APPROVAL SHEET

Title of Thesis: A Study of the Antagonistic Activity of *Bacillus subtilis* Strain T1 Against Shrimp Pathogen *Vibrio parahaemolyticus* Strain D4

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Master of Science, 2018

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Abstract

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Sarah Avery, Master of Science, 2018

Thesis directed by: Dr. Harold J. Schreier

Acute hepatopancreatic necrosis disease (AHPND), also known as early mortality syndrome (EMS), is caused by strains of *Vibrio parahaemolyticus* containing the PirAB toxin and has been devastating to shrimp aquaculture globally. Current methods for prevention rely on the use of antibiotics that leads to the development of antibiotic-resistant bacteria. *In vitro*, *Bacillus subtilis* strain T1 was found to possess antagonistic activity against EMS-causing *V. parahaemolyticus* strain D4 and is a candidate for use as a probiotic in the aquaculture industry. Competitive growth experiments examined the effect of T1 on D4 growth. Using qPCR to assess T1 and D4 growth, these studies showed that T1 was capable of inhibiting D4 growth in a density-dependent manner, with complete inhibition occurring when the T1 starting density was 10^4-fold higher than D4. Using a *mariner*-based transposon system, T1 mutants were generated to identify genes involved in D4-inhibitory activity. Of over 3,000 colonies screened using an overlay-based assay, 17 were identified as having either complete or partial loss of activity. Eleven mutants contained insertions within an ~30-kb DNA cluster that included lipopeptide and polyketide biosynthesis genes. One of these mutants, A3-41, which

contained an insertion within a non-ribosomal peptide synthetase gene utilized for lipopeptide biosynthesis, was found to have lost the ability to inhibit D4 growth in co-culture experiments. Two mutants were found to contain insertions within stationary phase regulators, *spo0A* (sporulation gene regulator) and *oppA* (first gene of the oligopeptide transporter system operon) suggesting that D4 inhibitory activity is associated with a stationary phase product. Consistent with the mutagenesis results, supernatant fractions prepared from stationary phase cultures of T1 were found to inhibit D4 growth in a dose-dependent manner while culture supernatants prepared from mutant A3-41 were not inhibitory. These results indicate that T1 produces and excretes a stationary phase metabolite(s) that has inhibitory activity against D4 and has implications for the use of T1 as a probiotic in shrimp aquaculture.

A Study of the Antagonistic Activity of *Bacillus subtilis* Strain T1 Against Shrimp Pathogen *Vibrio parahaemolyticus* Strain D4

by

Sarah E. Avery

Thesis submitted to the Faculty of the Graduate School of the University of Maryland Baltimore County in partial fulfillment of the requirements for the degree of Master of Science
2018

Advisory Committee:

Dr. Harold J. Schreier, Advisor
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DEDICATION

This thesis is dedicated to my mother, Mona Neaderhiser, who instilled in me the value of education and helped cultivate my love of science.

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I would like to thank Dr. Kathleen Cusick, Dr. Stephen Miller, Dr. Eric Schott and principally, Dr. Harold Schreier for serving as members of my committee. The insight and guidance given by my committee has shaped my thesis research. Additionally, I would like to thank Dr. Eric Schott for his expertise and guidance in the use of qPCR. I wish to give special thanks to Dr. Schreier for his continued support throughout my research and during the writing process. He has been instrumental in my growth as a research scientist and to the development of this thesis.

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I would like to acknowledge Sabeena Nazar of the BioAnalytical Services Lab for DNA sequence analysis.

Lastly, I am grateful to my family for their unwavering support in my pursuit of an advanced degree in biological sciences. I would like to especially thank my wife, Shannon Avery, for encouraging me to attend graduate school, for her support throughout the challenges of scientific research, and her seemingly limitless patience. I know I could not have finished without her support.

CONTRIBUTIONS

This study would not have been possible without contributions from members of the Schreier Lab group. Dr. Harold Schreier and Amanda Hise generated T1 mutants using the pDB384 plasmid (designated by the letter "A"). Susannah Ruzbarsky identified T1 mutant insertion sites for S16-13, S21-46, A13-56, S11-14, A3-41, S35-30, S31-22 and A8-11. Dr. Schreier also is credited with the genome analysis of T1.

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INTRODUCTION

Aquaculture, the farming of aquatic organisms such as fish and crustaceans, is a growing global industry. According to the Food and Agriculture Organization of the United Nations, in 2014, farm-raised aquaculture was valued at over 160 billion dollars, with shrimp farming accounting for nearly a quarter of the total value (1). Over the last decade, the shrimp aquaculture industry has been impacted by the emergence of acute hepatopancreatic necrosis disease (AHPND), also known as early mortality syndrome (EMS), caused by certain strains of the marine bacterium *Vibrio parahaemolyticus* (1). Currently, the best method for prevention of EMS along with other diseases, is through the use of antibiotics (2). This practice leads to increased antibiotic resistance in bacteria. Better methods are needed to prevent EMS outbreaks and one such possibility is the use of probiotics—living microorganisms that confer health benefits to the host.

This study investigates the activity of a *Bacillus subtilis* strain T1, against the EMS-causing *V. parahaemolyticus* strain D4. T1 is a potential probiotic candidate for use in shrimp aquaculture, however, a better understanding of how T1 affects D4 is needed. This study aims to identify the genes involved in the activity of T1 against D4 and to examine conditions necessary for stimulating the ability of T1 to inhibit D4.

Early Mortality Syndrome

First observed in China in 2009, early mortality syndrome (EMS), also known as acute hepatopancreatic disease (AHPND), has had devastating effects on the global shrimp aquaculture industry. The disease spread to Vietnam in 2010, Malaysia in 2011, Thailand in 2012, Mexico in 2013, the Philippines in 2015 and throughout South

America in 2016 (3). The prevalence of EMS is nearly 100% in all pond-reared stocks in south-eastern Asia and Mexico (1,4). Once an EMS causing strain has been introduced to a shrimp pond, there can be 100% mortality within 20-30 days post exposure (5). EMS has resulted in annual losses estimated to be over 1 billion dollars (1).

EMS is caused by strains of *Vibrio parahaemolyticus* that carry a plasmid with the binary toxin PirAB, also known as Pir-likeAB and Pir^{VP} (6). *V. parahaemolyticus* is a Gram-negative, halophilic bacterium that is readily found in marine and estuarine environments (7). The PirAB toxin is homologous to the *Photorhabdus* insect-related (Pir) toxin, produced by *Photorhabdus luminescens* to infect and kill caterpillars (6). PirAB is the product of two genes, *pirA* and *pirB*, which are located on the 69 kbp pVA1 plasmid (6). Further analysis of the PirAB toxin shows structural similarities to another insecticidal toxin produced by *Bacillus thuringiensis* known as Cry toxins (6). Both Cry and PirAB toxins contain domains that are consistent with other toxins capable of causing cell death through pore formation in the cell membrane (6). Characteristics of EMS infection in shrimp include a pale hepatopancreas, the organ that produces digestive enzymes and assists with absorption of digested food, and an empty stomach and midgut caused by damage to the tubule epithelial cells in the hepatopancreas (8). *V. parahaemolyticus* initially colonizes and replicates in the stomach prior to infiltrating the hepatopancreas (5). EMS is lethal in both *Penaeus monodon* and *Litopenaeus vannemei* (Whiteleg), the two most cultivated shrimp species in the aquaculture industry.

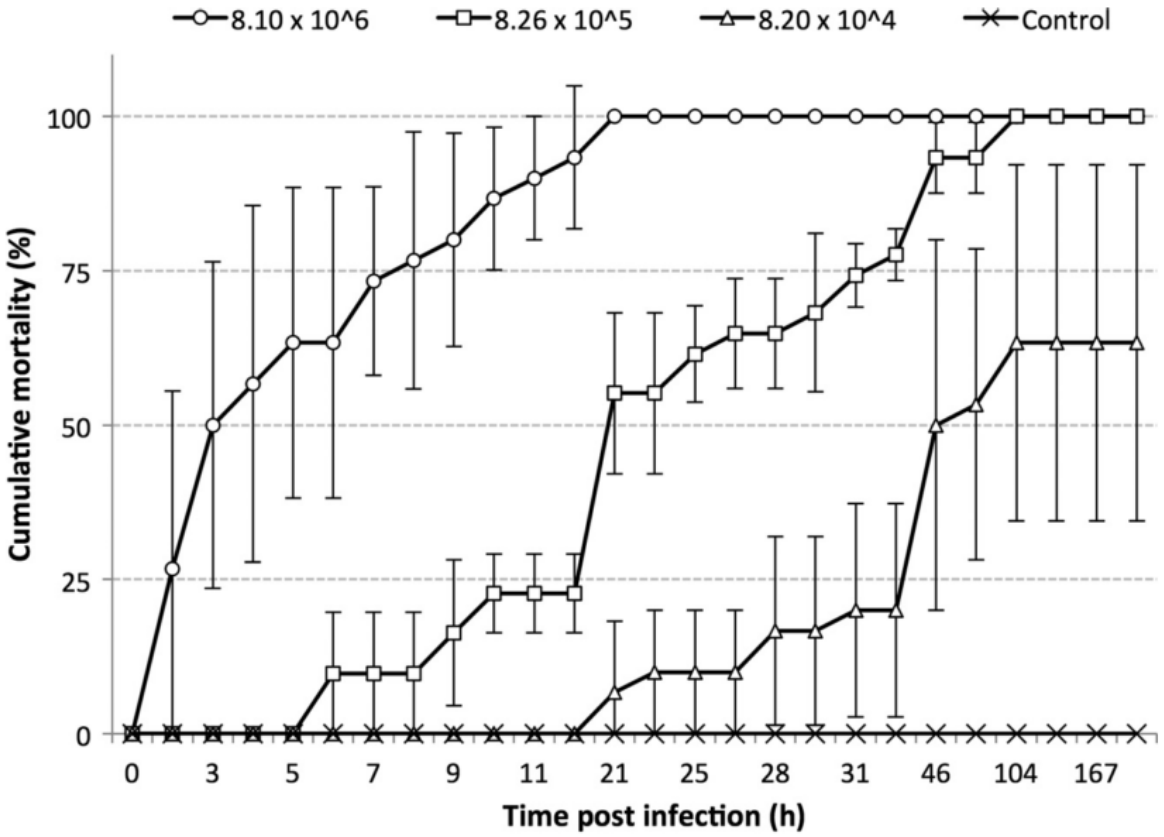
Penaeid shrimp undergo several stages of development including multiple larval stages, the first being the nauplii stage that lasts roughly 36 hours post hatching (9). Protozoea is the second larval stage and lasts 3-5 days followed by mysis, which also

lasts 3-5 days (9). Post-larval shrimp develop into juvenile shrimp over several weeks when the body reaches 1 cm in length (9). Sexual maturity is reached 4-5 months after hatching and the shrimp live approximately 2 years (9). Penaeid juvenile shrimp are highly susceptible to EMS infections (1).

A study conducted by Soto-Rodriguez (1) of the Research Center for Food and Development in Mazatlan, Mexico, showed that low cell densities (10^4 CFU/ml) of EMS causing *V. parahaemolyticus* causes high levels of mortality within days of exposure to the pathogen. Figure 1 shows the percentage of shrimp mortality post-immersion in EMS-causing strain M09-04 at 8.10×10^6, 8.26×10^5, and 8.20×10^4 CFU/ml. In less than 24 hours, 100% of shrimp exposed to the highest concentration of M09-04 had died. In 4 days, over 60% of the shrimp exposed to the lowest concentration of M09-04 had died.

In addition to directly affecting the hepatopancreas, colonization by EMS-causing *V. parahaemolyticus* strains creates a major shift in the microbiota of the shrimp gut (10). *Rhodobacterales* and *Rhizobiales*, members of the Alphaproteobacteria class along with Planctomycetales phylum are the major contributors to the gut microbiome in healthy shrimp. Post-infection, members of the orders Mycoplasmatales and Vibrionales are the dominant gut inhabitants (10). The practice of draining and disinfecting ponds between shrimp stocks may increase the risk of an EMS outbreak by removing beneficial bacteria (11). The use of "microbially mature" water has been shown to increase survival of fish larvae over the use of filter-sterilized water (12). Thus, an approach that involves the manipulation of the microbial community could be one way to combat this disease and, as described below, such an approach is the basis for the present studies.

Figure 1. Mortality of shrimp challenged with *V. parahaemolyticus* strain M09-04 at varying cell densities. Figure taken from Soto-Rodriguez *et al*. (1)



Disease Prevention in Aquaculture

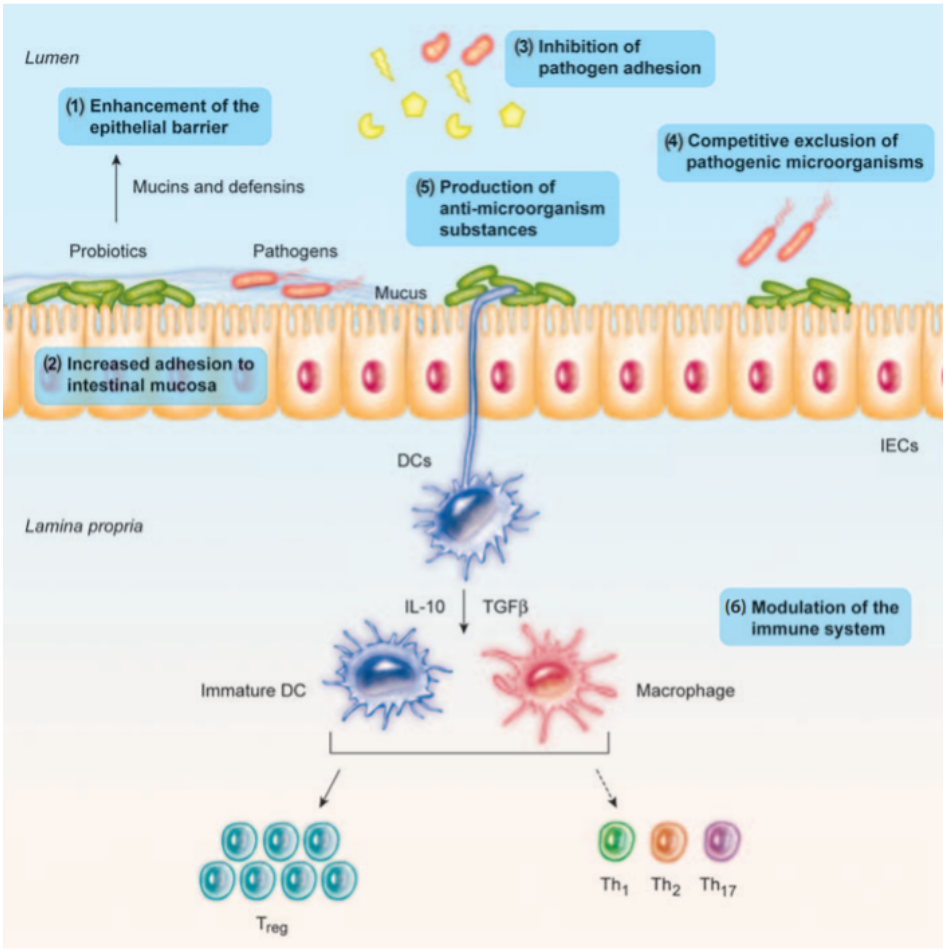
Aquaculture relies heavily on the use of antibiotics to prevent disease. Regulation of the use of antibiotics in aquaculture varies greatly depending on the country (2). Ninety percent of aquaculture occurs in developing countries where regulations and enforcement are lenient or non-existent (2). Overuse of antibiotics leads to the development of antibiotic resistance in bacteria through enrichment of populations containing spontaneously generated mutations or via horizontal gene transfer (HGT). HGT can occur in bacteria through transformation, the up-take of exogenous DNA available in the surrounding environment, or through conjugation between donor and recipient cells. Aquaculture/farm systems have been labeled "hotspots for antimicrobial resistance genes" due to the regular use of antibiotics as prophylactics, resulting in the increase of bacteria resistant to oxytetracycline, quinolones, sulfa/trimethroprim, florfenicol, and amoxicillin (2, 13). *V. parahaemolyticus* EMS strains are universally resistant to ampicillin, streptomycin, sulfamethoxazole, fosfomycin, and bicozamycin (5).

