2014

CHARACTERIZATION OF THE PROBIOTIC MECHANISM OF PHAEOBACTER GALLAECIENSIS S4 AGAINST BACTERIAL PATHOGENS

Wenjing Zhao

University of Rhode Island, mayzhwj@gmail.com

CHARACTERIZATION OF THE PROBIOTIC MECHANISM OF PHAEOBACTER

GALLAECIENSIS S4 AGAINST BACTERIAL PATHOGENS

BY

WENJING ZHAO

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

CELL AND MOLECULAR BIOLOGY

UNIVERSITY OF RHODE ISLAND

2015

DOCTOR OF PHILOSOPHY DISSERTATION

OF

WENJING ZHAO

APPROVED:

Dissertation Committee:

Major Professor   David R. Nelson

                    Paul S. Cohen

                    David C. Rowley

                    Nasser H. Zawia
        DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

2015

Abstract

Infections by pathogenic marine bacteria are a major problem for both the shellfish and finfish aquaculture industries, causing severe disease and high mortality, which seriously affect aquaculture production and cause significant economic loss. Marine pathogens like *Vibrio tubiashii* and *Roseovarius crassostreae* frequently cause disease in a variety of shellfish. With the understanding that the use of antibiotics in large-scale aquaculture leads to the development and transfer of antibiotic resistance, investigation of probiotic approaches for the prevention of infectious disease has become important. In manuscript I, screening of bacterial isolates from Rhode Island marine organisms and environment using agar-based assay methods for detection of antimicrobial activity against oyster pathogens led to the isolation of candidate probiotic bacteria *Phaeobacter gallaeciensis* S4. *P. gallaeciensis* S4 is a gram-negative α-*Proteobacteria* within the *Roseobacter* clade. Pretreatment of larval and juvenile oysters for 24 h with 10^4 CFU / mL of *P. gallaeciensis* S4 protected larval oysters against mortality resulting from challenge with *R. crassostreae* and *V. tubiashii*. Probiotic isolates had no negative impact on oyster survival. These results suggest the potential of marine bacterial isolate *P. gallaeciensis* S4 to serve as probiotic bacterium to control the infection and disease by bacterial pathogens in the culture of *Crassostrea virginica*.

The probiotic bacterium *P. gallaeciensis* S4, isolated from the inner shell surface of a healthy oyster, secretes the antibiotic tropodithietic acid (TDA), is an excellent biofilm former, and increases oyster larvae survival when challenged with bacterial

pathogens. In manuscript II, we investigated the specific roles of TDA secretion and biofilm formation in the probiotic activity of S4Sm (a spontaneous streptomycin-resistant mutant of the parental S4). For this purpose, mutations in *clpX* (ATP-dependent ATPase) and *exoP* (an exopolysaccharide biosynthesis gene) were created by insertional mutagenesis using homologous recombination. Mutation of *clpX* resulted in the loss of TDA production, no decline in biofilm formation, and loss of the ability of S4Sm to inhibit the growth of *Vibrio tubiashii* and *Vibrio anguillarum in vitro*. Mutation of *exoP* resulted in a ~70% decline in biofilm formation, no decline in TDA production, and delayed inhibitory activity towards *Vibrio* pathogens *in vitro*. Both *clpX* and *exoP* mutants exhibited reduced ability to protect oyster larvae from death when challenged by *Vibrio tubiashii*. Complementation of the *clpX* and *exoP* mutations restored the wild type phenotypes. We also found that pre-colonization by S4Sm was critical for this bacterium to inhibit pathogen colonization and growth. Our observations suggest that probiotic activity by S4Sm involves contributions from both biofilm formation and the production of the antibiotic TDA.

In manuscript III, we found that culture supernatant of S4Sm down-regulates protease activity in *V. tubiashii* cultures. The effects of S4Sm culture supernatant on the transcription of several genes involved in protease activity, including *vtpA*, *vtpB*, and *vtpR* (encoding metalloproteases A and B and their transcriptional regulator, respectively), were examined by qRT-PCR. Expression of *vtpB* and *vtpR* were reduced to 35.9% and 6.6%, respectively, compared to an untreated control. In contrast, expression of *vtpA* was not affected. A *V. tubiashii* GFP-reporter strain was constructed to detect the inhibitory compounds. Three molecules responsible for *V.*

tubiashii protease inhibition activity were isolated from S4Sm supernatant and identified as N-acyl homoserine lactones (AHLs): *N*-(3-hydroxydecanoyl)-L-homoserine lactone, *N*-(dodecanoyl-2,5-diene)-L-homoserine lactone and *N*-(3-hydroxytetradecanoyl-7-ene)-L-homoserine lactone, and their half maximal (50%) inhibitory concentrations (IC) against *V. tubiashii* protease activity are 0.264 μM, 3.713 μM and 2.882 μM, respectively. Our qRT-PCR data demonstrated that exposure to the individual AHL reduced transcription of *vtpR* and *vtpB*, but not *vtpA*. Treatment with a combination of three AHLs (any two AHLs or all three AHLs) on *V. tubiashii* showed that there were additive effects among these three AHL molecules upon protease inhibition activity. These AHL compounds may act by disrupting the quorum-sensing pathway that activates protease transcription of *V. tubiashii*.

ACKNOWLEDGEMENTS

PREFACE

This dissertation has been prepared in the Manuscript Format according to the guidelines of the Graduate School of the University of Rhode Island. The dissertation includes an introduction and the following three manuscripts:

The first manuscript: "Probiotic Strains for Shellfish Aquaculture: Protection of Eastern Oyster, *Crassostrea virginica*, Larvae and Juveniles Against Bacterial Challenge" was published in Journal of Shellfish Research in 2013.

The second manuscript: "Contributions of tropodithietic acid and biofilm formation to the probiotic activity of *Phaeobacter gallaeciensis*" will be resubmitted to PLOS ONE.

The third manuscript: "The probiotic bacterium, *Phaeobacter gallaeciensis* S4, down-regulates protease virulence factor transcription in the shellfish pathogen, *Vibrio tubiashii*, by quorum quenching" has been written in the same form as the second manuscript and will be submitted for publication.

TABLE OF CONTENTS

CONTENT	PAGE

LIST OF TABLES

MANUSCRIPT III

LIST OF FIGURES

LITERATURE REVIEW

Overview of *Phaeobacter gallaeciensis*

Abstract

Phaeobacter gallaeciensis, a member of the gram-negative α-*Proteobacteria*, belongs to the *Roseobacter* clade. The *Roseobacter* clade, an important member of the marine microbiota, accounts for as much as ~40% of prokaryotic DNA from the ocean and plays an important role in the organic sulfur cycle of the ocean. *P. gallaeciensis* exhibits a diverse ecological distribution and is found to be both free-living and host-associated. This indicates that *P. gallaeciensis* is metabolically versatile, able to survive and thrive in a variety of environments. Also these traits can be supported by their genome sequences. Analysis of completed genomes indicated the ability to produce various secondary metabolites. *P. gallaeciensis* is able to secrete tropodithietic acid (TDA), a broad spectrum antibiotic. In addition to antibiotics, *P. gallaeciensis* also produce N-acyl homoserine lactones, which are commonly used by Gram-negative bacteria for quorum sensing. Moreover, genes encoding for roseobacticides and siderophore can also be found in *P. gallaeciensis* genomes. Comparison of *P. gallaeciensis* genomes with other *Roseobacter* strains revealed unique or characteristic features for *P. gallaeciensis*. *P. gallaeciensis* has been demonstrated to exhibit probiotic activity with many marine host species, and TDA plays an important role in probiotic activity of *P. gallaeciensis*.

Introduction

Members of the genus *Phaeobacter* are gram-negative α-*Proteobacteria*, and belong to the *Roseobacter* clade. The *Roseobacter* clade, an important member of the marine microbiota, accounts for as much as ~40% of prokaryotic DNA from the ocean and plays an important role in the organic sulfur cycle of the ocean [1-3]. Although *Phaeobacter* is exclusively isolated from marine or hypersaline environments [4], it has a diverse and broad ecological distribution, from coastal environments to fish farms [5]. *Phaeobacter* are found to be free-living, particle associated (with microalgae and rotifers [6]), or in commensal relationship with marine phytoplankton, invertebrates (cephalopods [7]) and vertebrates (turbot larva [8] and seahorses [9]). Several species from *Roseobacter* clade, *Phaeobacter gallaeciensis*, *Phaeobacter inhibens* and *Ruegeria mobilis*, have been shown to exhibit inhibitory activity against marine pathogens and protect fish larvae from infections by these pathogenic bacteria [10].

Roseobacter gallaeciensis was first reported in 1998 [11], and reclassified as the type species of a new genus, *Phaeobacter*, as *Phaeobacter gallaeciensis* in 2006 [12]. *P. gallaeciensis* strains are generally isolated from alga or larval cultures of marine fish [10]. It is a short rod with 1-2 flagella on one or both poles [10,13,14]. It can form rosettes, is an excellent biofilm former, and a dominant colonizer of surfaces in marine environments [13,14]. *P. gallaeciensis* has been used as a probiotic treatment to reduce the density of the fish pathogen *Vibrio anguillarum*, resulting in the prevention of vibriosis in the cod [6] or turbot larvae [15]. Due to its ecological and aquaculture importance, this review will focus on the current research progress about *P. gallaeciensis*, including knowledge about

genomes, metabolism, secondary metabolite secretions and potential probiotic mechanisms.

Main body

1. Genomes of *P. gallaeciensis*

To date, only three *P. gallaeciensis* completed genomes are available, and they are *P. gallaeciensis* strains 2.10 [16], DSM 17395 [16] and DSM 26640 [17]. Additionally, two draft genomes from *P. gallaeciensis* strains ANG1 [18] and BS107 [16] are available. Comparison of those three completed genomes revealed that strains 2.10 and DSM 17395 share more similarities with each other than with DSM 26640. The genomes of 2.10 and DSM 17395 are 4.16 Mb and 4.23 Mb (< 2% difference in size), respectively, and each is composed of a single circular chromosome and three plasmids. On the nucleotide level, the genomes differ by only 3% and the chromosome and plasmids extensive synteny. Strain 2.10 has 3798 ORFs, and DSM 17395 harbors 3960 ORFs; they share a total of 3438 coding sequences (or ~87-91% of their ORFs, respectively). The DSM26640 genome is 7-9% larger at 4.54 Mb. It is organized into a single circular chromosome and seven plasmids, which has 4437 ORFs (Table 1). Comparison with other *Roseobacter* genomes revealed genomic traits that are characteristic for *P. gallaeciensis* strains. The two finished *P. gallaeciensis* genomes (2.10 and DSM 17395) possess 74 orthologous genes that are not present in other *Roseobacter* bacteria [16]. Most of these genes show no functional annotations; however, two sets of genes with functions can be potentially used as unique chemotaxonomic markers for this species. The two sets of genes are two copies of a chromosomally encoded D-alanine poly-ligase (*dltA*) and a cluster of genes for biosynthesis and

transport of siderophore. The *dltA* gene is involved in biosynthesis of lipoteichoic acid, which is a constituent of the Gram-positive bacteria cell wall in many organisms. This suggests that *P. gallaeciensis* may have an uncommon cell envelope composition or that the annotation is not correct. A second feature is the presence of a cluster of genes involved with siderophore synthesis. This suggests that *P. gallaeciensis* may use the siderophore to facilitate iron uptake from environment, perhaps allowing it to outcompete pathogenic bacteria [16]. The differences between *P. gallaeciensis* genomes with other members of the *Roseobacter* clade suggest *P. gallaeciensis* potential to adapt to a variety of marine environments and also allows it to associate with a wide variety of hosts.

2. Metabolism of *P. gallaeciensis*

P. gallaeciensis is strictly aerobic heterotrophic [19-21] and metabolically versatile [16,21], which could contribute to its wide ecological distribution. Here features of the central carbon and sulfur metabolisms in *P. gallaeciensis* will be reviewed.

2.1. Central carbon metabolism in *P. gallaeciensis*

2.1.1. Pathways for glucose catabolism

The carbon core metabolism of *P. gallaeciensis* possesses three potential routes for glucose catabolism, as predicted from the annotated genome sequence. Glucose can be alternatively catabolized through glycolysis (EMP), the Entner-Doudoroff pathway (EDP) and the pentose phosphate pathway (PPP) [19]. By using ^{13}C labeled isotopes the metabolic fluxes in the central carbon metabolism of *P. gallaeciensis* was obtained (Fig. 1) [19]. The use of $[1-^{13}C]$ glucose by each different pathway yields to

a different labeling pattern in specific amino acids, which can be used as a differentiation marker of the flux. Interestingly, *P. gallaeciensis* mainly uses the ED pathway during growth on glucose. The quantification of different fluxes revealed that the use of the ED pathway amounts to >99%, whereas glycolysis and PPP pathways only contribute <1% (Table 2). Most organisms, such as *E. coli* [22] and *B. subtilis* [23], use glycolysis and PPP pathways, though at different ratios. This should result from lack of phosphofructokinase, which converts fructose-6-phosphate to fructose 1,6-bisphosphate, in *P. gallaeciensis*. The exclusive utilization of ED pathway has been also observed in *Pseudomonas* and *Arthrobacter* for the same reason [24,25].

2.1.2. Anaplerotic reactions

Oxaloacetate, as a central metabolite, can be formed by two major pathways, carboxylation involving pyruvate carboxylase or via pyruvate dehydrogenase and the TCA cycle. By using the same isotope-labeling method both pathways are demonstrated to be active in *P. gallaeciensis* [19].

2.2. Sulfur metabolism in *P. gallaeciensis*

Genome data from sequenced bacteria from the *Roseobacter* clade revealed that pathways for the degradation of sulfur metabolic pathways are widespread [26]. The main source of marine organic sulfur is dimethylsulfoniopropionate (DMSP), which is produced by a wide range of marine organisms and especially large amounts by dinoflagellates [27]. DMSP is generally degraded by marine bacteria via two competing pathways, one is

lysis to volatile dimethyl sulfide (DMS), which in atmosphere is converted to condensation nuclei for water droplets, by a DMSP lyase; the other way is via demethylation to 3-(methylmercapto) propionic acid (MMPA), which may be transformed into methanethiol (MeSH) (Fig. 2) [28,29]. DMS and MeSH are important participants in the biogeochemical sulfur cycle [28,30]. DMSP in the ocean attracts *Roseobacter* bacteria, including *P. gallaeciensis*, which use it as carbon and sulfur source. Based on the genomic sequence, *P. gallaeciensis* possess genes involved in both DMSP degradation pathways, include a gene encoding the DMSP lyase DddP, which is encoded on its chromosome, and a *dmd*-gene cluster for the demethylation pathway located on a plasmid. Using deuterium labeled [^{2}H$_6$] DMSP and monitoring the derived volatile sulfur with the isotopic signal confirmed the prediction above based on the genomic sequence [29]. Degradation of DMSP by *P. gallaeciensis* contributes to sulfur cycling in the world's oceans [29].

3. Secondary metabolites

Genome sequence analysis suggested that *P. gallaeciensis* strains are able to produce several interesting secondary metabolites. Tropodithietic acid, a broad-spectrum antibiotic, is able to inhibit many human or marine pathogens, especially *Vibrio* species [10,31]. TDA production has been demonstrated to play an important role in probiotic activity of *P. gallaeciensis* [6,32]. In addition, other secondary metabolites, like N-acyl homoserine lactones (AHLs), can be produced by *P. gallaeciensis* strains and several AHL synthases are found in their genomes [33]. It was shown that *P. gallaeciensis* BS107 strain genome harbors a hybrid polyketide synthase / non-ribosomal peptide

synthetase cluster, which potentially encode enzymes involved in the synthesis of various pharmaceutically important natural products [34]. It was also reported that BS107 is able to produce potent but selective algicides (named roseobacticides A and B, small molecules modulating the symbiosis relationship between algae and bacteria) upon sensing the lignin-derived breakdown product p-coumaric acid [35]. Moreover, several *P. gallaeciensis* strains encode a cluster for biosynthesis and transport of an iron-chelating siderophore, which is located on one of their plasmids [16]. Production of various secondary metabolites suggested diverse interaction between *P. gallaeciensis* and other marine species, and adaptation of the strains to different ecological niches. Several important secondary metabolites are selected to be reviewed here.

3.1. TDA production in *P. gallaeciensis*

3.1.1. TDA biosynthesis and regulation

TDA is a sulfur-containing and broad-spectrum antibiotic with inhibitory activity towards a wide range of human- and marine-pathogens, including both gram-negative and gram-positive bacteria [2]. The TDA structure has been resolved [2]. A proposed model for biosynthesis of TDA in *P. gallaeciensis* DSM 17395 is presented in Figure 3, combining the results from several publications [16,36,37]. By using transposon insertion mutagenesis and screening for mutants with less yellow pigment from TDA, 26 genes were identified to be essential for TDA synthesis (Fig. 3) [16]. These genes are located in a cluster on a plasmid, including the well-known key TDA production genes *tdaABCEF* [2], and the rest genes are scattered

over the chromosome and affiliated with different pathways of the primary metabolism.

Several regulatory genes for TDA synthesis have also been identified, including *tdaA* [33], *iorR* [38], *clpX* [2] and *pgaR* [33]. TdaA was shown to be able to induce expression of *tdaBEF* in the same operon [33]. The *iorR* gene is located adjacent to gene *ior1* and was shown to be important for the transcription of *ior1* and phenylalanine catabolism [38]; ClpX is an AAA+ ATPase that functions as an unfoldase chaperon for ClpP (ATP-dependent protease) and with ClpP forms the multimeric ClpXP protease [39]. PgaR is part of PgaRI, a LuxRI-type quorum-sensing system, which up-regulate the transcription of *tdaA*. Mutations in *pgaR* or *pgaI* caused reduction of TDA production in *P. gallaeciensis* [33]. This indicated that QS is involved in regulation of TDA production. Interestingly, TDA may also function as an autoinducer, as addition of exogenous TDA into QS mutants increases the expression of TDA synthesis genes in *P. gallaeciensis* [33] and *Silicibacter sp.* TM1040 [40]. Cultivation conditions also influence TDA production. DSM 17395 produces 10-fold-higher amounts of TDA during shaken culture conditions than during static conditions [33]. The production of TDA is regulated by a complex regulatory network, which may act at different levels in a regulatory cascade. Such a complex regulatory network would be required by the need of *P. gallaeciensis* to detect, integrate, and respond to various environmental and physiological signals, and to adapt and colonize different niches in the environments [33].

3.1.2. TDA antibiotic mechanism and resistance

Although TDA is well known as a broad-spectrum antibiotic, against many human- and marine-pathogens, to our knowledge, the specific mechanism of action for this antibiotic has not yet been elucidated. Porsby et al. [41] reported that resistance to TDA was hard to select, and enhanced tolerance to TDA is difficult to gain. Further, the bacterial TDA-tolerant phenotype seems to confer to only low-level resistance and is very unstable. From this aspect, TDA is a promising broad-spectrum antimicrobial agent.

3.2. AHL production in *P. gallaeciensis*

Quorum sensing (QS) is a population-dependent chemical communication system used by bacteria to control various biological functions through the production of small signaling molecules, which interact with target cells to regulate the expression of sets of genes within certain bacterial species [42]. By far the most common intercellular signal molecules among Gram-negative are the N-acyl homoserine lactones (AHLs). AHLs are synthesized by a LuxI-type AHL synthase and directly or indirectly bind to their cognate LuxR type transcriptional regulator proteins, thus activating the expression of target genes mediating a specific response [43,44]. Homologs of LuxR and LuxI have been found in *P. gallaeciensis* genomes. The genome of strain 2.10 encodes two LuxI-type AHL synthases and four LuxR-type transcriptional regulators; strain BS107 also has two LuxI-type AHL synthases and three LuxR-type transcriptional regulators. PgaRI, one of the homologs of LuxRI and encoded by *P. gallaeciensis*, has been demonstrated to be important for TDA production. PgaR is the QS regulator and PgaI is responsible for synthesis of N-3-hydroxydecanoyl homoserine lactone (3OHC10-HSL) in *P. gallaeciensis*

[33,45]. Additionally, multiple putative acyl-homoserine lactone synthases have been found in *P. gallaeciensis* genomes, which indicated more than one AHL compound can be produced by *P. gallaeciensis*.

A new class of homoserine lactones, the p-coumaroyl-homoserine lactone (pCA-HSL) [46], is produced by *Silicibacter pomeroyi*, a member of *Roseobacter* clade, in the presence of *p*-coumaric acid, which is secreted by *Emiliania huxleyi*, an environmentally important marine microalga [35]. However, at this time there are no reports demonstrating that *P. gallaeciensis* is able to produce pCA-HSL.

3.3. Roseobacticides production in *P. gallaeciensis*

P. gallaeciensis BS107 and 2.10 are symbionts associated with marine algae [47]. In these symbiont-host relationship, *P. gallaeciensis* secrete antibiotics and auxins that inhibit the growth of potential parasitic bacteria and promote algal growth [2,36,48,49], respectively. Marine algae, in turn, could provide a suitable surface for *P. gallaeciensis* colonization, and also secrete DMSP into the environment. DMSP attracts *P. gallaeciensis*, which use it as carbon and sulfur source [50]. However, Seyedsayamdost et al. [47] reported that *Phaeobacter* symbionts might switch to opportunistic parasites of their hosts due to the roseobacticides secretion from these bacteria. *p*-Coumaric acid, a small molecule secreted by microalgae, *E. huxleyi*, induces *P. gallaeciensis* to produce potent but selective algaecides, which are named as roseobacticides A and B [35]. Roseobacticides A and B are able to kill *E. huxleyi*, and affect two other microalgal strains, the cryptomonad *Rhodomonas salina* and the diatom *Chaetoceros muelleri*, at nM concentrations [35]. A proposed biosynthesis

pathway and structure of these roseobacticides were presented in recent studies [35]. Seyedsayamdost et al. also suggested that roseobacticide biosynthesis might involve an alternative use of compounds within the antibiotic and auxin production pathways and could transform a bacterial symbiont into an opportunistic pathogen [35,47].

3.4. Indigoidine production in other *Phaeobacter* species

Some *Phaeobacter* strains are able to produce indigoidine, an antimicrobial secondary metabolite, other than TDA [51]. *Phaeobacter sp.* strain Y4I produces the blue pigment indigoidine via a nonribosomal peptide synthase (NRPS)-based biosynthetic pathway encoded by a series of genes: *igiBCDFE*. Interestingly, the loss of indigoidine production in an *igiD* null mutant appears to have pleiotrophic effects in strain Y41. The *igiD* null mutant cells gain resistance to hydrogen peroxide, have decreased motility and colonize surfaces more rapidly when compared to the wild type strain [51]. Additionally, competitive co-cultures of *V. fischeri* and *Phaeobacter* Y4I show that the secretion of indigoidine by Y4I strain significantly reduces colonization of *V. fischeri* on artificial surfaces [51].

4. Probiotic mechanisms of *P. gallaeciensis*

Probiotics used in aquaculture have been proposed to have several modes of action: competition for colonization sites with pathogenic bacteria, competition for chemicals or available energy, production of antimicrobial compounds, improvement of the nutritional status of host, enhancement of immune responses of host species and improvement of water quality [52]. Although *P. gallaeciensis* has been shown to exhibit probiotic activity on many marine organisms [6,15], the

probiotic mechanism (or mechanisms) has not been investigated extensively.

TDA production has been demonstrated to play an essential role in *P. gallaeciensis* probiotic activity [1,3,10]. TDA shows inhibitory activity towards a wide range of human- and marine-pathogens, such as *Vibrio* species, *Bacillus subtilis* and *Salmonella typhimurium* [41]. D' Alvise et al. [6,31] demonstrated that a TDA null mutant was no longer able to protect cod larvae against bacterial pathogens.

Analysis of the completed genome sequences revealed that *P. gallaeciensis* is able to synthesis a siderophore [16], an iron chelating agent. Siderophores can dissolve precipitated iron and make it available for microbial growth. Non-pathogenic bacteria, which can produce siderophores, are able to compete for iron with pathogens, whose pathogenicity are known to be based on siderophore production, and outcompete other bacteria requiring iron for growth, especially under iron-limited environment such as ocean [52]. Holmstrøm et al. [53] reported that *Pseudomonas fluorescens* AH2 strain was able to secrete siderophores that efficiently chelate iron resulting in iron deprivation of the pathogen *Vibrio anguillarum* and complete growth arrest. It is reported that *P. gallaeciensis* 2.10 and DSM17395 are able to secrete siderophores in laboratory conditions [16], but the roles of siderophore production in *P. gallaeciensis* on the probiotic activity has not been demonstrated.

Quorum sensing has been demonstrated to regulate a diversity of physiological activities in Gram-negative bacteria. These processes include virulence, symbiosis, competence, conjugation, antibiotic production, motility and

biofilm formation [54]. It has been demonstrated that mutations in *pgaR* or *pgaI* genes caused delayed and reduced TDA production, which is important for *P. gallaeciensis* probiotic activity. Therefore, PgaRI QS system modulates TDA synthesis and, as a result, influence probiotic activity under specific conditions [55].

Goals of this study

Based on the current progress in *P. gallaeciensis* study, the overall goal of our research was to elucidate the probiotic mechanisms of *P. gallaeciensis* S4 against several bacterial pathogens of Eastern oyster, *Crassostrea virginica*, including *Vibrio tubiashii* and *Roseovarius crassostreae*.

P. gallaeciensis S4 was previously isolated from inner shell surface of healthy oyster. The first specific aim of our study was to characterize the physiology, morphology and probiotic activity of the *P. gallaeciensis* S4 strain. A series of experiments were carried out to determine the growth curve, cell shape, inhibitory activity against pathogenic bacteria *in vitro*, and its probiotic activity in oyster larvae challenge assay *in vivo*.

The second specific aim of our research was to determine the specific contributions and roles of tropodithietic acid (TDA) production and biofilm formation to the probiotic mechanisms of *P. gallaeciensis* S4. To investigate this question we constructed TDA production and biofilm formation mutant strains. Interactions between these mutants and pathogens on glass coverslip *in vitro* were characterized. Furthermore, these mutants were tested for their probiotic ability to protect oyster larvae against *V. tubiashii* compared to wild type S4.

The third specific aim of our research was determine the mechanism by which *P. gallaeciensis* S4 uses to down-regulate virulence gene expression in *V. tubiashii*. *P. gallaeciensis* S4 supernatant showed inhibitory activity against protease activity in *V. tubiashii*. Protease activity is regarded as the major virulence factor in *V. tubiashii* [56]. In order to elucidate the mechanism by which S4 down-regulates *V. tubiashii* virulence gene expression, effects of the S4 culture supernatant upon the expression of several *V. tubiashii* virulence genes including *vtpR* (a regulator of protease activity), *vtpA* (encodes metalloprotease A) and *vtpB* (encodes metalloprotease B) were determined by using qRT-PCR. Further, a high-throughput screen for molecules with the ability to repress the *vtpB* protease gene was developed and the active molecules were isolated and identified by using NMR, MS and HPLC.

Reference:

1. Bruhn, J.B., et al., *Ecology, inhibitory activity, and morphogenesis of a marine antagonistic bacterium belonging to the Roseobacter clade.* Appl Environ Microbiol, 2005. **71**(11): p. 7263-70.
2. Geng, H., et al., *Genetic dissection of tropodithietic acid biosynthesis by marine roseobacters.* Appl Environ Microbiol, 2008. **74**(5): p. 1535-45.
3. Brinkhoff, T., H.A. Giebel, and M. Simon, *Diversity, ecology, and genomics of the Roseobacter clade: a short overview.* Arch Microbiol, 2008. **189**(6): p. 531-9.
4. Buchan, A., J.M. Gonzalez, and M.A. Moran, *Overview of the marine roseobacter lineage.* Appl Environ Microbiol, 2005. **71**(10): p. 5665-77.
5. Prado, S., et al., *Inhibitory activity of Phaeobacter strains against aquaculture pathogenic bacteria.* Int Microbiol, 2009. **12**(2): p. 107-14.
6. D'Alvise, P.W., et al., *Phaeobacter gallaeciensis reduces Vibrio anguillarum in cultures of microalgae and rotifers, and prevents vibriosis in cod larvae.* PLoS One, 2012. **7**(8): p. e43996.
7. Barbieri, E., et al., *Phylogenetic characterization of epibiotic bacteria in the accessory nidamental gland and egg capsules of the squid Loligo pealei (Cephalopoda:Loliginidae).* Environ Microbiol, 2001. **3**(3): p. 151-67.
8. Hjelm, M., et al., *Seasonal incidence of autochthonous antagonistic Roseobacter spp. and Vibrionaceae strains in a turbot larva (Scophthalmus maximus) rearing system.* Appl Environ Microbiol, 2004. **70**(12): p. 7288-94.
9. Balcazar, J.L., et al., *Phylogenetic characterization and in situ detection of bacterial communities associated with seahorses (Hippocampus guttulatus) in captivity.* Syst Appl Microbiol, 2010. **33**(2): p. 71-7.
10. Porsby, C.H., K.F. Nielsen, and L. Gram, *Phaeobacter and Ruegeria species of the Roseobacter clade colonize separate niches in a Danish Turbot (Scophthalmus maximus)-rearing farm and antagonize Vibrio anguillarum under different growth conditions.* Appl Environ Microbiol, 2008. **74**(23): p. 7356-64.
11. Ruiz-Ponte, C., et al., *Roseobacter gallaeciensis sp. nov., a new marine bacterium isolated from rearings and collectors of the scallop Pecten maximus.* International Journal of Systematic Bacteriology, 1998. **48**(2): p. 537-542.
12. Martens, T., et al., *Reclassification of Roseobacter gallaeciensis Ruiz-Ponte et al. 1998 as Phaeobacter gallaeciensis gen. nov., comb. nov., description of Phaeobacter inhibens sp. nov., reclassification of Ruegeria algicola (Lafay et al. 1995) Uchino et al. 1999 as Marinovum algicola gen. nov., comb. nov., and emended descriptions of the genera Roseobacter, Ruegeria and Leisingera.* Int J Syst Evol Microbiol, 2006. **56**(6): p. 1293-1304.
13. Karim, M., et al., *Probiotic strains for shellfish aquaculture: protection of Eastern oyster, Crassostreac virginica, larvae and juveniles against bacterial.* Journal of Shellfish Research, 2013.

14. Bruhn, J.B., L. Gram, and R. Belas, *Production of antibacterial compounds and biofilm formation by Roseobacter species are influenced by culture conditions.* Appl Environ Microbiol, 2007. **73**(2): p. 442-50.

15. Prol-Garcia, M.J. and J. Pintado, *Effectiveness of probiotic Phaeobacter bacteria grown in biofilters against Vibrio anguillarum infections in the rearing of Turbot (Psetta maxima) larvae.* Mar Biotechnol (NY), 2013. **15**(6): p. 726-38.

16. Thole, S., et al., *Phaeobacter gallaeciensis genomes from globally opposite locations reveal high similarity of adaptation to surface life.* ISME J, 2012. **6**(12): p. 2229-44.

17. Frank, O., Petersen,J., Goeker,M., Klenk,H.-P., Rohde,M. and Scheuner,C., *Complete genome sequence of the Phaeobacter gallaeciensis type strain CIP 105210T.* Microbial Ecology and Diversity, 11-Dec-2013.

18. Collins, A.J. and S.V. Nyholm, *Draft genome of Phaeobacter gallaeciensis ANG1, a dominant member of the accessory nidamental gland of Euprymna scolopes.* J Bacteriol, 2011. **193**(13): p. 3397-8.

19. Furch, T., et al., *Metabolic fluxes in the central carbon metabolism of Dinoroseobacter shibae and Phaeobacter gallaeciensis, two members of the marine Roseobacter clade.* BMC Microbiol, 2009. **9**: p. 209.

20. Zech, H., et al., *Dynamics of amino acid utilization in Phaeobacter inhibens DSM 17395.* Proteomics, 2013. **13**(18-19): p. 2869-85.

21. Zech, H., et al., *Growth phase-dependent global protein and metabolite profiles of Phaeobacter gallaeciensis strain DSM 17395, a member of the marine Roseobacter-clade.* Proteomics, 2009. **9**(14): p. 3677-97.

22. Fischer, E., N. Zamboni, and U. Sauer, *High-throughput metabolic flux analysis based on gas chromatography-mass spectrometry derived 13C constraints.* Anal Biochem, 2004. **325**(2): p. 308-16.

23. Sauer, U., et al., *Metabolic fluxes in riboflavin-producing Bacillus subtilis.* Nat Biotechnol, 1997. **15**(5): p. 448-52.

24. Lessie, T.G. and P.V. Phibbs, Jr., *Alternative pathways of carbohydrate utilization in pseudomonads.* Annu Rev Microbiol, 1984. **38**: p. 359-88.

25. Lynn, A.R. and J.R. Sokatch, *Incorporation of isotope from specifically labeled glucose into alginates of Pseudomonas aeruginosa and Azotobacter vinelandii.* J Bacteriol, 1984. **158**(3): p. 1161-2.

26. Newton, R.J., et al., *Genome characteristics of a generalist marine bacterial lineage.* ISME J, 2010. **4**(6): p. 784-98.

27. Curson, A.R., et al., *Catabolism of dimethylsulphoniopropionate: microorganisms, enzymes and genes.* Nat Rev Microbiol, 2011. **9**(12): p. 849-59.

28. Dickschat, J.S., C. Zell, and N.L. Brock, *Pathways and substrate specificity of DMSP catabolism in marine bacteria of the Roseobacter clade.* Chembiochem, 2010. **11**(3): p. 417-25.

29. Brock, N.L., et al., *Isotopically labeled sulfur compounds and synthetic selenium and tellurium analogues to study sulfur metabolism in marine bacteria.* Beilstein J Org Chem, 2013. **9**: p. 942-50.

30. Kiene, R.P., L.J. Linn, and J.A. Bruton, *New and important roles for DMSP in marine microbial communities.* Journal of Sea Research, 2000. **43**(3–4): p. 209-224.

31.   D'Alvise, P.W., et al., *Inactivation of Vibrio anguillarum by attached and planktonic Roseobacter cells.* Appl Environ Microbiol, 2010. **76**(7): p. 2366-70.

32.   Wenjing Zhao, C.D., Murni Karim, *Contributions of tropodithietic acid and biofilm formation to the probiotic activity of Phaeobacter gallaeciensis.* 2014.

33.   Berger, M., et al., *Tropodithietic acid production in Phaeobacter gallaeciensis is regulated by N-acyl homoserine lactone-mediated quorum sensing.* J Bacteriol, 2011. **193**(23): p. 6576-85.

34.   Martens, T., et al., *Bacteria of the Roseobacter clade show potential for secondary metabolite production.* Microb Ecol, 2007. **54**(1): p. 31-42.

35.   Seyedsayamdost, M.R., et al., *The Jekyll-and-Hyde chemistry of Phaeobacter gallaeciensis.* Nat Chem, 2011. **3**(4): p. 331-5.

36.   Thiel, V., et al., *Identification and biosynthesis of tropone derivatives and sulfur volatiles produced by bacteria of the marine Roseobacter clade.* Org Biomol Chem, 2010. **8**(1): p. 234-46.

37.   Teufel, R., et al., *Studies on the mechanism of ring hydrolysis in phenylacetate degradation: a metabolic branching point.* J Biol Chem, 2011. **286**(13): p. 11021-34.

38.   Berger, M., et al., *Genetic analysis of the upper phenylacetate catabolic pathway in the production of tropodithietic acid by Phaeobacter gallaeciensis.* Appl Environ Microbiol, 2012. **78**(10): p. 3539-51.

39.   Baker, T.A. and R.T. Sauer, *ClpXP, an ATP-powered unfolding and protein-degradation machine.* Biochim Biophys Acta, 2012. **1823**(1): p. 15-28.

40.   Geng, H. and R. Belas, *TdaA regulates Tropodithietic acid synthesis by binding to the tdaC promoter region.* J Bacteriol, 2011. **193**(15): p. 4002-5.

41.   Porsby, C.H., et al., *Resistance and tolerance to tropodithietic acid, an antimicrobial in aquaculture, is hard to select.* Antimicrob Agents Chemother, 2011. **55**(4): p. 1332-7.

42.   Camilli, A. and B.L. Bassler, *Bacterial small-molecule signaling pathways.* Science, 2006. **311**(5764): p. 1113-6.

43.   Fuqua, C., S.C. Winans, and E.P. Greenberg, *Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators.* Annu Rev Microbiol, 1996. **50**: p. 727-51.

44.   Swift, S., et al., *Quorum sensing as a population-density-dependent determinant of bacterial physiology.* Adv Microb Physiol, 2001. **45**: p. 199-270.

45.   Zhao, W., et al., *The probiotic bacterium, Phaeobacter gallaeciensis S4, down-regulates protease virulence factor transcription in the shellfish pathogen, Vibrio tubiashii, by quorum quenching.* 2014.

46.   Schaefer, A.L., et al., *A new class of homoserine lactone quorum-sensing signals.* Nature, 2008. **454**(7204): p. 595-9.

47.   Seyedsayamdost, M.R., et al., *Roseobacticides: small molecule modulators of an algal-bacterial symbiosis.* J Am Chem Soc, 2011. **133**(45): p. 18343-9.

48.   Rao, D., et al., *Low densities of epiphytic bacteria from the marine alga Ulva australis inhibit settlement of fouling organisms.* Appl Environ Microbiol, 2007. **73**(24): p. 7844-52.

49.	Hold, G.L., et al., *Comparison of paralytic shellfish toxin (PST) production by the dinoflagellates Alexandrium lusitanicum NEPCC 253 and Alexandrium tamarense NEPCC 407 in the presence and absence of bacteria.* FEMS Microbiol Ecol, 2001. **36**(2-3): p. 223-234.

