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INVESTIGATING THE INHIBITORY PROPERTIES OF CANDIDATE PROBIOTICS TOWARDS VIBRIO PARAHAEMOLYTICUS

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INVESTIGATING THE INHIBITORY PROPERTIES OF CANDIDATE
PROBIOTICS TOWARDS VIBRIO PARAHAEMOLYTICUS

BY

JACQUELINE HILARY CAMM

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ABSTRACT

Acute Hepatopancreatic Necrosis Disease (AHPND) causes more than a billion dollars of losses to the shrimp farming industry annually. In 2013, strains of the marine pathogen *Vibrio parahaemolyticus* containing genes for the PirA^{Vp}/B^{Vp} toxins, encoded on a 70 Kb plasmid, were identified as the causative agent of AHPND. Antibiotics have been used to attempt to reduce losses, however their use in aquaculture is controversial. Additionally, the pathogen has demonstrated the ability to rapidly acquire antibiotic resistances through genetic mutation and lateral gene transfer. Probiotics, often used to prevent disease in aquaculture, may offer the opportunity to mitigate AHPND infections without the risks to consumers and the environment posed by antibiotic use. The goal of this study was to determine if the marine probiont *Phaeobacter inhibens* S4 protects *Litopenaeus vannamei* post-larvae (PLs) against fatal *V. parahaemolyticus* infections and to elucidate its protective mechanisms. A further goal of this study was to isolate and identify other potential probionts, including “*Bdellovibrio* and Like Organisms” (BALO), whose prey range includes *V. parahaemolyticus*.

P. inhibens S4 was tested in challenge assays using *Artemia* nauplii and *L. vannamei* for its ability to protect these organisms from *V. parahaemolyticus*-induced mortality. Zhao et al previously identified three key probiotic activities of *P. inhibens* S4: 1) the production of the antibiotic compound tropodithietic acid (TDA), 2) the formation of a copious biofilm, and 3) quorum sensing/quorum quenching activity through *N*-acyl homoserine lactone (AHL) signaling. Mutant strains of *P. inhibens* S4

with one or a combination of these three mechanisms altered were used in PL challenge assays to assess which probiotic functions were key to protecting shrimp. Competition assays, to assess in vitro growth of *V. parahaemolyticus* PSU5579 when co-cultured with *P. inhibens* S4 or one of the mutant strains, were also performed to determine the ability of S4 to inhibit the growth of the pathogen in biofilms and planktonic states. Results showed that *P. inhibens* S4SmKm protects *L. vannamei* PLs from AHPND, increasing survival from 45% in the infected control to 80% when the environment is pre-treated with *P. inhibens* S4SmKm. Assays with wild type and mutant strains of *P. inhibens* show protection whether or not TDA is produced; however, S4 no longer protects when biofilm production is reduced or quorum sensing/quorum quenching is disrupted. In support of these results, it was shown that *P. inhibens* S4 does not inhibit the growth of *V. parahaemolyticus* when co-cultured, nor is *V. parahaemolyticus* sensitive to the antibiotic TDA, even up to concentrations of 100 µg/ml.

To screen for other potential probionts and a BALO organism, bacteria isolated from seawater and shrimp tank water were screened using a zones of inhibition (ZOI) assay and for plaque forming capabilities in lawns of *V. parahaemolyticus*. A candidate BALO was isolated from Narragansett Bay that showed predatory activity against *V. parahaemolyticus*. Genomic DNA was extracted, and the genome was sequenced. The organism appears to be a previously unidentified species of *Halobacteriovorax*, given the strain name GB3. While *Halobacteriovorax* GB3 does not harm *L. vannamei* PLs, we did not test its ability to

protect shrimp from AHPND because it does not reduce *V. parahaemolyticus* concentrations below 1×10^6 CFU/ml, which is an infectious dose.

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DEDICATION

I dedicate this thesis to my three children: Evan, Annalise, and Jack Travis. For 15 years your love of inquiry and learning have amazed and inspired me to grow in my own love of learning. I am especially grateful for your support and encouragement, and for your investment in helping me achieve my goals.

I also dedicate this thesis to my partner in everything: Charles Leduc; and to our parents, Edgar, and Dorothy Leduc, and John Camm for the encouragement you have provided during my studies.

PREFACE

The following thesis has been prepared in manuscript format according to the guidelines of the Graduate School of the University of Rhode Island. This thesis contains a literature review and two manuscripts.

The first manuscript “Investigating the probiotic activities of *Phaeobacter Inhibens* S4 against AHPND-inducing *Vibrio parahaemolyticus*” will be submitted to Applied and Environmental Microbiology.

The second manuscript “Draft Genome Sequence of *Halobacteriovorax* sp. Strain GB3, a Predatory Bacterium Isolated from Narragansett Bay that Preys on Shrimp Pathogen *Vibrio parahaemolyticus* PSU5579” will be submitted to Microbiology Resource Announcements.

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REVIEW OF LITERATURE

Statement of the problem

Shrimp aquaculture is a multi-billion dollar global industry with production estimated between 2.9-3.5 billion tons in 2017 with 75-80% of production originating in South East Asia (1). In the United States alone, shrimp imports in 2017 were worth \$6.5 billion, an increase of 14% from the year before (1). Acute Hepatopancreatic Necrosis Disease (AHPND) is the major cause of Early Mortality Syndrome (EMS), responsible for significant losses in the shrimp farming industry. EMS emerged in 2009 affecting shrimp farms in southern China, causing up to 100% mortality in shrimp ponds within 30 days (2). The disease in shrimp is characterized by a pale and atrophied hepatopancreas and an empty gut (3). By 2014, AHPND had spread to Vietnam, Malaysia, Thailand, Mexico and the Philippines (2). The primary cause of AHPND has been identified as strains of *Vibrio parahaemolyticus* that produce PirA and PirB toxins (encoded by *pirA* and *pirB*, respectively) and contain an antibacterial Type VI secretion system (T6SS) (3). Utilizing antibiotics to control AHPND has not been a successful strategy (4). Multi-antibiotic resistant strains of *V. parahaemolyticus* have been isolated from shrimp farms world-wide (5). Additionally, there is public health concern about the rise of antibiotic resistant organisms. Many bacteria, including *V. parahaemolyticus*, acquire and transfer new antibiotic resistances through lateral gene transfer (6), demonstrating the dangers of wide-scale application of antibiotics in aquaculture. Furthermore, as invertebrates,

shrimp lack an acquired immune system, posing challenges to vaccine development. Probiotics, defined as “live microorganisms, conferring a healthy benefit on the host when being consumed in adequate amounts” (7) are becoming a preferred method to prevent bacterial infections in aquaculture (8). Recent data indicate that *Phaeobacter inhibens* S4Sm, which has shown significant inhibitory activity against other *Vibrio* species such as of *Vibrio anguillarum* and *Vibrio coralliilyticus* RE22 (9) (10) (11), also has potential as a probiotic treatment in shrimp aquaculture to prevent AHPND (12). In addition to *P. inhibens*, we have isolated and investigated two other bacterial strains: *Pseudoalteromonas piscicida* JC3, which has recently been shown to inhibit the growth of multiple *Vibrio* species including *V. parahaemolyticus* (13), and a previously unidentified member of the BALO genus, *Halobacteriovorax* strain GB3.