Better prophylactic methods are needed in the aquaculture industry to prevent further development of antibiotic-resistant bacteria and to quell the outbreaks of EMS. These methods might include the introduction of probiotic bacteria to aquaculture species through their feed. Probiotics are defined by the Food and Agriculture Association of the United Nations as live microorganisms, which when administered in adequate amounts confer health benefits to the host (13). The health benefits can come from stimulating the host's immune response, outcompeting pathogenic bacteria for nutrients or adhesion within the host, enhancement of the host's epithelial layer, modification of the microbial

population within the host, aggregation with pathogens, or through production of antimicrobial compounds (Fig. 2) (13). Probiotic activity of several genera of bacteria have been well-studied and documented, including *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Streptococcus*, and *Bacillus* spp. (14).

Much of what is known about the mechanisms of probiotic activity comes from the study of lactic acid bacteria (LABs) (Lactobacillales). LABs can bind to sites within the intestinal tract blocking the ability of pathogens to bind. Probiotic binding stimulates the release of defensins, small peptides produced by the host that have antimicrobial activity through membrane disruption (13). The intestinal epithelium of animals is the first line of defense against disease. Once that layer is damaged, it can lead to inflammation or infection. LABs increase mucin expression to enhance the epithelial barrier to prevent damage (13). Exclusion of pathogen binding is also due to the production of lactic or acetic acid, making the environment less hospitable to other bacteria. Intestinal epithelial cells are the most common host cell to interact with probiotic species. This interaction occurs through pattern recognition receptors including toll-like receptors and extracellular C-type lectin receptors (13). Probiotic bacterial interaction with receptors activates both the adaptive (in vertebrates) and innate immune systems within a host, triggering a complex cascade of immune responses (13). Probiotic bacteria can also reduce intestinal inflammation through downregulation of certain immune responses linked to toll-like receptor expression (13).

Figure 2. Mechanisms of action of probiotics in the animal intestine. Probiotic bacteria (depicted in green) can enhance the host's immune and defense systems through interaction with the pathogen or prevent pathogenic bacteria (depicted in orange) from colonizing. Figure taken from Bermudez-Brito *et al.* (13).



Lastly, many probiotic species, including *Bacillus*, produce antimicrobial substances such as antibiotics, small antimicrobial peptides called bacteriocins, and secondary metabolites such as lipopeptides and polyketides (15).

Bacillus species as Probiotics

Members of the genus *Bacillus* are Gram-positive, rod shaped, spore-forming, facultative anaerobic bacteria whose probiotic activity has been studied (15). While considered soil inhabitants, *Bacillus* species have been isolated from diverse environments, including fresh and saltwater, air, and the gastrointestinal tract of animals (15). Some *Bacillus subtilis* species have been shown to have probiotic activity (15). *Bacillus* species have higher acid tolerance compared to LABs and their ability to sporulate leads to survival during high heat processing and cold temperature storage (15). The food industry employs amylase, glucoamylase, cellulase, proteinase, and pectinase produced by *Bacillus* species (15). Several *Bacillus* species are used for the synthesis of vitamin and carotenoid supplements as well as gut probiotics (15). The under-utilization of *Bacillus* species in probiotic development is due in part to the pathogenic members of the *Bacillus* genera, including *B. anthracis*, which causes anthrax and *B. cereus* which can cause food-born illnesses (15).

All *B. subtilis* species are phenotypically similar and share 3299 open reading frames, however subspecies differ by the presence of multiple protein-coding gene sequences (16). *B. subtilis* is divided into three subspecies—*B. subtilis* subspecies *subtilis*, subspecies *spizizenii*, and subspecies *inaquosorum* (16). *Bacillus* subspecies produce different secondary metabolites including bacteriocins, antibiotics, lipopeptides,

and polyketides. Bacteriocins are narrow-spectrum antimicrobial peptides synthesized ribosomally (17). They are divided into three classes: Class I lantibiotics, which includes subtilin, Class II small linear peptides, which includes coagulin, and Class III large, heat-labile bacteriocins such as megacin (17). *Bacillus* species can also produce broad-spectrum antibiotics such as surfactin and bacilysin, which are synthesized non-ribosomally (15).

Lipopeptides and Polyketides from Bacillus

Under batch culture conditions, bacterial growth involves a lag phase, where adjustments are made to environmental changes, exponential phase, which results in a population doubling that occurs at a constant rate, and stationary phase, which is attained when nutrients or growth factors are limiting or exhausted. During the end of exponential and beginning of stationary phase bacteria will adjust their physiology and molecular components to deal with poor environmental conditions. For members of the genus *Bacillus*, this adjustment includes an increase in biofilm formation, motility, competence (transformation ability), hydrolytic enzyme excretion (lipases, amylases and proteases) and, the production of secondary metabolites such as lipopeptides (LPs) and polyketides (PKs). Nearly 5% of the *Bacillus subtilis* genome is involved in the production of secondary metabolites (18). LPs consist of a lipid attached to a small peptide between 6 and 13 amino acids in length and synthesized by non-ribosomal peptide synthetases (NRPS) (19). NRPSs are comprised of four main catalytic domains--adenylation, thiolation, condensation and theioesterase domain. Accessory domains include epimerization and methyl transferase (19). *Bacillus* LPs are linear or cyclic in

structure and are divided into four main families based on structure—surfactins, fengycins/plipastatins, iturins, and kurkstakins (Fig. 3) (19,20).

Surfactin, the namesake of the surfactin family, was one of the first lipopeptides isolated from *B. subtilis* (20). It is an excellent biosurfactant that led to its name. Surfactin is necessary for biofilm formation and swarming motility (21), it has antimicrobial activity through the disruption of cell membranes, acts as fibrin clot inhibitor, and possesses antitumor activity (20). There are 20 different members of the surfactin family that share the same structural traits—a heptapeptide with a chiral sequence of leucine and aspartate (LLDLLDL) interlinked with a β-hydroxy fatty acid (20).

The iturin family are similar in structure to surfactins, consisting of a heptapeptide with a chiral sequence LDDLLDL interlinked with a β-linked amino acid fatty acid 14 to 17 carbons in length (20). Mycosubtilin, an antifungal lipopeptide made by *B. subtilis*, was isolated from the Ituri region of the Congo in 1950, inspiring the name iturin (20). Bacillomycin is another representative of the iturin family and possesses antifungal properties. Synthesis of iturins involves a polyketide synthase (PKS)/NRPS hybrid complex (20).

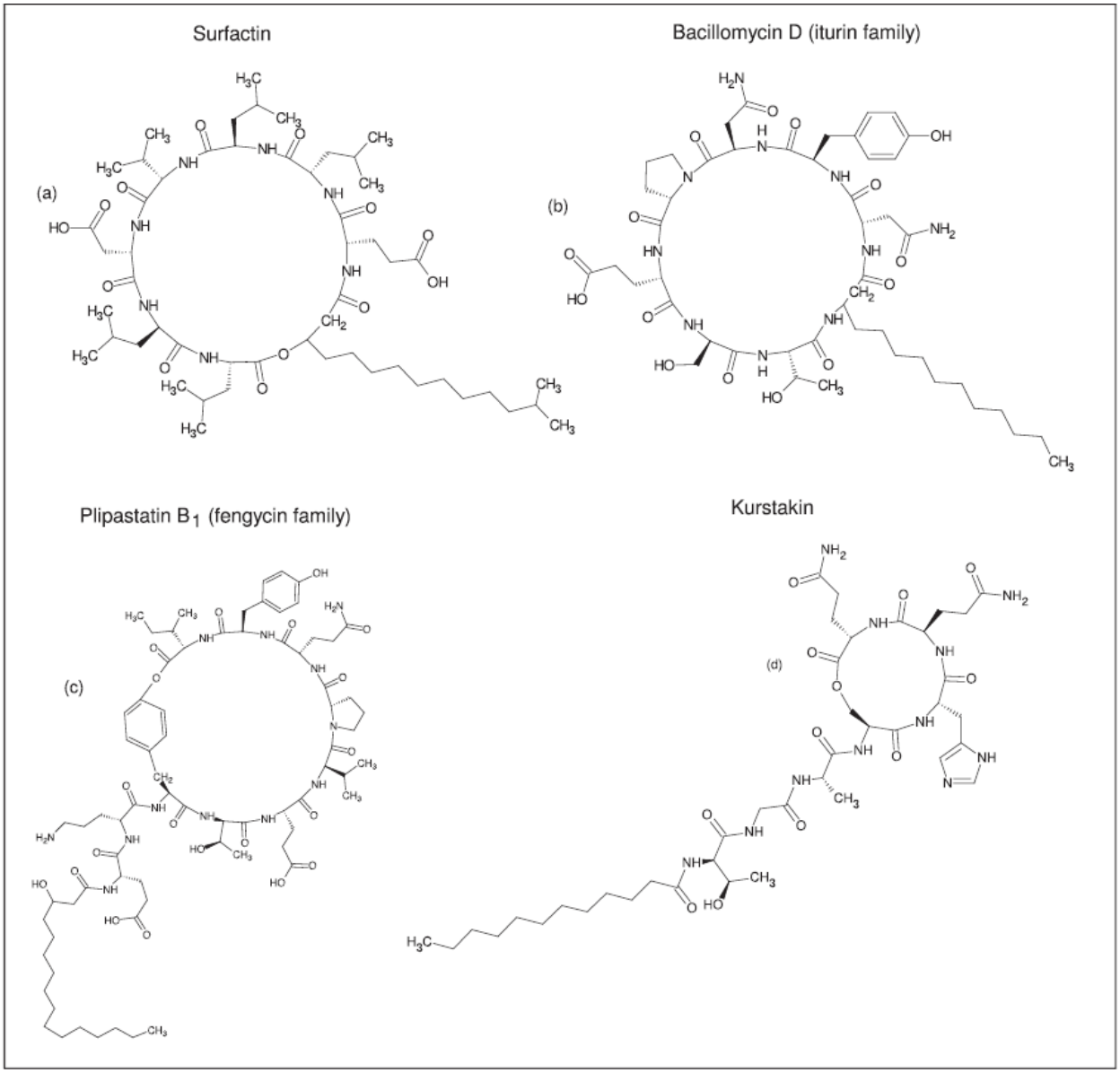
Fengycins and plipastatins, described as lipodecapeptides, differ from each other only by the substitution of 2 amino acids (20). Fengycin A and B are produced by *B. subtilis* and have antifungal activity while plipastatin is produced by *B. cereus* acts as a phopholipase A2 inhibitor (20).

In 2000, the Kurstakin family was added a the fourth family of lipopeptides. Characterized by a heptapeptide with a lactone ring linking the carboxy terminal amino

acidy and the hydoxyl group on the side chain of the serine residue, these compound are produced by *B. thuringiensis* subspecies *kurstaki* (20). Kurstakin coats the surface of *B. thuringiensis* spores and has antifungal activity (20).

PKs are synthesized by PK synthases (PKS) (19). Both NRPSs and PKSs are modular in design, having multiple domains that can be assembled into various combinations, resulting in the production of different compounds (19). PKS machinery has three core domains—the acyl transferase, the acyl carrier protein, and the ketosynthase. PKs are synthesized through repetitive decarboxylation and condensation cycles on the PKS enzyme (19). The acyl transferase activates and transfers malonyl coenzyme A to the acyl carrier protein. The ketosynthase catalyzes the decarboxylation and condensation reactions between the two malonates that are linked together by the acyl carrier proteins (19). PKS has accessory domains that include ketoreductases, dehydratases, enoyl reductases, and methyl transferases (19). The domains are interchangeable pieces that leads to the diversity in the PKs produced. PKs are divided into three main classes: type I, type II, and type III based on the order of the catalytic domains (19). Representative type I PKS are shown in Figure 4 (19). Some polyketide synthases work in conjunction with NRPS to form hybrid PKS-NRPS enzyme complexes, as in the production of the iturin family of lipopeptides (19, 20).

Figure 3. Reprsentative *Bacillus* LPs from the surfactin, iturin, fengycin, and kurkstakin families. Figure taken from Aleti *et al.* (19).



Bacillaene, difficidin, and macrolactin are three well studied type I polyketides from Bacillus species (Fig. 5). Bacillaene is produced from the *bae* gene cluster and inhibits protein biosynthesis in prokaryotic organisms. Difficin is encoded by the *dif* gene cluster and has activity against a broad spectrum of bacteria through the inhibition of protein synthesis (19). Macrolactins are 24-membered lactone rings with three dienes in the carbon skeleton. These, too, show antibacterial activity and have been effective against the Herpes simplex virus, HIV, and melanoma cancer cells (19).

B. subtilis subspecies *inaquosorum strain T1*

T1 is a *Bacillus* species that was provided by Epicore Bionetworks Incorporated, a multifaceted biotechnology company that develops specialized feed for aquaculture. Through genome sequencing, T1 was determined to be *B. subtilis* subspecies *inaquosorum* with a genome size of approximately 4.2 mbp (Schreier, unpublished). It has been previously shown that T1 produces an activity that inhibits growth of D4, an EMS-causing strain of *V. parahaemolyticus*, which is detected by an overlay assay, shown in Figure 6. A zone of clearance around a T1 colony is due to the absence of growth of D4, which is provided in a semi-solid agar overlay.

Figure 4. Type 1 PK synthases (PKS) are shown below. The domains are acyl carrier protein (ACP), peptidyl carrier protein (PCP), adenylation (A), ketosynthase (KS), dehydratase (DH), methyl transferase (MT), ketoreductase (KR), and thioesterase (TE). Gene names are listed above each model; (a) difficidin, (b) macrolactin, and (c) bacillaene. Modules and recruited enzymes are indicated below and gene names are indicated above each illustration. Figure taken from Aleti *et al.* (19)

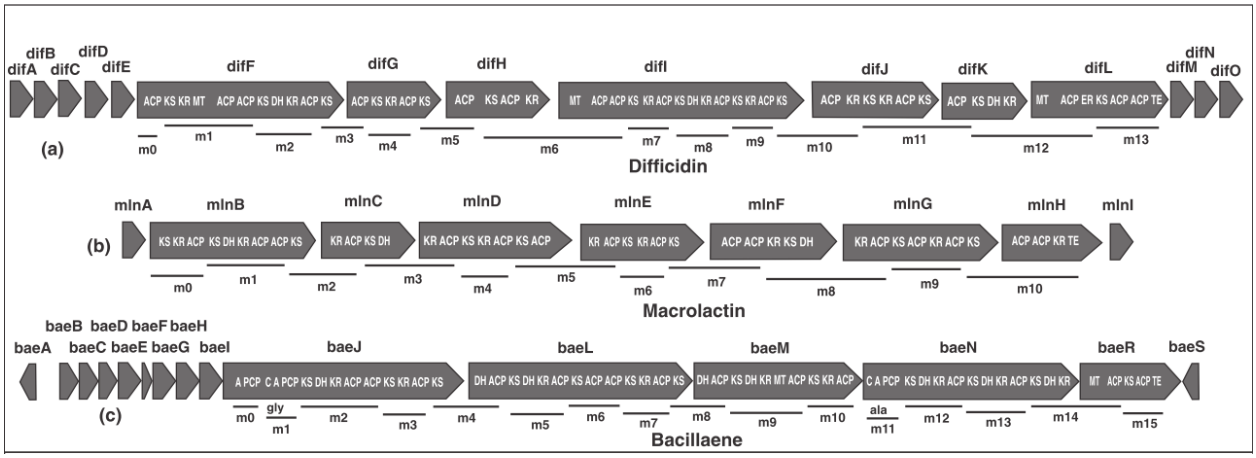
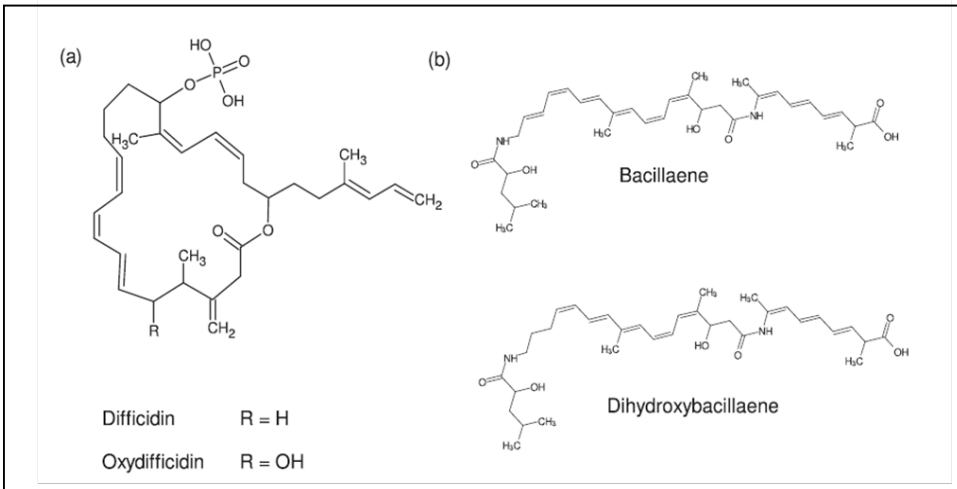
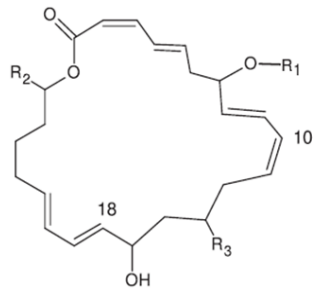


