IDENTIFICATION AND PATHOGENICITY TEST OF BACTERIAL SYMBIONTS FROM AN ENTOMOPATHOGENIC *OSCHEIUS*

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE IN

TROPICAL PLANT PATHOLOGY

JULY 2018

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ACKNOWLEDGMENT

I would like to thank the Almighty Allah for His blessings through the research project and protecting me to the successful completion of the research. My deep and sincere gratitude goes to the research committee that has guided me throughout this work. Specifically, I would like to appreciate my advisor, Dr. Brent S. Sipes for helping, encouraging, and being patient with me throughout the stages. I have been greatly inspired by his motivation, sincerity, dynamism, and commitment to research and educational programs. It was a privilege and honor to study and work under his guidance. He is indeed my role model and I look forward to working with him as I advance my career.

My appreciation also goes to Dr. Koon-Hui Wang and Dr. Zhiqiang Cheng for their invaluable input in my research project. I would also wish to thank the laboratory members – Donna Meyer and Garth Nagai and who were there to help me in the laboratory work. You have all been supportive of my career goals and vision and have been active in providing with the opportunity and facilities to pursue the goals and complete the thesis.

Nobody has been of more importance in the pursuit of this research project that my family members. There have been there for me, providing the required moral and financial support to ensure I complete my degree. Specifically, I thank my father, Ali A. Alhussaini, and my mother, Huda M. Alhussaini, for their love and guidance in whatever I pursue. My appreciation also goes to my brother, Abdullah A. Alhussaini, who has been listened and encouraged me throughout my studies and research. My special thanks also go to my friends and research colleagues. Specifically, I would wish to extend my appreciation to Firas Ahmed, Phillip Waisen, Ismet Acar, Shavale Boone, Ritz Gosai and Adriana Larrea. Their support and motivation during my research were immeasurable. They are a definition of what a true

friendship can be. I am grateful for the assistance in the field work and motivation that propelled me to complete the project.

I also extend my sincere gratitude to the University of Hawai‘i at Mānoa for the opportunity they accorded me to pursue my Master of Science degree and thesis work. The research project department was very supportive of the research project, providing the necessary materials and guidance that I needed for the project. I thank the entire academic staff in my department for their kindness and support in the thesis. Finally, I would wish to thank the government of Saudi Arabia under the Saudi Arabian Cultural Mission (SACM) and the King Saud University for the sponsorship they offered in support of my post-graduate degree program. The courses and project could not have been a success without your financial assistance. I am glad to have successfully completed my Master of Science degree and making my dream of advancing my post-undergraduate studies a reality.

ABSTRACT

The main objectives of this thesis research were to examine *Oscheius* nematodes for its 1) associated bacteria (to be identified), 2) associated bacteria pathogenicity on insects, and 3) status as entomopathogenic nematodes. Nematodes are unsegmented pseudocoelomic roundworms that exist in all environments and occupy all trophic levels including of bacterial-feeders, predators, and parasites. *Oscheius* spp. are clearly bacterial-feeding nematodes but some isolates have behaviors with entomopathogenicity. A series of tests were conducted to isolate, identify, and determine insect toxicity of bacteria associated with several Hawaii isolates of *Oscheius* sp. Bacteria were isolated from *Oscheius* isolates BI 1a, BI 12a, OJ 4a, OJ 5b and compared with that of a well-recognized entomopathogenic nematode, *Steinernema feltiae* MG14. Eleven bacterial isolates were identified and isolated from four different *Oscheius* populations through DNA sequencing. *Enterobacter* genus was most commonly isolated followed by *Pseudomonas* and *Enterococcus*. Pathogenicity of *Serratia* sp., *Enterococcus* sp., and *Pseudomonas* sp. isolated were evaluated on mealworm (*Tenebrio molitor*) larvae using two assays: 1) feeding assay on a bacteria-inoculated diet, or 2) directly assay by injecting the bacteria into the hemolymph. All assays showed that these three bacteria isolates were pathogenic to the mealworms within 48 hour after the feeding or injection. Among the three bacteria isolates tested, *Pseudomonas* was more lethal to mealworms than *Serratia* or *Enterococcus* in both assays. Injection the bacteria at 10^8 cfu/ml increased their pathogenicity compared to 10^2-10^6 cfu/ml regardless of the bacteria species. The association between the entomotoxic bacteria and the nematode confirms the entomopathogenic nature of these isolates of *Oscheius* from Hawaii. The implication of the findings from this study showed that *Oscheius* indigenous to Hawaii can offer effective biological control against some

insects without having to go through importation regulation and would be a good alternatives to conventional insecticides that are harmful to the environment.

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CHAPTER 1

ENTOMOPATHOGENIC NEMATODES

INTRODUCTION

Nematodes are unsegmented roundworms found in all the environments (Ingham, usda.gov). Nematodes occupy all trophic levels including that of parasites (first trophic level), fungal and bacterial-feeders (second trophic level), and predators (higher trophic levels) (Ruan *et al.,* 2013). Some nematode species cause life-threatening diseases of plants and animals (Lapage, 1938; Zuckerman *et al.,* 1971). According to Koenning *et al.* (1999), plant-parasitic nematodes alone cause over $US100 billion in damage worldwide to the agricultural industry. The most affected areas include subsistence farming, forestry, field and truck crops, ornamentals, and turf or an annual 12.3% reduction in yield (Sasser and Freckman, 1987; Koenning *et al.,* 1999). However, not all parasitic nematodes are undesirable; some, such as insect parasitic nematodes, effectively serve as biological pest control agents (Grewal *et al.,* 2005). Insect parasitic nematodes kill, sterilize, or seriously interfere with the insect host's development (Poiner, 1979).

Entomopathogenic nematodes (EPNs) are one group of insect parasitic nematodes of particular interest. According to Lacey and Georgis (2012), the word entomopathogenic is derived from the Greek *entomon* meaning insect and *pathogenic* meaning disease. EPN are obligate or facultative parasites on insects associated with mutualistic bacteria. EPN infect the hemocoel system of the host insect and eventually kill the insect (Kaya and Gaugler, 1993).EPNs occur in several different nematode families – but are predominately found in the Heterorhabditidae and the Steinernematidae (Poiner, 1979). Even though both of these families belong to the same order, Rhabditida, the families are not closely related. This significant observable distinction suggests

that the mutualistic insect parasitism arose independently on at least two occasions (Van Megan *et al.,* 2009).

Morales *et al.* (2016) states that Steinernematids were the first EPNs to be discovered. Steiner described *Steinernema kraussei* in 1923 and placed it in the family Steinernematidae. Currently, 95 species of *Steinernema* are recognized (Steiner, 1923; Morales *et al.,* 2016). The species are differentiated morphologically based on characteristics of the male tail, the infective stage juveniles (IJ), and through a biological species approach (Poinar and Veremtschuk, 1970; Poinar, 1986, 1990). Steinernematids generally reproduce sexually (Grewal *et al.,* 2005). A mutualistic association exists between all *Steinernema* and the bacteria *Xenorhabdus* spp. The bacteria symbiont is attached to the IJ, and upon IJ entry into the insect, the bacteria proceed to cause septicemia in the insects (Akhurst and Boemare, 1990; Boemare, 2002). The EPNs feed on both the bacterial tissues and the insect hemolymph. The infected insect cadavers become brown or tan due to the effects of the toxin secreted by *Xenorhabdus* (Kaya and Gaugler, 1993).

The second family of EPN, however, was not discovered until a quarter century after *Steinernema*. In 1975, Poinar reported on a new EPN family infecting *Heliothis punctigera* (Australian bollworm) in South Australia (Poinar, 1975). Poinar named this new family Heterorhabditidae. Moreover, Poinar identified the symbiotic bacterium of *Heterorhabditis* as *Photorhabdus* (Boemare *et al.,* 1993). Today, *Heterorhabditis* includes 18 species (Morales *et al.,* 2016). *Heterorhabditis* has a unique dorsal "hook" on the tip of the head. This structure allows the nematode to penetrate the insect body through the outer integument, through the trachea, and through the gut wall (Bedding and Molyneux, 1982; Poinar and Georgis, 1990). *Heterorhabditis* nematodes are hermaphroditic in general (Grewal *et al.,* 2005). In insects killed by *Heterorhabditis* sp., the cadaver becomes red (Kaya and Gaugler, 1993). *Photorhabdus* can fluoresce to such an

extent that the entire insect cadaver glows in the dark (Poinar *et al.*, 1980).Several studies have produced findings that aid in better understanding of nematode parasites of insects. Nematodes have undergone evolutionary changes to enhance their survival involving insects (Shapiro-Ilan *et al.*, 2006). The evolutionary stages consist of phases such as free-living, phoresy, necromeny, parasitism, and entomopathogeny (Gulcu *et al.*, 2012). The free-living stage is where microbotrophic nematodes only associate transiently with insects, such as *Rhabditis orbitalis* on muroid rodents (Schulte and Poinar, 1991). Phoresy is the relationship where nematodes adapt to using insects as means of dispersal and/or shelter; examples include the plant parasite *Bursaphelenchus* associates of burying beetles (*Nicrophorus* spp.) (Richter, 1993). The phoresy phase does not exhibit any nutritional relationship between the nematodes and insects. The third evolutionary phase is known as necromeny, the point where nematodes are morphologically adapted to feed saprophytically on the insect cadaver. An example of a necromenic nematode is *Pristionchus* on beetles. Necromenic nematodes do not the cause insect death. The final phase in the evolution entails advanced levels of necromeny – parasitism and entomopathogeny (Shapiro-Ilan *et al.*, 2006). In parasitism, the nematode infects and derives nutrients from the insect while the insect lives. The parasitic nematode may kill or simply injure the insect without causing its death. The ultimate result of nematode parasitism on insects depends on the virulence of the nematode towards the insect host. Entomopathogeny is the most advanced evolutionary stage. In entomopathogenicity, the nematode, specifically the infective juveniles, invade and rapidly kill the insect with the aid of bacteria (Gulcu *et al.*, 2012).

EPNs share similar life cycles. The free-living stage is the third-stage juvenile (J3), a dauer stage, also called the infective juvenile. The IJ invades the host insect through the spiracles, mouth, excretory system, or intersegmental membranes of the cuticle, and then moves into the hemocoel

(Bedding and Molyneux, 1982). A mutualistic bacterium is associated with a particular species of EPN. The bacteria are released from the IJ into the insect hemocoel. In the insect hemolymph, the bacteria multiply and cause septicemia within 24 to 72 hours (Smart, 1995). The nematodes feed on both the bacteria and host tissues. Nematode development continues to maturity and reproduction ensues. The availability of resources from the insect cadaver controls the number of EPN generations. Usually, 1 to 3 generations take place within the insect host cadaver after which the J3 enter into the dauer IJ and leave the insect cadaver to locate another insect (Kaya and Gaugler, 1993; Gulcu *et al*., 2012).

Two symbiotic bacteria are associated with *Heterorhabditis* and *Steinernema*, *Photorhabdus* and *Xenorhabdus,* respectively. These bacteria are phylogenetically related gammaproteobacteria (Akhurst and Boemare, 1990). Both bacteria infect a comparable range of insect hosts, however, each bacteria partner with an EPN from a different family. The bacteria provide advantageous commitments to the nematode by initiating disease in the insects, safeguarding the insect cadaver from predators and secondary invaders, and serving as food for the nematode (Goodrich-Blair and Clarke, 2007). The cooperation amongst EPNs and their bacterial microbes is mutualistic and symbiotic. The EPN bacteria produce antibiotics that prevent the reproduction of other pathogenic microorganisms on the insect cadaver and the nematode ensures that the bacteria find new hosts (Dillman *et al.,* 2012).

