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Paratransgenic control of vibriosis in shrimp aquaculture

Bobban Subhadra

BOBBAN SUBHADRA

Candidate

Biomedical Sciences, School of Medicine, UNM

Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

ROBERT L RUBIN

DAVID PEABODY

WILMER SIBBITT

KEVIN O'HAIR

PARATRANSGENIC CONTROL OF VIBRIOSIS IN SHRIMP AQUACULTURE

BY

BOBBAN SUBHADRA

B.S., Marine Biology, Kerala Agricultural University, Kerala, 2000
M.S., Food Processing Technology, Central Inst. Fisheries Edu. Mumbai, 2002
M.S., Fisheries, University of Arkansas at Pine Bluff, 2005

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

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DEDICATION

To my parents, sister, loving wife, Viji and lovely daughter Niya

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ABSTRACT

Infectious diseases are an increasing threat to shrimp farming industries worldwide and account for nearly $3 billion of annual economic loss. The need to devise novel approaches against these infectious agents is pressing, as traditional methods are insufficient to ward off infections. The main aim of this study was to apply paratransgenesis to control infectious agents mediating shrimp diseases. As proof-of-concept we showed that Artemia, a food source in shrimp aquaculture, internalized bacteria expressing recombinant proteins. Stable expression and retention of these marker molecules up to 10 hours after feeding with the transgenic bacteria were evident within the gut of Artemia, coincident with the time period of highest density of internalized microorganisms. Uptake of recombinant proteins by Artemia occurred during active feeding, with their rapid depletion during a non-feeding washout phase. Bioamplification of recombinant proteins through increasing trophic levels via the paratransgenic approach was then tested. For this trials were conducted aimed at delivery of recombinant proteins

to larval stages of the commercial white shrimp, *Litopenaeus vannamei*, via direct feeding of transgenic bacteria or via feeding transgenic bacteria-engorged Artemia. In both trials, shrimp extracts showed recombinant protein accumulation during the active feeding phase and decrease during the washout period. From these studies we concluded that transgenic expression of proteins in bacteria can be detected through their paratransgenic expression in Artemia and shrimp, suggesting that this commonly employed feed organism could be applied to target infectious agents in shrimp mariculture. For this purpose we identified cecropin and melittin as effective antimicrobial peptides against *vibrio* species, and their combination was particularly potent without toxicity toward probiotic bacteria or algal feed organisms. Furthermore, unlike mellitin alone antibiotic resistance did not develop in *Vibrio* strains exposed to the cecropin/melittin mixture. Finally, the melittin gene was transduced into *B.subtilis*, and expression of significant levels of melittin was detected, although this was insufficient to cause detectable anti-*vibrio* activity. Optimization of *B.subtilis* expression for higher antimicrobial peptide production or employment of algal strains for anti-infectious molecule expression are key future directions for developing a paratransgenic approach as an environmentally sustainable disease mitigation strategy in marine aquaculture.

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CHAPTER 1

GENERAL INTRODUCTION AND MAJOR AIMS OF DISSERTATION

This chapter is adapted from the following published article:

Durvasula, R., Subhadra, B., Matthews, S., Hurwitz, I., Subba Rao, D.V., 2009. Paratransgenic approaches to the control of infections of shrimp: Prospects for use of Dunaliella Pp. 385-402. In: Ben Amotz, A., Polle, A.E.W., Subba Rao, D.V. (Eds). *The Alga Dunaliella: Biodiversity, Physiology, Genomics and Biotechnology*. Science Publishers, New Hampshire, USA.