Figure 5. Representative *Bacillus* Type 1 PKs. Difficidin (a), bacillaene and dihydorxybacillaene (b), macrolactins (c). Figure taken from Aleti *et al.* (19)



(c)



Macrolactin A	$R_1 = H, R_2 = CH_3, R_3 = OH$
7- Malonylmacrolactin A	$R_1 = CO\text{-}CH_2\text{-}COOH, R_2 = CH_3, R_3 = OH$
7- Succinylmacrolactin A	$R_1 = CO\text{-}CH_2\text{-}CH_2\text{-}COOH, R_2 = CH_3, R_3 = OH$
Macrolactin B	$R_1 = \beta\text{-D-glucosyl}, R_2 = CH_3, R_3 = OH$
Macrolactin C	$R_1 = H, R_2 = CH_3, R_3 = O\text{-}\beta\text{-D-glucosyl}$
Macrolactin D	$R_1 = 6`\text{Succinyl-}\beta\text{-D-glucosyl}, R_2 = CH_3, R_3 = OH$
Macrolactin O	$R_1 = \beta\text{-D-glucosyl}, R_2 = CH_3, R_3 = O$
Macrolactin P	$R_1 = \beta\text{-D-glucosyl}, R_2 = CH_2CH_3, R_3 = OH$
Macrolactin Q	18 Z Macrolactin B
Macrolactin R	10 E Macrolactin B

Based on this activity, T1 could be considered a candidate for probiotic studies. The T1 genome sequence has identified an approximately 30 kb DNA regions that contains the coding capacity for PKs and LPs (Schreier, unpublished), which may be responsible for the observed antagonistic activity against EMS-causing *V. parahaemolyticus* strains. This activity appears to be specific for T1 since it is not observed in *B. subtilis* strain SMY, which is a *subtilis* subspecies and does not contain the lipopeptide/polyketide gene cluster.

In a dosing experiment, T1 was added to shrimp feed in a mixture with two other *Bacillus* species at concentrations of 0.1%, 0.2% and 0.4% total volume of feed. (The other *Bacillus* species had no activity against D4 per the overlay assay.) Juvenile shrimp (PL20) were provided with the treated feed for two weeks prior to exposure to *V. parahaemolyticus* strain D4. The shrimp were then challenged by immersion in water containing D4 at a concentration of 10^6 CFU/ml for 30 min, then transferred to 40 L aquaria, resulting in a final concentration of 10^4 CFU/ml. Figure 7 shows the results of the challenge experiment. Sixteen hours after exposure to D4, 18% of the shrimp that received the control feed survived. Shrimp that received the treated feed had higher survival rates in a dose-dependent manner, with the highest concentration of bacteria (0.4%) yielding the greatest survival at 16 hours (64%). Shrimp that were given 0.4% treated feed and not exposed to D4 had the same survival as the control group that were given untreated feed and were not exposed to D4, indicating that T1 had no negative effect on the shrimp.

Figure 6. Overlay assays showing the antagonistic activity of T1 and *B. subtilis* strain SMY against EMS-causing *V. parahaemolyticus* strains D4 (A), A3 (B), and non-EMS-causing *V. parahaemolyticus* strain (C). The zone of clearance around a colony indicates the absence of growth of the *Vibrio* spp. Details are described in Materials and Methods.

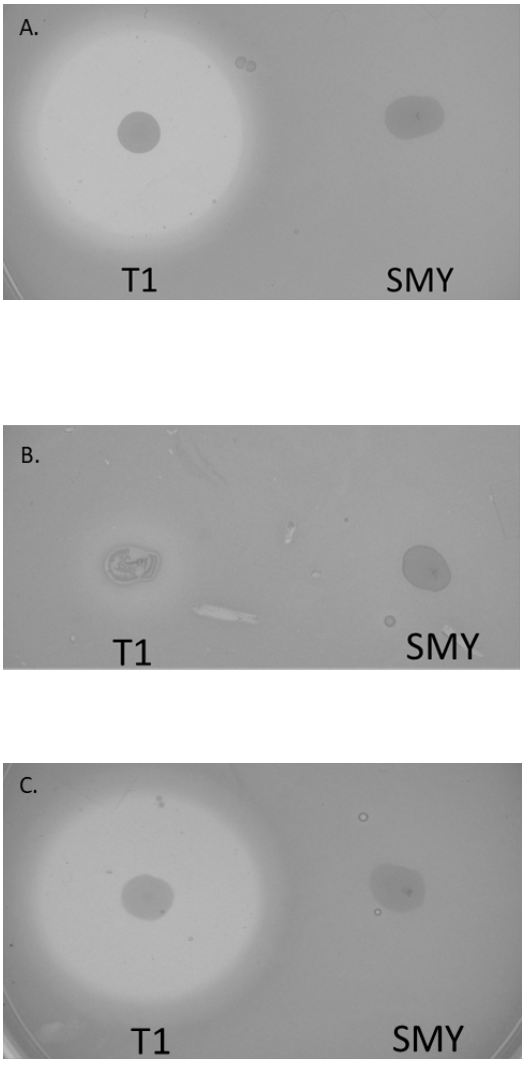
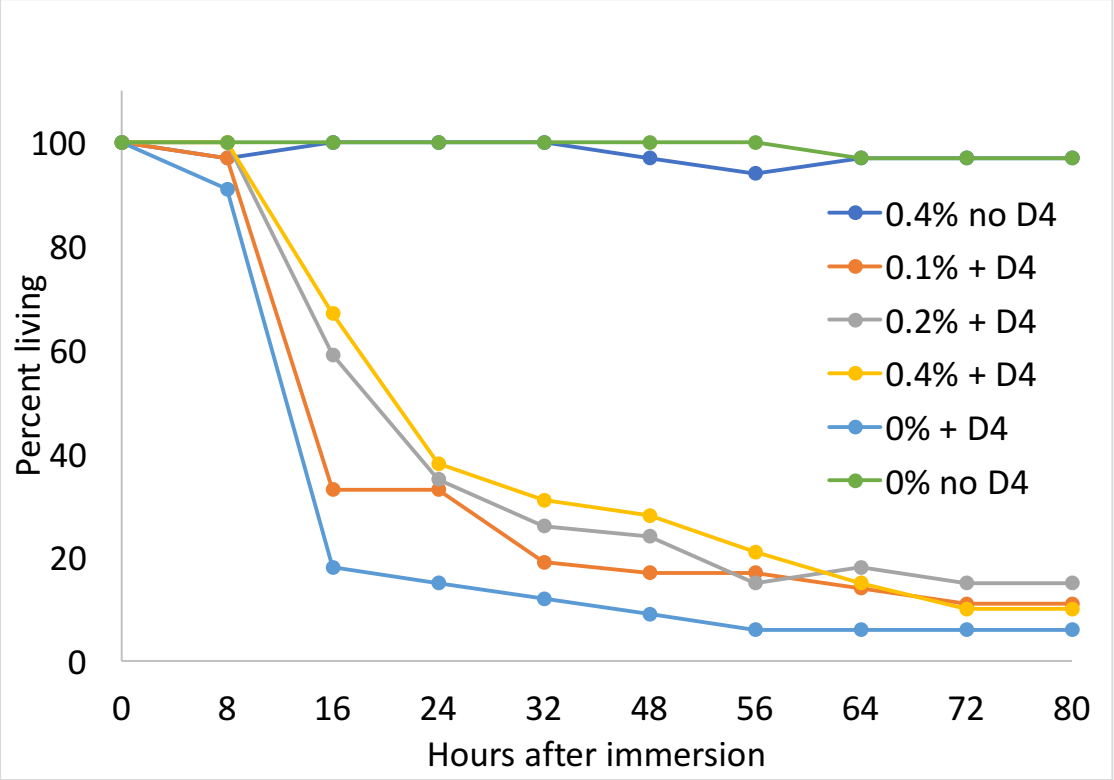


Figure 7. T1 dose-dependent challenge experiment. Shrimp were given feed treated with varying concentrations of *B. subtilis* strains including strain T1 at 0.1%, 0.2%, and 0.4% for two weeks prior to exposure to D4 (10^4 CFU/ml final concentration). A control group was given untreated feed. Shrimp receiving the 0.4% treated feed had the highest survival rate through 56 hours post-D4 exposure (Schreier and Schott, unpublished).



Purpose of This Study

In vitro assays have shown that T1 produces an extracellular antagonistic activity against *V. parahaemolyticus* strain D4. The focus of this study combines molecular genetic and physiological studies to examine how T1 inhibits D4, how growth conditions affect inhibition, and if D4 inhibition is due to the production of a secreted secondary metabolite. The objectives were as follows: 1) use a *mariner*-based transposition system to identify T1 genes involved in the antagonistic activity; 2) use competitive growth experiments to quantify effects on growth of D4 when co-cultured with T1; and 3) determine whether the antagonistic activity of T1 is due to the release of a secreted product. Results indicate that T1 produces a secreted product from the LP/PK cluster that can negatively impact the growth of D4 in a concentration-dependent manner.

MATERIALS and METHODS

Bacterial Strains and Culture Media. Bacterial strains used in this study and their source are listed in Table 1. *B. subtilis* strain T1g was constructed by transforming strain T1 by electroporation (22) with DNA from *B. subtilis* strain AR13 (*amyE*::*gfp*), which was obtained from the *Bacillus* Genetic Stock Center, Ohio State University, and selecting for spectinoycin-resistance (Sp^R). Insertion of *gfp* into the *amyE* locus was confirmed by the loss of amylase activity when growing on starch agar and by an increase in the size of the *amyE* PCR product. Plate overlay assays showed that Inhibition of D4 growth by strain T1g was not affected by disruption of *amyE* (not shown). Frozen stocks were made by adding glycerol to overnight cultures to a final concentration of 20% and stored at -80°C. Strains were grown in Zobell 2216 marine broth (HiMedia Laboratories), tryptic soy broth (Sigma-Aldrich) or agar supplemented with 2% NaCl (TSB2 or TSA2, respectively) and Luria-Bertani (LB) agar (23) augmented with antibiotic, when appropriate. SOC (24) and 2xYT (25) have been described previously. Antibiotics were added to media at concentrations of 100 μg spectinomycin (Sp) /ml and 1 μg erythromycin (Em)/ml (26).

Plasmids. Plasmids pDB384 and pEP4 were provided by Dr. Daniel Kearns, Indiana University, and are delivery vectors containing the *mariner*-derived *himar1* transposase that will randomly insert the transposon containing a Sp^R cassette into the T1 chromosome (26). These vectors harbor a temperature-sensitive *B. subtilis* origin of replication and Em-resistance (Em^R) gene located outside of transposon sequences. Plasmid pDB384 differs from pEP4 by the presence of a promoter-less *lacZ* gene within transposon sequences, which is expressed when inserted downstream of an active

transcription start-site (26). Plasmids were purified using a Wizard DNA Clean-Up Kit (Promega) according to manufacturer's standards.

Identification of T1 Genes Involved in Inhibition of D4 via Mutagenesis Study

Electroporation of T1 with Delivery Vectors and Mutagenesis. T1 was prepared for electroporation by growing in 250 ml in 2xYT to OD_{600}=0.8 at 37°C, 240 RPM, washing cells three times in ice cold 10% glycerol and suspending in 1 ml ice cold 10% glycerol, storing at -80°C in 200 µl aliquots. For electroporation, one aliquot was mixed with purified plasmid (at a concentration of 7 µg/ml) then incubated on ice for 5 min. The mixture was then transferred to a 2 mm electroporation cuvette and electroporation was done using the "Sta" program of a Bio-Rad MicroPulser electroporator (approx. 1.8 kV for 2.5 msec). After electroporation, 0.5 ml of SOC was added and cells were incubated at 28°C with aeration for 2 hr, followed by plating onto L+Sp+Em plates. The presence of the transposase gene in several Sp^R Em^R transformants was confirmed by PCR and one transformant was selected to carry out the mutagenesis. After growth in LB+Sp for 18 hr at 37°C, the cells were plated onto LB+Sp plates at a dilution of 10^{-6}. Because both delivery plasmids harbor a temperature-sensitive *B. subtilis* origin of replication, the elevated temperature ensured that Sp^R was the result of a transposition into the chromosome and not the consequence of a self-replicating delivery vector (26). Approximately 3000 colonies were screened for loss of D4 inhibitory activity using the plate overlay assay with TSB2 plates as described below.

Table 1. Bacterial strains used in this study.

Strain	Description	Source or Reference
T1	*Bacillus subtilis* subsp. *inaquosorum*	Epicore BioNetworks, Inc.
T1g	*amyE::gfp* (T1 x AR13 DNA), Sp^R	This study
D4	*Vibrio parahaemolyticus* isolate 13-306, PirAB	(4)
A3	*Vibrio parahaemolyticus* isolate 13-028, PirAB	(4)
LVS3	*Aeromonas hydrophila*	(27)
SMY	*Bacillus subtilis* subsp. *subtilis*	Laboratory strain
A1-20	T1 x pEP4, Lac^+, Sp^R	This study
A2-18	T1 x pEP4, Sp^R	
A3-41	T1 x pEP4, Lac^+, Sp^R	
A2-30	T1 x pEP4, Sp^R	
A11-79	T1 x pEP4, Lac^+, Sp^R	
A10-67	T1 x pEP4, Sp^R	
A20-86	T1 x pEP4, Lac^+, Sp^R	
A23-33	T1 x pEP4, Sp^R	
A8-11	T1 x pEP4, Sp^R	
S11-14	T1 x pDB384, Sp^R	
S16-13	T1 x pDB384, Sp^R	
S12-29	T1 x pDB384, Sp^R	
S21-46	T1 x pDB384, Sp^R	
S21-28	T1 x pDB384, Sp^R	
S31-22	T1 x pDB384, Sp^R	
S35-30	T1 x pDB384, Sp^R	
S2-30	T1 x pDB384, Sp^R	

Table 2. Primers used in this study.

Target	Forward Primer (5´-3´)	Reverse Primer (5´-3´)	Product Size (bp)
amyE	AGGCTGGGCAGTGATTGCTT	ACTTCCGCGGTCGCCTATTT	110
Sp^R	2569R: TCTGATTACCAATTAGAATGAAT	2570R: GAATATACGGAAATTATGACTTA	722
gfp	CGACATTGTGTGGACAGGTAA	CCCGAAGGTTATGTACAGGAAAG	353
toxR	AATCCATGGATTCCACGCGTTAT	CACCAATCTGACGGAACTGAGATTC	103
Transposon insertion	2569: ATATTCATTCTAATTGGTAATCAGA	2570: CTAAGTCATAATTTCCGTATATTC	Varies

Mutant Characterization and Identification of Transposon Insertion Site.