In the last 5 years, nematode species in the genus *Oscheius,* belonging to the Rhabditidae, have been recognized as having EPN characteristic (Dillman *et al.,* 2012). *Oscheius* has been considered a bacterial feeding nematode with necromantic behavior. The necromantic behavior, for which *Oscheius* is historically known, is now viewed as in a transition stage from a free-living stage with bacterial feeding behavior to an entomopathogenic behavior (Dillman *et al.*, 2012).

Oscheius chongmingensis and *O. carolinensis* have been classified as EPNs (Zhang *et al.,* 2008; Ye *et al.,* 2010; Torres-Barragan *et al.,* 2011; Dillman *et al.,* 2012).

Aside from the behavior of an *Oscheius* sp. found during EPN surveys in Hawaii, Myers and Bisel also confirm the same characteristic of an EPN (Myers *et al.,* 2015; Bisel, 2016). The *Oscheius* isolated in Hawaii was verified to behave like an EPN through Koch's Postulates. *Oscheius* infected mealworm (*Tenebrio molitor*) larvae. The insect cadavers turn dark brown and remain moist. The cadavers are full of lipids while retaining their original shape. These phenomena are similar to characteristics induced by other EPN mutualistic bacteria. Thus, Myers and Bisel concluded that this Hawaiian isolate of *Oscheius* is likely entomopathogenic (Myers *et al.,* 2015; Bisel, 2016).

EPNs are appropriate for IPM programs (Integrated pest management) because EPNs are specific to their target pest(s), beneficial as a biological control agent, and can be applied using standard spray equipment (Shapiro-Ilan *et al.,* 2006). Over 200 species of insects from several orders can be infected by steinernematid and heterorhabditid (Georgis and Manweiler, 1994). Therefore, the addition of a third family of EPNs holds promise of increasing the number of insect species susceptible to EPN as well as providing an additional tool for IPM.

OBJECTIVES

The knowledge gap of *Oscheius* from Hawaii is the understanding of its associated symbiotic bacteria. The specific objectives of this research were to:

1. Identify the symbiotic bacteria from *Oscheius* isolates from Hawaii; and
2. Determine the pathogenicity of the bacteria to insect larvae.

REFERENCES

Akhurst, R.J, and Boemare, N.E. 1990. Biology and taxonomy of *Xenorhabdus,* pp. 75–90 in R. Gaugler, and H. K. Kaya, eds. Entomopathogenic Nematodes in biological control. Boca Raton: CRC Press.

Bedding, R.A, and Molyneux, A.S. 1982. Penetration of insect cuticle by infective juveniles of *Heterorhabditis* spp. (Heterorhabditidae: Nematode). Nematologica 28:354–359.

Bisel, J. 2016. Potential of Endemic Entomopathogenic Nematodes against Emerging Insect Pests in Hawai‘i. Master of Science Thesis. University of Hawaii at Manao.

Boemare, N.E. 2002. Biology, taxonomy, and systematics of *Photorhabdus* and *Xenorhabdus*. pp. 35–56. In R. Gaugler, ed. Entomopathogenic Nematology. Wallingford, UK: CABI Publishing.

Boemare, N., Akhurst, R., and Mourant, R. 1993. DNA Relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. International Journal of Systematic Bacteriology 43:249-255.

Dillman, A.R., Guillermin, M.L., Lee, J.H., Kim, B., Sternberg, P.W., and Hallem, E.A. 2012. Olfaction shapes host-parasite interactions in parasitic nematodes. Proceedings of the National Academy of Science 108:E2324-E2333.

Elaine R. Ingham. Soil nematodes. Available online: https://www.nrcs.usda.gov/wps/portal/nrcs/detailfull /soils/health/biology/?did=nrcs142p2_053866. (Accessed 11 December 2017).

Georgis, R., and Manweiler, S.A. 1994. Entomopathogenic nematodes: a developing biological control technology. Agricultural Zoology Review 6:63–94.

Goodrich-Blair, H. and Clarke, D.J. 2007. Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: Two roads to the same destination. Molecular Microbiology 64:260-268.

Grewal, P.S., Ehlers R.-U., and Shapiro-Ilan, D.I. 2005. Nematodes as biocontrol agents. Wallingford, UK; Cambridge, MA: CABI Pub.

Gulcu, B., Hazir, S., and Kaya, H. 2012. Scavenger deterrent factor (SDF) from symbiotic bacteria of entomopathogenic nematodes. Journal of Invertebrate Pathology 110:326-333.

Kaya, H.K. and Gaugler, R. 1993. Entomopathogenic nematodes. Annual Review of Entomology 38: 181-206.

Koenning, S.R., Overstreet, C. Noling, J.W., Donald, P.A. Becker, J.O., and Fortnum, B.A. 1999. Survey of crop losses in response to phytoparasitic nematodes in the United States. Supplement to Journal of Nematology 31:587–618.

Lacey, L.A. and Georgis, R. 2012. Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. Journal of Nematology 44:218–225.

Lapage, G. 1938. Nematodes parasitic in animals (Methuen's monographs on biological subjects). New York: Chemical pub.

Morales, N., Morales-Montero, P., Puza, V., and San-Blas, E. 2016. The first report of *Heterorhabditis amazonensis* from Venezuela and characterization of three populations. Journal of Nematology 48:139–147.

Myers, R.Y., Sipes, B.S., Matsumoto Brower, T.K., Mello, C.L., and Mello, J.S. 2015. Occurrence and distribution of Heterorhabditid populations in the Hawaiian Islands. Nematropica. 45:198-207.

Poinar, G.O., Jr. 1990. Taxonomy and biology of Steinernematidae and Heterorhabditidae. Pg. 23–61. in R. Gaugler and H. K. Kaya, eds. Entomopathogenic Nematodes in Biological Control. Boca Raton. Florida: CRC Press.

Poinar, G.O., Jr. 1986. Recognition of *Neoaplectana* species (Steinernematidae: Rhabditida). Proceedings of the Helminthological Society of Washington 53:121–129.

Poinar, G. 1979. Nematodes for biological control of insects. Boca Raton, Fla.: CRC Press.

Poinar, G.O., Jr. 1975. Description and biology of a new insect parasitic rhabditoid, *Heterorhabditis bacteriophora* n. gen.., n. sp. (Rhabditida: Heterorhabditidae n. fam.). Nematologica 21:463–470.

Poinar, G.O., Jr., and Georgis, R. 1990. Characterization and field application of *Heterorhabditis bacteriophora* strain HP88 (Heterorhabditidae: Rhabditida). Reveu de Nematologie 13:387–393.

Poinar, G.O., Jr., Thomas, G., Haygood, M., and Nealson, K.H. 1980. Growth and luminescence of the symbiotic bacteria associated with the terrestrial nematode, *Heterorhabditis bacteriophora*. Soil Biology and Biochemistry 12:5–10.

Poinar, G.O., Jr., and Veremtshuk, G.V. 1970. A new strain of entomopathogenic nematodes and geographical distribution of *Neoaplectana carpocapsae* Weiser (Rhabditida, Steinernematidae). Zoological Journal 49:966–969.

Richter, S. 1993. Phoretic association between the dauerjuveniles of Rhabditis Stammeri (Rhabditidae) and life history stages of the Burying Beetle Nicrophorus Vespilloides (Coleoptera: Silphidae). Nematologica 39:346-355.

Ruan, Ren, Chen, Zhu, and Wang. 2013. Effects of conventional and reduced N inputs on nematode communities and plant yield under intensive vegetable production. Applied Soil Ecology 66:48-55.

Schulte, F. and Poinar, G. O. 1991. On the geographical distribution and parasitism of *Rhabditis (Pelodera) orbitalis* (Nematoda: Rhabditidae). Journal of the Helminthological Society of Washington 58:82–84.

Sasser, J.N. and Freckman, D.W. 1987. A world's perspective on nematology: The role of the society. pp. 7–14. In Vistas on Nematology, J.A. Veech and D.W. Dickson, eds. Society of Nematologists, Hyattsville, MD.

Shapiro-Ilan, D.I., Gouge, D.H., Piggott, S.J., and Patterson Fife, J. 2006. Application technology and environmental considerations use of entomopathogenic nematodes in biological control. Biological Control 38:124-133.

Smart, G. 1995. Entomopathogenic nematodes for the biological control of insects. Journal of Nematology 27(4S):529-34.

Steiner, G. 1923. *Aplectana kraussei* n. sp., einer in der Blattwespe *Lyda* sp. parasitierende Nematodenform, nebst Bemerkungen über das Seitenorgan der parasitischen nematoden. Zentralblatt fuer Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene 59:14–18.

Torres-Barragan, A., Suazo, A., Buhler, W.G., and Cardoza, Y.J. 2011. Studies on the entomopathogenicity and bacterial associates of the nematode *Oscheius carolinensis*. Biological Control 59:123-129.

Van Megen, H., van den Elsen, S., Holterman, M., Karssen, G., Mooyman, P., Bongers, T., Holovachov, O., Bakker, J., and Johannes Helder. 2009. A phylogenetic tree of nematodes

based on about 1200 full-length small subunit ribosomal DNA sequences. Nematology 11:927-950.

Ye, W.M., Torres-Barragan, A., and Cardoza, Y.J. 2010. *Oscheius carolinensis* n. sp. (Nematoda: Rhabditidae), a potential entomopathogenic nematode from vermicompost. Nematology 12:121-135.

Zhang, C., Liu, J., Sun, J., Yang, S., An, X., Gao, G., Lin, M., Lai, R., He, Z., and Wu, Y. 2008. *Heterorhabditoides chongmingensis* gen. nov., sp. nov (Rhabditida: Rhabditidae), a novel member of the entomopathogenic nematodes. Journal of Invertebrate Pathology 98:153-168.

Zuckerman, B., Mai, W. F, and Rohde, R.A. 1971. Plant parasitic nematodes. New York: Academic Press.

CHAPTER 2

IDENTIFICATION OF BACTERIA ASSOCIATED WITH *OSCHEIUS*

INTRODUCTION

Intimate relationships between microbes and eukaryotes exists in almost all ecological niches. Such a spectrum of interactions varies in complexity. Some associations exhibit a high level of obligatory symbioses whereas others are loosely held (Dillman *et al.,* 2012). Microbial symbiotic relationships are naturally part of ecosystems. Researchers have, therefore, developed interest of gaining insights into the fundamental processes and the role of symbiosis, especially the association between the eukaryotes and prokaryotes (Yedid, 2016). The diversity of symbioses between nematodes and bacteria, makes an outstanding model for understanding the critical aspects of such feeding relationships.

Entomopathogenic nematodes such as *Steinernema* and *Heterorhabditis* typically have mutual relationships with a particular strain of bacteria that facilitates successful infection of the host (Heve *et al*., 2016). Ulug *et al*. (2015) explain that symbiotic bacteria are carried by infective juveniles (IJ), the stage of EPNs that survive in the external environment without feeding for an extended period. The bacteria acquire protection and shelter in the body of the IJ during the time the IJs are outside an insect. After gaining entry into the insect body, the IJs release symbiotic bacteria into the insect hemocoel (Gulcu *et al*., 2012). The bacteria then undergo rapid multiplication and release toxins and hydrolytic enzymes which in turn damages the systems of the insect and kills the insect within 24 hour (Ulug *et al*., 2015). The IJs molt to a fourth-stage juvenile (J4) and feed on the damaged tissues of the cadaver and bacteria. This normal life cycle continues until cues trigger the development of IJ (Laznik and Trdan, 2015). Subsequently, IJ and bacteria re-associate (Laznik and Trdan, 2015).