AQUACULUTURE

Aquaculture is the fastest growing food sector globally and holds great promise for closing the nutritional gap for many of the world's population (Smith et al., 2010). Further, aquaculture-based animal production system has several advantages over other animal protein production systems (**Table 1**). World aquaculture production has increased to 59.4 million metric tons (MT) in 2008, with a value of $70 billion. While total production from capture fisheries is now declining, aquaculture, in contrast, has increased by ~9 percent per year since 1985 and now accounts for about half of all aquatic harvest by weight (SOFIA, 2008). Of this, farmed shrimp production accounts for 2.4 million MT, representing a value of nearly $10 billion (FAO, 2009). The intensive aquaculture has also several sustainability issues associated with it (Naylor et al., 2000). The reliance on use of wild-caught fish in aquaculture feeds, which could deplete food supplies for other marine life and the aquaculture industry itself over time, is a major sustainability issue (Aquaculture Task Force, 2007; Naylor et al., 2009). Emergence of drug-resistant bacteria due to indiscriminate use of antibiotics in aquafeed for disease control and growth promotion has also been a major public health issue related to sustainability of mariculture. The use of coastal and marine resources for aquaculture poses environmental threats including spread of disease from farmed to wild fish and nutrient discharge of effluents into surrounding waters (i.e. eutrophication).

The aquaculture industry's growth in the US has been less pronounced but just as steady as global production (Goldburg et al., 2001). Instead of depending largely on fish-farming to meet demand, the US has relied on high levels of seafood imports.

Table 1. Comparison of fish and shellfish meat production with other terrestrial meat production systems (Deutch et al., 2007).

Production System	FCR[1]	LUE[2]	CF[3]	HBI [4]
Beef	9.0	45.0	14.0	1
Pork	3.5	873.0	4.8	1
Poultry	2.0	7946.0	1.8	2
Fish/Shellfish	1.8	7941.0	2.0	3

[1] Food Conversion Ratio - Kg of feed required to produce/ kg of meat;
[2] Land Use Efficeincy - Kg/ha/year;
[3] Carbon Footprint - KgCO2-e/Kg;
[4] Health Benefit Index- An index based on availability, digestability and health benefits of nutrients such as protein, lipid, micronutrients, vitamins . 1 = good ; 2 = very good; 3 = excellent.

In the US, one of the world's fastest-growing seafood markets, the gap between demand and supply is even more pronounced, contributing in excess of $8 billion seafood trade deficit. About half of the trade deficit is in shrimp. Per capita consumption of seafood in the U.S. has jumped to 16.3 pounds, its highest level in more than 20 years, and is expected to continue rising. America's aquaculture industry currently meets only a meager 5-7% of domestic demand for seafood. Generally, more and more consumers are recognizing fish and shellfish as an excellent source of high-quality protein, containing lipids with high levels of health-promoting omega-3 fatty acids (Lee et al., 2009), which are increasing the demand of seafood. The US saw a rapid growth in its elderly population during the 20th century. The number of Americans aged 65 and older climbed above 34.9 million in 2000, compared to 3.1 million in 1900. Between 1990 and 2020, the population aged 65 to 74 is projected to grow 74% (US Bureau of Census, 2008). The elderly populations are consumers of seafood because of the health benefits of fish on many degenerative diseases and enhancement of health by reducing the risk of cardiovascular diseases. The growing elderly population will add an additional demand

for seafood in the coming decades suggesting the crucial need for local production and marketing of seafood.

INFECTIOUS DISEASE PROBLEMS IN AQUACULTURE

Becaue of the increased demand of seafood production the production practices in aquaculture industry were oriented towards high intensity production method. This method includes heavy stocking and high level of feeding. This is especially true for the production of shrimp as land and water suitable for shrimp production are limited and confined to coastal areas. The heavy nutrient content (e.g. high organic load) of the water, high biological carrying capacity of culture water and reduced water quality (e.g. high ammonia and less oxygen) of culture water are physiological stress factors and immune compromise factors for shrimp. These physiological conditions of culture shrimp caused the emergence of numerous infectious diseases in shrimp. Diseases caused by agents such as White Spot Syndrome Virus (WSSV) and Vibrio species have decimated shrimp farming industries in many parts of Asia and South America, and account for nearly $3 billion of economic loss annually. Unregulated uses of antibiotics in farmed shrimp and fish operations have widely been banned and contribute to the epidemic of drug-resistant bacteria in humans. Intensive practices that involve meticulous water exchange with strict standards of hygiene have been effective in reducing transmission of infectious pathogens in farmed shrimp (Otoshi et al., 2002; Otoshi et al., 2001) but are impractical in many lower-income settings of the world. Usually, appearance of disease is associated with loss of harvest for shrimp farmers and a staggering 30% of global production is lost annually. The economic impact of infectious diseases of mariculture is overshadowed only by their tremendous threat to global food security.

