 was sufficient to cause disease. This is similar to other Indo-Pacific coral pathogens that cause tissue loss diseases. *V. shiloi* and *V. coralliilyticus* are temperature-dependent pathogens of the corals *Oculina patagonica* and *Pocillopora damicornis*, respectively (53, 119). *V. coralliilyticus* has also been implicated in disease causation in acroporids and montiporids in other regions of the Indo-Pacific (39). These species are distinct from the Harveyi clade; *V. shiloi* is related to the unclassified *V. meditererranei* and the Splendidus clade, and *V. coralliilyticus* is
the namesake of the Coralliilyticus clade (53, 119, 126). *V. owensii* has also recently been isolated from *Acropora* white syndrome lesions in American Samoa, but its role as a coral pathogen there is yet to be determined (136). The American Samoa studies indicate that *V. owensii* could be acting as a coral pathogen in other areas in the Pacific as well.

Disease states in infection trials were consistent with those in the field. Field and laboratory diseased fragments both had a higher abundance of cultivable bacteria, particularly of the genus *Vibrio*. Infection by OCN002 under laboratory conditions had an average incubation period of 28 days, similar to the rate of cMWS transmission between fragments under laboratory conditions [34]. In the field, chronic MWS is a slow moving disease, killing colonies at a rate of approximately 3% of a colony per month [34]. In contrast, other coral tissue loss diseases progress far more quickly in the field and also have a shorter incubation period in infection trials (39, 110, 112).

In this study, it was conclusively demonstrated that bacteria re-isolated from diseased fragments infected in the laboratory were derived from the initial pure culture of OCN002 used to inoculate healthy corals. Coral specimens are brought in from the field with a natural bacterial flora, and *rrsH* gene sequencing is insufficient to differentiate between the resident bacterial flora and inocula used in infection trials. In addition to the *rrsH* gene sequence, the sequence of a second genomic region was compared between OCN002 and bacteria isolated from diseased coral. Inclusion of a sequence not expected to encode part of a protein or functional RNA molecule like the one used with OCN002 would be expected to increase the resolution.

Ultimately, a plasmid capable of replication in strain OCN002 was used to genetically tag cells of OCN002 used in infection trials to provide conclusive evidence that strain OCN002 had been re-isolated from laboratory infected coral fragments. Use of the tagged strain also allowed the
determination that OCN002 had not colonized the extracellular mucus of fragments that remained healthy, even those in the same aquarium as fragments that showed signs of MWS. Although it is technically possible that some bacteria lost the plasmid, a measurable curing rate for the plasmid was not observed under laboratory growth conditions.

The methods described in this study were developed to fulfill Henle-Koch’s four postulates of disease causation (123, 124). Henle-Koch’s postulates are a set of criteria developed to establish a causal relationship between a potential pathogen and a disease [36, 50]. The isolation of OCN002 and subsequent growth in an axenic culture fulfilled Henle-Koch’s second postulate. Induction of disease anew in healthy laboratory specimens with OCN002 fulfilled the third postulate. Re-isolation of OCN002 from experimentally diseased specimens fulfilled the fourth postulate. Fulfillment of the first postulate, finding the pathogen in all diseased organisms, is likely to be difficult due to the limited range of observable responses a coral can have to a stressor, e.g., bleaching, cell lysis, growth anomalies, and discoloration, which results in the ambiguity of identified “white syndrome” diseases (137–139). Hence, there may be different pathogens or stressors that are causing “white syndrome” for M. capitata. However, these postulates are thought of as guidelines, and for many diseases, such as typhoid fever, diphtheria, leprosy, and cholera, the accepted pathogens do not fulfill all the postulates (140, 141).

Currently, little is known of the virulence mechanisms used by strain OCN002 in the infection of M. capitata. However, three traits found in OCN002 (swimming motility, swarming motility and protease activity) have been shown to be important in the virulence of other bacteria. Swimming motility is typically dependent on polar flagella, which can mediate attachment to hosts (142). In V. coralliilyticus expression of the fhlA gene, which encodes a
membrane protein for transport of flagellar proteins to the cell surface, is important for pathogenesis (56). Swarming motility relies on lateral flagella, which has been extensively studied in *V. parahaemolyticus* and has since been shown in several other members of the Harveyi clade (143–145). Similar to some members of the Harveyi clade, the growth rate of OCN002 was exceptionally fast, yet average time to infection was 28 days. In this study, the rate of pathogen growth does not correlate with time to infection or rate of disease progression [34]. In contrast, other bacterial pathogens of coral have been found to infect after a comparatively shorter incubation period in infection trials as well as progress rapidly in the field [25]. Lastly, extracellular protease activity has been suggested to contribute to the pathogenesis of *V. coralliilyticus* in its interaction with coral [32, 60].

The concentration of bacteria used in infection assays in this study and others is orders of magnitude higher than that expected in seawater for the bacterium of interest, suggesting that either infection relies on introduction of a high concentration of bacteria from a source other than the surrounding seawater, or infection results when some event, such as compromised host tissue, permits growth of bacteria to a sufficient concentration after introduction. These observations point to the possibility of a vector providing an environment to reach infectious doses and transfer among hosts. The coral pathogen *V. shiloi* uses the fireworm *Hermodice caranxculata* as a winter reservoir (147), and human pathogens *V. cholerae* and *V. parahaemolyticus* attach to the chitin exoskeletons of copepods (148, 149). Pathogen transfer and damage to host tissue could also be accomplished by coral predators, (150, 151). Future research is planned to examine potential vectors associated with cMWS.
METHODS

Growth of bacteria

Marine bacteria were grown at 25°C in glycerol artificial seawater (GASW) medium (152) with the exception that Rila salts were replaced with Instant Ocean® (Spectrum Brands Inc., Atlanta, GA). Thiosulfate Citrate Bile Salts Sucrose (TCBS) solid medium was prepared according to the manufacturer’s instructions (BD, Franklin Lakes, NJ). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium. Concentrations of antibiotics were 100 µg/ml for ampicillin, 50 µg/ml for kanamycin and 100 µg/ml for spectinomycin.

Screen for a potential MWS pathogen

A fragment of *Montipora capitata* with cMWS from reefs surrounding Coconut Island in Kaneohe Bay was crushed, diluted 1:10 in FSW, and spread on GASW plates in triplicate. From the 1710 colonies that grew on plates overnight, 50 were isolated, grown with aeration for approximately 15 hours, washed twice and resuspended in FSW, and inoculated in groups of 5 into aquaria (9.4 L) containing 3 healthy *M. capitata* fragments in FSW. Control tanks were inoculated with FSW only. Corals were held under ambient light and temperature, elevated on plastic grates to promote water flow, and visually monitored daily for signs of tissue loss. Aquaria air pumps provided water circulation, and water was changed every 4 days. Experiments were terminated following mortality in infection tanks. All necessary permits were obtained for the described field studies, including special activity permit number 2011-68 from the Hawaii Department of Land and Natural Resources for the collection of corals.
Infection trials with OCN002

From the initial screening, one bacterium, OCN002, was found to produce tissue loss. Additional infection trials with strain OCN002 were as described above with minor modifications. Four fragments instead of three were present in each aquarium. Each run consisted of a water control tank (no bacteria added), a bacterial control tank (inoculated with strain OCN004), and experimental tanks to which the test bacterium OCN002 was added. Cultures of inocula were grown to approximately $10^9$ cells/ml and final concentrations after inoculation were approximately $6 \times 10^6$ cells/ml of tank water based on CFU produced on solid GASW. The inoculum was pipetted directly over each coral fragment, while care was taken not to cause physical damage to the fragment.

Control strain OCN004 was isolated from extracellular mucus of healthy M. capitata based on its ability to grow on GASW solid medium and classified as a member of the genus Alteromonas based on the sequence of the corresponding rrsh gene (Accession # JX152761). Coral fragments did not display signs of tissue loss when exposed to cultures of OCN004.

Bacterial abundances and proportions of Vibrios in MWS coral fragments

Mucus was collected from diseased and healthy M. capitata colonies from experimental corals in infection trials (n=13/treatment) as well as from the field (n=5/condition) and assessed for numbers of CFUs on GASW and TCBS media, the latter of which is selective for the genus Vibrio (153). Extracellular mucus was removed with a pipettor, vortexed, and plated either directly or diluted in FSW on GASW and TCBS solid media in triplicate. Colonies were counted after 24 hours. To determine changes during infection trials, CFUs per ml of mucus...
were determined immediately after collection from the field and subsequently upon completion of the infection trial.

Conjugation of plasmid pRL1383a and re-isolation of strain OCN002 post-infection

To allow identification of strain OCN002 post-infection, the RSF1010-derived plasmid pRL1383a was conjugated into strain OCN002 to add a genetic tag to the strain (154). Conjugation was by tri-parental mating with *E. coli* strains DH5α MCR with the self-transmissible plasmid pEVS104 or the mobilizable plasmid pRL1383a as previously described, with the exception that LB was used in place of heart infusion medium (155, 156). Transconjugants were selected on LB with spectinomycin, resistance to which is encoded on pRL1383a, and ampicillin, to which strain OCN002 is naturally resistant.

Strain OCN002 containing pRL1383a was used in infection trials as described above. Mucus from coral was plated in triplicate on GASW, TCBS, and GASW with spectinomycin, and resulting colonies were counted and recorded. Plasmids were isolated from 10 colonies per plate as described previously (157). The identity of pRL1383a was confirmed by PCR and subsequent sequencing of the product using the plasmid-specific primers pRL1383a MCS-F and pRL1383a MCS-R (Table 1).

DNA sequence analysis of strain OCN002

DNA primers used in this study are listed in Table S1. The *rrsH* gene of OCN002 was amplified using universal primers 8F and 1513R [72], sequenced with the same primers used for
PCR (Accession #JX127215), and aligned using BioEdit (158) with 17 other sequences from species in the *V. harveyi* clade (126) and others in GenBank. A maximum likelihood tree was constructed using the generalized time-reversible (GTR) algorithm (159), and 1000 bootstrap replicates were performed using MEGA5 (160). All positions containing gaps and missing data were eliminated.

For multilocus sequence analysis of OCN002, the *pyrH, gapA, mreB, ftsZ, gyrB*, and *topA* genes were amplified by PCR using primers described previously (126), sequenced (Accession #s JX127216, JX127217, JX127218, JX127219, JX127220, and JX127221, respectively), concatamerized (126), and aligned in BioEdit against corresponding concatamerized sequences from 16 other species of the Harveyi clade, Cholera clade, Vulnificus clade, and Coralliilyticus clade. Sequences were from NCBI and the open database resource <http://www.taxvibrio.lncc.br/> (130). A maximum likelihood tree was constructed with 1000 bootstrap replicates using the concatemers in MEGA5 and the GTR algorithm as described above (160).

To establish an identifier of strain OCN002, genomic DNA from OCN002 was partially digested with Sau3A1 and 2 to 4 kb size-fractionated fragments were cloned into the BamHI site of plasmid pBluescript S/K+. A 2976 bp genomic DNA fragment was sequenced (Accession # JX127222) from one clone using M13 forward and reverse priming sites (161) on the plasmid initially and subsequently within the fragment using primers VPR4-Walk1R, VPR4-Walk2R, and VPR4-Walk3R. From this sequence a 1201 bp region flanked by primers UTR-MoxR-UTR-F and UTR-MoxR-UTR-R was chosen to be used as an identifier of strain OCN002.
Metabolic characterization of strain OCN002

To aid in discrimination between Vibrio species, biochemical and metabolic tests were conducted. To test for extracellular protease, DNase, and hemolysin activity, OCN002 was streaked on Skim milk agar, Methyl green DNase agar, and 5% sheep blood TSA, respectively (BD, Franklin Lakes, NJ). Additional biochemical tests were performed with API 20NE strips (Biomérieux Durham, NC) in triplicate with the provided AUX media and artificial seawater (GASW without glycerol, tryptone, or yeast extract). The indicator plates and API 20NE strips were incubated at 25°C for 24 hours and visually evaluated according to the manufacturer’s guidelines.

Salt tolerance was measured in LB supplemented with the following concentrations of NaCl: 0, 0.1, 0.5, 1.0, 3.0, 5.0, 6.0, and 10.0% (w/v). 2 ml cultures of OCN002 at each NaCl concentration were inoculated in triplicate from an overnight culture. Growth was assessed after 12 h incubation at 25°C by measuring the optical density of cultures at 600 nm.

Examination of the effect of temperature on growth employed triplicate 200 ml cultures in GASW inoculated from an overnight culture and incubated at 23°C or 28°C. Numbers of CFU from cultures plated every hour for 24 hours were used to calculate generation times.

Statistical Analysis

Infection trial data was analyzed using McNemar’s test, a non-parametric method used for nominal data (162). Changes in bacterial abundances and proportions of Vibrio species in infection trials were calculated as the difference between colony counts from the pre-trial fragments and the same fragments at the end of the infection trials. Differences in bacterial
abundances within each group (control, control bacteria, pathogen exposed healthy, pathogen exposed diseased) were compared using a nonparametric Wilcoxon signed-rank test. The culturable bacterial abundances from field specimens and the between treatment changes in bacterial abundances, including the proportion of *Vibrios* for the laboratory specimens, were analyzed using a nonparametric Mann-Whitney U test.
### Table 1. Oligonucleotides used in this study.

<table>
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<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Description</th>
<th>Source</th>
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<td>5’ CGAAGTTATATCGATGCGG 3’</td>
<td>Forward primer for the vector pRL1383a</td>
<td>This study</td>
</tr>
<tr>
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<td>Reverse primer for the vector pRL1383a</td>
<td>This study</td>
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<td>Forward primer for the 16S rRNA gene</td>
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</tr>
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<td>[72]</td>
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</tr>
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<td>VftsZ800R</td>
<td>5’ GCCATTGTAGCTGTACCAAG 3’</td>
<td>Reverse primer for the Vibrio ftsZ gene</td>
<td>[39]</td>
</tr>
<tr>
<td>VgyrB274F</td>
<td>5’ GAAGTTATCATGACGGTACTTC 3’</td>
<td>Forward primer for the Vibrio gyrB gene</td>
<td>[39]</td>
</tr>
<tr>
<td>VgyrB1171R</td>
<td>5’ CCTTACGAGTTAATGCTTTTC 3’</td>
<td>Reverse primer for the Vibrio gyrB gene</td>
<td>[39]</td>
</tr>
<tr>
<td>VtopA400F</td>
<td>5’ GAGATCATCGGTGGTGATG 3’</td>
<td>Forward primer for the Vibrio topA gene</td>
<td>[39]</td>
</tr>
<tr>
<td>VtopA1200R</td>
<td>5’ GAAGGACGAATCGCTTCACTG 3’</td>
<td>Reverse primer for the Vibrio topA gene</td>
<td>[39]</td>
</tr>
<tr>
<td>M13-F</td>
<td>5’ GTAACACGGCCAGTGTG 3’</td>
<td>Forward primer for the M13 region for vector pBluescript SK(+) used in this study</td>
<td>[76]</td>
</tr>
<tr>
<td>M13-R</td>
<td>5’ GGAACAGTATGACACCAGT 3’</td>
<td>Reverse primer for the M13 region for vector pBluescript SK(+) used in this study</td>
<td>[76]</td>
</tr>
<tr>
<td>VPR4-Walk1R</td>
<td>5’ CCCTCTTCGTTTACCGATTTC 3’</td>
<td>Internal primer developed for sequencing of cloned section of OCN002 genome</td>
<td>This study</td>
</tr>
<tr>
<td>VPR4-Walk2R</td>
<td>5’ CTGAATACGGCAAGAGCTTG 3’</td>
<td>Internal primer developed for sequencing of cloned section of OCN002 genome</td>
<td>This study</td>
</tr>
<tr>
<td>VPR4-Walk3R</td>
<td>5’ GACTCTGGCAAGATCGTGT 3’</td>
<td>Internal primer developed for sequencing of cloned section of OCN002 genome</td>
<td>This study</td>
</tr>
<tr>
<td>UTR-MoxR-UTR-F</td>
<td>5’ GTAACGCAATACAAGCGGGATCG 3’</td>
<td>Forward primer developed from OCN002 chromosomal DNA</td>
<td>This study</td>
</tr>
<tr>
<td>UTR-MoxR-UTR-R</td>
<td>5’ GCCATTGCGAATTGCTTGTG 3’</td>
<td>Reverse primer developed from OCN002 chromosomal DNA</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 2. Summary of CFU counts and calculations for fragments used in infection trials.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Pre-trial GASW Average CFUs per ml of mucus</th>
<th>Average GASW CFUs per ml of mucus</th>
<th>Average Change (CFUs/ml)*</th>
<th>Test Statistic (W)</th>
<th>p-value**</th>
<th>Pre-trial TCBS CFUs per ml of mucus</th>
<th>Average TCBS CFUs per ml of mucus</th>
<th>Average proportion Vibrios</th>
<th>Percent Change in Vibrios (%)</th>
<th>Test Statistic (W)</th>
<th>p-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seawater</td>
<td>13</td>
<td>13.66</td>
<td>2.72</td>
<td>-1.0 X 10^6 (SE±3.79)</td>
<td>43</td>
<td>0.07</td>
<td>3.63</td>
<td>0.05</td>
<td>1.84%</td>
<td>-14.9% (SE±8.07%)</td>
<td>36</td>
<td>0.01</td>
</tr>
<tr>
<td>OCN004</td>
<td>13</td>
<td>13.66</td>
<td>1.26</td>
<td>-1.1 X 10^6 (SE±4.52)</td>
<td>67</td>
<td>0.01</td>
<td>3.63</td>
<td>&lt;0.01</td>
<td>&lt;1%</td>
<td>-16.0% (SE±7.72%)</td>
<td>36</td>
<td>0.01</td>
</tr>
<tr>
<td>OCN002-healthy</td>
<td>6</td>
<td>13.66</td>
<td>9.57</td>
<td>+1.3 X 10^3 (SE±466.98)</td>
<td>9</td>
<td>0.06</td>
<td>3.63</td>
<td>0.02</td>
<td>0.21%</td>
<td>-3.0% (SE±36%)</td>
<td>3</td>
<td>0.051</td>
</tr>
<tr>
<td>OCN002-diseased</td>
<td>7</td>
<td>13.66</td>
<td>2390.60</td>
<td>+2.5 X 10^7 (SE±69.55)</td>
<td>28</td>
<td>0.01</td>
<td>3.63</td>
<td>2203.57</td>
<td>92.18%</td>
<td>+78.9% (SE±26.35%)</td>
<td>28</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Changes in bacterial abundance and proportion of Vibrio species were calculated as the difference between colony counts from the pre-trial fragments and corresponding fragments at the end of the infection trials.

** The pre and post-trial levels of culturable bacteria and proportion of Vibrio species within each treatment (seawater control, bacterial control, fragments exposed to OCN002 that did and did not develop tissue loss) were examined using a Wilcoxon-signed rank test.
Table 3. Summary of metabolic and physiological characterization of *Vibrio owensii* strain OCN002, as compared to the type strains *V. owensii* strain DY05 (128) and *V. communis* strain R-40496 (129).

<table>
<thead>
<tr>
<th>Test</th>
<th>Vibrio owensii strain OCN002</th>
<th>Vibrio owensii strain DY05</th>
<th>Vibrio communis strain R-40496</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate Reduction to Nitrite</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indole production</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine dihydrolase production</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urease production</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-glucosidase production</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Gelatinase production</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-galactosidase production</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D-glucose assimilation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-arabinose assimilation</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-mannose assimilation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-mannitol assimilation</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-acetyl-glucosamine assimilation</td>
<td>+</td>
<td>ND</td>
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<td>D-maltose assimilation</td>
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<td>ND</td>
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<tr>
<td>Potassium gluconate assimilation</td>
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<td>+</td>
<td>ND</td>
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<td>Capric acid assimilation</td>
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<td>ND</td>
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<tr>
<td>Adipic acid assimilation</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Malic acid assimilation</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Trisodium citrate assimilation</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenylacetic acid assimilation</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Growth at 23°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 28°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 37°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) indicates a positive result and (-) indicates a negative result. ND indicates no data. API 20NE and indicator plate tests were quantified according to the manufacturer’s instructions, unless otherwise specified.
CHAPTER 3: VIBRIO CORALLIILYTICUS STRAIN OCN008 IS AN ETIOLOGICAL AGENT OF ACUTE MONTIPORA WHITE SYNDROME

INTRODUCTION

Bacteria have been implicated as etiological agents in many coral diseases; however, a causal relationship between a pathogen and disease has been established in only a limited number (15, 34, 39, 41, 53, 84, 114, 115, 119, 163, 164). Disease presentation is frequently characterized by one or both of two physical processes. Bleaching is the loss of photosynthetic symbiotic zooxanthellae from coral cells (165), and tissue loss is necrosis and eventual sloughing of infected tissue that exposes the calcium carbonate skeleton (101). Both of these responses can lead to the death of entire coral colonies. Most corals appear to have a limited number of gross morphological responses to disease; tissue loss or bleaching can also result from environmental stressors, so pathogen identification and precise disease description are critical first steps towards understanding disease processes (104).

Koch’s postulates of disease causation have been utilized in the field of coral disease research as guidelines for identifying a microorganism, rather than an environmental stressor, as the cause of a specific disease (123). In general, a causal relationship between disease and pathogen is inferred after isolation of the pathogen from diseased but not healthy hosts; isolation of the microorganism in pure culture; experimental induction of disease in healthy hosts by the microorganism; and re-isolation of the identical microorganism from experimentally infected hosts. Five coral pathogens have fulfilled Koch’s postulates: Aurantimonas coralica (14, 110), Serratia marcescens (15, 37), Vibrio shiloi (166), Vibrio owensii (84), and Vibrio coralliilyticus (38, 39).

**V. coralliilyticus** is a pathogen of particular concern because it has broad host and geographic ranges. Strains of **V. coralliilyticus** have been isolated from diseased corals in the Indian Ocean (53), Red Sea, Caribbean (40), the Great Barrier Reef, and Micronesia (39), and strains have been shown to infect coral of the genera *Pocillopora* (38), *Pachyseris, Acropora*, and *Montipora* (39). **V. coralliilyticus** has also been shown to infect non-coral hosts that include the mussel larva *Perna canaliculus* (49) and the rainbow trout *Oncorhynchus mykiss* (167).

Although various strains of **V. coralliilyticus** infect a broad range of hosts, pathogenesis has been studied predominantly in the strains BAA-450 and P1, which infect the corals *Pocillopora damicornis* and *Montipora aequituberculata*, respectively (38, 53). Water temperature plays a prominent role in the infection of *P. damicornis* by BAA-450 (17). Infections at temperatures from 24 to 25 °C cause bleaching of the coral host, whereas infections at 27 to 29 °C cause tissue loss, and BAA-450 is avirulent at temperatures below 22 °C. Increased concentrations of proteins that contribute to the virulence of other bacterial pathogens are present in, or secreted by, **V. coralliilyticus** strains BAA-450 and P1 at the higher temperature range, perhaps explaining the temperature-dependent change in disease presentation (58, 59). The level of one protein, FlhA, increased 10-fold when the temperature was shifted from 24 to 27 °C (59), and an intact *flhA* gene was required for chemotaxis, attachment and virulence with the coral *P. damicornis* (56).

In Kāneʻohe Bay, Hawaiʻi, *Montipora capitata*, one of the major reef-building corals, is affected by a tissue loss disease called *Montipora* white syndrome (MWS) (121). MWS has two disease presentations: a progressive infection designated chronic MWS (cMWS) that is caused by *Vibrio owensii* strain OCN002 and is characterized by subacute tissue loss, and a comparatively faster acute infection designated acute MWS (aMWS) (84, 121). The infectious
potential of aMWS was observed during outbreaks of rapid, wide-spread tissue loss in 2010 and 2011, with transmission observed between neighboring coral colonies in contact with one another (Aeby, unpublished data). Tissue loss from aMWS was hypothesized to be caused by an infectious agent like cMWS and not an environmental stressor.

This study focused on the pathogenesis of *V. coralliilysticus* strain OCN008, which contrasts with the temperature-dependent infections of BAA-450. Koch’s postulates were fulfilled for strain OCN008, establishing it as an etiological agent of acute *Montipora* white syndrome. OCN008 induced tissue loss on *M. capitata* fragments but not fragments of *Porites compressa*, suggesting host specificity for OCN008 infections. Comparative genomics showed that OCN008 possesses many of the putative virulence factors upregulated in strains BAA-450 and P1 in response to increases in temperature, despite the reduced influence of temperature on virulence. OCN008 is the first pathogenic *V. coralliilysticus* strain isolated from Hawai’i.