Background on shrimp farming

Aquaculture is the world’s fastest growing food production sector with an average annual growth rate of 8% (14). Shrimp, second only to salmon and trout, accounted for 17% of the global trade in aquaculture species by value in 2017 (approximately \$6 billion) (15). While shrimp farming has been a traditional means of growing food for thousands of years, the rise of large-scale farming for export began in the 1980s, driven by high demand and prices in the United States and Japan (14). The rapid rise in shrimp farming has had significant impacts on the coastal landscapes, especially in South East Asia, with the destruction of salt flats, coastal wetlands, mangroves, and agricultural land converted to brackish water ponds for

farming shrimp due to its value as a crop (16). Farming practices vary widely both regionally and within a given region, but shrimp ponds are either closed systems into which water is pumped and then pumped off when the ponds are emptied, or open systems which exchange water at some interval, up to 20% daily (17). Effluent from shrimp ponds degrades the water quality in the surrounding areas by adding suspended solids, nutrients, and organic matter, contributing to eutrophication of coastal waters (16). Outbreaks of disease in these ponds often lead to massive crop losses. Around the turn of the 21st century, hatcheries began captive breeding programs with *Litopenaeus vannamei* (whiteleg shrimp) to produce Specific Pathogen Free (SPF), Specific Pathogen Resistant (SPR), and Specific Pathogen Tolerant (SPT) brood stock. As a result, white leg shrimp are now the most commonly farmed species of shrimp (14). Ponds are stocked with Post Larval Shrimp (PLs), which are then harvested when mature.

Rise and spread of AHPND

Originally named Early Mortality Syndrome, AHPND was first reported in China in 2009. By 2011 it had spread to Malaysia and Vietnam and in 2012 to Thailand. The causative agent was isolated in 2013 (18), also in 2013 AHPND was confirmed in Mexico (19). Clinical appearance includes a pale to white hepatopancreas (HP), reduced HP size, empty stomach (ST), and empty midgut (MG) (Fig. 1). The impact of the disease was a rapid decline in the rate of production, such

as was seen in Thailand where production peaked in 2012 and decreased sharply with the emergence of AHPND (Fig 2).

Figure 1. Characteristics of AHPND. A and B show signs of disease post challenge with *V. parahaemolyticus* including atrophied, pale HP, empty ST and empty MG. C and D are examples of healthy shrimp. (18)

Figure 2: Farmed shrimp production in Thailand from 1970-2015. The steep decline in production between 2010 and 2015 coincides with the emergence of AHPND in 2012. (1)

***Vibrio parahaemolyticus* as pathogen**

V. parahaemolyticus is also known as a human pathogen; however, there are key differences between the strains that cause gastroenteritis in humans and those causing AHPND in shrimp. *V. parahaemolyticus* has two type six secretion systems (T6SS1 and T6SS2) located on chromosome one and two, respectively. T6SS1 is most active in high salinity conditions and appears to target bacteria, whereas T6SS2 is active in low salinity, such as inside the guts of host organisms (20). The antibacterial T6SS1, when activated by the high salinity of shrimp ponds, gives *V. parahaemolyticus* a competitive advantage as it is able to attack and kill other bacteria competing to fill niches in these aquatic environments. Additionally, strains pathogenic to shrimp contain a Type Three Secretion System 1 (T3SS1), but lack a T3SS2, which is present in all human clinical strains and considered an important factor for human virulence (21). Further, *V. parahaemolyticus* strains pathogenic to humans contain the thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*tdr*) genes; however, neither of these genes are found in AHPND-inducing strains (21, 22).

PirA/B-containing plasmid

The PirA^{VP}/B^{VP} toxins are encoded on a highly conserved 70 Kb plasmid and flanked by transposases. It has been described as a “selfish plasmid” (3) because the plasmid also encodes for conjugative transfer gene clusters and a post-segregational killing system (PSK) associated gene *pndA* (23,24) (Fig. 3). Selfish genetic elements

are those that enhance their own transmission without necessarily conferring a benefit to the individual (25); the conjugative transfer gene cluster increases the likelihood of transfer (24). The PSK ensures that any bacterial progeny without the plasmid will die; this system encodes for a highly stable antibacterial mRNA which persists following cell division, along with a less stable and complementary antisense RNA which quickly degrades so that cells without the plasmid-based PSK are killed by the antibacterial mRNA (26). It is well documented that lateral gene transfer occurs among *Vibrio* species of the same clade, so it is not surprising to note that this plasmid has also been isolated from other members of the *V. harveyi* clade including *Vibrio campbelli* and *Vibrio owensii* (23, 27, 28) (Fig. 4). However, it is notable that the plasmid has also been isolated from more distantly related species and the *pirA^{VP}/B^{VP}* genes have also been observed in *Micrococcus luteus* strains isolated in Mexico (29).

Figure 3. Genetic map of the 70 Kb AHPND associated plasmid. The genetic elements of the plasmid include *pirA^{VP}/pirB^{VP}* flanked by transposases as well as a Type II Secretion System. The presence of a conjugative transfer gene cluster and the

PSK associated *pndA* gene enhance the transfer of the plasmid and ensure its presence in subsequent progeny (24).

Figure 4. Sequence map and identity plot of AHPND associated plasmid extracted from *V. owensii*. Mapping shows greater than 99% identity with AHPND associated plasmids extracted from *V. parahaemolyticus* (blue) and *V. harveyi* (green) when compared to the reference sequence from *V. owensii* (grey) demonstrating that the plasmid is highly conserved across species. Used with permission.(23)

Issues with antibiotics

The use of antibiotics in aquaculture is widespread and problematic for many reasons. Residual antibiotics linger in the environment, altering microflora and selecting for antibiotic-resistant bacteria (30). There is a potential for human harm as well since residual antibiotics have been observed on aquaculture products from China, including shrimp. Though not well studied as a side effect of antibiotic use in aquaculture, one potential risk to humans includes allergic reactions (31). The greatest threat, however, to human populations is likely the rise in antibiotic resistance. The data suggest that there is a strong link between the use of antibiotics in aquaculture and the increased frequency of drug resistant strains of pathogenic bacteria (32). Furthermore, the use of fluoroquinolones has become increasingly common in aquaculture. Unfortunately, this class of antibiotics which do not readily biodegrade, also accumulate in the tissues of farmed aquatic species (32) increasing the potential for human adverse reaction and antibiotic resistance to a class of drugs originally developed to address the issue of drug-resistant pathogens (33).

Other novel therapies

One central dogma of evolutionary immunology is that shrimp, as invertebrates, lack a true adaptive immune system (34). However, research over the past two decades has demonstrated that shrimp can develop an immune “memory” or response to immune priming to both viral and bacterial pathogens following vaccination and other exposure to pathogens (34–37); however, it is important to note that *V. parahaemolyticus* does not appear to colonize the shrimp gut, and therefore vaccination or similar immune stimulus to the pathogen is not likely to increase survival. Recent studies have shown an ability to stimulate increased immune response to protect shrimp from AHPND through non-lethal heat shock, which upregulates heat shock proteins (38, 39), and through passive “vaccination” with feed-base exposure to the PirA toxin by adding anti-PirA-toxin immunoglobulin (IgY) from chicken egg yolk to feed (40) that render the shrimp less susceptible to the toxin itself. Another emerging area of research in control of bacterial illness is bacteriophage therapy, which is the application of bacteriophage viruses that infect and kill the pathogenic bacteria without harm to the host organism. There are many challenges to utilizing this treatment method, and phage therapy would be ineffective as a therapy for AHPND for two reasons. First, the viruses are species specific and sometimes strain specific, so as the *pirA^{vp}*/*pirB^{vp}* genes continue to be transferred to other *Vibrio* species, this treatment will become increasingly ineffective. Second, bacteria evolve resistance to phages quickly, and some strains of AHPND-inducing *V. parahaemolyticus* are naturally resistant to phage infection (41).

The application of bacteriophage may initially be an effective means to slow or stop some infections; however, repeated use will increasingly select for phage resistant strains of *V. parahaemolyticus*, requiring an ongoing search for new phage strains and rendering this therapy inefficient for long-term application.