50.	Howard, E.C., et al., *Bacterial taxa that limit sulfur flux from the ocean.* Science, 2006. **314**(5799): p. 649-52.

51.	Cude, W.N., et al., *Production of the antimicrobial secondary metabolite indigoidine contributes to competitive surface colonization by the marine roseobacter Phaeobacter sp. strain Y4I.* Appl Environ Microbiol, 2012. **78**(14): p. 4771-80.

52.	Verschuere, L., et al., *Probiotic bacteria as biological control agents in aquaculture.* Microbiol Mol Biol Rev, 2000. **64**(4): p. 655-71.

53.	Holmstrom, K. and L. Gram, *Elucidation of the Vibrio anguillarum genetic response to the potential fish probiont Pseudomonas fluorescens AH2, using RNA-arbitrarily primed PCR.* J Bacteriol, 2003. **185**(3): p. 831-42.

54.	Miller, M.B. and B.L. Bassler, *Quorum sensing in bacteria.* Annu Rev Microbiol, 2001. **55**: p. 165-99.

55.	Prol Garcia, M.J., P.W. D'Alvise, and L. Gram, *Disruption of cell-to-cell signaling does not abolish the antagonism of Phaeobacter gallaeciensis toward the fish pathogen Vibrio anguillarum in algal systems.* Appl Environ Microbiol, 2013. **79**(17): p. 5414-7.

56.	Hasegawa, H., et al., *The extracellular metalloprotease of Vibrio tubiashii is a major virulence factor for pacific oyster (Crassostrea gigas) larvae.* Appl Environ Microbiol, 2008. **74**(13): p. 4101-10.

57.	Furch, T., et al., *Comparative study on central metabolic fluxes of Bacillus megaterium strains in continuous culture using 13C labelled substrates.* Bioprocess Biosyst Eng, 2007. **30**(1): p. 47-59.

58.	Becker, J., C. Klopprogge, and C. Wittmann, *Metabolic responses to pyruvate kinase deletion in lysine producing Corynebacterium glutamicum.* Microb Cell Fact, 2008. **7**: p. 8.

Table 1. General genomic features of *P. gallaeciensis* strains

Strain	Length (kbp)	G+C content (%)	DNA scaffolds	Number of CDSs	Number of pseudo -genes	rRNA	tRNA	Ref.
2.10	4161	59.78	4	88.09	6	12	57	[16]
DSM 17395	4227	59.82	4	89.04	16	12	57	[16]
DSM 26640	4540	59.44	8	89.28	4	12	58	[17]

CDS: coding sequence

Table 2. Comparison of catabolic pathway activity and origins of metabolic intermediates in central carbon metabolism of *D. shibae*, *P. gallaeciensis* and other bacteria derived from carbon labeling experiments.

	Pathway activity / Fractional pool composition [%]					
	D. shibae [19]	*P. gallaeciensis* [19]	*B. subtilis* [23]	*B. megaterium* [57]	*C. glutamicum* [58]	*E. coli* [22]
Glycolysis	< 1	< 1	27	46	49	73
PPP	< 1	< 1	72	49	48	22
EDP	> 99	> 99	n.a.	n.a.	n.a.	4

n.a. = not available in the organism

PPP: pentose phosphate pathway

EDP: Entner-Doudoroff pathway

Figure 1. Metabolic network of the central carbonmetabolism of *Dinoroseobacter shibae* [1] and *Phaeobacter gallaeciensis* [25] as predicted from the annotated genome sequence. G6P: glucose-6-phosphate; F6P: fructose-6-phosphate; GAP: glyceraldehyde-3-phosphate; PEP: phosphoenolpyruvate; PYR: pyruvate; AcCoA: acetyl-Coenzyme A; OGA: 2-oxoglutarate; SUC: succinate; FUM: fumarate; OAA: oxaloacetate; MAL: malate; 6PG: 6-phosphogluconate; KDGP: 2-keto-3-deoxy-6-phosphogluconate; pycA: pyruvate carboxylase; pckA: phosphoenolpyruvate carboxykinase; ppdK: pyruvate orthophosphate dikinase.

Glucose

Glycolysis

G6P → 6PG

PP Pathway

F6P

GAP

PEP

PYR

CO_2

ppdK

pckA

pycA

ED Pathway

KDPG

AcCoA

CO_2

OAA/
MAL

**TCA
cycle**

AKG

CO_2

FUM

SUC

CO_2
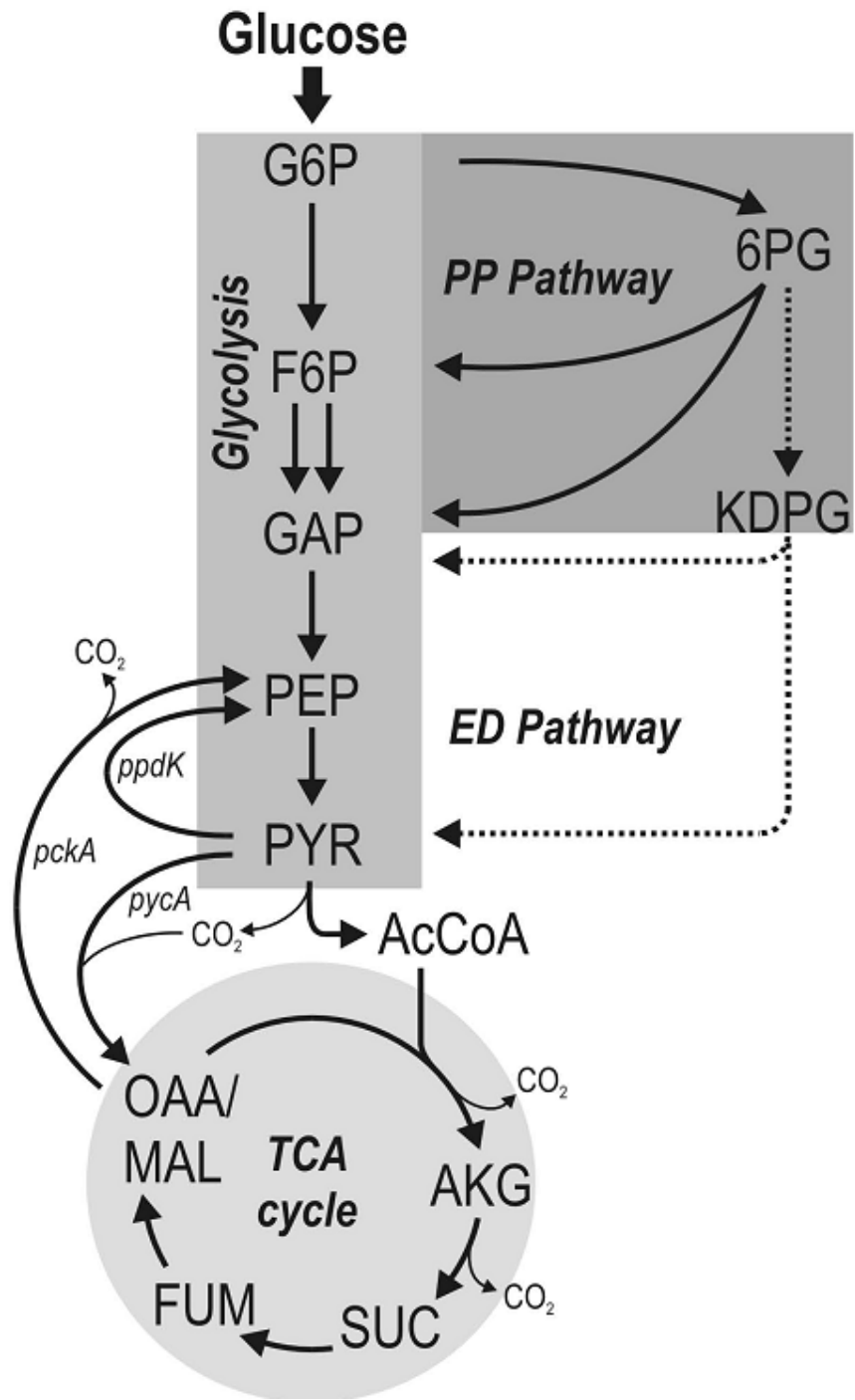
Figure 2. Degradation of DMSP via (A) demethylation pathway and (B) cleavage

pathways. FH4: tetrahydrofolate [28,29].
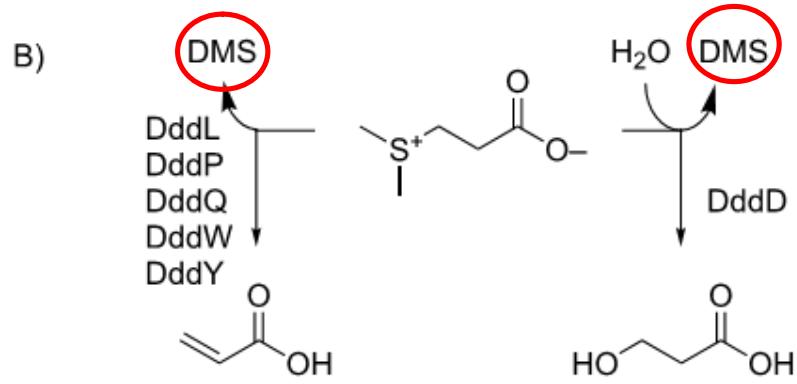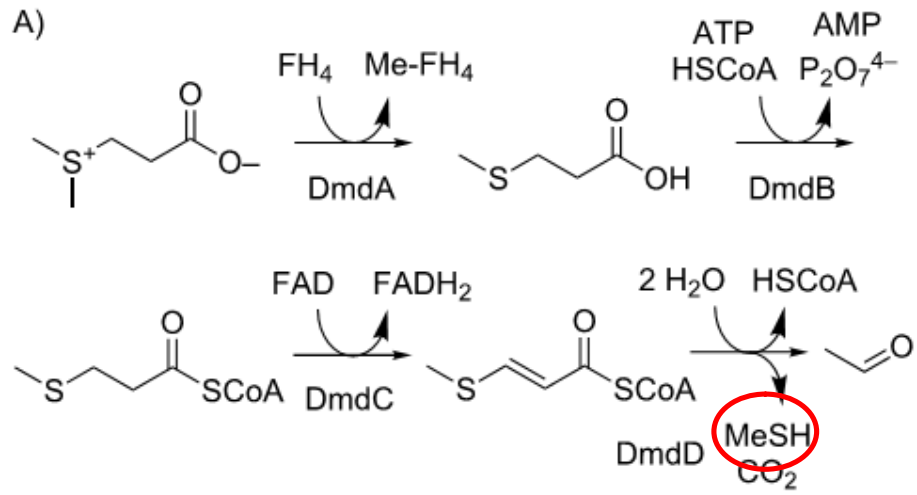
Figure 3. Proposed model for the biosynthesis of TDA in *P. gallaeciensis* DSM 17395. Integrated are the combined results of the transposon mutagenesis and the genome analysis presented in this study, as well as previously published data. Unknown reactions or ambiguities with respect to enzyme functions are indicated by question marks. Chemical structures: (1) phenylalanine; (2) phenylpyruvate; (3) phenylacetate; (4) phenyacetyl-CoA; (5) ring-1,2-epoxyphenylacetyl-CoA; (6) 2-oxepin-2(3H)-ylideneacetyl-CoA(oxepin-CoA); (7) 3-oxo-5,6-dehydrosuberyl-CoA semialdehyde; (8) 2-hydroxycyclohepta-1,4,6-triene -1-formyl-CoA; (9) tropolone; (10) tropone and (11) thiotropocin. Gene and protein names: cobA2: uroporphyrinogen-III C-methyltransferase; cysE: serine acetyltransferase; cysH: phosphoadenosine phosphosulfate reductase; cysI: putative sulfite reductase; cysK: cysteine synthase; hisC: histidinol-phosphate aminotransferase; ior1: indole pyruvate oxidoreductase (fused); paaA: ring-1,2-phenylacetyl-CoA epoxidase; paaC: ring-1,2-phenylacetyl-CoA epoxidase; paaD: ring-1,2-phenylacetyl-CoA epoxidase; paaE: ring-1,2-phenylacetyl-CoA epoxidase; paaF: 2,3-dehydroadipyl-CoA hydratase; paaG: ring-1,2-epoxyphenylacetyl-CoA isomerase (oxepin-CoA forming)/postulated 3,4-dehydroadipyl-CoA isomerase; paaH: 3-hydroxyadipyl-CoA dehydrogenase ; paaJ: 3-oxoadipyl-CoA/3-oxo-5,6-dehydrosuberyl-CoA thiolase; paaK1, paaK2: phenylacetate-CoA ligase; paaZ2: enoyl-CoA hydratase; patB: cystathionine betalyase; sat/cysC: putative bifunctional SAT/APS kinase; serB: phosphoserine phosphatase; serC: phosphoserine aminotransferase; tdaA: transcriptional regulator, LysR family; tdaB: -etherase; tdaC: prephenate dehydratase domain protein; tdaE: acyl-CoA dehydrogenase; tdaF: putative flavoprotein, HFCD family; thiG: thiazole biosynthesis protein and tyrB: aromatic amino-acid aminotransferase.

Manuscript I

Title: Probiotic strains for shellfish aquaculture: Protection of Eastern oyster, *Crassostrea Virginica,* larvae and juveniles against bacterial challenge

Authors: Murni Karim[1, 4], Wenjing Zhao[2], David Rowley[3], David Nelson[2], and Marta Gomez-Chiarri[1]

Author Affiliations: [1]Department of Fisheries, Animal and Veterinary Sciences, [2]Department of Cell and Molecular Biology, [3]Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI 02881, USA; [4]Universiti Putra Malaysia, Selangor, Malaysia.

Key Words: oyster larvae, oyster juveniles, probiotic bacteria, *Roseovarius* oyster disease, shellfish hatchery, shellfish nursery, vibriosis, *Vibrio tubiashii*.

*Corresponding author: Marta Gómez-Chiarri, Department of Fisheries, Animal and Veterinary Sciences, University of Rhode Island, 169 CBLS, 120 Flagg Road, Kingston, RI 02881, Phone 1-401-874-2917, Email Address: gomezchi@uri.edu

ABSTRACT

Bacterial pathogens, including several *Vibrio* spp. and *Roseovarius crassostreae* cause severe mortality of larval and juvenile Eastern oysters. The introduction of beneficial bacterial isolates in oyster hatcheries and nurseries for the biocontrol of bacterial diseases is a good alternative to the use of antibiotics. The goal of this study was to screen and characterize marine bacterial isolates as potential agents to prevent larval and juvenile mortality by the oyster pathogens *V. tubiashii* and *R. crassostreae*. Screening of bacterial isolates from Rhode Island marine organisms and environment using agar-based assay methods for detection of antimicrobial activity against oyster pathogens led to the isolation of candidate probionts *Phaeobacter* sp. S4 and *Bacillus pumilus* RI06-95. Pretreatment of larval and juvenile oysters for 24 h with 10^2 to 10^6 CFU ml^{-1} of *Phaeobacter* sp. S4 or *B. pumilus* RI06-95 protected larval oysters against mortality due to challenge with *R. crassostreae* and *V. tubiashii* (Relative Percent Survival, RPS, ranging from 9% to 56%). These probiotics also protected juvenile oysters against challenge with *V. tubiashii* (RPS 37 – 50%). Probiotic isolates had no negative impact on oyster survival. Protection conferred to larvae against bacterial challenge was short-lived, lasting only for 24 h after removal of the probiotics from the incubation water. These results suggest the potential of marine bacterial isolates *Phaeobacter* sp. S4 and *B. pumilus* RI06-95 to serve as biocontrol agents to reduce the impact of bacterial pathogens in the culture of *C. virginica*.

INTRODUCTION

The Eastern oyster, *Crassostrea virginica* (Gmelin), a bivalve species of the Gulf of Mexico and Atlantic coasts of North America, has significant economical and ecological value (Kennedy et al. 1996). However, this species suffers from the impact of different bacterial and parasitic diseases causing high mortalities in cultured and wild populations (Lee et al. 1996, Burreson & Ford 2004, Villalba et al. 2004). Bacterial infections are considered as a major problem for the shellfish aquaculture industry, causing mass mortality especially during larval and juvenile stages (Paillard et al. 2004). The pathogens *Roseovarius crassostreae* and several *Vibrio* spp. are amongst the major causative agents of bacterial disease in the culture of the Eastern oyster. As the causative agent of Juvenile or *Roseovarius* Oyster Disease (ROD), *Roseovarius crassostreae* causes high seasonal mortalities of oyster juveniles in the Northeast US (Boettcher et al. 2005, Maloy et al. 2007). Meanwhile, *Vibrio tubiashii* is a reemerging pathogen that causes vibriosis and severe losses of production in oysters during the larval stages (Tubiash 1965, Elston et al. 2008).

Disease outbreaks in shellfish aquaculture are managed using methods such as disease avoidance, frequent water changes, good husbandry, and the use of immunostimulants and antibiotics (Elston and Ford 2011). Antibiotics have been widely used in aquaculture systems as a method for disease control. However, due to the emergence of antibiotic resistance and concerns about environmental pollution, alternatives to the use of antibiotics are needed (Austin 1985, Vershuere et al. 2000). One of these alternative methods is the use of non-pathogenic microorganisms called probiotics.

A probiotic is generally defined as a live microbial food supplement which, when administered in a sufficient amount, confers a health benefit on the host (FAO 2006). In aquaculture, probiotics can be administered either as a food supplement or as an additive to the water (Moriarty 1998). Probiotics in aquaculture have been proposed to have several modes of action: improvement of water quality, enhancement of immune responses of host species, enhancement of nutrition of host species through the production of supplemental digestive enzymes, competition for space with pathogenic bacteria, and production of antimicrobial compounds (Kesacordi-Watson et al. 2008, Thompson et al. 1999, Verschuere et al. 2000). The potential for the beneficial impact of the use of probiotic bacteria on shellfish aquaculture has been shown for many different species, including oysters. Douillet and Langdon (1994) demonstrated that Pacific oyster larvae fed with algae and *Alteromonas* sp. show increased survival and growth compared with treatments fed with algae alone. The authors suggest that the bacteria may act as an essential nutrient to the larvae, which is not provided by the algae. Gibson et al. (1998) successfully isolated a bacterium producing bacteriocin-like inhibitory substances capable of inhibiting the growth of several pathogenic bacteria. This probiont, identified as *Aeromonas media* A199, significantly inhibits the growth of *V. tubiashii* in the culture of Pacific oyster larvae. The addition of *Vibrio* sp. probiotic candidate OY15 provides a beneficial effect in the culture of *C. virginica* larvae with and without the presence of the shellfish pathogen *Vibrio* sp. B183 (Kapareiko et al. 2011). Most recently, the use of *Pseudoalteromonas* sp. D41 and *Phaeobacter gallaeciensis* were found to provide 50% and 40% improved survival respectively in Pacific oyster larvae after being challenged with *Vibrio coralliilyticus* (Kesarcodi-Watson et al. 2012). The

introduction of selected beneficial bacterial isolates for biocontrol of *R. crassostreae* and vibriosis may help in combating diseases in the culture of Eastern oysters.

In this study, two potential probionts were isolated from two different local sources in Rhode Island. A Gram negative *Phaeobacter* sp. S4 was isolated from the inner shell of oysters and a Gram positive *Bacillus pumilus* RI06-95 was isolated from a marine sponge from Narrow River, a tidal estuary in Narragansett Rhode Island. Both of these candidate probionts showed promising results during *in vitro* screening of antibiotic activity against oyster and fish pathogens, as well as protecting larval and juvenile during *in vivo* challenge experiments with two oyster bacterial pathogens (*V. tubiashii* RE22 and *R. crassostreae* CV 919-312^T). We also describe the length of the protection conferred by the probiotic treatment.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains *Vibrio tubiashii* RE22 (Hasegawa et al. 2008) and *R. crassostreae* Cv919-312^T (Boettcher et al. 2005) were kindly supplied by H. Hasegawa, Department of Biomedical Sciences, Oregon State University (USA), and K. Boettcher, formerly at the University of Maine (USA), respectively. Strain *V. harveyi* BB120 (Bassler et al. 1997) was obtained from B. Bassler (Princeton University, USA). The marine bacteria *Phaeobacter* sp. S4 and *Bacillus pumilus* RI05-95 were identified as potential probiotics using the *in vitro* plate assays described below. The isolates were characterized to the level of species using 16S rDNA sequence analysis (Gauger and Gómez-Chiarri 2002) (GenBank Accession nus. KO625490 and KC625491). All the isolates were maintained and stored in 50 % glycerol stocks at -80°C. Probiotic candidates and pathogens were routinely

grown overnight in yeast peptone with 3% NaCl (YP3) broth (5 g L^{-1} of peptone, 1 g L^{-1} of yeast extract, 30 g L^{-1} of ocean salt, Instant Ocean) at 27 °C (*V. tubiashii, V. harveyi,* and *R. crassostreae*) or 25°C (*B. pumilus* RI06-95) with shaking.

***In vitro* screening of probiotic candidates**

A bacterium-bacterium competition assay described by Teasdale et al. (2009) was used in this assay with several modifications. In the 'colony on top' assay, 5 ml of 0.8% of YP3 soft agar containing 50 µl of approximately 10^8 CFU ml^{-1} of the pathogen from an overnight culture was poured atop YP3 agar plates. After the agar cooled, 2 µl of a solution of about 10^8 CFU ml^{-1} of the candidate probiotic from an overnight culture was spotted onto the plate and incubated at 27 °C for 12 – 16 h before the inhibition zones were measured. For the 'membrane overlay' assay, an aliquot of 2 µl of a solution of approximately 10^8 CFU ml^{-1} of the candidate probiotic was spotted onto YP3 agar plates and incubated at 27 °C for 48 h. After incubation, a sterile 12-14 kDa molecular-weight-cutoff (MWCO) dialysis membrane (Spectra/Por; Spectrum Medical Industries, Inc., Houston, TX) was laid atop the colonies and covered with 6 ml of 0.8% YP3 agar containing 60 µl of approximately 10^8 CFU ml^{-1} of pathogen from an overnight culture. Plates were incubated at 27 °C for 12 - 16 h after agar solidification and the diameter of the clear (inhibitory) zones around the probiont colonies were measured using a ruler.

Characterization of *Phaeobacter sp.* S4 growth and morphology

Single colonies of *Phaeobacter* sp. S4 were inoculated into YP3 media, grown for 48 h at 27 ˚C with shaking, and then backdiluted into fresh YP + 2% NaCl (YP2) or YP3 media at a 1: 1000 dilution. Cultures were incubated at 27˚C with shaking for up to 72 h and aliquots were taken at selected time points to

determine bacterial concentration (CFU ml^{-1}) by plating of serial dilutions. Aliquots of bacterial cells taken from cultures grown to late exponential (36 h) and stationary (48 h) phases were placed on glass coverslips and examined by phase contast microscopy at the Rhode Island Genomics and Sequencing Center at the University of Rhode Island with a Zeiss Axio Imager 2 microscope using phase contrast optics. Biofilm-containing samples were grown in static culture conditions for 48 h at 27°C and scraped from the walls of the glass culture tubes (15 × 150 mm) before being placed on glass slides and observed by phase contrast microscopy.

Preparation of bacterial isolates for challenge

Candidate probiotics and pathogens were cultured overnight with shaking in 10 ml YP3 broth. Overnight cultures were transferred to 50 ml sterile Falcon tubes and centrifuged at 2,300 x g for 10 min to harvest the cells. Cells were washed twice with 10 ml of filtered sterile seawater (FSSW) and the cell pellet was resuspended in 10 ml FSSW and mixed using a vortex mixer. The bacterial density was determined by measuring optical density at 550 nm using a spectrophotometer (Synergy^{TM} HT, BioTek, USA) and assuming that an optical density of 1.000 corresponds to 1.2 x 10^9 CFU ml^{-1} according to the McFarland standard (BioMerieux, Marcyl'Etoile, France). After the concentration of the bacteria was determined, the bacterial suspension was diluted to the target concentration in FSSW. The final target concentration was confirmed by plating serial dilutions of the bacterial cultures for each treatment on the appropriate agar plates and counting colony forming units (CFU) after overnight incubation at 25 or 27 °C. The commercial probiotic mix (Sanolife MIC, INVE Aquaculture, Belgium) was mixed by adding 0.1 g of Sanolife in 50 ml of FSSW following the manufacturer's

protocol. The solution was then adjusted to a stock concentration of 5 x 10^6 CFU ml^{-1} and used at a target concentration of 10^4 CFU ml^{-1}.

Larval oyster bacterial challenges

Experimental challenges were performed as previously described (Gómez-León et al. 2008) with minor modifications. Larvae of Eastern oysters *Crassostrea virginica* (12 to 20 days of age, 50 – 150 μm in size) were obtained from the Blount Shellfish Hatchery at Roger William University (Bristol, RI, USA). Oysters (25 to 30 larvae) were placed in each well of a 6 well plate containing 5 ml of FSSW at 28 psu. The candidate probiotics isolates S4 and RI06-95 were added to the wells at final concentrations ranging from 10^2 to 10^6 CFU ml^{-1}. The commercial probiotic Sanolife MIC was used at a final concentration of 10^4 CFU ml^{-1}. Larval oysters were fed with commercial algal paste (20,000 cells ml^{-1}; Reed Mariculture Inc., San Jose, CA, USA) in order to promote ingestion of the probiotics. Plates were incubated at 22-23°C for 24 h with gentle rocking. Water in the wells was then changed to remove the probiotics. Either *V. tubiashii* RE22 or *R. crassostreae* CV919-312^T was added to 5 ml of FSSW containing the larvae to achieve the target concentration of pathogen (10^5 or 10^6 CFU ml^{-1}). Control wells included non-treated larvae (with and without pathogen) and larvae incubated with probiotics but not with the pathogen. Each treatment was run in triplicate. Larval survival was determined 24 h after addition of the pathogen by adding 200 μl of neutral red to each well to a final concentration of 0.53 mg l^{-1} and incubation for 2 h before counting living and dead oysters. The neutral red staining technique distinguishes between live (stained) and dead (not stained) larvae (Figure 1; Gómez-León et al., 2008). The survival rate was calculated by using the formula: *Survival rate (%) = 100 x (number of live*

larvae/total number of larvae). The Relative Percent Survival (RPS) (Amend 1981) conferred by the probiont (treatment) with respect to the challenged larvae (control) was calculated by using the formula: *RPS (%) = [1 – (% mortality treatment / % mortality control)] x 100.* These experiments were run at least 3 times in triplicate for the candidate probionts S4 and RI06-95, once for the commercial probiont Sanolife MIC.

Length of protection conferred by candidate probionts

Larval oysters were placed in 6-well plates containing 5 ml of FSSW and candidate probionts were introduced to a final concentration of 10^4 CFU ml^{-1}. Plates were incubated at 22-23°C for 24 h with gentle rocking. At 24 h of incubation, FSSW was removed from the wells and exchanged with 5 ml of FSSW without the probiotics. Pathogen *V. tubiashii* RE22 (final concentration of 10^5 CFU ml^{-1}) was applied to the wells 24, 72, or 120 h after addition of the candidate probionts (equivalent to 0, 48, or 96 h after removal of the probiont). Larval oyster survival and RPS was determined as described above after 24 h of incubation with the pathogen. Larval oysters were fed daily with commercial algal paste (20,000 cells ml^{-1}). This assay was run only once with each treatment tested in triplicate.

Juvenile oyster bacterial challenges

Ten juvenile oysters (8 – 15 mm in shell height) per container were placed in 500 ml buckets containing 200 ml of FSSW and each container was provided with continuous aeration via airstones. Candidate probionts were applied at a final concentration of 10^5 CFU ml^{-1} and containers were incubated at 22-23°C for the length of the experiment. After 24 h of incubation with the probiont, *V. tubiashii* RE22 was applied to a final concentration of 10^5 CFU ml^{-1}. Mortalities were recorded every 2 - 3 d for 13 d and cumulative % survival was calculated. Water

was exchanged every 2 - 3 d and the oysters were fed daily with commercial algal paste (20,000 cells ml^{-1}). This experiment was performed once using duplicate containers per treatment.

Statistical Analysis

Survival and cumulative mortality data were analyzed using One or Two Way Analysis of Variance (ANOVA) and multiple comparison tests (Tukey test) was used to determine significance between groups. Data collected as percentage were arcsine of the square root-transformed before analysis. Results were considered significant at 95% level of confidence ($p<0.05$). All the statistics were run using Sigmastat 3.1 software (Systat).

RESULTS

Antibiotic activity against bacterial pathogens

In this study, amongst 64 bacteria strains isolated from the inner shell of healthy oysters, only *Phaeobacter* sp. S4 was found to have an antibiotic activity against *V. harveyi* BB120 by using two different plates assays. In the 'membrane overlay' assay, the use of the membrane prevents direct contact between probiont and pathogen, only allowing chemicals with a molecular mass <12-14 kDa to go through. This method allows observation of chemical interactions between probiont and pathogen. Meanwhile, the 'colony on top' assay allows for direct bacterial interaction between probiont and pathogen. The probiont candidate *Bacillus pumilus* RI06-95 inhibited the growth of pathogens *V. harveyi* BB120 at 27 °C and *R. crassostreae* CV 919-312^{T} at 20 and 27 °C using both the 'colony on top' and the 'membrane overlay' assays (Table 1). This isolate, however, showed no growth inhibitory activity against *V. tubiashii* RE22. The candidate probiont *Phaeobacter* sp. S4 inhibited the growth of all pathogens with the exception of *V.*

tubiashii RE22 at 20°C in the 'colony on top' assay (Table 1). Differences in the pattern of inhibition between the two assays for this probiont are probably due to differences in the length of the incubation times of the probiotic with the pathogen (12 - 16 h for the 'colony on top' and 48 h for the 'membrane overlay').

Characterization of *Phaeobacter sp.* S4 growth and morphology.

We characterized *Phaeobacter* sp. S4 with regard to some basic properties that might affect its ability to serve as a probiotic organism in marine aquaculture, namely growth curves in marine media and the ability to form biofilms. Briefly, S4 grew well in YP + 2% or 3% NaCl at temperatures from 18°C up to 30°C (not shown). Cells were unable to grow at 37°C. At 27°C there was no difference in the growth rate of S4 when cells were grown in either YP2 or YP3 (Figure 2). The average doubling time for each condition was 3.1 h for YP2 and 3.2 h for YP3. The final density of S4 in either YP2 or YP3 was ~2.0×10^9 CFU ml^{-1}.

While growth in 2% and 3% NaCl produces virtually identical growth rates and final cell densities, these two conditions resulted in two different morphologies for *Phaeobacter* sp. S4 (Figure 3). Growth in YP3 results in small, ovoid motile cells (Figure 3a), that when entering stationary phase form rosettes. Cells grown in YP2 elongate to spindle-shaped cells during late stationary phase, lose motility, and form rosettes (Figs. 3b,c). If grown in static culture, the cells formed a thick biofilm on glass surfaces (Figure 3d). Plastic surfaces (polycarbonate, polystyrene, and polypropylene) did not support the formation of a biofilm by S4 (not shown).

Effect of pre-treatment with probiotics on larval oysters' survival to bacterial challenge

Candidate probionts were not pathogenic to the host since the survival of oyster larval treated with the candidate probionts was not significantly different to

the control (Figure 4). Rapid deaths of larval oysters were seen after exposure to pathogens *V. tubiashii* RE22 and *R. crassostreae* CV 919-312^T for 24 h, with survival ranging from 14 - 31% depending on the pathogen and dose (>80% for unchallenged controls). Survival of oysters pre-treated with candidate probionts for 24 h and then exposed to the bacterial pathogens were significantly higher than those of larvae that had not been exposed to the probiont, increasing from a survival of 14 - 31% for non-treated larvae to 32 – 64% for probiotic-treated larvae (Figure 4).

The level of protection was different depending on the relative concentrations of candidate probionts and pathogen added (Table 2). Candidate probiont *Phaeobacter* sp. S4 was found to protect larval oysters more effectively against *V. tubiashii* RE22 than against *R. crassostreae* CV 919-312^T. This study also demonstrated *Phaeobacter* sp. S4 gave higher levels of protection against both pathogens than *B. pumilus* RI06-95. The optimal concentration for probionts *Phaeobacter* sp. S4 and *B. pumilus* RI6-95 was 10^4 CFU ml^{-1}. At this concentration, both probiotics were able to confer significant survival against *V. tubiashii* RE22 (p<0.05) and *R. crassostreae* CV 919-312^T (Table 2). On the other hand, no protection effect was found in larval oysters treated with commercial probiotic Sanolife MIC (INVE, Belgium) after challenge with *V. tubiashii* RE22 (survival of challenged larvae pretreated with Sanolife MIC of 2 ± 2 % compared to 98 ± 2 % for non-challenged larvae pretreated with Sanolife MIC, Table 2).

Length of protection conferred by probiotics

In order to determine the duration of protection provided by a 24 h exposure of larval oysters to the candidate probionts, we determined the survival of larval oysters challenged at different time points after exposure to the probionts (0,

48, and 96 h after removal of the candidate probionts). As observed above, larval oysters incubated with probionts for 24 h were significantly protected against a 24 h bacterial challenge with *V. tubiashii* when the pathogen was added immediately after the removal of the probiont (Figure 5). However, no significant protection was obtained when the larvae were challenged 48 and 96 h after removal of the probionts. The Relative Percent Survival of larval oysters exposed to *Phaeobacter* sp. S4 for 24 h dropped significantly from 78% when oysters were challenged immediately after removal of the probiont to 14% and 13% when oysters were challenged 48 and 96 h after removal of the probiont. The RPS of larval oysters exposed to *B. pumilus* RI06-95 dropped from 44% when oysters were challenged right after removal of the probiont to 1% (challenged at 48 h) and 4% (challenged at 96 h).

Effect of pre-treatment with probionts on juvenile oysters survival to bacterial challenge

We wanted to determine whether exposure to the probiotic bacteria would protect juvenile oysters from *V. tubiashii* in a manner similar to what was observed for larval oysters. While juvenile oysters showed a sharp increase in mortalities on day 6 after challenge with *V. tubiashii* RE22, oysters in containers to which probiotic strains were added 24 h before challenge showed relatively low levels of mortality (less than 15%) until day 8 after challenge (Figure 6). At the end of the assay (13 d), exposure to the probionts significantly reduced juvenile oyster mortalities after challenge with *V. tubiashii* ($p < 0.05$; RPS, *B. pumilus* RI06-95: 60 ± 0 % and *Phaeobacter* sp. S4: 67 ± 0 %). Co-incubation of juvenile oysters with both S4 and RI06-95 did not confer added levels of protection compared to preincubation with either one of the probiotics alone ($p > 0.05$).

DISCUSSION

This study successfully identified two potential bacterial candidates to be used as probiotics for disease management control in oyster aquaculture. Bacterial strains *Phaeobacter* sp. S4 and *B. pumilus* RI06-95 were selected as candidate probionts due to their antagonistic properties against the oyster pathogens *R. crassostreae* and *V. tubiashii*, and also the marine finfish and shellfish pathogen *V. harveyi*. We demonstrate here that they also conferred significant protection to larval oysters against experimental bacterial challenge. Furthermore, preincubation of juvenile oysters with these probionts led to significantly improved survival of juvenile oysters 13 days after challenge with *V. tubiashii*.

These two candidate probionts were able to protect oyster larvae and juveniles against the severe bacterial challenges used in our research, and show the potential to provide protection when used prophylactically in hatcheries, where the levels of pathogenic bacteria in seawater sometimes approach levels similar to the challenge doses used in our experiments (Elston et al. 2008). The bacterial pathogens *V. tubiashii* and *R. crassostreae* cause rapid mortalities in larval oysters in our experiments; oysters stopped swimming and most of the tissue was completely digested leaving an empty shell after 24 h of exposure to the pathogens. This is consistent with previous research on these pathogens (Elston et al. 2008, Gibson et al. 1998, Gómez-León et al. 2008).

The candidate probionts we have tested here are commensals of marine organisms and proved in our experiments to be safe to larval and juvenile oysters, since they had no significant effect on larval or juvenile survival at the concentrations tested (up to 10^6 CFU ml^{-1}). Although we have not direcly tested the effect of these probionts on algal cultures, previous research on another

Phaeobacter sp. (*Phaeobacter galleciensis*) with probiotic activity on cod larval cultures showed no negative effects of this probiont on the survival of the microalgae *Tetraselmis suecica,* a species commonly used in aquaculture hatcheries (D'Alvise et al. 2012). Our experiments also showed that significant levels of protection were obtained with a dose of probiotic of 10^4 CFU ml^{-1}, a dose easily achievable even in the large culture tanks used at commercial hatcheries. The length of protection conferred to larval oysters by exposure to the probionts, however, is short-term (24 h), suggesting that these probiotics may need to be supplied to larvae in the hatcheries daily to maintain their effectiveness. This is not uncommon for other probiotics, which are usually provided daily with the feed to host organisms to provide maximum benefits (Kesacordi-Watson et al. 2008, Verschuere et al. 2000). Interestingly, a single dose of probiotics added to the culture water of juvenile oysters 24 h prior to bacterial challenge provided significant levels of protection for at least 13 days, suggesting that the probionts may persist longer in juvenile oysters compared to larval oysters, or that additional mechanisms of protection are involved in juvenile oysters. More research should be done to determine the effectiveness and mechanisms of action of these probiotic bacterial strains in different developmental stages of oysters and different growing conditions.