**RESULTS**

*Isolation and identification of a strain of V. coralliilysticus from healthy P. compressa*

Strain OCN008 was isolated on solid GASW medium from the coral *P. compressa* (168). It was initially chosen for further study based on an apparent zone of growth inhibition of neighboring bacteria surrounding the colony. Details of the inhibitory activity will be presented elsewhere. OCN008 was identified as a strain of *V. coralliilysticus* based on the sequence of the
gene encoding the 16S rRNA (KF042020) and MLSA (Figure 6). Although not identical, OCN008 clustered with the previously described pathogenic V. coralliilyticus strains P1 and BAA-450.

**V. coralliilyticus strains isolated from diseased Montipora capitata**

Two strains of bacteria that produced zones of growth inhibition similar to that of OCN008 were cultured from an M. capitata fragment displaying signs of chronic Montipora white syndrome (cMWS). These strains, designated OCN018 and OCN019, shared 99% and
100% sequence identity with the 16S rRNA gene of OCN008, respectively (KF042021 and KF042022, respectively). Both strains clustered with strain OCN008 in MLSA, indicating that they are strains of *V. coralliilyticus* and are more similar to OCN008 than to other strains of *V. coralliilyticus* that have been described (Figure 6). Screening of bacteria from healthy *M. capitata* did not yield any *V. coralliilyticus* strains that were similar to OCN008.

**Infection trials with M. capitata and P. compressa**

In the field, colonies of *M. capitata* with aMWS are often observed adjacent to healthy colonies of *P. compressa* (Figure 7A), suggesting host specificity of the causative agent of aMWS. To determine if strain OCN008 could infect *M. capitata* or *P. compressa*, healthy coral was exposed to $10^8$ CFU of OCN008 per ml of water at 27 °C. All *M. capitata* fragments displayed acute tissue loss between 12 and 96 h post-exposure, indicative of a cause and effect relationship between exposure to OCN008 and aMWS (McNemar’s test, $p=0.001$, $n=12$; Figure 2BC; Table 4). In contrast, none of the *P. compressa* fragments ($n=6$) developed tissue loss under the same conditions. None of the control *M. capitata* fragments maintained in FSW without added bacteria ($n=12$) or fragments exposed to $10^8$ CFU/ml of the bacterial control OCN004, a strain of *Alteromonas* ($n=12$), displayed signs of tissue loss during the experiment. When healthy *M. capitata* was exposed to strain OCN019, 90% of fragments displayed tissue loss between 12 and 96 h post-exposure, supporting a relationship between exposure to a strain of *V. coralliilyticus* and the initiation of tissue loss (McNemar’s test, $p=0.007$, $n=10$). We conclude that OCN008 and similar strains are capable of infecting healthy *M. capitata* but not *P. compressa*. 
Minimum infectious dose

To determine the minimum concentration of OCN008 required for infection, *M. capitata* fragments were exposed to different concentrations of OCN008. All *M. capitata* fragments exposed to $10^8$ CFU/ml of OCN008 (n=12) developed tissue loss, 17% developed tissue loss with $10^7$ CFU/ml (n=12), and no tissue loss was observed in fragments exposed to $10^6$ or $10^5$ CFU/ml (n=12 for each concentration; Table 4). Therefore, the infectious dose (ID$_{50}$) of strain
OCN008 was estimated to be between $10^7$ and $10^8$ CFU/ml of seawater. All of the control fragments exposed to FSW alone (n=12) and all fragments exposed to $10^8$ CFU/ml of OCN004 (n=12) remained healthy for the duration of the experiment.

*Influence of water temperature on OCN008 infection*

Paired infection trials maintained at 23 and 27 °C were used to determine if OCN008 infections were temperature dependent in a manner similar to those of BAA-450. Consistent with previous trials, all of the coral fragments exposed to OCN008 at 27 °C were infected between 12 and 96 h post-inoculation (n=6; Figure 8; Table 4). Of the corresponding fragments kept at 23 °C, 83% became infected between 24 and 192 h, and no additional infections were observed for the duration of the 28-day trial (n=6). Analysis of the Kaplan-Meier survival curves for infections at 23 and 27 °C found a significant difference between the incubation periods of the disease at the two temperatures (logrank test, p=0.0002, n=6). However, there was no significant difference for the risk of infection, defined as the likelihood of infection under certain conditions,
at 23 or 27 °C (McNemar’s test, p= 1.0, n=6). This suggests that at elevated temperatures *M. capitata* was infected earlier after exposure to OCN008 or the incubation time was shorter, but the risk of infection was unaffected.

**Re-isolation of OCN008 from laboratory-infected coral**

To facilitate re-isolation of OCN008 and differentiate it from similar bacteria that may be naturally present on corals, plasmid pRL1383a was used to genetically tag strain OCN008 (154). Tagged-OCN008 was re-isolated from all four corals infected by this strain as evidenced by identical sequences of PCR products derived from the plasmid and a unique chromosomal region of the OCN008 genome.

In addition to recovery of the plasmid-tagged strain of OCN008, non-tagged OCN008 was recovered from all six of the corals tested from the infection trials described in the section above. For each of the corals, all five bacterial isolates tested yielded a PCR product with primers specific to a unique chromosomal region of the OCN008 genome. Subsequent PCR amplification and sequencing of the 16S rRNA genes from re-isolated OCN008 confirmed that they were identical to that of the OCN008 laboratory stock. No isolates identical to OCN008 were isolated from control fragments used in the infection trials.

**Phenotypic characterization of OCN008**

To better describe *V. coralliilyticus* strain OCN008, some basic properties that may relate to its pathogenicity were evaluated. After 15 h incubation at 27 °C, OCN008 formed circular, opaque, mucoid, non-luminescent colonies on GASW agar, and colonies on TCBS agar were yellow, indicative of sucrose fermentation. Swarming motility was not observed, but at both 23
°C and 27 °C, β-hemolytic, protease, lipase, and DNase activities were observed. OCN008 is naturally resistant to ampicillin (200 μg/ml) and kanamycin (25 μg/ml). The maximum doubling time was calculated to be 14 min at 23 °C and 11.5 min at 27 °C. Growth of OCN008 was observed at NaCl concentrations from 0.7% to 6.0% (w/v) after 15 h of incubation, with no growth outside this range of NaCl concentrations.

Comparison of the genomes of OCN008, BAA-450 and P1

In silico comparison of the BAA-450 genome, the draft P1 genome, and the recently released draft genome of OCN008 (168) revealed 84.30% ± 2.75% similarity between OCN008 and BAA-450, 89.40% ± 2.32% similarity between OCN008 and P1, and 83.20% ± 2.82% similarity between BAA-450 and P1 using the genome-to-genome distance calculator (35–38; Table 5). Proteomic analysis of BAA-450 by Kimes et al. (2011) identified genes encoding proteins upregulated after a temperature shift from 24 to 27 °C (59), a majority of which were also present in the OCN008 genome (Table 7). Sequence identity between proteins from BAA-450 upregulated at least 4-fold by temperature and the corresponding proteins from OCN008 was 97 to 100%. Additionally, RAST analysis identified 31 genes present in the OCN008 genome that were absent from BAA-450 (Table 8). Predicted amino acid sequences from virulence-associated genes annotated as encoding hemolysins, proteases, lipases, type III secretion systems, prophage-related proteins, type VI secretion systems, toxins, and toxin-antitoxin systems in OCN008 were compared to those from the BAA-450 and P1 genomes (Table 9). A homolog of the zinc-metalloprotease vcpA (AFK08686), a proposed virulence factor of P1 (58), was found in OCN008 (ERB62335) and BAA-450 (EEX33179). Two genes (ERB62950 and ERB62951) in OCN008 encode products similar to V. cholerae RTX-like proteins, which have
been suggested to be virulence factors for BAA-450 and P1 (58, 59). The genes for four potential quorum sensing (QS) systems were identified in the OCN008 genome by comparison to characterized genes in the well-studied models for QS, Vibrio campbellii strain BAA-1116 (formally V. harveyi strain BB120) and Vibrio cholerae O1 biovar El Tor strain N16961 (40, 41; Table S6). All putative QS proteins encoded in the OCN008 genome (ERB66061, ERB66059, ERB62645, ERB62643, ERB62642, ERB62963, ERB62962, ERB63170, ERB63172, ERB62567, ERB66350, ERB64458, ERB62341, ERB62638) shared 98 to 100% identity with corresponding proteins in BAA-450 and P1. Despite differences in the regulation of pathogenesis, the three V. coralliilyticus strains share similar QS and virulence-associated genes.

DISCUSSION

This report describes OCN008, a virulent strain of V. coralliilyticus that infects the coral M. capitata inducing the tissue loss disease aMWS. Strains very similar to OCN0008 were isolated in pure culture from diseased M. capitata, but none were isolated from healthy M. capitata. Healthy fragments of M. capitata were infected by OCN008 during controlled laboratory experiments, and OCN008 could be re-isolated from infected fragments. The disease lesions observed in laboratory fragments appeared identical to the tissue loss lesions observed during aMWS outbreaks (84). No tissue loss was observed from any of the fragments exposed to seawater alone or control bacterium OCN004, indicating that tissue loss was associated with exposure to OCN008 and not due to captivity or general exposure to a bacterial culture. Taken together, these results fulfill Koch’s postulates of disease causation and identify OCN008 as a pathogen of M. capitata responsible for the tissue loss disease aMWS.
The apparent resistance of *P. compressa* to concentrations of OCN008 that were lethal to *M. capitata* indicates host-specificity for this pathogen. Field observations of healthy *P. compressa* colonies in contact with *M. capitata* colonies with aMWS are consistent with the observed host specificity in infection trials. Host specificity has been observed before with *V. coralliilyticus*. Strain P1, the etiological agent of white syndrome in *M. aequituberculata*, did not infect the coral *A. millepora* in infection trials, whereas a pathogenic strain of *Alteromonas* was infectious under the same conditions (68). Clearly, differences in corals affect the susceptibility of each to infection by a given strain of *V. coralliilyticus*. *V. coralliilyticus* as a species is regarded as having a broad host range, however, individual strains such as P1 and OCN008 may be more limited in their ability to cause disease in multiple host genera.

A large infectious dose of $10^7$ to $10^8$ CFU/ml of OCN008 was required for laboratory infection of *M. capitata*, yet inoculation with the same concentration of the avirulent, coral-associated *Alteromonas* strain OCN004 did not result in infection. Studies with the other strains of *V. coralliilyticus*, BAA-450 and P1, also report the use of comparatively high inocula in infection trials (17). Why might such a high infectious dose be required? Corals have a large suite of defensive strategies for protection from settling organisms (175, 176). One such mechanism is thought to be mucous production and sloughing. Recent work suggests that the sloughing of mucus is effective at controlling levels of the pathogen BAA-450 on the surface of coral, but this shedding mechanism can be overcome at levels of BAA-450 similar to the ID$_{50}$ found for OCN008 (78). At concentrations at or below $5 \times 10^6$ cells/ml seawater, levels of *V. coralliilyticus* on the surface-layer of corals appeared to be controlled by shedding, but after exposure to $5 \times 10^7$ cells/ml, levels of BAA-450 remained consistently at $10^6$ cells/cm$^3$ on the coral surface. Therefore, a large infectious dose for infection may be required for persistence of
V. coralliilyticus in the presence of mucous sloughing and other host defenses. In response to bacterial challenge by P1, Acropora millepora upregulated components of the innate immune response suggesting that secondary defenses also protect coral from opportunistic pathogens (68). An additional level of protection against pathogens is thought to be conferred by antimicrobial compounds produced by the normal bacterial flora associated with corals. For instance, growth of the pathogens V. shiloi and V. coralliilyticus was inhibited by substances produced by bacteria isolated from the corals Oculina patagonica, Montastraea annularis, and Pseudopterogorgia americana (40, 87, 88). The combined defensive potential of coral and its associated flora suggests that coral are not easily infected by any given bacterium. It is unknown what concentration of V. coralliilyticus is required for infection in the field, or if a concurrent environmental stressor is a prerequisite for infection. Factors such as physical injury have been shown to be required for disease initiation in other coral diseases (150). It is likely that OCN008 is persistent in the environment, but specific conditions are required for disease infection to take place, a scenario consistent with sporadic outbreaks of aMWS in Kāne‘ohe Bay.

Strains similar to OCN008 were isolated from M. capitata fragments displaying signs of a chronic MWS infection, whereas no such strains were found in healthy M. capitata. This suggests that OCN008 may readily associate with compromised hosts but apparently not with healthy coral. Studies with coral from the field have shown that bacterial communities of diseased corals are enriched for Vibrios compared to communities on healthy coral (84, 121). V. coralliilyticus strains similar to OCN008 may readily colonize pre-existing cMWS lesions that have reduced host defenses, consistent with the observation that infected corals switch between cMWS and aMWS in the field (Aeby and Smith, unpublished data). We have shown that OCN008 can initiate acute tissue loss in M. capitata. The next step will be to determine the
complex relationship between coral host, multiple bacterial pathogens and the environmental conditions in nature that allow infections to occur.

All infections of *M. capitata* by OCN008 resulted in tissue loss regardless of temperature, and no significant difference in the number of coral infected by OCN008 at 23 or 27 °C was observed. This is in stark contrast to the association of temperature and disease state in strain BAA-450; at 27 °C BAA-450 causes tissue loss in *P. damicornis*, whereas bleaching is observed at temperatures of 24 to 25 °C (17). It has been suggested that BAA-450 invasion of *P. damicornis* cells only occurs at elevated water temperatures, perhaps accounting for tissue lysis only at temperatures above 27 °C (48). Despite the differences in infection by the two strains, numerous genes encoding proteins that were upregulated in BAA-450 at elevated temperature (59) were also present in OCN008. Although sets of shared genes do not necessarily suggest a conserved mechanism of infection, the different effects of temperature on disease states may be indicative of differences in the regulation of virulence factors in the two strains or of differences in the host corals. While specific environmental factors promoting OCN008 infections are not yet understood, water temperature is not a primary factor in the infection of *M. capitata* by OCN008, indicative of strain variation and different host responses to *V. coralliilyticus.*

**METHODS**

*Coral collection, bacterial strains, and growth conditions*

Samples of *Montipora capitata* and *Porites compressa* for infection trials measured approximately 3 x 3 x 1 cm and were collected from a fringing reef surrounding the island Moku o Lo‘e in Kāneʻohe Bay, Hawaiʻi, which is dominated by these two coral species (121). All
collected fragments were allowed to recover for three days in flow-through seawater tables prior to the start of experiments (84).

*V. coralliilyticus* strain OCN008 was originally isolated from a fragment of *P. compressa* as described previously (168). Strains OCN018 and OCN019 were isolated from diseased *M. capitata* displaying signs of chronic MWS (cMWS) in the same manner as OCN008. Control bacterium *Alteromonas* sp. strain OCN004 was isolated from healthy *M. capitata* as previously described (84).

Marine bacteria were grown in glycerol artificial seawater (GASW) medium (84, 152) and incubated at 25 °C with aeration unless otherwise stated. Thiosulfate citrate bile salts sucrose (TCBS) agar (Sigma-Aldrich) was prepared according to the manufacturer’s instructions and incubated at 25 °C for the growth of *Vibrio* strains. *Escherichia coli* strains used for conjugation were maintained on Luria-Bertani (LB) medium (Sigma-Aldrich). Conjugations were conducted as previously described (84) on LB supplemented with 2.0% NaCl. Concentrations of antibiotics were kanamycin, 50 µg/ml; spectinomycin, 100 µg/ml; and ampicillin, 100 µg/ml.

**Identification of bacterial strains**

The 16S rRNA gene of strains OCN008, OCN018, and OCN019 was amplified and sequenced using the primers 8F and 1513R (177). Multilocus sequence analysis (MLSA) employed concatemerized partial sequences of the genes *pyrH, gapA, mreB, fitsZ, gyrB, and topA*. PCR, sequencing, and MLSA were performed with primers and protocols described previously (84).
Phenotypic characterization

Extracellular enzyme activity potentially related to virulence was assessed at 23 or 27 °C in triplicate using skim milk agar (Becton, Dickinson and Company) for protease activity, methyl green DNase agar (Becton, Dickinson and Company) for DNase activity, and 5% sheep blood TSA (Becton, Dickinson and Company) for hemolysin activity. The results from the indicator plates were evaluated according to the manufacturer’s instructions. Salt tolerance was tested in triplicate by spectrophotometric assessment of growth in LB broth with final NaCl concentrations ranging from 0% to 1.0% in 0.1% increments and ranging from 1% to 10% in 0.5% increments. Growth rate was determined with aerated liquid GASW cultures at 23 or 27 °C in triplicate and inoculated 1:10,000 from an overnight culture. Every hour for 36 h, optical density at 600 nm (OD\textsubscript{600}) was recorded, and dilutions were plated on solid GASW to calculate CFU/ml.

Laboratory infection trials

Coral fragments were acclimatized to infection trial temperatures over the course of a day prior to inoculation. Seawater passed through a 0.2 µm filter (FSW) was used for all laboratory trials. Infection trials utilized a block design with fragments from the same colony exposed to FSW, control bacterium OCN004 or strain OCN008 as previously described with the following minor modifications (84). Each replicate in each infection trial was collected from a unique coral colony. Control bacterium OCN004 is a strain of *Alteromonas* previously isolated from healthy *M. capitata* (84). Each fragment was tested separately in its own primary 4 L container filled with 3 L of FSW. Each set of primary containers was maintained in a larger, freshwater-filled secondary container that was fitted with water pumps and heaters for temperature control. Water
was heated to 23 or 27 °C, the average low and high sea surface temperatures of Kāne‘ohe Bay, respectively (127). For infection trials testing the effect of temperature, fragments at 27 °C had corresponding fragments from the same colony at 23 °C to account for genotypic differences between coral colonies.

To prepare inocula of infection trials, an overnight culture of the desired strain was diluted to an OD$_{600}$ of approximately 0.03 in GASW and incubated at either 23 or 27 °C, depending on the infection temperature. Cultures grown to an OD$_{600}$ of 1.6 were washed and diluted with FSW to an OD$_{600}$ of 0.8 for inoculation. Each tank was inoculated to a final concentration of 10$^8$ CFU/ml of water unless indicated otherwise. For determination of the minimal infectious dose, serial 10-fold dilutions of the standard inoculum were used to generate final concentrations of 10$^7$, 10$^6$, and 10$^5$ CFU/ml of seawater. Cultures were diluted in FSW immediately preceding exposure to corals during trials and plated to determine CFU/ml. Corals were monitored daily, and individual experiments were run for a maximum of 28 days.

Re-isolation and identification of OCN008

Prior to inoculation of corals, the RSF1010-derived vector pRL1383a was introduced into OCN008 through conjugation as previously described (84, 154) to genetically tag the bacterium. Fragments infected by tagged OCN008 were crushed in FSW, and serial dilutions from 1:10 to 1:10$^6$ were plated on solid GASW supplemented with spectinomycin, resistance to which was encoded on the plasmid. From each crushed fragment, 30 colonies were screened for the plasmid by PCR using the primers pRL1383a-MCS-F and pRL1383a-MCS-R (84). From each set of 30, five of the PCR products were sequenced as previously described to verify the identity of the plasmid (84).
The primers OCN008-42310-F and OCN008-43080-R were designed to amplify 772 bp of an intergenic region of the OCN008 genome (Table S2). The intergenic region was located between two divergent genes encoding a putative carboxypeptidase (ERB67101) and a pyridoxal phosphate-dependent aspartate aminotransferase (ERB66117). The intergenic region did not share significant nucleotide sequence similarity with any other sequence in NCBI as determined by BLAST. All isolates from the four corals infected with the tagged OCN008 were also screened by PCR using the primers for the specified intergenic region. Six corals infected with the non-tagged OCN008 were crushed, plated on TCBS, and had 10 bacterial colonies from each coral screened with the primers for the intergenic region and primers for the 16S rRNA gene. The resulting PCR products for the 16S rRNA gene were sequenced using the same primers used for PCR.

Analysis and comparison of the V. coralliilyticus genomes

The genome used in this analysis was previously uploaded to GenBank under the accession number AVOO00000000 and annotated using the NCBI prokaryotic genome annotation pipeline (168). The OCN008 genome was prepared for secondary analysis and genome comparisons using the Rapid Annotation using Subsystem Technology (RAST) server and SEED viewer version 2.0 (178, 179). Gene-to-gene comparisons were done with predicted amino acid sequences and conducted using RAST and BLASTp. Whole genome in silico comparisons were done using the genome-to-genome distance calculator (GGDC) version 2.0 with the MUMmer alignment method (169–172).
### TABLES

**Table 4:** Summary of infection trials. Infected fragments represents the proportion of fragments infected under the conditions described.

<table>
<thead>
<tr>
<th>Coral Species</th>
<th>Virulence of OCN008</th>
<th>Infectious Dose of OCN008</th>
<th>Effect of Temperature on OCN008 Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. capitata</td>
<td>M. capitata</td>
<td>M. capitata</td>
</tr>
<tr>
<td></td>
<td>M. capitata</td>
<td>M. capitata</td>
<td>M. capitata</td>
</tr>
<tr>
<td></td>
<td>M. capitata</td>
<td>P. compressa</td>
<td>P. compressa</td>
</tr>
<tr>
<td></td>
<td>P. compressa</td>
<td>P. compressa</td>
<td>P. compressa</td>
</tr>
<tr>
<td>Treatment</td>
<td>Seawater</td>
<td>OCN004</td>
<td>OCN008</td>
</tr>
<tr>
<td></td>
<td>OCN008</td>
<td>Seawater</td>
<td>OCN004</td>
</tr>
<tr>
<td>CFU/ml</td>
<td>-</td>
<td>10^6 CFU/ml</td>
<td>OCN004</td>
</tr>
<tr>
<td></td>
<td>10^6 CFU/ml</td>
<td>-</td>
<td>OCN008</td>
</tr>
<tr>
<td>Temperature</td>
<td>27 °C</td>
<td>27 °C</td>
<td>27 °C</td>
</tr>
<tr>
<td></td>
<td>27 °C</td>
<td>27 °C</td>
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<td>Infected Fragments</td>
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<td>0/6</td>
</tr>
</tbody>
</table>


Table 5. General genome comparison of *V. coralliilyticus* strains BAA-450, P1, and OCN008 based on annotation and analysis by the RAST server.