Probiotics in aquaculture

Investigations into the use of probiotics in aquaculture date back to the 1980's (42). In aquaculture, probiotics may be applied and interact differently than in other systems. They may be administered with feed or applied directly to the water. In addition to inhibiting pathogen growth through the production antibiotic compounds, probiotics can limit pathogen growth by competing for nutrients (Fig. 5) (43). Probiotics can also protect host organisms by stimulating innate immune response and enhancing disease resistance, as multiple *Bacillus* species have been shown to do in shrimp aquaculture (35,44,45)

Figure 5. The applications and benefits of probiotics in shrimp aquaculture.

Probiotics can be applied directly to the environment or in feed-based supplements. Benefits include enhanced immunity, the inhibition of pathogens and increased growth (43).

***Phaeobacter inhibens* S4Sm**

P. inhibens S4Sm, isolated from a healthy oyster (10) is a Gram negative, motile rod-shaped marine member of the Roseobacter clade of Alphaproteobacteria (46). *P. inhibens* S4Sm is a spontaneous streptomycin-resistant (Sm^R) of S4 (9). It has strong biofilm formation capabilities and produces tropodithietic acid (TDA), which has been shown to be a potent antibiotic against multiple *Vibrio* species (9, 47). *P. inhibens* has been shown to be an effective probiotic against fish pathogen *V. anguillarum*, as well as oyster-associated pathogens *V. vulnificus* and *V. coralliilyticus* (9–11, 47, 48). Probiotic activity of *P. inhibens* S4Sm has been previously attributed to TDA production, biofilm formation, and acyl homoserine production (Fig. 6) (9, 49). Prior research by LaPorte and Nelson showed that *P. inhibens* S4Sm significantly increased shrimp survival from *V. parahaemolyticus* induced AHPND compared to the AHPND infection control and many other probiont candidates (Fig. 7) (12). Only *Pseudoalteromonas piscicida* provided similar protection against AHPND as *P. inhibens* S4Sm.

Figure 6. Oyster larvae survival in the presence of *P. inhibens* strains after challenge with *V. coralliilyticus*. The *P. inhibens* S4Sm strains (1×10^4 CFU/ml) were introduced 24 h before larvae were challenged with *V. coralliilyticus* RE22Sm (1×10^5 CFU/ml). *clpX* mutants have normal biofilm formation but no TDA production. *exoP* mutants produce TDA, but biofilm formation is reduced to 40% compared to wild type. Oyster larvae treated only with artificial seawater served as control (mock). Oyster larvae survival (% $\pm$ SD) was determined 24 h after challenge with RE22Sm. Bars marked with an asterisk (*) show significant differences (t-test, $p < 0.05$). Representative of at least 3 experiments. Error bars represent one standard deviation (9).

Figure 7. Effect of preincubation of *L. vannamei* PLs with candidate probionts on survival 48 h after challenge with *V. parahaemolyticus* PSU5579Sm. PL's were preincubated with *P. inhibens* S4Sm, *Ps. piscicida* JL1Sm, *Ps. piscicida* JL15Sm, *Ps. arabiensis* JL29Sm, *B. denitrificans* JL63Sm, and *B. pumilus* RI06-95Sm for 24 h prior to challenge with PSU5579Sm. Candidate probionts were added at 10^6 CFU/ml at the start of the experiment and every 24 h thereafter. Challenge with *V. parahaemolyticus* PSU5579Sm (at 10^6 CFU/ml) occurred 24 h after the addition of the probionts. Representative of three independent experiments with three technical replicates per experiment. Error bars equal one standard deviation. * Indicates statistically significant difference from the *V. parahaemolyticus* PSU5579Sm treatment (ANOVA, $p < 0.05$) (12).

Halobacteriovorax

Halobacteriovorax is a genus of predatory marine bacteria within the Deltaproteobacteria; it was first differentiated from freshwater genus *Bacteriovorax* in 2015 (50). Currently, two named species belong to this genus: *Halobacteriovorax litoralis* and *Halobacteriovorax marinus* (50, 51). This genus shares many

characteristics with the predatory genus *Bdellovibrio* including its unique, two-phase life cycle in which it attacks a Gram-negative bacterium in its prey range, penetrates the cell into the periplasm and replicates inside the periplasm using the nutrients provided by the prey cell (52). Organisms with this unique life cycle are often grouped together into a class called *Bdellovibrio* and *Like* *Organisms* (BALOs). It has been proposed that BALOs could help prevent AHPND in shrimp aquaculture (53). *Halobacteriovorax* sp. GB3 was isolated from Narragansett Bay and tested against a strain of AHPND-inducing *Vibrio parahaemolyticus*.

Research Goals

The overall goal of this research was to examine the interactions between *V. parahaemolyticus* and potential probiotic organisms that demonstrate ability to inhibit its growth. The first aim was to understand the mechanisms by which the probiont *P. inhibens* S4Sm protects shrimp from *V. parahaemolyticus* induced AHPND, and how the probiont mitigates the pathogenic activity of *V. parahaemolyticus*.

The second aim was to isolate and identify a BALO organism, which preys on *V. parahaemolyticus*, and to test its safety for use in shrimp aquaculture as a potential control to prevent the wide-scale growth of *V. parahaemolyticus* in aquaculture environments.

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MANUSCRIPT I

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Title: Investigating the probiotic activities of *Phaeobacter Inhibens* S4 against AHPND-inducing *Vibrio parahaemolyticus*

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Abstract

We have been investigating potential probiotic treatments for Acute Hepatopancreatic Necrosis Disease (AHPND), affecting farmed shrimp and caused by the marine pathogen *Vibrio parahaemolyticus*. In this study we screened over 300 organisms for key probiotic functions, including antibiosis against the pathogen and biofilm formation. Twelve candidate probionts were screened using *Artemia* nauplii and post-larval *Litopenaeus vannamei* for the *in vivo* ability to protect against fatal *V. parahaemolyticus* infections. We found that *Phaeobacter inhibens*, which has been shown to protect oysters and turbot from vibriosis with inhibitory activity against *Vibrio coralliilyticus* and *Vibrio anguillarum* also has potential as a preventative treatment for AHPND. In infection challenges, pre-treatment with *P. inhibens* S4Sm increased the relative survival of *L. vannamei* post-larvae to 80% as compared to 45% survival in the infected control. As identified in previous research, the mechanisms of *P. inhibens* S4Sm probiotic activity may include its ability to form robust biofilms, quorum sensing/quorum quenching through N-acyl homoserine lactone (AHL) signaling, and production of antibiotic tropodithietic acid (TDA). In this study, we find that *V. parahaemolyticus* forms biofilms with 3 to 5-fold increased cell density when co-cultured with *P. inhibens* S4Sm as compared to a monoculture control. Additionally, *V. parahaemolyticus* is not sensitive to TDA at concentrations up to 100 µg/ml. Mutations that disrupt biofilm formation and quorum sensing eliminate probiotic activity of *P. inhibens* S4Sm against *V. parahaemolyticus*.

Statement of Significance

In this investigation we found that while *P. inhibens* S4Sm protects *Artemia* nauplii and White Leg shrimp (*Litopenaeus vannamei*) from AHPND. However, it does not inhibit the growth of the pathogen, suggesting that antibiosis is not a probiotic mechanism of *P. inhibens* in preventing AHPND. Rather, probiotic activity is linked to N-acyl homoserine lactone (AHL) signal production and biofilm formation. The advantage of a probiotic that does not inhibit the growth of a pathogen is that there is no selective pressure for the pathogen to develop resistance to the probiont, making such an organism an attractive option for long-term use in the prevention of disease. Further, resistance to TDA has not previously been observed outside of the *Roseobacter* genus.

Introduction

Shrimp aquaculture is a multi-billion dollar global industry with production estimated between 2.9-3.5 billion tons in 2017 with 75-80% of production in South East Asia (1). The United States alone, imported \$6.5 billion of shrimp in 2017, an increase of 14% from the year before (1). AHPND is the major cause of Early Mortality Syndrome, responsible for significant losses in the shrimp farming industry. AHPND usually begins within seven to ten days after ponds are stocked with post larval shrimp and can cause up to 100% mortality within 30 days (2). Effective

probiotics may offer a disease control strategy to reduce losses in the shrimp farming industry without the potentially harmful effects of wide-scale antibiotic use (3).