Confirmation that Sp^R was due to the presence of the transposon in the chromosome of T1 mutants was done by PCR using primer set 2569R/2570R followed by visualization via agarose (1%) gel-electrophoresis. PCR was carried out in 25 µl reactions with Qiagen *Taq* polymerase using the Bio-Rad S1000 Thermal Cycle with the following parameters: 3 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and a final step at 72°C for 10 min. T1 mutants having multiple transposon insertion sites were re-transformed into wildtype T1 using the electroporation protocol described above with T1 mutant chromosomal DNA. Insertion site location was determined by amplification of the transposon and adjacent DNA using a inverse PCR strategy as follows. Chromosomal DNA isolated from T1 mutants using the Wizard Genomic DNA Purification Kit (Promega) was done according to manufacturer's specifications. One µg of chromosomal DNA was digested with *Taq*I for 2 hr at 37°C in 20 µl. One µl of digested DNA was ligated using T4 DNA Rapid Ligation Kit (Thermo Scientific) for 5 min at room temperature according to manufacturer's specifications. The ligation mixture was then used in an inverse PCR reaction with primers 2569/2570 and Phusion DNA polymerase (New England BioLabs). PCR was carried out in 50 µl reactions subjected to the following cycling conditions: 3 min at 98°C followed by 30 cycles of 1 min at 98°C, 1 min at 55°C and 1 min at 72°C, and a final step at 72°C for 10 min. PCR products were purified using the Wizard PCR Preps DNA Purification System (Promega) and DNA sequencing was performed by the Institute of Marine and Environmental Technology BASLab using primer 2569 (Table 2), which anneals to transposon

sequences adjacent to the insertion site. Comparison of DNA sequences interrupted by the transposon to database sequences was done by BLAST (28).

Plate Overlay Assay

T1 mutants were spotted onto TSA2 plates using sterile toothpicks (45 to 50 per plate) and incubated at 37°C overnight. In a chemical hood, plates were then placed uncovered in a rectangular pyrex baking dish containing a reservoir of chloroform; the baking dish was then sealed with plastic wrap to enable cell inactivation by chloroform vapors. After 30 min, the plates were removed and allowed to equilibrate under air for 30 min to allow the chloroform to dissipate. For each plate, 3 ml of semi-solid TSA2 agar (TSA2 medium with 0.75% Bacto agar) was heated until liquified, cooled to 42°C, inoculated with 10 µl of overnight D4 culture, and immediately poured over the TSA2 surface. Plates were incubated at 28°C overnight and examined for zones of clearance. Colonies exhibiting partial or no D4 inhibition were identified by reduced (relative to a wild-type control) or absent clearance zones, respectively.

Competitive Growth Experiments

Competitive growth experiments that examined the effect of T1 or T1 mutant on the growth of D4 under different cultural conditions were done by combining overnight cultures of T1 with D4 at various cell densities in fresh medium—either TSB2 or 2216—monitoring growth by quantitative PCR (qPCR) (see below). The qPCR target to assess densities of T1g, which was used in all competitive growth experiments, T1 mutant A3-41, and strain D4 were *gfp*, *amyE* and *toxR*, respectively, and primers are listed in Table

2. Inocula for co-cultures were based on colony forming units/ml (CFU/ml) for each strain in both TSB2 and Zobell 2216, which ranged from 2.0 x 10^{10} to 3.0 x 10^{10} CFU/ml for D4 and 2.0 x 10^{9} to 3.0 x 10^{9} CFU/ml for both strains T1g and A3-41. All samples were prepared for qPCR using the Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's specifications.

In general, overnight cultures of T1g and D4 were prepared either in TSB2 or Zobell 2216 and were used to inoculate 50 ml of either TSB2 or Zobell 2216, respectively. The initial cell density for T1g was 3.0 x 10^{6} CFU/ml and initial cell densities for D4 ranged from 3.0 x 10^{4} to 10^{6} CFU/ml to create D4 to T1g ratios of 1:100, 1:10, and 1:1 respectively. D4 was also grown without T1g at 3.0 x 10^{4}, 10^{5}, and 10^{6} CFU/ml as controls. Cultures were grown at 28°C and 240 RPM for 24 hr. At 3 hr after inoculation, 10 ml of each culture was centrifuged at 4°C, 4,000 x g for 10 min and after 24 hr, 0.5 ml was taken from each culture and centrifuged at 4°C, 10,000 x g for 5 min. DNA was extracted from all samples as described above.

Competitive growth experiments that compared wild-type and mutant T1 strains were carried out in 20 ml Zobell 2216 with initial cell densities for T1g and A3-41 ranging from 2.0 x 10^{6} to 2.0 x 10^{8} CFU/ml. Initial cell density of D4 was 2.0 x 10^{4} CFU/ml for all co-cultures. For some experiments T1g and A3-41 cells were either washed in fresh Zobell 2216 to remove spent media or resuspended in their respective filtered supernatant fractions resulting in a final supernatant concentration of 10% for each co-culture. All cultures were grown at 28°C and 240 RPM for 24 hours. Two one-ml samples were taken from each culture at 0, 3, and 24 hr timepoints. Autoclaved *Aeromonas hydrophila* (10^{5} CFU/ml final concentration) was added to each sample prior

to centrifugation to assist in D4 recovery. Samples were centrifuged at 4°C, 10,000 x g for 10 min followed by DNA extraction as described above.

Supernatant Fraction Concentration-Dependence

Overnight cultures of D4, T1g and A3-41 grown in Zobell 2216 were centrifuged at 0°C, 5,000 x g for 10 min and the supernatant fractions from each culture were removed and sterilized by passing through a 0.2 μm filter. The filtered supernatant fractions were then added to fresh Zobell 2216 medium in 50 ml sidearm flasks to a final concentration of 10%, 25% and 50% in a total volume of 12 ml. The control flask received 12 ml of fresh Zobell 2216 media. Each culture was then inoculated with D4 at initial concentration of 2.0×10^4 CFU/ml. Cultures were grown at 28°C and 240 RPM for 24 hr. Two 1 ml samples were taken from each flask at 0, 3, and 24 hr time points followed by DNA extraction as described above in preparation for qPCR. Growth was also measured using a Klett-Summerson Colorimeter with a Wratten 54 filter.

Quantitative PCR

qPCR analysis was performed in 10 μl reactions containing 5 μl of PefeCTa SYBR Green Fastmix (Quanta), 3.5 μl PCR-certified water, 0.25 μl of 1/10 diluted forward and reverse primers (0.5 μM) each, and 1 μl of the sample to be quantified. All qPCR reactions were performed using an Applied Biosystems 7500 Fast Real-Time PCR machine. The run method for each reaction was as follows: hot start 1 min at 95°C, denaturation for 1 min at 95°C, annealing for 30 sec at 60°C, repeated for 40 cycles,

followed by a melt-curve. PCR-certified water was used as the no template DNA control to ensure the reaction mixture was not contaminated.

DNA template for standard curves was prepared by PCR performed in 50 µl reactions containing 25 µl Taq PCR Mastermix (Qiagen), 19 µl PCR-certified water, 2 µl of 1/10 diluted forward and reverse primer (0.5 µM) for *toxR*, *gfp* and *amyE* (Table 2), and 2 µl of DNA template. Chromosomal DNA from D4, T1g, and T1 were used for the DNA templates for *toxR*, *gfp*, and *amyE* respectively. The reactions were subjected to the following cycling: 3 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C and a final step at 72°C for 10 min. Confirmation of PCR product was done by agarose gel-electrophoresis. PCR products were then purified using the MinElute PCR Purification Kit (Qiagen) according to the manufacturer's specifications. DNA concentration (ng/ml) was determined by the Qubit Fluorometer (ThermoFischer).

Preparation of standard curves to determine copy number was based on the following calculation:

$$\text{copy number} = ([\text{DNA (ng/ml)}] \times 6.02 \times 10^{23} \text{ molecules/mole}) / (\text{bp length} \times 10^{9} \text{ ng/g} \times 650 \text{ g})$$

Serial dilutions of *toxR*, *gfp,* and *amyE* templates between 0.4×10^{-2} and 0.4×10^{-9} copy/µl were used to generate standard curves for each primer set. Standard curves were performed in duplicate with each microtiter plate well receiving 2.5 µl of each dilution, followed by 7.5 µl of qPCR reaction mixture.

Statistical Analysis

Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) with the significance level of 0.01 ($p < .01$).

RESULTS

In vitro studies have shown that *B. subtilis* subspecies *inaquosorum* strain T1 possesses antagonistic activity against EMS-causing *V. parahaemolyticus* strain D4 as well as other pathogenic *Vibrio* species. To exploit the effect of T1 against EMS-causing bacteria, an understanding of the interaction between T1 and D4 is needed. The objectives of this study were to evaluate the effect T1 has on growth of D4 when co-cultured under varying conditions, to identify the T1 genes involved in the antagonistic activity against D4 and determine whether the antagonistic activity of T1 is due to the production of a secreted product.

Competitive Growth Experiments

To evaluate the effect of T1 on the growth of D4, co-culture experiments were done followed by qPCR. For these experiments, the *gfp*-tagged version of T1 was used (see Methods). It is important to note that copy number does not directly translate to CFU/ml. Copy number is affected by the efficiency of DNA extractions from co-culture samples and the concentration of the extracted DNA. Copy number can be inflated by the presence of multiple copies of the chromosome within a cell at any given time.

When examined by the overlay assay, T1 was found to yield a zone of clearance 5 to 10-fold larger against D4 when grown on Zobell 2216 marine medium compared to TSA2, a richer medium (not shown). To establish whether T1's ability to negatively affect growth of D4 was impacted by media conditions, co-culture experiments were conducted examining growth in Zobell 2216 and TSB2 media. In these experiments, the initial cell density for T1g was 3.0×10^6 CFU/ml for each co-culture. The initial cell

densities for D4 were 3.0 x 10^4, 3.0 x 10^5, and 3.0 x 10^6 CFU/ml to establish starting cell density ratios of T1g to D4 at 100:1, 10:1, and 1:1 respectively. Samples from each co-culture were taken at 3 hrs and 24 hrs after inoculation and prepared for qPCR analysis, targeting *gfp* and *toxR* for T1 and D4, respectively (see Methods). Figure 8 shows the growth of D4 reported as average *toxR* copy number. D4 was also grown alone at the same initial cell densities as controls. Tables 3 and 4 show the average *toxR* copy numbers for cultures grown in TSB2 and Zobell 2216, respectively.

In TSB2 media, T1g did not significantly inhibit the growth of D4 at any ratio or time point (Fig. 8A). However, significant D4 growth inhibition was seen after 24 hrs when T1g and D4 were co-cultured in Zobell 2216 at a 100:1 ratio (Fig. 8B). The *toxR* copy number for D4 grown alone at 10^4 CFU/ml was 2.9 x 10^9 ± 0.4 x 10^9. This was almost 3-fold higher than when D4 was co-cultured with T1g at 10^6 CFU/ml (1.1x 10^9 ± 0.4 x 10^9 *toxR* copies/ml) as shown in Table 3. There was no significant effect on growth of D4 at 3 hrs. These results indicate that the ability of T1g to inhibit growth of D4 is dependent upon culture conditions, with greater inhibition observed in the simpler, less complex 2216 medium than the nutrient richer TSB2 media after 24 hrs of growth. Furthermore, significant inhibition was only detected when the starting inoculum of T1g was 100-fold greater than D4.

When examining T1g cell densities grown in Zobell 2216 media in the presence of D4, there was a significant difference at 24 hrs when T1g and D4 were present in equal densities (6.0 x 10^7 ±. 1.1 x 10^7 *gfp* copies/ml) compared to when T1g was grown alone (1.1 x 10^8 ± 0.2 x 10^8 *gfp* copies/ml) as shown in Figure 9. Table 4 shows the average *gfp* copy number for cultures grown in Zobell 2216.

Figure 8. Average *toxR* copy number for D4 cultures grown in the absence and presence of T1 in different media. D4 had initial cell densities of 3.0 x 10^4, 3.0 x 10^5, and 3.0 x 10^6 CFU/ml. T1g had an initial cell density of 3.0 x 10^6 CFU/ml in each co-culture, creating ratios of T1g to D4 of 100:1, 10:1 and 1:1. Samples were taken at 3 and 24 hrs as described in Materials and Methods. (A) Co-cultures grown in TSB2. (B) Co-cultures grown in Zobell 2216 marine media. The experiment was repeated 3 times with 4 technical replicates for each sample. Error bars represent the standard deviation within the technical replicates of the representative data.

** Indicates a significant difference (p value < .01) from the corresponding control.

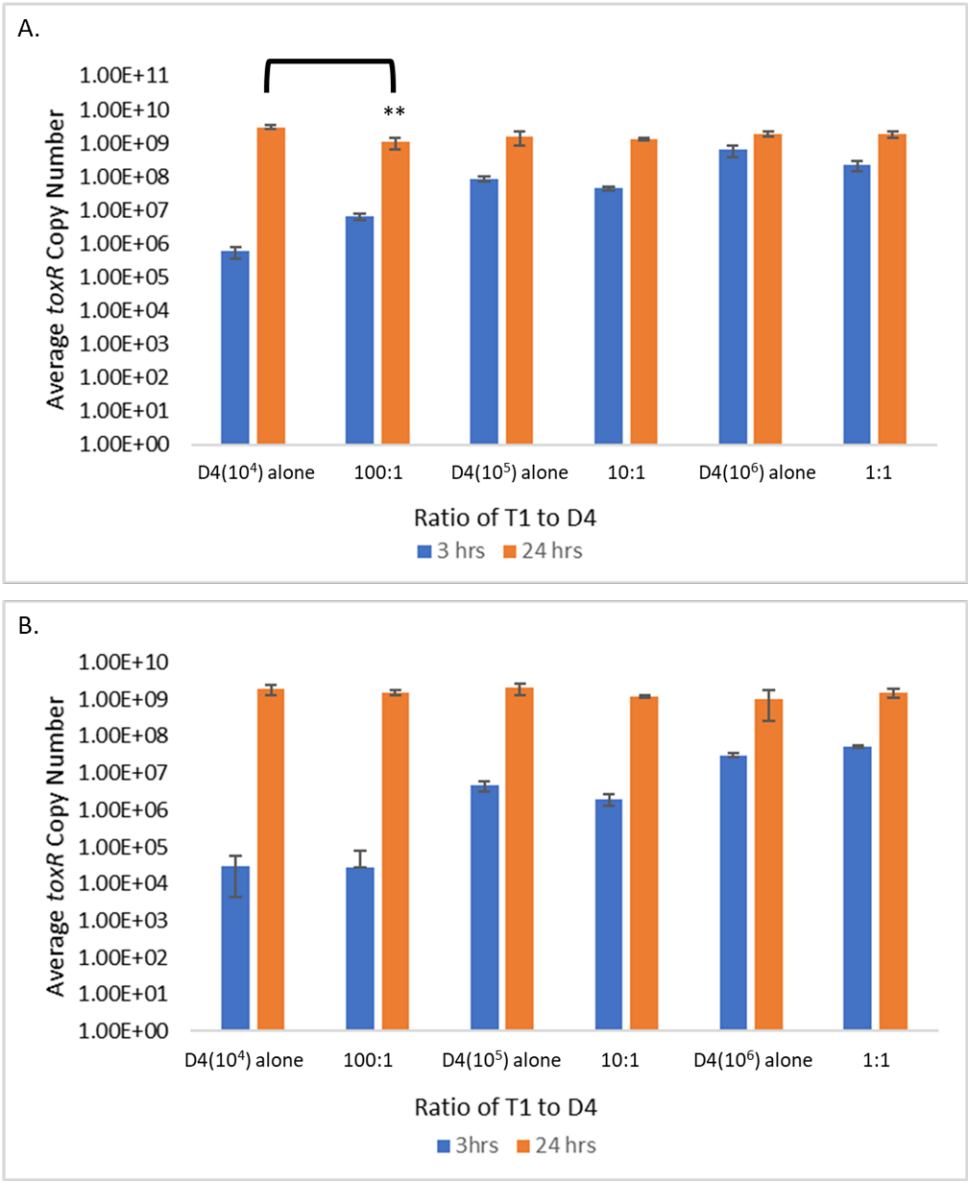


Table 3. Average *toxR* copy number of D4 grown in absence and presence of T1 in different media. Initial T1g cell density was 3.0 x 10^6 CFU/ml for all co-cultures. D4 had initial cell densities of 3.0 x 10^4, 3.0 x 10^5, and 3.0 x 10^6 CFU/ml to create T1g to D4 ratios of 100:1, 10:1 and 1:1 respectively. D4 was grown alone at the same initial densities as controls. The experiment was repeated 3 times with 4 technical replicates for each sample. Standard deviation within the technical replicates of the representative data is shown.