The Heterorhabditidae and Steinernematidae exhibit a symbiotic relationship with the bacterial genera *Xenorhabdus* and *Photorhabdus* respectively (Laznik and Trdan, 2015). By 2013, 25 species and 85 bacterial strains of *Xenorhabdus* had been identified. At the same time, three species (*P. luminescens*, *P. temperate*, and *P. asymbiotica*) and 58 strains of bacteria in the genus *Photorhabdus* have been associated with *Heterorhabditis* (Yedid, 2016).

Nematodes in the *Oscheius* genus are parasitic nematodes that prey on insects and slugs (Ye *et al.,* 2010). Agricultural experts have viewed *Oscheius* as possible biological control agents. *Oscheius* belongs to the Rhabditidae family. The phylogenetic trees based on the sequence of the internal transcribed spacer (ITS) and rDNA breakdown the *Oscheius* nematodes into three groups – the *Dolichura*, the *Insectivore,* and the *Heterorhabditidoides* groups (Serepa and Gray, 2014). According to Huang *et al.* (2015), some pathogenic nematodes can be found in the *Insectivore* group of *Oscheius.* These *Oscheius* are found in the soil and exhibit parasitic nutritional dependence on insects. The objective of this research was to identify the bacteria associated with an *Oscheius* species commonly isolated from Hawaii.

MATERIALS AND METHODS

Oscheius cultures. Cultures of *Oscheius* isolates BI 1a, BI 12a, OJ 4a, and OJ 5b were maintained in the lab which they were isolated from a statewide EPN survey near the coast lines of all the islands in Hawaii (Myers *et al.*, 2015; Bisel, 2016). Cultures were renewed weekly. *Oscheius* were inoculated onto Whatman #1 filter paper placed in 100-mm petri dishes. Ten mealworm larvae (*Tenebrio molitor*) were placed on top of the filter paper and the dish was covered. Forty-eight hours later, dead larvae were collect and placed on modified White traps (Kaya and Stock, 1997). Infective nematodes were collected 5-4 days later, counted, adjusted to 2000 nematodes/ml water, and stored in 100-mm petri plates sealed with parafilm. Cultures were

maintained in an incubator at 15℃. Cultures of *Steinernema feltiae* MG14 were maintained following similar procedures.

Tenebrio molitor. Mealworm was used as a test organism due to its susceptibility to most of EPN, ease of culture, wide availability, and ability to provide high EPN yields (Blinova and Ivanova, 1987; Shapiro-Ilan *et al.,* 2002). Larvae were purchased from pet stores and held at 15±2°C at 60±5% humidity in the dark until used in experiments. The larvae were provided a diet of Weetabix®, rolled oats, and apple slices.

Bacteria isolation from nematodes. An aliquot of 100 nematodes from freshly harvested cultures of *Oscheius* and *S. feltiae* were washed three times with sterile distilled water. A 10 µl drop of water that contain two to three nematodes was pipetted onto a clean glass microscope slide. Nematodes were fragmented by using a Roboz surgical micro 45° angle tool (model number RS-9421-06) with the aid of a dissecting microscope (Wild Heerbrugg, Heerbrugg, Switzerland). Water drops containing the fragmented nematodes were plated on TZC agar (Tripheny tetrazolium chloride) (Kelman, 1954), one agar plate for each one of the four *Oscheius* isolates and one *S. feltiae*. The plates were incubated in a BOD incubator at 28±2°C for 24 h. After 24 h, bacteria colonies were taken and subcultured repeatedly every 24 h on TZC medium until pure cultures or individual colonies were obtained.

Bacteria isolation from mealworm hemolymph. Larvae of *T. molitor* were individually infected with *Oscheius* isolates BI 1a, BI 12a, OJ 4a, OJ 5b, and *S. feltiae* MG14. Five larvae were placed into a sterilized petri plate (100 mm × 15 mm) containing a Whatman #1 filter paper on the top and bottom moistened with 50 µl of nematode suspension (about 1000 IJs or nematodes) and 3 ml of distilled water for each isolate individually (Mohammed *et al.,* 2012). The petri dishes were incubated at 25°C in the dark and observed every 24 h for insect mortality. The filter paper

was rehydrated as needed. The larvae were identified as dead or live by macroscopic examination. Larval cadavers were surface sterilized with a dip in 70% ethanol, rinsed three times in sterile distilled water, and placed in sterile dish in a laminar air flow chamber to dry. Thereafter, each cadaver from each EPN isolate was placed into a sterilized 1.5 ml tube and crushed with a sterile pestle (Iskender *et al.,* 2017). Each suspension was diluted from 10^{-1} to 10^{-8} and streaked on petri plates containing TZC. Plates were placed in a BOD incubator at 28 ± 2°C for 24 h. Bacterial colonies were subcultured repeatedly until pure cultures were obtained.

Extraction and sequencing of bacterial DNA. Bacterial isolates were plated onto TZC medium and the plates incubated for 18 h at 30ºC before DNA extraction. Bacterial cells were collected from the culture medium with a sterile loop. A DNA extraction kit (Promega, Madison, WI) was used to isolate the DNA according to the manufacturer's protocols. The DNA was quantified with a NanoDrop™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The forward primer fD1 (5′AGAGTTTGATCCTGGCTCAG3′) and the reverse primer rP2 (5′CGGCTACCTTGTTACGACTT3′) were used to amplify the 16S rRNA genes of the bacteria (Weisburg *et al.,* 1991). The PCR conditions were (T100™ Thermal Cycler, Bio-Rad, Hercules, CA, USA) 5 min initial denaturation at 94ºC; 35 cycles of denaturation (20 s at 94ºC), annealing (30 s at 85ºC) and extension (1 min at 72ºC) and a final extension at 72ºC for 3 min. After amplification, a 10 µl aliquot of the PCR product was loaded onto a 1.5% agarose gel containing 5 µl ethidium bromide, electrophoresed at 60 volts for 45 min, and then visualized under ultraviolet light. The remaining 25 µl of each PCR product was purified with ExoSAP-IT® (USB Products Affymetrix, Cleveland, OH). The sequence of the PCR products were obtained from GENEWIZ Laboratory (La Jolla, CA). The sequences were aligned using Genious (Biomatters Auckland, New Zealand), and compared with sequences from the National Center for Biotechnology Information

(NCBI) GenBank database by using the Basic Local Alignment Search Tool (BLAST) for determining the identity of the isolates. Isolation of bacteria for the nematode and sequencing of the isolated bacteria were conducted twice.

RESULTS

All *T. molitor* larvae inoculated were infected by the nematode isolate inoculated. Morbid *T. molitor* larvae were moist, retained their original shape and were filled with lipids. *T. molitor* larvae underwent a color change to dark brown or black.

Bacteria isolation from nematodes. Bacteria isolated from the nematodes differed by color and morphology. Based on color and morphology, 13 bacteria colonies were isolated from different nematodes. In BI 1a, three different colonies were isolated (BI1a-1, BI1a-2, and BI1a-3). In BI 12a, two colonies were isolated (BI12a-1, and BI12a-2). Six colonies were isolated from OJ 4a (OJ4a-1, OJ4a-2, OJ4a-3, OJ4a-4, OJ4a-5, and OJ4a-6). Only a single colony type was isolated from OJ 5b (OJ5b-1) and MG14 (Table 1.1) (Figure 1.1).

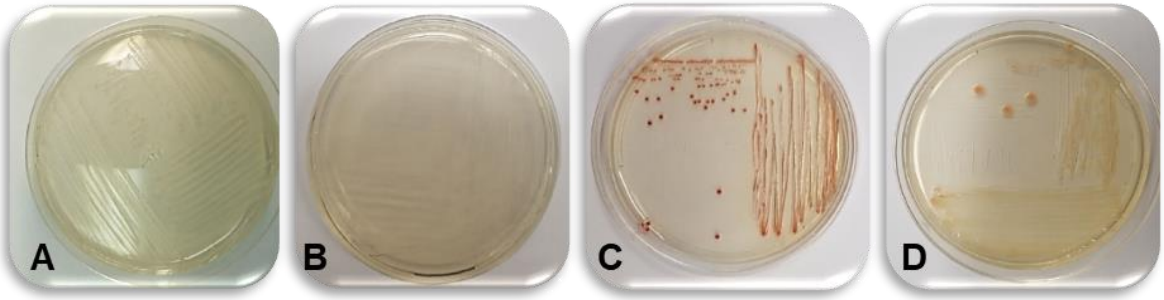

Figure1.1 Different colors of bacterial colonies isolated from *Oscheius* on TZC medium: (A) White, (B) Off-white, (C) Red, and (D) Yellow.

Bacteria isolation from mealworm hemolymph. Based on color and morphology, 16 different isolates were collected from mealworm hemolymph. Insects killed by BI 1a produced three colonies (BI1a-1h, BI1a-2h, and BI1a-3h). Four colonies were isolated from BI 12a infected larvae (BI12a-1h, BI12a-2h, BI12a-3h, and BI12a-4h). Seven different isolates were found in OJ 4a (OJ4a-1h, OJ4a-2h, OJ4a-3h, OJ4a-4h, OJ4a-5h, OJ4a-6h, and OJ4a-7h). Larvae killed by OJ 5b (OJ5b-1h) and MG14 produced only a single colony each (Table 1.1).

Sequencing of bacterial DNA. Twenty nine different bacterial isolates were found and sequenced. Twelve different bacterial species were identified from the 29 isolates sequenced. Based on 16S rDNA sequencing and BLAST, the identity of all bacterial species were 99% or 90% when compared to the GenBank database, and the query coverage in all isolates were ≥99% (Table 1.2).

From the insect hemolymph, *Pseudomonas* sp. was identified in isolates BI1a-1h, BI12a-2h and OJ5b-1h. Bacteria isolated from BI1a-2h, OJ4a-3h, OJ4a-5h, OJ4a-6h, BI12a-3h and BI12a-4h were identified as *Enterobacter* sp. Bacterial isolates OJ4a-4h, OJ4a-7h, and BI12a-1h carried an *Enterococcus* sp. A *Citrobacter* sp. was identified from hemolymph isolates OJ4a-1h and OJ4a-2h.

Several bacterial species were isolated from nematode. Nematode isolate BI1a-1 was the only nematode to be associated with a *Bacillus* sp. OJ4a-1 was the only nematode associated with *Delftia* sp. A *Chryseobacterium* sp. was identified associated with OJ4a-3 and an *Elizabethkingia* sp. associated with OJ4a-4. The BI12a-1 was associated with *Enterococcus* sp. *Ochrobactrum* sp. was identified in BI1a-2 and BI12a-2. A *Stenotrophomonas* sp. was found in association with OJ4a-2 and OJ4a-6. Both OJ5b-1 and OJ4a-5 were identified as *Pseudomonas* sp.

Table 1.1 Different colors of bacterial colonies isolated from *Oscheius* isolates and *Steinernema feltiae* MG14 from the nematode and from an infected insect hemolymph.