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (4). Research indicates that strong candidate probiotics are able to inhibit the growth of pathogens through the production of antibiotic compounds, biofilm formation and quorum sensing/quorum quenching such as through acyl homoserine lactone (AHL) signaling (5–9) to prevent disease. Candidate probiotics must also be able to tolerate growth conditions associated with the pathogen, such as the acidity of the digestive tract or, in the case of shrimp aquaculture, the salinity of the environment. Most importantly, a probiont must not reduce the overall health of the host organism while present in a concentration sufficient for preventing disease.

In this study, we screened ten newly isolated bacteria and two oyster probionts, *Phaeobacter inhibens* S4Sm and *Bacillus pumilus* RI06-95, for antibiotic activity against an AHPND-inducing strain of *V. parahaemolyticus*, as well as their ability to protect *Artemia* nauplii or *Litopenaeus vannamei* post-larvae (PLs) from AHPND *V. parahaemolyticus*. We found that *P. inhibens* S4Sm was the only candidate probiont that both significantly increased the survival of and did not harm either *Artemia* nauplii and *L. vannamei* PLs challenged with AHPND *V. parahaemolyticus*. To understand the mechanisms by which *P. inhibens* S4Sm protected *L. vannamei* PLs from AHPND, we performed a series of competitive growth assays using mutant strains that exhibited decreased production of biofilms, no production of the

ionophore antibiotic tropodithietic acid (TDA), or knockout mutations in the AHL signaling pathways. We also tested the ability of the mutant strains to protect PLs challenged with *V. parahaemolyticus*.

Results

Selection of an AHPND-inducing *V. parahaemolyticus* strain.

Ten strains of *V. parahaemolyticus*, isolated from shrimp farms in southern Thailand during an outbreak of AHPND were screened for the presence of the *priA* and *priB* genes by duplex PCR. PCR amplification was performed using primers VpPirA-284F/R and VpPirB-392F/R as described by Han et al. (10). Of these strains, six were identified to possess both genes (Fig. 1) and were then used to challenge *Artemia* nauplii (Fig. 2). Strain PSU5579 exhibited the highest rate of mortality (92%) in *Artemia* nauplii and was selected for use in future experiments. All other strains tested appeared to be avirulent.

Selection of probiotic organisms:

More than 300 bacterial isolates were screened for probiotic activity against *V. parahaemolyticus* with a zone of inhibition (ZOI) assay. Ten isolates produced ZOIs between 0.1mm and 6.4mm. These candidate organisms that produced a zone of inhibition, along with the two known marine probionts *P. inhibens* S4Sm and *B. pumilus* RI06-95, were then evaluated for biofilm formation via crystal violet staining (Fig. 3) to select organisms to test *in vivo*. Eleven of the 12 candidate probiotic

microbes produced biofilms significantly larger than the pathogen *V.*

parahaemolyticus PSU5579 with optical densities between 1.3 and 3.7 at 580 nm.

Only isolate HR-1 failed to produce copious biofilm. The oyster probiont, *P. inhibens* S4Sm, consistently produced the most abundant biofilm.

In an effort to identify the candidate probionts, genomic DNA was extracted from the ten isolates for PCR amplification and sequencing of the 16S rRNA gene as described in the Methods. The organisms were identified (Table 1) by comparing the resulting 16S rDNA sequences with the NCBI bacterial ribosomal database (11).

Seven of the isolates were found to be members of the genus *Pseudoalteromonas*, two were genus *Bacillus*, and one was *Bowmanella*.

***Artemia* nauplii assay**

Artemia are frequently used as a model organism to test for biotoxicity in marine systems (12). In this assay, *Artemia* were used to screen candidate probionts identified above for potential harmful effects in *Artemia* nauplii prior to their use in trials with *L. vannamei* PLs. *Pseudoalteromonas* strains (JL1, JL15, JL29) significantly decreased survival of *Artemia* when the *Artemia* nauplii were treated with concentrations at 1×10^5 CFU/ml, while *Bowmanella denitrificans* (JL63) did not (Fig. 4). *Artemia* nauplii were then pre-treated with candidate probionts at concentrations ranging between 1×10^4 to 1×10^7 CFU/ml for 24 hours and then challenged with *V. parahaemolyticus* PSU5579 at 10^5 CFU/ml. Potential probiotics were added every 24 hours and survivors were counted 48 hours post challenge. Pre-treatment with

Pseudoalteromonas species (isolates GR1, GR4, JL1, JL12, JL15, JL18 and JL29) or *B. denitrificans* (isolate JL63) followed by challenge with the pathogen PSU5579 resulted in the complete killing of the *Artemia* compared to 37% survival of *Artemia* the challenged with PSU5579 alone. (Fig. 5). When *Artemia* were pre-treated with *B. pumilus* strains RI06-95, JL70, and HR1 at 10^4 , 10^5 , or 10^6 CFU/ml for 24 hours and then challenged with *V. parahaemolyticus* PSU5579, there was no significant increase in survival 48 hours following the challenge when compared to the challenged control (Fig. 6). *Artemia* nauplii pre-treated for 24 hours with *P. inhibens* S4Sm and then challenged *V. parahaemolyticus* PSU5579 showed a 1.84-fold increase (to 70%) in their rate of survival 48 hours post challenge when compared with the challenged control with survival of 38%. Pre-treated and challenged survival was similar to survival when treated with *P. inhibens* S4Sm alone. (Fig. 7). This result suggested that since *P. inhibens* S4Sm was not harmful to *Artemia* and was able to reduce AHPND mortalities in *Artemia* challenged with *V. parahaemolyticus* PSU5579, it should be an excellent candidate probiont for *L. vannamei*.

Shrimp trials

In initial trials to examine the safety of the candidate probionts, *L. vannamei* PLs were treated with candidate probionts at 10^6 CFU/ml every 24 hours for 72 hours and survivors were counted. Most strains tested did not significantly decrease survival of shrimp PLs; however, *P. piscicida* JL1 significantly decreased survival to 83% compared to 97% survival in the untreated control (Fig. 8). To test the efficacy of the probiont candidate strains, shrimp pre-treated with the probiotic isolates were

challenged at 24 hours with *V. parahaemolyticus* PSU5579 at 10^6 CFU/ml. They were followed for 48 hours post-challenge and survivors were counted. Shrimp treated with either *P. inhibens* S4Sm or *P. piscicida* JL1 showed significant protection against AHPND when compared to the challenged control (Fig. 9); however, since JL1-treated shrimp exhibited decreased survival even when not challenged with PSU5579, S4Sm was selected as the best candidate probiont.

A series of experiments were performed to determine which probiotic characteristics of *P. inhibens* S4 were critical to protecting *L. vannamei* from *V. parahaemolyticus* PSU5579. These trials were altered from the original experimental design to prevent the *L. vannamei* microbiome from confounding experimental results. In this set of experiments, bottles containing 200 ml 3% artificial sea water (ASW) plus *P. inhibens* wild type or mutant strains at a concentration of $\sim 10^6$ CFU/ml were allowed to incubate for 24 hours at 29°C. Shrimp were added to the bottles after 24 hours and immediately challenged with *V. parahaemolyticus* PSU5579 at 10^6 CFU/ml. *P. inhibens* treatments were repeated every 24 hours and survivors were counted 48 hours post-challenge. The relative rates of survival for shrimp treated with wild type *P. inhibens* S4SmKm, or the *clpX* (WZ10) mutant strain (no TDA production) or the *luxO* (CS003) mutant (over-produces TDA) were the same (76.5 % to 80%). In contrast, the relative rates of survival for shrimp treated with the *exoP* (WZ20) mutant strain (reduced biofilm formation; 51.5%), or *pgaI* mutant strain (lacking biofilm, TDA production and AHL signal production; 59%), or the *pgaR* mutant strain (lacking biofilm, TDA production and AHL signal reception; 53%) were

not significantly different from the 45% survival rate in PSU5579 challenged control shrimp (Fig. 10). We did not test revertants or complements of these mutant strains; however, Zhao et al (6) found that complementation of the *clpX* and *exoP* genes in mutant strains restored function to wild type levels. These data strongly suggest that biofilm formation and quorum signaling are necessary for the probiotic activity of *P. inhibens* S4Sm, while TDA production is not necessary.