Culture	*toxR* Copy Number per ml Culture			
	TSB2		2216	
T1g to D4	3 hrs	24 hrs	3 hrs	24 hrs
D4(10^4)	3.0 x 10^4 ± 2.5 x 10^4	1.9 x 10^9 ± 0.6 x 10^9	5.9 x 10^5 ± 2.2 x 10^5	2.9 x 10^9 ± 0.4 x 10^9
100:1	2.9 x 10^4 ± 4.8 x 10^4	1.5 x 10^9 ± 0.2 x 10^9	6.5 x 10^6 ± 1.3 x 10^6	1.1 x 10^9 ± 0.4 x 10^9
D4(10^5)	4.5 x 10^6 ± 1.4 x 10^6	2.0 x 10^9 ± 0.7 x 10^9	8.8 x 10^7 ± 1.5 x 10^7	1.5 x 10^9 ± 0.7 x 10^9
10:1	2.0 x 10^6 ± 0.7 x 10^6	1.2 x 10^9 ± 0.1 x 10^9	4.5 x 10^7 ± 0.5 x 10^7	1.3 x 10^9 ± 0.1 x 10^9
D4(10^6)	3.1 x 10^7 ± 0.4 x 10^7	1.0 x 10^9 ± 0.8 x 10^9	6.3 x 10^8 ± 2.4 x 10^8	1.8 x 10^9 ± 0.3 x 10^9
1:1	5.2 x 10^7 ± 0.3 x 10^7	1.5 x 10^9 ± 0.4 x 10^9	2.2 x 10^8 ± 0.8 x 10^8	1.8 x 10^9 ± 0.4 x 10^9

Figure 9. Average *gfp* copy number for T1g cultures when grown with D4 in Zobell2216 at initial cell density of 3.0 x 10^6 CFU/ml. T1g was grown alone as a control. The experiment was repeated twice with four technical replicates for each sample. The error bars represent the standard deviation between technical replicates for representative data.

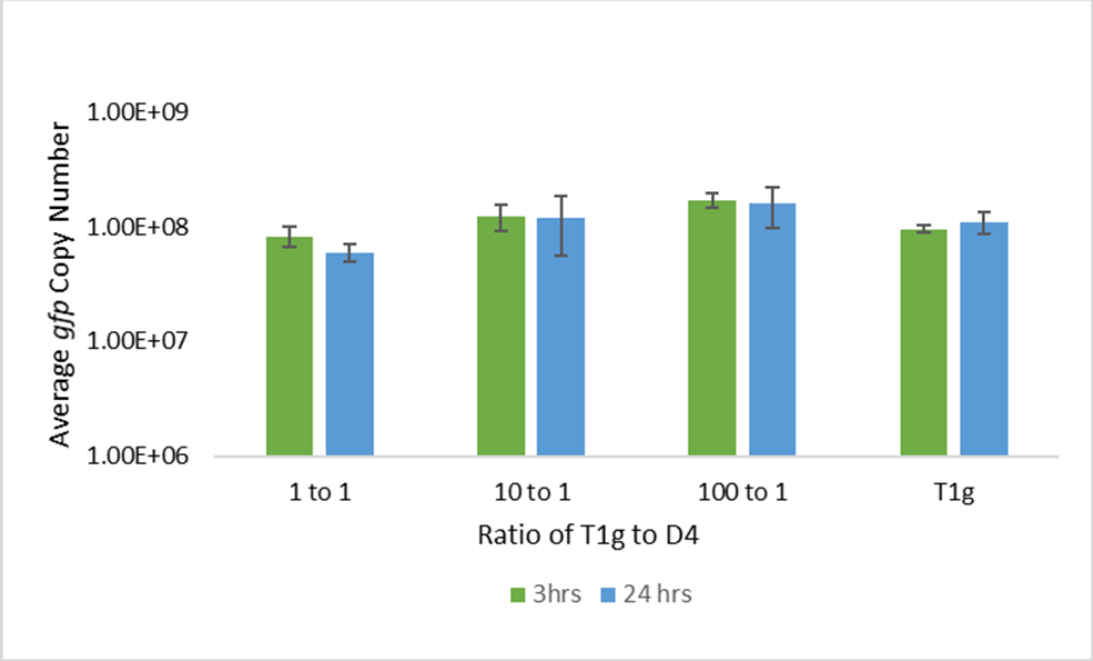


Table 4. Average *gfp* copy number for T1g when co-cultured with D4 in Zobell 2216. Initial cell density for T1g was 3.0×10^6 for all co-cultures. T1g was also grown alone at the same cell density as a control. This experiment was repeated twice with four technical replicates for each sample. Standard deviation between the technical replicates for representative data is shown.

Culture T1g to D4	*gfp* Copy Number per ml Culture	
	3 hrs	24 hrs
1:1	$8.3 \times 10^7 \pm 1.6 \times 10^7$	$6.0 \times 10^7 \pm 1.1 \times 10^7$
10:1	$1.2 \times 10^8 \pm 0.3 \times 10^8$	$1.2 \times 10^8 \pm 0.7 \times 10^8$
100:1	$1.7 \times 10^8 \pm 0.3 \times 10^8$	$1.6 \times 10^8 \pm 0.6 \times 10^8$
T1g (@10^6)	$9.6 \times 10^7 \pm 0.6 \times 10^7$	$1.1 \times 10^8 \pm 0.2 \times 10^8$

Identification of T1 Genes Involved in Inhibition of D4.

Co-culture growth experiments with T1 and D4 indicated that T1 produces some factor that is capable of inhibiting D4. To determine what this activity might be, a genetic approach using the *mariner*-derived *himar1* transposase was taken. Pozsgai *et al.* (26) described the use of this method for the generation of *B. subtilis* mutants. For this study, delivery vectors pDB384 and pEP4 were used to successfully introduce the transposon into the T1 chromosome as described in the Material and Methods, generating over 3,000 transposon-containing mutants. These were screened using the plate overlay assay (see Methods) for partial or complete loss of D4 inhibition activity compared to wild type T1 as shown in Figure 10. Seventeen T1 mutants were identified as having partial or complete loss of activity against D4 and are listed in Table 5. To verify that each T1 mutant was the result of a transposon insertion, amplification of the Sp^R cassette was done by PCR using primer set 2569R/2570R (results not shown).

Inverse PCR using primer set 2569/2570 (see Methods) was used to identify the transposon insertion site by amplifying the region of T1 chromosomal DNA adjacent to the insertion site of the transposon. DNA was sequenced (see Methods) and a database comparison was done through BLAST analysis (28). Thirteen T1 mutants with complete loss or a reduction in D4 inhibitory activity had transposon insertion sites within an ~30 kb region of DNA of *B. subtilis* subsp. *inaquosorum* that contains LP and PK biosynthesis operons. Shown in Figure 11, this "cluster" includes 17 open reading frames (ORFs); insertions were found in 8 unique ORFs in the LP/PK region, which is shown in Table 5. Mutants with insertion sites within the NRPS or PKS resulted in complete loss of inhibition of D4.

Three T1 mutants were found to contain transposon insertions outside of the LP/PK cluster: A8-11, A2-18, and S31-22. The mutations in A8-11 and A2-18 resulted in a partial loss of D4 inhibitory activity, while S31-22 had no D4 inhibitory activity. The sequence adjacent to the insertion for A8-11 was identified as *spo0A*, which encodes a master transcriptional regulator required for activating genes associated with early sporulation and is expressed prior to stationary phase and continues 1-2 hrs into stationary phase (29). Spo0A is also involved in control of secondary metabolism genes (29, 30). The mutation in A2-18 interrupted *oppA*, the first gene of a five-gene operon responsible for the synthesis of the oligopeptide-binding protein (Opp). The Opp operon encodes an ABC transporter that plays a role in a variety of stationary phase activities, including the initiation of sporulation and competence development (30). Of relevance to the D4 inhibitory activity, production of secondary metabolites by *B. subtilis* is impaired by mutations in the *opp* operon and *spo0A* (31). Mutants S31-22 has an insertion within sequences having high similarity to a glutamine amidotransferase and may be directly or involved in PK/LP biosynthesis; its role in the D4 inhibitory activity is presently unclear.

Taken together, the mutant analysis indicates that the LP/PK cluster of T1 plays a role in the inhibition of D4. More specifically, T1 is producing a secondary metabolite via the LP/PK cluster that has antagonistic activity against D4. Metabolite production may be regulated in part by Spo0A and effected by the functionality of the *opp* operon.

Elevated T1g Initial Cell Densities Lead to Increased Inhibition of D4.

Inhibition of D4 by T1g in Zobell 2216 was seen when initial cell densities for D4 were 10^4 and T1g was 10^6 CFU/ml. To determine the effect of D4 inhibition by T1g at

elevated initial cell densities, co-culture experiments were conducted in Zobell 2216 with T1g initial cell densities of 2.0×10^6, 2.0×10^7, and 2.0×10^8 CFU/ml. A3-41 is a T1 mutant strain with an interruption within *orfB* of the NRPS resulting in complete loss of ability to inhibit D4 via the overlay assay (Fig. 11A). This strain was used as an additional control at the same initial cell densities of T1g to determine if inhibition of D4 was caused specifically by T1g or by D4 being outcompeted for nutrients due to high cell concentrations of T1g and A3-41. In order to eliminate any effects associated with the medium produced by a stationary phase culture, the overnight T1g culture was harvested, washed, and resuspended in fresh 2216 prior to inoculation into fresh medium. The initial cell density for D4 was 2.0×10^4 CFU/ml for each co-culture and by itself, as a control. Co-cultures were grown for 24 hrs and samples were taken at 3 and 24 hrs for qPCR analysis as described above (Methods) and average *toxR* copy number for each culture is shown in Table 6.

As can be seen in Figure 12, T1g was found to significantly inhibit growth of D4 ($1.2 \times 10^6 \pm 0.2 \times 10^6$ *toxR* copies/ml) over 1,100-fold when the initial cell density of T1g was 10^8 CFU/ml compared to D4 grown alone ($1.3 \times 10^9 \pm 0.5 \times 10^9$ *toxR* copies/ml). Inhibition was not detected when D4 was co-cultured with A3-41, indicating that the deleterious effect on D4 is not due to higher cell concentrations alone. When present at 2.0×10^7 CFU/ml, T1g inhibited growth of D4 nearly 1.5-fold ($8.8 \times 10^8 \pm 1.4 \times 10^8$ *toxR* copies/ml). There was no significant inhibition seen at 3 hrs. These results indicate that increasing T1g initial cell density leads to an increase in growth inhibition of D4 at 10^4 CFU/ml, however, inhibition was not observed until the cultures were most likely in

Figure 10. Activity of T1 and select transposon mutants against *V. parahaemolyticus* D4. Mutants were grown on Zobell 2216 medium and examined for D4 growth inhibition using the overlay assay as describe in Methods. Mutants S21-46 (NRPS *orfA* insertion), A13-56 (Acyl CoA dehydrogenase insertion), A1-20 (PK *orfD* insertion), A3-41 (NRPS *orfB* insertion) and A8-11 (*oppA* insertion) were compared to T1 wild type, as shown.

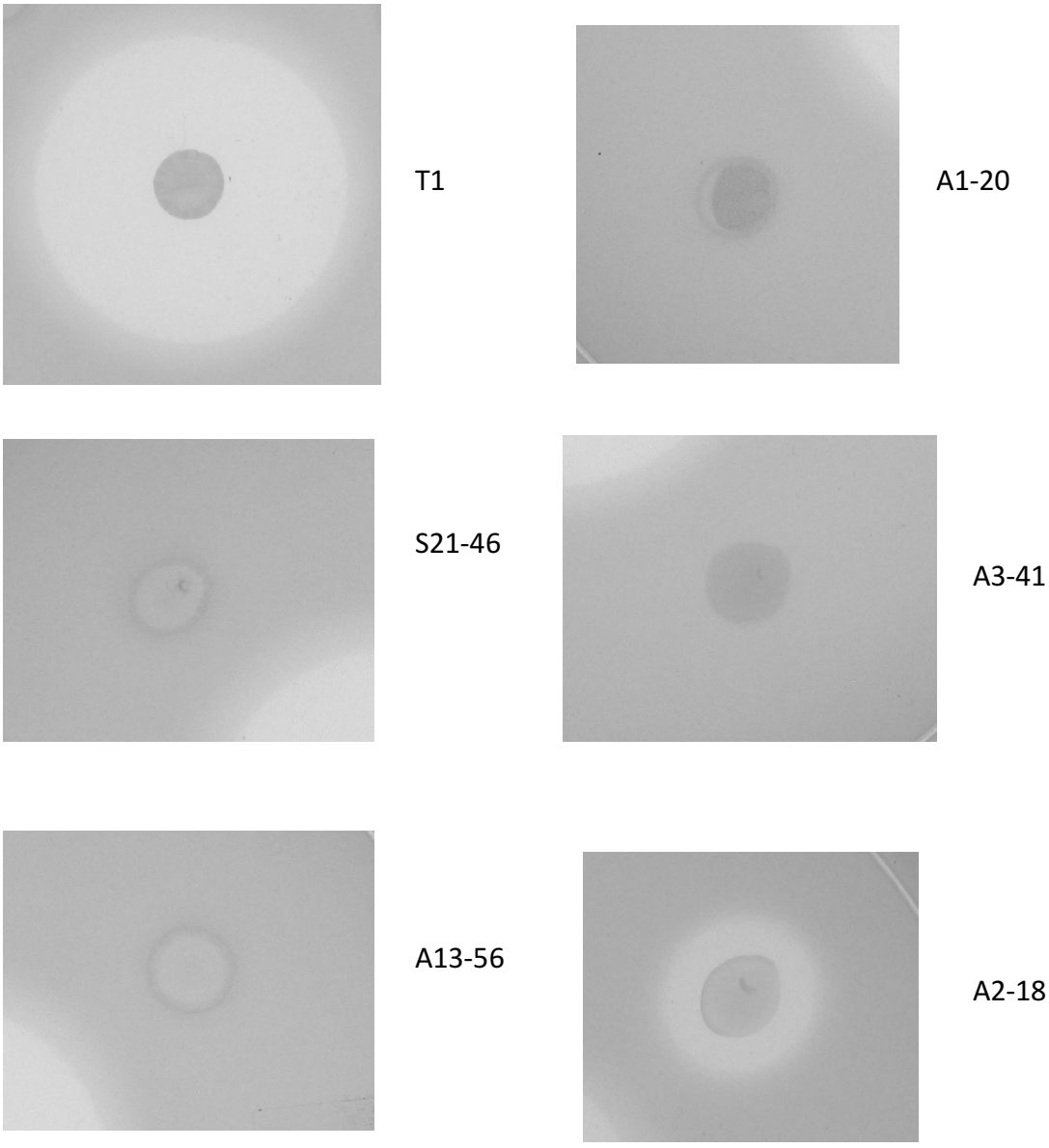
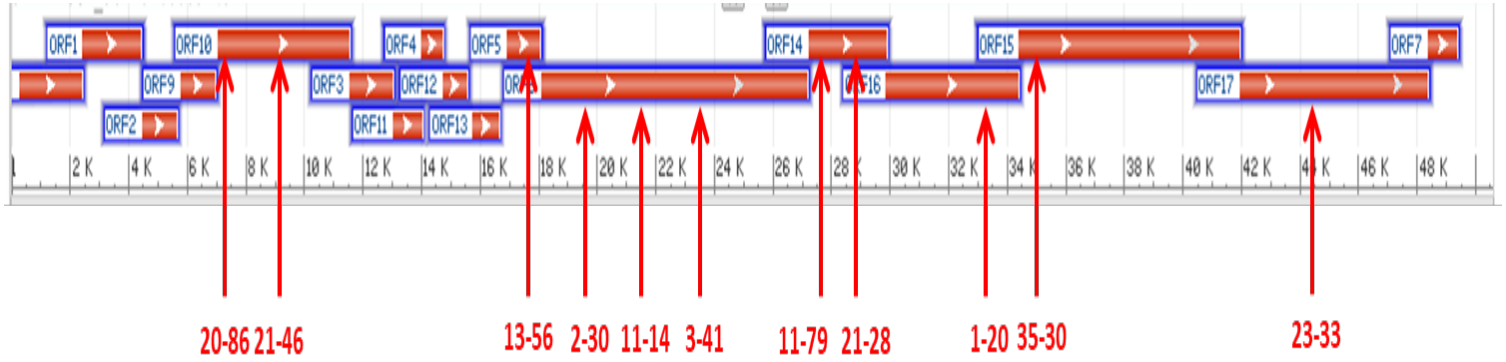


Table 5. Summary of transposon insertion sites in T1 mutants. Table includes the delivery vector used to create the mutant, phenotype based on partial (+/-) or complete (-) loss of inhibition of D4, the location of the insertion site, the gene locus and function (if known), the ORF number within the LP/PK cluster if applicable, and the ORF location on the chromosome. XX indicates an ORF outside of the LP/PK cluster.