WHITE SPOT SYNDROME VIRUS

WSSV is the most striking example of shrimp viral disease. This disease has devastated many parts of the world with grave economic consequences and reduction in available food supply. Infection of peneaid shrimp by WSSV can result in up to 100% mortality within 3 to 7 days. The virus is extremely virulent and has a broad host range including other marine invertebrates such as crayfish and crab. The global annual economic loss due to WSSV is estimated to be $3 billion (Hill, 2005). In much of the world, there is no effective method to control this disease. Entry and pathogenesis of WSSV in peneaid shrimp occur either via oral ingestion or water-borne contact (Chou et al., 1998). Work by several investigators has demonstrated that VP28, a structural protein found on the virion envelope, is responsible for viral attachment, penetration and consequently the systemic infection of shrimp (Chappel et al., 2004; van Hulten et al., 2001). Although studies on the shrimp immune response are limited, the presence of viral inhibiting proteins in both experimental and natural survivors of WSSV infections suggests that a primitive adaptive immune-like response called ‘quasi-immune’ response exists in shrimp (Venegas et al., 2000; Wu et al., 2002). In crustacea, the lymphoid organ (LO) is found exclusively in penaeid prawns and believed that the lymphoid organ is involved in defence against invading pathogens. The discovery of immune-related genes with the LO confirms some cells have immunological roles even though these genes might be from the haemocytes within this organ. The formation of the lymphoid organ spheroid (LOS) cells has been observed in many naturally or experimentally infected penaeid prawns with viral diseases. It has been suggested that these spheroid cells constitute a major site of viral degradation and is most likely that this cell type is

produced from exocytosed granular haemocytes and eliminated to the environment rather than in situ destruction via necrosis or apoptosis (Owens, 2010).

Venegas et al. (Venegas et al., 2000) were the first to report that Kuruma shrimp (*Penaeus japonicas*) survivors from WSSV outbreaks were able to survive a subsequent WSSV challenge with very little mortality when compared to naïve control shrimp. This was later shown to be associated with WSSV neutralizing activity in the shrimp hemolymph (Wu et al., 2002) although specificity was not tested using a different virus, for example. Other reports have shown that administration of inactivated WSSV (Bright Singh et al., 2005) or heterologously expressed WSSV coat proteins by either injection or feeding can give some protection against mortality from a subsequent WSSV challenge (Namikoshi et al., 2004; Witteveldt et al., 2004; Xu et al., 2006). Although these reports refer to the process as "vaccination," that designation may be inappropriate, since the underlying mechanisms have not been elucidated and shrimp are not known to possess antibodies. Several approaches using VP28 and another structural envelope protein, VP19, have been used to elicit an immune response in shrimp. Witteveldt et al. (2004) orally vaccinated *P. monodon* and *L. vannamei* (Witteveldt et al., 2006), two of the most important cultured shrimp species, using feed pellets coated with inactivated bacteria that were over-expressing VP28. In both cases, lower mortality was found in test versus control animals up to three weeks post vaccination. In a similar study, crayfish were protected fully from WSSV following injection with fusion VP19 + VP28 polyclonal antiserum (Li et al., 2005). Vaccination trials with VP292, a newly identified envelope protein, also resulted in significant resistance to WSSV for up to 30 days post initial vaccination (Vaseeharan et al., 2006). Using a different strategy, Robalino et al. (2005,

2007) demonstrated that the administration of dsRNA specific for WSSV genes induces a potent and virus-specific antiviral response in shrimp. Both studies revealed significant reduction in mortality in the shrimp population protected by vp28 and vp19 dsRNA injections.