Competitive biofilm assays

In an effort to understand the competition between pathogen (PSU5579) and probiont (S4Sm), we used a modification the glass coverslip assay developed by Zhao et al (6). We found that *P. inhibens* S4SmKm did not inhibit the biofilm formation of *V. parahaemolyticus* PSU5579 when the two organisms were co-cultured (Fig. 11). In fact, at all times the biofilm cell density of PSU5579 in co-culture with S4SmKm was increased compared to the PSU5579 monoculture biofilm. The increases in biofilm were 5.5-fold at 24 h, 3-fold at 48 h, and 3.7-fold at 72 h. The biofilm density of S4Sm was not affected by co-culture. This contrasts with the probiotic activity of *P. inhibens* S4Sm against other *Vibrio* species, where pathogen biofilm cell density was decreased when in co-culture (6).

Mutant strains of *P. inhibens* S4SmKm (*clpX*, *luxO*, or *pgal*) were tested for their ability to affect either biofilm formation or planktonic cell density of *V. parahaemolyticus* PSU5579. Co-culture of PSU5579 with any of the three mutants resulted in less robust biofilm formation (Fig. 12a). Planktonic cell concentration of

V. parahaemolyticus PSU5579 was not reduced when grown in co-culture with any of the mutant strains compared to co-culture with the wild type of *P. inhibens* S4SmKm (Fig. 12b).

TDA Assay

Since the ability of *P. inhibens* to provide protection against *V. parahaemolyticus* PSU5579 was not affected by their ability to produce TDA, we wanted to determine the minimum inhibitory concentration (MIC) of TDA against *V. parahaemolyticus* PSU5579. Briefly, *V. parahaemolyticus* PSU5579 (initial density 10^5 CFU/ml) was grown in mLB30 (27°C, 24 h) in the presence of different concentrations of TDA plus two controls, one containing 1% DMSO, (the solvent in which TDA is dissolved) and one without additions. Cell density was determined by serial dilution and spot plating. *Vibrio coralliilyticus* RE22 and *Vibrio anguillarum* NB10Sm are inhibited at 6.25 µg/ml and 1.25 µg/ml, respectively (6). In contrast, *V. parahaemolyticus* PSU5579 growth was not inhibited by TDA at concentrations up to 100 µg/ml. These data demonstrate that TDA production does not inhibit *V. parahaemolyticus* PSU5579 growth.

Discussion:

In this study we screened more than 300 isolates for probiotic activity. First, we examined antibiosis using a ZOI assay. Antibiosis is considered one of the key and necessary functions of probiotic organisms, enabling them to establish in the

host environment through competitive advantage and also to fend off potential pathogens (13). Next we considered biofilm formation. A biofilm is an assemblage of microbial cells that is associated with a surface and enclosed in a matrix of primarily polysaccharide material (14). Formation of dense biofilms by probiotics is considered protective because it allows the probiont to establish long-lasting colonization of the host and prevent colonization by pathogens (15). In some strains of Lactobacilli, it has been observed that the exopolysaccharide (EPS), which binds the bacteria to a surface, can also prevent biofilm formation by pathogens (15). The aquatic environment allows for the possibility that colonization of the environment may be an effective alternative to probiotic colonization of the gut (16). Perhaps the most important feature of a probiotic treatment is that it not cause harm to the host (17). After completing challenge assays in both *Artemia* nauplii and shrimp PLs, a single organism met all these criteria. *P. inhibens* S4Sm produces the potent antibiotic TDA, forms the most robust biofilms of the twelve organisms selected for trials, and it did not cause significant harm for either model organism when used as a monotreatment. Specifically, *P. inhibens* S4Sm had no negative effects on *L. vannamei* and had the least negative effect (decline of ~15%) on *Artemia* compared to all other candidates tested.

In challenge assays, we found that *P. inhibens* S4Sm protects *Artemia* nauplii and *L. vannamei* from the pathogen *V. parahaemolyticus* PSU5579. Zhao et al (6, 18) identified three key characteristics in *P. inhibens* S4Sm's ability to protect against *V. coralliilyticus* and *V. anguillarum* (TDA production, biofilm formation and AHL

signaling); however, these functions are not equally important in all systems. *V. anguillarum* is highly sensitive to TDA, at concentrations as low as 1.25 µg/ml (6), but the disruption of AHL signaling has little impact on the inhibitory activity of *Phaeobacter gallaenciensis* against this fish pathogen (19). In contrast, *V. coralliilyticus* is less sensitive to TDA at 6.25 µg/ml (6); however, AHL signaling plays a significant role in the downregulation of virulence factors, and is a component critical to the protective functions of *P. inhibens* S4Sm against this oyster pathogen (18).

Upon identifying *P. inhibens* S4Sm as a candidate probiotic to protect shrimp from AHPND, we investigated these three probiotic functions to determine which were critical to protect shrimp against the pathogenic activity of *V. parahaemolyticus*. Our data suggest that the reduction of quorum sensing/quenching signaling through mutation of the AHL synthase (*pgal*) or transcriptional activation/response regulation (*pgaR*) or reduced AHL concentration due to decreased biofilm formation (*exoP*) decreases the probiotic function of *P. inhibens* S4Sm. *P. inhibens* S4Sm mutant strains CS001 (Δ *pgal*) and CS002 (Δ *pgaR*) did not provide significant protection of PLs when compared to the infected control (Fig. 10). These mutant strains together encode for a *luxIR* homologous AHL mediated quorum sensing system. The *pgal* gene encodes for the N-3-hydroxydecanoylhomoserine lactone (3-OH C(10)-HSL) synthase and, therefore, a mutation in this gene disrupts AHL production. *pgaR* encodes for the AHL response regulator. While this mutant can produce the AHL signal, it does not engage in the positive feedback loop triggered by the reception of the AHL signal from neighboring

cells (20). Cell to cell signaling and biofilm formation are intricately connected (21). In general, cells in biofilms encounter much higher local cell densities, and therefore higher concentrations of AHL signals than free-floating planktonic cells (22); as a result, the disruption of the *pgaIR* system also reduces biofilm formation. Likewise the Δ exoP mutant WZ20, which has 40% or less of the biofilm formation of wild type cells (6) also has decreased AHL activity due to the reduced localized cell concentration. WZ20 does not, however, have reduced TDA activity and Zhao et al (6) found it had delayed inhibitory activity against TDA-sensitive *Vibrio* species. Given the common features of these three mutant strains, which are the interconnected functions of AHL signaling and biofilm formation, and their shared inability to protect shrimp from AHPND, we conclude that AHL signaling and biofilm formation are critical to the probiotic activity of *P. inhibens* S4Sm in protecting shrimp. As Zhao et al (18) showed that the AHL activity of *P. inhibens* S4Sm downregulates virulence factors in *V. coralliilyticus* RE22, we propose studying the effects of AHL signaling upon the regulation of AHPND related virulence factors.