<table>
<thead>
<tr>
<th>Strain</th>
<th>BAA-450</th>
<th>P1</th>
<th>OCN008</th>
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<tbody>
<tr>
<td>Size</td>
<td>5,680,628 bp</td>
<td>5,513,256 bp</td>
<td>5,534,904 bp</td>
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<tr>
<td>G+C content</td>
<td>46.5%</td>
<td>46.0%</td>
<td>45.7%</td>
</tr>
<tr>
<td>Total CDS</td>
<td>5,078</td>
<td>5,107</td>
<td>5,632</td>
</tr>
<tr>
<td>Identified CDS</td>
<td>1,199</td>
<td>1,245</td>
<td>3,952</td>
</tr>
<tr>
<td>Hypothetical CDS</td>
<td>3,873</td>
<td>3,862</td>
<td>1,680</td>
</tr>
<tr>
<td>Identified tRNAs</td>
<td>92</td>
<td>53</td>
<td>45</td>
</tr>
<tr>
<td>Identified rRNAs</td>
<td>30</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Contigs</td>
<td>20</td>
<td>230</td>
<td>210</td>
</tr>
<tr>
<td>GDCC similarity to OCN008</td>
<td>84.30%</td>
<td>89.40%</td>
<td>-</td>
</tr>
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</table>
Table 6. List of DNA oligos and plasmids used in this study.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Description</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRL1383a MCS-F</td>
<td>5’ CGAAGTTATATTCCGATCGG 3’</td>
<td>Forward primer for vector pRL1383a</td>
<td>(84)</td>
</tr>
<tr>
<td>pRL1383a MCS-R</td>
<td>5’ CATTATGGTAAAGTGGAAACC 3’</td>
<td>Reverse primer for vector pRL1383a</td>
<td>(84)</td>
</tr>
<tr>
<td>8F</td>
<td>5’ AGAGTTTGATCCTGGCTCAG 3’</td>
<td>Forward primer for 16S rRNA gene</td>
<td>(177)</td>
</tr>
<tr>
<td>1513R</td>
<td>5’ GGTACCTTTGGTACGACTT 3’</td>
<td>Reverse primer for 16S rRNA gene</td>
<td>(177)</td>
</tr>
<tr>
<td>VrecA130F</td>
<td>5’ GTCTACCAATGGGCTGATTC 3’</td>
<td>Forward primer for the <em>Vibrio recA</em></td>
<td>(126)</td>
</tr>
<tr>
<td>VrecA720R</td>
<td>5’ GCCATTTAGCTGATACCAAG 3’</td>
<td>Reverse primer for the <em>Vibrio recA</em></td>
<td>(126)</td>
</tr>
<tr>
<td>VpyrH80F</td>
<td>5’ GATCGTGATGGCTCAAGAAG 3’</td>
<td>Forward primer for the <em>Vibrio pyrH</em></td>
<td>(126)</td>
</tr>
<tr>
<td>VpyrH530R</td>
<td>5’ TAGGCATTGGTGTGGCAG 3’</td>
<td>Reverse primer for the <em>Vibrio pyrH</em></td>
<td>(126)</td>
</tr>
<tr>
<td>VgapA150F</td>
<td>5’ AACTCAGGCTGTTCAAC 3’</td>
<td>Forward primer for the <em>Vibrio gapA</em></td>
<td>(126)</td>
</tr>
<tr>
<td>VgapA899R</td>
<td>5’ CGTTGTCGTAACAGATAC 3’</td>
<td>Reverse primer for the <em>Vibrio gapA</em></td>
<td>(126)</td>
</tr>
<tr>
<td>VmrebB12F</td>
<td>5’ ACTTCGTCGGCATTGTTC 3’</td>
<td>Forward primer for the <em>Vibrio mreB</em></td>
<td>(126)</td>
</tr>
<tr>
<td>VmrebB999R</td>
<td>5’ CCGTGCAATCGACGATTC 3’</td>
<td>Reverse primer for the <em>Vibrio mreB</em></td>
<td>(126)</td>
</tr>
<tr>
<td>VftsZ75F</td>
<td>5’ GCTGTTGACACATGGTACG 3’</td>
<td>Forward primer for the <em>Vibrio ftsZ</em></td>
<td>(126)</td>
</tr>
<tr>
<td>VftsZ800R</td>
<td>5’ GCACCGAGCAAGATCGATATC 3’</td>
<td>Reverse primer for the <em>Vibrio ftsZ</em></td>
<td>(126)</td>
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<tr>
<td>VgyrB274F</td>
<td>5’ GAAGTTATCATGACGGTACTTC 3’</td>
<td>Forward primer for the <em>Vibrio gyrB</em></td>
<td>(126)</td>
</tr>
<tr>
<td>VgyrB1171R</td>
<td>5’ CCTTTAGGAGGTCATCTTC 3’</td>
<td>Reverse primer for the <em>Vibrio gyrB</em></td>
<td>(126)</td>
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<tr>
<td>VtopA400F</td>
<td>5’ GAGATCATCGGTGGTGATG 3’</td>
<td>Forward primer for the <em>Vibrio topA</em></td>
<td>(126)</td>
</tr>
<tr>
<td>VtopA350F</td>
<td>5’ AGCTATTCATGAGGCACCTTC 3’</td>
<td>Forward primer for the <em>V. coralliilyticus topA</em></td>
<td>(126)</td>
</tr>
<tr>
<td>VtopA1200R</td>
<td>5’ GAAGGACGAATCGCTTTCTG 3’</td>
<td>Reverse primer for the <em>Vibrio topA</em></td>
<td>(126)</td>
</tr>
<tr>
<td>OCN008-42310-F</td>
<td>5’ GGTAGTCAAAGTGATAAATCGTCC 3’</td>
<td>Forward primer for OCN008 intergenic region</td>
<td>This study</td>
</tr>
<tr>
<td>OCN008-43080-R</td>
<td>5’ CTGTTGATACACTGGTACG 3’</td>
<td>Reverse primer for OCN008 intergenic region</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid name</td>
<td>Description</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>pRL1383a</td>
<td>RSFI1010-derived, broad-host range vector; Sp’/Sm’</td>
<td></td>
<td>(154)</td>
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</table>
Table 7. Genes which had a 4-fold upregulation in product in *V. coralliilyticus* strain BAA-450 when temperature was shifted from 23 to 27 °C. Genes were compared based upon predicted amino acid sequences.

<table>
<thead>
<tr>
<th>#</th>
<th>BAA-450 Accession Number</th>
<th>Gene product</th>
<th>Amino acid identity to OCN008 gene</th>
<th>OCN008 Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ZP_05885687.1</td>
<td>Che cluster related two-component response regulator</td>
<td>100%</td>
<td>ERB63516.1</td>
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<tr>
<td>2</td>
<td>ZP_05885322.1</td>
<td>CheW domain protein</td>
<td>98%</td>
<td>ERB64269.1</td>
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<tr>
<td>3</td>
<td>ZP_05884966.1</td>
<td>Methyl-accepting chemotaxis protein</td>
<td>100%</td>
<td>ERB63940.1</td>
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<td>4</td>
<td>ZP_05886733.1</td>
<td>Methyl-accepting chemotaxis protein</td>
<td>99%</td>
<td>ERB64942.1</td>
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<td>5</td>
<td>ZP_05886914.1</td>
<td>Methyl-accepting chemotaxis protein</td>
<td>99%</td>
<td>ERB66087.1</td>
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<td>6</td>
<td>ZP_05886733.1</td>
<td>Methyl-accepting chemotaxis protein</td>
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<td>ERB64942.1</td>
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<tr>
<td>7</td>
<td>ZP_05886661.1</td>
<td>Methyl-accepting chemotaxis protein</td>
<td>98%</td>
<td>ERB65227.1</td>
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<td>8</td>
<td>ZP_05887140.1</td>
<td>Methyl-accepting chemotaxis protein</td>
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<td>ERB63658.1</td>
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<td>9</td>
<td>ZP_05887212.1</td>
<td>Methyl-accepting chemotaxis protein</td>
<td>99%</td>
<td>ERB66811.1</td>
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<tr>
<td>10</td>
<td>ZP_05887588.1</td>
<td>Methyl-accepting chemotaxis protein</td>
<td>96%</td>
<td>ERB64224.1</td>
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<tr>
<td>11</td>
<td>ZP_05886915.1</td>
<td>Methyl-accepting chemotaxis protein</td>
<td>99%</td>
<td>ERB66085.1</td>
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<td>12</td>
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<td>14</td>
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<td>15</td>
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<td>24</td>
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<td>Methyl-accepting chemotaxis sensory transducer</td>
<td>98%</td>
<td>ERB66094.1</td>
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**Flagellar Proteins**

<table>
<thead>
<tr>
<th>#</th>
<th>BAA-450 Accession Number</th>
<th>Gene product</th>
<th>Amino acid identity to OCN008 gene</th>
<th>OCN008 Accession Number</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>ZP_05883831.1</td>
<td>Flagellar biosynthesis protein flgN</td>
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<td>Flagellar biosynthesis protein flhA</td>
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<td>WP_019277846.1*</td>
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<tr>
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<td>ZP_05885373.1</td>
<td>Flagellar biosynthesis protein flIL</td>
<td>99%</td>
<td>ERB62290.1</td>
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<td>4</td>
<td>ZP_05883723.1</td>
<td>Flagellar biosynthesis protein flIL</td>
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<td>ERB63143.1</td>
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<tr>
<td>5</td>
<td>ZP_05888389.1</td>
<td>Flagellar hook protein flgE</td>
<td>97%</td>
<td>WP_019274765.1*</td>
</tr>
<tr>
<td>6</td>
<td>ZP_05885421.1</td>
<td>Flagellar motor rotation protein motB</td>
<td>99%</td>
<td>ERB65276.1</td>
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<tr>
<td>7</td>
<td>ZP_05885379.1</td>
<td>Flagellar M-ring protein flfF</td>
<td>100%</td>
<td>ERB62438.1</td>
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<tr>
<td>8</td>
<td>ZP_05885390.1</td>
<td>Flagellin protein flaF</td>
<td>100%</td>
<td>ERB63253.1</td>
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<td>9</td>
<td>ZP_05885387.1</td>
<td>Flagellin protein flaG</td>
<td>100%</td>
<td>ERB63257.1</td>
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<td>10</td>
<td>ZP_05883832.1</td>
<td>Negative regulator of flagellin synthesis flgM</td>
<td>100%</td>
<td>ERB64522.1</td>
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<tr>
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<td>ZP_05885328.1</td>
<td>RNA polymerase sigma factor for flagellar operon</td>
<td>100%</td>
<td>ERB64264.1</td>
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<tr>
<td><strong>Multidrug Efflux Pumps</strong></td>
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<td>1</td>
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<td>Multidrug resistance efflux pump</td>
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<tr>
<td><strong>Hemolysins</strong></td>
<td></td>
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<tr>
<td>1</td>
<td>ZP_05888459.1</td>
<td>Hemolysin</td>
<td>99%</td>
<td>ERB64020.1</td>
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<td><strong>Toxins</strong></td>
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<tr>
<td>1</td>
<td>ZP_05887752.1</td>
<td>Cholera toxin transcriptional activator (toxR-like)</td>
<td>99%</td>
<td>ERB64819.1</td>
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<tr>
<td>2</td>
<td>ZP_05883808.1</td>
<td>Transcriptional activator ToxR</td>
<td>99%</td>
<td>ERB64497.1</td>
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<tr>
<td><strong>Proteases</strong></td>
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<tr>
<td>1</td>
<td>ZP_05884175.1</td>
<td>Membrane-associated zinc metalloprotease</td>
<td>98%</td>
<td>ERB65389.1</td>
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<tr>
<td>2</td>
<td>ZP_05884287.1</td>
<td>Outer membrane stress sensor protease DegS</td>
<td>100%</td>
<td>ERB63319.1</td>
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<tr>
<td>3</td>
<td>ZP_05885130.1</td>
<td>Putative stomatin/prohibitin-family membrane protease subunit aq_911</td>
<td>100%</td>
<td>ERB64648.1</td>
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<tr>
<td>4</td>
<td>ZP_05883718.1</td>
<td>SOS-response repressor and protease LexA</td>
<td>100%</td>
<td>WP_006957296.1*</td>
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<td><strong>Ribosomal gene products</strong></td>
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<td>1</td>
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<td>LSU ribosomal protein L25p</td>
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<td>3</td>
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<td>LSU ribosomal protein L32p</td>
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<td>4</td>
<td>ZP_05884022.1</td>
<td>Ribosomal large subunit pseudouridine synthase D (EC 4.2.1.70)</td>
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<td>ERB62776.1</td>
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<td>5</td>
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<td>SSU ribosomal protein S17p (S11e)</td>
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<td>ERB66877.1</td>
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<tr>
<td>6</td>
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<td>SSU ribosomal protein S21p</td>
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<td>ERB64957.1</td>
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<td><strong>Type I Secretion Proteins</strong></td>
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<td>1</td>
<td>ZP_05884344.1</td>
<td>(GlcNAc)2 ABC transporter, periplasmic substrate-binding protein</td>
<td>100%</td>
<td>WP_006958092.1*</td>
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<tr>
<td>2</td>
<td>ZP_05887533.1</td>
<td>ABC transporter protein</td>
<td>N/A</td>
<td></td>
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<tr>
<td>3</td>
<td>ZP_05887406.1</td>
<td>ABC transporter substrate binding protein</td>
<td>100%</td>
<td>ERB63824.1</td>
</tr>
<tr>
<td>4</td>
<td>ZP_05885258.1</td>
<td>ABC transporter, ATP-binding protein</td>
<td>99%</td>
<td>ERB64355.1</td>
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<tr>
<td>5</td>
<td>ZP_05886569.1</td>
<td>ABC transporter, ATP-binding protein</td>
<td>100%</td>
<td>ERB64404.1</td>
</tr>
<tr>
<td>6</td>
<td>ZP_05887533.1</td>
<td>ABC transporter, ATP-binding/permease protein</td>
<td>N/A</td>
<td></td>
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<tr>
<td>7</td>
<td>ZP_05887946.1</td>
<td>ABC transporter, periplasmic substrate-binding protein YjB</td>
<td>97%</td>
<td>WP_019277060.1*</td>
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<tr>
<td>8</td>
<td>ZP_05887479.1</td>
<td>ABC-type amino acid transport/signal transduction system</td>
<td>99%</td>
<td>ERB65507.1</td>
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<tr>
<td>9</td>
<td>ZP_05887443.1</td>
<td>Amino acid ABC transporter, permease protein</td>
<td>99%</td>
<td>ERB65468.1</td>
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<tr>
<td>10</td>
<td>ZP_05885066.1</td>
<td>Arginine/ornithine ABC transporter, permease protein AotQ</td>
<td>99%</td>
<td>WP_019276060.1*</td>
</tr>
<tr>
<td>11</td>
<td>ZP_05888200.1</td>
<td>Excinuclease ABC subunit B</td>
<td>99%</td>
<td>ERB62964.1</td>
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<td>12</td>
<td>ZP_05886404.1</td>
<td>Nitrate ABC transporter, nitrate-binding protein</td>
<td>99%</td>
<td>ERB66044.1</td>
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<td>13</td>
<td>ZP_05884332.1</td>
<td>Outer membrane protein OmpU</td>
<td>100%</td>
<td>ERB66165.1</td>
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<td>14</td>
<td>ZP_05883981.1</td>
<td>Peptide ABC transporter, permease component</td>
<td>100%</td>
<td>ERB62508.1</td>
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<tr>
<td>15</td>
<td>ZP_05883982.1</td>
<td>peptide ABC transporter, permease protein</td>
<td>99%</td>
<td>ERB62507.1</td>
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<td>16</td>
<td>ZP_05886568.1</td>
<td>Predicted ABC-type transport system, permease component</td>
<td>99%</td>
<td>ERB64403.1</td>
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<tr>
<td>17</td>
<td>ZP_05886687.1</td>
<td>Putative ABC transporter ATP-binding protein</td>
<td>99%</td>
<td>ERB65208.1</td>
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<tr>
<td>18</td>
<td>ZP_05886092.1</td>
<td>Putative outer membrane protein</td>
<td>86%</td>
<td>ERB63108.1</td>
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<tr>
<td>19</td>
<td>ZP_05887876.1</td>
<td>Putative sugar ABC transporter, periplasmic sugar-binding</td>
<td>99%</td>
<td>ERB65859.1</td>
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<tr>
<td>20</td>
<td>ZP_05887271.1</td>
<td>Urea ABC transporter, permease protein UrtB</td>
<td>96%</td>
<td>ERB65683.1</td>
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<tr>
<td>21</td>
<td>ZP_05887272.1</td>
<td>Urea ABC transporter, urea binding protein</td>
<td>99%</td>
<td>ERB65682.1</td>
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<tr>
<td>22</td>
<td>ZP_05884075.1</td>
<td>Vitamin B12 ABC transporter, B12-binding component BtuF</td>
<td>98%</td>
<td>ERB65423.1</td>
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</tbody>
</table>

**Type II Secretion Proteins**

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>1</td>
<td>ZP_05884093.1</td>
<td>General secretion pathway protein B</td>
<td>96%</td>
<td>ERB62547.1</td>
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**Type IV Pili Proteins**

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<tbody>
<tr>
<td>1</td>
<td>ZP_05884490.1</td>
<td>MSHA biogenesis protein MshG</td>
<td>97%</td>
<td>ERB65812.1</td>
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<tr>
<td>2</td>
<td>ZP_05884482.1</td>
<td>MSHA biogenesis protein MshQ</td>
<td>69%</td>
<td>ERB65820.1</td>
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<tr>
<td>3</td>
<td>ZP_05885611.1</td>
<td>Flp pilus assembly protein RcpC/CpaB</td>
<td>84%</td>
<td>ERB66221.1</td>
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<tr>
<td>4</td>
<td>ZP_05885604.1</td>
<td>Flp pilus assembly protein TadD, contains TPR repeat</td>
<td>80%</td>
<td>ERB66231.1</td>
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<tr>
<td>5</td>
<td>ZP_05884461.1</td>
<td>Type IV pilus (Tfp) assembly protein, ATPase component PilU</td>
<td>95%</td>
<td>WP_019277261.1*</td>
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**Type VI Secretion Proteins**

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<tr>
<td>1</td>
<td>ZP_05887403.1</td>
<td>putative lipoprotein</td>
<td>100%</td>
<td>ERB63827.1</td>
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<tr>
<td>2</td>
<td>ZP_05887404.1</td>
<td>hypothetical protein VIC_003916</td>
<td>99%</td>
<td>ERB63826.1</td>
</tr>
<tr>
<td>3</td>
<td>ZP_05887406.1</td>
<td>ABC transporter substrate binding protein</td>
<td>100%</td>
<td>ERB63824.1</td>
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<td>4</td>
<td>ZP_05887409.1</td>
<td>hypothetical protein VIC_003921</td>
<td>100%</td>
<td>ERB63822.1</td>
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<tr>
<td>5</td>
<td>ZP_05887410.1</td>
<td>protein ImpG/VasA</td>
<td>99%</td>
<td>ERB63821.1</td>
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<td>6</td>
<td>ZP_05886644.1</td>
<td>uncharacterized protein ImpB</td>
<td>100%</td>
<td>ERB64085.1</td>
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**Regulators**

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<tbody>
<tr>
<td>1</td>
<td>ZP_05888198.1</td>
<td>Phosphorelay protein luxU</td>
<td>100%</td>
<td>ERB62962.1</td>
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<tr>
<td>2</td>
<td>ZP_05884374.1</td>
<td>Quorum-sensing regulator of virulence HapR</td>
<td>100%</td>
<td>ERB64458.1</td>
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<td>3</td>
<td>ZP_05887548.1</td>
<td>Two component transcriptional regulator, LuxR family</td>
<td>100%</td>
<td>ERB63040.1</td>
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<td>4</td>
<td>ZP_05886724.1</td>
<td>Anti-anti-sigma regulatory factor</td>
<td>100%</td>
<td>ERB66348.1</td>
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<tr>
<td>5</td>
<td>ZP_05885328.1</td>
<td>RNA polymerase sigma factor for flagellar operon</td>
<td>100%</td>
<td>ERB64264.1</td>
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<tr>
<td>6</td>
<td>ZP_05885306.1</td>
<td>RNA polymerase sigma-70 factor, ECF subfamily</td>
<td>99%</td>
<td>ERB64285.1</td>
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</table>

* Best match on NCBI: genes found from RAST analysis, but not annotated on NCBI. The gene sequence has been provided for that gene.

N/A = Not found in genome using RAST or BLAST
Table 8. Genes present in OCN008 but not in BAA-450 after RAST annotation of both genomes. Genes were compared through RAST using their predicted amino acid sequences.