Isolate	Color of the colony		
	Nematode	Isolate	Insect hemolymph
BI1a-1	Off-white	BI1a-1h	Off-white
BI1a-2	White	BI1a-2h	White
BI1a-3	Red	BI1a-3h	Red
BI12a-1	Yellowish	BI12a-1h	Yellowish
BI12a-2	White	BI12a-2h	Off-white
-	-	BI12a-3h	White
-	-	BI12a-4h	Off-white
OJ4a-1	Off-white	OJ4a-1h	Off-white
OJ4a-2	Off-white	OJ4a-2h	White
OJ4a-3	Off-white	OJ4a-3h	Off-white
OJ4a-4	Off-white	OJ4a-4h	Yellowish
OJ4a-5	White	OJ4a-5h	White
OJ4a-6	Off-white	OJ4a-6h	Off-white

Table 1.2 Identification of bacterial species based on sequencing of 16S rDNA. The bacteria were isolated from entomopathogenic *Oscheius* and *Steinernema feltiae* MG14 from the nematode and from an infected insect hemolymph nematodes. The available accession number in GenBank database is shown.

Isolate	Nematode			Isolate	Insect hemolymph		
	Bacterial genus	Identity	Accession number		Bacterial genus	Identity	Accession number
BI1a-1	*Bacillus* sp.	99%	MF418040.1	BI1a-1h	*Pseudomonas sp.*	99%	CP026675.1
BI1a-2	*Ochrobactrum* sp.	99%	KT425063.1	BI1a-2h	*Enterobacter* sp.	99%	CP010377.1
BI1a-3	*Serratia* sp.	99%	CP028946.1	BI1a-3h	*Serratia* sp.	99%	CP028946.1
BI12a-1	*Enterococcus* sp.	99%	CP027773.1	BI12a-1h	*Enterococcus* sp.	99%	MH111486.1
BI12a-2	*Ochrobactrum* sp.	99%	MH236108.1	BI12a-2h	*Pseudomonas* sp.	99%	GU991854.1
-	-	-	-	BI12a-3h	*Enterobacter* sp.	99%	KU359266.1
-	-	-	-	BI12a-4h	*Enterobacter* sp.	99%	KU359266.1
OJ4a-1	*Delftia* sp.	99%	JF309194.1	OJ4a-1h	*Citrobacter* sp.	99%	CP022273.1
OJ4a-2	*Stenotrophomonas* sp.	99%	MF170829.1	OJ4a-2h	*Citrobacter* sp.	99%	CP022273.1
OJ4a-3	*Chryseobacterium* sp.	99%	MG711812.1	OJ4a-3h	*Enterobacter* sp.	99%	KR232868.1
OJ4a-4	*Elizabethkingia* sp.	99%	CP016377.1	OJ4a-4h	*Enterococcus* sp.	99%	MH111486.1
OJ 4a-5	*Pseudomonas* sp.	99%	KU324481.1	OJ4a-5h	*Enterobacter* sp.	99%	KR232868.1
OJ4a-6	*Stenotrophomonas* sp.	99%	MG905308.1	OJ4a-6h	*Enterobacter* sp.	99%	KR232868.1
-	-	-	-	OJ4a-7h	*Enterococcus* sp.	99%	MH111486.1
OJ 5b-1	*Pseudomonas* sp.	99%	KM232740.1	OJ 5b-1h	*Pseudomonas* sp.	99%	MG855667.1
MG14	*Xenorhabdus* sp.	90%	DQ211709.1	MG14	*Xenorhabdus* sp.	90%	DQ211709.1

The isolate BI 1a from both the nematodes (BI1a-3) and insect hemolymph (BI1a-3h) was associated with the bacteria *Serratia* sp. MG14 isolate identified as expected which is *Xenorhabdus* sp. from both the nematodes and insect hemolymph. The greatest number of *Oscheius* isolates from Hawaii were associated with *Enterobacter* sp. followed by *Pseudomonas* sp. and *Enterococcus* sp.

DISCUSSION

Several species of bacteria were associated with the isolates of *Oscheius* from Hawaii. The presence of the EPNs in mealworm larval cadavers is an indication of the association of the bacteria with *Oscheius*. The *T. molitor* cadavers were moist and retained their original shape, an indication of the presence of bacteria and *Oscheius* (Blinova and Ivanova, 1987). Additionally, the change in cadaver color from tan to dark brown and black suggests the presence of bacteria. Twelve different bacterial species were associated with the different isolates. Studies have shown that the symbiotic bacteria associated with EPNs produce colonies of varied colors, such as greenish to blue green (Kaya and Stock 1997), bright red (Ortega-Estrada *et al*., 2012), bioluminescent (Babic *et al.*, 2000) and red (Zhang *et al.*, 2009) when grown on TZC media.

Our research has shown that different bacterial genera can be associated with *Oscheius* sp. Sangeetha *et al.* (2016) state that a close association exists between EPNs in Steinernematidae, Heterorhabditidae, and Rhabditidae and the bacteria that are isolated from *Oscheius* species. The association of bacteria outside the Enterobacteriaceae with *Oscheius* has been previously reported. In the Sangeetha study, sequence analysis identified 18 different bacteria associates – *Enterobacter* sp., *Proteus mirabilis, Providencia* sp., *Pseudomonas* sp., *Stenotrophomonas maltophilia* (class γ-*proteobacteria*), *Alcaligenes faecalis* (class β-*proteobacteria*), and *Bacillus cereus, Enterococcus faecalis, Lysinibacillus sphaeriscus* (class *Bacilli*). *Bacillus cereus* was associated with *Oscheius*

in work reported by Kumar *et al*. (2013). Kumar *et al*. (2014) also found *Comamonas testosteroni* associated with *Oscheius*. Serepa-Dlamini and Gray (2018) isolated both *Enterococcus* and *Acinetobacter* from *Oscheius*. Twelve different symbiotic bacteria have been isolated from *Oscheius* spp. belonging to the genera *Serratia, Acinetobacter, Bacillus, Comamonas, Stenotrophomonas, Achromobacter, Klebsiella* and *Brucellaceae* (Deepa *et al.*, 2011). Eighty percent of the bacterial isolates identified in the Hawaiian isolates of *Oscheius* belong to the Enterobacteriaceae, the same family as *Xenorhabdus* and *Photorhabdus* (Williams *et al*., 2010).

The high frequency of *Pseudomonas* sp. associated with the isolates and the bacteria's toxicity to insects suggests the association is important in the nematode-bacteria-insect interaction (Ulug and Hazir, 2015). Different species of *Pseudomonas* have been reported to carry toxin genes against insects (Chen *et al*., 2014; Péchy-Tarr *et al*., 2008; Vodovar *et al*., 2006). It is possible that the *Pseudomonas* sp. associated with the Hawaii isolates of *Oscheius* do also. Similarly, *Enterobacter* sp. occurred in high frequency and was toxic to insects. The association of *Pseudomonas* sp. and *Enterococcus* sp. with *Oscheius* suggests a useful pest control combination.

During the infection and colonization process of EPN attack, there is a cellular and molecular interface between the bacteria, the nematode, and the insect host that results in the nematode-bacterium relationships. A set of common DNA exists such as toxins, transcriptional regulators, proteases, putative membrane transporters, and genes encoding lipopolysaccharide production that kill the insect, protect the insect cadaver from other saprophytic organisms, and provide nutrition to the nematode. The process is important in ensuring a stable mutual relationship between the bacteria. Deepa *et al.* (2011) molecularly characterized EPN through the novel isolation and characterization of bacteria. Their findings are consistent with the results from the present study that confirms the existence of the bacteria in *Oscheius* from Hawaii. Moreover, the

study shows that there is a relatively lower similarity (90%) among the bacterial species an indication of the presence of different bacteria. According to Poretsky *et al*. (2014) the similarity or identity of bacterial isolates is effected by the limitation of the databases used for sequence comparisons. Deepa *et al.* (2011) proposed three major EPN clades from the isolation process. These EPN clades include the bacteriophora-argentinensis-hepialius group (Clade I), the baujardi-sonorensis-amazonensis group (Clade II), and the indica-brevicaudis-hawaiiensis group (Clade III). A similar scheme could be used to group isolates of *Oscheius* in the future.

REFERENCES

Babic, I., Fischer-Le Saux, M., Giraud, E., and Boemare, N. 2000. Occurrence of natural dixenic associations between the symbiont *Photorhabdus luminescens* and bacteria related to *Ochrobactrum* spp. in tropical entomopathogenic *Heterorhabditis* spp. (Nematoda, Rhabditida). Microbiology 146:709-18.

Bisel, J. 2016. Potential of Endemic Entomopathogenic Nematodes against Emerging Insect Pests in Hawai‘i. Master of Science Thesis. University of Hawaii at Manao.

Blinova, S.L., and Ivanova, E.S. 1987. Culturing the nematode-bacterial complex of *Neoaplectana carpocapsae* in insects. Pp. 22–26 *in* M. D. Sonin, ed. Helminths of Insects. New Delhi: American Publishing Co.

Chen, W.J., Hsieh, F.C., Hsu, F.C., Tasy, Y.F., Liu, J.R. and Shih, M.C. 2014. Characterization of an insecticidal toxin and pathogenicity of *Pseudomonas taiwanensis* against insects. PLoS Pathogens 10:e1004288.

Dillman, A., Chaston, J., Adams, B., Ciche, T., Goodrich-Blair, H., Stock, S. and Sternberg, P. 2012. An entomopathogenic nematode by any other name. PLoS Pathogens 8:e1002527.

Deepa, I., Mohandas, C., and Siji, J.V. 2011. Molecular characterization of novel symbiotic bacteria from entomopathogenic nematodes. In: National Seminar on Climate Change and Food Security January 20– 22:399–404.

Gulcu, B., Hazir, S., and Kaya, H. 2012. Scavenger deterrent factor (SDF) from symbiotic bacteria of entomopathogenic nematodes. Journal of Invertebrate Pathology 110: 326-333. doi: 10.1016/j.jip.2012.03.014.

Heve, W., El-Borai, F., Carrillo, D., and Duncan, L. 2016. Biological control potential of entomopathogenic nematodes for management of Caribbean fruit fly, *Anastrepha suspensa* Loew (Tephritidae). Pest Management Science 73:1220-1228. doi: 10.1002/ps.4447.

Huang, R., Ye, W., Ren, X., and Zhao, Z. 2015. The morphological and molecular characterization of Nematoda: Rhabditidae, a new rhabditid nematode from South China. PLOS ONE 10:e0144386. doi: 10.1371/journal.pone.0144386.

Iskender, N., Algur, O., Aksu, Y., and Saral, A. 2017. Isolation, identification and characterization of biotechnologically important bacteria from microflora of *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae). Biotechnology and Biotechnological Equipment 31:505-510.

Kaya, H.K., and Stock, S.P. 1997. Techniques in insect nematology. Pp. 281-324 *in* L.A. Lacey, ed. Manual of Techniques in Insect Pathology. London: Academic Press.

Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.

Kumar, Nishanth, Mohandas, C., Nambisan, Bala, Kumar, D., and Lankalapalli, Ravi. 2013. Isolation of proline-based cyclic dipeptides from *Bacillus* sp. N strain associated with

rhabitid entomopathogenic nematode and its antimicrobial properties. World Journal of Microbiology and Biotechnology 29:355-364.

Kumar, S., Mohandas, C., Nambisan, B., Sreerag, R., and Jayaprakas, C. 2014. Cyclo(L-Pro-D-Arg): A new antibacterial and antitumour diketopiperazine from *Bacillus cereus* associated with a rhabditid entomopathogenic nematode. Folia Microbiologica 59:197-202.

Laznik, Ž. and Trdan, S. 2015. Attraction behaviors of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) to synthetic volatiles emitted by insect-damaged carrot roots. Journal of Pest Science 89:977-984. doi: 10.1007/s10340-015-0720-9.

Mohammed, R.H. 2012. Biological control of imported cabbageworm (*Pieris rapae*, Lepidoptera: Pieridae) with *Steinernema feltiae*. M.S. Thesis, University of Hawai‘i at Mānoa, Honolulu, Hawaii.