These approaches to controlling WSSV involve induction of an immune response to virulence epitopes of WSSV and suggest that this could potentially control this disease. In each approach, however, vaccine delivery constrains implementation. The method used in the studies cited above, individual inoculation of shrimp, is highly impractical under field conditions. Given that a typical shrimp grow-out pond can harbor upwards of 300,000 post-larvae per hectare, labor costs imposed by this method rule out commercial application. The coating of dry feed with inoculum appears logical, but the feeding behavior of shrimp involves the slow nibbling of feed particles. This sluggish behavior can cause substantial losses of inoculum through leaching. It has been demonstrated that within an hour, shrimp feed can lose more than 20% of its crude protein, about 50% of its carbohydrates and 85 to 95% of its vitamin content (Rosenberry, 2005). In light of the tremendous global impact of WSSV on shrimp farming and the constraints of high-intensity cultivation, new strategies to impart immunity against WSSV are essential. It is also critical that such a technology be economically viable, scalable to large shrimp farming facilities and be easily delivered to the shrimp.

VIBRIOSIS

Vibriosis is one of the most devastating bacterial diseases caused by at least 14 species of Vibrio (Brock and Lightner, 1990). Highly virulent strains such as *V. harveyi, V. vulnificus, V. penaeicida, V. parahaemolyticus, V. alginolyticus and V.*

nigripulchritudo can cause up to 90% mortality within a day after infection in *Penaeus monodon*, *P. japonicus* and *L. vannamei* (Lavilla-Pitago et al., 1998). The term Vibriosis describes a spectrum of diseases and includes oral and enteric Vibriosis, appendage and cuticular Vibriosis, localized Vibriosis of wounds, shell disease, systemic Vibriosis and septic hepatopancreatitis (Brock and Lightner, 1990). Outbreaks may occur when environmental factors trigger the rapid multiplication of bacteria usually tolerated at low levels within shrimp blood (Sizemore and Davis, 1985), or by bacterial penetration of host barriers. The shrimp exoskeleton provides an effective physical barrier to pathogens, however, *Vibrio* spp. are chitinoclastic bacteria associated with shell disease and may enter through wounds in the exoskeleton or pores (Jiravanichpaisal and Miyazaki, 1994; Alday-Sanz et al., 2002). In recent years, Vibriosis caused by *V. nigripulchritudo* and *V. penaeicida* caused enormous losses in various shrimp grow-out facilities in New Caledonia (Reynaud et al., 2008; Sakai et al., 2007; Goarant et al., 2006). Many new techniques have been proposed for the control of Vibriosis but most of them are impractical and are not cost-effective under field conditions. The lack of a vertebrate – like adaptive immune response mediated by B and T cells limits the use of vibrio-specific vaccination strategies in shrimp (Hill, 2005). Use of prophylatic antibiotics in aquaculture is banned in many countries due to emergence of antibiotic-resistant microbes (Cabello, 2006; FAO-WHO, 2006). Emergence of novel, drug resistant forms of these pathogens compounds the crisis for many shrimp farmers. The need for new tools in the battle against Vibriosis is pressing.

USE OF PROBIOTICS IN AQUACULTURE

Probiotics are defined as microorganisms that are beneficial to the health of the host. They are not therapeutic agents but, instead, directly or indirectly alter the composition of the microbial community in the rearing environment or in the gut of the host. Although the mode of action of probiotics is not fully understood, it is likely that they function by competitive exclusion, that is, they antagonize the potential pathogen by the production of inhibitory compounds or by competition for nutrients and/or space (Verschuere et al., 2000). It is also likely that probiotics stimulate a humoral and/or cellular response in the host. The first report citing probiotics as a biological control in aquaculture was in the late 1980's. In this mode of farming, probiotics are usually introduced as part of the feeding regimen or applied directly to the water. A variety of microorganisms, ranging from aerobic Gram-positive bacteria (*Bacillus* spp. (Raengipat et al., 1998)), to Gram-negative bacteria (*Vibro* spp. (Irianto and Austin, 2002)) and yeast (Scholz et al., 1999) have been utilized successfully to increase the commercial yield of farmed marine animals. Several species of microalgae have also effectively been used for this purpose. Of note, the unicellular alga, *Tetraselmis suecica*, has been used as feed for penaeids and salmonids with significant reduction in the level of bacterial diseases. The probiotic activity of *T. suecica* was first reported by Austin et al. (1992). These investigators went on to show that when used as a food supplement, the algal cells inhibited laboratory-induced infections in Atlantic salmon by *Aeromonos hydrophila, A. salmonicida, Serratia liquefaciens, Vibrio anguillaram, V. salmonicida* and *Yersenia ruckeri type I* (Austin et al., 1992). Although the precise mode of action of *T. suecica* is unknown it is suspected that unspecified antimicrobial components in the algal cells