Our study showed that lack of growth inhibitory activity *in vitro* towards a particular pathogen is not necessarily predictive of how a candidate probiont would perform *in vivo.* Candidate probiont *Bacillus pumilus* RI06-95 was not able to inhibit the growth of *V. tubiashii in vitro* but showed a protective effect toward larval and juvenile oysters during *in vivo* challenge, suggesting that protection conferred by *B. pumilus* RI06-95 against *V. tubiashii* may not be due to antibiotic

activity or that the *in vitro* assays used in the screening process do not predict the production of the antibiotic *in vivo*. Probiotics are able to improve survival of the hosts by different mechanisms (Kesacordi-Watson et al. 2008, Verschuere et al. 2000). Beside secretion of antibiotic compounds, it is known that probiotics are capable of various other modes of action that give benefits to the host. In previous research, *Bacillus* sp. S11 has been reported to improve health by stimulating the immunity of the host organism (Rengpipat et al. 2000). This may be one of potential mechanism provided by *B. pumilus* RI06-95 in order to protect the oysters against *V. tubiashii* in our in vivo assay. This probiotic may also promote enhanced digestion in oysters. Research by Olmos et al. (2011) demonstrated the ability of *B. subtilis* to enhance carbohydrate digestion and improve the health of the shrimp *Litopenaeus vannamei*. Furthermore, Sun et al. (2010) demonstrated that grouper *Epinephelus coioides* consumed dietary nutrients better after supplementing the feed with *B. pumilus* or *B. clausii*.

In contrast, the results from the *in vitro* tests with *Phaeobacter* sp. S4 showed growth inhibitory activity against the two oyster pathogens and this coincided with increased protection seen in the *in vivo* assays. Research performed by Porsby et al. (2008) showed that members of *Roseobacter* clade such as *Phaeobacter gallaeciensis* and *P. inhibens* produce an antibiotic compound named tropodithietic acid (TDA), capable of inhibiting the growth of the bacterial pathogens *Vibrio anguillarum, V. splendidus, V. cholerae, B. subtilis*, and *Halomonas* spp. Furthermore, the application of bacterial cultures or cell extracts of *Phaeobacter* spp. improve survival of fish larvae (Makridis et al. 2005; Planas et al. 2006) and shellfish (Balcázar et al. 2007; Ruíz-Ponte et al. 1999) in rearing tanks. Recently, D'Alvise et al. (2012) demonstrated the ability of *P. galleciensis*

to protect cod larvae from vibriosis. Besides producing TDA, *Phaeobacter* spp. are known as primary colonizers of various inorganic and organic marine surfaces, including marine algae and dinoflagellates (Dang and Lovell, 2002; Mayali et al. 2008). Our observations of this bacterium confirm that *Phaeobacter* sp. S4 avidly forms rosettes and biofilms on inorganic surfaces, such as glass. Further, our results showing the ability of a *Phaeobacter* sp. isolated from the inner side of an adult oyster shell (and probably a member of the natural oyster microbiome) to protect larval oysters from bacterial challenge provide further evidence of the potential of *Phaeobacters* as probiotic species.

In conclusion, these studies successfully isolated two candidate probionts for diseases management in oyster hatcheries. *Phaeobacter* sp. S4 is a good probiont candidate showing clear antibiotic activity *in vitro* and protection *in vivo*. The relationship between probiotic activity *in vivo* and antibiotic activity *in vitro*, however, is not so strong in the case of protection of larval oysters against *V. tubiashii* conferred by *B. pumilus* RI06-95, suggesting that other mechanisms contribute to probiotic activity. Thus, in addition to good candidates for use in shellfish aquaculture, these candidate probionts will be useful in evaluating the relationship between antibiotic and probiotic activities in order to help establish rational strategies for the screening for potential probiotics.

ACKNOWLEDGEMENTS

REFERENCE:

Amend D. F. 1981. Potency testing of fish vaccines. Fish Biologies: Serodiagnostics and Vaccines. In: Anderson D. P. & H. Hennessen, editors. Dev. Biol. Standard. 49, Karger, Basel. Pp. 447-454.

Austin, B. 1985. Antibiotic pollution from fish farms and its effect on the aquatic microflora. *Microbiol Sci* 2: 113-118.

Balcázar, J.L., I. de Blas, I. Ruiz-Zarzuela, D. Cunningham, D. Vendrell & J.L. Muzquiz. 2006. The role of probiotics in aquaculture. *Vet Microbiol* 114: 173-186.

Bassler, B.L., E.P. Greenberg & A.M. Stevens. 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J Bacteriol* 179: 4043–4045.

Boettcher, K.J., K.K. Geaghan, A.P. Maloy & B.J. Barber. 2005. *Roseovarius crassostreae sp*. nov., a member of the *Roseobacter* clade and the apparent cause of juvenile oyster disease (JOD) in cultured Eastern oysters. *Int J Syst Evol Microbiol* 55: 1531-1537.

Burreson, E.M. & S. E. Ford. 2004. A review of recent information on the Haplosporidia, with special reference to Haplosporidium nelsoni (MSX). *Aquat Living Res* 17: 499-517.

Dang, H.Y. & C.R. Lovell. 2002. Seasonal dynamics of particle-associated and free-living marine *Proteobacteria* in a salt march tidal creek as determined using fluorescence in situ hybridization. *Environ Microbiol* 4: 287-295.

Douillet, P. A. & C. J. Langdon. 1994. Use of a probiotic for the culture of larvae of the pacific oyster (*Crassostrea gigas* Thunberg). Aquacult 119: 25-40.

D'Alvise, P. W., S. Lillebø, M. J. Prol-Garcia, H. I. Wergeland, K. F. Nielsen, Ø.

Bergh, & Gram, L., 2012. *Phaeobacter gallaeciensis* reduces *Vibrio anguillarum* in cultures of microalgae and rotifers, and prevents vibriosis in cod larvae. *PLoS ONE 7*:   e43996.

Elston, A. R. & S. E. Ford. 2011. Shellfish Diseases and Health Management, In: Shumway, S. editior. Shellfish Aquaculture and the Environment. Ames, Iowa: Wiley-Blackwell. pp. 359-394.

Elston, R. A., H. Hasegawa, K.L. Humphrey, I. K. Polyak & C. C. Häse. 2008. Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and management. *Dis Aquat Org* 82:   119-134.

Food and Agriculture Organization of the United Nations (FAO). 2006. Probiotics in Food: Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. Food and Nutrition Technical Paper 85.   World Health Organization, Rome.

Gauger, E.J. & M. Gómez-Chiarri. 2002. 16S ribosomal DNA sequencing confirms the synonymy of *Vibrio harveyi* and *V. carchariae. Dis Aqua Org* 52:   39-46.

Gibson, L. F., J. Woodworth & A. M. George.   1998. Probiotic activity of *Aeromonas media* on the Pacific oyster, *Crassostrea gigas*, when challenged with *Vibrio tubiashii. Aquaculture* 169:   111–120.

Gómez-León, J., L. Villamil, S. A. Salger, R. H. Sallum, A. Remacha-Trivino, D. F. Leavitt & M. Gómez-Chiarri. 2008. Survival of eastern osyters *Crassostrea virginica* from three lines following experimental challenge with bacterial pahtogens. *Dis Aqua Org* 79: 95-105.

Hasegawa, H., E.J. Lind, M.A. Boin & C.C. Häse. 2008. The Extracellular Metalloprotease of *Vibrio tubiashii* Is a Major Virulence Factor for Pacific

Oyster (*Crassostrea gigas*) Larvae. *Appl Environ Microbiol* 74: 4101-4110.

Kapareiko, D., H.J. Lim, E.J. Schott, A. Hanif & G.H. Wikfors. 2011. Isolation and evaluation of new probiotic bacteria for use in shellfish hatcheries: II. Effects of a *Vibrio* sp. probiotic candidate upon survival of oyster larvae (*Crassostrea virginica*) in pilot-scale trials. *J Shellfish Res* 30: 617–625.

Kennedy, V.S., R.I. Newell & E.F. Eble. 1996. The eastern oyster, *Crassostrea virginica*. Maryland Sea Grant College, College Park, Maryland, 734 pp.

Kesarcodi-Watson, A., P. Miner, J.L. Nicolas & R. Robert. 2012. Protective effect of four potential probiotics against pathogen-challenge of the larvae of three bivalves: Pacific oyster (*Crassostrea gigas*), flat oyster (*Ostrea edulis*) and scallop (*Pecten maximus*).   *Aquaculture* 344: 29–34.

Kesarcodi-Watson, A., H. Kaspar, M. J. Lategan & L. Gobson. 2008. Probiotics in aquaculture: the need, principles and mechanisms of action and screening processes. *Aquaculture* 274:1–14.

Lee, M., G.T. Taylor, V.M. Bricelj, S.E. Ford & S. Zahn. 1996. Evaluation of *Vibrio* spp. and microplankton blooms as causative agents of juvenile oyster disease in *Crassostrea virginica* (Gmelin).   *J Shellfish Res* 15: 319–329.

Makridis, P., S. Martins, M. Tsalavouta, L.C. Dionisio, G. Kotoulas, A. Magoulas & M.T. Dinis. 2005. Antimicrobial activity in bacteria isolated from Senegalese sole, *Solea senegalensis*, fed with natural prey. *Aquatic Res* 36: 1619-1627.

Maloy, A.P., S.E. Ford, R. Karney & K. J. Boettcher. 2007. *Roseovarius crassostreae*, the etiological agent of Juvenile Oyster Disease in *Crassostrea virginica*. *Aquaculture* 269: 71-83.

Mayali, X., P.J.S. Franks & F. Azam. 2008. Cultivation and ecosystem role of a

marine *Roseobacter* clade-affiliated cluster bacterium. *Appl Environ*

Microb 74: 2595-2603.

Moriarty, D.J.W., 1998. The role of microorganism in aquaculture ponds.

Aquaculture 151: 333-349.

Olmos, J., L. Ochoa, P. Michel, R. Contreras. 2011. Functional feed assessment

on *Litopenaeus vannamei* using 100% fish meal replacement by soybean

meal, high levels of complex carbohydrates and *Bacillus* probiotic strains.

Mar Drugs 9: 1119-1132.

Paillard, C., F. Le Roux & J.J. Borreg. 2004. Bacterial disease in marine

bivalves, a review of recent studies: trends and evolution. Aquat. Living.

Resour. 17: 477–498.

Planas, M., M. Pérez-Lorenzo, M. Hjelm, L. Gram, I.U. Fiksdal, Ø. Bergh & J.

Pintado. 2006. Probiotic effect in vivo of *Roseobacter* strain 27-4 against

Vibrio (Listonella) anguillarum infections in turbot (*Scophthalmus*

maximus L.) larvae. Aquacult 255: 323-333.

Porsby, C.H., K.F. Nielsen & L. Gram. 2008. *Phaeobacter* and *Ruegeria* species

of the *Roseobacter* clade colonize separate niches in a Danish turbot

(*Scophthalmus maximus*)-rearing farm and antagonize *Vibrio anguillarum*

under different growth conditions. *Appl Environ Microb* 74: 7356-7364.

Rengpipat, S., S. Rukpratanporn, S. Piyatiratitivorakul, P. Menasaveta. 2000.

Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a

probiont bacterium (*Bacillus* S11). *Aquaculture* 191: 271–288.

Ruiz-Ponte, C., J.F. Samain, J.,L. Sánchez & J.L. Nicolas. 1999. The benefit of

Roseobacter species on the survival of scallop larvae. *Mar Biotechnol* 1:

52–59.

Sun, Y. Z., H. L. Yang, R. L. Ma & W. Y. Lin. 2010. Probiotic applications of two dominant gut *Bacillus* strains with antagonistic activity improved the growth performance and immune responses of grouper *Epinephelus coioides.* Fish Shellfish Immunol. 29: 803-809.

Teasdale, M.E., J. Liu, J.   Wallace, F. Akhlaghi & D.C. Rowley. 2009. Secondary metabolites produced by the marine bacterium *Halobacillus salinus* that inhibit quorum sensing-controlled phenotypes in Gram-negative bacteria. *Appl Environ Microb* 75: 567-572.

Thompson, F.L., P.C. Abreu & R.O. Cavalli, 1999. The use of microorganisms as food source for *Penaeus paulensis larvae*. *Aquaculture* 174: 139-153.

Tubiash, H. S., P. E. Chanley & E. Leifson. 1965. Bacillary necrosis, a disease of larval and juvenile bivalve mollusks. I. Etiology and epizootiology. J. Bacteriol. 90: 1036-1044.

Verschuere, L., G. Rombaut, P. Sorgeloos & W. Verstraete. 2000. Probiotic bacteria as biological control agents in aquaculture. *Microbiol Mol Biol Rev* 64: 655-671.

Villalba, A., K. S. Reece, M.C. Ordas, S. M. Casas, S.M. & A. Figueras. 2004.
Perkinsois in molluscs: A Review. *Aquat Living Res* 17: 411-432

Table 1: Antibiotic activity of candidate probionts *Phaeobacter* sp. S4 and *Bacillus pumilus* RI06-95 against selected bacterial pathogens of finfish (*Vibrio harveyi* BB120) and shellfish (*V. tubiashii* RE22 and *Roseovarius crassostreae* CV919-312^T) as determined by 2 plate diffusion assays at 2 temperatures. The antibiotic activity is reported as the diameter of the inhibition zone in mm ± standard error of the mean (SEM), including the size of the colony for the candidate probiont (3 mm).

Probiotics Temp.	Colony-on-top (mm)			Membrane overlay (mm)		
	BB120	RE22	CV919-312^T	BB120	RE22	CV919-312^T
RI06-95 20°C	-	0	11 ± 1	-	0	4 ± 1
RI06-95 28°C	10 ± 1	0	13 ± 1	7 ± 1	0	5 ± 1
S4 20°C	-	0	16 ± 0	-	6 ± 1	15 ± 1
S4 28°C	6 ± 1	7	13 ± 1	6 ± 1	6 ± 1	13 ± 2

- = Not Tested

Table 2. Effect of preincubation with candidate probionts *Bacillus pumilus* RI06-96, *Phaeobacter* sp. S4, and commercial probiotic mix Sanolife MIC (INVE Aquaculture, Belgium) on larval oyster survival 24 h after challenge with bacterial pathogens *Roseovarious crassostreae* CV919-312[T] and *Vibrio tubiashii* RE22. The candidate probionts were introduced 24 h before larvae were challenged. Data are expressed as Relative Percent Survival (RPS, % ± SEM) of challenged oysters pre-treated with probiotic to control challenged oysters. Different letters in superscript indicate statistical differences between treatments for each probiont (1 way ANOVA, p<0.05)

Bacterial pathogens and concentration (CFU ml⁻¹)		Relative Percent Survival (RPS, %)						
		Probiotics and concentration (CFU/ml)						
		RI06-95			S4			INVE
		10^6	10^4	10^2	10^6	10^4	10^2	10^6
RE22	10^6	31 ± 2^a	-	-	53 ± 3^b	-	-	-
	10^5	29 ± 3^a	29 ± 3^a	-	44 ± 3^b	55 ± 2^b	-	0
CV919-312[T]	10^6	11 ± 2^x	-	-	14 ± 3^x	-	-	-
	10^5	22 ± 2^y	42 ± 3^z	20 ± 1^{xy}	43 ± 5^z	49 ± 3^z	29 ± 2^y	-

(-) Not tested.   *RPS (%) = [1 – (% survival control / % survival treatment)] x 100.*

Figure 1. Effect of preincubation with candidate probiont *Phaeobacter* sp. S4 on the morphology of larval oysters 24 h after challenge with the bacterial pathogen *Vibrio tubiashii* RE22. The candidate probionts were introduced 24 h before pathogen challenge. (A) Larva challenged with RE22 showing clumping of cilia (arrow). (B) Group of larva challenged with RE22, showing cell debris (arrowhead) and dead larvae as indicated by empty shells not stained with neutral red (arrow). (C & D) Larvae preincubated with S4 and challenged with RE22 were viable, showing staining with neutral red (arrow) and normal cilia (arrowhead).
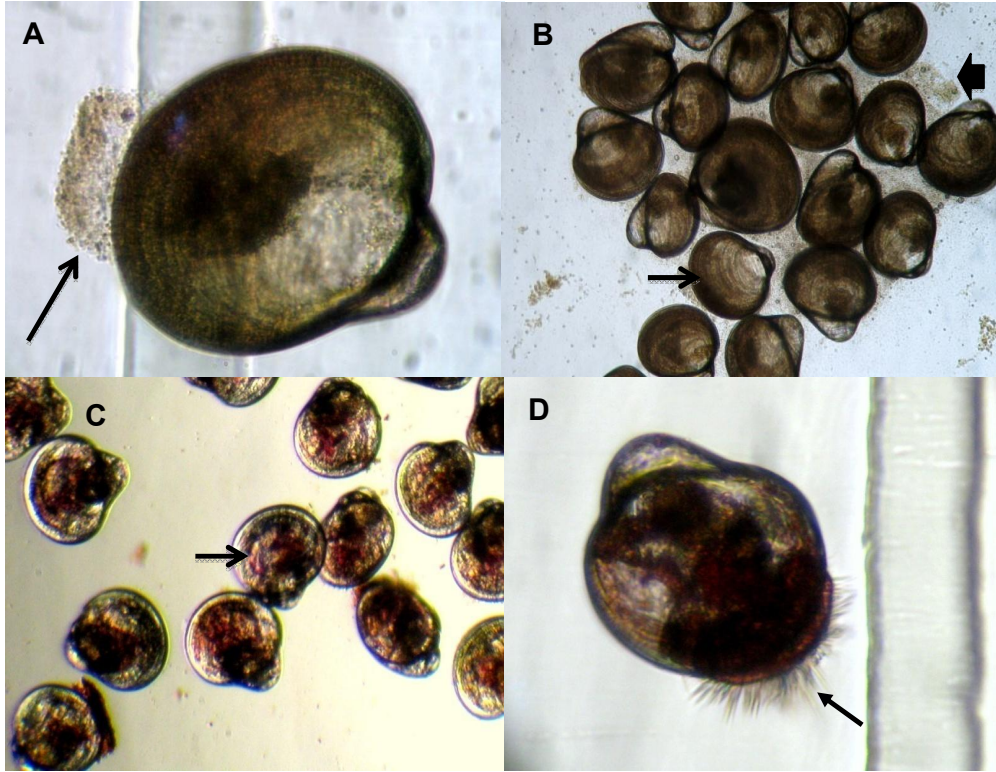
Figure 2. Growth curve of *Phaeobacter* sp. S4 in YP2 and YP3 at 27°C. Cells were grown for 48 h in YP3 and then back diluted into fresh YP2 (△) or YP3 (■) at a 1:1000 dilution. Samples were taken at the indicated times and the cell density determined by serial dilution and plating onto YP3.
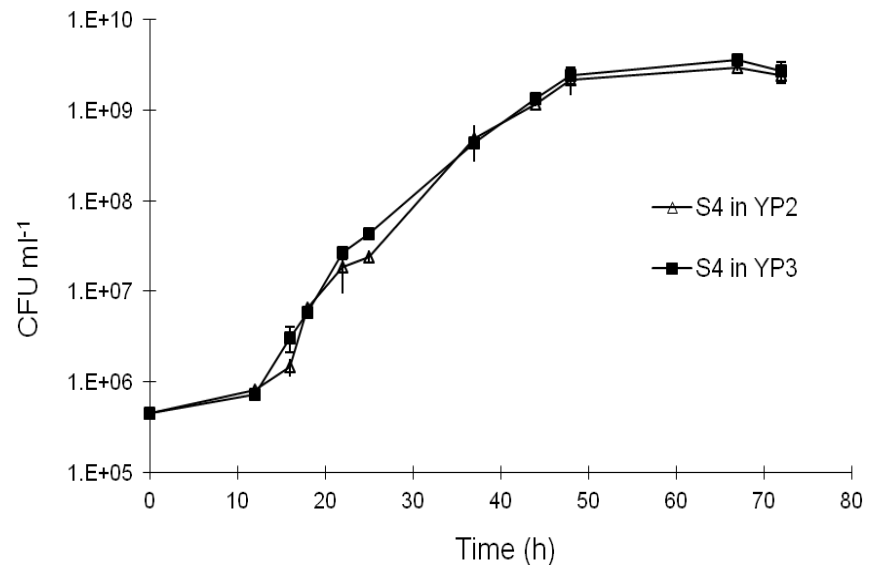
Figure 3. Phase contrast micrographs showing the morphology of *Phaeobacter* sp.

S4 in different growth phases. (A) Late exponential phase cells grown in YP3; (B)

Late exponential phase cells grown in YP2; (C) YP2-grown cells in rosettes; (D)

S4 cells grown in YP2 in a biofilm. Size bar = 10 μm.
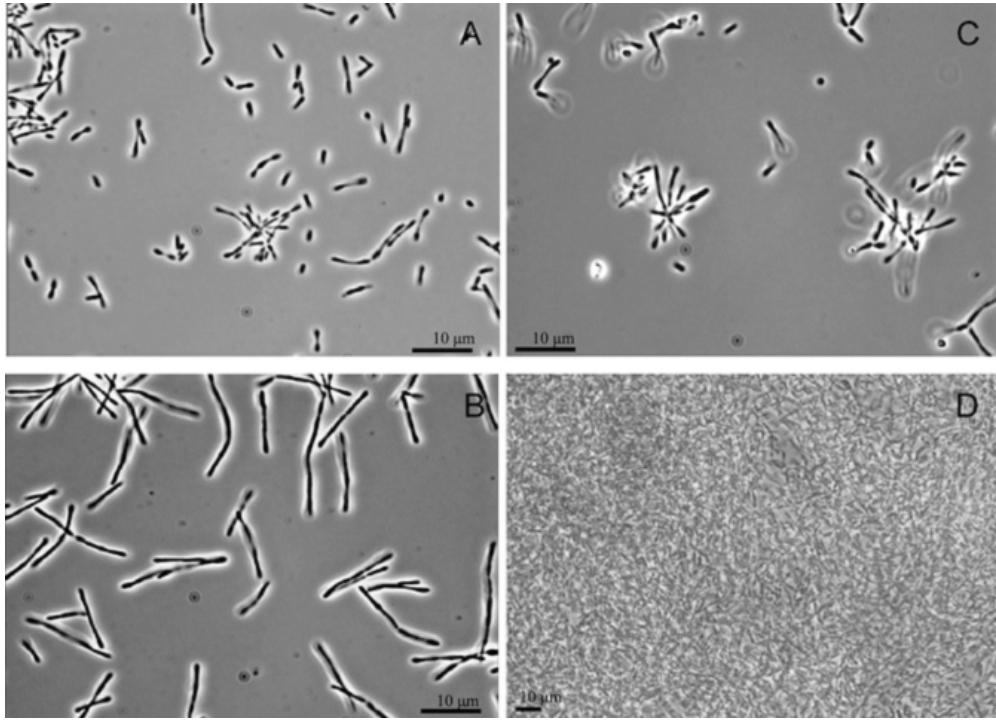
Figure 4. Effect of preincubation of larval oysters with candidate probionts RI06-95 and S4 at 10^4 CFU ml^{-1} on survival (%± SEM) 24 h after challenge with bacterial pathogens *Roseovarious crassostreae* CV919-312^T and *Vibrio tubiashii* RE22 at 10^5 CFU ml^{-1}. The candidate probionts were introduced 24 h before larvae were challenged. Representative of at least 3 experiments; different letters indicate statistical significance between groups (One Way ANOVA, $p<0.05$).
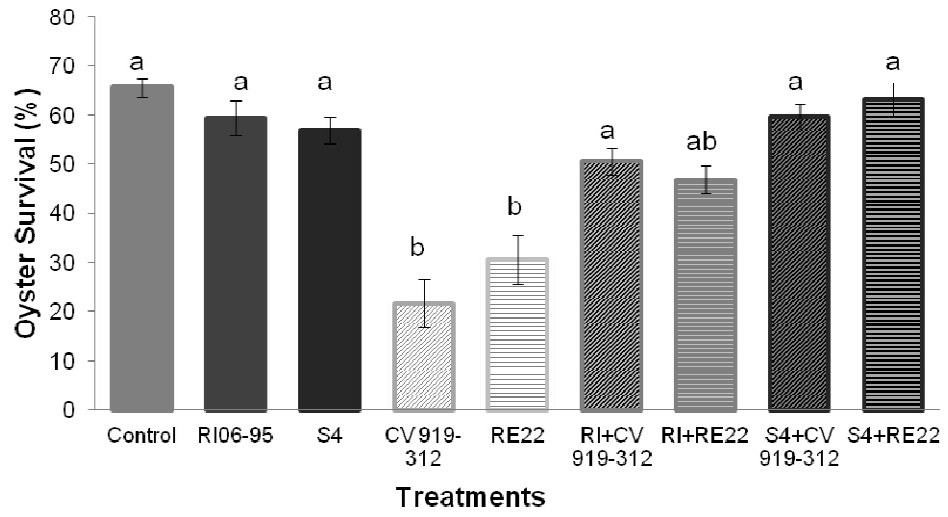
Figure 5. Length of protection to bacterial challenge provided by preincubation of larval oysters with candidate probionts RI06-95 and S4. Larval oysters were preincubated for 24 with 10^4 CFU ml^{-1} of the probiont, washed, placed in filtered sterile seawater and then challenged by adding 10^5 CFU ml^{-1} of pathogen *Vibrio tubiashii* RE22 0, 48, or 96 h after removal of the probionts. Different letters indicate statistical significance between treatments and times (2 way ANOVA, $p<0.05$).
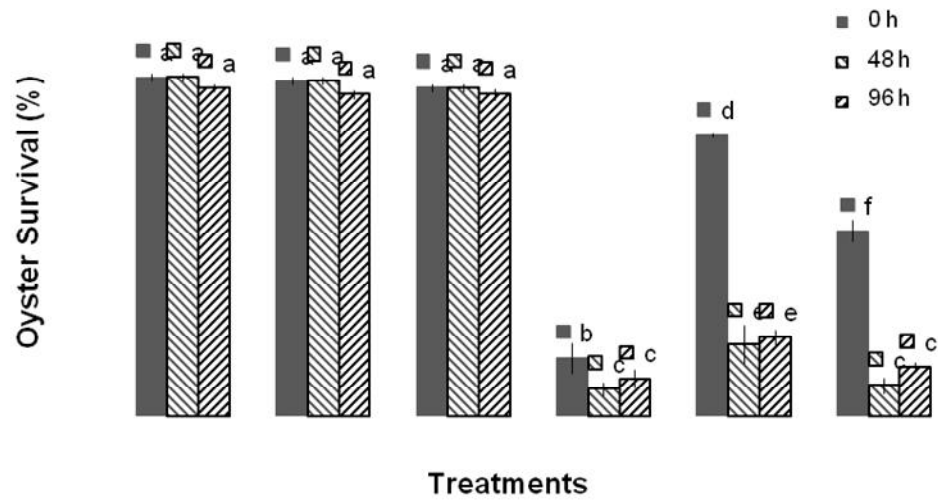
Figure 6. Effect of preincubation of juvenile oysters with candidate probionts on oyster survival after bacterial challenge. Oysters were preincubated with 10^4 CFU ml^{-1} of probionts *Bacillus pumilus* RI06-95 or *Phaeobacter* sp. S4 for 24h, then 10^5 CFU ml^{-1} of pathogens *Vibrio tubiashii* RE22 were added to the incubation seawater and survival was determined every 2 – 3 days for 13 days. Different letters indicate statistical significance between treatments (One way ANOVA, $p<0.05$).
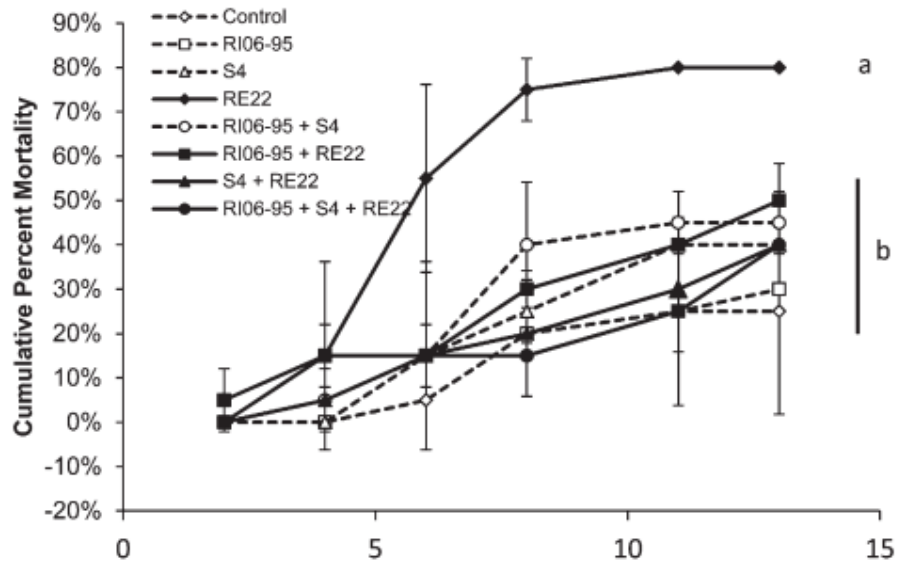
Title: Contributions of tropodithietic acid and biofilm formation to the probiotic activity of *Phaeobacter gallaeciensis*.

Short Title: Probiotic activity of *Phaeobacter gallaeciensis*

Authors: Wenjing Zhao[1], Christine Dao[2], Murni Karim[3], Marta Gomez-Chiarri[3], David Rowley[2] and David R. Nelson[1*]

Affiliation: [1]Department of Cell and Molecular Biology, [2]Biomedical and Pharmaceutical Sciences, [3]Fisheries, Animal and Veterinary Sciences, University of Rhode Island, Kingston, RI 02881

*Corresponding author: David R. Nelson, Department of Cell and Molecular Biology, 120 Flagg Rd., University of Rhode Island, Kingston, RI 02881, USA; E-mail: dnelson@uri.edu; Phone: 1-401-874-5902

Abstract

The probiotic bacterium *Phaeobacter gallaeciensis* strain S4Sm, isolated from the inner shell surface of a healthy oyster, secretes the antibiotic tropodithietic acid (TDA), is an excellent biofilm former, and increases oyster larvae survival when challenged with bacterial pathogens. In this study, we investigated the specific roles of TDA secretion and biofilm formation in the probiotic activity of S4Sm. For this purpose, mutations in *clpX* (ATP-dependent ATPase) and *exoP* (an exopolysaccharide biosynthesis gene) were created by insertional mutagenesis using homologous recombination. Mutation of *clpX* resulted in the loss of TDA production, no decline in biofilm formation, and loss of the ability of S4Sm to inhibit the growth of *Vibrio tubiashii* and *Vibrio anguillarum in vitro*. Mutation of *exoP* resulted in a ~70% decline in biofilm formation, no decline in TDA production, and delayed inhibitory activity towards *Vibrio* pathogens *in vitro*. Both *clpX* and *exoP* mutants exhibited reduced ability to protect oyster larvae from death when challenged by *Vibrio tubiashii*. Complementation of the *clpX* and *exoP* mutations restored the wild type phenotype. We also found that pre-colonization of surfaces by S4Sm was critical for this bacterium to inhibit pathogen colonization and growth. Our observations suggest that probiotic activity by S4Sm involves contributions from both biofilm formation and the production of the antibiotic TDA.

Introduction

Infections by pathogenic marine bacteria are a major problem for both the shellfish and finfish aquaculture industries, causing severe disease and high mortality, which seriously affect aquaculture production and cause significant economic loss [1]. This problem particularly affects the survival and growth of fish and shellfish during the larval and juvenile stages [1,2]. Opportunistic pathogens from the Vibrionaceae and at least one member of the *Roseobacter* clade cause disease in a variety of shellfish [3-5]. For example, *Vibrio tubiashii*, a reemerging pathogen of larval bivalve mollusks that causes invasive and toxigenic disease has been responsible for massive mortalities among larval oysters in hatcheries on the west coast of the United States [5]. Additionally, *Roseovarius crassostreae*, a member of the *Roseobacter* clade and the causative agent of juvenile or *Roseovarius* oyster disease (JOD or ROD), infects juvenile oysters in the summer when water temperatures are ≥20°C causing high mortalities [6]. Although antibiotics and vaccines can be used to control some infectious diseases in aquaculture, they have some distinct disadvantages and limitations. Use of antibiotics increases the risk of development and transfer of antibiotic resistance [7]. Vaccines, which rely on an adaptive immune response, are only effective for vertebrate organisms and cannot be used to protect shellfish [8].

Probiotics represent a promising alternative strategy to control infection and some probiotic strains are already used commonly in aquaculture as biological control agents in finfish and shellfish [9,10]. For example, the probionts *Bacillus subtilis* and *Bacillus licheniformis* are widely used in shrimp aquaculture to

provide beneficial effects potentially including improved health and water quality, control of pathogenic bacteria and their virulence, stimulation of the immune system and improved growth [11]. With the understanding that the use of antibiotics in large-scale aquaculture leads to the development and transfer of antibiotic resistance, investigation of probiotic approaches for the prevention of infectious disease has become important. For example, D'Alvise et al [12] have demonstrated that *Phaeobacter gallaeciensis* can be used as a probiotic treatment to reduce the density of the fish pathogen *Vibrio anguillarum* in cultures of cod larvae, resulting in the reduction of mortality by vibriosis in the cod larvae. The probiotic activity was dependent upon the production of tropodithietic acid (TDA) by *P. gallaeciensis*. Further, D'Alvise et al [13] previously demonstrated that a different TDA-producing strain of *Phaeobacter* was able to reduce or eliminate *V. anguillarum* from a combined liquid-surface system. These and other studies strongly suggest that antagonistic interactions by probiotic bacteria against marine pathogens may be useful in protecting commercially important species of shellfish and finfish from infectious disease.

Phaeobacter gallaeciensis are gram-negative α-*Proteobacteria* from the *Roseobacter* clade. The *Roseobacter* clade, an important member of the marine microbiota, accounts for ~5% to as much as ~40% of bacterial DNA from the ocean and plays an important role in the organic sulfur cycle of the ocean [14,15]. As noted above, several species in this clade have been shown to produce TDA and to exhibit inhibitory activity against the growth of marine pathogens, including *V. anguillarum*, *V. tubiashii* and *R. crassostreae* [16]. Additionally, many of these species from *Roseobacter* clade are routinely isolated from alga or larval cultures

of marine fish and shellfish [17]. Further, *Phaeobacter* species are typically excellent biofilm formers, colonizing a variety of surfaces including the walls of rearing tanks, microalgae, the skin of finfish, and the shells of mollusks [13,18,19]. Biofilm formation is thought to be essential for probiotic activity by a variety of mechanisms including competition for adhesion sites, oxygen, nutrients, and by preventing contact between pathogens and hosts [20].

Previously, we isolated *P. gallaeciensis* S4 from the inner shell surface of a healthy oyster [16]. This bacterium is a short rod with 1-2 flagella on one or both poles [16]. It has pleiomorphic morphology and will elongate into long rods and filaments under specific conditions (low salt concentration, static incubation, stationary phase [16]). It can form rosettes and is an excellent biofilm former and a dominant colonizer of surfaces in marine environments [16]. *P. gallaeciensis* S4Sm is a spontaneous streptomycin-resistant mutant of the parental S4 [16]. When S4Sm was used as a potential probiotic treatment of oyster larvae, it showed strong anti-pathogen activity and increased host survival. S4Sm can be used to antagonize diseases, but little is known about the actual mechanisms of action of this and other probiotic species [16].

In this study we examine the roles of biofilm formation and TDA production in probiotic activity of *P. gallaeciensis* S4Sm on oyster challenged by marine pathogen, *V. tubiashii*. In order to determine the contributions of TDA production and biofilm formation to the probiotic activity of S4Sm, mutations in *clpX* (a regulator of TDA biosynthesis pathway [21]) and an exopolysaccharide biosynthesis gene (*exoP*) were created by insertional mutagenesis. The effects of these mutations upon TDA production, biofilm formation and probiotic activity

were determined.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this report are listed in Table 1. *P. gallaeciensis* strains were routinely grown in yeast extract (0.5%)-peptone (0.1%) broth plus 3% sea salts, pH7.6 (YP30) [16], supplemented with the appropriate antibiotic, in a shaking water bath at 27°C. Overnight cultures of *P. gallaeciensis*, grown in YP30, were harvested by centrifugation (8,000 × *g*, 2 min) and the pelleted cells were washed twice with nine-salt solution (NSS) [22]. Washed cells were resuspended to appropriate cell densities in experimental media. Cell densities were estimated by optical density at 600 nm (OD_{600}) and more accurately determined by serial dilution and spot plating. Specific conditions for each experiment are described in the text. *Escherichia coli* strains were routinely grown in Luria-Bertani broth plus 1% NaCl (LB10) [23]. *Vibrio anguillarum* strains were routinely grown in LB20 at 27°C [24]. *V. tubiashii* and *R. crassostreae* strains were routinely grown in YP30 at 27°C [16]. Antibiotics were used at the following concentrations: streptomycin, 200 μg/ml (Sm^{200}); ampicillin, 100 μg/ml (Ap^{100}) for *E. coli* and *Vibrio* strains; chloramphenicol, 20 μg/ml (Cm^{20}) for *E. coli* and 5 μg/ml (Cm^{5}) for *P. gallaeciensis* and *Vibrio* strains; kanamycin, 50 μg/ml (Km^{50}) for *E. coli* strains and 200 μg/ml (Km^{200}) for *P. gallaeciensis*; and tetracycline, 15 μg/ml (Tc^{15}) for *E. coli* and 1 μg/ml (Tc^{1}) for *V. anguillarum*.

Insertional mutagenesis

Insertional mutagenesis by homologous recombination was used to create

interruptions within specific genes using a modification of the procedure described by Milton and Wolf-Watz [25,26]. Primers (Table 2) were designed to amplify specific *Phaeobacter* genes based on homologous sequences from *P. gallaeciensis* 2.10 (GenBank accession No.CP002972.1). A fragment of the selected gene was PCR amplified, then digested with SacI and XbaI restriction enzymes, and the DNA fragments separated on a 1% agarose gel. Subsequently, the gel-purified PCR fragment was ligated into the suicide vector pNQ705 after digestion with SacI and XbaI and the ligation mixture was introduced into *E. coli* Sm10 (λ pir) by electroporation with Bio-Rad Gene Pulser II. The resulting recombinant plasmids were confirmed by both PCR amplification and sequencing. The mobilizable suicide vector was transferred from *E. coli* Sm10 (λ pir) into S4Sm by conjugation. Transconjugants were selected by utilizing the chloramphenicol resistance gene located on the suicide plasmid. The incorporation of the suicide vector into the gene of interest was confirmed by PCR analysis and DNA sequencing.