Myers, R.Y., Sipes, B.S., Matsumoto Brower, T.K., Mello, C.L., and Mello, J.S. 2015. Occurrence and distribution of Heterorhabditid populations in the Hawaiian Islands. Nematropica. 45:198-207.

Ortega-Estrada M D J, Rincón-Castro M C D, Regina Basurto-Ríos R, Toledo J and Ibarra J E. 2012. Phoresis between *Serratia marcescens* and *Steinernema carpocapsae* (Rhabditida: Steinernematidae) during infection of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae. Florida Entomologist 95:120-27.

Péchy-Tarr, M., Bruck, D.J., Maurhofer, M., Fischer, E., Vogne, C., Henkels, M.D., Donahue, K.M., Grunder, J., Loper, J.E. and Keel, C. 2008. Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*.

Environmental Microbiology 10:2368-2386.

Poretsky, R., Rodriguez-R, L. M., Luo, C., Tsementzi, D., and Konstantinidis, K. T. 2014. Strengths and Limitations of 16S rRNA Gene Amplicon Sequencing in Revealing Temporal Microbial Community Dynamics. PLoS ONE, 9(4), e93827. http://doi.org/10.1371/journal.pone.0093827

Sangeetha, B. G., Jayaprakas, C. A., Siji, J. V., Rajitha, M., Shyni, B., and Mohandas, C. 2016. Molecular characterization and amplified ribosomal DNA restriction analysis of entomopathogenic bacteria associated with *Rhabditis* (*Oscheius*) spp. Biotechnology 6*:*32.

Serepa-Dlamini, M., and Gray, V. 2018. A new species of entomopathogenic nematode *Oscheius safricana* n. sp. (Nematoda: Rhabditidae) from South Africa. Archives of Phytopathology and Plant Protection 1-13. doi: 10.1080/03235408.2018.1475281

Serepa, M., and Gray, V. 2014. Draft whole-genome sequence of *Serratia marcescens* strain MCB, associated with *Oscheius* sp. MCB (Nematoda: Rhabditidae) isolated from South Africa. Genome Announcements 2: e00911-14-e00911-14. doi: 10.1128/genomea.00911-14.

Shapiro-Ilan, D., Gaugler, R., Tedders, W., Brown, I., and Lewis, E. 2002. Optimization of inoculation for in vivo production of entomopathogenic nematodes. Journal of Nematology 34:343-50.

Ulug, D., Hazir, C., and Hazir, S. 2015. A new technique for the isolation of symbiotic bacteria associated with entomopathogenic nematodes (Heterorhabditidae and Steinernematidae). Turkish Journal of Zoology 39:365-367. doi: 10.3906/zoo-1404-63.

Vodovar, N., Vallenet, D., Cruveiller, S., Rouy, Z., Barbe, V., Acosta, C., Cattolico, L., Jubin, C., Lajus, A., Segurens, B. and Vacherie, B. 2006. Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium *Pseudomonas entomophila*. Nature Biotechnology 24:673.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. 1991. 16S ribosomal DNA amplification for phylogenetic study. The Journal of Bacteriology 173:697-703.

Williams, Kelly P., Gillespie, Joseph J., Sobral, Bruno W. S., Nordberg, Eric K., Snyder, Eric E., Shallom, Joshua M., and Dickerman, Allan W. 2010. Phylogeny of Gammaproteobacteria. The Journal of Bacteriology, *192*(9), 2305-2314.

Ye, W., Torres-Barragan, A., and Cardoza, Y. 2010. *Oscheius carolinensis* n. sp. (Nematoda: Rhabditidae), a potential entomopathogenic nematode from vermicopost. Nematology 12:121-135. doi: 10.1163/156854109x458464.

Yedid, G. 2016. Molecular characterization and isolation of symbiotic bacteria *Xenorhabdus* associated with entomopathogenic nematodes in Ghaziabad and Meerut, India. International Journal of Science and Research 5:1998-2000. doi: 10.21275/v5il.nov153189.

Zhang, C., Yang, X., Xu, S. Y., Sun, M. X., Liu, J., Liu, H., J. R., Liu, H., Kan, F., Sun, J., Lai, R., and Zhang, K.Y. 2009. *Serratia nematodiphila* sp. nov., associated symbiotically with the entomopathogenic nematode *Heterorhabditidoides chongmingensis* (Rhabditida: Rhabditidae). International Journal of Systematic and Evolutionary Microbiology 59: 1603-08.

CHAPTER 3

PATHOGENICITY OF BACTERIA ASSOCIATED WITH *OSCHEIUS*

INTRODUCTION

Various and unique pathogenic bacteria are associated with the nematodes that kill insects. The lethality of these nematode-associated bacteria determines whether the nematodes are classified as entomopathogenic nematodes (EPNs) or not. EPNs exhibit a symbiotic relationship with specific bacteria as the nematodes infect an insect host (Laznik and Trdan, 2015). The relationship between EPNs and the strain of bacteria is often specific in *Steinernema* and *Heterorhabditis*. However, the molecular characterization of EPB (EntomoPathogenic Bacteria) showed 18 bacterial isolates associated with *Oscheius* including *Enterobacter* sp., *Pseudomonas* sp., *Providencia* sp., *Proteus mirabilis, Alcaligenes faecalis, Stenotrophomonas maltophilia,* and *Bacillus cereus* (Yedid, 2016).

Ye *et al*. (2010) argue that a myriad of well-researched species of *Oscheius* have the characteristics of a necromenic lifestyle. For example, *Oscheius myrophila* and *O. colombiana* are clearly necromenic in their feeding behavior. However, related species, like *O. carolinensis,* exhibit a facultative entomopathogenic feeding behavior. *Oscheius carolinensis* can access the insect body and kill the insect with the assistance of the bacteria *Serratia marcescens* (Serepa and Gray, 2014). These bacteria, after multiplying within the insect hemolymph, secrete enzymes such as proteases and lipases that contribute to the death of the infected insect. Contrary to the internally symbiotic bacteria of the Heterorhabditid and Steinernematid EPNs, *S. marcescens* only has an external association with *O. carolinensis* (Zhou *et al*., 2017). The fact that entomophilic nematodes can cooperate with pathogenic bacteria is an indication that entomopathogenicity may advance from non-parasitic to parasitic nematodes through an initial intervention of bacteria.

Furthermore, some recent studies have emphasized the point that species of *Oscheius* use bacterial pathogens to parasitize insect hosts. According to Huang *et al*. (2015), *O. carolinensis* and *O. chongmingensis* have potential as entomopathogens that attack insects in the soil. Contemporary studies also confirm that species of *Oscheius* associate with *Serratia*. More specifically, *Oscheius* species partner with *S. marcescens* to gain access to the target insect. *Oscheius chongmingensis* is reported to have the ability to associate with other bacteria in addition to *S. marcescens* (Ye *et al*., 2010). The objective of these experiments was to determine the pathogenicity of bacteria associated with Hawaiian isolates of *Oscheius* against mealworm larvae.

MATERIALS AND METHODS

Bacterial isolates. Isolates of *Serratia* sp., *Enterococcus* sp., and *Pseudomonas* sp., originally isolated from nematodes and insect hemolymph BI 1a, OJ 4a, and OJ 5b were tested for insect pathogenicity. After isolation and purification from nematodes or insects, the bacteria were stored at −80°C in glycerol stocks before use. Bacterial isolates were grown on TZC medium for 4 days in a BOD incubator at 30°C before being used (Kelman, 1954; Harding *et al*., 2013). A bacterial loop full of cells of each isolate was individually transferred into a 100 ml tube filled with liquid TZC medium and incubated on a rotary shaker (180 rpm) at 24°C for 24 hours before use in the experiments (Flury *et al.,* 2016). In preparation for insect exposure, bacterial cells were washed in sterile 0.9% NaCl and the absorbance (OD_{600}) was measured at 0.1 nm. The cultures were adjusted with NaCl to a concentration of 10^8 colony forming units (CFU) per ml. Cell suspensions were diluted to the desired concentrations.

Injection assay. A 10 µl wash of bacterial cells was adjusted to concentrations of 0, 10^2, 10^4, 10^6, and 10^8 cfu/ml using 0.9% sterile NaCl buffer (Péchy-Tarr *et al.,* 2008). Using a sterile 3-ml syringe with a 31-gauge needle (EXELINT, Redondo Beach, CA), the bacterial suspensions

were injected into the hemolymph of mealworm larvae (Flury *et al.*, 2017). Injected larvae were placed in 10-cm petri plates and held in a dark incubator at 27°C. Larvae were observed daily for death. Mortality was described as the failure of larvae to move or react to poking. The experiment consist of four replicates of 10 larvae per bacterial concentration for each of the three bacterial isolates (*Serratia*, *Enterococcus*, and *Pseudomonas*). As a control, mealworm larvae were injected with an empty needle and water and NaCl. The experiment was repeated twice.

Feeding assay. A feeding assay was conducted with slight modifications as described by Ruffner *et al.* (2013). Whatman #1 filter paper was moistened and placed into the bottom of a 100-mm petri plate onto which a modified insect diet pellet was placed. The pellet consisted of 100 ml sterile ddH_2O containing 100 ml glycerin, 100 ml honey, 5 ml baby vitamins (Enfamil, Evansville, IN), and 50 g of baby cereal (GERBER® cereals, Florham Park, NJ). The ingredients were mixed and a 10 µl of suspension of washed bacterial cells at concentrations of 0, 10^2, 10^4, 10^6, and 10^8 cfu/ ml was placed on each pellet (Gupta *et al.*, 2005; Ruffner *et al.*, 2013). Sterile distilled water served as control. Ten mealworm larvae were introduced into each plate containing one food pellet. The plates were placed in a 27°C incubator in the dark. Plates were observed 24, 48, 72, and 96 hours later for insect mortality. When the larvae of *T. molitor* did not respond to poking, they were considered dead. The experiment consist of four replicates of 10 larvae per bacterial concentration for each of the bacterial isolates. The experiment was repeated two times.

Statistics. Data were subjected to ANOVA using SAS (SAS Inc, Cary, NC) for the effect of time, bacterial isolate, concentration and their interactions on mortality rates. The lethal concentrations (LC_{50} and LC_{90}) were calculated at 24, 48, 72, and 96 hours for each isolate by using regressions analysis.

RESULTS

Injection assay. All the three bacteria isolates tested kill 100% of mealworms at 48 hours after injection at 10^8 cfu/ml (Table 2.1). However, *Pseudomonas* sp. was more lethal to mealworms than *Enteroccocus* sp. or *Serratia* sp. based on mealworm mortality at 24 hours after injection (Table 2.1, Fig. 2.1). *Pseudomonas* sp. achieved LC_{50} and LC_{90} at 10^2 cfu/ml within 48 and 72 hours after bacteria injection, respectively (Table 2.1). In addition, *Enteroccocus* and *Serratia* achieved LC_{50} and LC_{90} at 10^4 cfu/ml within 48 and 72 hours, respectively (Table 2.1). *Pseudomonas* was significantly more lethal to mealworm larvae than *Enteroccocus* or *Serratia* ($P \leq 0.01$).