might have contributed to its probiotic activities. Along these lines, Avedano and Riquelme (1999) reported the possibility of incorporating bacteria with the ability to produce inhibitory substances into an axenic culture of *Isochrysis galbana*. Thus, this microalga could serve as a vector for transmitting inhibitory substances into cultures of larval bivalves to antagonize pathogenic bacteria.

USE OF VACCINES IN AQUACULTURE

In recent years, vaccines based on recombinant DNA technology appear to be a promising approach to controlling infectious diseases in farmed fish and certain shellfishes (Biering et al., 2005; Clark and Cassidy-Hanley, 2005; Heppel et al., 1998). By intramuscular injection of eukaryotic expression vectors encoding the sequence of a pathogen antigen, DNA vaccines offer a method of immunization that overcomes many of the disadvantages such as risk of infection and high costs of traditional live attenuated, killed or subunit protein-based counterparts. They induce strong and long-lasting humoral and cell mediated immune responses in fish which have made them attractive for the aquaculture industry (Heppel and Davis, 2000). DNA vaccination has already been proven to be effective in rainbow trout for infectious haematopoietic necrosis virus (Boudinot et al., 1998; Corbeil et al., 1999; Kim et al., 2000; Kurath et al., 2006; Lorenzen et al., 2001; Lorenzen et al., 1999) and viral haemorrhagic septicemia virus (Lorenzen et al., 2002) as well as channel catfish for ictalurid herpes virus 1 (Nusbaum et al., 2002). After intramuscular injection of plasmid DNA carrying promoter-driven reporter genes, protein expression has been achieved in tilapia (Rahman and Maclean, 1992), goldfish (Kanellos et al., 1999), zebrafish (Heppel et al., 1998), Japanese flounder (Takano et al., 2004) and gilthead seabream (Verri et al., 2003).

Although there are several ways to administer vaccines, most young fish continue to be vaccinated by hand. In Norway, for example, over 200 million fish are vaccinated each year. Each fish is removed from the water, anesthetized and vaccinated. This method is highly stressful for the fish, and in some circumstances rather impractical. Another method of vaccination is by dip immersion into a solution containing the vaccine. Dip immersion is usually used in fish stocks that are too young or small for manual handling. Unfortunately, this method alone is not sufficient to achieve a long duration of protection. Thus, the fish are usually subjected to intra-peritoneal re-vaccination injection as soon as their size allows. Oral vaccine delivery systems are by far the most desirable method for immunizing fish. However, this system has proven ineffective. A vaccine approach has not been explored in disease control in shrimp in farm conditions because of the lack of adaptive immune system, although, recent approaches to induce quasi-immune response using viral proteins and double stranded DNA is somewhat successful in lab-based studies. However, vaccination of individual shrimp is simply impractical in field condition because of the fragile body nature and high physiological stress compared to fish when taken out of water. All these hurdles point to the need for the development of a more user-friendly and field oriented methodologies for infection control particularly in shrimp aquaculture (Lin et al., 2005).