<table>
<thead>
<tr>
<th>Gene function</th>
<th>Gene with highest amino acid identity to [Bacterium]</th>
<th>Accession Number</th>
<th>AA similarity</th>
<th>OCN008 Accession Number</th>
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<tr>
<td><strong>Carbohydrate metabolism</strong></td>
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<tr>
<td>Beta-glucanase precursor (EC 3.2.1.73)</td>
<td>beta-glucanase [Vibrio genosp. F6]</td>
<td>WP_017054793</td>
<td>329/522(63%)</td>
<td>ERB64603.1</td>
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<tr>
<td>Periplasmic beta-glucosidase (EC 3.2.1.21)</td>
<td>1,4-beta-D-glucan glucohydrolase [Vibrio shilonii]</td>
<td>WP_006069517</td>
<td>565/848(67%)</td>
<td>ERB64600.1</td>
</tr>
<tr>
<td>Maltose O-acetyltransferase (EC 2.3.1.79)</td>
<td>hypothetical protein [Vibrio corallilyticus strain P1]</td>
<td>WP_019275206</td>
<td>179/181(99%)</td>
<td>ERB64548.1</td>
</tr>
<tr>
<td>Maltose operon transcriptional repressor MalR, LacI family</td>
<td>transcriptional regulator [Vibrio sp. 16]</td>
<td>WP_005469176</td>
<td>272/331(82%)</td>
<td>ERB64604.1</td>
</tr>
<tr>
<td>GDP-mannose 4,6-dehydratase (EC 4.2.1.47)</td>
<td>GDP-D-mannose dehydratase [Francisella philomiragia subsp. philomiragia ATCC 25017]</td>
<td>WP_001677973</td>
<td>316/378(84%)</td>
<td>ERB62487.1</td>
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<tr>
<td>N-acetyleneuraminic acid outer membrane channel protein NanC</td>
<td>membrane protein [Vibrio tubiashii]</td>
<td>WP_004744130</td>
<td>205/228(90%)</td>
<td>ERB63648.1</td>
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<tr>
<td><strong>Hypothetical Proteins</strong></td>
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<tr>
<td>FIG116849: hypothetical protein</td>
<td>hypothetical protein VC0395_A2355 [Vibrio cholerae O395]</td>
<td>WP_001218255</td>
<td>444/458(97%)</td>
<td>ERB64825.1</td>
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<tr>
<td>FIG131328: Predicted ATP-dependent endonuclease of the OLD family</td>
<td>hypothetical protein VC0395_A2354 [Vibrio cholerae O395]</td>
<td>WP_001218254</td>
<td>548/565(97%)</td>
<td>ERB64826.1</td>
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<td>Type IV fimbrial biogenesis protein FimT</td>
<td>hypothetical protein [Vibrio corallilyticus P1]</td>
<td>WP_019274191</td>
<td>47/48(98%)</td>
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<tr>
<td>Similar to TadZ/CpaE, associated with Flp pilus assembly</td>
<td>pilus assembly protein TadE [Vibrio corallilyticus P1]</td>
<td>WP_019274095</td>
<td>63/63(100%)</td>
<td>ERB66226.1</td>
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<tr>
<td>Flp pilus assembly protein</td>
<td>pilus assembly protein [Vibrio corallilyticus P1]</td>
<td>WP_019274093</td>
<td>309/389(79%)</td>
<td>ERB66224.1</td>
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<td><strong>Aromatic compound degradation proteins</strong></td>
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<td>Salicylate hydroxylase (EC 1.14.13.1)</td>
<td>hypothetical protein [Vibrio corallilyticus P1]</td>
<td>WP_019275914</td>
<td>366/369(99%)</td>
<td>ERB65063.1</td>
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<td>toluenesulfonate zinc-independent alcohol dehydrogenase</td>
<td>S-ketoacyl-ACP reductase [Hyphomicrobium zavarzinii]</td>
<td>WP_020084432</td>
<td>153/249(61%)</td>
<td>ERB66389.1</td>
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<td><strong>Phage-associated proteins</strong></td>
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<tr>
<td>Phage capsid scaffolding protein</td>
<td>capsid scaffolding protein [Vibrio corallilyticus P1]</td>
<td>WP_019274347</td>
<td>269/271(99%)</td>
<td>ERB66310.1</td>
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<td>Phage major capsid protein</td>
<td>capsid protein [Vibrio corallilyticus P1]</td>
<td>WP_019274348</td>
<td>345/345(100%)</td>
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<td>Peptide chain release factor homolog</td>
<td>hypothetical protein [Vibrio corallilyticus P1]</td>
<td>WP_019275950</td>
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<td>Protein with similarity to RtcB</td>
<td>release factor H-coupled RctB family protein [Vibrio corallilyticus P1]</td>
<td>WP_019275949</td>
<td>293/299(98%)</td>
<td>ERB66517.1</td>
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<tr>
<td>Protein with similarity to RtcB</td>
<td>release factor H-coupled RctB family protein [Vibrio corallilyticus P1]</td>
<td>WP_019275949</td>
<td>52/53(98%)</td>
<td>ERB66518.1</td>
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<td>WP_019277566</td>
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<td>Translation elongation factor Tu</td>
<td>protein translation elongation factor Tu (EF-TU), partial [Vibrio tubiashii]</td>
<td>WP_004749174</td>
<td>353/356(99%)</td>
<td>ERB62177.1</td>
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<td><strong>RNA encoding</strong></td>
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<tr>
<td>tRNA (guanosine(18)-2'-O-)methyltransferase (EC 2.1.1.34)</td>
<td>tRNA methyltransferase [Microbulbifer agariyiticus S89]</td>
<td>WP_010131545</td>
<td>153/194(79%)</td>
<td>ERB62181.1</td>
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<tr>
<td><strong>Toxin-antitoxin system</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Death on curing protein, Doc toxin</td>
<td>Death on curing protein, Doc toxin [Vibrio parahaemolyticus BB22OP]</td>
<td>WP_007275327</td>
<td>98/100(98%)</td>
<td>ERB63072.1</td>
</tr>
<tr>
<td>Protein Type</td>
<td>Description</td>
<td>Species</td>
<td>Accession Number</td>
<td>Identity</td>
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<td>--------------</td>
<td>-------------</td>
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<td>-----------------</td>
<td>----------</td>
</tr>
<tr>
<td>Death on curing protein, Doc toxin</td>
<td>Plasmid stabilization system protein</td>
<td>[Vibrio vulnificus CMCP6]</td>
<td>NP_761257</td>
<td>96/100(96%)</td>
</tr>
<tr>
<td>Prevent host death protein, Phd antitoxin</td>
<td>Antitoxin of toxin-antitoxin stability system</td>
<td>[Vibrio parahaemolyticus BB22OP]</td>
<td>YP_007275328</td>
<td>83/85(98%)</td>
</tr>
<tr>
<td>Prevent host death protein, Phd antitoxin</td>
<td>Antitoxin of toxin-antitoxin stability system</td>
<td>[Vibrio vulnificus CMCP6]</td>
<td>NP_761256</td>
<td>84/85(99%)</td>
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<tr>
<td>ParD protein (antitoxin to ParE)</td>
<td>Prevent-host-death protein</td>
<td>[Vibrio harveyi]</td>
<td>WP_005442033</td>
<td>80/80(100%)</td>
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<tr>
<td>ParE toxin protein</td>
<td>ParE toxin protein</td>
<td>[Vibrio parahaemolyticus BB22OP]</td>
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<td>95/99(96%)</td>
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<tr>
<td>Motility</td>
<td>Twitching motility protein PilT</td>
<td>[Vibrio brasilensis]</td>
<td>WP_006880589</td>
<td>113/131(86%)</td>
</tr>
<tr>
<td>Stress response</td>
<td>Glutathione S-transferase-like protein</td>
<td>[Vibrio coralliilyticus P1]</td>
<td>WP_019275947</td>
<td>211/216(98%)</td>
</tr>
</tbody>
</table>
Table 9. Comparison of virulence-associated genes in OCN008 to homologs in BAA-450 and P1. Genes were compared through RAST using their predicted amino acid sequences.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>OCN008 Contig</th>
<th>Shared identity with BAA-450</th>
<th>Shared identity with P1</th>
<th>OCN008 Accession Number</th>
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</thead>
<tbody>
<tr>
<td></td>
<td><strong>Hemolysins, cytolysins, and porins</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Cytolysin and hemolysin, HlyA, Pore-forming toxin</td>
<td>contig00118</td>
<td>607/616 (98%)</td>
<td>609/616 (98%)</td>
<td>ERB62649.1</td>
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<tr>
<td>2</td>
<td>Cytolysin secretion protein</td>
<td>contig00023</td>
<td>167/168 (99%)</td>
<td>167/168 (99%)</td>
<td>ERB65555.1</td>
</tr>
<tr>
<td>3</td>
<td>Cytolysin precursor</td>
<td>contig00023</td>
<td>471/471 (100%)</td>
<td>471/471 (100%)</td>
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<tr>
<td>4</td>
<td>Phospholipase/lecithinase/hemolysin</td>
<td>contig00064</td>
<td>407/413 (98%)</td>
<td>410/413 (99%)</td>
<td>ERB63813.1</td>
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<tr>
<td>5</td>
<td>21 kDa hemolysin precursor</td>
<td>contig00082</td>
<td>188/189 (99%)</td>
<td>187/189 (98%)</td>
<td>ERB63310.1</td>
</tr>
<tr>
<td>6</td>
<td>Hemolysin</td>
<td>contig00002</td>
<td>83/85 (97%)</td>
<td>84/85 (98%)</td>
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<tr>
<td>7</td>
<td>Leukocidin/Hemolysin toxin family protein</td>
<td>contig00138</td>
<td>302/302 (100%)</td>
<td>302/302 (100%)</td>
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<td>OGI1272: Predicted membrane protein hemolysin III homolog</td>
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<td>217/217 (100%)</td>
<td>ERB64687.1</td>
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<tr>
<td>9</td>
<td>Hemolysins and related proteins containing CBS domains</td>
<td>contig00034</td>
<td>423/424 (99%)</td>
<td>423/424 (99%)</td>
<td>ERB64943.1</td>
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<td>10</td>
<td>Putative hemolysin</td>
<td>contig00034</td>
<td>581/584 (99%)</td>
<td>583/584 (99%)</td>
<td>ERB64972.1</td>
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<tr>
<td>11</td>
<td>Hemolysin</td>
<td>contig00049</td>
<td>903/910 (99%)</td>
<td>904/910 (99%)</td>
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<tr>
<td>12</td>
<td>Putative hemolysin</td>
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<td>154/154 (100%)</td>
<td>154/154 (100%)</td>
<td>WP_006961773.1*</td>
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<td>13</td>
<td>Putative hemolysin</td>
<td>contig00069</td>
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<td>407/409 (99%)</td>
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<td>14</td>
<td>Outer membrane protein N, non-specific porin</td>
<td>contig00002</td>
<td>335/346 (96%)</td>
<td>345/346 (99%)</td>
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<tr>
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<td>Outer membrane protein N, non-specific porin</td>
<td>contig00092</td>
<td>301/347 (86%)</td>
<td>329/333 (98%)</td>
<td>ERB63108.1</td>
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<tr>
<td></td>
<td><strong>Protease, metalloproteases, and petidases</strong></td>
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<td></td>
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<tr>
<td>1</td>
<td>Outer membrane stress sensor protease DegQ, serine protease</td>
<td>contig00082</td>
<td>450/455 (98%)</td>
<td>454/455 (99%)</td>
<td>ERB63318.1</td>
</tr>
<tr>
<td>2</td>
<td>Outer membrane stress sensor protease DegS</td>
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<td>355/355 (100%)</td>
<td>354/355 (99%)</td>
<td>ERB63319.1</td>
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<tr>
<td>3</td>
<td>Zinc protease, insulinase family</td>
<td>contig00068</td>
<td>760/783 (97%)</td>
<td>720/740 (97%)</td>
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<tr>
<td>4</td>
<td>Zinc protease, insulinase family</td>
<td>contig00068</td>
<td>106/111 (95%)</td>
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<tr>
<td>5</td>
<td>Zn-dependent protease with chaperone function PA4632</td>
<td>contig00047</td>
<td>251/262 (95%)</td>
<td>261/262 (99%)</td>
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<tr>
<td>6</td>
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<td>contig00047</td>
<td>343/360 (95%)</td>
<td>353/360 (98%)</td>
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<tr>
<td>7</td>
<td>Putative ATP-dependent Lon protease</td>
<td>contig00113</td>
<td>156/156 (100%)</td>
<td>155/156 (99%)</td>
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<tr>
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<td>Probable protease htpX homolog (EC 3.4.24.-)</td>
<td>contig00036</td>
<td>155/155 (100%)</td>
<td>155/155 (100%)</td>
<td>ERB64841.1</td>
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<tr>
<td>9</td>
<td>Protease II (EC 3.4.21.83)</td>
<td>contig00041</td>
<td>690/696 (99%)</td>
<td>694/696 (99%)</td>
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<tr>
<td>10</td>
<td>Hemagglutinin</td>
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<td>660/667 (98%)</td>
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<td>11</td>
<td>Putative membrane-bound ClpP-class protease associated with q911</td>
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<td>454/459 (98%)</td>
<td>452/459 (98%)</td>
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</tr>
<tr>
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<td>Putative stomatin/prohibitin-family membrane protease subunit q911</td>
<td>contig00041</td>
<td>255/256 (99%)</td>
<td>256/256 (100%)</td>
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<td>13</td>
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<td>266/267 (99%)</td>
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<tr>
<td>14</td>
<td>Cold-active serine alkaline protease</td>
<td>contig00003</td>
<td>292/299 (97%)</td>
<td>292/299 (97%)</td>
<td>ERB66971.1</td>
</tr>
<tr>
<td>15</td>
<td>Cold-active serine alkaline protease</td>
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<td>292/299 (97%)</td>
<td>292/299 (97%)</td>
<td>ERB66971.1</td>
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<tr>
<td>16</td>
<td>Tail-specific protease precursor (EC 3.4.21.102)</td>
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<td>659/664 (99%)</td>
<td>659/664 (99%)</td>
<td>ERB65485.1</td>
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<td>ATP-dependent protease La (EC 3.4.21.53) Type II</td>
<td>contig00024</td>
<td>543/547 (99%)</td>
<td>538/547 (98%)</td>
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<td>18</td>
<td>Inactive homolog of metal-dependent proteases, putative molecular chaperone</td>
<td>contig00006</td>
<td>231/233 (99%)</td>
<td>233/233 (100%)</td>
<td>ERB66706.1</td>
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<td>19</td>
<td>Possible protease s0hB (EC 3.4.21.-)</td>
<td>contig00006</td>
<td>349/349 (100%)</td>
<td>349/349 (100%)</td>
<td>ERB66741.1</td>
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<tr>
<td>20</td>
<td>ATP-dependent protease HsIV (EC 3.4.25.-)</td>
<td>contig00053</td>
<td>69/69 (100%)</td>
<td>69/69 (100%)</td>
<td>WP_006963336.1*</td>
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<tr>
<td>21</td>
<td>ATP-dependent protease HsIV (EC 3.4.25.-)</td>
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<td>102/103 (99%)</td>
<td>102/103 (99%)</td>
<td>WP_006963336.1*</td>
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<td>22</td>
<td>ATP-dependent hsl protease ATP-binding subunit HsU</td>
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<td>437/444 (98%)</td>
<td>442/446 (99%)</td>
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<td>23</td>
<td>Exported zinc metalloprotease YfgC precursor</td>
<td>contig00039</td>
<td>430/431 (99%)</td>
<td>481/484 (99%)</td>
<td>ERB64715.1</td>
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<td>24</td>
<td>Membrane-associated zinc metalloprotease</td>
<td>contig00026</td>
<td>447/452 (98%)</td>
<td>451/452 (99%)</td>
<td>ERB65389.1</td>
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<tr>
<td>25</td>
<td>ATP-dependent protease La (EC 3.4.21.53) Type II</td>
<td>contig00029</td>
<td>781/786 (99%)</td>
<td>782/786 (99%)</td>
<td>ERB65201.1</td>
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<td>26</td>
<td>Tricorn protease N-terminal domain-containing protein</td>
<td>contig00029</td>
<td>341/385 (88%)</td>
<td>N/A</td>
<td>ERB65231.1</td>
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<td>27</td>
<td>Tricorn protease N-terminal domain-containing protein</td>
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<td>308/381 (80%)</td>
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<tr>
<td>28</td>
<td>SOS-response repressor and protease LexA (EC 3.4.21.88)</td>
<td>contig00090</td>
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<td>205/205 (100%)</td>
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<td>29</td>
<td>Protease VII (Omp85) precursor (EC 3.4.33.49)</td>
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<td>297/303 (98%)</td>
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<td>30</td>
<td>Secreted trypsin-like serine protease</td>
<td>contig00049</td>
<td>300/335 (89%)</td>
<td>317/341 (92%)</td>
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<tr>
<td>31</td>
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<td>contig00049</td>
<td>325/329 (98%)</td>
<td>325/329 (98%)</td>
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<td>32</td>
<td>VtpB zinc metalloprotease</td>
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<td>774/779 (99%)</td>
<td>ERB66478.1</td>
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<td>566/569 (99%)</td>
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<td>181/181 (100%)</td>
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<tr>
<td>35</td>
<td>predicted phosphohydrolase</td>
<td>contig00037</td>
<td>781/786 (99%)</td>
<td>770/779 (98%)</td>
<td>ERB64837.1</td>
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<td>36</td>
<td>ABC-type protease exporter</td>
<td>contig00072</td>
<td>441/463 (95%)</td>
<td>459/463 (99%)</td>
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<td>37</td>
<td>VtpA zinc metalloprotease (vcpA)</td>
<td>contig00151</td>
<td>474/475 (99%)</td>
<td>475/475 (100%)</td>
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<td>38</td>
<td>Alkaline serine exprotease A precursor (EC 3.4.21.-)</td>
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<td>480/490 (97%)</td>
<td>ERB66833.1</td>
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<td>39</td>
<td>ATP-dependent Clp protease adaptor protein ClpS</td>
<td>contig00001</td>
<td>106/106 (100%)</td>
<td>106/106 (100%)</td>
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</tr>
<tr>
<td>40</td>
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<td>contig00001</td>
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<td>757/757 (100%)</td>
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<td>41</td>
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<td>contig00001</td>
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<td>244/244 (100%)</td>
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<td>909/924 (98%)</td>
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<td>43</td>
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<td>208/208 (100%)</td>
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<td>44</td>
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<td>133/133 (100%)</td>
<td>133/133 (100%)</td>
<td>WP_006959348.1*</td>
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<td>45</td>
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<td>284/284 (100%)</td>
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<td>753/755 (99%)</td>
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<td>437/443 (98%)</td>
<td>ERB64365.1</td>
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<td>48</td>
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<td>202/209 (96%)</td>
<td>ERB63840.1</td>
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<tr>
<td>49</td>
<td>Protease, insulinase family/protease, insulinase family</td>
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<td>412/414 (99%)</td>
<td>411/414 (99%)</td>
<td>WP_019278135.1*</td>
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<td>contig00124</td>
<td>505/508 (99%)</td>
<td>503/508 (99%)</td>
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<td>464/466 (99%)</td>
<td>464/466 (99%)</td>
<td>ERB63737.1</td>
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<tr>
<td>52</td>
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<td>contig00013</td>
<td>207/212 (97%)</td>
<td>204/212 (96%)</td>
<td>WP_006958057.1*</td>
</tr>
<tr>
<td>No.</td>
<td>Description</td>
<td>Contig/Protein</td>
<td>Similarity 1</td>
<td>Similarity 2</td>
<td>Accession</td>
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<tr>
<td>53</td>
<td>Putative protease</td>
<td>contig00013</td>
<td>69/73 (94%)</td>
<td>70/73 (95%)</td>
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</tr>
<tr>
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<td>contig00013</td>
<td>47/48 (97%)</td>
<td>47/48 (97%)</td>
<td>WP_019274462.1*</td>
</tr>
<tr>
<td>55</td>
<td>Putative protease</td>
<td>contig00013</td>
<td>275/284 (96%)</td>
<td>275/284 (96%)</td>
<td>WP_006958056.1*</td>
</tr>
<tr>
<td>56</td>
<td>Putative stomatin/prohibitin-family membrane protease subunit PA4582</td>
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<td>N/A</td>
<td>ERB62862.1</td>
</tr>
<tr>
<td>57</td>
<td>Putative stomatin/prohibitin-family membrane protease subunit PA4582</td>
<td>contig00104</td>
<td>N/A</td>
<td>N/A</td>
<td>ERB62863.1</td>
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<td>58</td>
<td>Zinc-dependent protease with chaperone function</td>
<td>contig00033</td>
<td>79/391 (96%)</td>
<td>386/391 (98%)</td>
<td>ERB65035.1</td>
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<tr>
<td>59</td>
<td>Metalloprotease, putative zinc-binding domain</td>
<td>contig00064</td>
<td>917/918 (99%)</td>
<td>917/918 (99%)</td>
<td>ERB63812.1</td>
</tr>
<tr>
<td>60</td>
<td>Alkaline serine protease</td>
<td>contig00030</td>
<td>171/171 (100%)</td>
<td>170/171 (99%)</td>
<td>ERB65167.1</td>
</tr>
<tr>
<td>61</td>
<td>Alkaline serine protease</td>
<td>contig00030</td>
<td>564/584 (96%)</td>
<td>564/584 (96%)</td>
<td>ERB65168.1</td>
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<tr>
<td>62</td>
<td>Uncharacterized protein, similar to the N-terminal domain of Lon protease</td>
<td>contig00084</td>
<td>171/174 (98%)</td>
<td>171/174 (98%)</td>
<td>ERB63278.1</td>
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<td></td>
<td><strong>Lipase</strong></td>
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<td>1</td>
<td>Lipase (EC 3.1.1.3)</td>
<td>contig00014</td>
<td>238/242 (98%)</td>
<td>16/218 (99%)</td>
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<td>2</td>
<td>Lipase-related protein</td>
<td>contig00047</td>
<td>283/283 (100%)</td>
<td>282/283 (99%)</td>
<td>ERB64388.1</td>
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<td>3</td>
<td>Lipase chaperone</td>
<td>contig00036</td>
<td>280/285 (98%)</td>
<td>283/285 (99%)</td>
<td>ERB64880.1</td>
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<td>4</td>
<td>Lipase precursor (EC 3.1.1.3)</td>
<td>contig00036</td>
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<td>154/155 (99%)</td>
<td>WP_019277067.1*</td>
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<td>1</td>
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<td>contig00023</td>
<td>322/328 (98%)</td>
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<td>29/333 (98%)</td>
<td>329/333 (98%)</td>
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<td>948/958 (98%)</td>
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<td>334/337 (99%)</td>
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<td>197/216 (91%)</td>
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<td>119/119 (100%)</td>
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<td>9</td>
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<td>contig00094</td>
<td>69/69 (100%)</td>
<td>69/69 (100%)</td>
<td>ERB63036.1</td>
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<td>69/69 (100%)</td>
<td>ERB63036.1</td>
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<td>261/263 (99%)</td>
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<td>157/157 (100%)</td>
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<td>477/479 (99%)</td>
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<td>ERB66322.1</td>
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<td>contig00011</td>
<td>N/A</td>
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<td>ERB66326.1</td>
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<td>contig00128</td>
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**Type VI secretion system**

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<th>Coverage (% of contig)</th>
<th>Accession ID</th>
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<td>----------------</td>
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<td>2</td>
<td>VgrG protein</td>
<td>contig00055</td>
<td>630/633 (99%)</td>
<td>631/633 (99%)</td>
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<td>582/582 (100%)</td>
<td>582/582 (100%)</td>
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<td>582/582 (100%)</td>
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<td>protein ImpH/VasB</td>
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<td>172/173 (99%)</td>
<td>171/173 (98%)</td>
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<td>Transcriptional regulator, AsnC family</td>
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<td>149/149 (100%)</td>
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<td>contig00055</td>
<td>719/727 (98%)</td>
<td>720/727 (99%)</td>
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<td>contig00055</td>
<td>375/376 (99%)</td>
<td>375/376 (99%)</td>
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<td>442/445 (99%)</td>
<td>315/316 (99%)</td>
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<td>200/200 (100%)</td>
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<td>11</td>
<td>protein Impl/VasC</td>
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<td>458/461 (99%)</td>
<td>458/461 (99%)</td>
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<td>166/166 (100%)</td>
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<td>425/426 (99%)</td>
<td>426/427 (99%)</td>
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<td>262/263 (99%)</td>
<td>262/263 (99%)</td>
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**Toxin/antitoxin systems, toxins**

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<td>N/A</td>
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<td>contig00208</td>
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<td>N/A</td>
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<td>SibE replicon stabilization toxin</td>
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<td>contig00099</td>
<td>2363/2413 (97%)</td>
<td>2381/2413 (98%)</td>
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<td>RTX toxins and related Ca2+ binding proteins</td>
<td>contig00099</td>
<td>274/280 (97%)</td>
<td>277/281 (98%)</td>
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<td>ERB63073.1</td>
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</table>

* Best match on NCBI; genes found from RAST analysis, but not annotated on NCBI. The gene sequence has been provided for that gene.

N/A = Not found in genome using RAST or BLAST
Table 10: Comparison of putative quorum sensing genes from different species and strains to OCN008. Gene comparisons are with translated amino acid sequences. NCBI accession numbers are in parenthesis.

<table>
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<th>Percentage Identity to <em>V. campbellii</em> BAA-1116</th>
<th>Percentage Identity to <em>V. cholerae</em> O1 biovar El Tor strain N16961</th>
<th>Percentage Identity to <em>V. coralliilyticus</em> P1</th>
<th>Percentage Identity to <em>V. coralliilyticus</em> BAA-450</th>
</tr>
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* Found with RAST analysis but not annotated on NCBI

** *V. cholerae* strain O395 was used since strain N16961 has a naturally occurring frame shift in *hapR*

*** OCN008 appears to have two genes annotated as *luxN*; they are denoted as *luxN*-1 and *luxN*-2
CHAPTER 4: MUTATION OF THE TOXR OR MSHA GENES FROM
VIBRIO CORALLIILYTICUS STRAIN OCN014 REDUCES INFECTION OF
THE CORAL ACROPORA CYTHEREA

INTRODUCTION

Disease, overfishing, pollution, and climate change threaten the existence of the world’s coral reefs (2, 3). Roughly 33% of reef-building coral species currently face an increased risk of extinction due to climate change, primarily as a result of the associated elevated sea surface temperatures (SST) (4, 5). Prolonged exposure to elevated SST promotes both coral bleaching, the loss of photosynthetic symbiotic dinoflagellates that provide the main source of fixed carbon to many coral species (6), and infection by pathogens to cause disease that can result in tissue loss and mortality of coral colonies (10, 20–22). The link between increased incidence of disease and elevated SST is not well understood; are increased infection levels caused by decreased fitness of the coral host at higher temperatures, increased virulence capabilities of the pathogen, or both?

A tight association between pathogenesis and elevated temperature is best exemplified by the coral pathogen *Vibrio coralliilyticus* strain BAA-450 (hereafter BAA-450) and its coral host *Pocillopora damicornis* (17, 38, 53). When *P. damicornis* was infected by BAA-450 in laboratory experiments conducted at 24 °C, the intracellular dinoflagellates were degraded and the host bleached (17). At 27 °C, infections resulted in tissue lysis and increased host mortality. And at temperatures below 23 °C, BAA-450 was incapable of infecting *P. damicornis*. Electron microscopy studies suggest that tissue invasion of *P. damicornis* by BAA-450 occurs only when water temperatures reach 27 °C (52). During invasion, BAA-450 proliferates in the *P.*
*damicornis* ectodermal cells and then penetrates into deeper tissue layers causing tissue lysis. Increased production of an extracellular metalloprotease at elevated growth temperatures may contribute to the disparity between BAA-450 infections at different temperatures. This metalloprotease displays homology to the *V. cholera* hemagglutinin involved in attachment to mammalian hosts, and in a purified form is capable of lysing *P. damicornis* tissue, suggesting it may be involved in infection (17, 57). However, deletion of the gene encoding the BAA-450 metalloprotease homolog in another pathogenic strain of *V. coralliilyticus*, strain P1, did not result in reduced virulence (58) leaving the role of metalloproteases in this association open. It is hypothesized that periods of elevated SST result in the upregulation of multiple genes associated with pathogenesis in BAA-450. Proteomic analysis of BAA-450 identified 136 gene products with significant upregulation at 27 °C (59). Proteomic analysis of BAA-450 identified 136 gene products with significant upregulation at 27 °C that fit into categories known to contribute to virulence in other pathogenic *Vibrio* species, including hemolysins, an RTX toxin, pili for adhesion, and secretion systems. Though shown to be upregulated, these putative virulence factors were not demonstrated to be involved in pathogenesis. The increased incidence of fatal coral infections caused by BAA-450 during times of elevated water temperature illustrates the danger posed by climate change.

The 2009 El Niño event caused a 2 °C increase in SST, within the bleaching threshold stated above, around Palmyra Atoll (99), an atoll surrounded by pristine reefs that is part of the Northern Line Islands and included in a National Wildlife Refuge under US federal protection (180). A minor bleaching event ensued and was followed by an outbreak of the coral disease *Acropora* white syndrome (AWS) that spread throughout Palmyra Atoll’s *Acropora* population (99, 100). AWS is a tissue loss disease that destroys healthy coral tissue and can lead to complete
colony mortality (100, 181). From October 2009 to July 2010, the prevalence of AWS increased from 0% to 25% among the *A. cytherea* population, a major reef-building species at the atoll (100). This was the first incidence of AWS at Palmyra Atoll since monitoring began in 2007. The reefs at Palmyra Atoll are free from high levels of anthropogenic stressors such as overfishing, excessive nutrients, and dredging that are commonly found at reefs around highly populated pacific islands and are thought to contribute to incidences of coral disease (182). As such, the reefs at Palmyra present an opportunity to study the effects of natural stressors such as increased SST on coral health and disease in the absence of significant anthropogenic stress.