Complementation of mutants

P. gallaeciensis mutants were complemented by cloning the appropriate gene fragment into the shuttle vector pBBR1MCS4 (GenBank accession No. U25060), using a modification of the method described previously by Rock and Nelson [27]. Primers (Table 2) were designed with a SacI or XbaI site added to the 5' end of the appropriate primer. The primer pair was then used to amplify the entire gene plus ˜500 bp of the 5′ and 3′ flanking regions from genomic DNA sequences of *P. gallaeciensis* 2.10 (GenBank accession No.CP002972.1). The resulting amplicon was ligated into the pBBR1MCS4 plasmid after digestion with SacI and XbaI and the ligation mixture introduced into *E. coli* Sm10 (λ pir) by electroporation with

Bio-Rad Gene Pulser II. Transformants were selected on LB10-Amp100 agar plates and the recombinant plasmids confirmed by both PCR amplification and sequencing. The complementing plasmid, pBBR1MCS4-*clpX* or pBBR1MCS4-*exo*P, was transferred from *E. coli* Sm10 into *clpX* or *exoP* mutants by conjugation using the procedures described previously. The transconjugants were confirmed by PCR amplification.

Fluorescence tagging of *P. gallaeciensis* strains and *Vibrio species*

P. gallaeciensis strains were tagged by pRhokHi-2-OFP and *V. tubiashii* was tagged by pRhokHi-2-GFP. The orange fluorescence protein gene (*ofp*) and the green fluorescence protein gene (*gfp*) were PCR amplified by using the appropriate primer pair (Table 2) designed according to the sequence of pmOrange vector (Clontech, Cat. No. 632529) and pSUP202p/P*flaB-gfp* vector. The PCR product was digested with NdeI and BamHI restriction enzymes and the DNA fragments separated on a 1% agarose gel. Subsequently, the gel-purified *ofp* or *gfp* PCR fragment was ligated into pRhokHi-2 after digestion with NdeI and BamHI and the ligation mixture was introduced into *E. coli* Sm10 (λpir) by electroporation with Bio-Rad Gene Pulser II. Transformants were selected on LB10-Cm^{20} agar plates. The resulting plasmids, pRhokHi-2-OFP or pRhokHi-2-GFP, were transferred from *E. coli* Sm10 into S4Sm, *clpX* and *exoP* mutants or *V. tubiashii* and *R. crassostreae* by conjugation using the procedures described previously. pSUP202p/P*flaB-gfp* was transferred from *E. coli* Sm10 into *V. anguillarum* by conjugation using the procedures described previously [28]. The transconjugants were confirmed by fluorescence microscopy.

TDA purification, identification and detection.

P. gallaeciensis S4Sm was cultured in 7 x 1 L volumes of YP30 culture medium at 27°C with shaking at 175 rpm. After 96 h, the cells were pelleted by centrifugation at 10,000 rpm for 10 min. The resulting culture supernatants were acidified to pH 3 with formic acid (FA) and extracted with acidified (0.1% FA) ethyl acetate. The organic extract was concentrated *in vacuo* to yield 0.673 g of crude extract. The extract was fractionated using C18 flash chromatography (Redisep Rf high performance gold 30g hp combiflash column; linear gradient elution 5% - 100% CH_3OH in H_2O, 0.1% FA, 35 ml/min, 45 min). Fractions containing TDA (t_R= 15 min) were further purified by reversed-phased HPLC (Xterra 5μm C18 100 x 3.0 mm column, 0.5 ml/min, 5% to 100% CH_3OH in H_2O over 24 min). Pure TDA (10 mg) was identified based on comparison of ^{1}H NMR (Varian 500 MHz spectrometer) and mass spectral data in comparison to previously reported values [29].

Culture supernatants from various *P. gallaeciensis* strains were analyzed by HPLC for the presence of TDA. *P. gallaeciensis* strains were cultivated in 50 ml YP30 broth until stationary phase (OD_{600} = 0.8). Cells were pelleted by centrifugation at 5000 ×*g* for 10 min, and the resulting supernatant was acidified to pH3 with FA and then partitioned with acidified ethyl acetate (0.1% FA). The organic layer was concentrated to dryness *in vacuo* and then reconstituted as a 10mg/ml solution in methanol (Pharmco-AAPER). HPLC chromatography was performed on a Hitachi LaChromUltra UHPLC equipped with a Fortis C18 UHPLC Column (1.7 μm, 2.1 x 50 mm). Method: 0.25 ml/min flow rate, 5% methanol in H_2O (both acidified with 0.1% FA) for 1 min, linear gradient to 100% CH_3OH over 6.2 min, 100% CH_3OH for 2 min.

Minimum inhibitory concentrations of TDA against *V. anguillarum*, *V. tubiashii*, and *R. crassostreae*

The minimal inhibitory concentrations (MIC) of TDA against the marine pathogens were determined using a broth dilution method in microtiter plates [30]. Overnight bacterial cultures were diluted to 10^5 CFU/ml in YP30 and treated with serial dilutions of pure TDA. After 24 h incubation, MICs were determined as the lowest concentration where there was no visible growth. Two independent experiments were done and each independent experiment had three replicates.

Biofilm formation

Biofilm formation was assessed using a modification of the crystal violet (CV) staining method [19]. Bacteria were grown for 2 days in YP30 (27°C with shaking) to an cell density $\sim 2 \times 10^9$ CFU/ml (2 μl; 0.1% inoculum) were transferred into 2 ml of fresh YP30 broth in 30 mm × 100 mm borosilicate (Pyrex) glass culture tubes containing 2 ml of YP30 broth and allowed to grow at 27°C without shaking. When sampling, the liquid culture was discarded and each tube rinsed twice with NSS to remove loosely attached cells. The biofilm attached to the test tube wall was stained with 2 ml of CV solution (0.2%) for 20 min at room temperature. Unbound dye was removed with two washes of NSS. The bound dye was eluted with 95% (vol/vol) ethanol for 30 min and then the amount of eluted crystal violet was measured by spectroscopy at 580 nm using a VERSA-MAX microplate reader.

Inhibition zone assay

Anti-bacterial activity of *P. gallaeciensis* strains was measured by a growth

inhibition assay using *V. anguillarum*, *V. tubiashii*, and *R. crassostreae* as the target organisms. Briefly, an aliquot (100 μl) from a stationary phase overnight culture of the appropriate *Vibrio* or *R. crassostreae* culture was spread onto YP30 agar plates, then 10 μl of a 2-day-old culture (OD600 = 0.8) of a *P. gallaeciensis* strain was spotted in triplicate onto the pathogen cell lawn. After incubation at 27°C for 24 h, the level of antibacterial activity was determined by the diameter of the inhibition zone around the *P. gallaeciensis* colonies.

P. gallaeciensis culture supernatant killing assay

In order to determine the bactericidal activity of culture supernatants, *P. gallaeciensis* strains were grown for 2 days in YP30 (27°C with shaking). Cultures were centrifuged (8,000 × *g*, 10 min) and filtered through 0.2 μm pore membrane filters to collect filter sterilized cell-free supernatants. Overnight cultures of *V. anguillarum* (NB10Sm) cells were then serially diluted in filter sterilized, cell-free *P. gallaeciensis* culture supernatant obtained from the various strains of *P. gallaeciensis* or NSS, and then spotted (10 μl/spot of diluted *V. anguillarum* cells) in triplicate onto YP30 plates. The time of exposure to S4Sm supernatant was during serial dilution (<5min) followed by time on plate with diffusion of TDA. Killing percentage was calculated as follows: Killing % = [(no. of colonies in NSS control) − (no. of colonies in S4 supernatant treated)/ (no. of colonies in NSS control)] × 100

Glass coverslip colonization competition assay between *P. gallaeciensis* strains and *V. tubiashii* WZ103 or *V. anguillarum* WZ203.

For all competition experiments, *P. gallaeciensis* strains (S4Sm, *clpX* mutant

and *exoP* mutant) were grown for 2 days in YP30 (27°C with shaking) to an OD_{600} ~0.8. Cells were harvested by centrifugation, washed twice in NSS, resuspended in fresh YP30, and then transferred into 6-well plates (Costar 3516). Each well contained a glass coverslip, 4 ml YP30 broth supplemented with the appropriate antibiotics, and was inoculated with the appropriate *P. gallaeciensis* strain tagged with orange fluorescence protein (OFP) (final concentration ~1×10^4 CFU/ml). For experiments examining the effects of pretreatment with *P. gallaeciensis*, after 24 h incubation at 27°C with no shaking (pretreatment with *P. gallaeciensis*) all coverslips were washed twice with NSS. Each coverslip was transferred into a fresh well containing 4 ml of YP30 broth supplemented with the appropriate antibiotic plus the green fluorescence protein (GFP)-tagged *V. tubiashii* WZ103 or GFP-tagged *V. anguillarum* WZ203 (final concentration ~1×10^5 CFU/ml). After another 24 h incubation at 27°C with no shaking, all coverslips were removed, washed twice on a rotary shaker (LAB-LINE instrument, Inc.) for 2 min (200 rpm) with NSS, and then transferred into clean wells with fresh YP30 broth and allowed to incubate as before. Two coverslips were removed at each sampling time (24, 48, 72 h). One was used for determination of the cell density of the strains on the coverslip; the second one was used for confocal imaging. Glass coverslips were washed with NSS twice on a rotary shaker for 2 min. After draining excess water, coverslips used for confocal imaging were placed on depression slides and cells on the upside of coverslip were wiped off with Kimwipes™. Coverslips used for CFU determinations were immersed in 50 ml plastic tubes containing 10 ml NSS and glass beads (0.5 g, 1 mm), then vortexed for 1 minute. Cell densities (CFU/ml) in the wells or suspended from the coverslip were determined by serial dilution and spot plating. For experiments without pretreatment with *P. gallaeciensis*, all

procedures were identical to those described above except that GFP-tagged *V. tubiashii* WZ103 or *V. anguillarum* WZ203 were added at the same time as OFP-tagged *P. gallaeciensis*. Additionally, in the *V. anguillarum* competition experiments, both *P. gallaeciensis* and *V. anguillarum* were inoculated at ~10^6 CFU/ml.

Effects of TDA supplementation on pathogen growth in a co-culture system containing the *clpX* mutant and a *Vibrio* species.

OFP-tagged *P. gallaeciensis* strains (S4Sm, *clpX* mutant) grown for 2 days in YP30 (27°C with shaking) to an OD_{600} ~0.8, cells were transferred into 6-well plates (Costar 3516). Each well was inoculated with the appropriate OFP-tagged *P. gallaeciensis* strain (initial concentration at ~ 10^4 CFU/ml) and contained 4 ml of YP30 broth supplemented with the appropriate antibiotic and one glass coverslip. After 24 h incubation (pre-treatment with *P. gallaeciensis*), all coverslips were washed twice in NSS. Each coverslip was transferred into a clean well containing 4 ml YP30 broth and either GFP-tagged *V. anguillarum* WZ203 or *V. tubiashii* WZ103 at a concentration of ~ 10^5 CFU/ml plus TDA (5 μg/ml for *V. anguillarum* WZ203 or 10 μg/ml for *V. tubiashii* WZ103; based on calculated MIC). The biofilms on the coverslips were imaged as described below and cell densities were determined as described above.

Laser confocal scanning microscopy

Laser confocal scanning microscopy was performed in the Rhode Island Genomic Sequencing Center using Zeiss LSM 700 laser scanning confocal imaging system and Zeiss Axio Imager 2 microscope.

Challenge trial

Oyster larvae (n=21-28 per well, veliger stage, ~0.060-0.150 mm in diameter) were placed in wells of a 6-well plate containing 5 ml of sterile filtered seawater (28 psu). *P. gallaeciensis* strains were added to a concentration of ~10^4 CFU/ml. Plates were incubated at 20°C for 24 h with shaking. Water was changed and *V. tubiashii* RE22 was added at a concentration of ~10^5 CFU/ml and incubated for an additional 24 h before counting living and dead oysters. Oyster larvae treated only by artificial seawater serve as control (mock). The survival rate was calculated by using the formula: *Survival rate (%) = 100 x (number of live larvae/total number of larvae).* These experiments were run at least 2 times in triplicate [16]. As invertebrates, oysters are exempt from approval from IACUC.

Statistical analysis

Data statistical analysis was performed using SPSS v16.0 with general linear model (univariate or multivariate) for Windows and P<0.05 was considered to indicate a statistically significant difference.

Results

***P. gallaeciensis* S4Sm secretes the antibiotic tropodithietic acid.**

Bioassay-guided fractionation of *P. gallaeciensis* supernatants resulted in the purification of a single secondary metabolite possessing antimicrobial activity. The molecule was identified as tropodithietic acid (TDA) based upon a molecular ion of $[M+H]^+ = 211$ [14] and comparison of ^{1}H NMR chemical shift data (500 MHz, C_6D_6) with literature values (not shown). UHPLC analysis data (Fig. 1A) confirmed that TDA was present in S4Sm supernatant.

Biofilm formation by *P. gallaeciensis* S4Sm.

It was previously reported that *Phaeobacter* spp. are effective surface colonizers [13,17]. Using the crystal violet staining assay [19] to determine biofilm formation ability of S4Sm, we observed that S4Sm formed thick biofilms on glass. In this assay, the OD_{580} value for the S4Sm biofilm after 60 h was ~4.0 at 27°C under static conditions (Table 3). In contrast, all three pathogens (*V. anguillarum, V. tubiashii,* and *R. crassostreae*) used in this study had biofilms that were between 13.4-14.9% of the S4Sm (Table3) ($P< 0.05$).These data suggested that S4Sm was able to form a thick, dense biofilm matrix on glass coverslips and tubes.

Differential sensitivities of marine pathogens to TDA.

It was previously reported that *Phaeobacter* species antagonized and killed the fish pathogen *V. anguillarum* [13,17]. Since we had previously shown that *P. gallaeciensis* S4 was able to protect oyster larvae against mortalities caused by *V. tubiashii* and *R. crassostreae* [16], we examined the relative sensitivities of the three pathogens (*V. anguillarum* NB10Sm, *V. tubiashii* RE22Sm, and *R. crassostreae* CV919Sm) to *P. gallaeciensis* S4Sm (a spontaneous streptomycin-resistant mutant of S4) by looking at the inhibition of growth around a colony of S4Sm. *V. anguillarum* NB10Sm was most sensitive to S4Sm with largest zone of inhibition (ZOI) (diameter = 12.5±0.5 mm); *R. crassostreae* exhibited slightly less sensitivity to S4Sm (ZOI =11.2±0.3 mm); and the least sensitive pathogen to S4Sm was *V. tubiashii* RE22Sm (ZOI = 9.2±0.6 mm) (Fig. 1C) These data corresponded with the results for minimum inhibitory concentration (MIC) of TDA against each of the three pathogens: the MIC for

TDA against NB10Sm was 1.25 µg/ml, against *R. crassostreae* the MIC was 5 µg/ml, and against RE22Sm the MIC was 6.25 µg/ml.

Effect of *clpX* gene mutation on TDA production.

In order to examine the roles of TDA production and biofilm formation in the probiotic activity of S4, we performed mutations on genes involved in the TDA biosynthesis pathway. Our data showed that *tdaA*, *tdaB*, and *tdbD* mutants not only lost TDA production, but were also defective for biofilm formation (Fig. S1). In order to examine the separate roles of TDA production and biofilm formation with respect to probiotic activity, we needed mutants that were deficient in either TDA synthesis or biofilm production. It was previously shown that mutation in *clpX* by transposon mutagenesis resulted in the loss of TDA production in *Phaeobacter sp*. strain 27-4 [21]. The *clpX* gene was identified in S4Sm and found to encode a 408 amino acid ATP-dependent protease ATP-binding subunit and is part of the ClpXP multimer. Mutation of *clpX* by insertional mutagenesis resulted in the loss of TDA production. UHPLC analysis data (Fig. 1B) showed that no TDA was present in *clpX* mutant supernatant. Further, there were no inhibition zones around the *clpX* mutant cells when tested against the three pathogens, *V. anguillarum* NB10Sm, *V. tubiashii* RE22Sm, and *R. crassostreae* CV919Sm (Fig. 1C). Additionally, culture supernatant from the *clpX* mutant was no longer able to kill NB10Sm cells (Table 4). Complementation of the *clpX* gene restored TDA production (Fig. 1B) and anti-*Vibrio* activity (Fig. 1C and Table 4). Mutation of *clpX* did not result in defective biofilm formation (Table 3).

Effect of *exoP* gene mutation on biofilm formation

In order to develop a strain of S4 defective in biofilm formation but able to produce TDA, the *exoP* gene, which encodes an exopolysaccharide biosynthesis domain protein, was identified in *P. gallaeciensis* S4Sm strain. Mutation of *exoP* resulted in decreased biofilm formation, with the *exoP* mutant exhibiting only 25% to 50% of the wild type level (Table 3) (P < 0.05). Complementation of *exoP* gene restored biofilm formation to wild type level (Table 3). Mutation of *exoP* did not result in defective TDA production (Fig. 1B).

Effect of *clpX* and *exoP* mutations on the ability of *P. gallaeciensis* to antagonize *Vibrio* species in a mixed culture colonization assay.

The *clpX* mutant is characterized by the inability to produce TDA, but is able to form a normal biofilm, while the *exoP* mutant is characterized by its reduced ability to form a biofilm while producing TDA at wild type levels. This allowed us to examine the relative roles of biofilm formation and TDA production on the ability of S4 to antagonize colonization of glass surfaces by the pathogens used in this study, as well as decrease the levels of pathogen in the culture media. When a co-colonized glass coverslip was examined after 72 h of growth by laser scanning confocal microscopy, more RE22Sm cell clusters were observed in the biofilm matrix of the *clpX* mutant than in the biofilm matrix of either S4Sm wild type or *exoP* (Fig. 2A). These observations were reflected in the viable cell counts of the *V. tubiashii* RE22Sm in both biofilms (sessile) and in suspension (planktonic) when grown in the presence of biofilms of *P. gallaeciensis* S4Sm wild type, the *clpX* mutant or the *exoP* mutant (Fig 2B-E). For example, as shown in Fig 2B and 2C at 123 h, the number of viable RE22Sm in the biofilm on a coverslip was 1×10^4 CFU when precolonized with S4Sm. In contrast, the number of RE22Sm cells increased

180-fold (to 1.8×10^6 CFU/coverslip) when grown in the presence of the *clpX* mutant. This was about the same number of cells on a coverslip as when RE22Sm was allowed to colonize alone. Further, when grown in the presence of the *exoP* mutant the number of viable RE22 cells was 4.5-fold higher (4.5×10^4 CFU/coverslip) than in the presence of S4Sm cells. In suspension, the cell density of RE22Sm reached 2×10^8 CFU/ml under conditions of precolonization by the *clpX* mutant; this was similar to the density of RE22Sm grown alone (1.8×10^8 CFU/ml), but about two orders of magnitude higher than when RE22Sm was co-cultured with either S4Sm (3.1×10^6 CFU/ml) or *exoP* (2.6×10^6 CFU/ml) (P < 0.05) (Fig. 2E). These data indicated that the *clpX* mutant was not able to inhibit RE22Sm growth or biofilm formation under the tested conditions, while the *exoP* mutation had little effect on the ability to inhibit RE22Sm growth or biofilm formation. When the same experiments were performed using *V. anguillarum* NB10, the results were very similar. The wild type S4 almost completely eliminated NB10 from the coverslip and from suspension by 40 to 48 h, respectively (Fig. S2B-D). The *exoP* mutant inhibited NB10 biofilm formation and survival in suspension almost as well as S4Sm. In contrast, the *clpX* mutant (TDA deficient) exhibited almost no inhibition on either biofilm formation or survival of NB10, compared to the ones when NB10 grown alone. These observations are also illustrated by the confocal images of biofilms formed by OFP-tagged *P. gallaeciensis* strains and GFP-tagged NB10 cells (Fig. S2A).

Addition of exogenous TDA restores the antagonistic activity of the *clpX* mutant.

The correlation between the loss of TDA production and that the *clpX* mutant was unable to block biofilm formation by any of the tested pathogens strongly suggested that the loss of TDA biosynthesis was responsible for the defect in antagonistic activity in the *clpX* mutant. In order to confirm the hypothesis, we repeated the coverslip colonization experiments with the addition of exogenous TDA. TDA (10 μg/ml) was added to the co-culture system at the same time as the pathogens; distilled water was added to the non-exogenous-TDA control group. As expected, 24 h after RE22Sm addition, the amount of RE22 in the biofilm formed by both RE22 and WZ10 (*clpX* mutant) was significantly (15-fold) higher than RE22Sm co-cultured with S4Sm $(1.8 \times 10^6 \pm 4 \times 10^5 \text{CFU/coverslip}$ vs $1.2 \times 10^5 \pm 3.5 \times 10^4$ CFU/coverslip, P=0.019) (Fig. 3A) in the non-exogenous-TDA group. However, when the cultures were supplemented with 10 μg/ml of exogenous TDA, the amount of RE22Sm (at T=24 h) in the biofilm of RE22Sm and *clpX* mutant showed no significant difference from the amount of RE22Sm (at T=24 h) in the biofilm of RE22Sm and S4Sm $(2.27 \times 10^3 \pm 288$ CFU/coverslip vs. $1.37 \times 10^3 \pm 321$ CFU/coverslip, $P = 0.099)$. Further, the amount of RE22Sm in the biofilm (at T=24 h) in a monoculture in the presence of TDA $(1.5 \times 10^3 \pm 600$ CFU/coverslip) (Fig. 3A) was not significantly different from the amounts found in either of the two TDA-treated mixed culture biofilms. Similar results were observed for planktonic RE22 cells in culture of the co-culture system (Fig. 3C).

The effects of exogenous TDA were transitory. By 48 h after TDA addition, amounts of sessile RE22Sm cells, which was co-cultured with *clpX* mutant, increased nearly two log units (24 h vs. 48 h, $2.27 \times 10^3 \pm 288$ CFU/coverslip vs. $3.67 \times 10^5 \pm 1.53 \times 10^5$ CFU/coverslip). However, amount of sessile RE22Sm, which was co-cultured with S4Sm, only exhibited ~2.3-fold increase from $1.37 \times 10^3 \pm 321$

CFU/coverslip (24 h) to $3.13\times10^3\pm1.07\times10^3$ CFU/coverslip (48 h). Additionally, at 48 h the amounts of RE22Sm cells in both the biofilm and in suspension, which were co-cultured with the *clpX* mutant, were not significantly different with the values for RE22Sm cultured alone (Fig. 3A and C). Further, the confocal micrographic images of biofilms from 48 h cultures confirmed that more RE22Sm cells (green) were observed in the *clpX* mutant biofilm than in S4Sm biofilm (Fig. 3B). The explanation for the transient effect of exogenous TDA upon RE22Sm cells could be that TDA is unstable over the period of the assay. Taken together, our data strongly suggest that the loss of TDA biosynthesis is responsible for the defect in antagonistic activity in the *clpX* mutant.

The effects of *V. tubiashii* on growth of *P. gallaeciensis* strains in competition assays.

Our study showed that *P. gallaeciensis* strains (S4Sm wild type, *clpX* or *exoP* mutants) exhibited different inhibitory effects against *V. tubiashii* in competition assays. In order to see if *V. tubiashii* would affect the growth of our various *P. gallaeciensis* strains, we compared the growth of *P. gallaeciensis* strains in the presence of *V. tubiashii* with monoculture controls. The growth of wild type S4Sm and *exoP* mutant were not affected by *V. tubiashii* in either suspension or on the coverslip (Fig. 4). In contrast, the growth of the *clpX* mutant was affected by *V. tubiashii*. At each time point tested, the density of the *clpX* mutant (grown with RE22Sm) was lower than that of the monoculture control (Fig. 4). For example, at 72 h the biofilm density of *clpX* mutant cells grown in the presence of RE22 was 13.2% of *clpX* mutant cells grown axenically ($3.3\times10^6\pm5.3\times10^5$ CFU/coverslip vs. $2.5\times10^7\pm1.2\times10^6$ CFU/coverslip, P< 0.05). Similarly, the planktonic cell density of *clpX* mutant cells grown in the presence of RE22 was 13.5% of *clpX* mutant

cells grown axenically ($3.1\times10^7\pm6.0\times10^6$ CFU/ml vs. $2.3\times10^8\pm6.9\times10^7$ CFU/ml, P< 0.05). These data suggest that *P. gallaeciensis clpX* mutant was affected differently by *V. tubiashii* when co-cultured; however, the wild type S4Sm and *exoP* mutant were not affected by co-culture with *V. tubiashii*. Additionally, when *V. anguillarum* NB10Sm was co-cultured with either S4Sm or the *exoP* mutant, it did not affect their growth; however, NB10Sm did inhibit the growth of the *clpX* (Fig. S3).

Pre-colonization of *Phaeobacter* is important for probiotic activity of S4Sm against *V. tubiashii in vitro.*

Karim et al. [16] and D' Alvise et al. [12] showed that pretreatment with probiotic *Phaeobacter* species protected oyster and cod larvae, respectively, from infections caused by pathogenic bacteria. In order to determine if pre-colonization was important for the probiotic/antagonistic activity of *P. gallaeciensis* S4Sm against *Vibrio* pathogens, the effects of a 24 h pre-colonization by the probiont followed by addition of the pathogen were tested (Fig. 5). On the coverslip, the amount of RE22Sm cells in the biofilm (without pre-colonization:RE22Sm and S4Sm were introduced into system at the same time) was $\sim8.3\times10^7$ CFU/coverslip at 48h (Fig. 5A). This was 830-fold more RE22 cells than the one detected in the biofilm of a coverslip, which was pre-colonized with S4 (1×10^4 CFU/coverslip) (Fig. 2B). Similarly, without pre-colonization by the *P. gallaeciensis* mutants (*clpX* or *exoP* mutants) RE22Sm exhibited 10- to 100-fold more cells in the mixed biofilm compared to biofilms formed with pre-colonization by the *P. gallaeciensis* mutants (for *exoP*: the amount of RE22Sm with pre-colonization is $\sim1\times10^5$ CFU/coverslip, without pre-colonization $\sim1.1\times10^7$ CFU/coverslip; for *clpX*: with

pre-colonization~1.2×10^6 CFU/coverslip, without pre-colonization~$1.0^5\times10^7$ CFU/coverslip) (Fig. 2B, Fig. 5A). Further, as shown above, in experiments where S4Sm was allowed to pre-colonize coverslips the density of planktonic RE22Sm cells (~2.8×10^7 CFU/ml) was only 8.7% of the density of plankton S4Sm cells (3.2×10^8 CFU/ml) in the same co-culture system at 48h (Fig. 2D); however, without pre-colonization, cell density of planktonic RE22Sm (at 48 h) was~ 9.3×10^8 CFU/ml. This was >30-fold higher than the density of RE22Sm observed in the pre-colonized culture (2.8×10^7 CFU/ml). This was also 66-fold higher than the growth of S4Sm (1.4×10^7 CFU/ml) in the same co-culture system (Fig. 5B). In contrast, pre-colonization with S4Sm was not necessary to antagonize *V. anguillarum* (NB10Sm). In experiments where S4Sm and NB10 were inoculated together, NB10Sm was eliminated from both the coverslip biofilm and the liquid culture by 48 h (Fig. S2). Further, NB10 was also more sensitive to both the *exoP* and *clpX* mutants than RE22 in the co-culture experiments. These data indicate that pre-colonization facilitated S4Sm inhibition of *V. tubiashii* RE22, but was not necessary for inhibition against *V. anguillarum* NB10. Further, the data also suggest that *V. anguillarum* NB10 is more sensitive to *P. gallaeciensis* S4Sm than is *V. tubiashii* RE22.

Mutations in *clpX* and *exoP* affect probiotic activity of *P. gallaeciensis* against *V. tubiashii* in oyster larvae.

It was reported previously that *P. gallaeciensis* was able to reduce *V. anguillarum* in cultures of microalgae and rotifers, and prevent vibriosis in cod larvae [12]. Also our previous study showed that S4Sm provided protection to oyster larvae against *V. tubiashii* RE22 [16]. In order to determine if mutations in

TDA production or biofilm formation would affect the probiotic activity of S4Sm against *V. tubiashii in vivo*, oyster challenge assays were performed and the survival of oyster larvae was determined (Fig. 6). *P. gallaeciensis* mutants showed a significant reduction in their ability to protect oysters against *V. tubiashii* challenge compared to wild type S4Sm. The *clpX* mutant exhibited a >50% decline in oyster larvae survival compared to S4Sm (S4Sm: 72.4% ± 1.4% vs. *clpX*: 35.7% ± 3.3%, P< 0.05), while the *exoP* mutant provided almost 70% of the protection as S4Sm (S4Sm: 72.4% ± 1.4% vs. e*xoP*: 50.6% ± 8.3 %, P<0.05) (Fig. 6). However, both *P. gallaeciensis* mutants still provided partial protection. Survival in larvae pretreated with either the *clpX* or *exoP* mutant (35.7% ± 3.3% and 50.6% ± 8.3%, respectively) was significantly higher than the survival of larvae treated only with RE22 (20.3% ± 1.9%, P < 0.05) (Fig. 6). These data show that both the *clpX* and *exoP* mutant exhibited decreased ability to protect oyster larvae against *V. tubiashii*.

Discussion

Several *Phaeobacter* species are known to have probiotic activity and are able to protect fish species against bacterial pathogens [12]. The production of the broad-spectrum antibiotic, tropodithietic acid (TDA) is regarded as one of the major factors contributing to probiotic activity [12]. We recently reported that the new isolate *P. gallaeciensis* S4Sm protects the Eastern oyster (*Crassostrea virginica*) from infection by two oyster pathogens, *V. tubiashii* and *R. crassostreae* [16]. In this report, we dissect the roles of TDA biosynthesis and biofilm formation in promoting probiotic activity by *P. gallaeciensis* S4Sm, showing that both mechanisms are involved.

Although the TDA biosynthetic pathway has not been fully elucidated, many of the genes required for the formation of TDA and much of the pathway have been discovered [21,31,32]. One gene reported to be involved in the regulation of TDA biosynthesis is *clpX* (encoding ClpX) [21]. ClpX is an AAA+ ATPase that functions as an unfoldase chaperon for ClpP (ATP-dependent protease) and with ClpP forms the multimeric ClpXP protease [33]. We created an insertional mutation in the *clpX* gene and this mutation specifically blocked the biosynthesis of TDA in S4Sm (Fig. 1A) without affecting biofilm formation. In contrast, mutations in *tdaA*, *tdaB*, and *tdbD*, all block TDA biosynthesis and also affect biofilm formation. The mechanism by which ClpX affects TDA production is still unknown. Additionally, the reasons why mutations in *tdaA*, *tdaB*, and *tdbD* decrease biofilm formation, as well as TDA biosynthesis, are not understood.

The *clpX* mutant was unable to inhibit *V. tubiashii* growth in either liquid or as a biofilm on a glass coverslip (Fig. 2); however, when cultures were supplemented with TDA, *V. tubiashii* growth was inhibited (Fig. 3). These data strongly suggest that the loss of TDA production is responsible for the defect in antagonistic activity in the *clpX* mutant. Further, 48 h after the addition of TDA into the co-culture the inhibitory effect of TDA disappeared, likely due to instability of TDA over time. Except for the loss of TDA synthesis, the *clpX* mutant exhibits no other defects in growth or biofilm formation compared to the S4Sm wild type. Moreover, the results reported here confirm the role of TDA as an antibiotic promoting probiotic activity of *Phaeobacter* species described previously by D'Alvise et al [12].

P. gallaeciensis, a member of the abundant marine Roseobacter clade, is

known to be an excellent colonizer of environmental surfaces [31]. However, no study of the effects of biofilm formation on the probiotic mechanism of *Phaeobacter* has been reported. The *exoP* gene was identified in S4Sm (using RAST [34]) as an exopolysaccharide biosynthesis gene, which is thought to be involved in biofilm formation. Mutation in *exoP* results in a large decrease in biofilm formation (Table 3). While the *exoP* mutant is defective in its ability to inhibit *Vibrio* species in competition assays (Figs. 2 & 5) and also exhibits decreased probiotic activity in oyster challenge assay against *V. tubiashii* (Fig. 6), these declines are less than those seen in the *clpX* mutant. These data suggest that biofilm formation is important for S4Sm probiotic activity. Biofilms may contribute to probiotic activity in two ways. First, biofilms would allow *P. gallaeciensis* to physically occupy potential sites of colonization and prevent the oyster pathogens from gaining access to the oyster. Second, the formation of an extensive biofilm with cells at high density may induce the production of TDA. A more extensive biofilm would produce more TDA and, therefore, more effectively inhibit the ability of pathogens to infect the oyster host. It should also be noted that other than decreased biofilm formation, the *exoP* mutant exhibits no other defects in growth or TDA formation.

As a broad spectrum antibiotic TDA inhibits the growth of several marine pathogens [35]. However, in the ocean environment TDA will be rapidly diluted once it is secreted. We suggest that *P. gallaeciensis* requires both TDA production and biofilm formation for effective probiotic activity. The biofilm matrix creates a micro-environment, within which TDA can accumulate, without rapid dilution by seawater, to reach concentrations high enough to inhibit pathogens. In the absence

of TDA, a *P. gallaeciensis* biofilm does not eliminate pathogens and provides only modest protection against disease. Further, *P. gallaeciensis* growing with a diminished biofilm also exhibits significantly reduced probiotic activity probably due to the decreased mass of cells producing TDA and the increase in available sites for pathogens to colonize. Our data indicate that maximum probiotic activity requires both TDA production and biofilm formation.

Karim et al. [16] reported that oyster larvae were best protected when *P. gallaeciensis* S4Sm was added 24 h prior to challenge by either of the two oyster pathogens, *V. tubiashii* and *R. crassostreae*. The data presented in this report correspond with those previous observations and reveal that pre-colonization of a surface by S4Sm is most effective at inhibiting *V. tubiashii* RE22 from either colonizing the glass coverslip surface or from growing planktonically. The generation time of *Vibrio* species in YP30 is less than 1 hour (*V. tubiashii* is ~0.53 h, *V. anguillarum* is ~0.89 h); however, the doubling time for *P. gallaeciensis* S4Sm is ~3.1 h. These observations suggest that successful probiotic activity by S4Sm is also dependent upon growth rate and having enough TDA producing cells in the biofilm to successfully antagonize and out-compete the oyster pathogens. Practically, this means that it is necessary for oysters to be pretreated with *P. gallaeciensis* S4Sm prior to challenge by potential oyster pathogens, such as *V. tubiashii* and *R. crassostreae*. In contrast, D' Alvise et al. [12] showed that it was not necessary for *P. gallaeciensis* to precolonize the wells containing cod larvae in order to antagonize *V. anguillarum* and significantly reduce cod larvae mortalities. However, they added 10-fold more *P. gallaeciensis* cells (10^7 CFU/ml) than *V. anguillarum* cells (10^6 CFU/ml) and *V. anguillarum* is more sensitive to TDA than

is *V. tubiashii*. Further, this density of *P. gallaeciensis* is 10^3-fold higher than the density used in our experiments reported here and previously by Karim et al [16] . Additionally, we used 10-fold more *V. tubiashii* (10^5 CFU/ml) than *P. gallaeciensis* (10^4 CFU/ml). This suggests that cell density and, ultimately for *P. gallaeciensis*, biofilm formation contributes to probiotic activity.

References

1. Austin B, Austin DA (2012) Bacterial Fish Pathogens. Disease of Farmed and Wild Fish.
2. Paillard C, Le Roux F, Borrego JJ (2004) Bacterial disease in marine bivalves, a review of recent studies: trends and evolution. Aquatic Living Resources 17: 477-498.
3. Maloy AP, Ford SE, Karney RC, Boettcher KJ (2007) Roseovarius crassostreae, the etiological agent of Juvenile Oyster Disease (now to be known as Roseovarius Oyster Disease) in Crassostrea virginica. Aquaculture 269: 71-83.
4. Elston RA, Hasegawa H, Humphrey KL, Polyak IK, Hase CC (2008) Re-emergence of Vibrio tubiashii in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and management. Dis Aquat Organ 82: 119-134.
5. Hasegawa H, Lind EJ, Boin MA, Hase CC (2008) The extracellular metalloprotease of Vibrio tubiashii is a major virulence factor for pacific oyster (Crassostrea gigas) larvae. Appl Environ Microbiol 74: 4101-4110.
6. Boettcher KJ, Geaghan KK, Maloy AP, Barber BJ (2005) Roseovarius crassostreae sp. nov., a member of the Roseobacter clade and the apparent cause of juvenile oyster disease (JOD) in cultured Eastern oysters. Int J Syst Evol Microbiol 55: 1531-1537.
7. Cabello FC (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ Microbiol 8: 1137-1144.
8. Bachère E (2003) Anti-infectious immune effectors in marine invertebrates: potential tools for disease control in larviculture. Aquaculture 227: 427-438.
9. Wang YB, Li JR, Lin J (2008) Probiotics in aquaculture: challenges and outlook. Aquaculture 281: 1-4.
10. Balcazar JL, de Blas I, Ruiz-Zarzuela I, Cunningham D, Vendrell D, et al. (2006) The role of probiotics in aquaculture. Vet Microbiol 114: 173-186.
11. Decamp O, Moriarty DJW, Lavens P (2008) Probiotics for shrimp larviculture: review of field data from Asia and Latin America. Aquaculture Research 39: 334-338.
12. D'Alvise PW, Lillebo S, Prol-Garcia MJ, Wergeland HI, Nielsen KF, et al. (2012) Phaeobacter gallaeciensis reduces Vibrio anguillarum in cultures of microalgae and rotifers, and prevents vibriosis in cod larvae. PLoS One 7: e43996.
13. D'Alvise PW, Melchiorsen J, Porsby CH, Nielsen KF, Gram L (2010) Inactivation of Vibrio anguillarum by attached and planktonic Roseobacter cells. Appl Environ Microbiol 76: 2366-2370.
14. Bruhn JB, Nielsen KF, Hjelm M, Hansen M, Bresciani J, et al. (2005) Ecology, inhibitory activity, and morphogenesis of a marine antagonistic bacterium belonging to the Roseobacter clade. Appl Environ Microbiol 71: 7263-7270.
15. Gram L, Melchiorsen J, Bruhn JB (2010) Antibacterial activity of marine culturable bacteria collected from a global sampling of ocean surface waters and surface swabs of marine organisms. Mar Biotechnol (NY) 12: 439-451.