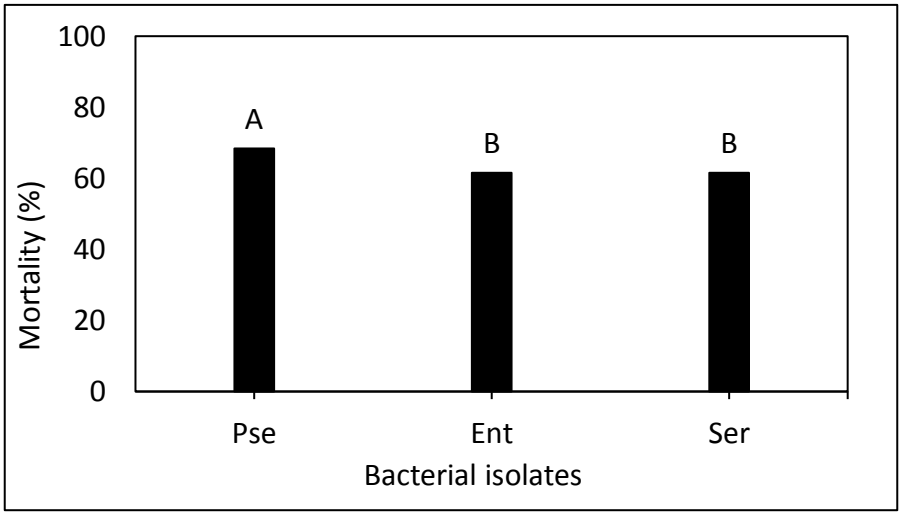

Figure 2.1 Average mortality (%) of mealworm injected with 10^2, 10^4, 10^6, and 10^8 cfu/ml of *Pseudomonas* (Pse), *Enteroccocus* (Ent) and *Serratia* (Ser) after 24 hours. Bars with the same letter are not different.

Table 2.1 Mortality of mealworms affected by bacteria associated with isolate of *Oscheius* from Hawaii at different concentrations over time after bacteria injection.

	Enteroccocus	*Pseudomonas*	*Serratia*
Cfu/ml	Mean (%)	Mean (%)	Mean (%)
----------0 hr----------			
10^0	0.0±0.0[z] A	0.0±0.0 A	0.0±0.0 A
10^2	0.0±0.0 A	0.0±0.0 A	0.0±0.0 A
10^4	0.0±0.0 A	0.0±0.0 A	0.0±0.0 A
10^6	0.0±0.0 A	0.0±0.0 A	0.0±0.0 A
10^8	0.0±0.0 A	0.0±0.0 A	0.0±0.0 A
----------24 hr----------			
10^0	0.0±0.0 E	0.0±0.0 E	0.0±0.0 E
10^2	12.5±2.5 D	37.5±2.5 D	12.5±2.5 D
10^4	32.5±2.5 C	52.5±2.5 C	27.5±2.5 C
10^6	42.5±2.5 B	65.0±2.9 B	45.0±2.9 B
10^8	90.0±0.0 A	92.5±2.5 A	90.0±0.0 A
----------48 hr----------			
10^0	0.0±0.0 E	0.0±0.0 D	0.0±0.0 D
10^2	40.0±4.1 D	62.5±2.5 C	32.5±2.5 C
10^4	67.50±2.5 C	75.0±5.0 B	60.0±4.1 B
10^6	85.00±2.9 B	92.5±4.8 A	95.0±2.9 A
10^8	100.00±0.0A	100.0±0.0A	100.0±0.0A

	Enteroccocus	*Pseudomonas*	*Serratia*
Cfu/ml	Mean (%)	Mean (%)	Mean (%)
----------72 hr----------			
10^0	0.00±0.0 C	0.00±0.0 C	0.00±0.0 C
10^2	65.00±2.9 B	90.00±0.0 B	72.50±2.5 B
10^4	97.50±2.5 A	100.00±0.0A	97.50±2.5 A
10^6	100.00±0.0A	100.00±0.0A	100.00±0.0A
10^8	100.00±0.0A	100.00±0.0A	100.00±0.0A
----------96 hr----------			
10^0	0.0±0.0 B	0.00±0.0 B	0.00±0.0 B
10^2	100.0±0.0A	100.0±0.0A	100.0±0.0A
10^4	100.0±0.0A	100.0±0.0A	100.0±0.0A
10^6	100.0±0.0A	100.0±0.0A	100.0±0.0A
10^8	100.0±0.0A	100.0±0.0A	100.0±0.0A

[z]Means (n = 4) in a column at each time followed by the same letter are not different based on Waller-Duncan *k*-ratio (*k*=100) *t*-test.

Table 2.2 Lethal concentration of *Enteroccocus*, *Pseudomonas* and *Serratia* achieving LC_{50} and LC_{90} on mealworms in a bacterial injection experiment.

	Enteroccocus		*Pseudomonas*		*Serratia*	
Time (hr)	LC 50	LC 90	LC 50	LC 90	LC 50	LC 90
	----------10^X cfu/ml of suspension----------					
0	0	0	0	0	0	0
24	5.00[Z]	9.00	4.00	8.00	5.00	9.00
48	3.00	6.00	2.00	6.00	3.00	6.00
72	2.00	5.00	1.00	5.00	2.00	5.00
96	1.00	5.00	1.00	5.00	1.00	5.00

[Z] Value of X

Trails I and II shared homogeneity of variance, so the data were combined. Based on the linear regression, the LC_{50} and LC_{90} were calculated. The LC_{50} for *Pseudomonas* was at 10^4 cfu/ml and the LC_{90} was at 10^8 cfu/ml at 24 hr (Fig. 2.2). For *Enteroccocus* and *Serratia*, the LC_{50} was achieved at 10^5 cfu/ml and the LC_{90} was at 10^9 cfu/ml at 24 hr (Fig. 2.2). In both trials, *Pseudomonas* required 10 fold less bacteria to achieve the LC_{50} than *Enteroccocus* or *Serratia* (Table 2.2). *Pseudomonas* also required 10 fold fewer cfu/ml to achieve the LC_{90} compared to *Enteroccocus* and *Serratia* at 24 hour after bacteria injection.

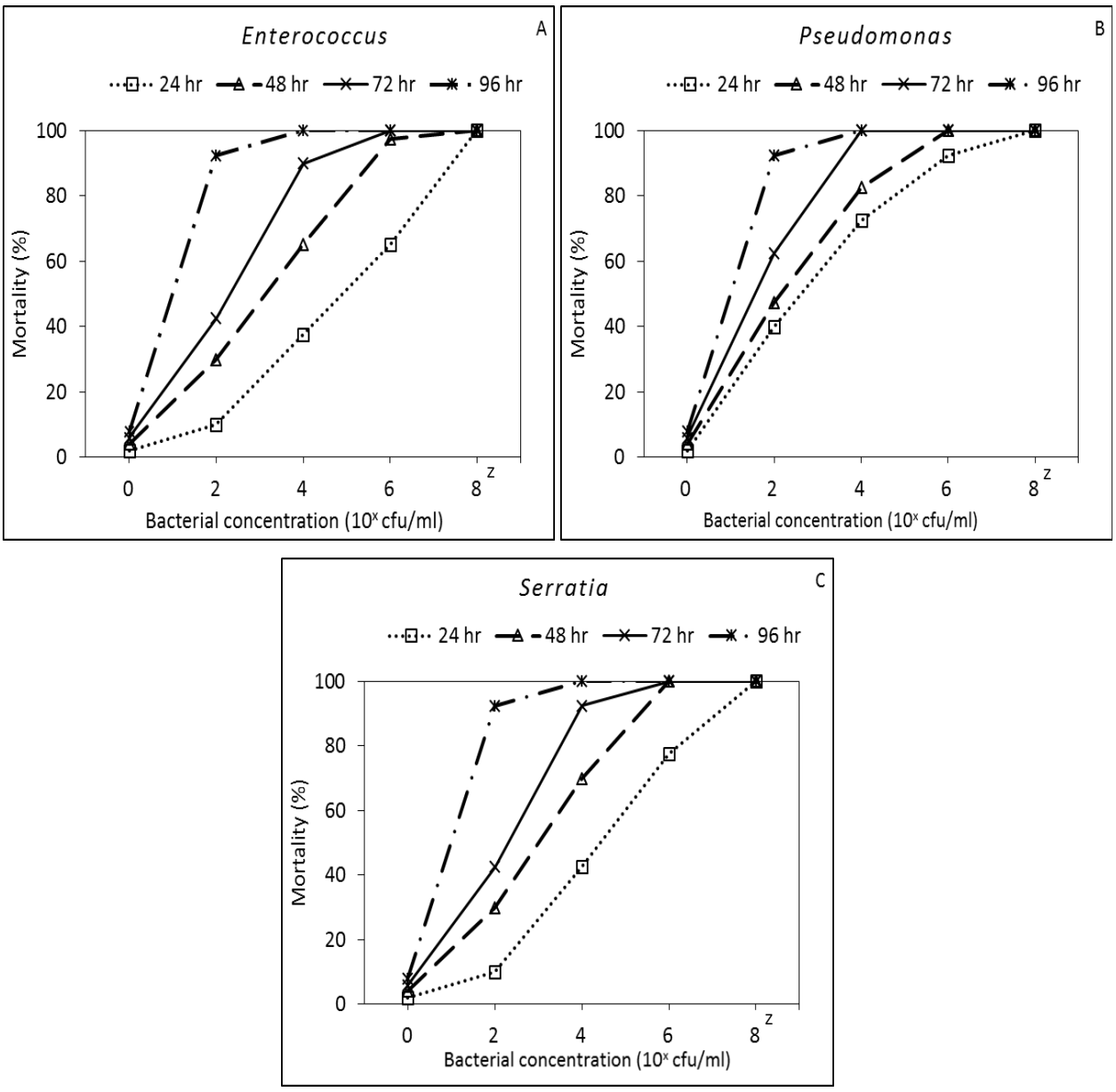

Figure 2.2 Effect of three bacterial isolates: A) *Enteroccocus.* B) *Pseudomonas.* C) *Serratia* on mortality rates of mealworm larvae, over time after injection bioassay.

[z] Value of X

Feeding assay. Similar to the results from the injection test, *Pseudomonas* was more lethal to the *T. molitor* larvae compared to *Enteroccocus* and *Serratia* (Fig. 2.3). Table 2.3 showed that *Pseudomonas* had higher mealworm mortality within 24 hrs than *Enteroccocus* or *Serratia.* The interaction between time, bacterial isolate, and concentration was significant ($P \leq 0.01$). The general trend appeared to be that percent of mealworm mortality was directly proportional to bacterial concentration (Table 2.3). Mealworm mortality was significantly greater than 10^4 cfu/ml for all isolates on 24 and 48 hrs after feeding an inoculated food pellet to *T. molitor* larvae ($P \leq 0.01$). Although *Pseudomonas* achieved LC_{50} at 10^4 and 10^6 cfu/ml in 24 hr, all isolates reached LC_{90} within 24 hours at 10^8 cfu/ml (Table 2.3). At 72 and 96 hr, mealworm mortality at 10^2 cfu/ml became significantly higher than the no bacteria control ($P \leq 0.01$).