In this work, a coral pathogen, *V. coralliilyticus* strain OCN014 (hereafter OCN014), was isolated from coral at Palmyra Atoll during the 2009 disease outbreak and shown to cause infection of *A. cytherea* in a temperature-dependent fashion in a manner similar to that of BAA-450, with the notable exception that bleaching was not induced by OCN014. Two genes with expression levels responsive to temperature were demonstrated to reduce infection when mutated in strain OCN014, indicative of increased virulence capabilities of the pathogen at increased temperatures. The genes for the associated virulence factors were shared between geographically isolated pathogenic strains, both of which are responsible for tissue lysis in corals.

**RESULTS**

*V. coralliilyticus strain OCN014 is an etiological agent of disease for Acropora white syndrome at Palmyra Atoll.*

Bacterial isolates were cultivated from *A. cytherea* fragments displaying disease signs of *Acropora* white syndrome (AWS) and were assessed for virulence to identify potential etiological agents. After screening 125 isolates, only one, strain OCN014, was observed to
induce tissue loss in *A. cytherea* fragments (Figure 9AB). The minimum infectious dose of OCN014 was calculated to be between $10^5$ and $10^6$ CFU/ml of tank water; coral fragments exposed to concentrations of at least $10^6$ CFU/ml of OCN014 developed tissue loss, while inocula of $10^5$ CFU/ml or less were non-infectious. Following inoculation with $10^6$ CFU/ml of OCN014, 88% of the healthy *A. cytherea* fragments developed tissue loss within 12 to 72 h post-
inoculation (McNemar’s test, \( n = 18, p < 0.01 \); Figure 9C) and disease lesions were similar to AWS lesions previously observed in the field. After inoculation with OCN014, tissue loss only manifested on *A. cytherea* fragments within 96 h post-exposure. For negative controls, *A. cytherea* fragments were exposed to FSW alone or inoculated with *Vibrio nereis* strain OCN044, neither of which induced disease on any of the coral fragments treated (\( n = 18 \) per treatment). The infection trial results suggest that exposure to cultures of OCN014 results in infection of healthy *A. cytherea* fragments and recapitulates the disease signs of AWS under laboratory conditions.

In previous studies with bacterial coral pathogens, the etiological agent could be recovered from experimentally infected coral fragments (38, 39, 42, 84); OCN014 was also re-isolated from experimentally infected *A. cytherea* fragments. Isolates with 16S rRNA gene sequences identical to the sequence from OCN014 were cultivated from all 10 experimentally infected *A. cytherea* fragments screened. However, to ensure that the presumed pathogen re-isolated from infected fragments originated from the laboratory culture, OCN014 was genetically tagged with a non-self-transmissible plasmid as previously described (42, 84), which conferred resistance to spectinomycin to facilitate antibiotic-mediated selection and identification. The infection rate of the genetically-tagged OCN014 was not significantly different from wild-type, 87% of fragments were infected within 72 h of inoculation (Mantel-Cox test, \( n = 8, p = 0.94 \)), and tagged OCN014 was re-isolated from all of the experimentally infected fragments. Together, these results demonstrate that OCN014 is an etiological agent of AWS.
The complete genome of isolate OCN014 was sequenced previously (43), and sequencing data [CP009264, CP009265, and CP009266] was used to identify OCN014 as a strain of *V. coralliilyticus*. The 16S rRNA gene of OCN014 shared 99% nucleotide identity to the sequence from *V. coralliilyticus* strain OCN008 [KF042020], another experimentally-verified coral pathogen (42). However, due to the genetic plasticity of *Vibrio* species, multilocus sequence analysis (MLSA) was utilized to more precisely demarcate the species classification of OCN014. Applying a previously described MLSA protocol (84, 126), OCN014 clustered together with *V. coralliilyticus* strains OCN008, P1, and BAA-450 (Figure 10), which confirmed its identification as a strain of *V. coralliilyticus*.

**Figure 10.** Phylogenetic tree of MLSA shows that OCN014 is a strain of *Vibrio coralliilyticus*. Evolutionary history was inferred using the maximum likelihood method. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to each branch. The scale bar represents 0.02 nucleotide substitution per site.
Infection of A. cytherea by OCN014 is abrogated at decreased water temperatures

Coral infections by the V. coralliilyticus type strain, BAA-450, are characteristically sensitive to water temperature, which prompted investigation on the effect of water temperature on OCN014 infection of A. cytherea. Though the average water temperature at Palmyra Atoll ranges from 28 to 29 °C, some reefs experienced sustained water temperatures as high as 31 °C during the 2009 bleaching event (99). At either 31 °C or 28 °C, no significant difference in infection rate (Mantel-Cox test, n = 6, p = 0.744) or minimum infectious dose of OCN014 during the 12-day experiment was observed. Though these results indicate that the upper thermal limits observed at Palmyra Atoll do not greatly influence infection in the short term, it is not known whether prolonged exposure to elevated water temperatures affects susceptibility. Conversely, when the water temperature was lowered to 23 °C, OCN014 infection was completely abrogated (Mantel-Cox test, n = 6, p = <0.01; Figure 9D). No negative effects relating to coral health were observed during treatments in elevated or reduced water temperatures, and no fragments were visibly affected by the control bacterium. The abrogation of virulence was not believed to be due to a reduced growth rate at lower water temperatures because the doubling times of OCN014 cells, about 10 minutes, were identical when grown at 23 or 29 °C. It appears that infection by OCN014 may be modulated by water temperature in a manner similar to that of BAA-450. However, in contrast to infection by BAA-450 at reduced temperature, no signs of bleaching were observed over the range of water temperatures at which OCN014 is infectious.

A transposon-based screen for promoters in OCN014 that are influenced by temperature

OCN014 is virulent at 29 °C and avirulent at 23 °C. Therefore, genes contributing to virulence were hypothesized to have differential expression at 23 versus 29 °C. A transposon-
based genetic screen was developed to define promoters that are differentially expressed at 23 and 29 °C to identify potential virulence-associated genes in OCN014. The system used was a version of the Himar-1 *mariner* transposon (183) containing the *lacZ* reporter gene. The transposon delivery vector, pBU121, contains the Himar-1 *mariner* transposase under the control of the arabinose-inducible *araBAD* promoter to allow inducible induction of transposition. To select for transposon mutants, genetic complementation of a *thyA* deletion introduced into OCN014 was used instead of antibiotic resistance because antibiotic selection routinely resulted in the growth of spontaneously resistant mutants. The gene encoding the enzyme thymidylate synthase, *thyA*, is required for the enzymatic reactions involved in nucleotide synthesis, and deletion of this gene is lethal unless growth medium is supplemented with deoxythymidine (DT) or the mutation is complemented with a functional copy of *thyA* (184). The *thyA* gene from wild-type OCN014 [AIU67794] was introduced into the transposon as a transcriptional fusion to the constitutively-expressed gentamicin resistance cassette, which was already present on the transposon. Expression of *thyA* on the transposon allowed for clean selection of transposon mutants via complementation of a Δ*thyA* mutation in the recipient strain on media without DT supplementation. A promoterless *lacZ* gene was cloned into the transposon as a transcriptional reporter and used for differential selection of transposon mutants with temperature-sensitive β-galactosidase activity on media supplemented with X-gal. Transposon mutant Tn1-1 (Table 13), which harbors an insertion in a constitutively expressed gene annotated as *cdd*, which displays homology to cytidine daminase [AIU67028], was used as a positive control for β-galactosidase activity. Wild-type OCN014 did not display β-galactosidase activity at any temperature and was used as a negative control.
Over 9,000 transposon mutants were screened for temperature-sensitive β-galactosidase activity. Fifteen unique transposon hits resulted in increased β-galactosidase activity at 29 °C (Table 13). Two mutants designated Tn3-3 and Tn4-8 had transposon insertions in genes with annotated functions other than those involved in thermal stress responses. Mutant Tn4-8 had a transposon insertion in a toxR homolog [AIU66105] that shares 99% amino acid identity with the gene product of toxR in V. coralliilyticus strain BAA-450 [EEX35320]. Mutant Tn3-3 had an insertion in a putative mshA homolog [AIU65959] that shares 79% amino acid identity with the gene product of mshA in the environmental isolate V. cholerae strain RC385 [BAD06384]. The mannose-sensitive hemagglutinin (MSHA) type IV pili operon is comprised of 16 genes, mshA is the fifth gene, and is required for biofilm formation by V. cholerae in aquatic environments (185).

**Mutants with transposon insertions in toxR and mshA had reduced virulence**

Virulence of the Tn3-3 and Tn4-8 transposon mutants was assessed by conducting infection trials with healthy fragments of A. cytherea. Each transposon mutant displayed an 88% reduction in virulence (Mantel-Cox test, $n = 9$ for each mutant, $p = <0.01$) compared to wild-type infection (Figure 11). Of the few fragments infected by the transposon mutants, the incubation period and signs of disease resulting from inoculation with each transposon mutant were comparable to infection by the wild type strain. Mutant Tn3-3 and Tn4-8 showed no observable defects in growth in GSW medium (data not shown).
**Defined mutations in toxR and mshA reduce virulence in a surrogate infection model**

Due to the logistical constraints of conducting manipulative studies at Palmyra Atoll, a surrogate coral host, the Hawaiian coral *Montipora capitata*, was evaluated for susceptibility to OCN014 infection. *M. capitata* has been reported to be susceptible to infection by *V. coralliilyticus* strain OCN008 (42), however a $10^6$ CFU/ml dose of OCN014 was not infectious to *M. capitata* (McNemar’s test, $n = 12$, $p = 1.0$). When OCN014 was inoculated at a higher concentration of $10^8$ CFU/ml, the infectious dose of OCN008 (Figure 12), roughly 40% of the *M. capitata* fragments developed tissue loss, which was significantly lower than the infection rate of OCN008 (Mantel-Cox test, $n = 18$, $p = 0.024$). As a positive control, *M. capitata* fragments were
exposed to OCN008, which had a consistent infection rate of 88% (McNemar’s test, \( n = 24, p < 0.01 \)). These results demonstrate that, although *M. capitata* was susceptible to disease by OCN014 at a drastically increased inoculum, it would not make an efficient infection model for OCN014. To investigate the roles of *mshA* and *toxR* in coral infection further, OCN008 infection of *M. capitata* was pursued as a surrogate system.

The *mshA* [ERB65815] and *toxR* [ERB64497] homologs in OCN008 were identified and clean deletions of the *toxR* gene or the entire MSHA operon were created resulting in strains OCN121 and OCN128, respectively. The MSHA operon was deleted rather than *mshA* alone because it is not known whether deletion of *mshA* or potential polar effects of the transposon
insertion on the entire operon were responsible for the reduced virulence. Strains OCN121 and OCN128 had a significant 70% (Mantel-Cox test, \( n = 9 \), \( p = 0.011 \)) and 100% (Mantel-Cox test, \( n = 9 \), \( p = < 0.01 \)) reduction in virulence (Figure 13A), respectively, compared to OCN008, which had an 80% infection rate. Neither of the deletion mutants displayed obvious defects in growth.
rate (data not shown). These results indicate that the MSHA operon and toxR are virulence factors required for wild-type levels of coral infection, and that OCN008 can serve as a surrogate infection system for results generated by the transposon-mediated OCN014 screen for temperature-responsive transcription. It also suggests that there may be a conserved set of virulence factors utilized by V. coralliilyticus coral pathogens.

*Temperature controlled gene expression may influence temperature-dependent V. coralliilyticus infection*

Strains OCN014 and OCN008 have been shown to infect coral in temperature-dependent and –independent manners, respectively, though infection by both strains requires toxR and the MSHA operon for wild-type levels of virulence. It has been postulated that the basis of temperature-dependent V. coralliilyticus infection may be due to the differential expression of virulence genes in a response to temperature fluctuations (58, 59). To determine if temperature-dependent infection correlated with temperature-dependent expression of the toxR and mshA genes, expression of each gene was investigated at 23 and 29 °C. As mentioned earlier, the transposon used in the genetic screen to identify the toxR, mshA and cdd genes carried a promoterless copy of lacZ that created a transcriptional fusion with each of these genes in the OCN014 strain background to create strains Tn4-8, Tn3-3 and Tn1-1. Analogous strains were created in the OCN008 background by inserting a promoterless copy of lacZ into the toxR, mshA or cdd genes of that strain to create strains OCN175, OCN176 and OCN174, respectively. All strains were grown at 23 and 29 °C to OD$_{600} = 0.8$ and β-galactosidase activity was measured to assess transcriptional activity. As expected, strains Tn1-1 and OCN174 harboring lacZ fusions within cdd and the wild-type strains OCN014 and OCN008 did not display significant variation
in β-galactosidase levels between the temperatures tested (Figure 13B). Strains Tn4-8 and Tn3-3, containing toxR- and mshA-lacZ transcriptional fusions, displayed significantly higher β-galactosidase activity at 29 °C than 23 °C (t-test, n = 3, p < 0.05). In contrast, β-galactosidase activity did not differ significantly between growth at 23 and 29 °C in strains of OCN008 harboring analogous fusions, OCN175 and OCN176. These results show that expression of the toxR and mshA genes is temperature-dependent in OCN014 but not in OCN008. We infer that temperature-dependent expression of genes required for virulence, and thus a temperature-dependent regulatory mechanism, may be the underlying cause of temperature-dependent V. coralliilyticus coral infections.

DISCUSSION

The work presented here describes the isolation of V. coralliilyticus strain OCN014 and its fulfillment of Koch’s postulates of disease causation, suggesting its role as an etiological agent of the 2009 AWS outbreak at Palmyra Atoll. Infection of A. cytherea was temperature-dependent in controlled laboratory conditions. Utilizing a transposon-based screen developed for this study, fifteen genes expressed in a temperature-dependent manner (upregulated at 29 °C) were initially identified. Assessment of the virulence of strains harboring mutations in the toxR and the MSHA operon, likely involved in pathogenesis due to their homology to virulence factors in other pathogenic Vibrio strains, determined that these genes were required for wild-type levels of coral infection by both V. coralliilyticus strains OCN014 and OCN008. Additional assessment of the transcription of toxR and mshA indicated that these genes are expressed in a temperature-dependent manner in OCN014 but not another pathogenic strain, OCN008.
In humans, infections by individual bacterial pathogens can result in a range of disease symptoms, and each presentation may be designated as its own disease (i.e. gastroenteritis vs. necrotizing fasciitis vs. septicemia, all caused by *V. parahaemolyticus*) (186–188). Coral species display a comparatively limited repertoire of disease signs, so classification of coral infections must employ other criteria. Based on the results presented here and in previous work, we propose a system of classifying *V. coralliilyticus* infections based on temperature dependence and the type of disease state elicited. Implementation of a categorical classification system will facilitate comparison of virulence determinants between strains based on modes of infection and suggests a set of measurements necessary for assignment of newly identified pathogenic *V. coralliilyticus* strains. As *V. coralliilyticus* infections become more prevalent as a result of climate change, broad utilization of such a system will provide an information baseline for each new pathogenic strain and facilitate the organization of related groups of results from labs worldwide, future meta-analyses, and epidemiological studies. To date, several pathogenic *V. coralliilyticus* strains have been vetted as etiological agents of coral disease: BAA-450, P1-7, OCN008, and OCN014 (39, 42, 53). Infections caused by pathogenic *V. coralliilyticus* strains include tissue loss or bleaching of the coral host and may be modulated in response to temperature. Because infection by pathogenic strains has been reported from different coral species in disparate locations, classification based on geography or host strain is impractical. Rather, we propose a four-category system of coral infection based on temperature dependence and the induction of bleaching: category I is comprised of infections in which the coral host bleaches in a temperature-dependent manner; category II is comprised of infections in which the coral host bleaches in a temperature-independent manner; category III is comprised of infections in which the coral host does not bleach but tissue lysis occurs in a temperature-dependent manner;
category IV is comprised of infections in which the coral host does not bleach and tissue lysis occurs in a temperature-independent manner.

Category I infections are typified by the temperature-dependent bleaching of *P. damicornis* by BAA-450, which was the first *V. coralliilyticus* strain identified as a coral pathogen and has been the subject of some study (17, 53–56, 59, 63, 189–195). Category II infections result in bleaching without the temperature-dependence observed from BAA-450 infections. It is possible that infections by strains P1-7 could belong to either category I or II but, because infection trials with these strains were conducted at a single temperature, it is not known whether the bleaching response observed was temperature-dependent (39, 146). Based on the results presented here, *A. cytherea* infection by strain OCN014 fits within Category III because infection does not occur at 23 °C, and only tissue lysis was observed. Finally, Category IV infections result from the interaction of OCN008 and *M. capitata* because no bleaching was reported and tissue lysis occurred at every temperature tested (42). It is possible that this classification system could be expanded to include *V. coralliilyticus* infections of other organisms, but because bleaching is specific to coral, classification would need to be based on temperature dependence and conserved methods of infection, assuming that the mechanisms underlying bleaching and tissue lysis differ at some fundamental level and these differences are conserved among strains.

Several studies have speculated on genes thought to be involved in *V. coralliilyticus* pathogenesis (17, 56, 58, 59). Initial work on BAA-450 found that a purified protease produced by the bacterium caused coral tissue lysis (17). Following genome sequencing of strain P1, genes thought to contribute to infection were identified in silico, but mutation of a zinc-metalloprotease did not reduce P1 infectivity (58). Analysis of the BAA-450 proteome at 24 and 27 °C described
a host of proteins that were differentially regulated between these two conditions, a subset of which are potential virulence factors (59). A recent bioinformatics study of the BAA-450, P1, OCN008, and OCN014 genomes suggested that the integration of prophage harboring suspected virulence factors, especially toxins, could account for the diseases elicited by these strains (194). Unlike previous work that has utilized bioinformatics-based and omics based approaches, the work presented here employed random mutagenesis to identify genes that were upregulated at 29 °C. Out of the 15 unique temperature-dependent genes identified herein, 11 have homologs in BAA-450 and three of these were shown to be upregulated in a previous proteomics study; genes within the MSHA operon were upregulated 3- to 11-fold and toxR displayed a 60-fold upregulation at 27 °C (59). Like their homologs in BAA-450, we confirmed that toxR and mshA are upregulated at increased temperatures in OCN014. This study is unique in employing mutation of toxR and the MSHA operon to demonstrate decreased virulence, thus providing a direct link between virulence and temperature-dependent gene regulation. Our results suggest that data from bioinformatics research can be used to prioritize genes to test for involvement in virulence and exemplifies the complementarity of these approaches for studying coral disease.

Since we were unable to conduct long-term research at Palmyra Atoll, and OCN014 did not infect M. capitata as well as A. cytherea, it was necessary to utilize M. capitata infection by OCN008 as a surrogate system to continue studying temperature-dependent virulence genes identified in OCN014. Interestingly, expression of toxR and the MSHA operon did not correlate with temperature in strain OCN008, but mutation of these genes individually still decreased virulence in strains isolated from geographically distant areas (Palmyra Atoll for OCN014 and Hawai‘i for OCN008). These results are consistent with differential regulation of a core set of
virulence factors required for coral infection by pathogenic *V. coralliilyticus* strains as the basis for temperature-dependent coral infection.

**METHODS**

*Bacterial growth conditions*

All bacterial strains used in this study are listed in Table 1. *V. coralliilyticus* strain OCN014 was isolated from a diseased *A. cytherea* fragment as described previously (43), and *Vibrio nereis* strain OCN044 was isolated from healthy *A. cytherea* in the same manner. Marine bacteria were grown in glycerol seawater medium (GSW) that was prepared by re-suspending 4 g/l tryptone, 2 g/l yeast extract, and 2 ml/l glycerol in a liter of seawater that had been previously filtered through a 0.22 µm pore membrane filter, and autoclaving the mixture before use. For solid medium, 15 g/l of agar was added prior to autoclaving. Marine bacterial cultures were incubated at 29 °C unless otherwise stated. Growth rate experiments were conducted in triplicate using GSW media at 23 and 29 °C. Optical density measurements were taken at 600 nm (OD$_{600}$) and culture dilutions were plated on GSW plates every hour for 24 h.

Strains of *Escherichia coli* were grown in LB broth incubated at 37 °C. Conjugations with marine bacteria were performed on glycerol artificial seawater (GASW) agar prepared according to a previously described protocol (84). Antibiotics for selection with *E. coli* were used at the following concentrations unless otherwise stated: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; streptomycin, 25 µg/ml; spectinomycin, 50 µg/ml; and chloramphenicol, 15 µg/ml. Antibiotics for selection with *Vibrio* strains were used at the following concentrations unless otherwise stated: ampicillin, 200 µg/ml; gentamicin, 15 µg/ml; streptomycin, 50 µg/ml; spectinomycin, 100 µg/ml; and chloramphenicol, 10 µg/ml. Growth media for *E. coli*
auxotrophic strains were supplemented with deoxythymidine (DT) or diaminopimelate (DAP) at a final concentration of 0.3 mM as required. Arabinose-inducible expression of the ccdB gene was achieved by the addition of 0.3% L-arabinose to the growth medium, and expression was repressed by addition of 1% D-glucose (196).

Coral collection and infection trials

Fragments of *A. cytherea* were collected from a reef at Palmyra Atoll under special use permits 12533-11025, 12533-12025, 12533-14025 granted by the U.S. Fish and Wildlife Service. Fragments of *M. capitata* were collected from a fringing reef surrounding the island Moku o Lo‘e in Kāne‘ohe Bay, O‘ahu, Hawai‘i under permit 2015-48 granted by the Hawai‘i Department of Aquatic Resources. All coral fragments were allowed to recover from collection in a flow-through water table at ambient temperature for at least two days prior to experimentation.

Screens to identify infectious bacterial isolates and infection trials at Palmyra were conducted essentially as previously described (42, 84), with the following modifications. Infection trials with *A. cytherea* were conducted in closed systems to prevent any release of bacterial cultures into the environment at Palmyra Atoll. Each coral fragment was individually housed in a 15 l tank, except during etiological-agent screening when three fragments were used per tank. Tanks were filled with 13 l of filtered seawater (FSW) that had been passed through a 0.22 μm pore membrane filter. The secondary containment surrounding the tanks was an 800 l tub with flow-through seawater and heaters to maintain water temperatures at 28 to 29 °C, unless otherwise stated. A recirculating chiller unit was used to maintain water temperatures lower than ambient temperature when needed. For coral inoculation, overnight cultures of OCN014 were
diluted 1:100 and grown to an OD$_{600}$ of 0.8 before being washed once and resuspended in sterilized FSW. OCN014 was inoculated to a final concentration of $10^6$ CFU/ml unless otherwise stated. Infection trials using *M. capitata* and OCN008 were conducted as previously described (42).

**Identification of bacterial isolates and phylogenetic analysis**

All primers used in this study are listed in Table 2. Sequences for the OCN014 16S rRNA gene and genes for multilocus sequence analysis (MLSA) were obtained from the publically-available OCN014 genome sequence on NCBI (43). PCR amplification and sequencing of the 16S rRNA gene from other bacterial isolates was done as previously described (42). MLSA was conducted on 20 Vibrio isolates that were aligned and analyzed as previously described. (42, 84).

**Re-isolation of OCN014 from infected coral fragments**

Re-isolation of genetically-tagged OCN014 with the non-self-transmissible vector pRL1383a was done as previously described (42, 84), with the following modifications. A total of 70 isolates were screened from the seven *A. cytherea* fragments infected by the genetically-tagged OCN014 strain. Isolates that grew on TCBS plates supplemented with ampicillin, spectinomycin, and streptomycin were screened using primers specific to pRL1383a as well as the primers OCN014unique-F and OCN014unique-R that are specific to *V. coralliilyticus*. The species-specific primers OCN014unique-F and OCN014unique-R were designed as previously described (42, 84), and used to amplify a 557 bp intergenic region in the OCN014 genome between the divergently expressed coding sequences for a Fis family transcriptional regulator [AIS57356] and an aldehyde dehydrogenase [AIS57357]. In addition, the 16S rRNA gene of
each isolate was amplified and sequenced as above to ensure that they were all identical to the OCN014 stock culture. Another 100 isolates, which grew on TCBS plates supplemented with ampicillin and streptomycin, cultivated from ten *A. cytherea* fragments experimentally infected by non-tagged OCN014, were also screened using the species-specific primers and primers for the 16S rRNA gene.

**Plasmid creation**

All plasmids used in this study are listed in Table 11. All PCR-derived products were verified by sequencing. Plasmid pAHB122 is a suicide vector based on pSW7848 (196) that lacks the *Sma*I site within the *ccdB* gene. Plasmid pSW7848 was amplified with the primers pSW7848-delta-SmaI-F and pSW7848-delta-SmaI-R, which inserted a G to C transversion at the third position of the G29 codon of the *ccdB* gene, and the mutation was introduced using the previously described QuickChange protocol (197) to create pAHB122.