16. Karim M, Zhao W, Nelson DR, Rowley D, Marta Gomez-Chiarri (2013) Probiotic strains for shellfish aquaculture: protection of Eastern oyster, Crassostreac virginica, larvae and juveniles against bacterial. Journal of Shellfish Research.
17. Porsby CH, Nielsen KF, Gram L (2008) Phaeobacter and Ruegeria species of the Roseobacter clade colonize separate niches in a Danish Turbot (Scophthalmus maximus)-rearing farm and antagonize Vibrio anguillarum under different growth conditions. Appl Environ Microbiol 74: 7356-7364.
18. Prado S, Montes J, Romalde JL, Barja JL (2009) Inhibitory activity of Phaeobacter strains against aquaculture pathogenic bacteria. Int Microbiol 12: 107-114.
19. Belas R, Horikawa E, Aizawa S, Suvanasuthi R (2009) Genetic determinants of Silicibacter sp. TM1040 motility. J Bacteriol 191: 4502-4512.
20. Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiology and molecular biology reviews 64: 655-671.
21. Geng H, Bruhn JB, Nielsen KF, Gram L, Belas R (2008) Genetic dissection of tropodithietic acid biosynthesis by marine roseobacters. Appl Environ Microbiol 74: 1535-1545.
22. Varina M, Denkin SM, Staroscik AM, Nelson DR (2008) Identification and characterization of Epp, the secreted processing protease for the Vibrio anguillarum EmpA metalloprotease. J Bacteriol 190: 6589-6597.
23. Sezonov G, Joseleau-Petit D, D'Ari R (2007) Escherichia coli physiology in Luria-Bertani broth. J Bacteriol 189: 8746-8749.
24. Denkin SM, Nelson DR (2004) Regulation of Vibrio anguillarum empA metalloprotease expression and its role in virulence. Appl Environ Microbiol 70: 4193-4204.
25. Milton DL, O'Toole R, Horstedt P, Wolf-Watz H (1996) Flagellin A is essential for the virulence of Vibrio anguillarum. J Bacteriol 178: 1310-1319.
26. Li L, Mou X, Nelson DR (2011) HlyU is a positive regulator of hemolysin expression in Vibrio anguillarum. J Bacteriol 193: 4779-4789.
27. Rock JL, Nelson DR (2006) Identification and characterization of a hemolysin gene cluster in Vibrio anguillarum. Infect Immun 74: 2777-2786.
28. Mou X, Spinard EJ, Driscoll MV, Zhao W, Nelson DR (2013) H-NS is a negative regulator of the two hemolysin/cytotoxin gene clusters in Vibrio anguillarum. Infect Immun 81: 3566-3576.
29. Liang L (2003) Investigation of Secondary Metabolites of North Sea Bacteria: Fermentation, Isolation, Structure Elucidation and Bioactivity.
30. Wiegand I, Hilpert K, Hancock RE (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3: 163-175.
31. Thole S, Kalhoefer D, Voget S, Berger M, Engelhardt T, et al. (2012) Phaeobacter gallaeciensis genomes from globally opposite locations reveal high similarity of adaptation to surface life. ISME J 6: 2229-2244.
32. Berger M, Brock NL, Liesegang H, Dogs M, Preuth I, et al. (2012) Genetic analysis of the upper phenylacetate catabolic pathway in the production of tropodithietic acid by Phaeobacter gallaeciensis. Appl Environ Microbiol 78: 3539-3551.

33. Baker TA, Sauer RT (2012) ClpXP, an ATP-powered unfolding and protein-degradation machine. Biochim Biophys Acta 1823: 15-28.
34. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, et al. (2008) The RAST Server: rapid annotations using subsystems technology. BMC Genomics 9: 75.
35. Porsby CH, Webber MA, Nielsen KF, Piddock LJ, Gram L (2011) Resistance and tolerance to tropodithietic acid, an antimicrobial in aquaculture, is hard to select. Antimicrob Agents Chemother 55: 1332-1337.

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Description	Resistance	Reference
P. gallaeciensis			
S4	Previously _Phaeobacter sp._ S4; wild type isolate from the inner shell of oysters		Karim et al, 2013
S4Sm	Spontaneous Sm^r mutant of S4	Sm^r	this study
WZ10	_clpX_ insertional mutant of S4Sm	$Sm^r Cm^r$	this study
WZ11	_clpX+_, clpX _in trans_ complement of WZ10	$Sm^r Cm^r Ap^r$	this study
WZ20	_exoP_ insertional mutant of S4Sm	$Sm^r Cm^r$	this study
WZ21	_exoP+_, exoP _in trans_ complement of WZ20	$Sm^r Cm^r Ap^r$	this study
WZ02	S4Sm (pRhokHi-2-_ofp_)	$Sm^r Cm^r Km^r$	this study
WZ12	_clpX_, WZ10 (pRhokHi-2-_ofp_)	$Sm^r Cm^r Km^r$	this study
WZ22	_exoP_, WZ20 (pRhokHi-2-_ofp_)	$Sm^r Cm^r Km^r$	this study
V. tubiashii			
RE22	Wild type isolate from oyster larvae		Estes et al, 2004
RE22Sm	Spontaneous Sm^r mutant of RE22	Sm^r	this study
WZ103	RE22Sm (pRhokHi-2-_gfp_)	$Sm^r Ap^r$	this study
V. anguillarum			
NB10	Wild type, serotype O1, clinical isolate from the Gulf of Bothnia		Norqvist et al, 1989
NB10Sm	Spontaneous Sm^r mutant of NB10	Sm^r	this study
WZ203	NB10Sm (pSUP202P-P_flaB_-gfp)	$Sm^r Ap^r Tet^r$	this study
R. crassostreae			
CV919-312[T]	Wild type isolate from a JOD-affected oyster		Boettcher et al, 1999
CV919Sm	Spontaneous Sm^r mutant of CV919-312T	Sm^r	this study
E. coli			
Sm10	_thi thr leu tonA lacY supE recA_ RP4-2 Tc::Mu::Km (λpir)	Km^r	Simon et al, 1983
S100	Sm10 harboring pNQ705-1		this study
WQ10	Sm10 harboring pNQ705-_clpX_		this study
WQ20	Sm10 harboring pNQ705-_exoP_		this study
WB01	Sm10 harboring pBBR1MCS4		this study
WB11	Sm10 harboring pBBR1MCS4-_clpX_		this study
WB21	Sm10 harboring pBBR1MCS4-_exoP_		this study
S122	Sm10 harboring pSUP202P-_gfp_(ORF)		this study
S136	Sm10 harboring pSUP202P-P_flaB_-gfp		this study
W900	Sm10 harboring pRhokHi-2-FbFP		this study

WR03	Sm10 harboring pRhokHi-2-*gfp*	this study
WR02	Sm10 harboring pRhokHi-2-*ofp*	this study
W901	Sm10 harboring pmOrange	this study
Plasmids		
pNQ705-1	Cm^r; suicide vector with R6K origin	Mcgee, 1996
pNQ705-*clpX*	Cm^r; derivative from pNQ705-1 for *clpX* insertional mutant	this study
pNQ705-*exoP*	Cm^r; derivative from pNQ705-1 for *exoP* insertional mutant	this study
pBBR1MCS4	Ap^r; derivative from pBBR1MCS (a broad-host-range cloning vector)	Kovach et al, 1995
pBBR1MCS4-*clpX*	Apr; derivative from pBBR1MCS4 for *clpX in trans* complement	this study
pBBR1MCS4-*exoP*	Apr; derivative from pBBR1MCS4 for *exoP in trans* complement	this study
pBS(gfp)-Pcampy	Template for *gfp* ORF PCR amplification	Eggers et al, 2004
pCE320(gfp)-PflaB	Template for *PflaB* PCR amplification	Eggers et al, 2004
pSUP202P	Ap^r Cm^r Tc^r; broad host shuttle vector	Simon et al, 1983
pSUP202P-*gfp*(ORF)	Ap^r Tc^r; derivative from pSUP202 for GFP tagging	this study
pSUP202P-P*flaB*-*gfp*	Ap^r Tc^r; derivative from pSUP202 for GFP tagging	this study
pRhokHi-2-FbFP	Cm^r Km^r; derivative from pBBR1MCS (a broad-host-range cloning vector) with promoter PaphII	Piekarski et al, 2009
pRhokHi-2-*gfp*	Cm^r Km^r; derivative from pRhokHi-2-FbFP with *gfp* under the control of P*aphII*	this study
pmOrange	Template for *ofp* ORF PCR amplification	Clontech Laboratories, Inc.
pRhokHi-2-*ofp*	Cm^{r}Km^r; derivative from pRhokHi-2-FbFP with *ofp* under the control of P*aphII*	this study

Table 2. Primers used in this study

Primer	Sequence (5' to 3', underlined sequences are engineered restriction sites)	Description
pw108	GAA<u>GAGCTC</u>GGACGACTATGTGATTGGTCAGGC	For *clpX* insertional mutation, forward, with *Sac*I site
pw109	GGG<u>TCTAGA</u>CGACGTTATATTCCGACGCCTGCA	For *clpX* insertional mutation, reverse, with *Xba*I site
pw153	GTATTA<u>GAGCTC</u>GAGCATAACCGCTTTGCCCGCCGCCCA	For *exoP* insertional mutation, forward, with *Sac*I site
pw154	CGACTA<u>TCTAGA</u>CCATGCTGAGTGCAAGGTTGACGGCGG	For *exoP* insertional mutation, reverse, with *Xba*I site
pw127	GCATTA<u>GAGCTC</u>GTCAGATTGGCCGAAGCCCCTTTT	For *clpX in trans* complement, forward, with *Sac*I site
pw128	CGGCTA<u>TCTAGA</u>CGAACTCACCACCTGAGGAGATACGT	For *clpX in trans* complement, reverse, with *Xba*I site
pw166	GTATTA<u>GAGCTC</u>CCCGTCCGATGTGTCAAAATAGGT	For e*xoP in trans* complement, forward, with *Sac*I site
pw165	CGTCTT<u>TCTAGA</u>GGTGCCTGCGGTCATCACCATGAC	For *exoP in trans* complement, reverse, with *Xba*I site
pwGFP-F	GCGGTA<u>CATATG</u>TAAGGAGGAAAAACATATG	For amplification of *gfp* ORF, forward, with *Nde*I site
pwGFP-R	CTATAT<u>GGATCC</u>CAGATCTATTTGTATAGTTCATCCA	For amplification of *gfp* ORF, reverse, with *BamH*I site
Pm113	<u>GGTACC</u>TGTCTGTCGCCTCTTGT	For amplification of PflaB, forward, with *Kpn*I site
Pm114	<u>GGTACC</u>ATATCATTCCTCCATGAT	For amplification of PflaB, forward, with *Kpn*I site
pwmO-F	GCGGTA<u>CATATG</u>ATGGTGAGCAAGGGCGAGGAGAAT	For amplification of *ofp* ORF, forward, with *Nde*I site
pwmO-R	CTATAT<u>GGATCC</u>CTTGTACAGCTCGTCCATGCCGCC	For amplification of *ofp* ORF, reverse, with *Bam*HI site

Table 3. Quantification of biofilm formation by measuring OD_{580} of crystal violet dye attached to the cells forming biofilms on glass tubes at 27°C under static condition at 60h. The data presented are average of two independent experiments and each independent experiment has three replicates.

Strains	OD_{580}[a]
P. gallaeciensis S4Sm	3.89±0.06
P. gallaeciensis WZ10 (*clpX*-)	3.90±0.12
P. gallaeciensis WZ11 (*clpX*+)	4.0±0.06
P. gallaeciensis WZ20 (*exoP*-)	1.60±0.09[b]
P. gallaeciensis WZ21 (*exoP*+)	3.90±0.10
V. anguillarum NB10Sm	0.58±0.02[b]
V. tubiashii RE22Sm	0.54±0.02[b]
R. crassostreae CV919Sm	0.52±0.08[b]

[a]Biofilm formation quantified by crystal violet dye assay as described in the Materials and Methods. The data presented are the average of two independent experiments and each independent experiment has three replicates.

[b]Statistically significant difference compared to S4Sm.

Table 4. Killing ability of culture supernatant of various *P. gallaeciensis* strains against *V. anguillarum* NB10Sm cell. Culture supernatant from each strain collected after two-day inoculation. The data presented is from a representative experiment of two independent experiments.

Treatment	Surviving *V. anguillarum* cell density (CFU/ml) after the treatment
NSS (negative control)	$40.67\ (\pm3.79)\times10^7$
S4Sm culture supernatant	<10
WZ10 (*clpX*-) culture supernatant	$41.33\ (\pm1.53)\times10^7$
WZ11 (*clpX*+) culture supernatant	<10
WZ10 (*clpX*-) culture supernatant plus TDA	<10
WZ20 (*exoP*-) culture supernatant	<10
WZ21 (*exoP*+) culture supernatant	<10

Figure 1. A and B) Reversed-phase HPLC chromatograms of ethyl acetate extracts from *Phaeobacter* strains showing presence (A) or absence of TDA (B). C) Inhibition zone assay of S4Sm, *clpX* mutant (*clpX*), *clpX* complement (*clpX*+), *exoP* mutant (*exoP*) or *exoP* complement (*exoP*+) on YP30 plates coated by *V. anguillarum* (NB10Sm), *V. tubiashii* (RE22Sm) or *Roseovarius crassostreae* (CV919Sm) after 24 h at 27°C. The data presented are averages of two independent experiments and each independent experiment has three replicates.
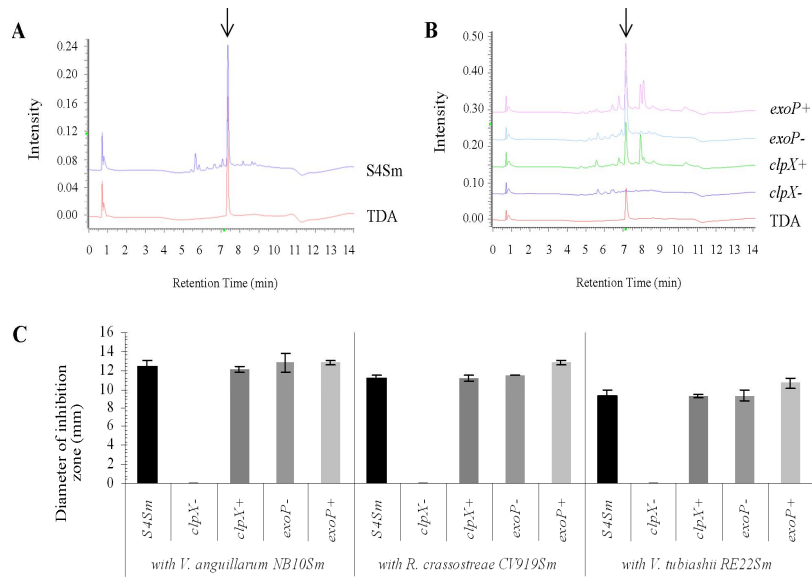
Figure 2. Competition assay between *P. gallaeciensis* strains and *V. tubiashii* with pre-colonization of glass surfaces by *P. gallaeciensis* for 24h. The mixed cultures are S4Sm-OFP with RE22Sm-GFP, *clpX*-OFP with RE22Sm-GFP and *exoP*-OFP with RE22Sm-GFP. A) Merged confocal microscopy images of mixed biofilm development by OFP-producing strains (S4Sm, *clpX* mutant *and exoP* mutant) and GFP-producing *V. tubiashii* (RE22Sm) strain on the surface of glass coverslip at 72 h. The data presented are from a representative experiment of two independent experiments. B) Growth of sessile *P. gallaeciensis* and *V. tubiashii* (RE22Sm) in a co-culture system. C) Comparison of growth of sessile *V. tubiashii* (RE22Sm) in different co-culture systems and monoculture control. D) Growth of planktonic *Phaeobacter* cells and *V. tubiashii* (RE22Sm) in a co-culture system. E) Comparison of growth of planktonic *V. tubiashii* (RE22Sm) in different co-culture systems and monoculture control. The data presented are average of two independent experiments and each independent experiment has three replicates.
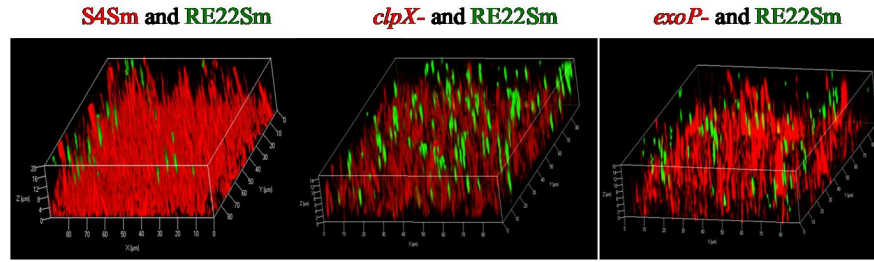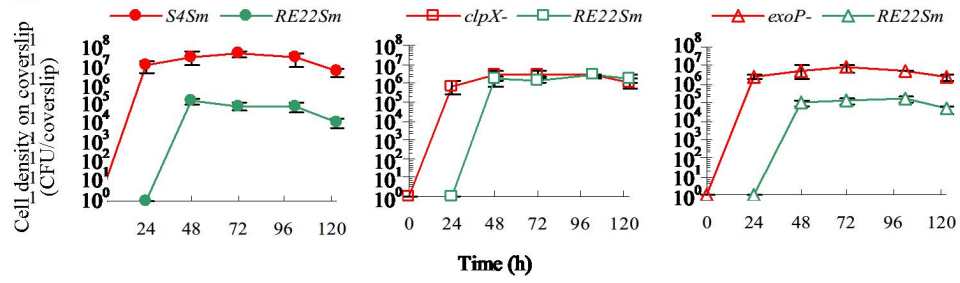
A

S4Sm and RE22Sm   *clpX-* and RE22Sm   *exoP-* and RE22Sm

B

D

C

E
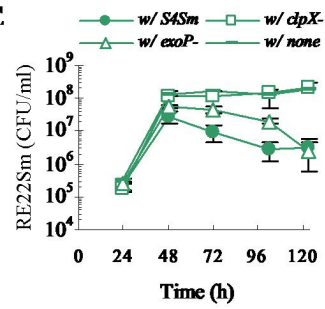
Figure 3. TDA supplementation in co-culture system between *clpX* mutant and *V. tubiashii*. A) Growth of sessile *V. tubiashii* on a glass coverslip in co-culture system (supplemented without or with 10μg/ml TDA) with different *Phaeobacter* strains. The data presented are average of two independent experiments and each independent experiment has three replicates. B) Single channel and merged confocal microscopy images of mixed biofilm development by OFP-producing strains (S4Sm, *clpX* mutant) and GFP-producing *V. tubiashii* (RE22Sm-GFP) on the surface of glass coverslip at 48 h after addition of RE22Sm and TDA. The data presented is from representative experiments of two independent experiments. C) Growth of planktonic *V. tubiashii* in co-culture system (supplemented without or with 10 μg/ml TDA) with different *Phaeobacter* strains. The data presented are averages of two independent experiments and each independent experiment has three replicates.

A

Legend:
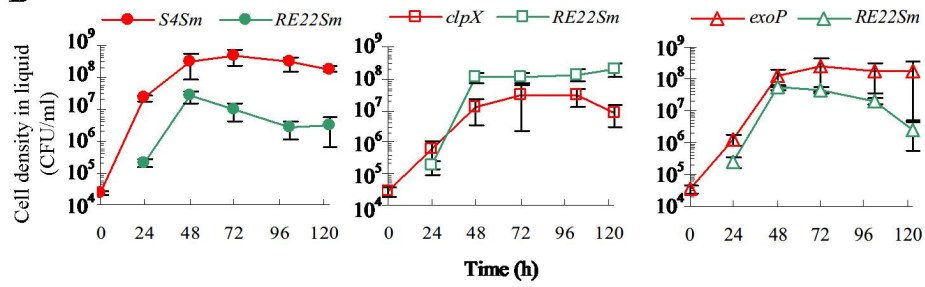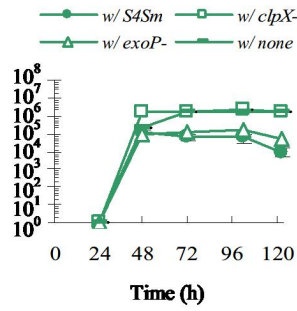- 24h after addition of RE22Sm
- 24h after addition of RE22Sm and TDA
- 48h after addition of RE22Sm
- 48h after addition of RE22Sm and TDA

Y-axis: RE22Sm Cell density on coverslip (CFU/ coverslip)

X-axis categories: RE22Sm only | w/ S4Sm | w/ clpX- | RE22Sm only | w/ S4Sm | w/ clpX-

No exogenous TDA added | TDA added

B

Left column labels: RE22Sm, S4Sm, Merged image
Right column labels: RE22Sm, clpX-, Merged image

C

Legend:
- 24h after addition of RE22Sm
- 24h after addition of RE22Sm and TDA
- 48h after addition of RE22Sm
- 48h after addition of RE22Sm and TDA

Y-axis: RE22Sm Cell density in liquid (CFU/ml)

X-axis categories: RE22Sm only | w/ S4Sm | w/ clpX- | RE22Sm only | w/ S4Sm | w/ clpX-

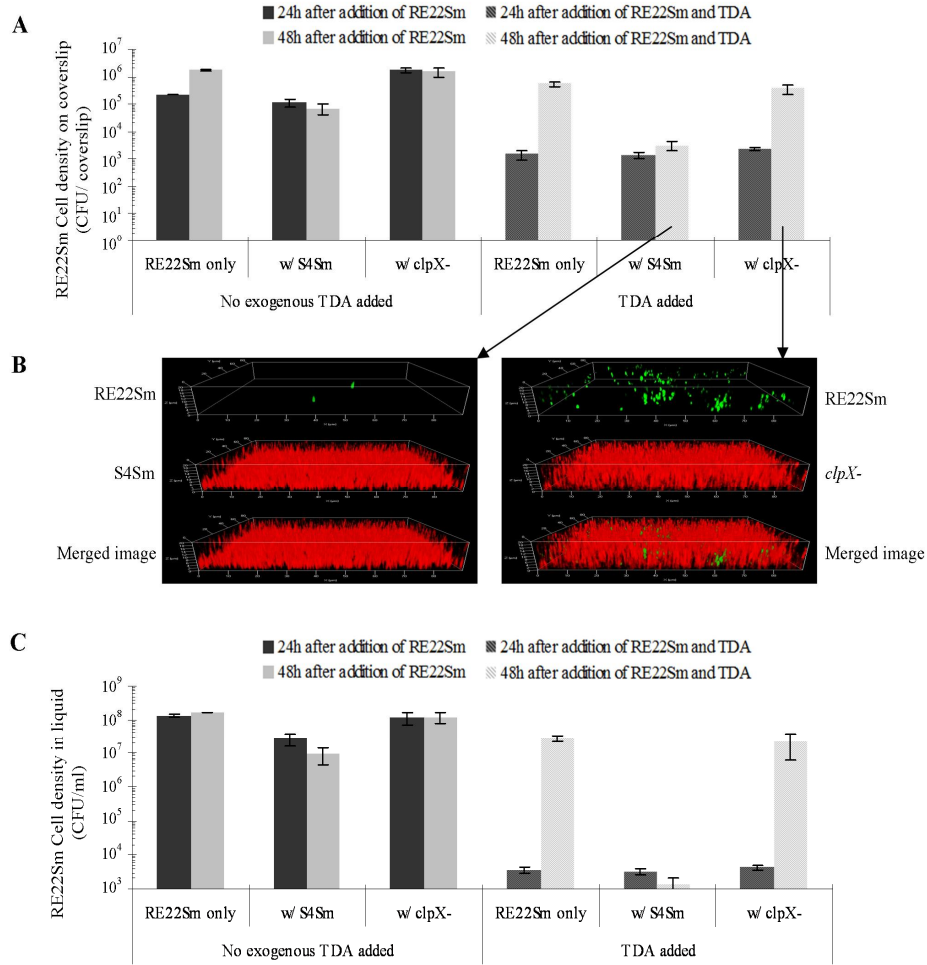No exogenous TDA added | TDA added

104

Figure 4. Effects of *V. tubiashii* on the growth of *P. gallaeciensis* strains in a competition assay with a 24 h pre-colonization by *P. gallaeciensis*. The mixed cultures are: S4Sm-OFP with RE22Sm-GFP, *clpX*-OFP with RE22Sm-GFP and *exoP*-OFP with RE22Sm-GFP. Colonization and initial cell densities were as described in the Materials and Methods. A) Growth of sessile *P. gallaeciensis* cells (with RE22Sm) in a co-culture system and a monoculture control. B) Growth of planktonic *P. gallaeciensis* cells (with RE22Sm) in a co-culture system and a monoculture control. The data presented are average of two independent experiments and each independent experiment has three replicates.

A

S4Sm only · w/ RE22Sm · clpX- only · w/ RE22Sm · exoP- only · w/ RE22Sm

Cell density on coverslip (CFU/coverslip)

Time (h)

B

S4Sm only · w/ RE22Sm · clpX- only · w/ RE22Sm · exoP- only · w/ RE22Sm
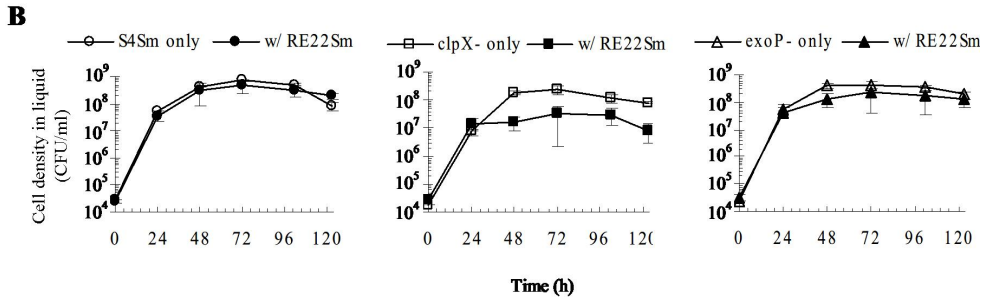
Cell density in liquid (CFU/ml)

Time (h)

Figure 5. Competition assay between *Phaeobacter* strains and *V. tubiashii* without pre-colonization by *Phaeobacter*. The mixed cultures areS4Sm-OFP with RE22Sm-GFP, *clpX*-OFP with RE22Sm-GFP, and *exoP*-OFP with RE22Sm-GFP. A) Growth of sessile *P. gallaeciensis* and *V. tubiashii* (RE22Sm) in a co-culture system. B) Growth of planktonic *P. gallaeciensis* and *V. tubiashii* (RE22Sm) in a co-culture system. The data presented are average of two independent experiments and each independent experiment has three replicates.

A
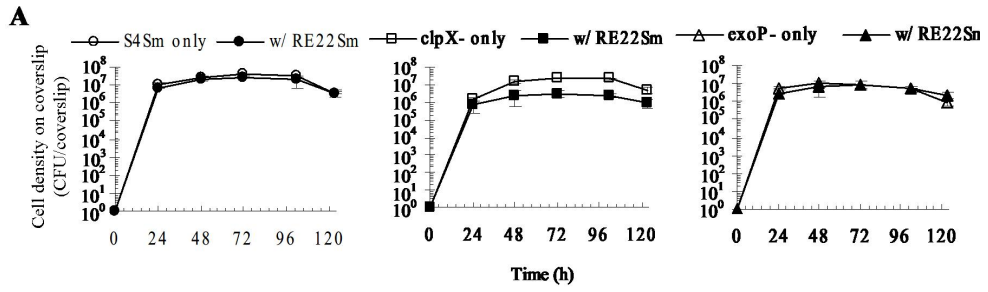

B
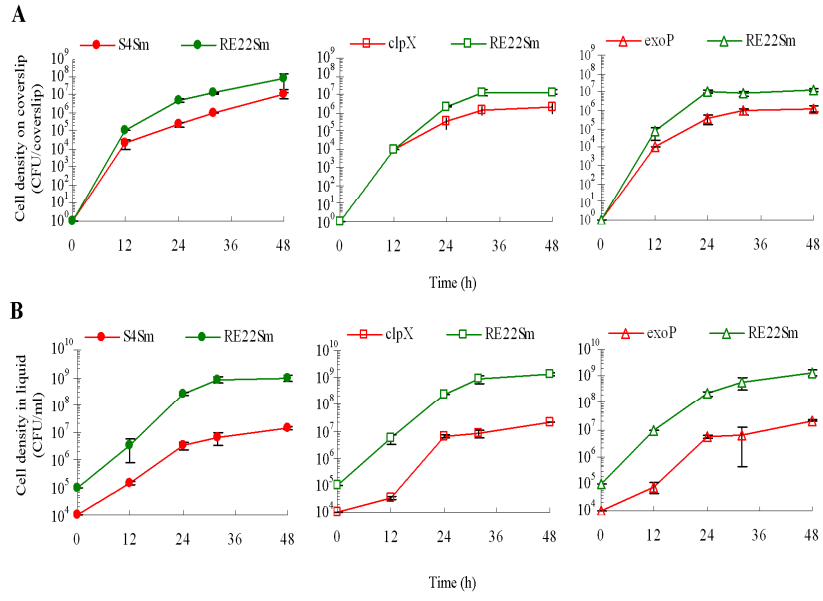
Figure 6. Effect of pre-incubation of larval oysters with *P. gallaeciensis* strains at 10^4 CFU/ml on survival (%± SE) 24 h after challenge with bacterial pathogen *Vibrio tubiashii* RE22 at 10^5 CFU/ml. The *P. gallaeciensis* strains were introduced 24 h before larvae were challenged. Oyster larvae treated only by artificial seawater serve as control (mock). Bars marked with an asterisk (*) show significant differences (p<0.05). Representative of at least 3 experiments.
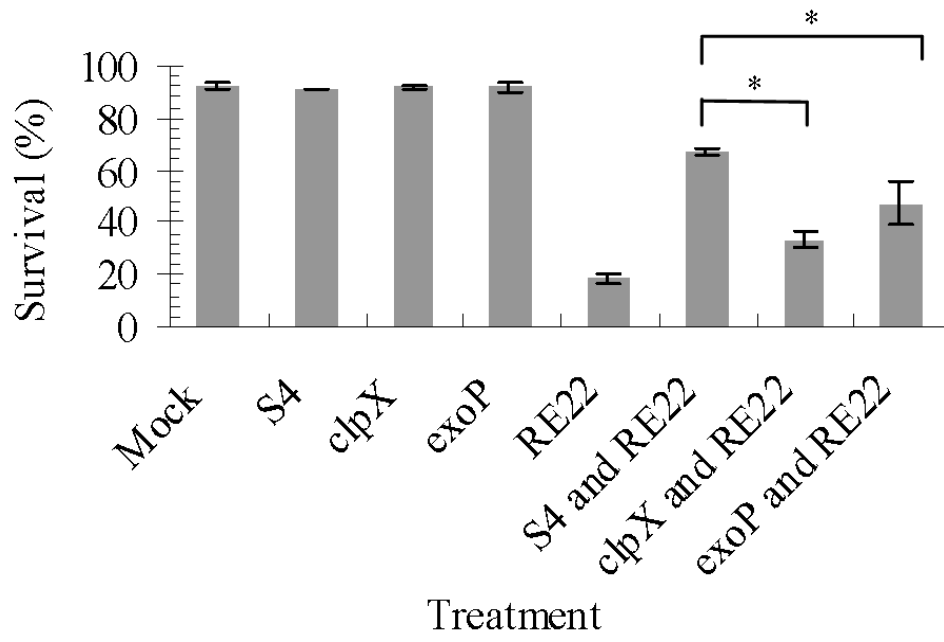
Supplemental Data

Table S1. Generation time of *Phaeobacter gallaeciensis* (S4Sm), *Vibrio anguillarum* (NB10Sm), and *Vibrio tubiashii* (RE22Sm) strains growing in YP30 during mid-log phase.

Strains	S4Sm	NB10Sm	RE22Sm
Generation Time (h)	3.098	0.892	0.528

Table S2. Competition assay between *Phaeobacter* strains and *V. tubiashii* (RE22Sm) with pre-colonization of *Phaeobacter*. The mixed culture are S4Sm with RE22Sm, *clpX* mutant with RE22Sm, *clpX* complement strain with RE22Sm, *exoP* mutant with RE22Sm, and *exoP* complement strain with RE22Sm. A) Growth of planktonic *V. tubiashii* (RE22Sm) in co-culture system. B) Growth of sessile *V. tubiashii* (RE22Sm) in co-culture system. The data presented are average of two independent experiments and each independent experiment has three replicates. [a]Statistically significant difference compared to RE22 only.

A

RE22 cell density in liquid					
RE22 only	RE22 w/S4sm	RE22 w/clpX-	RE22 w/exoP-	RE22 w/clpX-C	RE22 w/exoP-C
100%	5.7%	93.1%	27.4%	6.8%	6.3%

B

RE22 cell density on coverslip					
RE22 only	RE22 w/S4sm	RE22 w/clpX-	RE22 w/exoP-	RE22 w/clpX-C	RE22 w/exoP-C
100%	4.4%	86.5%	6.9%	3.6%	5.7%

Figure S1. A) Reversed-phase HPLC chromatograms of ethyl acetate extracts from *Phaeobacter* mutant strains. B) Quantification of biofilm formation by measuring OD580 of crystal violet dye attached to the cells forming biofilms on glass tubes at 27°C under static condition. The data presented are average of two independent experiments and each independent experiment has three replicates.
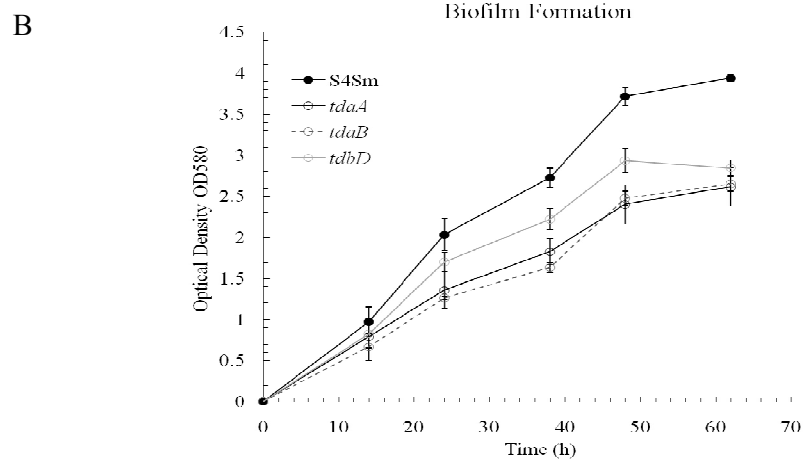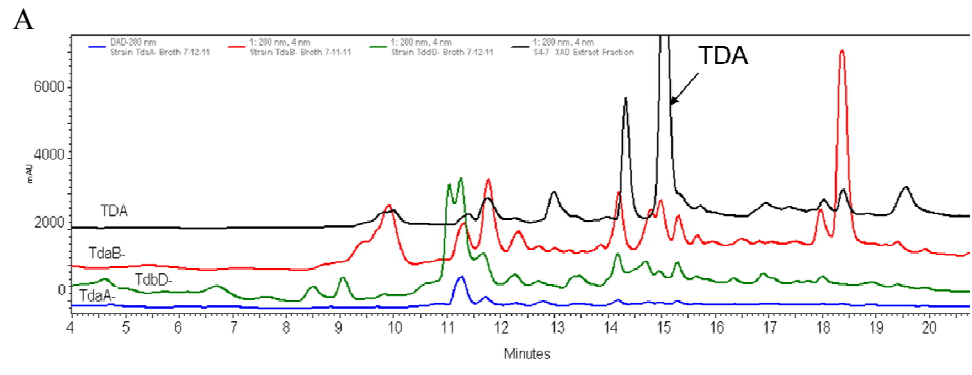
A

B

Biofilm Formation

114

Figure S2. Competition assay between *Phaeobacter* strains and *V. anguillarum* (NB10Sm) without pre-colonization of *Phaeobacter*. The mixed cultures are S4Sm-OFP with NB10Sm-GFP, *clpX*-OFP with NB10Sm-GFP and *exoP*-OFP with NB10Sm-GFP. A) Single channel and merged confocal microscopy images of mixed biofilm development by OFP-producing strains (S4Sm, *clpX* mutant or*exoP*mutant) and GFP-producing *V. anguillarum* NB10Sm-GFP on the surface of glass coverslip at 48 h. The data presented is from a representative experiment of two independent experiments. B) Growth of sessile*V. anguillarum* (NB10Sm) in co-culture system with different *Phaeobacter* strains. C) Comparison of growth of sessile *V. anguillarum* (NB10Sm) in different co-culture system and monoculture control. D) Growth of planktonic *V. anguillarum* (NB10Sm) in co-culture system with different *Phaeobacter* strains. E) Comparison of growth of planktonic *V. anguillarum* (NB10Sm) in different co-culturesystemand monoculture control.The data presented are average of two independent experiments and each independent experiment has three replicates.