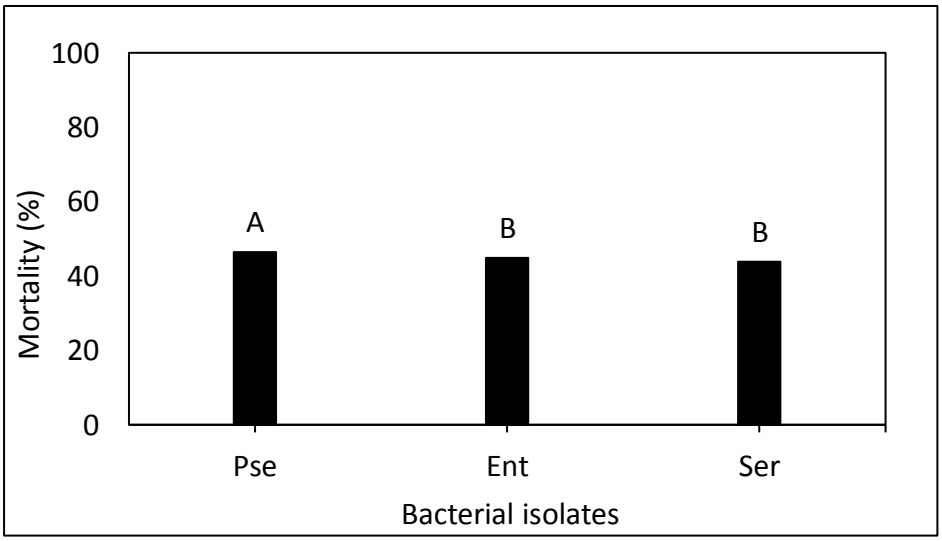

Figure 2.3 Average mortality (%) of mealworm fed on modified insect diet with 10^2, 10^4, 10^6, and 10^8 cfu/ml of *Pseudomonas* (Pse), *Enteroccocus* (Ent) and *Serratia* (Ser) after 24 hours. Bars with the same letter are not different.

Table 2.3 Mortality of mealworms feeding on food pellets with different concentrations of bacteria associated with *Oscheius* isolate from Hawaii over time.

	Enteroccocus	*Pseudomonas*	*Serratia*
Cfu/ml	Mean (%)	Mean (%)	Mean (%)
----------0 hr----------			
10^0	0.0±0.0[z] A	0.0±0.0 A	0.0±0.0 A
10^2	0.0±0.0 A	0.0±0.0 A	0.0±0.0 A
10^4	0.0±0.0 A	0.0±0.0 A	0.0±0.0 A
10^6	0.0±0.0 A	0.0±0.0 A	0.0±0.0 A
10^8	0.0±0.0 A	0.0±0.0 A	0.0±0.0 A
----------24 hr----------			
10^0	0.0±0.0 D	0.0±0.0 D	0.0±0.0 D
10^2	0.0±0.0 D	0.0±0.0 D	0.0±0.0 D
10^4	32.5±2.5 C	52.5±2.5 C	27.5±2.5 C
10^6	42.5±2.5 B	65.0±2.9 B	45.0±2.9 B
10^8	90.0±0.0 A	92.5±2.5 A	90.0±0.0 A
----------48 hr----------			
10^0	0.0±0.0 D	0.0±0.0 C	0.0±0.0 C
10^2	5.0±2.9 D	2.5±2.5 C	5.0±2.9 C
10^4	67.50±2.5 C	75.0±5.0 B	60.0±4.1 B
10^6	85.00±2.9 B	92.5±4.8 A	95.0±2.9 A
10^8	100.00±0.0A	100.0±0.0A	100.0±0.0A

	Enteroccocus	*Pseudomonas*	*Serratia*
Cfu/ml	Mean (%)	Mean (%)	Mean (%)
----------72 hr----------			
10^0	0.00±0.0 C	0.00±0.0 C	0.00±0.0 C
10^2	25.00±5.0 B	27.50±0.0 B	30.00±2.5 B
10^4	97.50±2.5 A	100.00±0.0A	97.50±2.5 A
10^6	100.00±0.0A	100.00±0.0A	100.00±0.0A
10^8	100.00±0.0A	100.00±0.0A	100.00±0.0A
----------96 hr----------			
10^0	0.0±0.0 C	0.00±0.0 C	0.00±0.0 C
10^2	52.50±4.8B	52.50±2.5B	60.0±5.8 B
10^4	100.0±0.0A	100.0±0.0A	100.0±0.0A
10^6	100.0±0.0A	100.0±0.0A	100.0±0.0A
10^8	100.0±0.0A	100.0±0.0A	100.0±0.0A

[z]Means (n = 4) in a column at each time point followed by the same letter are not different based on Waller-Duncan *k*-ratio (*k*=100) *t*-test.

Table 2.4 Lethal concentration (LC_{50} and LC_{90}) of *Enteroccocus, Pseudomonas* and *Serratia* to mealworms in a bacterial feeding experiment.

	Enteroccocus		*Pseudomonas*		*Serratia*	
Time (hr)	LC 50	LC 90	LC 50	LC 90	LC 50	LC 90
----------10^X cfu/ml of suspension----------						
0	0	0	0	0	0	0
24	5.00[Z]	9.00	4.00	8.00	5.00	9.00
48	4.00	6.00	3.00	6.00	4.00	6.00
72	2.00	5.00	2.00	5.00	2.00	5.00
96	2.00	5.00	2.00	5.00	2.00	5.00

[Z] Value of X

The LC_{50} and LC_{90} values for *Pseudomonas* were estimated to be 10^4 and 10^8 cfu/ml, respectively at 24 hour (Table 2.4). The LC_{50} and LC_{90} for *Enteroccocus* and *Serratia* were estimated via regression analysis at 10^5 and 10^9 cfu/ml on 24 hours. No difference was detected between the LC_{50} and LC_{90} values for *Pseudomonas, Enteroccocus* and *Serratia* at 48, 72, and 96 hours (Table 2.4) (Fig. 2.4).

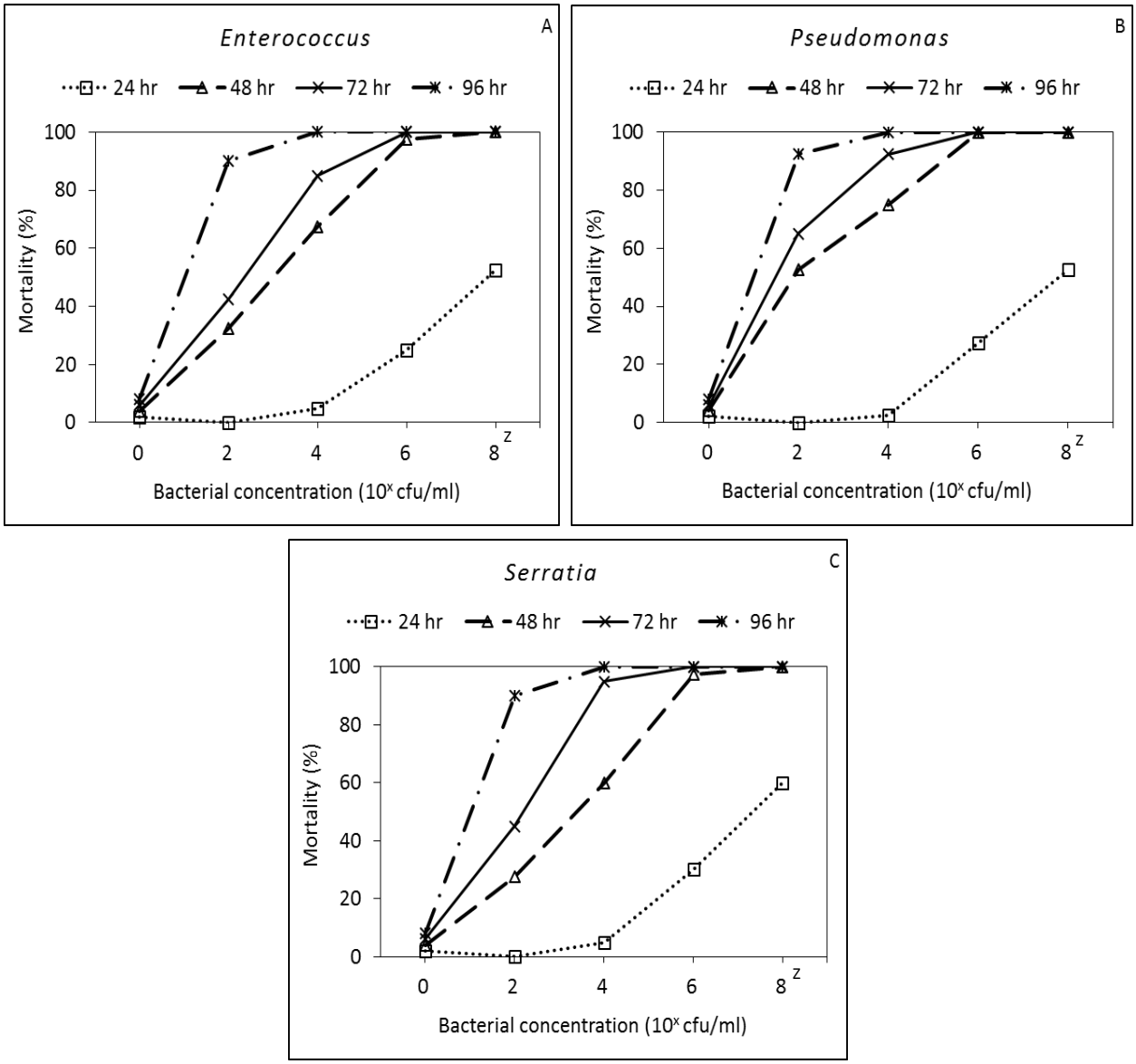

Figure 2.4 Effect of three bacterial isolates: A) *Enteroccocus.* B) *Pseudomonas.* C) *Serratia* on mortality rates of mealworm larvae, over time after feeding bioassay.

[Z] Value of X

DISCUSSION

The *Oscheius*-associated bacteria *Enterococcus, Pseudomonas,* and *Serratia* are toxic to mealworms. From the current study results, it is evident that all the three bacteria isolates are able to kill insects without the nematode. The findings are consistent with the former studies that the entomopathogenic bacteria associated with entomopathogenic *Oscheius* are able to kill the insect without the nematode (Al-Own, 2013; Hameed *et al*., 2014; Li *et al.*, 2017; Ruiu, 2015). Direct injection of the bacteria increased their effectiveness as entomopathogen than insect exposure through feeding. The extreme lethality of *Pseudomonas* compared to *Enterococcus* and *Serratia* in killing the mealworms from this research is consistent with the findings of Al-Own (2013). Mona and Aly (2015) showed that EPB *Enterococcus, Pseudomonas,* and *Serratia* have been widely used by nematodes to kill insects. This finding confirmed that isolates of *Oscheius* in Hawaii have developed symbiotic relationship with entomopathogenic bacteria.

It may be possible to formulate these entomopathogenic bacteria as biological control agents, independent of the nematode host. The use of pathogenic bacteria such as *Enterococcus, Pseudomonas*, and *Serratia* have been widely pursued by different scholars and government agencies as alternative to pest control and management. Mahmoud (2016) in a study of the use of entomopathogenic bacteria for insect pest biocontrol argue that there has been a growing crusade for environmentally safe chemicals, with limited impact on the non-target organisms, low toxicity to human, and short-term persistence in the field. *Enterococcus, Pseudomonas,* and *Serratia* present such characteristics and are therefore suitable for the use in pest control in different settings. Mahmoud (2016) also recognizes the differences in the strength of each of the pathogenic bacteria in the pest control and management. For instance, according to this study, there is an evidence that *Pseudomonas* is most effective in killing the mealworms and possibly other insect

pests. In most civilizations, there is a growing concern over the use of insecticides due to the public health, environmental pollution, rising costs, and resistance of pests associated with pest control (Lacey *et al*., 2015). This creates the need to explore the biological alternatives that are efficient and environmentally friendly to manage the target pests. The use of *Enterococcus, Pseudomonas,* and *Serratia* evaluated in this study offer opportunities to be explored for their biocontrol potential.