Plasmid pAHB166 is a suicide vector based on pSW4426T (196) that was used to cleanly delete the MSHA operon by removing all but the first 30 nucleotides of the *mshH* coding region and all but the last 30 nucleotides of the *mshQ* coding region in OCN008; the first and last genes of the MSHA operon. Regions upstream of *mshH* and downstream of *mshQ* were amplified from OCN008 chromosomal DNA with the primer pairs mshOP-up-F and mshOP-up-R and mshOP-dn-F and mshOP-dn-R, respectively. The up- and downstream fragments were fused together by overlap extension PCR (198) and cloned into the *Sma*I site of pAHB122. The fused product was excised from the resulting construct as a *Kpn*I-*Spe*I fragment and cloned into the *Kpn*I-*Xba*I sites of pSW4426T to create pAHB166.
Plasmid pAHB173 is a suicide vector based on pSW4426T that was used to cleanly delete all but the first 30 and last 30 nucleotides of the toxR coding region in OCN008. Regions up- and downstream of toxR were amplified from OCN008 chromosomal DNA by PCR with the primer pairs toxR-up-F and toxR-up-R and toxR-dn-F and toxR-dn-R, respectively. The up- and downstream fragments were fused together by overlap extension PCR and cloned into the SmaI site of pBlueScript SK+ (Stratagene). The fused product was excised from the resulting construct as an EcoRI-SpeI fragment and cloned into the EcoRI-XbaI sites of pSW4426T to create pAHB173.

Plasmid pBU113 is a suicide vector based on pSW7848 that was used to cleanly delete all but the first nine and last nine nucleotides of the thyA coding region in OCN014. Regions up- and downstream of thyA were amplified by PCR from OCN014 chromosomal DNA with the primer pairs thy-up-MunI-F and thy-up-R and thy-down-F and thy-down-SpeI-R, respectively. The up- and downstream fragments were fused together by overlap-extension PCR, digested with MunI-SpeI, and cloned into EcoRI-SpeI sites of pSW7848 to create pBU113.

Plasmid pBU118 is a source of the mariner transposon that contains a transcriptional fusion of the OCN014 thyA gene to the gentamicin resistance cassette. The thyA gene was amplified from OCN014 DNA by PCR with the primers ThyA-SmaI-F and ThyA-SmaI-R, digested with SmaI, and cloned into pBU124 following linearization by PCR with the primers pBT20-Gm-F and pBT20-Gm-R to create pBU118.

Plasmid pBU119 is a source of the mariner transposon that contains the thyA gene from OCN014 divergently transcribed from a promoterless copy of lacZ. A fragment containing lacZ was amplified by PCR from pUC18-mini-Tn7-gm-lacZ (199) using the primers lacZ-SmaI-F and
lacZ-SmaI-R, digested with SmaI, and cloned into pBU118 following linearization by PCR with the primers pBT20-F and pBT20-R to create pBU119.

Plasmid pBU120 is a suicide vector based on pSW7848 that lacks the DNA gyrase-inhibiting gene ccdB. Plasmid pSW7848 was amplified by PCR with the primers pSW7848-F-XhoI and pSW7848-delta-ccdB-XhoI, digested with XhoI, which removed ccdB, and self-ligated to create pBU120.

Plasmid pBU121 is a suicide vector based on pSW7848 that contains an arabinose-inducible mariner transposase. The mariner transposase was amplified from pBT20 (183) by PCR using the primers TNase-SalI-F and TNase-SalI-R, digested with SalI, and cloned into the XbaI site downstream of the arabinose-inducible araBAD promoter in pBU120 to create pBU121.

Plasmid pBU122 is a suicide vector based on pSW7848 used for transposon-based screening for genes differentially expressed at different temperatures in strain OCN014. A 5.9 kb fragment with the mariner transposon containing the thyA gene from OCN104 and a promoterless copy of lacZ was amplified by PCR from pBU119 using the primer pBT20-IR, which binds to the inverted repeats flanking the transposon, and cloned into the SmaI site of pBU121 to create pBU122.

Plasmid pBU124 is a source of the mariner transposon. A 1.7 kb fragment containing the mariner transposon was amplified from pBT20 by PCR with the primer pBU20-IR and cloned into the SmaI site of pBlueScript SK+ to create pBU124.

Plasmid pBU187 is a suicide vector based on pSW4426T with the araBAD promoter and operator region and ccdB removed. The plasmid pSW4426T was used as template for PCR using
the primers pSW4426T-up-AraC-SacI and pSW4426T-down-ccdB. The resulting PCR product was digested with SacI and DpnI and then self-ligated to create pBU187.

Plasmid pBU195 is a suicide vector based on pBU187 used to introduce a promoterless lacZ within the cdd coding region of OCN008 as a single recombinant to mimic the transposon insertion in Tn1-1. Regions upstream and within the cdd coding region was amplified with PCR from OCN008 chromosomal DNA using the primers cdd-up-SpeI-F and 008cdd-OEX-up-R. The lacZ coding region with its Shine-Dalgarno sequence and the gentamicin resistance cassette was amplified with PCR from the plasmid pUC18-mini-Tn7-gm-lacZ using the primer pairs cdd-lacZ-OEX-F with lacZ-down-SpeI-R and GmR-lacZ-OEX-F with GmR-SpeI-R, respectively. The three PCR products were used as template for an overlap extension PCR reaction using the primers cdd-up-SpeI-F and GmR-SpeI-R. The PCR product was cloned as a SpeI fragment into the SpeI-XbaI sites of pBU187.

Plasmid pBU196 is a suicide vector based on pBU187 used to introduce a promoterless lacZ within the toxR coding region of OCN008 as a single recombinant to mimic the transposon insertion in Tn4-8. Regions upstream and within the toxR coding region were amplified with PCR from OCN008 chromosomal DNA using the primers toxR-up-SpeI-F and PtoxR-OEX-up-R. The lacZ-coding region with its Shine-Dalgarno sequence and the gentamicin resistance cassette were amplified with PCR from the plasmid pUC18-mini-Tn7-gm-lacZ using the primer pairs cdd-lacZ-OEX-F with lacZ-down-SpeI-R and GmR-lacZ-OEX-F with GmR-SpeI-R, respectively. The three PCR products were used as template for an overlap extension PCR reaction using the primers cdd-up-SpeI-F and GmR-SpeI-R. The PCR product was cloned as a SpeI fragment into the SpeI-XbaI sites of pBU187.
Plasmid pBU198 is a suicide vector based on pBU187 used to introduce a promoterless lacZ within the mshA coding region of OCN008 as a single recombinant to mimic the transposon insertion in Tn3-3. Regions upstream and within the mshA coding region was amplified with PCR from OCN008 chromosomal DNA using the primers mshA-up-SpeI-F and mshA-OEX-up-R. The lacZ coding region with its Shine-Dalgarno sequence and the gentamicin resistance cassette was amplified with PCR from the plasmid pUC18-mini-Tn7-gm-lacZ using the primer pairs cdd-lacZ-OEX-F with lacZ-down-SpeI-R and GmR-lacZ-OEX-F with GmR-SpeI-R, respectively. The three PCR products were used as template for an overlap extension PCR reaction using the primers cdd-up-SpeI-F and GmR-SpeI-R. The PCR product was cloned as a SpeI fragment into the SpeI-XbaI sites of pBU187.

Conjugation of suicide vectors into V. coralliilyticus

All V. coralliilyticus suicide vectors were introduced using tri-parental conjugations with E. coli. Suicide vectors were maintained in E. coli strain β3914 and the self-transmissible vector pRK2013 (200) was maintained in strain π3813 (196). For conjugation, donor and recipient strains were grown overnight with the appropriate antibiotics for plasmid maintenance and DAP or DT as required. Overnight cultures were diluted 1:1000 in fresh culture medium without antibiotics, grown to an OD<sub>600</sub> of 0.7, and washed three times with either GASW or LB for Vibrio or E. coli strains, respectively. The strains were then combined, resuspended in GASW to a total volume of 50 µl, and spotted onto GASW plates supplemented with DAP, DT, and D-glucose, unless otherwise stated. Conjugation spots were incubated at 29 °C for 15 h before being resuspended in GASW, washed three times with GASW, diluted, and plated onto GASW supplemented with D-glucose and chloramphenicol or spectinomycin and streptomycin in
combination, but lacking DAP or DT for counterselection against the ΔdapA and ΔthyA *E. coli* donor strains. Antibiotic-resistant colonies, which contained the suicide vector introduced through a single recombination event, were isolated and grown overnight in GASW broth supplemented with D-glucose. Overnight cultures were washed with GASW three times, diluted, and plated onto GASW plates supplemented with L-arabinose to isolate mutants that had completed the second recombination event.

**Strain creation**

The *thyA* gene in OCN014, except for the first nine and last nine nucleotides of the coding region, the MSHA operon in OCN008, except for the first 30 nucleotides of *mshH* and the last 30 nucleotides of *mshQ* coding regions, and the *toxR* gene in OCN008, except for the first 30 and last 30 nucleotides of the coding region, were cleanly deleted from chromosomal DNA by allelic exchange as previously described (196) with the plasmids pBU113, pAHB166, and pAHB173, respectively. The resulting strains, OCN121 and OCN123, with deletions of the MSHA operon and the *toxR* gene, respectively, were verified by PCR with the primer pairs OCN008-mshOP-out-F and OCN008-mshOP-out-R, and OCN008-toxR-out-F and OCN008-toxR-out-R, respectively, which anneal outside the region of DNA used to make the mutations. Strain OCN053, with a deletion in the gene *thyA*, was verified by the strain’s inability to grow on media lacking supplementation with DT.

**Transposon mutagenesis to identify temperature-regulated genes**

A *mariner* transposon-based screen for genes differentially expressed at 23 and 29 °C was conducted by introducing pBU113 into OCN053 via conjugation using the protocol
described above with minor modifications. Conjugation mixes were spotted onto GASW plates without D-glucose and supplemented with DAP, DT, and L-arabinose. Following overnight conjugation, spots were washed three times with GASW, plated onto GASW plates spread with 60 µl of a 20 mg/ml stock of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and then incubated for 24 h at 29 °C to select for transposon mutants that were able to grow in the absence of DT by complementation with thyA on the transposon. Blue colonies were then patched onto GASW plates containing X-gal and incubated at 23 °C for 24 h. Colonies without observable β-galactosidase activity were screened again to ensure activity was observed at 29 °C, but not at 23 °C. Mutants with differential β-galactosidase activity were grown in liquid culture and DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). DNA extractions were digested with HpyCH4IV (New England Biolabs), self-ligated, and then used as templates for inverse PCR (201) with the primers pBU122-iPCR-F and pBU122-iPCR-R. PCR products were sequenced with the same primers used for amplification to identify transposon insertion sites.

**Transcriptional analysis by β-galactosidase assay**

Relevant strains were grown in overnight liquid cultures in LB-salt (LBS) medium supplemented with gentamycin (196). Overnight cultures were used to inoculate fresh 2 ml LBS cultures supplemented with gentamycin to an OD<sub>600</sub> ~0.05, which were grown at either 23 °C or 29 °C with shaking. Once OD<sub>600</sub> = 0.8 was reached, 1 ml aliquots were pelleted by centrifugation at 13 x g for 2 min, decanted, and stored at -80 °C until use. Pellets were resuspended in 1 ml of Z-buffer and β-galactosidase assays and Miller Unit conversions were conducted as previously described (202). Data was analyzed using GraphPad Prism (version 6.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).
## TABLES

**Table 11.** Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or citation</th>
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<td><strong>Vibrio coralliilyticus strains</strong></td>
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<td>OCN008</td>
<td>Wild type; isolated in Hawai’i; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>OCN014</td>
<td>Wild type; isolated at Palmyra Atoll; Ap&lt;sup&gt;+&lt;/sup&gt;, Sm&lt;sup&gt;-&lt;/sup&gt;</td>
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<td>OCN053</td>
<td>ΔthyA mutant derived from OCN014; Ap&lt;sup&gt;+&lt;/sup&gt;, Sm&lt;sup&gt;-&lt;/sup&gt;</td>
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<td>OCN108</td>
<td>OCN044 with replicative vector pBU147; Ap&lt;sup&gt;+&lt;/sup&gt;, Sp&lt;sup&gt;+&lt;/sup&gt;, Sm&lt;sup&gt;-&lt;/sup&gt;, Cm&lt;sup&gt;-&lt;/sup&gt;</td>
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<td>OCN121</td>
<td>OCN008 Δmsha mutant; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>OCN128</td>
<td>OCN008 ΔtoxR mutant; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>OCN174</td>
<td>OCN008 with cdd-lacZ transcriptional fusion; Ap&lt;sup&gt;+&lt;/sup&gt;, Sp&lt;sup&gt;+&lt;/sup&gt;, Sm&lt;sup&gt;-&lt;/sup&gt;, Cm&lt;sup&gt;-&lt;/sup&gt;, Gm&lt;sup&gt;-&lt;/sup&gt;</td>
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<td>OCN176</td>
<td>OCN008 with mshA-lacZ transcriptional fusion; Ap&lt;sup&gt;+&lt;/sup&gt;, Sp&lt;sup&gt;+&lt;/sup&gt;, Sm&lt;sup&gt;-&lt;/sup&gt;, Cm&lt;sup&gt;-&lt;/sup&gt;, Gm&lt;sup&gt;-&lt;/sup&gt;</td>
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<td>Tn1-1</td>
<td>OCN053 with transposon insertion in cdd; Ap&lt;sup&gt;+&lt;/sup&gt;, Sm&lt;sup&gt;-&lt;/sup&gt;, Gm&lt;sup&gt;-&lt;/sup&gt;</td>
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<td><strong>Marine bacterial strains</strong></td>
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<td><em>V. nereis</em> strain from Palmyra Atoll; control bacterium</td>
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<td>n3813</td>
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<td>pRK2013</td>
<td>Self-transmissible vector for conjugation; Km&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pRL1383a</td>
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<td>pSW4426T</td>
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<td>pSW7848</td>
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<td>pUC18-mini Tn7T-Gm-lacZ</td>
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<td>pAHB122</td>
<td>Modified version of pSW7848 with unique <em>Sma</em>I site</td>
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<td>pAHB166</td>
<td>Suicide vector used to create OCN121; Cm&lt;sup&gt;+&lt;/sup&gt;, Sp&lt;sup&gt;-&lt;/sup&gt;, Sm&lt;sup&gt;-&lt;/sup&gt;</td>
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<td>Transposon shuttle vector; Cm&lt;sup&gt;r&lt;/sup&gt;, Gm&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pBU147</td>
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*Abbreviation for antibiotic resistance cassettes: Ap<sup>r</sup>, ampicillin resistance; Sm<sup>r</sup>, streptomycin resistance; Sp<sup>r</sup>, spectinomycin resistance; Gm<sup>r</sup>, gentamicin resistance; Km<sup>r</sup>, kanamycin resistance; Em<sup>r</sup>, erythromycin resistance; Tc<sup>r</sup>, tetracycline resistance; Cm<sup>r</sup>, chloramphenicol resistance.
Table 12. Oligonucleotide primers used in this study.

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Table 13. Unique transposon hits in OCN014.

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CHAPTER 5: PRODUCTION OF THE ANTIBIOTIC ANDRIMID IS A
NOVEL VIRULENCE MECHANISM USED BY THE CORAL PATHOGEN

VIBRIO CORALLIILYTICUS STRAIN OCN008

INTRODUCTION

Mutualistic and commensal microorganisms can serve as a complex and adaptable defense system against pathogens that threaten their hosts. Symbiotic relationships with probiotic microflora, microorganisms that provide health benefits to the host, are vital for the wellbeing of all levels of host organisms, from invertebrates and plants to humans (204–210). Probiotic microflora protect their hosts from opportunistic pathogens, infectious organisms not normally found on the host, or members of the pathobiont, organisms found on the host that are only pathogenic under certain conditions (211, 212). Several mechanisms used by probiotic microorganisms to protect their hosts include the production of antagonistic compounds, sequestering resources from pathogens, and modulating the host immune system (204–210, 213–216).

The human gut microflora exemplify the effects of resident microorganisms on maintaining the health of their host by regulating differentiation and activity of the human immune system and sequestering available carbon and amino acids to prevent pathogen colonization (207–209). Disruption of the gut microflora following a course of prescribed broad-spectrum antibiotics, like clindamycin, enables infection by the pathogen Clostridium difficile, a causative agent of pseudomembranous colitis and a common member of the human pathobiont (217–219). The healthy gut microflora will inhibit C. difficile growth in vitro, and the direct
transfer of bacteria or fecal samples from healthy hosts to antibiotic-treated hosts will restore resistance to this pathogen (220, 213, 207, 221, 222, 215). Studies using high-throughput sequencing have identified several members of the gut microflora that, when lost from the population, correlate with susceptibility to C. difficile infection (222, 215). Several other pathogens, including Klebsiella oxytoca and Staphylococcus aureus, have taken advantage of in vitro antibiotic treatments to opportunistically infect humans, further demonstrating the essential protective properties of the gut microflora (214, 223–226).

The protective properties of probiotic strains are not limited to the human gut, there are similar situations observed with a range of plant and invertebrate hosts. For example, the marine alga, Ulva lactuca, and sea squirt, Ciona intestinalis, are colonized by the bacteria Pseudoalteromonas ulvae and P. tunicate, respectively, and are protected from fouling microorganisms by the antifungal and antibacterial compounds produced by these species (206, 205, 227, 228). Some of the research on probiotics from environmental sources is focusing on identifying biological controls for agrigutural and aquaculture crops. For example, different strains of Pseudomonas fluorescens have been identified to produce an array of antifungal compounds that can protect food crops from fungal infection (204, 229, 230). Similarly, biological controls for the shellfish industry are currently being investigated for their feasibility. Vibrio sp. PP05 and Pseudoalteromonas sp. PP107 have been identified to produce antibacterial compounds and can colonize the Ornate Spiny Lobster, Panulirus ornatus, which protect these crops from V. owensii infection, a devastating lobster pathogen (128, 231, 232). Using GFP-labeled bacteria, strains PP05 and PP107 were demonstrated to colonize different areas of P. ornatus and when used together, provide effective protection to lobsters in aquaculture settings.
Analogous to the examples mentioned above, the microorganisms present on corals are thought to form a network of antagonistic interactions that prevents domination by any single group of bacteria, including pathogens (76, 87, 80, 88, 40, 89–91, 81). The relatively slow growth and lack of an adaptive immune system impair corals from adapting to rapidly evolving threats. It has been hypothesized, however, that corals rely on the protective properties of the microflora that colonize their extracellular mucus layer to keep pathogens from infecting the host (40, 76, 80, 83, 85–87). According to the coral probiotic hypothesis, corals are unable to adapt to sudden stressors or environmental changes, however, the coral microflora is able to adapt through rearrangement of the microbial community or by the comparably faster evolution of prokaryotic organisms (85). Consistent with this hypothesis, bacterial strains isolated from healthy corals inhibited the growth of the coral pathogens *V. coralliilyticus* and *V. shiloi* during *in vitro* assays. Many of these inhibitory strains belonged to the genus *Pseudoalteromonas*, which includes multiple species that are known to produce a wide variety of antimicrobial compounds (76, 92, 87–89, 91).

The protective properties of the coral microflora are believed to be responsible for the acquired immunity of the coral *Oculina patagonica* to infection by the pathogen *V. shiloi* (93). In 1997, *V. shiloi* was discovered to infect and cause disease in *O. patagonica*, however, in 2004, laboratory stocks of *V. shiloi* no longer infected coral and could not be isolated from inoculated specimens. However, when corals were treated with the broad-spectrum antibiotic nalidixic acid, they became susceptible to *V. shiloi* infection and the pathogen could be isolated from infected coral tissue (93, 119, 166). Therefore, it was suggested that the coral animal did not develop resistance to *V. shiloi*, but the normal microflora evolved to prevent infection by this pathogen. Together, these results suggest that the coral mucus microflora may serve a protective role.
similar to the human gut microflora and disruption of the microflora may have analogous consequences.

In this work, the coral pathogen *V. coralliilyticus* strain OCN008 (42), hereafter OCN008, was shown to synthesize the broad-spectrum antibiotic andrimid, which it uses as a novel virulence mechanism to infect the coral *Montipora capitata* by disruption of the protective microflora. A mutant strain unable to produce andrimid was incapable of colonizing *M. capitata* with the same efficiency at wild type; treatment of coral with purified andrimid restored infectivity of the mutant. Coral fragments that underwent treatment with ampicillin and spectinomycin also had increased susceptibility to *V. coralliilyticus* infection, which provides evidence for the protective role of the normal microflora. This system demonstrates the influence of the normal microflora on an organism’s susceptibility to bacterial infection and a novel mechanism utilized by a pathogen to overcome this defense.
RESULTS

**OCN008 produces the antibiotic andrimid**

During the study to define *V. coralliilyticus* strain OCN008 as a coral pathogen, it was noted that a zone of growth inhibition was produced around OCN008 colonies possibly due to the production of an antimicrobial compound (42) (Figure 14A). To determine whether OCN008 produced an antimicrobial compound, OCN008-conditioned media and its extracts were assessed for growth inhibition of a susceptible target strain, *Pseudoalteromonas* sp. strain OCN003 (233). Growth of OCN003 was strongly inhibited by OCN008-conditioned medium and the inhibitory activity was not abrogated by enzymatic digestion or thermal stress (Figure 14E, Table 16). The inhibitory activity from OCN008 cultures began in log phase and peaked during stationary phase (Figure 15). Supplementation of GASW with boric acid increased OCN008 inhibitory activity by

![Figure 14. Antibacterial activity of OCN008.](image)

(A) Growth of OCN008 after being spotted onto a plate spread with the target bacterium and displaying a zone of growth inhibition. (B) Growth of OCN002 and (C) the target bacterium after being spotted onto plate spread with the target bacterium. The black scale bar represents 10 mm. (D) Optical density readings at 600 nm of a dilution series of OCN008-conditioned media inoculated with the target strain and incubated for 24 h.
three-fold, suggesting regulation by quorum sensing (Figure 16) (234). Chloroform extracts of conditioned medium impaired the growth of various Gram-positive and Gram-negative bacteria, but not yeast, which indicates that OCN008 likely produces a broad-spectrum antibacterial compound (Table 17).

To identify genes involved in OCN008 antibacterial production, 11,195 transposon mutants were screened for the inability to inhibit growth of OCN003. Of the transposon mutants screened, 25 unique insertions were mapped to genes potentially involved in the production of an antibiotic (Table 18). Two mutants lacking inhibitory activity harbored transposon insertions in

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**Figure 15. Correlation of growth phase and antibacterial activity.** Production of antimicrobial activity and phase of growth. Solid lines indicate OD$_{600}$ of wild type, and dashed lines indicate antimicrobial activity. Error bars indicate standard error for growth measurements or the standard deviation of three independent trials for antimicrobial activity. Units of antimicrobial activity are represented as the average reciprocal of the highest dilution capable of complete growth inhibition.
genes homologous to \textit{admP} and \textit{admS}; genes within the andrimid (\textit{adm}) biosynthetic gene cluster (235).

Analysis of the OCN008 genome identified 20 co-oriented genes (\textit{adm\textsubscript{ABCDEF}}HG\textsubscript{IJKLMONPRST}) [ERB66413 - ERB66393] (Figure 17) that shared an
average 80% amino acid identity with the gene products from the andrimid biosynthetic gene cluster of *Pantoea agglomerans*, the bacterium from which andrimid was first isolated (236, 237). In addition, these genes also shared 67 to 90% amino acid similarity to homologs from *Serratia marcescens*, *Serratia plymuthica*, and *Serratia* sp. C-1, suggesting these strains also contain the andrimid gene cluster. This gene cluster is absent from the genomes of other sequenced *V. coralliilyticus* strains that infect coral (43, 58, 59). The 2859 bp upstream and 8753 bp downstream of *admA* and *admT*, respectively, did not share nucleotide similarity to any of the *V. coralliilyticus* coral pathogens, while the sequence beyond those regions shared greater than 97% nucleotide identity to other *V. coralliilyticus* strains. Several ORFs within the region surrounding the andrimid gene cluster (Table 19) have predicted protein sequences similar to homologs in other bacteria, ranging from marine species, a member of Vibrionaceae, the plant pathogen *Dickeya dianthicola*, and the insect endosymbiont *Enterobacter* sp. Ag1. In all, this is suggestive that the andrimid gene cluster was acquired by horizontal gene transfer and made have passed through a variety of different bacterial hosts.