PARATRANSGENESIS

Paratransgenesis is a relatively novel approach for the control of vector-borne infectious disease transmission. Paratransgenesis is a 'Trojan horse' approach to control of disease transmission by insects. It employs the interactions between disease-transmitting vectors, bacterial symbionts of the vectors, and the pathogenic agent (Beard

et al., 2002; Dotson et al., 2003; Durvasula et al., 1997; Durvasula et al., 1999). Symbiotic bacteria are isolated and genetically transformed in vitro to export molecules that interfere with pathogen transmission. The genetically altered symbionts are then introduced into the host vector, where expression of engineered molecules affects the host's ability to transmit the pathogen, i.e., its vector competence. This approach attempts to decrease pathogen transmission without adverse effects on the vectors themselves. Furthermore, it employs, as a gene delivery mechanism, bacterial flora native to the host vector. There are several requirements for classical paratransgenic approach to be successful and the most important ones are listed below:

1. An appropriate symbiotic association must exist within a given disease-transmitting vector.
2. Bacterial symbionts should be amenable to culture and genetic manipulation.
3. Genetically altered symbionts should remain stable.
4. Fitness of the genetically altered symbionts to re-infect host vectors should not be compromised. Furthermore, their normal symbiotic functions should not be altered.
5. Transgene products released from the genetically altered symbionts should interact effectively with the target pathogen.
6. A method must exist for dispersal of the genetically altered symbionts amongst naturally occurring populations of vectors.

Durvasula et al. (1997) conducted the initial proof of concept studies on this approach to combat transmission of the Chagas disease parasite, *Trypanosoma cruzi*, by reduviid bug vectors **(Fig. 1)**.

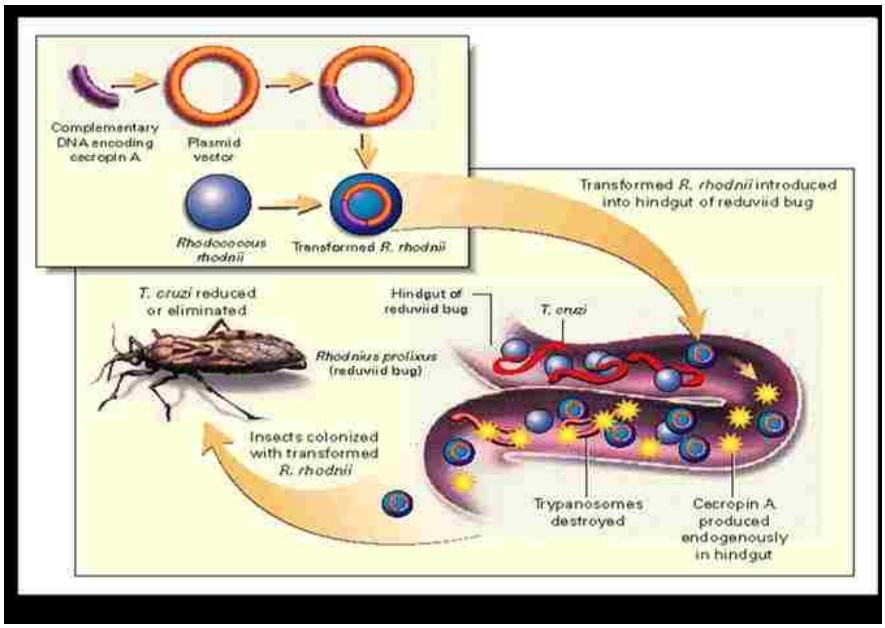

Figure 1. Paratransgenic disease control against Chagas disease. Complementary DNA encoding cecropin A, an antiparasitic peptide, is cloned into a plasmid vector. After transformation of *Rhodococcus rhodnii* with the vector, this bacterial symbiont that normally lives in the intestine of the reduviid bug (*Rhodnius prolixus*), expresses cecropin A, and the hindgut of these nymphs becomes colonized with transgenic *R. rhodnii*. The colonized nymphs are then allowed to feed on human blood containing living *Trypanosoma cruzi*, the agent of Chagas' disease. *T. cruzi* are reduced or eliminated in insects colonized with the transformed *R. rhodnii* (Conte, 1997)

Many research groups have since applied this concept for the intervention of numerous infectious diseases such as for the commensal bacteria of the human respiratory tree (Sundaram et al., 2006), sharpshooter vectors of the grape bacterial disease, Pierce's Disease (Bextine et al., 2004), sandfly vectors of leishmaniasis (Hillesland et al., 2008), and mosquito vectors of malaria (Favia et al., 2007; Ren et al., 2008).