The widespread testing of EPB has yielded success in most areas with a fast response to the control of pests compared to the traditional approaches (Hashem *et al*., 2015). Unlike the other alternatives, the biological alternatives do not have a negative effect on the crops. In a separate study, Lauzon *et al.* (2013) illustrate the effectiveness of the EPB, specifically *Serratia marcescens* and *Pseudomonas* sp. in the biological pest control of healthy apple maggot flies in the lab trial. From the findings, the EPB were effective in the elimination of the pests within 24 hours. With an increase in the concentration of 4.7×10^4 cfu/ ml, there is a shorter period to the elimination of the pests unlike when a lower concentration of the EPB is used in the experiment (Hameed *et al*., 2014). We have clearly established that the isolates of *Oscheius* tested in Hawaii are entomopathogenic in nature and have the potential to be developed into biological control agents.

REFERENCES

Al-Own, F. 2013. Population Structure of Insect Pathogenic Bacteria in UK Soil and their Associated Nematodes. Doctoral Dissertation. University of Bath.

Flury, P., Aellen, N., Ruffner, B., Pechy-Tarr, M., Fataar, S., Metla, Z. 2016. Insect pathogenicity in plant-beneficial pseudomonads: Phylogenetic distribution and comparative genomics. Journal of International Society for Microbial Ecology 10:2527-2542.

Flury, P., Vesga, P., Péchy-Tarr, M., Aellen, N., Dennert, F., Hofer, N., and Maurhofer, M. 2017. Antimicrobial and insecticidal: cyclic lipopeptides and hydrogen cyanide produced by plant-beneficial strains CHA0, CMR12a, and PCL1391 contribute to insect killing. Frontiers in Microbiology 8:100.

Gupta, G., Rani, S., Birah, A., and Raghuraman, M. 2005. Improved artificial diet for mass rearing of the tobacco caterpillar, *Spodoptera litura* (Lepidoptera: Noctuidae). International Journal of Tropical Insect Science 25:55-58.

Hameed, A. M., Malla, S., and Kumar, R. S. 2014. Molecular characterization of *Pseudomonas* sp. isolated from milk samples by using RAPD-PCR. European Journal of Experimental Biology, *4*(4), 78-84.

Harding, C., Schroeder, G., Collins, J., and Frankel, G. 2013. Use of *Galleria mellonella* as a model organism to study *Legionella pneumophila* infection. Journal of Visualized Experiments 81:E50964.

Hashem, Y. A., Yassin, A. S., and Amin, M. A. 2015. Molecular characterization of *Enterococcus* spp. clinical isolates from Cairo, Egypt. Indian journal of medical microbiology, *33*(5), 80.

Huang, R., Ye, W., Ren, X., and Zhao, Z. 2015. Morphological and molecular characterization of Nematoda: Rhabditidae a new Rhabditid nematode from South China. PLOS ONE 10:e0144386. doi: 10.1371/journal.pone.0144386

Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., and Goettel, M. S. 2015. Insect pathogens as biological control agents: back to the future. Journal of Invertebrate Pathology 132:1-41.

Lauzon, C. R., Bussert, T. G., Sjogren, R. E., and Prokopy, R. J. 2013. *Serratia marcescens* as a bacterial pathogen of Rhagoletis pomonella flies (Diptera: Tephritidae). European Journal of Endocrinology 100(1), 87-92.

Li, W., Jin, D., Shi, C., and Li, F. 2017. Midgut bacteria in deltamethrin-resistant, deltamethrin-susceptible, and field-caught populations of *Plutella xylostella*, and phenomics of the predominant midgut bacterium *Enterococcus mundtii*. Scientific reports, 7(1), 1947.

Laznik, Ž., and Trdan, S. 2015. Attraction behaviors of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) to synthetic volatiles emitted by insect-damaged carrot roots. Journal of Pest Science 89:977-984. doi: 10.1007/s10340-015-0720-9.

Mahmoud, M. F. 2016. Biology and Use of Entomopathogenic Nematodes in Insect Pests Biocontrol, A Generic View. Cercetari Agronomice in Moldova, 49(4), 85-105.

Mona, H. A., and Aly, N. A 2015. Insecticidal Activity and Genetic Characterization of Four Bacterial Isolates of *Xenorhabdus* and *Photorhabdus* Associated with Entomopathogenic Nematodes. Pest Technology, 3(1), 50-57.

Péchy-Tarr, M., Bruck, D.J., Maurhofer, M., Fischer, E., Vogne, C., Henkels, M.D., Donahue, K.M., Grunder, J., Loper, J. E. and Keel, C. 2008. Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. Environmental Microbiology 10:2368–2386. doi:10.1111/j.1462-2920.2008.01662.x

Ruffner, B., Péchy-Tarr, M., Ryffel, F., Hoegger, P., Obrist, C., Rindlisbacher, A., Keel, C. and Maurhofer, M. 2013. Oral insecticidal activity of plant-associated pseudomonads. Environmental Microbiology 15:751-763.

Ruiu, L. 2015. Insect pathogenic bacteria in integrated pest management. Insects, 6(2), 352-367.

Serepa, M., and Gray, V. 2014. Draft whole-genome sequence of *Serratia marcescens* strain MCB associated with *Oscheius* sp. MCB (Nematoda: Rhabditidae) isolated from South Africa. Genome Announcements 2:e00911-14-e00911-14. doi: 10.1128/genomea.00911-14.

Ye, W., Torres-Barragan, A., and Cardoza, Y. 2010. *Oscheius carolinensis* n. sp. (Nematoda: Rhabditidae), a potential entomopathogenic nematode from vermicompost. Nematology 12:121-135. doi: 10.1163/156854109x458464

Yedid, G. 2016. Molecular characterization and isolation of symbiotic bacteria *Xenorhabdus* associated with entomopathogenic nematodes in Ghaziabad and Meerut, India. International Journal of Science and Research 5:1998-2000. doi: 10.21275/v5il.nov153189.

Zhou, G., Yang, H., Wang, F., Bao, H., Wang, G., Hou, X., Lin, J., Yedid, G. and Zhang, K. 2017. *Oscheius microvilli* n. sp. (Nematoda: Rhabditidae): A facultatively pathogenic nematode from Chongming Island, China. Journal of Nematology 49:33-41. doi: 10.21307/jofnem-2017-044

CONCLUSION

The study on entomopathogenic nematodes (EPNs) has attracted various interests from researchers as an option for environmental friendly, classical, or conservation biological controls. The growing interests are associated with the improvement in mass production of EPB for pest control (Li *et al.*, 2017).

Lacey and Georgis (2012) in a study of using the EPNs to control the insect pests above and below ground summarized that there has been a significant technological improvement in the developments of EPNs for pest control that has reduced the use of pesticides and insecticides in pest control (Batalla-Carrera *et al.,* 2010). The growth has supported an increase in the development and commercial application of the EPNs in pest control. Furthermore, the commercially produced EPNs have been widely used in the control of scarab larvae and fungus gnats (Shapiro-Ilan *et al.*, 2012). According to Dillman *et al.* (2012), there is a diverse insect-parasitic nematodes effective in management of insect pests. As the EPNs infect the hemocoel system of the host insect, the bacteria associated with them kill the insect hosts (Batalla-Carrera *et al.*, 2010). Entomopathogenic bacteria might be effective than insecticides due to less negative impact on the environment and less likely for the insect pests to develop resistance as in the case of many pesticides (Ye *et al.*, 2010).

Ruiu (2015) in a study of the insect of pathogenic bacteria in pest management recognizes the development and existence of varied bacteria that helps in the pest control. Some of the species according to the study include *Pseudomonas entomophila*, *Serratia* species, *Betaproteobacteria* species *Burkholderia* spp. and *Chromobacterium* spp. (Ruiu, 2015). The pathogenicity of bacteria associated with *Oscheius* varies across the nematodes. The differences in their pathogenicity explain the effectiveness difference in pest control. The infection of the insect hots makes the EPNs

application in the pest management (Park *et al*., 2011). The enzymes proteases and lipases contribute to the deaths of insects. Other species such as *O. carolinensis* and *O. chongmingensis* have potential as entomopathogens that attack insects, making them an ideal option for biological pest control (Vijayakumari *et al.,* 2013). Additionally, the association of *Oscheius chongmingensis* with other bacteria explains the pathogenicity of the bacteria that acts against the mealworm larvae in this study. Base on the current experiments, the *Pseudomonas* is more lethal against insect compared to *Enterococcus* and *Serratia*.

Shapiro-Ilan *et al.* (2012) provide an elaborate review of the concepts and technological applications on the mass production of EPNs. This technology is a key element in the success of EPNs for pest control (Torres-Barragan *et al.,* 2011). The mixing of EPNs with polymers, sprayable gels, or surfactants are some of the recent developments that have been recorded in the EPN formulations for the above ground applications (Shapiro-Ilan *et al.,* 2012). The *in vitro* culture technology has been established to be an appropriate mechanism for the production of the EPNs. Shapiro-Ilan *et al.* (2012) further suggests that the efficacy in EPN applications can be enhanced by utilizing better delivery mechanisms. For instance, the optimization of delivery equipment or the cadaver applications. Future work should explore formulating entomopathogenic strains of *Oscheius* sp. isolated in Hawaii as biological control agent for local use in Hawaii as currently quarantine restriction against importation of foreign commercial EPNs are prohibited.

REFERENCES

Batalla-Carrera, L., Morton, A., and García-del-Pino, F. 2010. Efficacy of entomopathogenic nematodes against the tomato leafminer *Tuta absoluta* in laboratory and greenhouse conditions. Biocontrol, 55(4), 523-530.

Dillman, A. R., Chaston, J. M., Adams, B. J., Ciche, T. A., Goodrich-Blair, H., Stock, S. P., and Sternberg, P. W. 2012. An entomopathogenic nematode by any other name. PLoS Pathogens, *8*(3), e1002527.

Lacey, L. A., and Georgis, R. 2012. Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. Journal of Nematology, 44(2), 218.

Li, W., Jin, D., Shi, C., and Li, F. 2017. Midgut bacteria in deltamethrin-resistant, deltamethrin-susceptible, and field-caught populations of *Plutella xylostella*, and phenomics of the predominant midgut bacterium *Enterococcus mundtii*. Scientific reports, *7*(1), 1947.

Park, H. W., Kim, Y. O., Ha, J. S., Youn, S. H., Kim, H. H., Bilgrami, A. L., and Shin, C. S. 2011. Effects of associated bacteria on the pathogenicity and reproduction of the insect-parasitic nematode *Rhabditis blumi* (Nematoda: Rhabditida). Canadian Journal of microbiology, 57(9), 750-758.

Ruiu, L. 2015. Insect pathogenic bacteria in integrated pest management. Insects, 6(2), 352-367.

Shapiro-Ilan, D. I., Han, R., and Dolinksi, C. 2012. Entomopathogenic nematode production and application technology. Journal of Nematology, 44(2), 206.

Torres-Barragan, A., Suazo, A., Buhler, W. G., and Cardoza, Y. J. 2011. Studies on the entomopathogenic and bacterial associates of the nematode *Oscheius carolinensis*. Biological control, 59(2), 123-129.

Vijayakumari, S. J., Sasidharannair, N. K., Nambisan, B., and Mohandas, C. 2013. Optimization of media and temperature for enhanced antimicrobial production by bacteria associated with *Rhabditis* sp. Iranian journal of microbiology, 5(2), 136.

Ye, W., Torres-Barragan, A., and Cardoza, Y. J. 2010. *Oscheius carolinensis* n. sp. (Nematoda: Rhabditidae), a potential entomopathogenic nematode from vermicompost. Nematology, 12(1), 121-135.