Deletion of the entire andrimid gene cluster resulted in the creation of strain OCN041, which was unable to inhibit the growth of OCN003 in liquid- or plate-based culture and boric acid supplementation did not induce observable antibacterial activity (Figure 18EG, Figure 16). Strains harboring clean deletions of *admAB*, *admS*, or *admP* were similarly unable to inhibit OCN003 growth from plate or liquid assays (Figure 18BCD). Isolation of this compound from
large-scale cultures of OCN008 followed by $^1$H NMR analysis confirmed that it was andrimid (238). Taken together, these data demonstrate that OCN008 produces the broad-spectrum antibiotic andrimid and its production is responsible for all observed OCN003 growth inhibition.

Figure 18. Andrimid mutants. Growth of (A) wild type, (B) the $\Delta admAB$ mutant (OCN049), (C) the $\Delta admP$ (OCN033) mutant, (D) the $\Delta admS$ mutant (OCN035), (E) the $\Delta adm$ mutant (OCN041), and (F) OCN120 after being spotted onto a plate spread with the target bacterium. The black scale bar represents 10 mm. (G) Optical density readings at 600 nm of a dilution series of conditioned media from different mutant strains that were inoculated with the target strain and incubated for 24 h. (H) Kaplan-Meier survival curve of M. capitata in infection trials comparing the virulence of wild type to OCN041. The percentage of surviving corals over time in days is plotted. Dotted line with open squares, fragments with the control bacterium or FSW alone (results for each were identical) ($n = 20$); solid line with solid triangles, OCN008 ($n = 20$); dashed line with open triangles, OCN041 ($n = 20$). Concentration of bacteria was $10^8$ CFU/ml of seawater.
The Δadm mutant has reduced virulence

A reduction in virulence observed with OCN041 would be indicative that andrimid production is involved with OCN008 infection of *M. capitata*. OCN041 displayed a significant decrease in virulence (Mantel-Cox test, \( p < 0.01, n=20 \)), wild type infected 90% of exposed *M. capitata* fragments, while OCN041 infected 30% of fragments over a period of 21 days (Figure 18H). The reduced virulence of OCN041 could be attributed to a defect in other activities known to contribute to virulence in other pathogens including extracellular enzyme activities. OCN041 did not display any obvious growth defects, with a generation time and final culture density identical to wild type (Figure 19), and displayed wild-type levels of hemolysin, protease, lipase,

![Figure 19. Growth rate of the Δadm mutant vs wild type. Average OD<sub>600</sub> measurements of wild type and OCN041 cultures plotted over time (h).](image-url)
and DNase activity on indicator plates (Table 20). It is plausible that introduction of the gentamicin resistance cassette into OCN041 may have altered virulence, however introduction of the same cassette into wild type did not significantly alter virulence (Mantel-Cox test, $p = 0.92$, $n = 12$; Figure 20; Table 20). We infer that the reduced virulence of OCN041 was due to the absence of the andrimid biosynthesis genes, which resulted in the inability to produce a broad-spectrum antibiotic.
Figure 17. Schematic of the andrimid gene cluster in OCN008. Black arrows represent the genes for andrimid biosynthesis and resistance. The green arrows represent genes shared among the different *V. coralliilyticus* strains. The grey arrows represent ORFs encoding predicted proteins that are not found in other *V. coralliilyticus* strains pathogenic to coral. Arrows are to scale and the black scale bar represents 1000 bp.
The Δadm mutation can be complemented with treatment using purified andrimid

It is possible that mutagenesis of the adm operon could have affected other cellular processes relating to infection, therefore, OCN041 was complemented with exogenous andrimid to demonstrate that the abrogation of OCN041 virulence is due solely to the loss of antibiotic production. Treatment with andrimid extracted from wild type cultures, described above, reduced the average concentration of cultivable bacteria in *M. capitata* mucus by roughly 100-fold (n = 5

![Figure 21. Effect of antibiotic treatment on the cultivable microflora of *M. capitata*.](image-url)

(A) CFU/ml of coral mucus from *M. capitata* directly from the field, after four days in FSW alone, FSW with extract from OCN008 cultures, or FSW with extract from OCN041 (n = 5 coral genotypes). The final concentration of andrimid was 0.125 µg/ml. (B) CFU/ml of coral mucus from *M. capitata* directly from the field, after one day in FSW alone, or FSW with combinations of final concentrations of ampicillin at 50 µg/ml (Ap50), 100 µg/ml (Ap100), or 150 µg/ml (Ap150) and spectinomycin at 50 µg/ml (Sp50) or 100 µg/ml (Sp100). Average CFU/ml was calculated from triplicate plates counts on GASW (light grey) or TCBS agar (dark grey).
coral colonies) compared with coral fragments treated with FSW alone or with extract from OCN041, which does not contain andrimid (Figure 21A). Pre-treatment of the *M. capitata* fragments also allowed OCN041 to infect at wild type levels (Mantel-Cox test, \( p = 0.01, n = 12 \)), while fragments pre-treated with FSW alone or with extract from OCN041 still displayed resistance to OCN041 infection (Figure 22). These results demonstrate that andrimid activity effects the microflora present on *M. capitata* and is sufficient to complement OCN041.

**Figure 22. Complementation of the Δadm mutation with purified andrimid.** Kaplan-Meier survival curve of *M. capitata* in infection trials where the corals were previously kept for four days in (A) FSW only, (B) FSW with extract from wild type cultures for a final andrimid concentration of 0.125 \( \mu \)g/ml, and (C) FSW with extract from OCN041 cultures. The percentage of surviving corals over time in days is plotted. Dotted line with open squares, fragments with the control bacterium, FSW alone, or FSW with extract (results for each were identical) \( n = 12 \); solid line with solid triangles, wild type \( n = 12 \); dashed line with open triangles, OCN041 \( n = 12 \). Concentration of bacteria was 10^8 CFU/ml of seawater.
Functional complementation of the Δadm mutant restores virulence to wild-type levels

To determine if general antibiotic activity, and not just specifically andrimid, would complement OCN041, coral fragments were pre-treated with ampicillin and spectinomycin before inoculation with bacteria to clear the normal microflora (Figure 21B). Infection trials with coral fragments not treated with antibiotics resembled previous trials, wild type infected 80% of fragments \((n = 10)\), and OCN041 infected 20% of fragments \((n = 10)\) (Figure 23A). When these same coral genotypes were pre-treated for 24 h with the ampicillin/spectinomycin cocktail, 100% of fragments exposed to either the wild type or OCN041 were infected (Figure 23B). None of the control fragments became diseased or displayed any adverse side effects from antibiotic treatment. Pre-treatment with antibiotics resulted in a significant increase in virulence for OCN041 (Mantel-Cox test, \(p < 0.001, n = 10\)), and demonstrates general antibiotic activity complements OCN041. In addition, antibiotic pre-treatment increased wild type virulence (Figure 23B) and, interestingly, the minimum infectious concentration decreased from between \(10^7\) and \(10^6\) CFU/ml down to \(10^6\) to \(10^5\) CFU/ml of tank water (Table 21). These results suggest that the microflora present on *M. capitata* is hindering OCN041 infection, and provides some level of protection against wild type.
The Δadm mutant has reduced ability to colonize *M. capitata*

Antibiotic treatment alters the *M. capitata* microflora, but does not induce tissue lysis without pathogen present; therefore, complementation of OCN041 could be the result of improved pathogen colonization from antibiotic-induced disruption of the coral microflora. The ability to colonize *M. capitata* mucus was evaluated between genetically-tagged strains able and unable to produce andrimid, OCN122 and OCN119, respectively, using plate counts and

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**Figure 23. Pre-treatment with antibiotics increases disease susceptibility.** Kaplan-Meier survival curve of *M. capitata* in infection trials where the corals were pre-treated with (A) FSW only and (B) ampicillin (150 µg/ml) and spectinomycin (100 µg/ml) for 24 h. The percentage of surviving corals over time in days is plotted. Dotted line with open squares, fragments with the control bacterium, FSW alone with and without antibiotic pre-treatment (results for each were identical) (*n* = 10); solid line with solid triangles, OCN008 (*n* = 10); dashed line with open triangles, OCN041 (*n* = 10). Concentration of bacteria was $10^8$ CFU/ml of seawater.
selective media. By 5 h post-inoculation, the average concentration of OCN122 and OCN119 in coral mucus increased to 3.05 x 10^6 CFU/ml (SEM ±7.52 x 10^5) and 7.72 x 10^5 CFU/ml (SEM ±1.26 x 10^5), respectively (Figure 24A). After 5 h post-inoculation, the concentration of OCN122 steadily increased to an average of 6.63 x 10^7 CFU/ml (SEM ±1.05 x 10^7) at 23 h post-inoculation. Conversely, OCN119 concentrations reached a maximum of 7.72 x 10^5 CFU/ml (SEM ±1.26 x 10^5), then started to decrease by 9 h post-inoculation and averaged 3.87 x 10^6 CFU/ml (SEM ±2.12 x 10^4) at 23 h post-inoculation, which is below the minimum infectious dose of wild type. The concentrations of both OCN122 and OCN119 in the tank water maintained as a relatively constant concentration of roughly 10^6 CFU/ml of seawater throughout the experiment (Figure 24B). Two of the three coral genotypes exposed to OCN122 developed tissue loss within 48 h post-inoculation, while these same genotypes were not infected by OCN119 over the duration of the experiment.

To evaluate the total number of pathogen between infected and non-infected coral, the total concentration of pathogen was determined for 12 coral genotypes exposed to OCN122 and OCN119 at 96 h post-inoculation (Figure 24C). Nine of the 12 fragments exposed to OCN122 and three of the 12 fragments exposed to OCN119 developed tissue loss within the 96 h period. Regardless of exposure to OCN122 or OCN119, all diseased fragments had comparably similar concentrations of pathogen recovered, 1.38 x 10^7 CFU/ml (SEM ± 1.08 x 10^7) and 5.6 x 10^6 CFU/ml (SEM ±1.29 x 10^6), respectively. The coral fragments inoculated with OCN122 and OCN119 that remained healthy also shared similar concentrations of pathogen recovered, 2.9 x 10^5 and 8.3 x 10^2 CFU/ml, respectively. This data demonstrates that andrimid production enables this pathogen to successfully colonize the mucus of *M. capitata*. However, a proportion of coral genotypes are susceptible to infection or naturally resistant regardless of the pathogen’s ability to
produce andrimid, suggesting extraneous factors that influence susceptibility or variation in important microflora constituents that influence protection.

Figure 24. Andrimid production involved with the ability to colonize *M. capitata*. The average CFU/ml in (A) coral mucus and (B) tank water of the genetically-tagged, non-andrimid producing strain OCN119 and andrimid producing strain OCN122. The average CFU/ml over time (h) post-inoculation of the coral is plotted (*n* = 3 coral genotypes) and was calculated from triplicate plate counts on TCBS agar supplemented with chloramphenicol (10 µg/ml) and gentamicin (15 µg/ml). (C) the average CFU/ml of mucus of OCN119 and OCN122 from diseased and healthy fragments after four days during an infection trial (*n* = 12 coral genotypes).
DISCUSSION

This work demonstrates that *V. coralliilyticus* strain OCN008 produces the antibiotic andrimid to disrupt the bacterial microflora present on *M. capitata*, which is essential for effective OCN008 colonization. To our knowledge, this is the first description of a pathogen that requires the *in vivo* production of a broad-spectrum antibiotic to achieve normal host infection. The most analogous system is antibiotic-associated pseudomembranous colitis in humans caused by gastrointestinal pathogens including *C. difficile*, which rely on the intake of exogenous broad-spectrum antibiotics to weaken the healthy gut microflora facilitating colonization and host infection (217). Though the distribution and prevalence of OCN008-like strains remains unknown, this pathogen could be part of the *M. capitata* pathobiont, similar to the classification of *C. difficile* in humans, or it seldom associates with healthy hosts.

The combined effects of ampicillin/spectinomycin treatment were sufficient to increase the infection rate of both OCN008 and OCN041 to 100%, which suggests that ~15% of untreated corals maintain a microflora that is able to effectively prevent OCN008 colonization, possibly by andrimid resistance. Difference in microflora is likely the factor mediating infectivity between *M. capitata* and *A. cytherea* by the non-antibiotic-producing *V. coralliilyticus* strain OCN014; OCN014 infects roughly 80% of *A. cytherea* fragments, its natural host, but its infection rate with *M. capitata* is similar to that of OCN041. This could be akin to what is believed to have happened with the *O. patagonica/V. shiloi* infection model, in which the microfloral structure changed or evolved to prevent *V. shiloi* colonization and invasion (93).

Pathogens of the Vibrionaceae family are known to acquire new infection mechanisms through horizontal gene transfer (HGT). For example, pathogenic *Vibrio cholerae* strains evolved from non-pathogenic environmental strains following lysogenic conversion by the
CTXφ phage (239, 240). Strains of *Vibrio parahaemolyticus* harboring the hemolysin-encoding genes *tdh* and *trh* cause gastroenteritis in humans (241–243). These genes can be acquired through conjugation with *Vibrio alginolyticus*, which acts as a genetic reservoir for virulence factors for other *Vibrio* species (244–246). Though other antibiotic-producing *V. coralliilyticus* strains have been isolated (247, 248), only OCN008 has been shown to be pathogenic and horizontal acquisition of the *adm* gene cluster by an ancestral *V. coralliilyticus* strain likely conferred the fitness advantage required to extend its host range to *M. capitata*.

HGT extends well beyond the Vibrionaceae and is responsible for the pervasive spread of many human pathogens. Pathogenic strains of *Enterobacter cloacae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *E. coli*, and *Salmonella enterica* serotype Typhimurium (249) acquired genes encoding extended-spectrum beta-lactamases (ESBL), like NDM-1 and KPC-1, and spread them to other pathogens, which leads to nosocomial infections that are extremely difficult to treat (250, 251). It is well documented that antagonistic interactions between bacteria in soil systems have a constant flow of genes conferring antibiotic production and resistance, and similar interactions are thought to happen within the human gut. Based on the bacterial propensity to exchange genes that confer a selective advantage, as is evident from the spread of ESBLs and the incorporation of andrimid production in OCN008 infection, it will soon be shown that antimicrobial production by human pathogens modulates infectivity. This new paradigm of infection will pose a unique set of challenges for the management and prevention of both human and coral disease.
METHODS

Bacterial growth conditions

Marine bacteria were grown in glycerol artificial seawater (GASW) medium (42) and incubated at 25 °C with aeration unless otherwise stated. GASW media was supplemented with 50 mM Tris-Base and adjusted to a pH of 8.3 using concentrated HCl. Thiosulfate citrate bile salts sucrose (TCBS) agar was prepared according to the manufacturer’s instructions, supplemented with NaCl for a final concentration of 2% (w/v), and incubated at 25 °C for the growth of Vibrio strains. Escherichia coli strains were maintained on Luria-Bertani (LB) medium. Antibiotics were used at the following concentrations for E. coli unless otherwise stated: ampicillin 100 µg/ml; kanamycin 50 µg/ml; streptomycin 25 µg/ml; spectinomycin 50 µg/ml; chloramphenicol 15 µg/ml; gentamicin 30 µg/ml. Antibiotics were used at the following concentrations for Vibrio unless otherwise stated: ampicillin 200 µg/ml; streptomycin 50 µg/ml; spectinomycin 100 µg/ml; chloramphenicol 10 µg/ml; gentamicin 15 µg/ml. Growth media for E.coli auxotrophic strains were supplemented with deoxythymidine (DT) or diaminopimelate (DAP) at a final concentration of 0.3 mM. In suicide vectors, expression of ccdB, under control of the P_BAD promoter, was achieved by the addition of 0.3% L-arabinose to the growth media, while expression was repressed by addition of 1% D-glucose.

Inhibition assays

Antibacterial activity of OCN008 was evaluated against the target strain, the marine bacterium Pseudoalteromonas sp. strain OCN003 (233). GASW was used for inhibition assays on both solid and liquid inhibition assays. For solid media assays, 200 µl of a culture of the target
strain roughly at an optical density read at 600 nm (OD\textsubscript{600}) of 2.0 was spread over the plate, \textit{V. coralliilyticus} strains were then patched or streaked on the plate, and then incubated at 28 °C for 24 h before being observed for growth inhibition of the target strain.

For liquid inhibition assays, 24 h cultures of OCN008 (OD\textsubscript{600} = 3.5) were centrifuged at 10,000 x g for five minutes before being filter sterilized using a 0.2 µm pore membrane filter. The resulting cell-free supernatant (conditioned media) inhibited the growth of the target strain. Cultures of a non-inhibitory coral pathogen, \textit{V. owensii} strain OCN002 (84), was prepared as described above and used as a negative control. Conditioned media, or dilutions thereof, were inoculated with 10\textsuperscript{3} CFU/ml of the target bacterium and OD\textsubscript{600} was measured after incubation for 24 h.

Treatments of conditioned media for characterization of the OCN008 antibacterial were performed prior to inoculation with the target strain and using OCN002-conditioned media as a control. Proteinase K and RNase A stocks were prepared according to the manufactures instructions, and conditioned media was incubated for 15 h with 20 µg/ml of either enzyme before heat-inactivation and inhibition assays. The cellular contents of OCN008 was screened for antibacterial activity by centrifuging 500 ml of a 24 h culture of OCN008, running the cellular pellet though a French-press, then tested via the liquid inhibition assays described above.

\textit{Plasmid construction}

All plasmids used in this study are listed in Table 14. All PCR-derived products were verified by sequencing. Plasmid pPJAV217 is a replicative plasmid containing a gentamicin resistance cassette. The gentamicin resistance cassette was amplified by PCR from pUC18-mini-
Tn7T-Gm-\textit{lacZ} (199) with the primers Gm-SmaI-F and FRT-Gm-R. The PCR product was then cloned into the \textit{SmaI} site of pBlueScript SK+ (Stratagene).

Plasmid pPJAV241 is a suicide plasmid based on pSW4426T (196) used to delete all but the first 30 and last 30 nucleotides of the coding region of the \textit{admP} gene. Regions upstream and downstream of \textit{admP} were amplified by PCR from chromosomal DNA with the primer sets admP-up-EcoRI-F and admP-up-EcoRV-R and admP-down-EcoRV-F and admP-down-XbaI-R. The up- and downstream fragments were fused together by overlap extension PCR (198) and the product was cloned as an \textit{EcoRI-XbaI} fragment into the \textit{EcoRI-SpeI} sites of pSW4426T.

Plasmid pPJAV242 is a suicide plasmid based on pSW4426T used to delete all but the first 30 and last 30 nucleotides of the coding region of the \textit{admS} gene. Regions upstream and downstream of \textit{admS} were amplified by PCR from chromosomal DNA with the primer sets admS-up-MunI-F and admS-up-SmaI-R and admS-down-SmaI-F and admS-down-SpeI-R. The up- and downstream fragments were fused together by overlap extension PCR and the product was cloned as a \textit{MunI-SpeI} fragment into the \textit{EcoRI-XbaI} sites of pSW4426T.

Plasmid pPJAV259 is a suicide plasmid based on pSW4426T used to replace all but first 30 nucleotides of \textit{admA} and last 30 nucleotides of \textit{admS} of the andrimid biosynthesis operon with a gentamicin resistance (Gm\textsuperscript{r}) cassette. The \textit{admS} downstream fragment was amplified by PCR from chromosomal DNA with the primers admS-down-SmaI-F and admS-down-SpeI-R, cloned into the \textit{SmaI} site of pBlueScript SK+, and screened for directionality with PCR. The \textit{admA} upstream fragment was amplified by PCR from chromosomal DNA with the primers admA-up-F and admA-up-R, cloned into the \textit{EcoRV} site of the previous plasmid, and screened for directionality with PCR. The Gm\textsuperscript{r} cassette from pPJAV217 was moved as a \textit{SmaI} fragment into the \textit{SmaI} site between the \textit{admA} upstream and \textit{admS} downstream regions in pBlueScript
SK+. The entire region containing the Gm\(^r\) cassette flanked by the *admA* upstream and *admS* downstream regions was excised as an *XhoI-SacI* fragment, blunt-ended, and cloned into the blunt-ended *EcoRI* site of pSW4426T.

Plasmid pBU115 is derived from pEVS78 (155) and used as a replicative vector in OCN008 and contains a unique *SmaI* site. The vector pEVS78 was amplified by PCR using the primers pEVS78-SmaI-F and pEVS78-SmaI-R. The resulting PCR product was digested with *DpnI* and *SmaI* then self-ligated together.

Plasmid pBU153 is a suicide plasmid based on pSW4426T used to replace all but first 30 nucleotides of *admA* and last 30 nucleotides of *admB* of the andrimid biosynthesis operon. The region upstream of *admA* was amplified by PCR from chromosomal DNA with the primer sets admA-up-SpeI-F & admA-up-OEX-R. The region downstream of *admB* was amplified by PCR from chromosomal DNA with the primers admC-down-OEX-F & admC-down-SpeI-R. The up- and downstream fragments were fused together by overlap extension PCR and the product was cloned as a *SpeI* fragment into the *XbaI* site of pSW4426T.

Plasmid pBU155 is a replicative plasmid containing a 1.6 kb intergenic region downstream of the *glmS* gene in OCN008 with a unique *SmaI* site. The upstream and downstream regions of the intergenic site was amplified by PCR from chromosomal DNA with the primer sets 008glmS-nut-up-SpeI-F & 008 glmS-nut-up-OEX-R and 008glmS-nut-down-OEX-F & 008glmS-nut-down-SpeI-R. The up- and downstream fragments were fused together by overlap extension PCR and the product was then cloned into the *SmaI* site of pBlueScript SK+.
Plasmid pBU164 is a replicative plasmid with \textit{yfpmut2} as a transcriptional fusion to the constitutive \textit{bla} promoter. \textit{yfpmut2} was amplified with PCR from the plasmid pKL183 (252) using the primers \textit{yfpmut2-EcoRI-up-F} and pKL183-\textit{yfp-PstI-R}. The PCR product was cloned as a \textit{EcoRI-PstI} fragment into \textit{EcoRI-PstI} sites of pBlueScript SK$^+$ to create pBU159. The \textit{bla} promoter region was amplified by OCR from pBBR1MCS-4 using the primers \textit{bla-SmaI-F} and \textit{bla-SmaI-R}. The PCR product was cloned as a \textit{SmaI} fragment into the \textit{EcoRV} site of pBU159 to create pBU160. The \textit{P_{bla-yfpmut2}} transcriptional fusion was amplified from pBU160 with PCR using the primers M13-F and pKL183-\textit{yfp-PstI-R}. The PCR product was then cloned into the \textit{SmaI} site of pBU115.

Plasmid pBU179 is a suicide plasmid used to introduce the gentamicin resistance cassette into an intergenic region downstream of \textit{glmS} in the OCN008 genome. The gentamicin resistance cassette was amplified with PCR from the vector pUC18-mini-Tn7T-Gm-lux using the primers GmR-up-SacI-F and GmR-BamHI-R. The PCR product was then cloned into the \textit{SmaI} site of pBU155. The intermediate plasmid was then amplified with PCR using the primers 008glmS-up-SpeI-F and 008glmS-up-SpeI-R. The PCR product was cloned as a \textit{SpeI} fragment into the \textit{XbaI} site of pSW4426T.

\textit{Bacterial conjugation and transposon mutagenesis}

All \textit{V. coralliilyticus} suicide vectors and replicative vectors were introduced using tri-parental conjugations with \textit{E. coli}, unless otherwise stated. Suicide and replicative vectors were maintained in \textit{E. coli} strain $\beta$3914 and the self-transmissible vector pRK2013 (200) was maintained in strain $\pi$3813 (196). For conjugation, donor and recipient strains were grown
overnight with the appropriate antibiotics for plasmid maintenance and DAP or DT as required. Overnight cultures were diluted 1:1000 in fresh culture media without antibiotics, grown to an OD$_{600}$ of 0.7, and washed three times with either GASW or LB for *Vibrio* or *E. coli* strains, respectively. The donor and recipient strains were then mixed together in a 10:10:1 ratio of *E. coli* to *Vibrio* and 25 μl aliquots of the conjugation mixed was spotted onto GASW plates supplemented with DAP, DT, and D-glucose, unless otherwise stated. Conjugation spots were incubated at 28 °C for 15 h before being resuspended in GASW, washed three times with GASW, diluted, and plated onto GASW supplemented with D-glucose, when introducing suicide vectors, and chloramphenicol or spectinomycin and streptomycin in combination, but lacking DAP or DT for counterselection against the ΔdapA and ΔthyA *E. coli* donor strains. Antibiotic-resistant colonies, which contained the suicide vector introduced through a single recombination event, were isolated and grown overnight in GASW broth supplemented with D-glucose.