Mutant	Delivery Vector Used	D4 Inhibition	Transposon insertion site (*B. subtilis* subsp. inaquosorum)	Gene	ORF #	ORF Location
S16-13	pEP4	-	250,383 (?)	Unknown	XX	
A20-86	pDB384	-	668,534	Non-ribosomal peptide synthetase (*orfA*)	10	667,036 to 671,532
S21-46	pEP4	-	669,917			
A13-56	pDB384	-	678,057	Acyl CoA dehydrogenase	5	676,920 to 678,062
A2-30	pEP4	-	679,316	Non-ribosomal peptide synthetase (*orfB*)	6	678,084 to 687,179
A10-67	pDB384	-	679,316			
S11-14	pEP4	-	681,936			
S12-29	pEP4	-	681,936			
A3-41	pDB384	-	684,490			
A11-79	pDB384	-	687,466	Non-ribosomal peptide synthetase (*orfC*)	14	687,199 to 689,868
S21-28	pEP4	-	688,790			
A1-20	pDB384	-	692,220	Polyketide synthase (*orfD*)	16	689,861 to 694,387
S35-30	pEP4	-	694,499	Polyketide synthase (*orfE*)	15	694,405 to 701,949
A23-33	pDB384	-	704,976	Polyketide synthase (*orfF*)	17	701,951 to 708,379
A2-18	pDB384	+/-	1,198,408	*oppA*; ABC transporter substrate-binding protein	XX	1,197,981 to 1,199,618
S31-22	pEP4	-	1,973,040	Glutamine amidotransferase	XX	1,972,834 to 1,973,466
A8-11	pDB384	+/-	2,378,497	*Spo0A*	XX	2,377,760 to 2,378,563

Figure 11. The LP/PK cluster in *B. subtilis* subsp. *inaquosorum* and the identified transposon insertions from T1 mutant strains.



stationary phase. Thus, the action of T1g on D4 growth is conceivably due to an activity that is produced during late exponential or stationary phase.

To determine whether the inhibition by T1g observed at a density of 10^8 CFU/ml was due to the presence of the higher cell concentration or carry-over of stationary phase medium from the overnight culture, T1g and D4 co-cultures were grown using cells from overnight cultures that were resuspended in cell-free supernantant fractions obtained from stationary phase cultures grown in Zobell 2216 medium for the initial inocula. When the suspended cultures were added to fresh media, 10% of the culture medium was composed of the overnight supernatant fraction. As noted above, T1g initial cell densities were 2.0 x 10^6, 2.0 x 10^7, and 2.0 x 10^8 CFU/ml and D4 was maintained at 2.0 x 10^4 CFU/ml. Results for these experiments are shown in Figure 13 and average *toxR* copy number for each culture at the 3 and 24 hr time point is listed in Table 7. As can be seen, D4 growth was significantly inhibited when T1g was present at 2 x10^8 CFU/ml when examined after 24 hr (8.3 x 10^4 ± 5.3 x 10^4 *toxR* copies/ml compared to 4.6 x 10^9 ± 1.2 x 10^9 *toxR* copies/ml for D4 alone). There was only a 1.8-fold change in growth of D4 from the 3 hr to 24 hr time points when co-cultured with T1g at 10^8 CFU/ml. There was a 4-fold decrease in D4 growth when co-cultured with T1g at 10^6 CFU/ml (2.2 x 10^4 ± 1.1 x 10^4 *toxR* copy/ml) and nearly a 2-fold decrease when co-cultured with T1g at 10^8 CFU/ml (4.6 x 10^4 ± 1.4 x 10^4 *toxR* copy/ml) compared to D4 growth alone (8.8 x 10^4 ± 3.4 x 10^4 *toxR* copy/ml), however there was no inhibition seen with T1g at 10^7 CFU/ml. D4 co-cultured with A3-41 at 10^8 CFU/ml had the same effect (5.0 x 10^4 *toxR* copy/ml) as T1g at the same concentration, indicating that any inhibition seen at 3 hrs was most likely due to the addition of the high cell density rather than a product contained in the supernatant

fraction. The pronounced growth inhibition of D4 at 24 hrs, when T1g was at its highest concentration, indicates that the increased T1g cell density lead to increased inhibition of D4.

Effect of Supernatant Fraction Prepared from Overnight Cultures on D4 Growth

Based on the results from the overlay assays and from the identification of T1 genes involved in inhibition of D4, T1 appears to produce a metabolite from the LP/PK cluster that inhibits D4 growth. To determine if T1 supernatant fraction alone can influence growth of D4, D4 cultures were treated with cell-free supernatant fractions from T1g and D4 at 10%, 25%, and 50% total volume (see Methods). Growth of D4 cultures treated with supernatant fractions are shown in Figure 14. Average *toxR* copy number at 5.5 hrs for cultures treated with 50% total volume supernatant fractions are shown in Figure 15 and Table 8 below.

All D4 cultures grown in medium containing supernatant fractions prepared from overnight T1g cultures grew to a density that was similar to the control D4 culture, i.e. containing fresh medium. However, complete growth inhibition was observed for D4 for the first 12 hrs after inoculation in medium that contained 50% T1g supernatant fraction (Fig 15A) and, after 5.5 hrs of growth, D4 *toxR* copies/ml was 184 ± 7, compared to 5.3 x 10^7 ± 0.7 x 10^7 for the control culture, a 10^5-fold difference shown in Figure 15 and Table 9. Decreasing the concentration of T1g supernatant fraction in the growth medium to 25%, resulted in reduced D4 growth 2.7-fold for the first 6 hr after inoculation. D4 treated with 10% T1g supernatant fraction, on the other hand, grew similarly to the

Figure 12. Average *toxR* copy number for D4 cultures grown in the presence of T1g or A3-41 at initial washed cell densities of 2.0 x 10^6, 2.0 x 10^7, and 2.0 x 10^8 CFU/ml. D4 initial cell density was 2.0 x 10^4 CFU/ml for each culture. T1g and A3-41 cells were washed in fresh 2216 media to remove any spent media containing toxins. D4 was grown alone as a control. D4 was grown alone as a control. Samples were taken at 3 and 24 hrs. The experiment was repeated twice with four technical replicates per sample. Error bars represent standard deviation within technical replicates of representative data.

** Indicates a significant difference (p value < .01) from D4 grown alone.

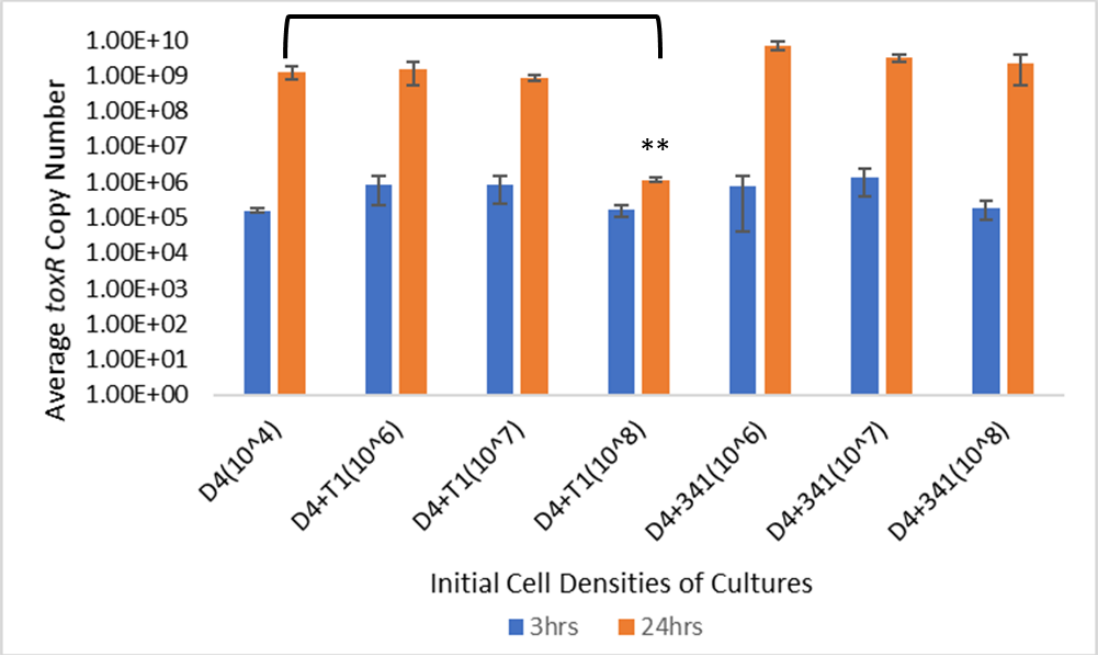


Table 6. Average *toxR* copy number for D4 cultures grown in the presence of T1g or A3-41 at initial washed cell densities of 2.0×10^6, 2.0×10^7, and 2.0×10^8 CFU/ml. Initial cell concentration for D4 is 2.0×10^4 CFU/ml for each culture. T1g and A3-41 cells were washed in fresh 2216 media to remove any spent media containing toxins. D4 was grown alone as a control. The experiment was repeated twice with four technical replicates per sample. Standard deviation within the technical replicates for representative data is shown.

Initial Cell Densities CFU/ml	*toxR* Copy Number per ml Culture	
	3 hrs	24 hrs
D4 x 10^4	$1.6 \times 10^5 \pm 0.3 \times 10^5$	$1.3 \times 10^9 \pm 0.5 \times 10^9$
T1g x 10^6	$8.8 \times 10^5 \pm 6.5 \times 10^5$	$1.5 \times 10^9 \pm 0.9 \times 10^9$
T1g x 10^7	$8.8 \times 10^5 \pm 6.3 \times 10^5$	$8.8 \times 10^8 \pm 1.5 \times 10^8$
T1g x 10^8	$1.7 \times 10^5 \pm 0.6 \times 10^5$	$1.2 \times 10^6 \pm 0.2 \times 10^6$
A3-41 x 10^6	$7.5 \times 10^5 \pm 7.1 \times 10^5$	$7.0 \times 10^9 \pm 2.0 \times 10^9$
A3-41 x 10^7	$1.4 \times 10^6 \pm 1.0 \times 10^6$	$3.1 \times 10^9 \pm 0.7 \times 10^9$
A3-41 x 10^8	$1.9 \times 10^5 \pm 1.0 \times 10^5$	$2.1 \times 10^9 \pm 1.6 \times 10^9$

Figure 13. Average *toxR* copy number for D4 cultures grown in the presence of T1g or A3-41 at initial cell densities of 2.0 x 10^6, 2.0 x 10^7, and 2.0 x 10^8 CFU/ml with the addition of 10% total volume cell-free supernatant fractions. T1g and A3-41 cells were harvested and resuspended in their respective filtered supernatant fractions, resulting in a final supernatant concentration of 10%. D4 initial cell density was 2.0 x 10^4 CFU/ml for each culture. D4 was grown alone as a control. Samples were taken at 3 and 24 hrs. The experiment was repeated twice and four technical replicates per sample. Error bars represent standard deviation within technical replicates of representative data.

** Indicates a significant difference (p value < .01) from D4 grown alone.

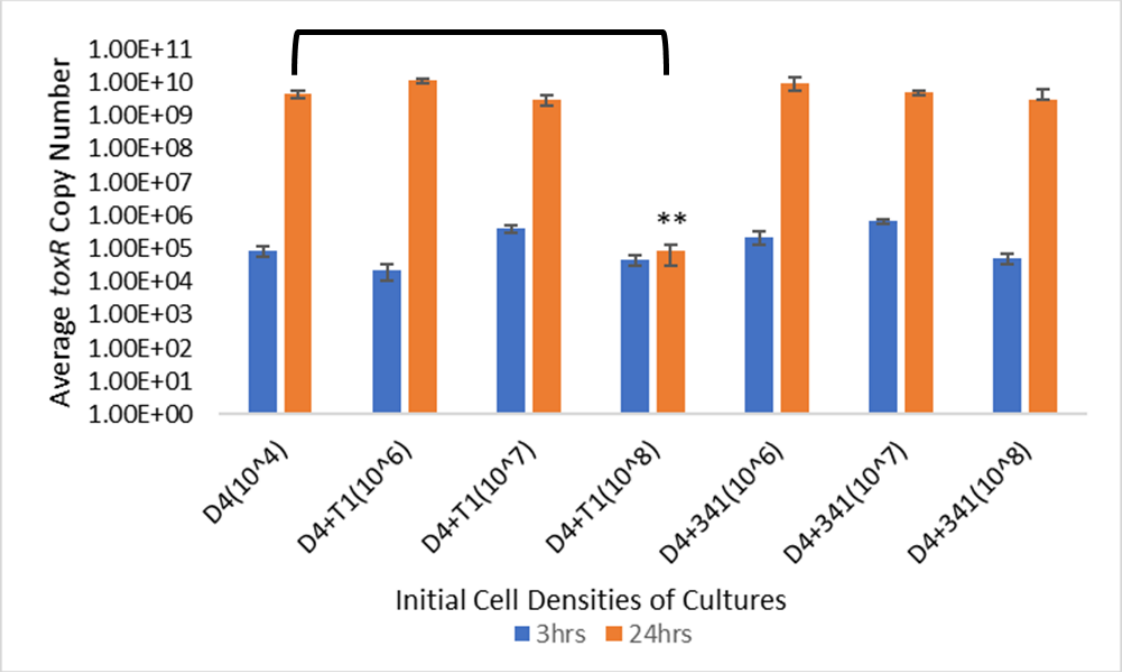


Table 7. Effect of overnight culture supernatant fraction on D4 growth. Average *toxR* copy number per ml for D4 cultures grown in the presence of T1g or A3-41 at initial cell densities of 2.0 x 10^6, 2.0 x 10^7, and 2.0 x 10^8 CFU/ml with the addition of 10% total volume cell-free supernatant fractions. Initial cell concentration for D4 is 2.0 x 10^4 CFU/ml for each culture. T1g and A3-41 cells were resuspended in their respective filtered supernatant fractions prior to inoculation. D4 was grown alone as a control. The experiment was repeated twice with four technical replicates per sample. Standard deviation within the technical replicates for representative data is shown.

Initial Cell Densities CFU/ml	*toxR* Copy Number per ml Culture	
	3 hrs	24 hrs
D4 x 10^4	8.8 x 10^4 ± 3.4 x 10^4	4.6 x 10^9 ± 1.2 x 10^9
T1g x 10^6	2.2 x 10^4 ± 1.1 x 10^4	1.1 x 10^{10} ± 0.2 x 10^{10}
T1g x 10^7	3.9 x 10^5 ± 1.1 x 10^5	3.0 x 10^9 ± 0.9 x 10^9
T1g x 10^8	4.6 x 10^4 ± 1.4 x 10^4	8.3 x 10^4 ± 5.3 x 10^4
A3-41 x 10^6	2.2 x 10^5 ± 0.9 x 10^5	9.9 x 10^9 ± 4.5 x 10^9
A3-41 x 10^7	6.8 x 10^5 ± 1.1 x 10^5	5.1 x 10^9 ± 0.8 x 10^9
A3-41 x 10^8	5.0 x 10^4 ± 1.8 x 10^4	3.1 x 10^9 ± 3.3 x 10^9

control. When examined using supernatant fractions from overnight cultures of mutant A3-41, D4 growth was not affected at any time or in the presence of any concentration of A3-41 supernatant fraction (yielding $1.5 \times 10^7 \pm 0.5 \times 10^7$ *toxR* copies/ml) as seen in Figure 15. These results support the assertion that T1g produces a substance that is inhibitory to D4 and that product is likely derived from the PK/LP cluster.