Having explored the functions of AHL signaling and biofilm formation, the remaining known probiotic feature of *P. inhibens* S4Sm is the production of the antibiotic ionophore, TDA. In contrast to the effects of *P. inhibens* on the oyster and fish pathogens (*Alliioseovarius crassostreae*, *V. coralliilyticus*, and *V. anguillarum*) (6, 23–25), the growth of *V. parahaemolyticus* PSU5579 is not inhibited by either *P. inhibens* S4Sm or pure TDA. The negative results of both the TDA growth inhibition assay and the competition assays suggest that *V. parahaemolyticus* PSU5579 is

resistant to TDA produced by *P. inhibens* S4Sm. This observation is notable for two reasons. First, because *P. inhibens* S4Sm does not inhibit the growth of PSU5579, it is potentially an excellent probiotic for long-term use in shrimp aquaculture as there is no selective pressure for the pathogen to develop resistance to the probiotic mechanisms of *P. inhibens*. In fact, competition assay data suggest these two organisms are mutualistic, growing as well, if not better when co-cultured in biofilms (Fig. 11). Second, naturally occurring resistance to TDA has not previously been observed outside the *Roseobacter* genus (26, 27). This unexpected result suggests that *P. inhibens* S4Sm is a particularly strong candidate as a probiotic, because, without growth inhibition, there is no selective advantage for *V. parahaemolyticus* PSU5579 to develop resistance to this probiont.

Materials and Methods:

Bacterial strains and culture conditions

Bacterial species and strains used in this study are listed in Table 1. All bacteria were grown at 27°C with shaking. *P. inhibens* S4Sm wild type and mutant strains were routinely grown to stationary phase in marine yeast peptone 30 (mYP30; 5 g/L peptone, 1 g/L yeast extract, 30 g/L Instant Ocean®, pH7.5) for 48 h. *V. parahaemolyticus* PSU5579Sm was grown to stationary phase in LB30 (10 g/L tryptone, 5 g/L yeast extract, 30 g/L Instant Ocean®, pH7.5) for 24 h.

Duplex PCR for the detection of *pirA*- and *pirB* genes

Ten strains of *V. parahaemolyticus* isolated from shrimp farms in the Pattani and Songkla provinces of southern Thailand during an AHPND outbreak were screened for the presence of the *pirA*- and *pirB* genes. The *V. parahaemolyticus* strains were gifted by Dr. John Mekalanos (Harvard Medical School). Genomic DNA was isolated using the Bio Basic EZ-10 Spin Column Bacterial Genomic DNA Miniprep Kit. Primers VpPirA-284F (5'-TGACTATTCTCACGATTGGACTGR-3'), VpPirA-284R (5'-CACGACTAGCGCCATTGTTA-3'), VpPirB-392F (5'-TGATGAAGTGATGGGTGCTC-3'), and VpPirB-392R (5'-TGTAAGCGCCGTTTAACTCA-3') were used to amplify the *pirA*- and *pirB* genes (10). 1 µl of extracted gDNA (10-60 ng/µl) was combined with 1 µl of each primer (10 µmol), 7.5 µl nuclease-free water, and 12.5 µl of QIAGEN *Taq* PCR master mix. Reaction conditions were 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. PCR products were separated on a 2% agarose gel containing ethidium bromide (0.4 µg/ml) and visualized on a UV transilluminator. Presence of *pirA* is indicated by a band at 284 bp, while presence of *pirB* is indicated by a band at 392 bp (28).

16S rRNA gene sequencing

Genomic DNA was isolated using the Bio Basic EZ-10 Spin Column Bacterial Genomic DNA Miniprep Kit. Primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') were used to amplify the 16S rRNA gene (11). Extracted gDNA (1 µl at a concentration of 10-60 ng/µl) was combined with 1 µl of

each primer (stock solution, 10 μ mol), 9.5 μ l nuclease-free water, and 12.5 μ l of QIAGEN *Taq* PCR master mix. Reaction conditions were 95 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 53 °C for 30 s, and 72 °C for 1.5 min; and a final extension at 72 °C for 5 min. PCR products were sequenced at the University of Rhode Island Genomics and Sequencing Center. Sequences were compared to the National Center for Biotechnology Information (NCBI) database of bacterial genomes using nucleotide to nucleotide basic local alignment search tool (blastn)

Zone of inhibition assay:

V. parahaemolyticus PSU5579Sm was grown for 24 h in mLB30, diluted 100-fold, and 100 μ l of the diluted culture was spread on mYP30 agar (YP30 + 1.6% Bacto Agar). The inoculated agar plates were allowed to dry for 5-15 min and then candidate probiotic cultures (grown to stationary phase) were spotted onto the plates (10 μ l/spot). Plates were incubated at 27 °C for 24-48 h. Zones of inhibition, defined as the distance between the growth of the probiotic organism and the lawn of *V. parahaemolyticus*, were measured in mm (28).

Biofilm assay

Biofilm formation was quantified using a modification of the crystal violet staining method (6). Bacteria were grown for 24 h before being diluted 10³-fold into 200 μ l of mYP30 in a polystyrene 96-well plate which was then incubated at 27 °C for 24 h under static conditions. Biofilms in the wells were washed with ASW twice,

stained with 0.2% crystal violet for 20 min, washed twice with ASW, and biofilm-bound crystal violet was eluted with 95% ethanol for 30 min before being measured at OD₅₈₀. Each candidate probiont was tested in three wells per experiment and each experiment was repeated twice.

***Artemia nauplii* challenge**

Artemia cysts (0.075 g) were hatched in an inverted 60 ml syringe, covered with perforated plastic wrap, containing 60 ml HEPPS-buffered (10 mM, pH 8.2) sterile ASW at 28 °C for 24 h with constant aeration and fluorescent light. To maintain pH and maximize *Artemia* hatching rate, an increased buffer capacity is required to avoid a drop in the pH due to the acid produced by cysts during hatching (29). Hatched nauplii (2 ml, containing approximately 400 nauplii) were transferred to 50 ml centrifuge tubes containing 26.3 ml HEPPS-buffered (10 mM, pH 8.2) ASW. Nauplii were fed autoclaved *Escherichia coli* K-12 cells at a final concentration of 10⁷ cells/ml during hatching and every 24 h. Candidate probionts were washed twice in sterile ASW by centrifugation at 6,000 × *g* for ten minutes at 4 °C. Nauplii were treated with candidate probionts after hatching and every 24 h. The centrifuge tubes were placed in a rotator set to 4 rpm at 30 °C. After incubation for 24 h, 1.6 ml mYP30 was added to each tube and nauplii were challenged with 1×10⁵ CFU/ml washed *V. parahaemolyticus* cells. *V. parahaemolyticus* cells were washed twice in sterile ASW by centrifugation at 5,000 × *g* for five minutes at room temperature. To count nauplii, the tubes were inverted five times to mix the nauplii suspension, 1 ml

of the nauplii suspension was transferred into each well of a tri-well petri dish containing 5 ml of ASW with 0.05% agar (to slow nauplii movement during counting), and nauplii were viewed under a dissecting microscope. Nauplii were counted at 24 h and 72 h to quantify survival. Nauplii which showed any signs of movement were counted as alive. Each treatment was tested in three tubes and was repeated twice. Water in each tube was not changed during the experiment.

White leg shrimp (*L. vannamei*) post-larvae challenge:

L. vannamei post-larvae (PL) were purchased from Miami-Aquaculture (Boynton Beach, Florida) and maintained in 3% ASW in a 20L aquarium tank with filtration and weekly water changes. PLs were fed Hagen® Fluval® Shrimp Granules daily. PLs were transferred to 250 ml glass bottles with 200 mL 3% ASW and provided aeration through air stones. PLs were pre-treated with potential probionts at 10^6 CFU/ml every 24 hours. After the initial 24 h incubation, PLs were challenged with *V. parahaemolyticus* cells at a concentration of 10^6 CFU/ml, which were grown for 24 h in mLB30 and harvested by centrifugation (6,000 x *g*, 5 min, 4C), and washed twice in ASW. PLs were incubated at 29 °C and survivors were counted at 24, 48 and 72 h time points (28).