A

S4Sm + NB10Sm	*clpX* + NB10Sm	*exoP* + NB10Sm	
			V. anguillarum
			P. gallaeciensis
			Merged

B

D

C

E
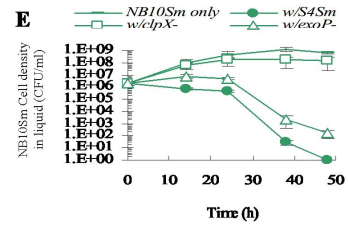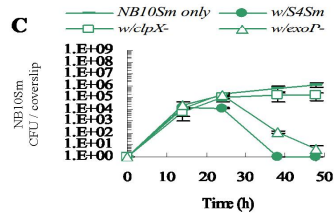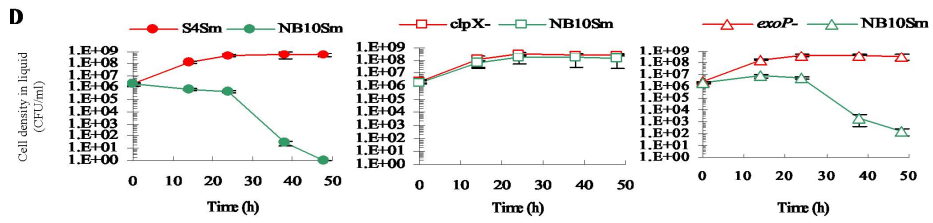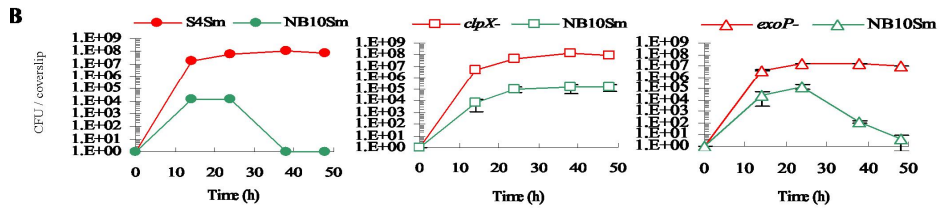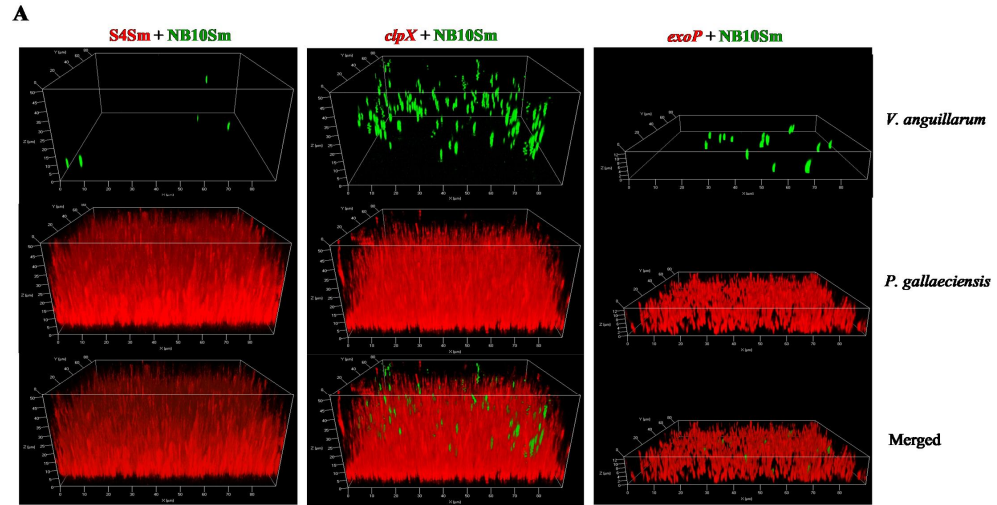
Figure S3. Effects of *V. anguillarum* NB10 on the growth of *P. gallaeciensis* strains in a competition assay with a 24 h without pre-colonization by *P. gallaeciensis*. The mixed cultures are: S4Sm-OFP with NB10Sm-GFP, *clpX*-OFP with NB10Sm-GFP and *exoP*-OFP with NB10Sm-GFP. Colonization and initial cell densities were as described in the Materials and Methods. A) Growth of sessile *P. gallaeciensis* cells (with NB10Sm) in a co-culture system and a monoculture control. B) Growth of planktonic *P. gallaeciensis* cells (with NB10Sm) in a co-culture system and a monoculture control. The data presented are average of two independent experiments and each independent experiment has three replicates.

A.

B.
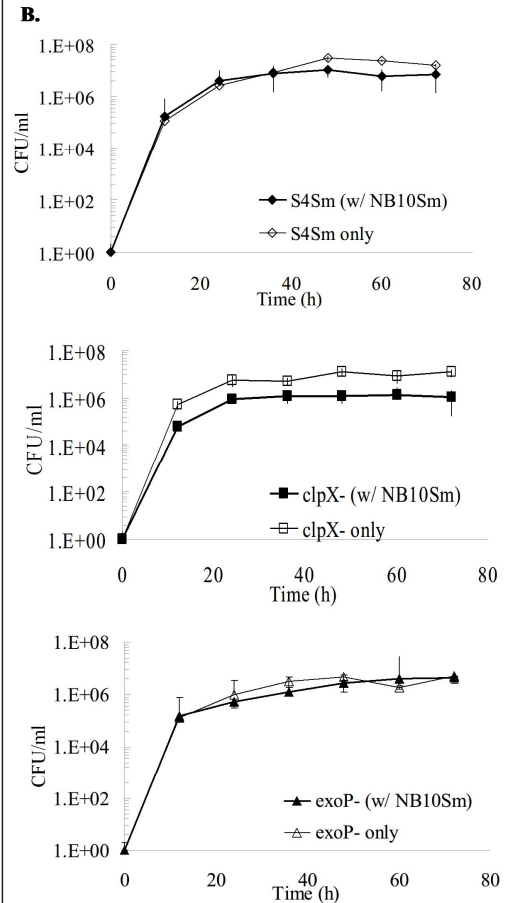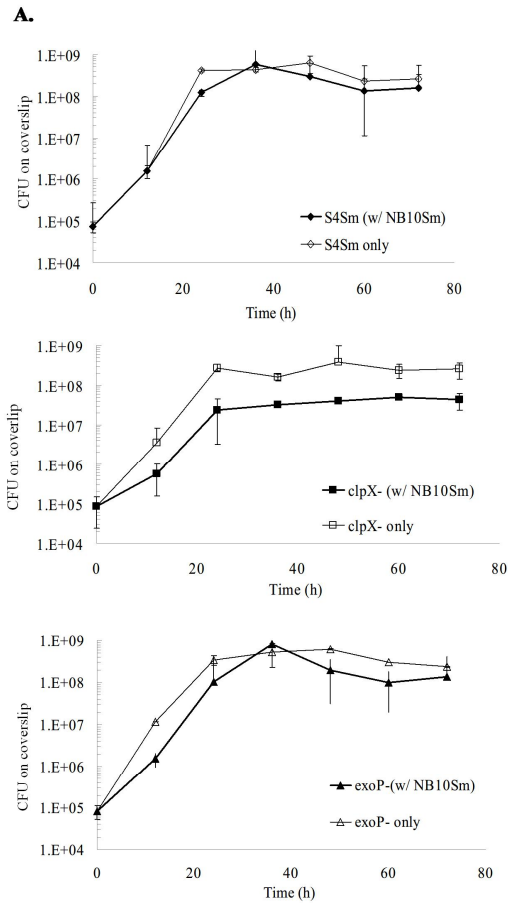
Manuscript III

Title: The probiotic bacterium, *Phaeobacter gallaeciensis* S4, down-regulates protease virulence factor transcription in the shellfish pathogen, *Vibrio tubiashii*, by quorum quenching.

Authors: Wenjing Zhao[1], Tao Yuan[2], Christine Dao[2], David Rowley[2] and David R. Nelson[1*]

Affiliation: [1]Department of Cell and Molecular Biology, [2]Biomedical and Pharmaceutical Sciences,University of Rhode Island, Kingston, RI 02881

Key Words: *Phaeobacter gallaeciensis*, N-acyl homoserine lactones, quorum sensing, metalloprotease, probiotic, marine pathogens, *Vibrio tubiashii*, oyster disease

*Corresponding author: David R. Nelson, Department of Cell and Molecular Biology, 120 Flagg Rd., University of Rhode Island, Kingston, RI 02881, USA; E-mail: dnelson@uri.edu; Phone: 1-401-874-5902

Abstract

Phaeobacter gallaeciensis S4Sm acts as a probiotic bacterium against the oyster pathogen, *Vibrio tubiashii*. S4Sm is an excellent biofilm former and produces the antibiotic tropodithietic acid (TDA). These two factors play important roles in probiotic activity. Here we report that culture supernatant of S4Sm down-regulates protease activity in *V. tubiashii* cultures. The effects of S4Sm culture supernatant on the transcription of several genes involved in protease activity including *vtpA*, *vtpB*, and *vtpR* (encoding metalloproteases A and B and their transcriptional regulator, respectively) were examined by qRT-PCR. Expression of *vtpB* and *vtpR* were reduced to 35.9 % and 6.6 %, respectively, compared to an untreated control. In contrast, expression of *vtpA* was not affected. We constructed a *V. tubiashii* GFP-reporter strain to detect the activity of inhibitory compounds. Using a bioassay-guided approach, the molecules responsible for *V. tubiashii* protease inhibition activity were isolated from S4Sm supernatant and identified as three N-acyl homoserine lactones (AHLs). The three AHLs are *N*-(3-hydroxydecanoyl)-L-homoserine lactone, *N*-(dodecanoyl-2,5-diene)-L-homoserine lactone and *N*-(3-hydroxytetradecanoyl-7-ene)-L-homoserine lactone, and their half maximal (50%) inhibitory concentrations (IC) against *V. tubiashii* protease activity are 0.264 μM, 3.713 μM and 2.882 μM, respectively. Our qRT-PCR data demonstrated that exposure to the individual AHLs reduced transcription of *vtpR* and *vtpB*. Combinations of the three AHLs (any two AHLs or all three AHLs) on *V. tubiashii* showed additive effects among upon protease inhibition activity.

These AHL compounds may act by disrupting the quorum-sensing pathway that activates protease transcription of *V. tubiashii*.

Introduction

Infections caused by pathogenic marine bacteria are a major problem for both the shellfish and finfish aquaculture industries, causing severe disease and high mortality, which seriously affect aquaculture production and cause significant economic loss [1, 2]. Opportunistic pathogens from the *Vibrionaceae* frequently cause disease in a variety of shellfish [3, 4]. For example, *Vibrio tubiashii*, a reemerging pathogen of larval bivalve mollusks, that causes invasive and toxigenic disease, has been responsible for high mortalities among oysters on the west coast of the United States [4]. The vibriosis is characterized by a rapid and large reduction in larval motility, detached vela, and necrotic soft tissue, which result in high mortality within one day of infection [5].

While *V. tubiashii* virulence almost certainly involves several factors, the hemolysin and extracellular protease activities are thought to play important roles during pathogenesis in oysters [4, 6]. *V. tubiashii* RE22 encodes two metalloproteases, VtpA and VtpB [4] and at least one hemolysin locus [6]. The hemolysin locus contains two genes, *vthA* and *vthB*; *vthA* encodes a hemolysin, while *vthB* is thought to encode a chaperone for the VthA protein [6]. The extracellular metalloproteases facilitate bacterial invasion and the infection process, acting to enhance vascular permeability, leading to necrotic tissue damage and cytotoxicity in the host [7]. Mutations in the two protease genes resulted in significantly reduced protease activity of RE22 supernatant and toxicity to oyster larvae, thus these two proteases are considered as major virulence factors in *V. tubiashii* [4]. Although the regulation of the virulence factors in RE22 is not fully understood, Hasegawa et al. [8] reported that VtpR was a member of the TetR

family of transcriptional regulators and positively regulates several virulence factors, including VtpA, VtpB and VthBA in *V. tubiashii* RE22. VtpR was also found to exhibit high homology to several quorum sensing regulators, including LuxR (*V. harveyi*, 84%) and HapR (*V. cholerae*, 75%), suggesting that VtpR functions as quorum sensing regulator in *V. tubiashii* RE22. However, the mechanism(s) of VtpR regulation of various virulence factors in RE22 has not been fully investigated.

Phaeobacter gallaeciensis is a gram-negative α-*Proteobacteria* of the *Roseobacter* clade [9-11]. Previously, we isolated *P. gallaeciensis* S4 from inner surface of an oyster shell and showed that S4Sm (a spontaneous streptomycin-resistant mutant) could function as a probiotic treatment for oyster larvae. S4Sm exhibited strong anti-pathogen activity and increased host survival against *V. tubiashii* RE22 and *Roseovarius crassostreae* challenge [12]. We also demonstrated that tropodithietic acid (TDA) production, a broad spectrum antibiotic active against many marine pathogens, and biofilm formation play important roles in S4Sm probiotic activity [13]. However, the complete mechanisms for the probiotic activities of *P. gallaeciensis* S4Sm are still not fully understood.

Besides antibiotic production, probiotic bacteria are able to secrete various types of secondary metabolites, some of which are used to antagonize pathogenic bacteria. Holmstrøm et al. [14] reported that *Pseudomonas fluorescens* AH2 strain secreted siderophores into the culture supernatant, which efficiently chelate iron, resulting in the cessation of growth due to iron deprivation for the pathogen *Vibrio*

anguillarum. Li et al. [15] reported that *Lactobacillus reuteri* produced cyclic dipeptides, which were able to quench *agr*-mediated expression of toxic shock syndrome toxin-1 in staphylococci. Bayoumi et al. [16] discovered that bioactive molecules produced by *Bifidobacterium bifidum* showed inhibitory activity against regulatory and virulence genes in the major pathogenicity islands of *Salmonella typhimurium* and *E. coli* O157:H7.

In this report, we investigate the ability of *P. gallaeciensis* S4Sm to produce secondary metabolites inhibiting RE22 protease activity. The compounds were identified as N-acyl homoserine lactones (AHLs) by UHPLC, mass spectroscopy, and NMR. The active AHLs function to suppress the transcription of *vtpR*, the quorum sensing regulator that positively regulates protease (VtpB) production. These results contribute to a better understanding of interspecies cell-to-cell communication between *P. gallaeciensis* and *V. tubiashii*, and provide an additional mechanism by which probiotic bacteria may attenuate virulence factor production by bacterial pathogens.

Materials and Methods

Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. S4 was isolated from the inner surface of an oyster shell [12]. *P. gallaeciensis* strains and *V. tubiashii* strains were routinely grown in yeast extract (0.5%)-peptone (0.1%) broth plus 3% sea salts (YP30) [12], supplemented with the appropriate antibiotic, in a shaking water bath at 27°C. Overnight cultures of *P. gallaeciensis* or *V. tubiashii*, grown in YP30, were harvested by centrifugation (8,000 × *g*, 2 min)

and the pelleted cells were washed twice with nine-salt solution (NSS) [17]. Washed cells were resuspended to appropriate cell densities in experimental media. Cell densities were estimated by optical density at 600 nm (OD_{600}) and more accurately determined by serial dilution and spot plating. Specific conditions for each experiment are described in the text. *Escherichia coli* strains were routinely grown in Luria-Bertani broth plus 1% NaCl (LB10) [18]. Antibiotics were used at the following concentrations: streptomycin, 200 μg/ml (Sm^{200}) for *P. gallaeciensis* and *Vibrio* strains; ampicillin, 100 μg/ml (Ap^{100}) for *E. coli* and *Vibrio* strains; chloramphenicol, 20 μg/ml (Cm^{20}) for *E. coli* and 5 μg/ml (Cm^{5}) for *P. gallaeciensis* and *Vibrio* strains; kanamycin, 50 μg/ml (Km^{50}) for *E. coli* and *Vibrio* strains; and tetracycline, 15 μg/ml (Tc^{15}) for *E. coli*.

P. gallaeciensis supernatant challenge assay.

The *P. gallaeciensis* supernatant challenge assay was a modification of the method described previously by Holmstrom and Gram [14]. *P. gallaeciensis* strain S4Sm was grown in YP30 at 27°C with shaking to stationary phase (20-24 h). The supernatant was collected by centrifugation at 8,000×g for 5 min, filtered through a 0.22-μm-pore-size cellulose-acetate filter, and used immediately. S4Sm supernatant challenge experiments were initially conducted by using exponentially growing *V. tubiashii* cultures (OD_{600} = 0.6 to 0.8) and transferring 1 ml of a culture to 4 ml of YP30 medium containing 2.5 ml of S4Sm supernatant and 1.5 ml of fresh YP30. For the control, 1 ml of an exponentially growing *V. tubiashii* culture was added to 4 ml of fresh YP30. Cell densities (CFU/ml) were determined at different times after challenge, also 1 ml *V. tubiashii* RE22 supernatant and mRNA were collected at each time point, and stored at -20°C and -74°C for future use,

respectively. All experiments were repeated at least twice.

Detection and quantification of protease activity

The protease activity of culture supernatants was quantified using the azocasein method of Windle and Kelleher [19], as modified by Denkin and Nelson [20]. Culture supernatant was incubated with azocasein (6 mg/ml), which previously was dissolved in Tris-HCl (50 mM [pH 8.0]) containing 0.04% sodium azide (NaN_3). Culture supernatant was prepared by centrifuging 1 ml of cells (12,000rpm, 10 min). Supernatant was removed and filtered through a 0.22-μm-pore-size cellulose-acetate filter. Filtered supernatant (100 μl) was incubated for 30 min at 27°C with 100 μl of azocasein solution. Reactions were terminated by the addition of trichloroacetic acid (TCA) (10% [wt/vol]) to a final concentration of 6.7% (wt/vol). The mixture was allowed to stand for 2 min and centrifuged (12,000g, 8 min) to remove unreacted azocasein, and supernatant containing azopeptides was suspended in 700 μl of 525 mM NaOH. The absorbance of the azopeptide supernatant was measured at 442 nm. Protease activity units (U) were calculated with the following equation: $U = [1,000 (OD_{442})/CFU] \times 10^9$, where OD_{442} is the optical density at 442 nm.

mRNA extraction

mRNA extraction was performed using the protocol previously described by Mou and Nelson [21]. Exponential-phase cells (5×10^7 CFU/ml) and stationary-phase cells (2×10^9 CFU/ml) of *V. tubiashii* under different treatments were treated with RNA protect bacterial reagent (Qiagen, Valencia, CA) following the manufacturer's instructions. Total RNA was isolated using an RNeasy kit and QIAcube (Qiagen) following the manufacturer's instructions. All purified RNA

samples were quantified spectrophotometrically by measuring absorption at 260 nm and 280 nm, using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA), and were stored at −74°C for future use.

Real-time quantitative RT-PCR.

Real-time quantitative RT-PCR was performed using the protocol previously described by Li and Nelson [22]. Quantitative reverse transcriptase PCR (qRT-PCR) was used to quantify various mRNAs by use of a Roche480 multiplex quantitative PCR system and Brilliant II SYBR green single-step qRT-PCR master mix (Agilent Technologies, Wilmington, DE) with 10 ng of total RNA in 20 μl reaction mixtures. The thermal profile was 50 °C for 30 min, 95 °C for 15 min, and then 40 cycles of 95 °C for 30 s and 55 °C for 30 s. Fluorescence was measured at the end of the 55 °C step during every cycle. Samples were run in triplicate along with no-RT and no-template controls. All experiments were repeated at least twice.

Ethyl acetate extraction of *P. gallaeciensis* S4Sm strain supernatant

P. gallaeciensis supernatant was collected and partitioned by ethyl acetate (volume ratio, 1:1) twice. Ethyl acetate was allowed to evaporate via rotary evaporator and ethyl acetate crude extract was obtained and stored.

Challenge of *V. tubiashii* cells with ethyl acetate extract of S4Sm supernatant

Ethyl acetate crude extract from S4Sm supernatant was obtained by using the protocol described above and re-dissolved into fresh YP30 medium. In this experiment instead of using S4Sm supernatant, ethyl acetate extract of S4Sm supernatant was used to challenge *V. tubiashii* RE22 cells. CFU/ml of RE22 cells were determined at different time points after challenge (1, 2 and 3 hours), also *V. tubiashii* RE22 supernatant was collected at each time point, and stored at -20 °C

for future use, respectively.

Construction of reporter strain *V. tubiashii* WZ112

V. tubiashii RE22 was tagged by pSUP203P/P*vtpB-gfp*. Kanamycin resistant gene (*kan*) was amplified by PCR using the appropriate primer pair (Table 2) designed according to the sequence of pCR2.1-TOPO (Invitrogen, Carlsbad, CA). The PCR product was digested with HindIII and BamHI restriction enzymes and the DNA fragments separated on a 1% agarose gel. Subsequently, the gel-purified *kan* PCR fragment was ligated into pSUP202P-PflaB-*gfp* [13] after digestion with HindIII and BamHI and the ligation mixture was introduced into *E. coli* Sm10 (λ pir) by electroporation with Bio-Rad Gene Pulser II. Transformants were selected on LB10-Kan[50] agar plates. The resulting plasmid was designated as pSUP203P-PflaB-*gfp*. The promoter region of *vtpB* gene was amplified by PCR using the appropriate primer pair (Table 2) designed according to the sequence of *vtpB* gene (accession no. GQ121132). The PCR product was digested with KpnI restriction enzyme and the DNA fragments separated on a 1% agarose gel. The gel-purified P*vtpB* PCR fragment was further digested by alkaline phosphatase (Promega, Madison, WI). Subsequently, the kit-purified P*vtpB* PCR fragment was ligated into pSUP203P-P*flaB-gfp* [13] after digestion with KpnI, and the ligation mixture was introduced into *E. coli* Sm10 (λ pir) by electroporation with Bio-Rad Gene Pulser II. Transformants were selected on LB10-Kan[50] agar plates. The resulting plasmid was designated as pSUP203P-PvtpB-*gfp*. The resulting clones were sequenced at the University of Rhode Island Genomics and Sequencing Center. Only pSUP203P-P*vtpB-gfp* with the correct orientation of P*vtpB* was transferred from *E. coli* Sm10 into RE22Sm by conjugation using the procedures described previously [21]. The transconjugants were confirmed by antibiotic

selection and fluorescence microscopy.

Protease inhibitory activity guided extraction and isolation of ethyl acetate extract of *P. gallaeciensis* S4Sm

The ethyl acetate extract of *P. gallaeciensis* S4Sm was separated using a Sephadex LH-20 column (3 × 80 cm) and eluted with chloroform-methanol (1:1, v/v) to give 60 fractions. Aliquotes were transferred into a 96-well plate and dried. Reporter strain *V. tubiashii* WZ112 culture (200 μl, OD_{600} = 0.3) was added into each well containing the dried ethyl acetate extract fractions. After 1.5 h incubation at 27°C, the OD_{600} and fluorescence signals in each well were measured (Mx3005 multiplex quantitative PCR system, plate read function) and the relative fluorescence (RF, fluorescence unit/OD_{600}) of each well was calculated. Wells with lower RF values than the negative controls were considered to contain potential quorum quenching activity and were saved for further analysis. After the first round of screening, fractions 13 to 27 (of 60 fractions) had lower RFU and were pooled to yield Fraction A for further analysis. Fraction A (123.2 mg) was separated by medium pressure liquid chromatography (MPLC) and eluted with gradient methanol-water (from 10:90 to 100:0, v/v, 3 mL/min) to give 29 fractions. Fractions 21 to 29 had lower RF activity than the negative controls and were combined into Fraction B for further purification. Fraction B (48.5 mg) was purified by the semi-preparative high performance liquid chromatography (HPLC) and eluted with methanol-water (0-25 min: from 40:100 to 100:0; 25-30 min: 100:0; v/v, 3 ml/min) to give active compounds DR-8 (2.0 mg), DR-11 (600 μg), and DR-12 (500 μg) (Fig. 4).

Concentration–Response Analyses of three AHL compounds

Exponentially growing *V. tubiashii* cultures (OD$_{600}$ = 0.6 to 0.8) were treated with a series of dilutions of the purified AHL molecules from *P. gallaeciensis*. 1 volume of *V. tubiashii* culture was added to 4 volumes of fresh YP30 medium containing a specific concentration of AHL (dissolved in methanol, 0.4% final concentration). Additions containing only methanol (0.4%, final concentration) were used as controls. *V. tubiashii* RE22 supernatant were collected at 1.5 h, and used for examining the protease activity. A series of concentration-response data (drug concentrations x1, x2... xn and relative protease activities y1, y2 ...yn) were obtained and then plotted. According to the concentration-response curve half maximal inhibitory concentrations (IC50) were calculated for each purified AHL.

Treatment of *V. tubiashii* cells with purified AHLs

Exponentially growing *V. tubiashii* cultures (OD$_{600}$ = 0.6 to 0.8) were treated with AHL molecules at the IC50 individually and in combinations of two or three AHL molecules. Briefly, 1 volume of *V. tubiashii* culture was added to 4 volumes of fresh YP30 medium containing the appropriate concentration of AHL(s) dissolved in methanol (0.4%, final concentration). Additions containing only methanol (0.4%, final concentration) were used as controls. Cell densities (CFU/ml) were determined at 15 min and 90 min after addition of AHL(s), also *V. tubiashii* RE22 culture supernatant and mRNA were collected at each time point, and stored at -20°C and -74°C for future analysis, respectively.

Statistical analysis

Two-tailed Student's *t* tests assuming unequal variances were used for statistical analyses for all experiments. *P* values of <0.05 were considered statistically significant.

Results

Supernatant of *P. gallaeciensis* S4Sm inhibited protease activity of RE22, but not growth

Prior work with *P. gallaeciensis* S4Sm showed that tropodithietic acid (TDA) production and biofilm formation play very important roles in probiotic activity of S4Sm against the marine pathogens, *V. tubiashii* and *V. anguillarum* [13]. In an effort to more completely understand the probiotic activity of *P. gallaeciensis* S4Sm, we investigated the effects of S4Sm culture supernatant against *V. tubiashii* protease activity, a major virulence factor. When a *V. tubiashii* culture ($\sim1\times10^8$ CFU/ml) was incubated in fresh YP30 containing an equal volume of sterile-filtered culture supernatant from a stationary phase *P. gallaeciensis* culture (grown to $\sim2\times10^9$ CFU/ml) the protease activity of RE22Sm was suppressed in response to growth with S4Sm supernatant (Fig. 1A). More specifically, at 1 h no protease activity was detected in the sample incubated with S4Sm supernatant, while the control culture had 149 ± 33 U of protease activity. At later times protease activity from the S4Sm-treated sample increased, but was still significantly lower than that of control. In contrast, the growth of *V. tubiashii* RE22Sm in YP30 (control) and in YP30 supplemented with S4Sm supernatant was nearly identical (Fig. 1B).

Supernatant of *P. gallaeciensis* S4Sm inhibited transcription of *vtpB* and *vtpR*, but not *vtpA*

Previous studies with *V. tubiashii* indicated that VtpR, a member of the TetR family of transcriptional regulators and a homolog of HapR and LuxR, functioned

as a global regulator controlling an array of potential virulence factors, including the two metalloproteases VtpA and VtpB [8]. VtpA and VtpB have been demonstrated to be major virulence factors and responsible for the protease activity of *V. tubiashii* RE22Sm [4]. Since S4Sm culture supernatant inhibited induction of RE22Sm protease activity, we first examined whether there is a protease inhibitor in S4Sm culture supernatant, which could inhibit RE22Sm protease activity. Equal volumes of a RE22Sm culture supernatant with protease activity were mixed with either sterile YP30 medium or with sterile filtered S4Sm culture supernatant (grown in YP30) and the relative protease activity was determined (Fig. S1). Sterile YP30 and sterile filtered S4Sm culture supernatant served as controls. Our data demonstrated that a S4Sm culture supernatant did not directly inhibit RE22Sm protease activity. The mixture of S4Sm and RE22Sm cell-free culture supernatants (1:1 mixture) had the same protease activity as the mixture of fresh YP30 medium and RE22Sm supernatant (1:1 mixture) (Fig. S1), indicating that there was no direct protease inhibitor in the S4Sm supernatant. Next, we examined, using real-time qRT-PCR, whether this inhibition resulted from inhibition of transcription of the *vtpA*, *vtpB* and *vtpR* genes. RE22Sm cells grown in YP30 to late exponential phase ($\sim 10^8$ CFU/ml) were treated with sterile-filtered S4Sm culture supernatant (1:1 mixture) and samples were collected after 15 min and 60 min of incubation for determination of *vtpA*, *vtpB*, and *vtpR* transcription (Fig. 2). Transcription from both *vtpB* and *vtpR* was reduced in the RE22 cultures treated with the S4Sm culture supernatant when compared to the control cultures (Fig. 2A&B). Specifically, transcription of *vtpB* and *vtpR* in the S4Sm supernatant-treated sample were 35.9% and 6.6%, respectively, of the control. After 60 min of incubation in S4Sm supernatant, transcription of *vtpB* and *vtpR*

was 41% and 29%, respectively, of the control. In contrast, there appeared to be no significant difference between the transcription of *vtpA* in RE22 cultures treated with S4Sm culture supernatant and the control (Fig. 2C). After 15 min of incubation the treated culture showed 136% transcription of *vtpA* compared to the control, while at 60 min transcription of *vtpA* in the treated culture was 89% of the control. Additionally, the amount of *vtpA* transcription was two to three orders of magnitude lower than transcription of *vtpB* in the control sample. The data presented here suggest that one or more compounds found in the S4Sm culture supernatant inhibited RE22Sm protease activity by suppressing transcription from the *vtpR* transcriptional regulator; this would decrease *vtpB* transcription and decrease protease activity. Further, our data suggest that VtpB, not VtpA, is the major metalloprotease when RE22 in late exponential phase (Fig. 2C).

Ethyl acetate extract of S4Sm supernatant inhibited protease activity of RE22Sm

We confirmed that TDA (0.5-1.0 µg/ml) did not inhibit protease activity, but did inhibit RE22 growth when cells (2×10^8 CFU/ml) were grown in YP30 (27 °C) for 1 h (Fig. S2). This observation strongly suggested that molecules other than TDA secreted by S4Sm were responsible for the inhibition of protease transcription and activity. In order to narrow down the possible molecules, we tested an ethyl acetate extract of S4Sm supernatants. The ethyl acetate and aqueous soluble fractions were added to RE22Sm cells to determine their effects on protease activity and growth (Fig. 3). Although growth of *V. tubiashii* RE22Sm in YP30 medium (control), YP30 supplemented with S4Sm supernatant, YP30 supplemented with ethyl acetate extract of S4Sm supernatant or YP30

supplemented with aqueous layer extract of S4Sm supernatant were similar (Fig. 3A), the protease activity of RE22Sm was dramatically reduced in response to exposure to S4Sm supernatant or the ethyl acetate extract of S4Sm supernatant (Fig. 3B). These results suggested that the compounds that inhibit RE22Sm protease expression were in the ethyl acetate extract and are likely non-polar molecules.

Screening for active compounds

In order to guide the isolation of the active compounds, a reporter strain, *V. tubiashii* WZ112, was constructed (Table 1). WZ112 harbors a plasmid, which containing the promoter region of *vtpB* fused to a promoterless *gfp*. WZ112 was cultivated in 96-well plates in the presence of YP30 supplemented with HPLC-derived fractions of ethyl acetate extract of S4Sm culture supernatant. Wells with fractions containing the active compounds had lower fluorescence signals (Fig. 4). Three rounds of screening resulted in the isolatation of three active compounds, designated DR-8, DR-11 and DR-12 (Fig. 5).

Identification of the structures of the active compounds

Bioassay guided isolation of the ethyl acetate extract of *P. gallaeciensis* S4 (Fig. 4) resulted in isolating three compounds (DR-8, DR-11, and DR-12) with potent protease inhibitory activities. Their chemical structures were elucidated by the extensive analysis of NMR and MS spectra. All three compounds were identified as *N*-acyl-homoserine lactones (AHLs) (Fig. 5).

The mass spectrum of compound DR-8 showed pseudomolecular ions at m/z [M+H]+ and [M+Na]$^+$, and its ^{1}H and ^{13}C NMR data were identical to those of

previously reported AHL compound (3*R*)-*N*-(3-hydroxydecanoyl)-L-homoserine lactone. The molecular formula of compound DR-11 was determined as $C_{16}H_{25}NO_3$ by HRESIMS at *m/z* 302.1729 [M+Na]$^+$ (calcd for $C_{16}H_{25}NO_3Na$, 302.1732). Compound DR-11showed the similar ^{1}H and ^{13}C NMR data with compound DR-8, the difference between them resided in the side chain. Four olefinic protons signal at δ6.80 (1H, dt, *J* = 15.6, 6.3 Hz, H-3), 5.95 (1H, dt, *J* = 15.6, 1.7 Hz, H-2), 5.55 (1H, m, H-6) and 5.41 (1H, m, H-5), were observed in the ^{1}H NMR spectrum, which indicated the presence of two double bonds in the side chain. Analysis of 2D NMR (^{1}H-^{1}H COSY and HMBC) data allowed the assignment of the position of double bonds. The 2*E*, 5*Z* configurations were determined by the coupling constant ($J_{2,3}$ = 15.6 Hz) and the NOESY correlation between H-5 and H-6. Thus, the compound DR-11 was elucidated as (2*E*, 5*Z*)-*N*-(dodecanoyl-2,5-diene)-L-homoserine lactone, which is a new compound. Compound DR-12 was identified as (3*R*, 7*Z*)-*N*-(3-hydroxytetradecanoyl-7-ene)-L-homoserine lactone by the comparison NMR data with those of previously reported data [32] (Fig. 5).

Concentration–response analyses of the three AHLs

A series of different concentrations of each AHL were used to treat late exponential phase *V. tubiashii* RE22Sm and the protease activities were measured. All three AHLs showed inhibition effects on the protease activity produced by RE22Sm, without effects on *V. tubiashii* growth (Fig. S3). Inhibition activities of the three AHLs are concentration-dependent (Fig. 6). According to concentration-response curve, the half maximal (50%) inhibitory concentration (IC50) of DR-8, DR-11, and DR-12 were determined to be 0.264 μM, 3.713 μM

and 2.882 μM, respectively. 3OHC10-HSL was the most potent inhibitor of RE22Sm protease activity when compared to the other two AHL molecules. Since the AHLs were dissolved in methanol (0.4%, final concentration), we also treated RE22 cells with methanol only as a control. This treatment did not inhibit RE22 protease activity (data not shown).

Exposure to the individual AHL (IC50) reduced transcription of *vtpR* and *vtpB*, but not *vtpA*

Since the IC50 values for the individual AHLs were based on protease activity (Table 3), we were interested in determining whether transcription of the individual genes involved with *V. tubiashii* RE22 protease activity was similarly affected. Cultures of RE22 were grown to late exponential phase (~10^8 CFU/ml) and exposed to the three individual S4Sm-derived AHLs at their IC50. After 15 min, cells were harvested, extracted for total RNA, and the amount of mRNA copies for *vtpA*, *vtpB*, and *vtpR* was determined by real time qRT-PCR. The data presented in Fig. 7 demonstrate that transcription of *vtpB*, was reduced to ~42-51% of the untreated control, and transcription of *vtpR* was reduced to 13.6-14.7% of the untreated control. In contrast, transcription of *vtpA* was not affected by treatment with the purified AHLs. Transcription of *vtpA* in the treated samples was 98-110% of the untreated control. Further, transcription of *vtpA* was about two to three orders of magnitude lower than *vtpB* (8.9 ×10^3 vs. 7.3 ×10^6). These data demonstrate that the three AHLs produced by *P. gallaeciensis* S4Sm inhibit the transcription of the transcriptional regulator, VtpR, to down-regulate transcription of the metalloprotease VtpB.

Effects of the three AHLs upon protease inhibition activity were additive

In order to obtain more information about the mechanism by which *vtpR* and *vtpB* transcription is inhibited, we used different combinations of any two or three AHLs to treat RE22Sm cells to determine whether their effects were additive. The concentration of each AHL was set at its calculated IC50 (see above). Single AHL treatment by 3OHC10-HSL, C12:2-HSL and 3-OHC14:1-HSL suppressed RE22Sm protease activity to ~50% of the untreated control (Fig.8). Treatment of RE22 cells with a combination of any two AHLs (each at IC50) decreased protease activities further: 32.4% for DR8 + DR11, 26.9% for DR8 + DR12 and 34.1% for DR11 + DR12 (Fig. 8). Treatment of RE22 cells with all three AHLs (each at IC50) resulted in the greatest inhibition of protease activity with the treated cells having only 18.0% of the control cell activity (Fig. 8).

Discussion

P. gallaeciensis has been shown to exhibit probiotic activity that protects fish species such as turbot and cod against the bacterial pathogen, *V. anguillarum* [23, 24]. D'Alvise et al [24] demonstrated that TDA production by *P. gallaeciensis* plays a key role in probiotic activity. Recently, our group successfully isolated *P. gallaeciensis* S4 from inner shell of a healthy Eastern oyster (*Crassostrea virginica*), and showed that this isolate has probiotic activity and is able to protect oyster larvae from challenge with two oyster pathogens, *V. tubiashii* and *R. crassostreae* [12]. We also demonstrated that *P. gallaeciensis* S4 probiotic activity not only relies upon TDA production, but also biofilm formation [13].