Figure 14. Growth of D4 cultures treated with 10%, 25%, or 50% total volume cell-free supernatant fractions from (A) T1 and D4 or (B) A3-41 and D4. D4 cultures had initial starting cell density of 2.0 x 104 CFU/ml. Cultures were treated with 10%, 25%, or 50% of T1g or D4 supernatant fractions that passed through a 0.2 µm filter. D4 was grown in Zobell 2216 as a control. Dotted lines represent simulated growth of culture. The experiment was repeated twice with four technical replicates for each sample.

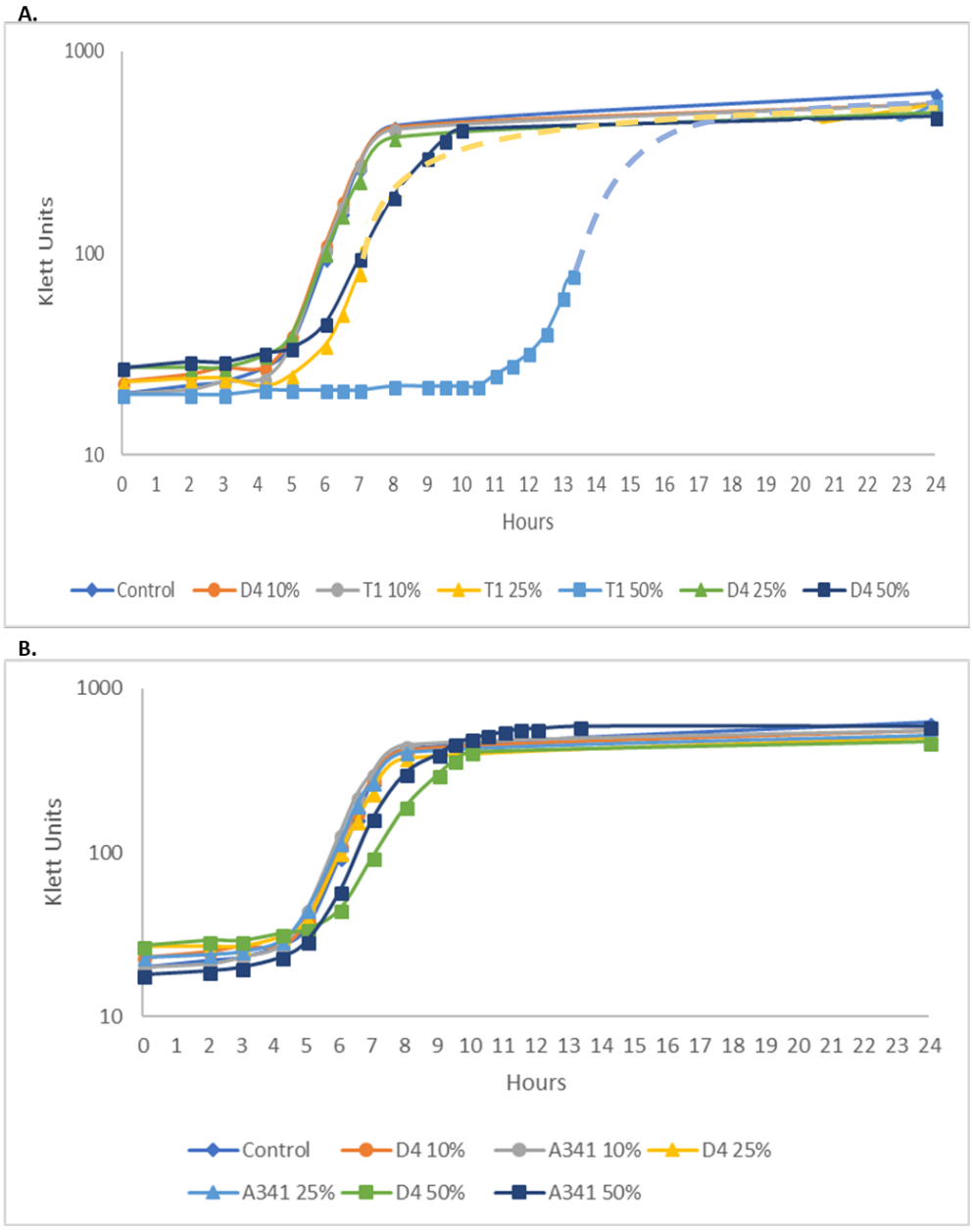


Figure 15. Average *toxR* copy number per ml for D4 cultures treated with 50% total volume cell-free supernatant fractions from D4, A3-41, and T1. D4 was grown in 2216 only as a control. Samples were taken 5.5 hrs post-inoculation. This experiment was repeated twice with four technical replicates for each sample. Error bars represent the standard deviation within technical replicates for representative data. ** Indicates a significant difference (p value < .01) from D4 grown in 2216 only.

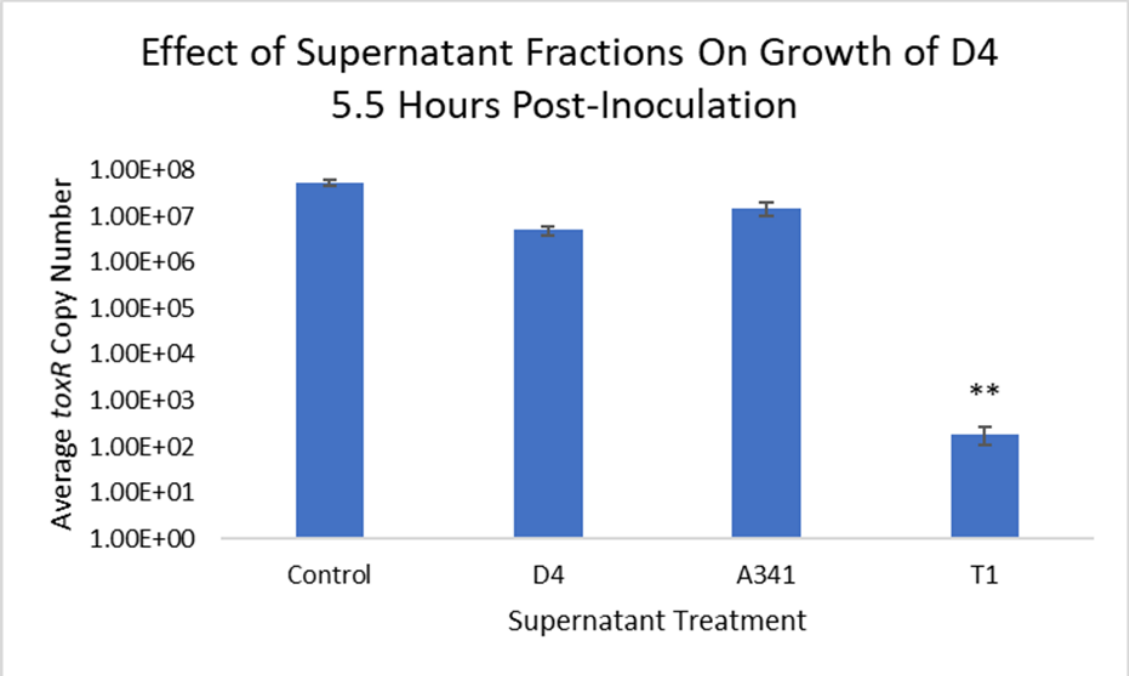


Table 8. Average *toxR* copy number for D4 cultures treated with 50% total volume cell-free supernatant fractions. Samples were taken 5.5 hrs after inoculation. This experiment was repeated twice with four technical replicates for each sample. Standard deviation within the technical replicates for representative data is shown.

Supernatant Fraction	*toxR* copy number per ml Culture
Control (2216 only)	$5.3 \times 10^7 \pm 0.7 \times 10^7$
D4	$5.0 \times 10^6 \pm 1.2 \times 10^6$
A3-41	$1.5 \times 10^7 \pm 0.4 \times 10^7$
T1	$1.8 \times 10^2 \pm 7$

DISCUSSION

Aquaculture is a multi-billion dollar global industry that has far reaching socioeconomic impacts (1). In 2016, 7.9 million tons of crustaceans were farmed, valued at 57.1 billion US dollars (32). The shrimp farming industry has been ravaged by EMS outbreaks, also known as AHPND, since its emergence in China in 2009 (1, 4, 31). EMS outbreaks are caused by strains of *V. parahaemolyticus* containing the PirAB toxin and can lead to 100% mortality of shrimp stocked ponds within 30 days of exposure to the pathogen (33). EMS outbreaks have impacted production of farmed shrimp in Asia, North America, and South America, in some cases crippling the economy. Recently, Thailand has suffered 33% drop in shrimp exports due to widespread EMS outbreaks (33). According to Nunan *et al.* (4) and Soto-Rodriguez *et al.* (1), the prevalence of EMS is nearly 100% in all pond-reared stocks in south-eastern Asia and Mexico.

Currently, the best method for preventing EMS is through the use of antibiotics (2). The majority of shrimp farming occurs in developing countries where regulation and enforcement of antibiotic usage is less stringent than in developed countries (34). Unfortunately, this practice leads to increased antibiotic resistant pathogens and exacerbates the problem of preventing disease outbreaks (2). Alternatives to the overuse of antibiotics are needed in aquaculture. The use of probiotics in shrimp aquaculture as a means to prevent EMS outbreaks could reduce mortality, potentially saving billions of dollars annually.

This study investigated the activity *B. subtilis* subsp. *inaquosorum* strain T1, a potential probiotic, on D4, an EMS-causing *V. parahaemolyticus* strain. Competitive

growth experiments examined the effect T1 has on growth of D4. Mutagenesis studies using the *mariner*-derived *himar1* transposase identified T1 genes involved in antagonistic activity against D4. Lastly, cell-free T1 supernatant fractions were examined for their ability to inhibit growth of D4.

Competitive growth experiments. To exploit the ability of T1 to inhibit D4, co-culture conditions were examined to determine if media affected inhibition and to establish initial cell densities of T1 and D4 that produced detectable D4 growth inhibition. Initial experiments using the overlay assay to test T1 activity demonstrated that inhibition of D4 was medium dependent. Competitive growth experiments were conducted using marine medium Zobell 2216 as well as richer nutrient medium, TSB2, to directly evaluate the ability of T1 to inhibit D4 growth. As shown in Figure 8, D4 growth inhibition was not detected in TSB2 at 3 or 24 hrs, regardless of the initial cell density ratios of T1g to D4. D4 growth was inhibited 3-fold at the 24 hr time point in Zobell 2216 when the ratio of cell density of T1g to D4 was 100:1. However, D4 inhibition was not detected at 3 hrs in Zobell 2216, when T1g was most likely in growth phase (exponential growth). These results suggest: 1) the antagonistic activity that T1g has on D4 is amplified in media that is limited by nutrients; 2) inhibition of D4 by T1g occurs when T1g cultures have entered stationary phase 3) and, initial T1g cell densities must be 10^6 CFU/ml and at least 100-fold greater than initial D4 cell densities for significant inhibition to occur in co-culture conditions. It is also likely that T1 is secreting a product that influences growth of D4.

Based on the competition experiment results, T1 may be producing a secreted product(s) during stationary phase that inhibits D4. To examine this possibility, co-

culture experiments were conducted using overnight T1g cultures that were washed and resuspended in fresh media to remove any inhibitors or toxins present in the overnight culture medium. Initial cell densities of T1g ranged from 2.0 x 10^6 to 2.0 x 10^8 CFU/ml. No inhibition was detected at 3 hrs, even when the concentration of T1g was 10^4-fold greater than D4 (Fig. 12), however, after 24 hrs, significant inhibition of D4 was observed when T1g was 10^4-fold greater (1,100-fold). Cultures with elevated initial cell densities will deplete available nutrients more rapidly than cultures with lower initial cell densities, driving the culture into stationary phase. A3-41 has a transposon insertion site within the NRPS and was unable to inhibit growth of D4 in co-culture experiments (Figs. 12 and 13). This provides evidence that inhibition of D4 by T1g at initial cell densities of 10^8 CFU/ml is caused by a product(s) of the LP/PK cluster and not due to D4 being outcompeted for nutrients.

Identification of T1 genes involved in inhibition of D4. Seventeen T1 mutants were identified as having complete or partial loss of ability to inhibit D4. Thirteen mutants have transposon insertion sites within the LP/PK cluster (Table 5) providing additional evidence that a product of the LP/PK cluster of T1 is important for the inhibition of D4. It is noted that preliminary experiments using pDB384-generated mutants A1-20 and A11-79, which harbor promoter-less *lacZ* gene polyketide synthase *orfD* and NRPS *orfC*, respectively, were found to be Lac+ after 48 hours of growth on plates containing X-gal, suggesting that these genes are actively transcribed (Avery, unpublished).

The LP/PK cluster of T1 is a ~30 kb region of DNA that includes 17 open reading frames (ORFs) and insertions were found in 8 unique ORFs (Figure 11). T1 mutants with insertions sites within the LP/PK cluster resulted in complete loss of ability to inhibit D4.

When environmental conditions change, bacteria will alter their physiology and molecular components to survive. *Bacillus* species will make adjustments that include biofilm formation, motility, competence, as well as the production of secondary metabolites such as lipopeptides and polyketides (18, 19, 21). T1 loses the ability to inhibit D4 when a transposon insertion occurs within any one of several genes in the LP/PK cluster, supporting the assertion that T1 is producing lipopeptide(s) and/or polyketide(s) that inhibit D4. This is further supported by the results from competitive growth experiments in Zobell 2216, showing that inhibition of D4 by T1g occurs when T1g is most likely in stationary phase. Lack of inhibition of D4 by T1g in TSB2 is likely due to the availability of more nutrients allowing T1g to stay in exponential growth phase longer, thus reducing stationary phase activities such as the production of secondary metabolites.

The mutagenesis study also yielded two T1 mutants that are involved in the regulation of secondary metabolite production. T1 mutant strains A2-18 and A8-11 have transposon insertion sites within *spo0K* (*oppA*) and *spo0A* respectively, two genes involved in the regulation of stationary phase activities (35, 36). Surfactin, a lipopeptide synthesized by NRPS of *B. subitilis* (Fig. 3), is the most powerful biosurfactant known and it also possesses antimicrobial activity as well as tumor-suppressing properties (37). Surfactin production is reliant upon *spo0A* (37), a transcription factor necessary for the initiation of early sporulation (38). When Spo0A is phosphorylated, it represses *abrB*

which is a phase regulator in *B. subtilis* (35) shown in Figure 16. AbrB is a small DNA binding protein that represses various genes involved in stationary phase activities like sporulation and antibiotic production (39). Disruptions in *spo0A* lead to overproduction of AbrB, which leads to inhibition of expression of stationary phase genes (35). Mutant A8-11 has a reduced ability to inhibit D4, indicating that *spo0A* is involved in the regulation of the stationary phase product(s) from the LP/PK cluster that has inhibitory activity against D4. The reduced loss of inhibition rather than complete loss of inhibition may be due to low levels of expression from the LP/PK cluster in the absence of *spo0A*.