Competitive Biofilm assay:

The competitive biofilm assay was carried out using a modification of the assay developed by Zhao et al (6). Probiotic bacteria were grown for 24 h, added to 5

mL of mYP30 in tri-well plates containing UV sterilized glass coverslips at a concentration of 10^4 CFU/mL, and incubated at 27 °C for 24 h. After 24 h the coverslips were removed, washed in sterile 3% artificial seawater and placed into fresh mYP30 in a new tri-well plate. At this time, *V. parahaemolyticus* PSU5579Sm was added at a concentration of 10^5 CFU/ml. A cover slip was removed each subsequent 24 h period, washed in 3% ASW and vortexed for 2 minutes with sterile glass beads to remove the biofilm. This mixture was serially diluted and spot plated (10 µl/spot) on mYP30 agar plates to determine concentration in CFU/cover slip. The supernatant was sampled at the same time to determine planktonic cell concentration of both organisms using serial dilution and differential media to plate and count CFU/ml. Samples were taken for up to 96 hours to track the changes in planktonic and biofilm cell concentrations (6).

Minimum Inhibitory Concentration Assay:

The minimal inhibitory concentrations (MIC) of TDA against *V. parahaemolyticus* was assessed using a broth dilution method in microtiter plates. Overnight bacterial cultures were diluted to 10^5 CFU/ml in LB30 and treated with serial dilutions of pure TDA. After 24 h incubation at 27 °C, growth was assessed using OD600 and serial dilution with spot plating to determine CFU/ml. Three independent experiments were done and each independent experiment had two replicates (6).

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Table 1. Bacterial Strains used

Strain	Origin	Characteristics	Reference
<i>Phaeobacter inhibens</i> S4Sm	Healthy oyster	Spontaneous Sm ^R ,	[1]
<i>Phaeobacter inhibens</i> S4SmKm CS4KN		Harboring pSUP203 Km ^r	C. Schuttert
<i>Phaeobacter inhibens</i> $\Delta clpX$ WZ10	Insertional mutagenesis	No TDA production	[1]
<i>Phaeobacter inhibens</i> $\Delta exoP$ WZ20	Insertional mutagenesis	Reduced Biofilm formation	[1]
<i>Phaeobacter inhibens</i> $\Delta pgaI$ CS001	Insertional mutagenesis	No TDA production, no biofilm formation, no AHL production	C. Schuttert
<i>Phaeobacter inhibens</i> $\Delta pgaR$ CS002	Insertional mutagenesis	No TDA Production, no biofilm formation	C. Schuttert
<i>Phaeobacter inhibens</i> $\Delta luxO$ CS003	Insertional mutagenesis	Increased TDA production	C. Schuttert
<i>Vibrio parahaemolyticus</i> PSU5579Sm	Shrimp farms, Thailand	Spontaneous Sm ^R	(28)
<i>Bacillus pumilus</i> RI06-95	Marine Sponge		
<i>Pseudoalteromonas piscicida</i> GR1	Seawater		This study
<i>Pseudoalteromonas flavipulchra</i> GR4	Seawater		This study
<i>Pseudoalteromonas piscicida</i> JL1	Seawater		This study
<i>Pseudoalteromonas piscicida</i> JL12	Seawater		This Study
<i>Pseudoalteromonas piscicida</i> JL15	Brine Shrimp		This study
<i>Pseudoalteromonas flavipulchra</i> JL18	Brine Shrimp		This Study
<i>Pseudoalteromonas arabiensis</i> JL29	Brine Shrimp		This Study

<i>Bowmanella denitrificans</i> JL63	Whiteleg Shrimp	This Study
<i>Bacillus pumilus</i> JL70	Gulf Shrimp intestines	This Study
<i>Bacillus pumilus</i> HR1	Lobster Shell	(28)

Figures

Figure 1. Detection of *pirA*- and *pirB*-like genes found on the pVPA3-1 plasmid. Imaged on a 2% agarose gel with ethidium bromide (0.4 $\mu\text{g}/\text{ml}$). PCR amplification was performed using primers VpPirA-284F/R and VpPirB-392F/R as described by Han et al. (10). A band at 284 bp indicates a *pirA*, while presence of a *pirB* amplicon is indicated by a band at 392 bp (28).

Figure 2. Survival rates of *Artemia* nauplii when treated with *V. parahaemolyticus* at 10^5 CFU/ml 24 hours after hatching. Strain PUS5579 caused the highest mortality and was selected for use in further experiments (28).

Figure 3. Biofilm formation quantified by the crystal violet method. Cultures were grown in 96-well plates for 24 hours at 27 °C. Biofilms in the wells were washed with ASW, stained with 0.2% crystal violet, washed again, and biofilm-bound crystal violet was eluted using 95% ethanol and then optical density at 580 nm was measured. These data are an average of three biological replicates with three technical replicates per experiment. Error bars equal one standard deviation. * Indicates statistically significant difference from *V. parahaemolyticus* PSU5579 (ANOVA $p < 0.05$) (28).

Figure 4. *Artemia* nauplii survival when treated with *Pseudoalteromonas* strains alone at 10^5 CFU/ml daily for 72 h. These data are an average of three biological replicates with three technical replicates per experiment. Error bars equal one standard deviation. * Indicates statistically significant difference from the Control (ANOVA, $p < 0.05$) (28).

Figure 5. *Pseudoalteromonas* and *Bowmanella* strains decreased *Artemia* nauplii survival when pretreated for 24 hours at 10^4 CFU/ml and then challenged with PSU5579 at 10^5 CFU/ml. Error bars equal one standard deviation. These data are an average of three biological replicates with three technical replicates per experiment. * Indicates statistically significant difference from the *V. parahaemolyticus* PSU5579 treatment (ANOVA, $p < 0.05$) (28).

Figure 6. *B. pumilus* strains did not improve survival of *Artemia* nauplii. *Artemia* were pre-treated with *B. pumilus* at concentrations of 10^4 CFU/ml (1E4), 10^5 CFU/ml (1E5), or 10^6 CFU/ml (1E6) and then challenged with *V. parahaemolyticus* PSU5579 at 10^5 CFU/ml. Candidate probionts were added every 24 hours and survivors were counted at 48 hours. These data are an average of three biological replicates with three technical replicates per experiment. Error bars equal one standard deviation. (28).

Figure 7. Pre-treatment of *Artemia* with *P. inhibens* S4Sm improves survival.

Artemia were pre-treated with *P. inhibens* S4Sm at 10^6 CFU/ml for 24 h improves survival 48 h after challenge with *V. parahaemolyticus* PSU5579 at 10^5 CFU/ml. *P. inhibens* S4Sm was added at the start of the experiment and every 24 h. These data are an average of three biological replicates with three technical replicates per experiment. Error bars equal one standard deviation. Different letters indicate statistical significance among groups (ANOVA, $p < 0.05$)(28).

Figure 8. *L. vannamei* PL survival when treated with probiotic strains alone at 10^6 CFU/ml daily for 72 h. These data are an average of three independent experiments with three technical replicates per experiment. Error bars equal one standard deviation. * Indicates statistically significant difference from the control ANOVA, $p < 0.05$) (28).

Figure 9. *P. inhibens* S4Sm and *P. piscicida* JL1Sm provide significant protection to *L. vannamei* PLs. When *L. vannamei* PLs were pre-treated with *P. inhibens* S4Sm and *P. piscicida* JL1Sm at 10^6 CFU/ml and challenged with *V. parahaemolyticus* PSU5579 at 10^6 CFU/ml after 24 hours. These data are an average of three biological replicates with three technical replicates per experiment. Error bars equal one standard deviation. * Indicates statistically significant difference from the challenged control (ANOVA, $p < 0.05$) (28).