Overnight cultures were washed with GASW three times, diluted, and plated onto GASW plates supplemented with L-arabinose to isolate double recombinants.

The donor strain, *E. coli* SM10 λPir, containing the transposon vector pBT20 (183) and the recipient strain, OCN008, were used in a bi-parental conjugation for transposon mutagenesis. The same conjugation protocol described above was used, except a 10:1 ratio of donor to recipient strain was used. The conjugation mix was then plated onto TCBS agar supplemented with gentamicin and incubated for 24 h at 30 °C. Potential transposon mutants were screened against the target bacterium using the solid medium inhibition assays described above.
**Identification of transposon insertion sites**

Genomic DNA was isolated from transposon mutants lacking antibacterial activity using a phenol-chloroform extraction method. DNA extractions were then digested with *HpyCH4IV* then circularized using T4 DNA ligase. Circularized DNA fragments were used as template for inverse PCR (201) using the primers pBT20-HPYCH4IV-F and pBT20-HPYCH4IV-R. Sequencing of the PCR products with the primer pBT20-HPYCH4IV-F was used to map transposon insertion sites to the OCN008 genome (237).

**Coral collection and infection trials**

Fragments of *M. capitata* for infection trials measured approximately 3 by 3 by 1 cm and were collected from a fringing reef surrounding the island Moku o Lo‘e in Kane‘ohe Bay, Hawai‘i. Coral was collected under Special Activities Permit 2015-48 issues by the Hawai‘i Department of Aquatic Resources. Handling of coral specimens and infection trials were carried out as previously described unless otherwise stated (42).

Antibiotic treatments and bacterial inoculations of *M. capitata* fragments were conducted in separate aquariums. After any antibiotic treatment, prior to bacterial inoculation, coral fragments were rinsed in FSW to remove any excess antibiotics that would interfere with an inoculum. For treatment, ampicillin and spectinomycin powder was suspended in FSW and then filter-sterilized through a 0.2 μm pore filter before being mixed with tank water for final concentrations of 150 μg/ml and 100 μg/ml of tank water, respectively. Coral fragments were exposed to the duel antibiotic treatment for 24 h, the minimum time required for treatment to prevent the recovery of CFUs on GASW and TCBS agar. Corals were treated with 2x the
minimal inhibitory concentration (MIC) of andrimid for the target strain; the MIC for the target strain was calculated to be 62.5 ng/ml. Andrimid extracts were resuspended in DMSO and stock solutions of 1.0 μg/μl were mixed thoroughly with tank water for a final concentration of 0.125 μg/ml of tank water for coral treatment. To control for the extraction process and introduction of any inorganic solvents into the system, comparable volumes of extract from OCN041 cultures were used as a negative control treatment. Corals were exposed to the extract from wild type and OCN041 for 96 h, the median time for tissue loss to manifest after wild type inoculation. All corals treated with antibiotics has corresponding fragments from the same colony undergoing the same procedure with FSW or OCN041 extract.

**Purification of andrimid**

Extraction and purification of andrimid from OCN008 cultures was performed according to a modified version of a previously described protocol (238). OCN008 cultures grown on GASW agar were used for extractions. For one extraction, four aluminum trays (23 x 33 x 5 cm) each containing 500 ml of GASW agar supplemented with 6 mM boric acid was inoculated with one ml of a liquid culture of OCN008 at an OD$_{600}$ of 3.0 spread on the surface of the medium. The bacterial tray cultures were incubated for 48 h at 27 °C. After incubation, the cells were scraped off of the surface of the agar, and the solid medium was sectioned into 3 x 3 cm pieces and extracted in ethyl acetate for 48 h at room temperature. The ethyl acetate was then decanted, dried with anhydrous sodium sulfate, and then concentrated *in vacuo* to obtain a crude oily extract. The extract was resuspended in an equal volume of a 90:10 methanol/water solution that was then extracted with an equal volume of toluene. After the toluene-soluble portion was decanted off, the remaining solution was adjusted to 65:35 methanol/water and then extracted.
with an equal volume of chloroform. The methanol/water partition was decanted off, while the remaining chloroform partition was resuspended in 100% ethanol and concentrated in vacuo to obtain an extract containing andrimid. Each crude extract of andrimid was resuspended in 500 µl DMSO. An identical protocol was used for OCN041 cultures.

Colonization studies

The effect of andrimid production on the colonization potential was assessed via CFU counts using genetically tagged strains and fluorescence microscopy of inoculated *M. capitata* fragments. The strains used in the colonization study, OCN119 and OCN122 (Table 1), each contained the vector pBU164 and a chromosomal copy of the gentamicin resistance cassette, but OCN119 does not have the genes for andrimid biosynthesis (a Δadm mutation). Each strain was inoculated onto healthy *M. capitata* fragments as described above, then a mucus and tank water sample was collected at regular intervals post-inoculation using a sterile pipette. Dilutions of the water and mucus samples were plated onto TCBS agar supplemented with chloramphenicol (10 µg/ml) and gentamicin (15 µg/ml) to calculate CFU/ml of mucus or tank water.
Table 14. Strains and plasmids used in this study

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<tr>
<td>pBU179</td>
<td>Suicide vector used to make OCN120; Cm', Sp', Sm', Gm'</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 15. PCR primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’ → 3’ orientation)</th>
<th>Source or citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13-F</td>
<td>GTAAACGACGCGCCAGTG</td>
<td>(161)</td>
</tr>
<tr>
<td>M13-R</td>
<td>GAAACGCTATGACCATATG</td>
<td>(161)</td>
</tr>
<tr>
<td>pBT20-HPYCH4-V-F</td>
<td>GAGATCAATTGTTGCACTTCTCGAG</td>
<td>This study</td>
</tr>
<tr>
<td>pBT20-HPYCH4-V-R</td>
<td>TGGTTAGTGGTGGGTACTTGGGTCG</td>
<td>This study</td>
</tr>
<tr>
<td>pSW4426T-MCS-F</td>
<td>CTCAACCGGAATCTGCTCTGCGAG</td>
<td>This study</td>
</tr>
<tr>
<td>pSW4426T-MCS-R</td>
<td>ACTGCTTGGTGCAATTGCAGCAATGAG</td>
<td>This study</td>
</tr>
<tr>
<td>admP-up-EcoRI-F</td>
<td>ATATAGAATTCGGTGTTCAACACACGCTGCTAG</td>
<td>This study</td>
</tr>
<tr>
<td>admP-up-EcoRV-F</td>
<td>CAGCTTAGATATCCCAAGAGAAACATACGTATCTTCAG</td>
<td>This study</td>
</tr>
<tr>
<td>admP-down-EcoRV-F</td>
<td>GTAGATCCGGATATCGAATCATCTGAACCTGAACTGAG</td>
<td>This study</td>
</tr>
<tr>
<td>admP-down-XbaI-R</td>
<td>ATATACTAGTGTACGTTGACTTTTAAATGGCAG</td>
<td>This study</td>
</tr>
<tr>
<td>admS-up-MumI-F</td>
<td>ATATCACAATTGCTGGTCATCTGTTAATTGCAG</td>
<td>This study</td>
</tr>
<tr>
<td>admS-down-SmaI-R</td>
<td>TTGCCGGAAACCCGAGATTCTCTCGCCGTTCTCAACTCAG</td>
<td>This study</td>
</tr>
<tr>
<td>admS-down-SmaI-F</td>
<td>AAGAGGAATCCCGGGTTCCCGAAGAGAAACCTGTTGAGC</td>
<td>This study</td>
</tr>
<tr>
<td>admS-down-SpeI-R</td>
<td>ATATACTAGTGAACATGACTCTTCACCCGAG</td>
<td>This study</td>
</tr>
<tr>
<td>admA-up-SpeI-F</td>
<td>ATATATACTAGTGCCTCCTCATTACACATGATGAG</td>
<td>This study</td>
</tr>
<tr>
<td>admC-down-OEX-SmaI-R</td>
<td>TAGGAAATGATGAAATGTAGTGCTCAGGAGGAGGAGCAT</td>
<td>This study</td>
</tr>
<tr>
<td>glmS-down-SpeI-R</td>
<td>TTGCCCTCGTTTTTCCCGGGATTTTAACTGTTACCTTGTTACCTCGT</td>
<td>This study</td>
</tr>
<tr>
<td>pEVS78-SmaI-F</td>
<td>GGGAACGCGTGTCCTGCTGTCGACGC</td>
<td>This study</td>
</tr>
<tr>
<td>pEVS78-SmaI-R</td>
<td>GGGATGAAAATCTAACATGCGCTC</td>
<td>This study</td>
</tr>
<tr>
<td>pEVS78-MCS-F</td>
<td>GCCCACTTACACGTACTCCCTCC</td>
<td>This study</td>
</tr>
<tr>
<td>pEVS78-MCS-R</td>
<td>CAAATGTAGCAGGAACTCAG</td>
<td>This study</td>
</tr>
<tr>
<td>yfpmut2-EcoR1-up-F</td>
<td>ATATAGGAATTCGCTCTGAGGCGATTATTGAGAT</td>
<td>This study</td>
</tr>
<tr>
<td>pKL183-yfPst-I</td>
<td>GATGCTCTGAGTCTGGGATTTAT</td>
<td>This study</td>
</tr>
<tr>
<td>bla-SmaI-F</td>
<td>ATATATCCCGGAGCGTTTCTCTGTGTAAT</td>
<td>This study</td>
</tr>
<tr>
<td>glmS-up-SpeI-F</td>
<td>ATATATCTAGGAAACCGTGATCGTTCAGCGCTC</td>
<td>This study</td>
</tr>
<tr>
<td>glmS-up-OEX-SmaI-R</td>
<td>GCTCCCACTCCGGGAAAAAACCGGCCAATGCGC</td>
<td>This study</td>
</tr>
<tr>
<td>glmS-down-OEX-F</td>
<td>TTTGCCGTTTTTCCCGGGATTTTAACTGTTACCTGTTACCTCGT</td>
<td>This study</td>
</tr>
<tr>
<td>glmS-down-SpeI-R</td>
<td>ATATATCTAGTCTGCTAGGTTATATGCAATCTGAAACG</td>
<td>This study</td>
</tr>
<tr>
<td>GmR-up-SacI-F</td>
<td>ATATATGACGCTCAAGATCCCGCTGACCCTC</td>
<td>This study</td>
</tr>
<tr>
<td>GmR-BamHI-R</td>
<td>ATATATGACGCTCAAGATCCCGCTGACCCTC</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 16. Characteristics of the antimicrobial compound produced by *Vibrio coralliilyticus* OCN008. All treatments were conducted for 24 h unless otherwise specified.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>OCN002-conditioned Media</th>
<th>OCN008-conditioned media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculated with OCN008</td>
<td>Inoculated with tester strain</td>
</tr>
<tr>
<td>RNase digest</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Protinase K digest</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Media conditioned with 0.1 mM FeCl₂</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Addition of 0.25 mM FeCl₂</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Storage at -80°C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Boiling (10 min)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Autoclaving (121.8°C, 30 min)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UV irradiation (125 mJ, 10 min)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acidification (pH 1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkalization (pH 10)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cytoplasmic contents</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>No Treatment</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) represents growth over OD<sub>600nm</sub> 1.0, while (-) represents an OD<sub>600nm</sub> below 0.05 after incubation overnight at 23°C with shaking.
Table 17. Antimicrobial susceptibility of various microorganisms to DCM-extracted OCN008 conditioned media concentrated 6.5X.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Growth in DCM-extracted OCN008 Conditioned Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter baumannii ATCC 19606</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus megaterium ATCC 14581</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus subtilis ATCC 6051</td>
<td>-</td>
</tr>
<tr>
<td>Bordetella bronchiseptica ATCC 4617</td>
<td>-</td>
</tr>
<tr>
<td>Citrobacter freundii ATCC 8090</td>
<td>-</td>
</tr>
<tr>
<td>E. coli B-strain ATCC 23848</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacter aerogenes ATCC 13848</td>
<td>+</td>
</tr>
<tr>
<td>Klebsiella pneumonia ATCC 13883</td>
<td>-</td>
</tr>
<tr>
<td>Proteus mirabilis ATCC 52933</td>
<td>+</td>
</tr>
<tr>
<td>Providencia stuartii ATCC 33672</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC 27853</td>
<td>+</td>
</tr>
<tr>
<td>Salmonella typhimurium TA 98</td>
<td>-</td>
</tr>
<tr>
<td>Shigella boydii ATCC 9207</td>
<td>-</td>
</tr>
<tr>
<td>Shigella flexneri ATCC 12022</td>
<td>-</td>
</tr>
<tr>
<td>Staphylococcus aureus ATCC 25923</td>
<td>-</td>
</tr>
<tr>
<td>Staphylococcus saprophyticus ATCC 15305</td>
<td>-</td>
</tr>
<tr>
<td>Anabaena sp. PCC7120</td>
<td>-</td>
</tr>
<tr>
<td>Vibrio nereis OCN044</td>
<td>+</td>
</tr>
<tr>
<td>Vibrio coralliilyticus OCN014</td>
<td>+</td>
</tr>
<tr>
<td>Vibrio owensii OCN002</td>
<td>-</td>
</tr>
<tr>
<td>Pseudoalteromonas sp. OCN003</td>
<td>-</td>
</tr>
<tr>
<td>Alteromonas sp. OCN004</td>
<td>-</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae REE3072a</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) represents growth over OD<sub>600nm</sub> 1.0, (-) represents an OD<sub>600nm</sub> below 0.05 after incubation.
Table 18. Unique genes disrupted by transposon mutagenesis

<table>
<thead>
<tr>
<th>Gene disrupted according to genome annotation</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>admP/andrimid biosynthetic operon</td>
<td>admS/andrimid biosynthetic gene</td>
</tr>
<tr>
<td>luxT/ quorum sensing protein</td>
<td>luxN/ quorum sensing protein</td>
</tr>
<tr>
<td>hapR quorum-sensing regulator</td>
<td>5’-methylthioadenosine nucleosidase</td>
</tr>
<tr>
<td></td>
<td>CRP/cyclic-amp receptor protein</td>
</tr>
<tr>
<td></td>
<td>5’-methylthioadenosine nucleosidase</td>
</tr>
<tr>
<td></td>
<td>GDP-mannose 4,6-dehydratase</td>
</tr>
<tr>
<td></td>
<td>Smf/ rossmann fold nucleotide-binding protein</td>
</tr>
<tr>
<td></td>
<td>sulfate adenylyltransferase subunit 2</td>
</tr>
<tr>
<td></td>
<td>mannitol-specific phosphotransferase system</td>
</tr>
<tr>
<td>sucB/ dihydrolipoamide succinyltransferase</td>
<td>aceI/ pyruvate dehydrogenase</td>
</tr>
<tr>
<td>capK/ hypothetical protein</td>
<td>GDP-mannose 4,6-dehydratase</td>
</tr>
<tr>
<td>hpt/ hypoxanthine-guanine phosphoribosyltransferase</td>
<td>NfuA/Fe-S protein maturation</td>
</tr>
<tr>
<td></td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td></td>
<td>fructose-specific phosphotransferase</td>
</tr>
<tr>
<td></td>
<td>sulfate adenylyltransferase subunit 2</td>
</tr>
<tr>
<td></td>
<td>sulfite reductase flavoprotein alpha-component</td>
</tr>
<tr>
<td>padR/ family protein</td>
<td>vpsF/ hypothetical protein</td>
</tr>
<tr>
<td>yjbH/ outer membrane lipoprotein</td>
<td></td>
</tr>
</tbody>
</table>
Table 19. Homologs of the apparent non-*V. coralliilyticus* predicted proteins from ORFs flanking the andrimid operon in OCN008.

<table>
<thead>
<tr>
<th>ORF</th>
<th>Predicted protein function</th>
<th>Percent amino acid similarity to closest homolog</th>
<th>Homolog origin</th>
<th>Homolog accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF1</td>
<td>hypothetical protein</td>
<td>95%</td>
<td><em>Vibrio</em> sp. SWAT-3</td>
<td>[EDK27207]</td>
</tr>
<tr>
<td>ORF2</td>
<td>LysR family transcriptional regulator</td>
<td>100%</td>
<td><em>V. coralliilyticus</em> S2043 <em>V. coralliilyticus</em> S2052</td>
<td>[KJY73479] [KJY74894]</td>
</tr>
<tr>
<td>ORF3</td>
<td>3-methyl-2-oxobutanoate hydroxymethyltransferase</td>
<td>80%</td>
<td>Marine γ-proteobacterium NOR5-3</td>
<td>[EED31400]</td>
</tr>
<tr>
<td>ORF4</td>
<td>aminotransferase, class III</td>
<td>76%</td>
<td>Marine γ-proteobacterium HTCC2143</td>
<td>[EAW31835]</td>
</tr>
<tr>
<td>ORF5</td>
<td>3-ketoacyl-ACP reductase</td>
<td>73%</td>
<td>Marine α-proteobacterium BRH_c36</td>
<td>[KUO53877]</td>
</tr>
<tr>
<td>ORF6</td>
<td>ATP-grasp domain-containing protein</td>
<td>33%</td>
<td><em>Pseudoalteromonas</em> sp. R3</td>
<td>[KUO53877]</td>
</tr>
<tr>
<td>ORF7</td>
<td>2-dehydrodantoate 2-reductase</td>
<td>40%</td>
<td><em>Moritella</em> sp. JT01</td>
<td>[KXO08505]</td>
</tr>
<tr>
<td>ORF8</td>
<td>hypothetical protein</td>
<td>45%</td>
<td><em>Dickeya dianthicola</em></td>
<td>[WP_024108547]</td>
</tr>
<tr>
<td>ORF9</td>
<td>major facilitator superfamily protein</td>
<td>44%</td>
<td><em>Enterobacter</em> sp. Ag1</td>
<td>[EJF32951]</td>
</tr>
</tbody>
</table>
Table 20. Exoenzyme activity of *V. coralliilyticus* strains on indicator plates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibiotic Activity (GASW with target strain)</th>
<th>Hemolysin Activity (TSA with 5% sheep blood, 1.5% NaCl)</th>
<th>Protease Activity (GASW with 1% skim milk)</th>
<th>Lipase Activity (GASW with 0.1 µg/ml Rhodamine B, 2.4% olive oil)</th>
<th>DNase Activity (Methyl green agar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCN008</td>
<td>5 - 9 mm</td>
<td>1 - 4 mm</td>
<td>4 - 5 mm</td>
<td>2 - 3 mm</td>
<td>3 - 5 mm</td>
</tr>
<tr>
<td>OCN041</td>
<td>0 mm</td>
<td>1 - 4 mm</td>
<td>4 - 5 mm</td>
<td>2 - 3 mm</td>
<td>3 - 5 mm</td>
</tr>
<tr>
<td>OCN119</td>
<td>0 mm</td>
<td>1 - 4 mm</td>
<td>4 - 5 mm</td>
<td>2 - 3 mm</td>
<td>3 - 5 mm</td>
</tr>
<tr>
<td>OCN120</td>
<td>5 - 9 mm</td>
<td>1 - 4 mm</td>
<td>4 - 5 mm</td>
<td>2 - 3 mm</td>
<td>3 - 5 mm</td>
</tr>
<tr>
<td>OCN122</td>
<td>5 - 9 mm</td>
<td>1 - 4 mm</td>
<td>4 - 5 mm</td>
<td>2 - 3 mm</td>
<td>3 - 5 mm</td>
</tr>
</tbody>
</table>

*Measurements are the ranges of radii of antibiotic/ enzyme activity on the indicator plates after incubation at 27 °C for 24 h in triplicate*
### Table 21. Summary of Infection Trials

<table>
<thead>
<tr>
<th>Coral pre-treatment</th>
<th>Inoculum</th>
<th>Inoculum concentration</th>
<th>Total diseased coral/total coral inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>The effect of the Δadm mutation on virulence</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSW</td>
<td>OCN008</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>18/20</td>
</tr>
<tr>
<td>FSW</td>
<td>OCN041</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>6/20</td>
</tr>
<tr>
<td>FSW</td>
<td>OCN004</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>0/20</td>
</tr>
<tr>
<td>FSW</td>
<td>FSW</td>
<td>N/A</td>
<td>0/20</td>
</tr>
<tr>
<td><strong>Complementation with purified andrimid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSW</td>
<td>OCN008</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>9/12</td>
</tr>
<tr>
<td>FSW</td>
<td>OCN041</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>1/12</td>
</tr>
<tr>
<td>FSW</td>
<td>OCN004</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>0/12</td>
</tr>
<tr>
<td>FSW</td>
<td>FSW</td>
<td>N/A</td>
<td>0/12</td>
</tr>
<tr>
<td>Purified andrimid (125 ng/ml)</td>
<td>OCN008</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>8/12</td>
</tr>
<tr>
<td>Purified andrimid (125 ng/ml)</td>
<td>OCN041</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>9/12</td>
</tr>
<tr>
<td>Purified andrimid (125 ng/ml)</td>
<td>OCN004</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>0/12</td>
</tr>
<tr>
<td>Purified andrimid (125 ng/ml)</td>
<td>FSW</td>
<td>N/A</td>
<td>0/12</td>
</tr>
<tr>
<td>Extract from OCN041 cultures</td>
<td>OCN008</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>9/12</td>
</tr>
<tr>
<td>Extract from OCN041 cultures</td>
<td>OCN041</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>1/12</td>
</tr>
<tr>
<td>Extract from OCN041 cultures</td>
<td>OCN004</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>0/12</td>
</tr>
<tr>
<td>Extract from OCN041 cultures</td>
<td>FSW</td>
<td>N/A</td>
<td>0/12</td>
</tr>
<tr>
<td><strong>Function complementation with general antibiotics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSW</td>
<td>OCN008</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>8/10</td>
</tr>
<tr>
<td>FSW</td>
<td>OCN041</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>2/10</td>
</tr>
<tr>
<td>FSW</td>
<td>OCN004</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>0/10</td>
</tr>
<tr>
<td>FSW</td>
<td>FSW</td>
<td>N/A</td>
<td>0/10</td>
</tr>
<tr>
<td>Ampicillin (150 µg/ml) and spectinomycin (100 µg/ml)</td>
<td>OCN008</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>10/10</td>
</tr>
<tr>
<td>Ampicillin (150 µg/ml) and spectinomycin (100 µg/ml)</td>
<td>OCN041</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>10/10</td>
</tr>
<tr>
<td>Ampicillin (150 µg/ml) and spectinomycin (100 µg/ml)</td>
<td>OCN004</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>0/10</td>
</tr>
<tr>
<td>Ampicillin (150 µg/ml) and spectinomycin (100 µg/ml)</td>
<td>FSW</td>
<td>N/A</td>
<td>0/10</td>
</tr>
<tr>
<td><strong>Antibiotic treatment and minimum infectious dose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSW</td>
<td>OCN008</td>
<td>$10^8$ CFU/ml of seawater</td>
<td>6/6</td>
</tr>
<tr>
<td>FSW</td>
<td>OCN008</td>
<td>$10^7$ CFU/ml of seawater</td>
<td>1/6</td>
</tr>
<tr>
<td>FSW</td>
<td>OCN008</td>
<td>$10^6$ CFU/ml of seawater</td>
<td>0/6</td>
</tr>
<tr>
<td>FSW</td>
<td>OCN008</td>
<td>$10^5$ CFU/ml of seawater</td>
<td>0/6</td>
</tr>
<tr>
<td>FSW</td>
<td>OCN004</td>
<td>$10^6$ CFU/ml of seawater</td>
<td>0/6</td>
</tr>
<tr>
<td>FSW</td>
<td>FSW</td>
<td>N/A</td>
<td>0/6</td>
</tr>
<tr>
<td>Ampicillin (150 µg/ml) and spectinomycin (100 µg/ml)</td>
<td>OCN008</td>
<td>$10^6$ CFU/ml of seawater</td>
<td>6/6</td>
</tr>
<tr>
<td>Ampicillin (150 µg/ml) and spectinomycin (100 µg/ml)</td>
<td>OCN008</td>
<td>$10^5$ CFU/ml of seawater</td>
<td>6/6</td>
</tr>
<tr>
<td>Ampicillin (150 µg/ml) and spectinomycin (100 µg/ml)</td>
<td>OCN008</td>
<td>$10^4$ CFU/ml of seawater</td>
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<td><strong>Infection rate of genetically tagged strains</strong></td>
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LITERATURE CITED