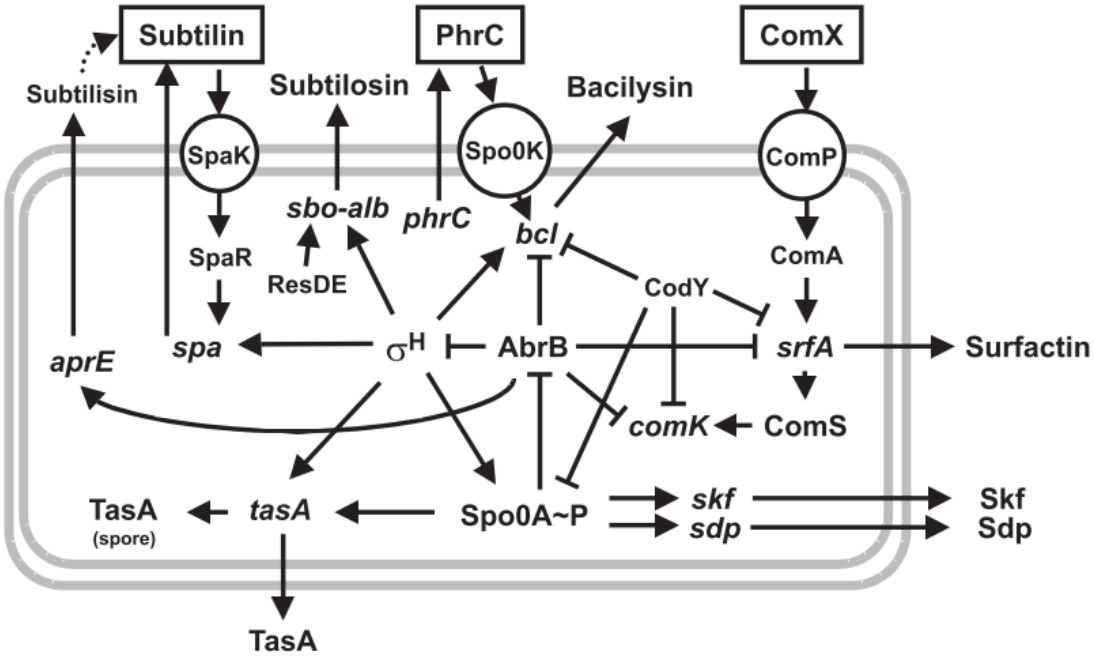
The *spo0K* operon (*opp* operon) is a five gene operon—*spo0KA-spo0KE*— that is homologous to the oligopeptide permease operon in *Salmonella typhimurium* (30). Spo0K is an ATP-binding cassette (ABC) transporter that also functions as a receptor in signaling (30, 36). The *spo0K* operon has been shown to be involved in competence and sporulation via the competence and sporulation stimulating factor (CSF), also known as PhrC, which is also involved in quorum-sensing for *B. subtilis* (36). Certain *B. subtilis* species also produce ComX which is similar to CSF in that both are pheromones that stimulate the activity of ComP (36). ComX is a strain-dependent signaling molecule, however CSF is recognized universally by *B. subtilis* (40). Both CSF and ComX enter the cell via Spo0K as shown in Figure 16 (21). ComX activates ComP, which phosphorylates ComA, in turn triggering the production of surfactin. Surfactin production increases when CSF accumulates extracellularly due to high cell densities (36). Mutant A2-18 has reduced activity against D4 due to a transposon insertion site within *spo0KA* (*oppA*), the first gene in the operon. This result supports the assertion that

Spo0K may be involved in regulation of the production of secondary metabolite(s) that inhibit D4.

Supernatant fraction activity. The supernatant fraction assay in conjunction with the overlay assay support the assertion that T1 is producing a secreted product that inhibits D4. T1 cell-free supernatant fractions prepared from overnight cultures were found to inhibit D4 growth for nearly 12 hrs (Fig. 14A), when present in culture media at a concentration of 50%, an effect not seen by the supernatant fractions prepared from overnight culture of D4 or A3-41 (Fig. 14B). These results provide strong evidence that the observed inhibition of D4 comes from a secreted product of T1.

The inhibitory activity present in T1 supernatant fraction from overnight cultures functions in a concentration-dependent manner. When T1 supernatant fraction is 50% of the total culture volume, growth of D4 was inhibited for nearly 12 hours and the *toxR* copy number/ml of culture 5.5 hrs post-inoculation was 10^5-fold difference from untreated D4 culture (Table 8). Reducing the concentration of T1 supernatant fraction to 25% reduced the inhibition of growth of D4, resulting in a 2.7-fold difference in Klett units after 6 hrs of growth. T1 supernatant fraction at 10% total culture volume had no observed effect on growth of D4. Similarly, competitive growth experiments with supernatant fractions accounting for 10% of total culture volume yielded no detectable inhibition at 3 hrs (Fig. 13). Cell-free supernatant fraction prepared from an overnight culture of A3-41 was unable to inhibit growth of D4, even at 50% concentration (Fig. 14B). These results indicate that the LP/PK cluster of T1 is producing a secondary metabolite(s) that has inhibitory effects on D4.

Figure 16. Antibiotic biosynthesis regulatory pathways in *B. subtilis*. Arrows indicate positive regulation of gene expression while T-bars indicate negative regulation. Pheromones (signaling molecules) are depicted as boxes while histidine kinases are depicted as circles. Figure taken from Stein (21).



Supernatant fractions prepared from mid-exponential T1 cultures were not examined for the ability to inhibit D4. However, no detectable inhibition was seen in any competitive growth experiments at 3 hrs, indicating that the inhibitory compound(s) was either absent or not at sufficient levels for inhibition.

D4 cultures treated with supernatant fractions from overnight T1 cultures were capable of overcoming inhibition and recovered to their maximum density within 24 hrs (Fig. 13). It is possible that D4 has the ability to degrade the secondary metabolite(s) made by T1. *Vibrio* species produce extracellular products such as proteases, lipases, and phospholipases(41, 42) and *V. parahaemolyticus* produces a serine protease, a metalloprotease, as well as a thermolysin-family protease (43), which could degrade lipopeptides and polyketides. Studies have shown that some proteases produced by *Vibrio* species are controlled by the LuxO-LuxR operon involved in quorum-sensing (43). Protease production has also been linked to stress responses in *V. anguillarum* (44). Protease production typically peaks during stationary phase in response to changes in the environment (43), which could explain results from the supernatant fraction assays; D4 cultures overcame inhibition after 12 hrs when cultures were most likely in stationary phase.

Alternatively, the secondary metabolite(s) T1 produces could be unstable, allowing D4 to recover and grow to maximum density. There is great variability in the structures of lipopeptides and polyketides (19), which could affect overall stability. For instance, bacitracin, a cyclic polyketide produced by certain *Bacillus* species is most active against susceptible bacteria 24-48 hrs incubation at pH level of 8.0-9.0 (45). The

secondary metabolite(s) of T1 could be affected by changes in pH as D4 cultures enter into stationary phase.

How is D4 affected by T1? T1 negatively impacts the growth of D4, but the mechanism of inhibition is unclear. *Bacillus pumilus* strain H2, a probiotic candidate for use in aquaculture (46), has been shown to possess antagonistic activity against a broad range of *Vibrio* species, including *V. vulnificus*, *V. harveyi*, and non-EMS-causing *V. parahaemolyticus* (46). Like T1, the cell-free supernatant fraction produced from H2 inhibits growth of several *Vibrio* species (46). Gao *et al.* (46) isolated antimicrobial compounds from the supernatant fraction of H2 using solid-phase extraction and purified the extract through reversed-phase high performance liquid chromatography. H2 was found to produce a compound identical to amicoumacin A, an antibiotic that was isolated from *B. pumilus* strain BN-103 (47). The exact mechanism of antibiotic activity that amicoumacin A has against *Vibrio* species is unknown, however, Gao *et al*. (46) demonstrated that the antibiotic damages the membrane integrity of *V. vulnificus*, creating holes in the membrane, leading to cell lysis.

Gao *et al.* (48) also found that *Bacillus velezensis* strain V4 produced multiple secondary metabolites with activity against *Aeromonas salmonicida*, a pathogen responsible for furunculosis in the salmon family. V4 was found to produce the lipopeptide iturin A, as well as polyketides macrolactin A and difficidin (48). Iturin A disrupts the plasma membrane and forms small vesicles using intramembranous particles (48). Macrolactin A is bacteriostatic, arresting cell division in Gram-positive bacteria, however, activity against Gram-negative bacteria such as *A. salmonicida* has not been observed (49). Difficidin is a broad spectrum antibiotic that has been shown to inhibit

protein synthesis (50). When *A. salmonicida* was treated with cell-free supernatant fraction from V4, there were visible particles interacting with the cell surface, wedge shaped membrane pores, and loss of cellular contents, and eventual cell lysis (48). The genome of V4 includes several gene clusters responsible for the production of secondary metabolites, including a 38-kb NRPS for iturin production, a 53-kb PKS for macrolactin A production, and a 71-kb PKS for difficidin production (48). Whether T1 is producing multiple secondary metabolites through its LP/PK cluster is presently unknown.

Because D4 cultures overcame inhibition in the supernatant fraction assay and were found to reach maximum density within 24 hr, the T1 inhibitory product is likely bacteriostatic towards D4 rather than bactericidal. This is supported by the observation that D4 could be revived from zones of clearing in overlay assays (Schreier, unpublished).

Examining the relationship between T1 and host. Co-culture experiments with T1 and D4 support further investigation of T1 as a probiotic to prevent EMS outbreaks. However, it is necessary to examine the effects T1 has upon its host organism to determine its value as a probiotic. The mechanisms of probiotic activity include stimulating the host's immune response, outcompeting pathogenic bacteria for nutrients or adhesion within the host, enhancement of the host's epithelial layer, modification of the microbial population within the host, aggregation with pathogens, or through production of antimicrobial compounds (13). Aquaculture systems present different challenges for the administration of probiotics, as organisms are constantly interacting with their environments through water intake. Verschuere *et al* (51) developed a unique definition specifically for probiotics in aquaculture which addressed the constant

interaction with the ambient environment: A probiotic used in aquaculture must have a beneficial effect on the host by modifying the host-associated or ambient microbial community through improvement of feed or nutritional value, by enhancing the host's response to disease, or by improving the quality of the ambient environment (51).

Determining the efficacy of a particular probiotic involves understanding its effect on the host. Invertebrates lack an adaptive immune system (52) meaning there are no immunoglobins and no memory of pathogen encounters (53). However, there are several enzymatic markers used to measure the immune response in Penaeid shrimp. Prophenoloxidase (proPO) is part of the immune-recognition process of defense mechanisms in invertebrates (54). It is activated in response to lipids, detergents, and organic solvents as well as bacterial components such as lipopolysaccharides and peptidoglycan, found in the outer membrane of Gram-negative bacteria and the cell wall of Gram-positive bacteria respectively (54). In crustaceans, proPO is found within haemocytes, the blood cells of invertebrates, where it is converted to its active form, phenoloxidase (PO), which is a required enzyme for response to infection and directly involved in the production of quinones and melanin (54–56). Haemocytes are classified into three forms: hyaline, semi-granular, and large granular cells where proPO is typically found (54, 57). Elevated proPO levels is indicative of an immune response (54).

Glutathione peroxidase (GPx) is an enzymatic antioxidant that acts on reactive oxygen species or free radicals (57). Oxidative bursts that generate large amounts of reactive oxygen species are an effective immune response for animals (57). GPx levels rise when there is an increase of reactive oxygen species due to the immune response to infection, making them a useful marker for measuring host immune response (57). GPx

is found in hyaline haemocytes which are responsible for phagocytosis in crustaceans (57). Additionally, lysozyme and serine protease inhibitors both play a role in the innate immune system for Penaeid shrimp resulting in increased levels of expression when shrimp are stressed (58, 59). These enzymatic markers have proven useful in studies of probiotic activity (60, 61) and could be used to evaluate the effects of T1 within the shrimp during *in vivo* trials.

Artemia as a model organism for probiotic studies. Artemia, a collective term for brine shrimp, are small crustaceans that possess similar immune responses as Penaeid shrimp (62–64) and provide more rapid and cost-effective means for probiotic studies, as well as a gnotobiotic system to remove the natural microbiome of the organism (63). Brine shrimp are also susceptible to *Vibrio* infections (27, 63–65). In a recent study, *Artemia franciscana* were fed the probiotics *Lactobacillus plantarum* and *L. lactis* and challenged by the fish and shrimp pathogen *V. anguillarum* (64). Immune responses of *A. franciscana* were measured by levels of antioxidant enzymes pro-PO, superoxide dismutase, and glutathione reductase and transferase (64), similar to previous studies with Penaeid shrimp (57, 58). There were detectable differences in enzyme levels between treated and untreated groups of artemia as well as increased survival rate post pathogen-challenge for artemia treated with probiotics (64). Artemia could be used to examine the effects T1 has on its host to determine what mechanisms of probiotic activity it may employ.

Feasibility of using T1 for commercial aquaculture. T1 is a spore-forming bacterium that is beneficial for its use as a probiotic, forgoing the need for additional treatment required for long-term storage such as lyophilization or encapsulation (66).

Requirements for use as a probiotic supplement for shrimp feed include the ability to be stored over time as well as heat and cold tolerance (48). It is important to note that spores need favorable environmental conditions to return to vegetative cells (67). In preliminary *in vivo* shrimp trials, T1 reduced mortality for shrimp challenged with D4, suggesting a return to vegetative cells (Fig. 7). Some members of the *Bacillus* genera have been well studied as probiotic candidates. *B. subtilis* strain P11 has antagonistic activity against *V. harveyi* and has been investigated as a shrimp probiotic (68). P11 was active after six months of storage at 4°C as well as at room temperature with viable cell counts at 10^9 CFU/g (68). P11 was still capable of reducing mortality in *P. monodon* challenged with *V. harveyi* by 20% after two years of cold storage (68). Cell-free supernatant fractions from potential probiotic *B. subtilis* strain H2 *and B. velezensis* strain V4 maintained their respective activities against pathogens after heat treatments of 121°C, enzyme digestions, exposure to organic solvents, and over a pH values ranging from 2 to 10 (46, 48). The ability of T1's supernatant fraction to retain activity against D4 under varying environmental conditions has not yet been tested.

T1 significantly inhibits growth D4 when its initial cell density is 10^4-fold higher than D4. This may prove a limiting factor for commercial probiotic use in aquaculture. However, the secondary metabolite(s) produced by T1 could potentially be provided to shrimp directly as an antibiotic. Since D4 eventually overcomes inhibition by the T1 product, the possibility that this product loses activity with time may be advantageous for its use as an anti-microbial. Its decreasing activity would prevent its accumulation in water and sediment and development of resistance would be significantly reduced.

Future Directions

Identifying the products of the LP/PK cluster. T1 could be producing a lipopeptide in conjunction with a polyketide that act synergistically to inhibit D4, similar to the activity of *B*. *velezensis* V4 (48). While it is unknown if the product(s) from the T1 LP/PK cluster are novel, the region is unique to *B. subtilis* subsp. *inaquosorum*, and not found in any other Bacillus species. Isolation and purification using a method similar to Gao *et al.* (46), will be informative. The diversity of lipopeptides is remarkable due to the variations in length, branching of the fatty acid chains, as well as amino acid substitutions (69). Polyketides have a great capacity for variation due to the interchangeable, modular assembly of the required and accessory domains (Fig. 4) as well as secondary modifications such as adenylation (19). It is likely that the secondary metabolite(s) produced by T1 are unique and may have broader applications as antibiotics or antimicrobials.

Engineering T1 to optimize LP/PK production. T1 mutant A8-11 provides some insight into the regulation of the LP/PK cluster. A8-11 has a transposon insertion site within *spo0A*, a master transcriptional regulator. *B. subtilis* has been genetically manipulated to have constitutively active *spo0A*, bypassing the need for starvation signals to activate stationary phase activities (70). Through in-frame deletion, *B. subtilis* mutants known as *spo0A* dominant or *sad* mutants were generated, resulting in a mutation in the amino-terminal domain of Spo0A, negating the need of phosphorylation to activate Spo0A (70). The *sad* mutants are capable of repressing *abrR*, the transcriptional repressor that inhibits expression of stationary phase genes, resulting in increased expression of stationary phase genes (21, 70). In particular, *sad67* produced 3-4 orders of

magnitude fewer spores than a culture with wildtype *spo0A* (70). This approach could be applied to T1 to increase the production of any secondary metabolite(s) with activity against D4.

The addition of a strong constitutive promoter could increase metabolite production (71). SPO1, a constitutive phage promoter, has been successfully introduced into *B. subtilis* to increase riboflavin production via the *rib* operon (71). This approach may prove useful in genetically manipulating T1 to overproduce desired secondary metabolites.

Conclusion

This study investigated the inhibitory effect that probiotic candidate *B. subtilis* strain T1 has against the shrimp pathogen EMS-causing *V. parahaemolyticus* strain D4. Growth competition experiments and mutant analysis indicate that T1 produces a secondary metabolite(s) derived from a LP/PK gene cluster, which is secreted into the medium, inhibiting growth of D4. Inhibition of D4 can be increased by driving T1 cultures into stationary phase through elevated initial cell density and reduced nutrient availability. Based on these results, T1 remains a promising probiotic candidate for the prevention of EMS outbreaks in shrimp aquaculture.

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