Figure 10: Relative shrimp survival when environment was pre-treated with *P. inhibens* S4smkm and mutant strains of *P. inhibens* S4. Shrimp were added at 24 hours and challenged with *V. parahaemolyticus* PSU5579. These data are an average of three biological replicates with three technical replicates per experiment. Error bars equal one standard deviation. * Indicates statistically significant difference from the uninoculated control shrimp (ANOVA, $p < 0.05$).

Figure 11. Biofilm formation by *V. parahaemolyticus* PSU5579 and *P. inhibens* S4SmKm is not inhibited during co-culture. These data are a log-transformed average of at least four biological replicates with three technical replicates per experiment. Error bars equal one standard deviation.

Figure 12

Figure 12. The effects of co-culture of *V. parahaemolyticus* PSU5579 with *P. inhibens* S4SmKm wild type (grey) or mutants (*luxO* (light blue), *clpX* (green), or *pgaR* (dark blue)) on PSU5579 biofilm (panel a) or planktonic (panel b) cell densities. Biofilm or planktonic cell density for the *V. parahaemolyticus* PSU5579 only control is shown in yellow. Time points represent the time after the start of the experiment (addition of *P. inhibens* cells, 10^4 CFU/ml). *V. parahaemolyticus* cells were added (10^5 CFU/ml) at 24 h. These data are log-transformed averages, Error bars represent one standard deviation.

Figure 13. Growth of *V. parahaemolyticus* PSU5579 in LB30 containing various concentrations of TDA. Cells were inoculated at an initial concentration of 1×10^5 CFU/ml and incubated at 27°C for 24 h. Final cell densities were determined by serial dilution and plating. Data are a log-transformed average of two biological replicates with at least four technical replicates each. Error bars equal one standard deviation. (28).

MANUSCRIPT II

Publication status: Preparing to submit to Microbiology Resource Announcements

Title: Draft Genome Sequence of *Halobacteriovorax* sp. Strain GB3, a Predatory Bacterium Isolated from Narragansett Bay that Preys on Shrimp Pathogen *Vibrio parahaemolyticus* PSU5579

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Key Words: *Halobacteriovorax*, BALO

Abstract

The draft genome of *Halobacteriovorax* sp. GB3, isolated after enrichment on the Acute Hepatopancreatic Necrosis Disease (AHPND)-inducing *Vibrio parahaemolyticus* PSU5579, is presented. The 3.62-Mbp genome of this predatory bacterium contains 3493 coding sequences. Sequence comparisons between 15 different proteins suggest that this represents a new species of *Halobacteriovorax*.

Body

Halobacteriovorax, a genus of marine *Bdellovibrio* and like organisms (BALOs) within the Deltaproteobacteria, was first differentiated from freshwater *Bacteriovorax* in 2015 (1). Currently, two named species belong to this genus: *Halobacteriovorax litoralis* and *Halobacteriovorax marinus* (1, 2). It has been proposed that BALOs could help prevent AHPND in shrimp aquaculture (3). *Halobacteriovorax* sp. GB3 was isolated from Narragansett Bay after enrichment on a strain of AHPND-inducing *Vibrio parahaemolyticus*. Sea water was filtered through a 0.45 µm filter to remove sediments and larger organisms. The filtrate was added to nutrient-free soft agar containing 10⁶ CFU/ml *V. parahaemolyticus* PSU5579 and poured onto mYP30 (5 g/L peptone, 1 g/L yeast extract, 30 g/L Instant Ocean®, pH7.5) + 1.5% agar plates. Plates were observed for plaque formation after 5 days incubation at 27°C. Picked plaques were added to stationary phase cultures of *V. parahaemolyticus* PSU5579 growing in mLB30 (10 g/L tryptone, 5 g/L yeast extract,

30 g/L Instant Ocean[®], pH7). This process was repeated; co-cultures were visualized by microscopy to determine if contamination was present. After 7 passes through the plaque process, the co-cultures were centrifuged at $6,000 \times g$ for 5 minutes at 4°C and the supernatant filtered through a 0.22 µm filter. Filtrate was added to a stationary phase culture of *V. parahaemolyticus* PSU5579 and grown shaking at 27°C. After 24 hours the co-culture was observed to be contaminant-free via microscopy.

Genomic DNA was isolated using Promega's Wizard genomic DNA purification kit. *Halobacteriovorax* sp. GB3 was grown shaking overnight at 27°C in a stationary phase culture of *V. parahaemolyticus* in mLB30 broth. Cultures were centrifuged for 5 minutes at $6,000 \times g$; supernatant was filtered through a 0.22 µm filter to remove prey cells. Isolated DNA was resuspended in 2 mM Tris-HCl buffer (Bio Basic). Sequencing was performed at the Rhode Island Genomics and Sequencing Center using an Illumina MiSeq Sequencer. Sequence trimming and de novo assembly was performed using CLC Genomics Workbench (version 12). The resulting contigs were processed using CLC Microbial Genome Finishing module. The draft genome contains 9 contigs, with a total sequence length of 3,622,901 bp and 36.9% GC content. Gene annotation was performed using Rapid Annotations using Subsystems Technology (RAST) resulting in 3493 coding sequences and 39 RNAs (4). 227 SEED subsystems were identified containing 19% of the coding sequences (5).

The 16S rRNA gene was PCR-amplified from genomic DNA using 16S primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') (6).

Comparison using the National Center of Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST), determined the 16S rRNA gene of *Halobacteriovorax* sp. GB3 shares 93.47% nucleotide identity with *H. litoralis* JS5, and 92.87% identity with *H. marinus* BE01 and *H. marinus* SJ respectively. A RAST comparison of 15 conserved genes revealed *Halobacteriovorax* sp. GB3 shares an average of 80.6% amino acid identity with *H. marinus* BE01 and *H. marinus* SJ, whereas the two *H. marinus* species are 100% identical across these genes (Table 2). For these reasons, we propose that *Halobacteriovorax* sp. GB3 is a novel species.

Accession number(s). The draft genome of *Halobacteriovorax* sp. GB3 has been deposited in NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA613698

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Table 1. Bacterial strains compared using BLAST analysis

STRAIN NAME	REFERENCE
<i>HALOBACTERIOVORAX MARINUS</i> BE01	(7)
<i>HALOBACTERIOVORAX MARINUS</i> SJ	(8)
<i>HALOBACTERIOVORAX SP.</i> BALO_7	(9)
<i>HALOBACTERIOVORAX SP.</i> GB3	This Study

Table 2. Comparison of conserved genes

Gene name	Percent amino acid identity				
	GB3 Vs BE01	GB3 vs SJ	GB3 vs BALO_7	BE01 vs SJ	BE01 vs BALO_7
Na(+)-translocating NADH-quinone reductase subunit E	91.13	91.13	90.15	100	88.67
NADH-ubiquinone oxidoreductase chain B	95.62	95.62	95	100	32.12
Rod shape-determining protein MreB	93.04	93.04	91.04	100	91.62
LSU ribosomal protein L14p	92.62	92.62	92.62	100	88.52
LSU ribosomal protein L16p	91.11	91.11	95.62	100	88.97
SSU ribosomal protein S10p	93.14	93.14	92.16	100	90.2

Gene name	GB3 Vs BE01	GB3 vs SJ	GB3 vs BALO7	BE01 vs SJ	BE01 vs BALO7
Cytochrome b/b6, N-terminal	95.77	95.77	95	100	94.23
DNA-directed RNA polymerase omega subunit	91.43	91.43	90	100	88.57
SSU ribosomal protein S12p	94.35	94.35	91.94	100	92.74
RNA polymerase sigma factor RpoD	86.11	86.11	81.03	100	79.69
DNA polymerase III beta subunit	67.92	67.92	61.99	100	60.38
RecA protein	81.77	81.77	84.12	100	82.02
Chaperone protein DnaK	33.21	33.21	33.21	100	86.53
Heat shock protein GroEL	88.83	88.83	86.36	100	83.52
DNA gyrase subunit A	82.68	82.68	79.29	100	78.64