In this study, we present data that in addition to TDA, S4 produces secondary metabolites, identified as AHLs, which have specific anti-virulence activity and may contribute to *P. gallaeciensis* probiotic activity against the marine pathogen, *V.*

tubiashii, in oyster larvae. Specifically, we found that stationary phase culture supernatant of *P. gallaeciensis* S4 had the ability to repress protease activity, but not the growth of *V. tubiashii* RE22 (Fig. 1). The repression of protease activity was specifically due to the repression of the protease gene *vtpB* and its transcriptional activator *vtpR* (Fig. 2) [8]. A *vtpB-gfp* reporter strain of *V. tubiashii* was constructed and used to screen the ethyl acetate-soluble fractions of the culture supernatant of stationary phase *P. gallaeciensis* S4 cells (Fig. 3) and the successive fractionations by liquid chromatography to isolate three compounds of interest (Fig. 4). The three compounds were found to be AHLs by a combination of NMR and mass spectroscopy analysis (Fig. 5) and identified as *N*-(3-hydroxydecanoyl)-L-homoserine lactone, *N*-(dodecanoyl-2,5-diene)-L-homoserine lactone and *N*-(3-hydroxytetradecanoyl-7-ene)-L-homoserine lactone. While each AHL is able to repress *vtpR* and *vtpB*, DR8 is the most active compound (lowest IC50 value), followed by DR12 and DR11, respectively (Figs. 6). Further, the effects of the AHLs are additive (Fig. 8). Since the isolated compounds are AHLs, this suggests that they function as QS compounds for *P. gallaeciensis* S4 and as quorum quenching (QQ) compounds against *V. tubiashii* RE22.

Quorum sensing (QS) has been demonstrated to regulate diverse physiological activities in Gram-positive and Gram-negative bacteria including virulence, symbiosis, competence, conjugation, antibiotic production, motility, and biofilm formation [25]. Gram-negative bacteria generally use AHLs as autoinducers for QS. Quorum quenching (QQ), which is disruption of QS pathways by any of several mechanisms (including antagonist binding to the native AHL receptor, degradation native AHL signals or suppression of AHL synthetase

and receptor activities, stabilities and productions [26]) can affect QS-regulated bacterial physiological functions [27]. It has been shown that synthetic antagonists are able to block pathogenic bacterial quorum sensing in *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* [28, 29]. Some bacteria, like *Bacillus* and *Streptomyces* strains, are able to produce AHL-degrading enzymes, such as lactonase [30] and acylase [31]. The degraded AHL products by those enzymes are no longer active in quorum sensing. Thus, in pathogenic bacteria, whose virulence is AHL-mediated, QQ of QS-regulated virulence factors can control infection and disease caused by those bacteria.

Our data suggests that AHLs from *P. gallaeciensis* S4 strain work as antagonists to the AHL receptor in *V. tubiahsii* (our proposed model showed in Fig. 9). By using two AHL indicator strains, *E. coli* JB525, which detects C6- to C8-HSL; and *E. coli* JM109L, which detects C10- to C12-HSL [20], we found that *V. tubiashii* RE22 and *P. gallaeciensis* S4 secrete C10- to C12-HSL, but no C6- to C8-HSL. This suggested that since RE22 and S4 share the same range of AHLs, AHL molecules from one of them may act as antagonist to the other. The protease inhibition activity of S4 supernatant is transient, as it gradually diminished during the experiment (Fig. 1A & 3B). One possibility for this observation is competitive inhibition of RE22-AHL-mediated QS regulation of *vtpR* gene expression. Specifically, as *V. tubiashii* cells grow, increasing concentrations of native RE22-AHLs are produced, diluting the exogenous S4-AHLs, and resulting in decreased antagonizing effects from S4-AHL molecules towards RE22 protease activity. Studies on AHL production profile of *V. tubiashii* and comparison between S4 and RE22 AHLs, as well as AHL binding to AHL receptor proteins

need to be carried out in order to determine the QQ mechanism applied by *P. gallaeciensis* AHL molecules.

Similarly, in *Chromobacterium violacium*, a Gram-negative bacterium commonly found in soil and water, longer chain AHLs (C10-C14-HSL) showed transient inhibition of purple pigment violacein production, which could be induced by HHL (C6-HSL), a shorter chain AHL [32]. The inhibition mechanism by longer chain AHLs towards shorter chain AHL has not been described, but it may also be an example of QQ by competitive inhibition.

Previously, Berger et al [33] showed that *P. gallaeciensis* produces N-3-hydroxydecanoylhomoserine lactone, which is involved in the QS-induction of the TDA biosynthesis genes in *P. gallaeciensis*. Similarly, *N*-(3-hydroxytetradecanoyl-7-ene)-L-homoserine lactone is produced by *Rhizobium leguminosarum*, where it is regulates several QS-mediated pathways, including growth inhibition, adaptation to stationary phase, and shorter chain AHL production [34]. To our knowledge, the production of *N*-(dodecanoyl-2,5-diene)-L-homoserine lactone has not been previously reported and is a newly discovered AHL. No previous studies have described any biological functions of either *N*-(dodecanoyl-2,5-diene)-L-homoserine lactone or *N*-(3-hydroxytetradecanoyl-7-ene)-L-homoserine lactone in *P. gallaeciensis*.

Generally, AHL molecules contain a common homoserine lactone moiety and a specific fatty acid side chain. The side chain varies within different species. Therefore, the specificity for AHL signals is conferred by the length and modifications to the acyl groups [26]. Basically, different AHL receptors only recognize specific AHL molecules. Our data suggest that the protease inhibition effect of S4 AHLs is species-specific. We found that expression of *vanT*, a

homolog of *vtpR* in *V. anguillarum* M93 strain [35], was not affected by addition of S4 supernatant into M93 cell culture (Fig. S4). This might be because regulation of *vanT* expression is different in *V. anguillarum* compared to regulation of *vtpR* expression in *V. tubiashii*. By using AHL indicators mentioned above, we found that *V. anguillarum* M93 secreted both C6-C8 and C10-C12 HSLs (Table. S1). Therefore, *vanT* expression (and protease activity) is not influenced by S4 AHLs because M93 and S4 use different ranges of AHLs for QS regulation.

Interestingly, treatment of *P. gallaeciensis* S4 with RE22 culture supernatant did not affect the expression of RaiR, the quorum sensing regulator in S4 strain [33]. RaiR of S4 is a homolog of PgaR in *P. gallaeciensis* DSM17395, and their sequence share almost 96% identity at amino acid level. Cell-free culture supernatant of stationary phase *V. tubiashii* cells was collected and used to treat *P. gallaeciensis* cells during late exponential phase. Both the S4 growth rate and RaiR transcription were the same in the treated and control cultures (Fig. S5). This might be because that native S4-AHLs have a higher binding affinity for the S4 AHL receptors than do the exogenous RE22-AHLs.

To our knowledge we describe here for the first time that a gram negative probiotic bacterium is able to not only efficiently colonize its host, and secrete an antibiotic, but also produce anti-virulence agents, AHLs, to cause QQ in a specific pathogen, which shares the same niche with the probiotic bacterium; as a result, the probiotic bacterium inhibits the growth of and kills the pathogen using antibiotics and it blocks expression of virulence factors in the pathogen, eventually benefiting the oyster host.

Acknowledgements

We thank R. Elston for providing *Vibrio tubiashii* RE22.

Reference

1. Jackson, J.B., et al., *Historical overfishing and the recent collapse of coastal ecosystems.* Science, 2001. **293**(5530): p. 629-37.
2. Lenihan, H.S. and C.H. Peterson, *How habitat degradation through fishery disturbance enhances impacts of hypoxia on oyster reefs.* Ecological Applications, 1998. **8**(1): p. 128-140.
3. Maloy, A.P., et al., *Roseovarius crassostreae, the etiological agent of Juvenile Oyster Disease (now to be known as Roseovarius Oyster Disease) in Crassostrea virginica.* Aquaculture, 2007. **269**(1): p. 71-83.
4. Elston, R.A., et al., *Re-emergence of Vibrio tubiashii in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and management.* Dis Aquat Organ, 2008. **82**(2): p. 119-34.
5. Sindermann, C.J., *Vibriosis of larval oysters.* Disease diagnosis and control in
    North American aquaculture, 1988: p. 271-274.
6. Hasegawa, H. and C.C. Hase, *The extracellular metalloprotease of Vibrio tubiashii directly inhibits its extracellular haemolysin.* Microbiology, 2009. **155**(Pt 7): p. 2296-305.
7. Miyoshi, S. and S. Shinoda, *Microbial metalloproteases and pathogenesis.* Microbes Infect, 2000. **2**(1): p. 91-8.
8. Hasegawa, H. and C.C. Hase, *TetR-type transcriptional regulator VtpR functions as a global regulator in Vibrio tubiashii.* Appl Environ Microbiol, 2009. **75**(24): p. 7602-9.
9. Bruhn, J.B., et al., *Ecology, inhibitory activity, and morphogenesis of a marine antagonistic bacterium belonging to the Roseobacter clade.* Appl Environ Microbiol, 2005. **71**(11): p. 7263-70.
10. Geng, H., et al., *Genetic dissection of tropodithietic acid biosynthesis by marine roseobacters.* Appl Environ Microbiol, 2008. **74**(5): p. 1535-45.
11. Brinkhoff, T., H.A. Giebel, and M. Simon, *Diversity, ecology, and genomics of the Roseobacter clade: a short overview.* Arch Microbiol, 2008. **189**(6): p. 531-9.
12. Karim, M., et al., *Probiotic strains for shellfish aquaculture: protection of Eastern oyster, Crassostreac virginica, larvae and juveniles against bacterial.* Journal of Shellfish Research, 2013.
13. Wenjing Zhao, C.D., Murni Karim, *Contributions of tropodithietic acid and biofilm formation to the probiotic activity of Phaeobacter gallaeciensis.* 2014.
14. Holmstrom, K. and L. Gram, *Elucidation of the Vibrio anguillarum genetic response to the potential fish probiont Pseudomonas fluorescens AH2, using RNA-arbitrarily primed PCR.* J Bacteriol, 2003. **185**(3): p. 831-42.

15. Li, J., et al., *Lactobacillus reuteri-produced cyclic dipeptides quench agr-mediated expression of toxic shock syndrome toxin-1 in staphylococci.* Proc Natl Acad Sci U S A, 2011. **108**(8): p. 3360-5.

16. Bayoumi, M.A. and M.W. Griffiths, *In vitro inhibition of expression of virulence genes responsible for colonization and systemic spread of enteric pathogens using Bifidobacterium bifidum secreted molecules.* Int J Food Microbiol, 2012. **156**(3): p. 255-63.

17. Varina, M., et al., *Identification and characterization of Epp, the secreted processing protease for the Vibrio anguillarum EmpA metalloprotease.* J Bacteriol, 2008. **190**(20): p. 6589-97.

18. Sezonov, G., D. Joseleau-Petit, and R. D'Ari, *Escherichia coli physiology in Luria-Bertani broth.* J Bacteriol, 2007. **189**(23): p. 8746-9.

19. Windle, H.J. and D. Kelleher, *Identification and characterization of a metalloprotease activity from Helicobacter pylori.* Infect Immun, 1997. **65**(8): p. 3132-7.

20. Denkin, S.M. and D.R. Nelson, *Regulation of Vibrio anguillarum empA metalloprotease expression and its role in virulence.* Appl Environ Microbiol, 2004. **70**(7): p. 4193-204.

21. Mou, X., et al., *H-NS is a negative regulator of the two hemolysin/cytotoxin gene clusters in Vibrio anguillarum.* Infect Immun, 2013. **81**(10): p. 3566-76.

22. Li, L., X. Mou, and D.R. Nelson, *HlyU is a positive regulator of hemolysin expression in Vibrio anguillarum.* J Bacteriol, 2011. **193**(18): p. 4779-89.

23. Porsby, C.H., K.F. Nielsen, and L. Gram, *Phaeobacter and Ruegeria species of the Roseobacter clade colonize separate niches in a Danish Turbot (Scophthalmus maximus)-rearing farm and antagonize Vibrio anguillarum under different growth conditions.* Appl Environ Microbiol, 2008. **74**(23): p. 7356-64.

24. D'Alvise, P.W., et al., *Phaeobacter gallaeciensis reduces Vibrio anguillarum in cultures of microalgae and rotifers, and prevents vibriosis in cod larvae.* PLoS One, 2012. **7**(8): p. e43996.

25. Miller, M.B. and B.L. Bassler, *Quorum sensing in bacteria.* Annu Rev Microbiol, 2001. **55**: p. 165-99.

26. Hirakawa, H. and H. Tomita, *Interference of bacterial cell-to-cell communication: a new concept of antimicrobial chemotherapy breaks antibiotic resistance.* Front Microbiol, 2013. **4**: p. 114.

27. Tang, K. and X.H. Zhang, *Quorum quenching agents: resources for antivirulence therapy.* Mar Drugs, 2014. **12**(6): p. 3245-82.

28. Passador, L., et al., *Functional analysis of the Pseudomonas aeruginosa autoinducer PAI.* J Bacteriol, 1996. **178**(20): p. 5995-6000.

29. Geske, G.D., et al., *Modulation of bacterial quorum sensing with synthetic ligands: systematic evaluation of N-acylated homoserine lactones in multiple species and new insights into their mechanisms of action.* J Am Chem Soc, 2007. **129**(44): p. 13613-25.

30. Dong, Y.H., et al., *Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase.* Nature, 2001. **411**(6839): p. 813-7.

31. Park, S.Y., et al., *Identification of extracellular N-acylhomoserine lactone acylase from a Streptomyces sp. and its application to quorum quenching.* Appl Environ Microbiol, 2005. **71**(5): p. 2632-41.

32. McClean, K.H., et al., *Quorum sensing and Chromobacterium violaceum: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones.* Microbiology, 1997. **143 (Pt 12)**: p. 3703-11.

33. Berger, M., et al., *Tropodithietic acid production in Phaeobacter gallaeciensis is regulated by N-acyl homoserine lactone-mediated quorum sensing.* J Bacteriol, 2011. **193**(23): p. 6576-85.

34. Lithgow, J.K., et al., *The regulatory locus cinRI in Rhizobium leguminosarum controls a network of quorum-sensing loci.* Mol Microbiol, 2000. **37**(1): p. 81-97.

35. Denkin, S.M., P. Sekaric, and D.R. Nelson, *Gel shift analysis of the empA promoter region in Vibrio anguillarum.* BMC Microbiol, 2004. **4**: p. 42.

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Description	Resistance	Reference
P. gallaeciensis			
S4	Previously named as *Phaeobacter sp.* S4; wild type isolate from the inner shell of oysters		Karim et al, 2013
S4Sm	Spontaneous Sm^r mutant of S4	Sm^r	Zhao et al, 2014
V. tubiashii			
RE22Sm	Spontaneous Sm^r mutant of RE22	Sm^r	Zhao et al, 2014
WZ112	Reporter strain, RE22Sm tagged by pSUP203P-P*vtpB-gfp*	Sm^{r}Km^r Ap^r	this study
V. anguillarum			
M93sm	Spontaneous Sm^r mutant of M93 (serotype J-O-1)	Sm^r	Denkin et al, 2004
E. coli			
Sm10	*thi thr leu tonA lacY supE recA* RP4-2 Tc::Mu::Km (λpir)	Km^r	Simon et al, 1983
WS203	Sm10(pSUP203P)	Cm^{r}Km^r Ap^r	this study
WS203-vtpB	Sm10(pSUP203P-PvtpB-gfp)	Km^r Ap^r	this study
JB525	MT102(pJBA132)	Tc^r	Andersen et al. 2001
JM109L	JM109(pSB1075)	Ap^r	Winson et al. 1998
Plasmids			
pSUP202P-P*flaB-gfp*	Ap^{r}Tc^r;		Zhao et al, 2014
pSUP203P-P*vtpB-gfp*	Ap^{r}Km^r; derivative from pSUP202P-PflaB-gfp		this study
pJBA132	luxR-PluxI-RBSII-gfp (ASV) in pME6031 (detects C6- to C8-AHLs)		Andersen et al. 2001
pSB1075	luxCDABE fused to lasRI′ in pUC18 (detects C10- to C12-AHLs)		Winson et al. 1998

Table 2. Primers used in this study

Primer	Sequence (5' to 3', underlined sequences are engineered restriction sites)	Description
pw200 Kan202 F	GCGGTA<u>AAGCTT</u>GAACACGTAGAA AGCCAGTCC	amplification of *kan* ORF, forward, with HindIII site
pw201 Kan202 R	CTATAT<u>GGATCC</u>CGTTTCTGGGTG AGCAAAAAC	amplification of *kan*ORF, reverse, with BamHI site
pw204 PvtpB F	GATGTT<u>GGTACC</u>TGTGGATCTCTG CCATTGGCT	amplification of *vtpB* promoter region, forward, with KpnI site
pw205 PvtpB R	CTGCAT<u>GGTACC</u>TATGATTCTCCT TATCGTGGC	amplification of *vtpB*promotor region, reverse, with KpnI site

Figure 1. Effects of supernatant of *P. gallaeciensis* S4Sm upon protease activity and growth of *V. tubiashii* RE22Sm. A) Determination of protease activities of *V. tubiashii* RE22Sm strain treated with S4Sm supernatant or fresh YP30 medium (control) by measuring OD_{442} of azopeptide from azocasein degradation caused by protease activity. B) Growth of *V. tubiashii* RE22Sm cells treated with S4Sm supernatant or fresh YP30 medium (control). The data presented are average of two independent experiments and each independent experiment has three replicates.

A

- ◆ treated w/ medium
- △ treated w/ S4Sm supernatant

B

- ◆ treated w/ medium
- △ treated w/ S4Sm supernatant
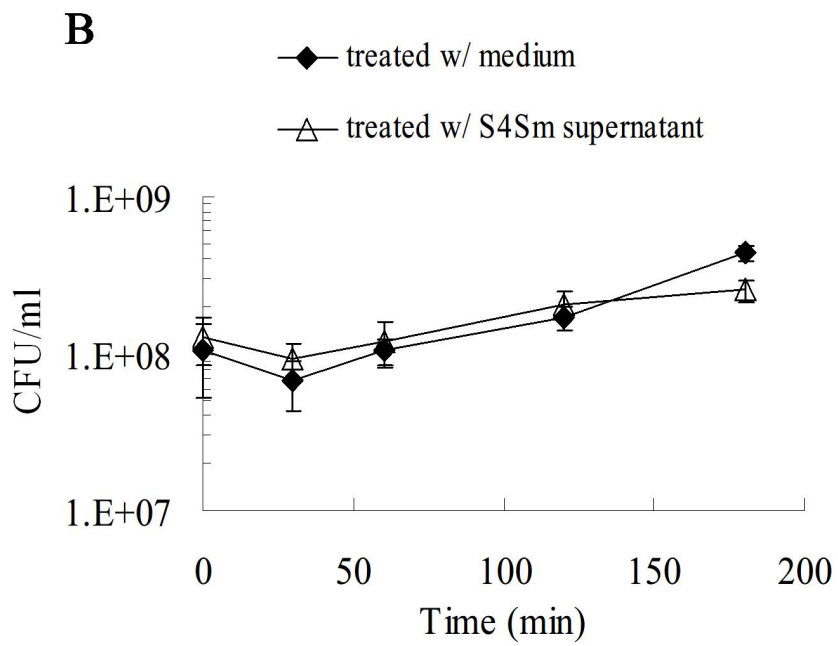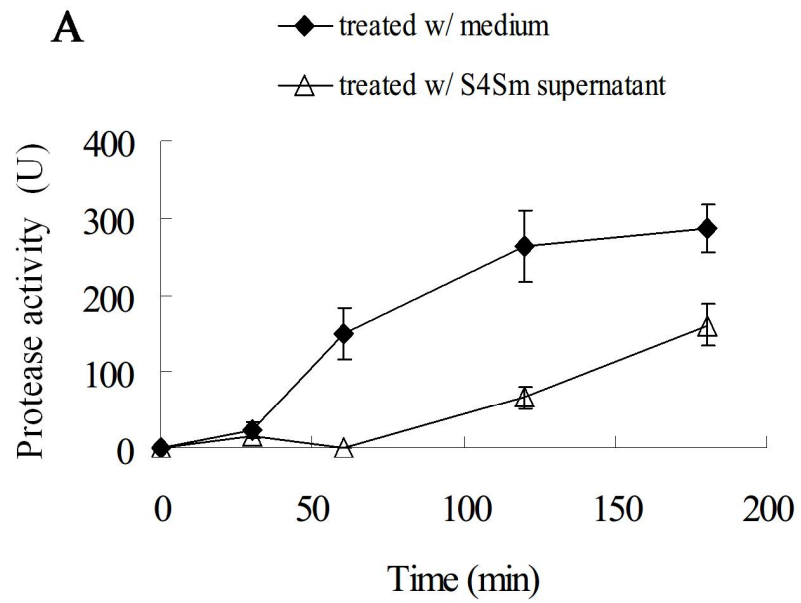
Figure 2. Effects of a supernatant of stationary phase *P. gallaeciensis* S4Sm upon transcription of *vtpA*, *vtpB* and *vtpR* in *V. tubiashii* RE22Sm. Expression of *vtpA*, *vtpB* and *vtpR* determined by qRT-PCR analysis of *V. tubiashii* RE22Sm during late logarithmic phase growth under *P. gallaeciensis* supernatant treatment. The data presented are representative of two independent experiments. Each value is the average for three replicates.

A   ***vtpB***

B   ***vtpR***

C   ***vtpA***
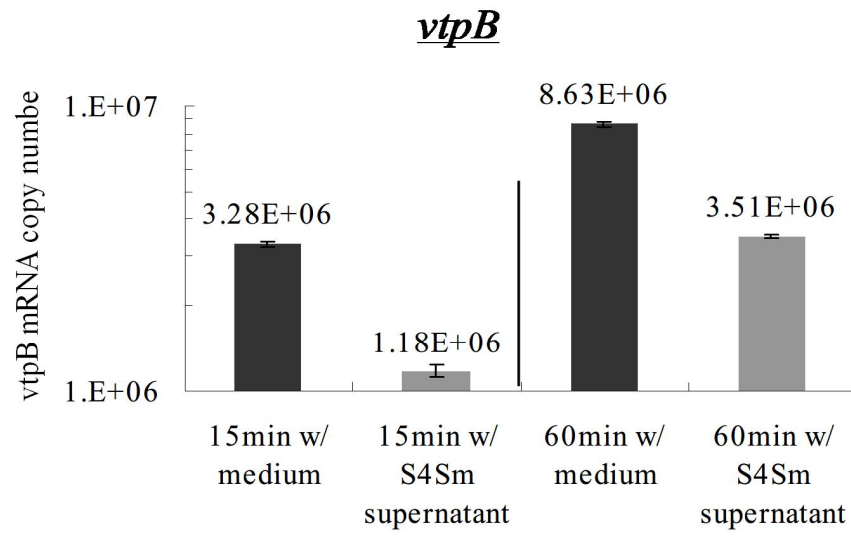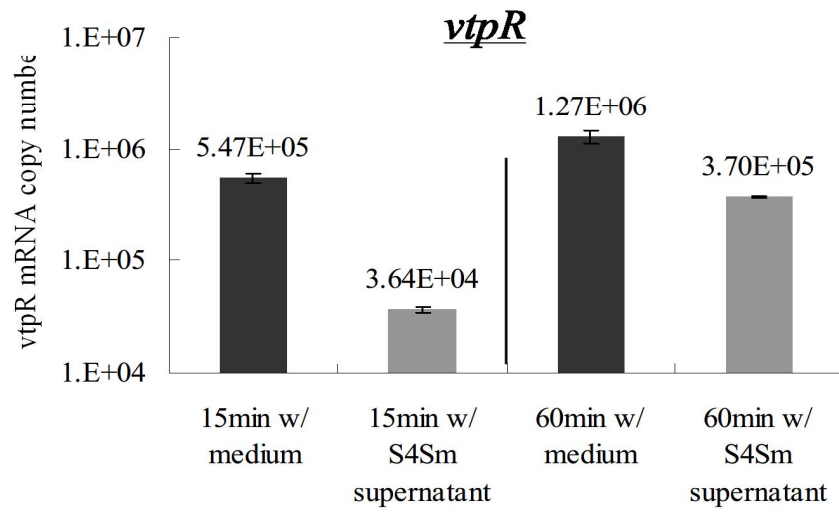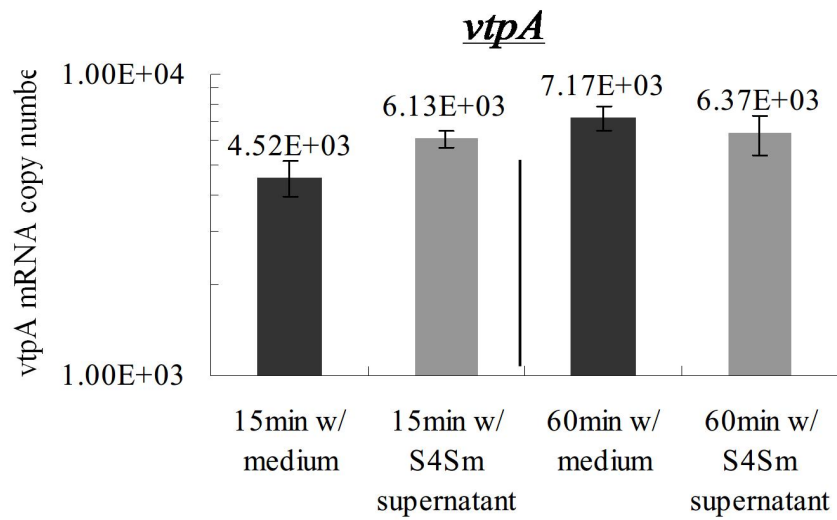
Figure 3. Effects of different fractions of *P. gallaeciensis* S4Sm supernatant upon protease activity and growth of *V. tubiashii* RE22Sm. A) Determination of protease activities of *V. tubiashii* RE22Sm strain treated with S4Sm supernatant or fresh YP30 medium (control) by measuring OD_{442} of azopeptide from azocasein degradation caused by protease activity. B) Growth of *V. tubiashii* RE22Sm cells treated with S4Sm supernatant or fresh YP30 medium (control). The data presented are average of two independent experiments and each independent experiment has three replicates

A
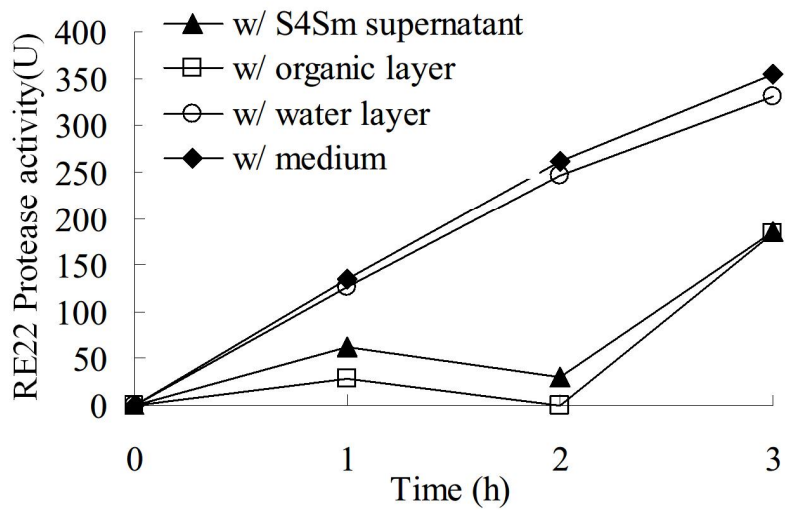
B
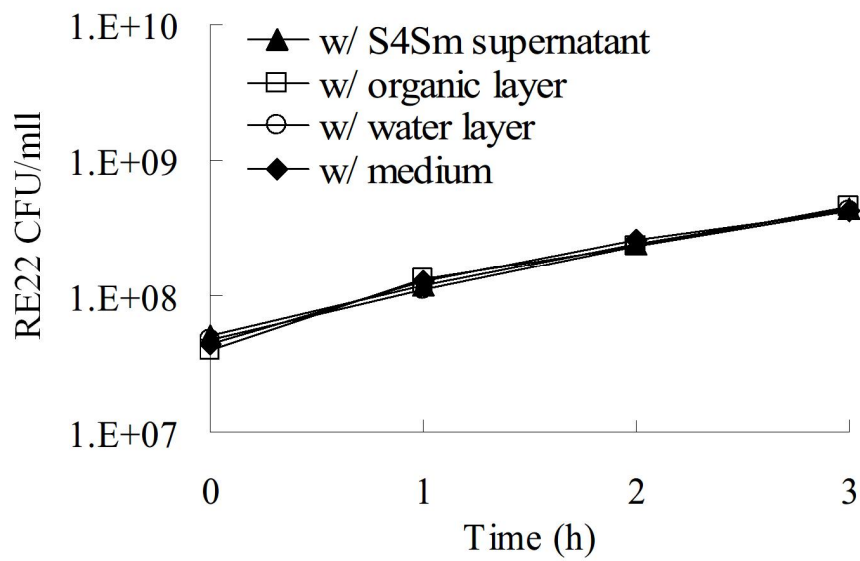
Figure 4. Schematic diagram of isolation process of three N-acyl homoserine lactones. In order to guide the isolation of the active compounds, a reporter strain, *V. tubiashii* WZ112, was constructed (Table 1). WZ112 harbors a plasmid, which contains the promoter region of *vtpB* fused to a promoterless *gfp*. WZ112 was cultivated in 96-well plates in the presence of YP30 supplemented with HPLC-derived fractions of ethyl acetate extract of S4Sm culture supernatant. Wells with fractions containing the active compounds had lower fluorescence signals.

```
                    ┌─────────────────────────────┐
                    │   Culture supernatant (80L)  │
                    └─────────────────────────────┘
                                    │  Ethyl acetate partitioning (80
                                    │         L * 2 times)
              ┌──────────────────────────┐      ┌──────────────────────────┐
              │ Ethyl acetate extract     │      │  Aqueous part (inactive) │
              │      (active)             │      └──────────────────────────┘
              └──────────────────────────┘
                          │  Sephadex LH-20 (CHCl3-
                          │         MeOH)
                 ┌─────────────────┐
                 │   60 fractions  │
                 └─────────────────┘
                          │  Screening
              ┌──────────────────────┐      ┌──────────────────────┐
              │ Fraction # 13-27     │      │  Others (inactive)   │
              │      (active)        │      └──────────────────────┘
              └──────────────────────┘
                  │  MPLC (MeOH-H2O)
                 ┌─────────────────┐
                 │   29 fractions  │
                 └─────────────────┘
                          │  Screening
              ┌──────────────────────┐      ┌──────────────────────┐
              │ Fractions # 21-29    │      │  Others (inactive)   │
              │      (active)        │      └──────────────────────┘
              └──────────────────────┘
                  │  Semi-HPLC (MeOH-H2O)
                 ┌─────────────────┐
                 │  17 compounds   │
                 └─────────────────┘
                          │  Screening
              ┌──────────────────────┐      ┌──────────────────────┐
              │   Three (active)     │      │  Others (inactive)   │
              └──────────────────────┘      └──────────────────────┘
```
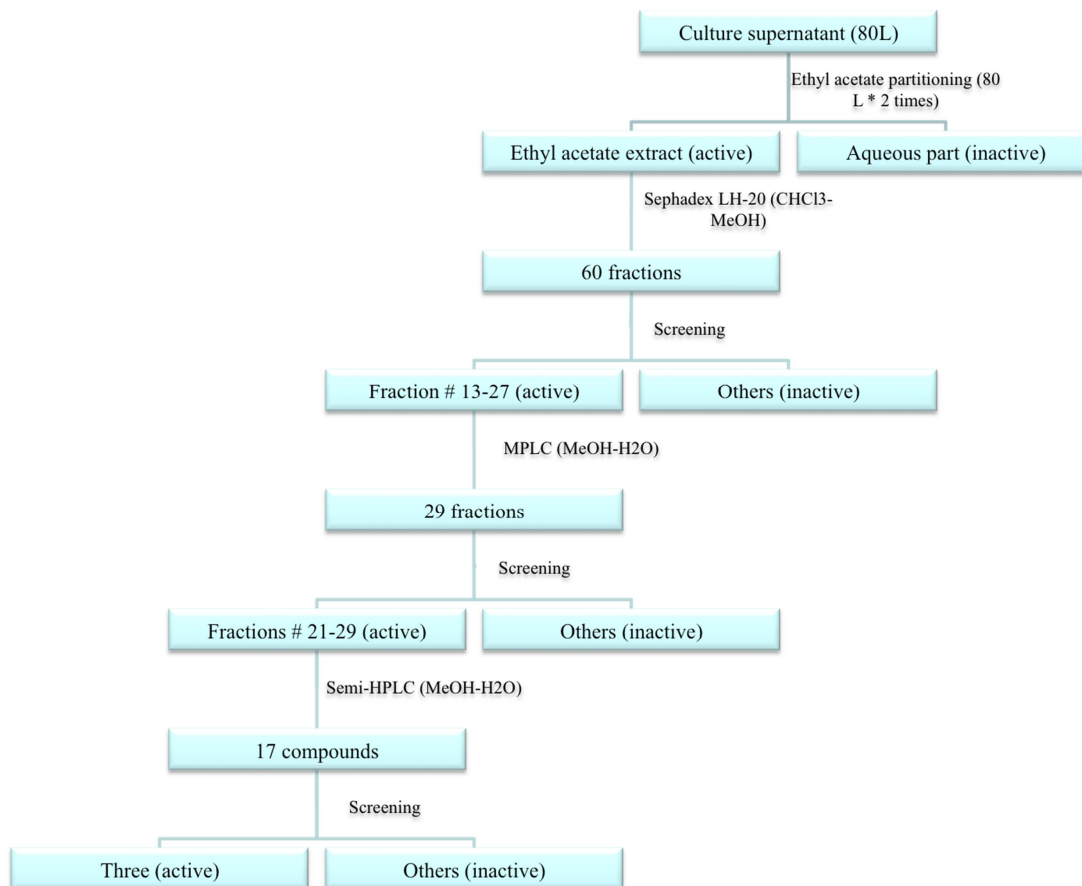
Figure 5. Chemical structures of three N-acyl homoserine lactones. Chemical structures for the three active compounds with potent protease inhibition activity were elucidated by the extensive spectra analysis including NMR and MS spectrum. All three compounds were identified as *N*-acyl-homoserine lactones (AHLs).
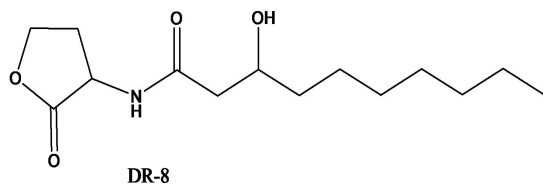
N-(3-hydroxydecanoyl)-L-homoserine lactone

DR-8
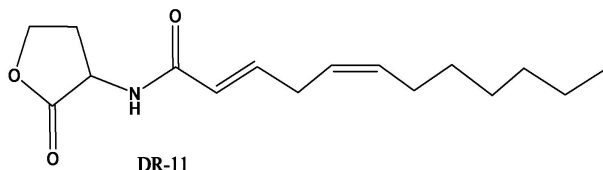
N-(dodecanoyl-2,5-diene)-L-homoserine lactone

DR-11
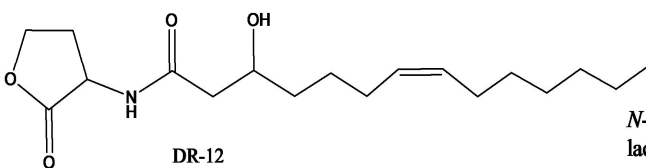
N-(3-hydroxytetradecanoyl-7-ene)-L-homoserine lactone

DR-12

156

Figure 6. Concentration–response analyses of the three AHLs. A series of different concentrations of each AHL were used to treat *V. tubiashii* RE22Sm and the protease activities were measured. Concentration-response curves (trendlines) of each AHL were obtained.
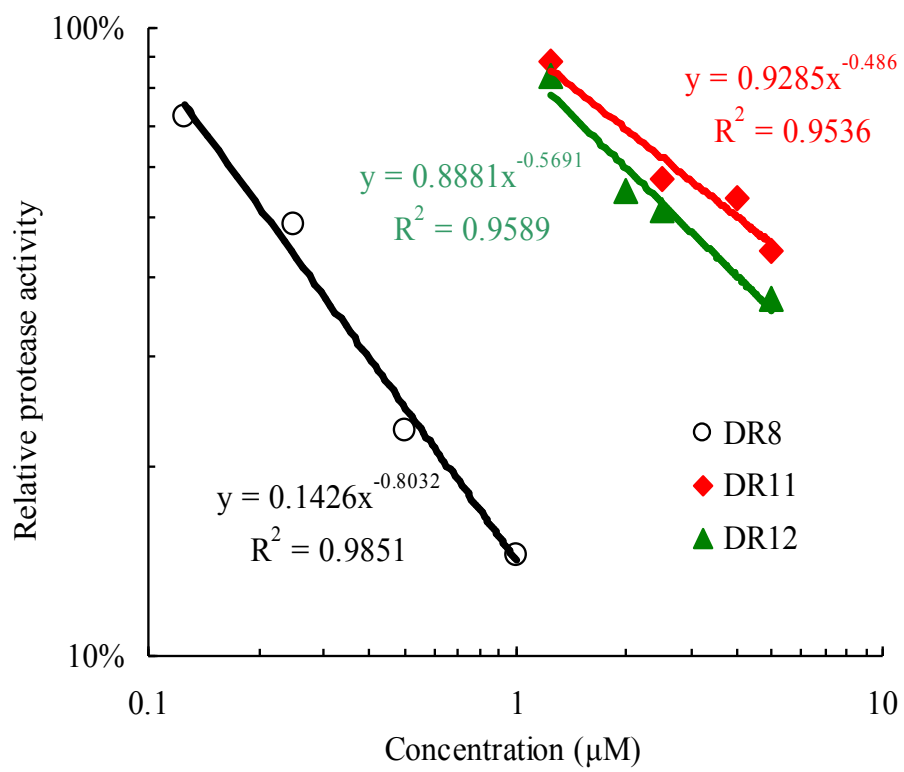
Figure 7. Effects of single AHL treatment upon transcription of *vtpA*, *vtpB*, and *vtpR* in *V. tubiashii* RE22Sm. Expression of *vtpR*, *vtpB*, and *vtpA* determined by qRT-PCR analysis of *V. tubiashii* RE22Sm treated by individual AHL (at their own IC_{50}) during late logarithmic phase growth (~1-2×10^8 CFU/ml). The data presented are representative of two independent experiments. Each value is the average for three replicates.

A

vtpR

B

vtpB

C

vtpA
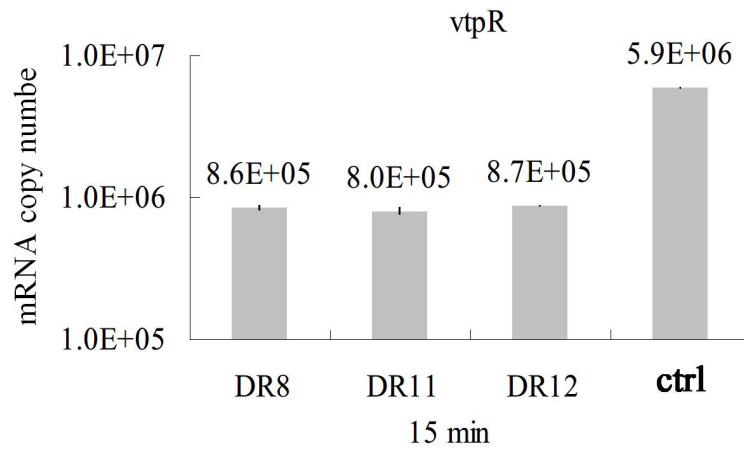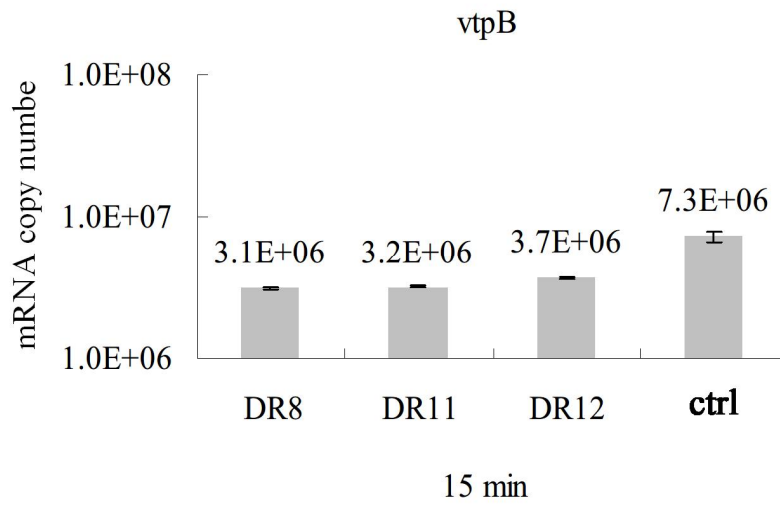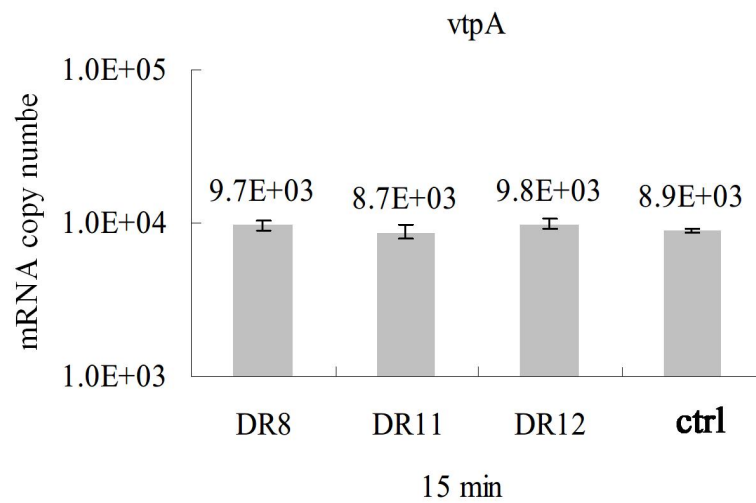
Figure 8. Effects of different combinations of three AHLs upon protease activity of *V. tubiashii* RE22Sm. Determination of protease activities of *V. tubiashii* RE22Sm strain treated with single AHL, various combinations of AHLs or appropriate amount of methanol (control) by measuring OD442 of azopeptide from azocasein degradation caused by protease activity. The data presented are representative of two independent experiments. Each value is the average for three replicates.
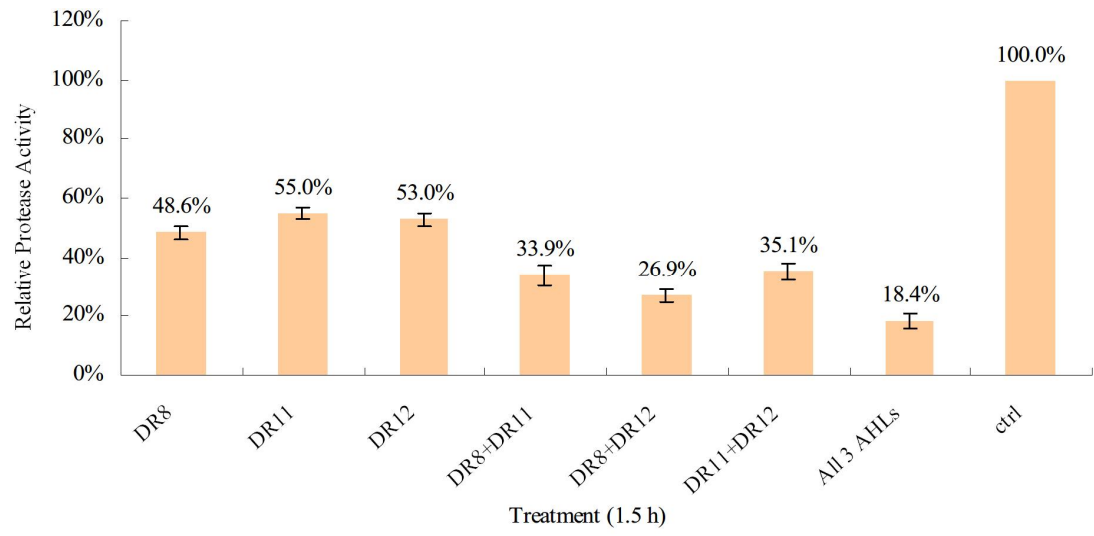
Figure 9. Proposed model: inhibition mechanism of *P. gallaeciensis* AHLs. A) Proposed quorum sensing pathway of *V. tubiashii* cells at late exponential phase. B) Proposed quorum quenching on *V. tubiashii* cells at late exponential phase in response to *P. gallaeciensis* S4 AHL molecules. Green solid circle represents *V. tubiashii* native AHLs, red solid circle represents *P. gallaeciensis* AHLs.

Late exponential phase *V. tubiashii* cell

A

Quorum Sensing

B

Quorum Quenching

Antagonist for
AHL receptor

OM

IM

AHL receptor

AHL synthetase

P

?

P

VtpR

VtpB

Metalloprotease

OM

IM

AHL receptor

AHL synthetase

?

P

VtpR

VtpB

Metalloprotease
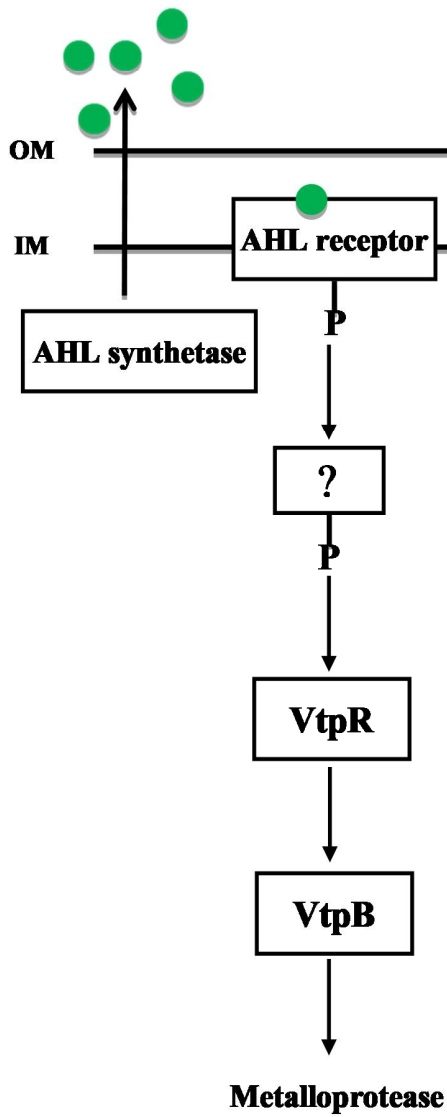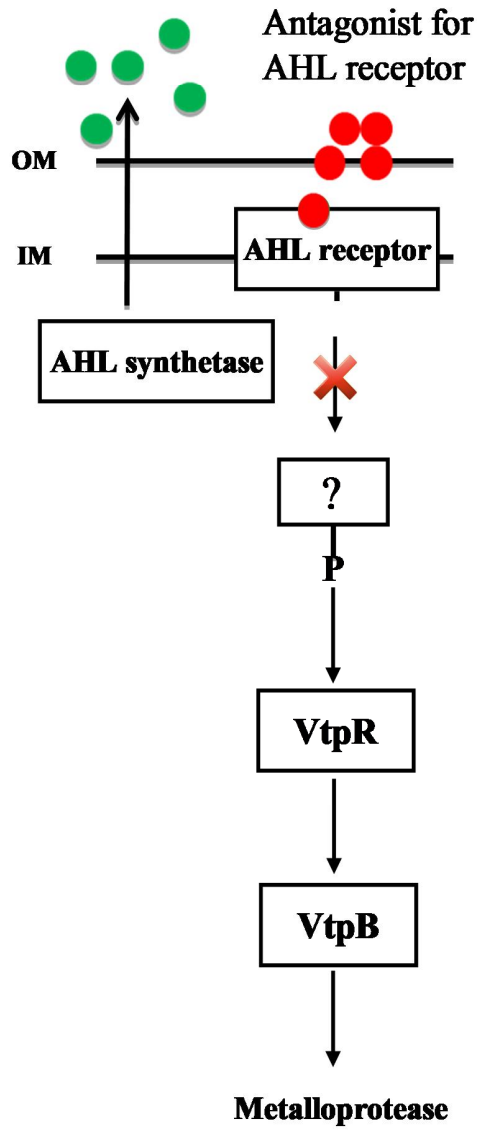
Table S1. AHLs secreted by various bacterial strains detected using AHL indicator strains*

Organism	AHL	
	C6-C8-HSL	**C10-C12-HSL**
P. gallaeciensis S4Sm	No	Yes
V. tubiashii RE22Sm	No	Yes
V. anguillarum M93Sm	Yes	Yes

*: AHL production measured with *E. coli* JB525 *gfp*-based (which responds to C6-C8-HSL) and *E. coli* JM109L *lux*-based (which responds to C10-C12-HSL) AHL sensor strains. Cell-free supernatant from S4Sm, RE22Sm and M93Sm growing in YP30 or LB20 were added to AHL indicator strains and fluorescence or luminescence signal, respectively, were measured by appropriate microtiter plate readers [20].

Figure S1. Direct protease inhibitor detection in *P. gallaeciensis* S4 supernatant. Determination of protease activities from different bacterial cell supernatant or supernatant mixtures by measuring OD442 of azopeptide from azocasein degradation caused by protease activity. The data presented are representative of two independent experiments. Each value is the average for three replicates.
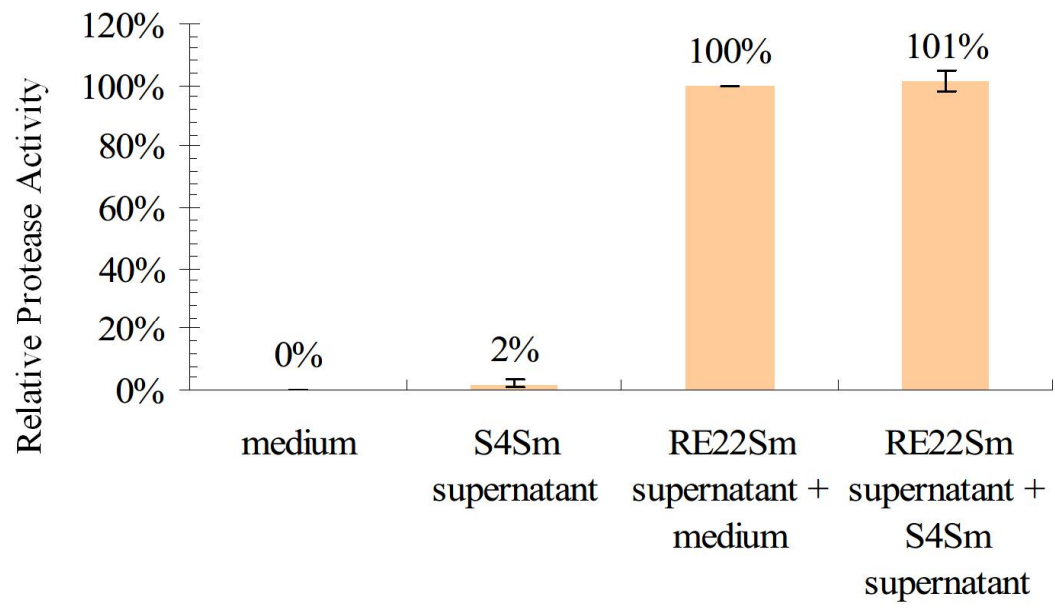
Figure S2. Effects of TDA at different concentration (0.5 µg/ml or 1.0 µg/ml) upon protease activity and growth of *V. tubiashii* RE22Sm. A) Determination of protease activities of *V. tubiashii* RE22Sm strain treated with TDA or fresh YP30 medium (control) by measuring OD442 of azopeptide from azocasein degradation caused by protease activity. B) Growth of *V. tubiashii* RE22Sm cells treated with TDA or fresh YP30 medium (control).

A

V. tubiashii Protease
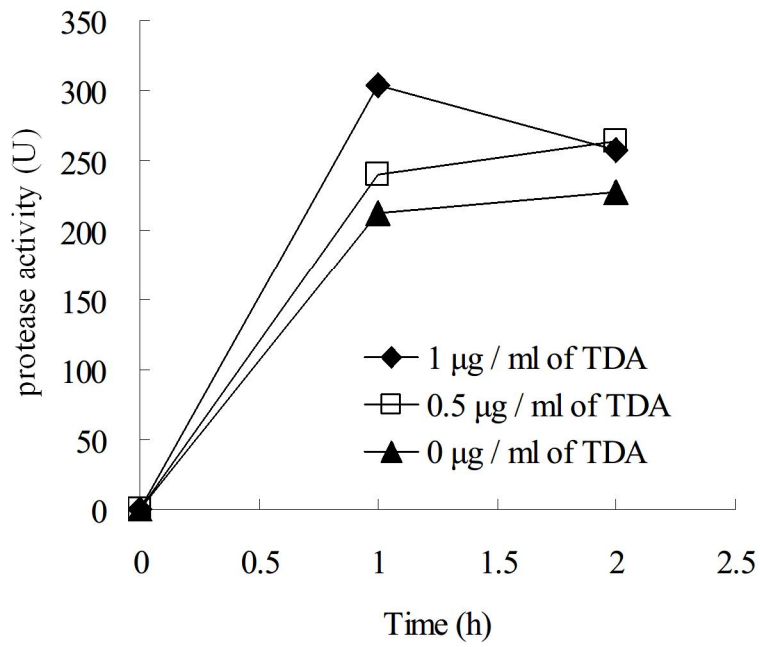
B

V. tubiashii Growth
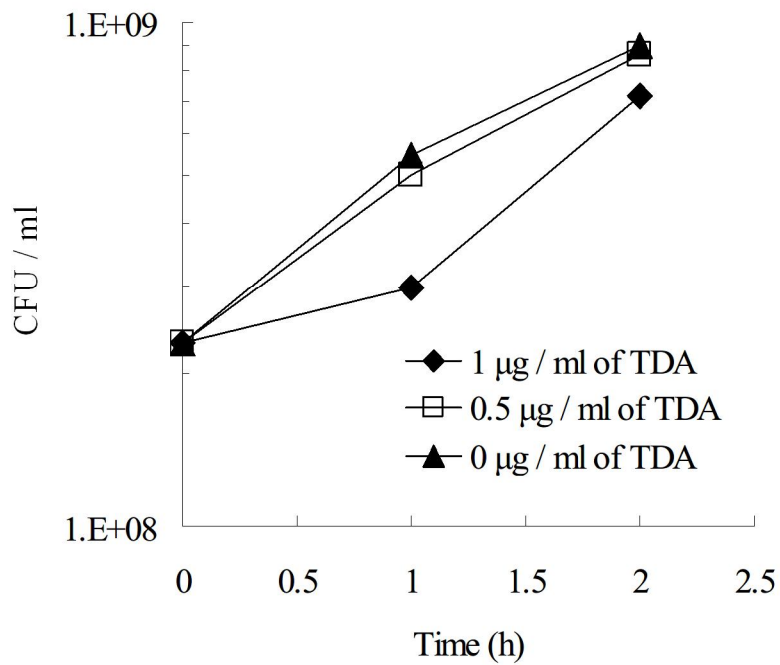
Figure S3. Effects of *P. gallaeciensis* AHLs upon growth of *V. tubiashii* RE22Sm. A series of different concentrations of each AHL were used to treat *V. tubiashii* RE22Sm and cell density (OD600) was measured. The relative growth of RE22 compared to the control (methanol) was calculated.
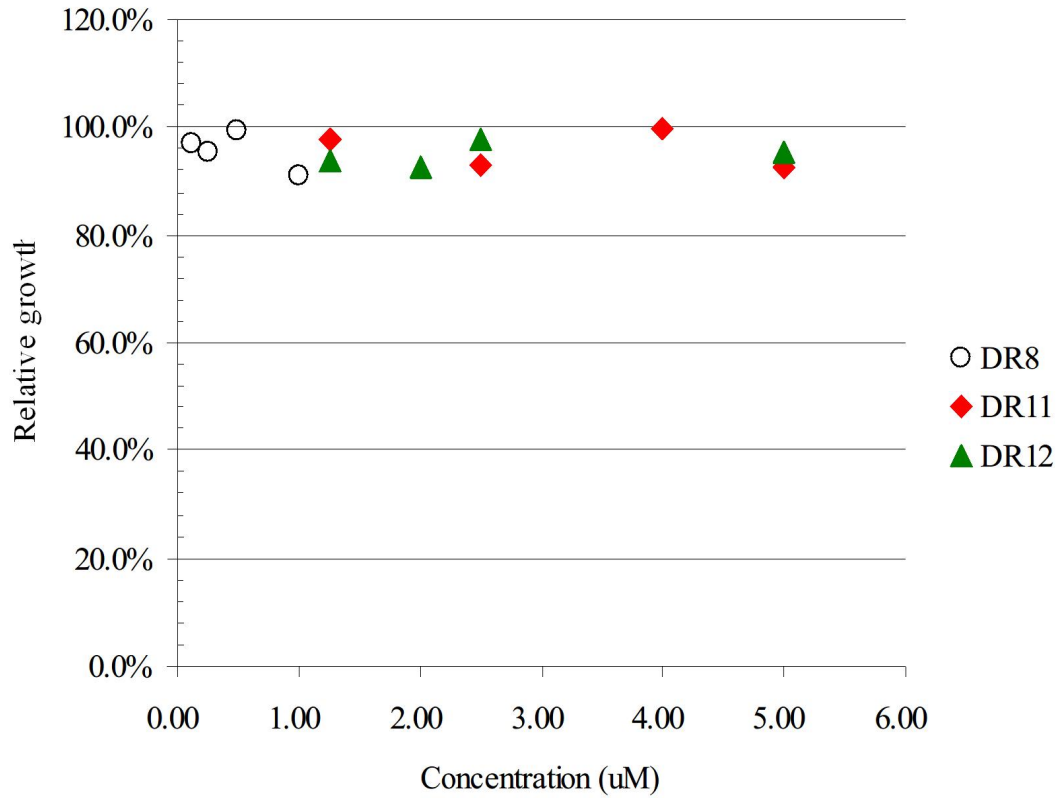
Figure S4. Effects of supernatant of *P. gallaeciensis* S4Sm upon transcription of *vanT* in *V. anguillarum* M93Sm. Expression of *vanT* determined by qRT-PCR analysis of *V. anguillarum* M93Sm during late logarithmic phase growth under *P. gallaeciensis* supernatant treatment.

Expression of *vanT*
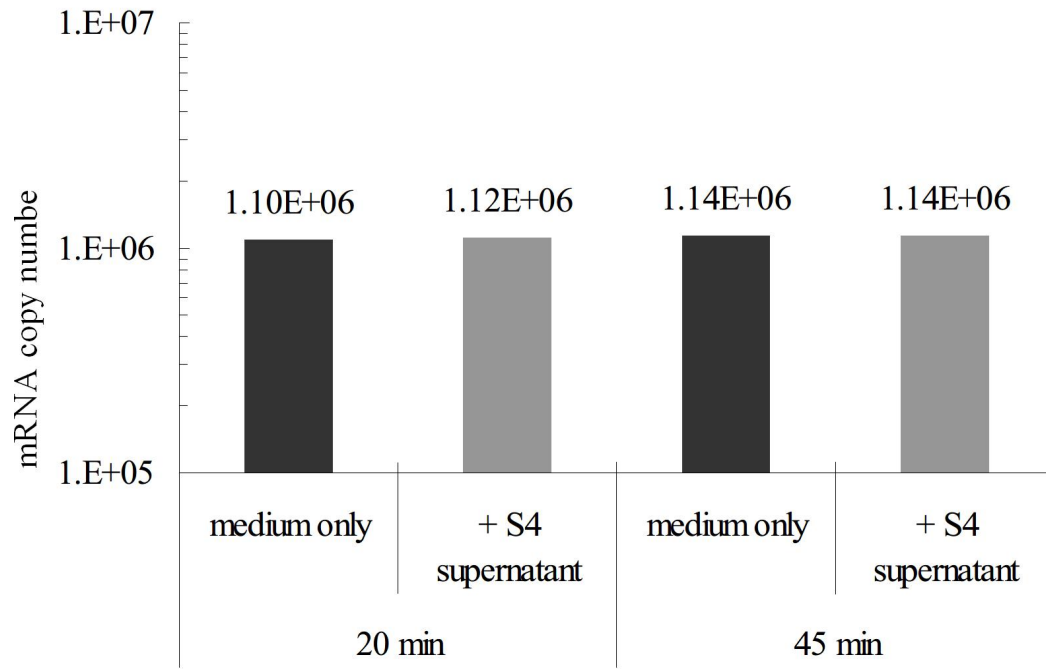
Figure S5. Effects of supernatant of *V. tubiashii* RE22Sm upon growth and transcription of *raiR* in *P. gallaeciensis* S4Sm. A) Growth of *P. gallaeciensis* S4Sm cells treated with RE22Sm supernatant or fresh YP30 medium (control). B) Expression of *raiR* determined by qRT-PCR analysis of *P. gallaeciensis* S4Sm during late logarithmic phase growth ($\sim 4 \times 10^8$ CFU/ml) under RE22Sm supernatant treatment. The data presented are average of two independent experiments and each independent experiment has three replicates.

A

Growth of *P. gallaeciensis*

B
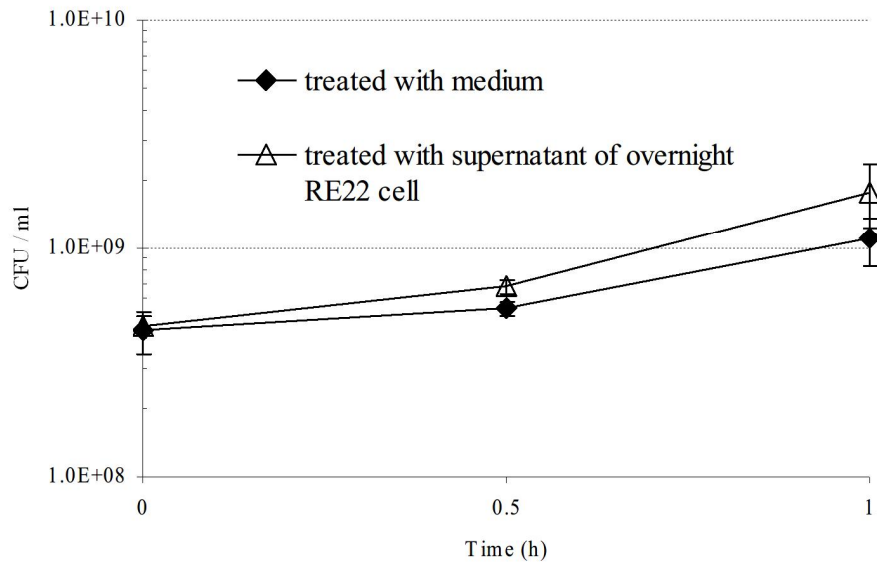
Expression of *raiR*
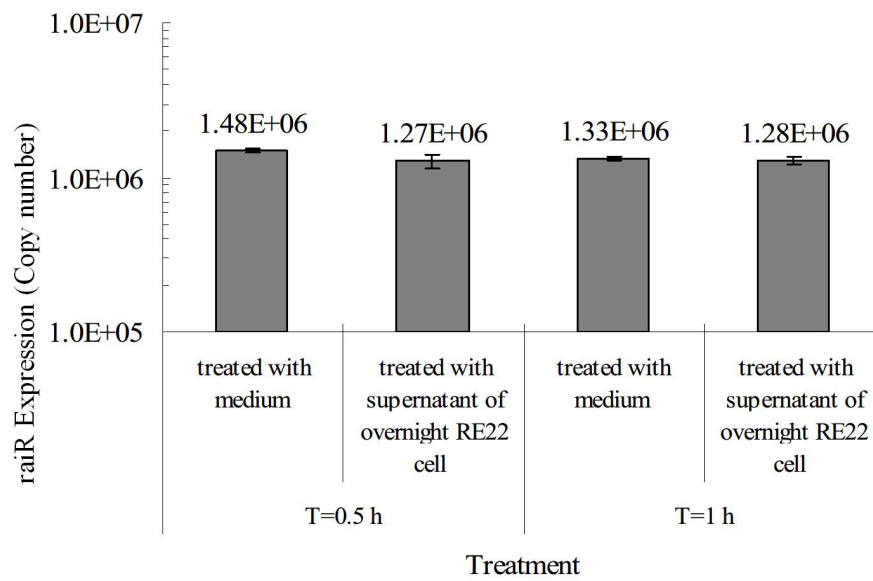
Appendix A (additional table for Manuscript I)

Inhibition zone assay

Probiotic	Pathogens	Strain	Inhibition zone (mm)
Phaeobacter gallaeciensis S4	*Roseovarius crassostreae*	CV919-312	12
	Vibrio harveyi	BB120	17
	Vibrio harveyi	DN01	7
	Vibrio tubiashii	RE22	7
	Vibrio anguillarum	M93	13
	Vibrio anguillarum	NB10	13

Anti-bacterial activity of *P. gallaeciensis* strains was measured by a growth inhibition assay using *V. anguillarum*, *V. tubiashii*, and *R. crassostreae* as the target organisms. Briefly, an aliquot (100 µl) from a stationary phase overnight culture of the appropriate *Vibrio* or *R. crassostreae* culture was spread onto YP30 agar plates, then 10 µl of a 2-day-old culture (OD600 = 0.8) of a *P. gallaeciensis* strain was spotted in triplicate onto the pathogen cell lawn. After incubation at 27°C for 24 h, the level of antibacterial activity was determined by the diameter of the inhibition zone around the *P. gallaeciensis* colonies
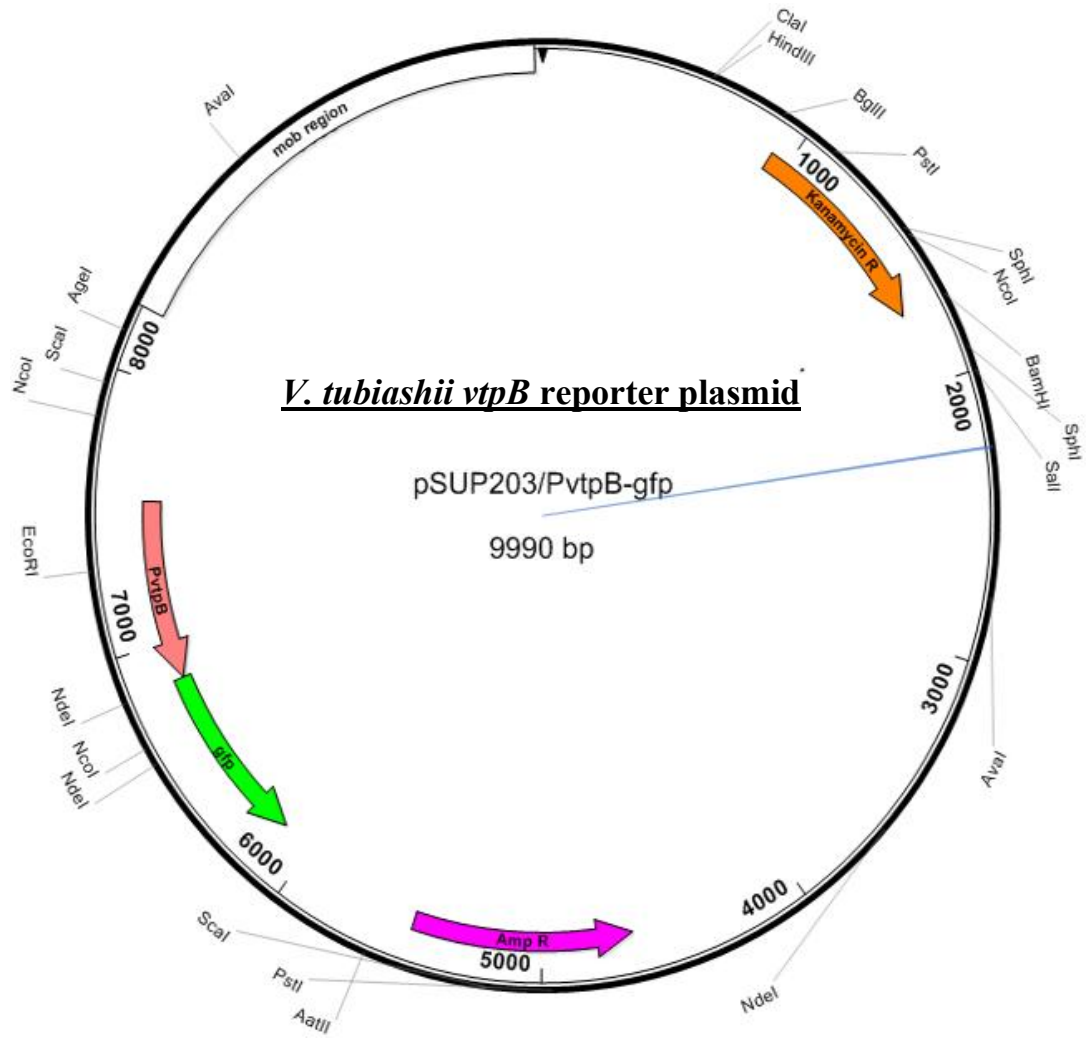
Figure 1. Map of *V. tubiashii vtpB* reporter plasmid

Figure 2. Proposed model to explain the observation that S4 AHLs at IC50 were not able to affect protease production of *V. tubiashii* stationary phase cells; however, S4 AHLs at 2 × IC50 exhibited protease inhibition activity upon *V. tubiashii* stationary cells.

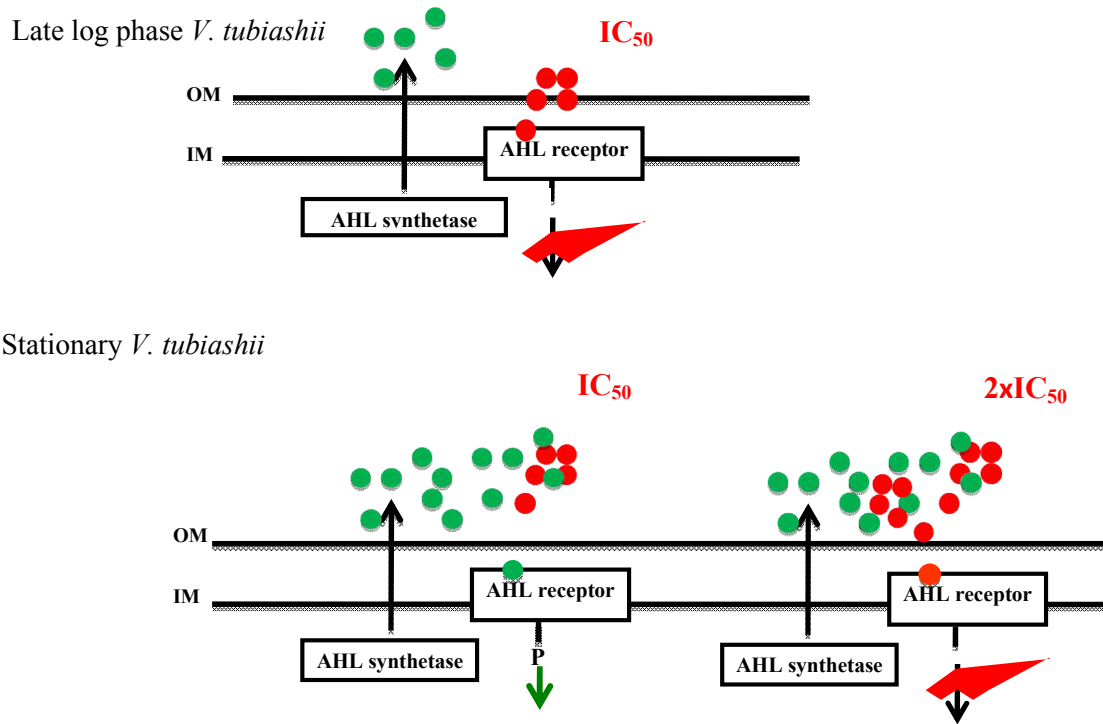Figure 3. Effects of S4 AHLs at different concentrations upon protease production of

V, tubiashii stationary phase cells.

Effects of AHL at IC50
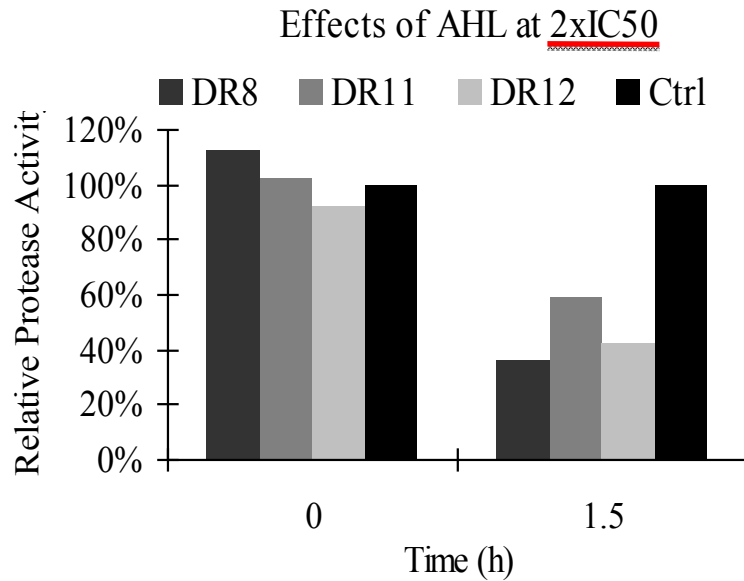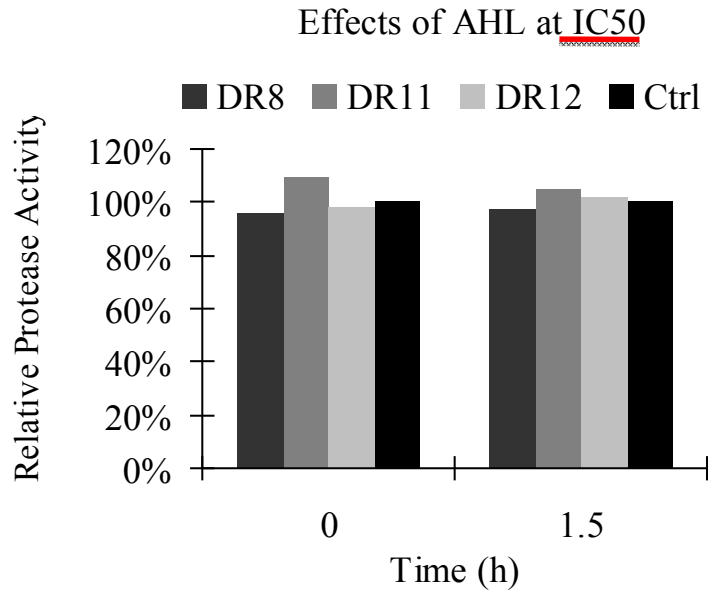

Effects of AHL at 2xIC50

Figure 4. Proposed model to explain the observation that S4 AHLs had no effects on protease production of *V. anguillarum* cells