MARINE PARATRANSGENESIS

The classical paratransgenesis consisted of four elements; the arthropod insect vector, the symbiotic bacteria which resides in the vector, the disease causing agent and human, the host which succumbs to the disease. A closer look at the shrimp culture system also reveals a similar biological system. In large-scale shrimp rearing, larval stages are fed with Artemia -an aquatic crustacean feed organism- to boost nutrition and improve survival in hatcheries. Artemia, which are reared separately, are fed with microalgae and other probiotic bacteria. Artemia is a nearly ideal feed organism: economical, hardy, and readily available worldwide. For the nutritional purpose of Artemia, they are fed with microalgae and probiotic bacteria. So similar to the classical paratransgenesis, there are four elements associated with shrimp culture system; the Artemia which is the arthropod intermediate, the microalgae feed organisms, the disease-causing bacteria such as Vibrio, Aeromonas etc, and finally the host, shrimp, which succumbs to the disease.

The supplementation or bioencapsulation of nutritional components, such as vitamins or calcium, into Artemia has been practiced by aquaculture hatcheries for over 10 years. Artemia nauplii were initially fed with emulsified fish oils containing highly unsaturated fatty acids (HUFA), to eventually be used as feed for marine finfish and crustacean larvae. Today, live nauplii of the brine shrimp have been used as vectors for delivering compounds of diverse nutritional (Dhert et al., 1999) and/or therapeutic (Campbell et al., 1993) value to larval stages of aquatic animals. Inoculating the digestive tracts of target organisms with probiotic bacteria through bioencapsulation is another alternative use for Artemia nauplii. Bacteria with various

characteristics have also been incorporated into Artemia nauplii prior to oral challenge of turbot larvae with a pathogenic *Vibrio anguillarum* strain (Chair et al., 1994a; Grisez et al., 1996). This route has also been used to vaccinate sea bass fry (Chair et al. 1994b); juvenile carp (Joosten et al., 1995) and fish fry (Campbell et al., 1993).

We propose a modified paratransgenic approach to control infectious diseases of shrimp culture. Lines of marine cyanobacteria, algae and diatoms - common components of feed for farmed shrimp and fish - can be transformed to produce antibodies that neutralize infectious pathogens such as WSSV and Vibrio. Delivery of these feed organisms, either directly in slurry preparations or via a bioamplification strategy with Artemia, will result in passive immunization of the alimentary tract of farmed marine animals. This is the portal of entry for many infectious agents and the delivery of neutralizing antibodies would either abort the infectious process or delay it sufficiently to permit harvest. We have demonstrated that a marine cyanobacterium, *Synechococcus bacillarus*, could be genetically transformed to express a functional recombinant antibody (Durvasula et al., 2006). We transformed *S. bacillarus* to produce a murine antibody (rDB3) against progesterone, using a heterologous expression system. In competitive ELISA studies, the rDB3 antibody bound progesterone in a dose- dependent and specific manner. No cross-reactivity with testosterone, a structurally similar steroid, was detected (Durvasula et al., 2006). This study demonstrated that a transgenic cyanobacterium could elaborate an active recombinant antibody, and serves as a model for future applications of this technology. An alternate strategy for delivery of transgenic *Dunaliella* to the target animal is via bioamplification. In this strategy a feed organism such as *Artemia* initially consumes

the transgenic *Dunaliella*. The engorged Artemia is then fed to the target animal. In this manner, the supplement is bioamplified as it progresses up the food-chain.

Artemia are non-selective filter feeders and therefore will ingest a wide range of foods. The main criteria for food selection are particle size, digestibility, and nutrient levels (Dobbeleir et al., 1980). Possibly the best foods for Artemia are live microalgae such as *Nannochloropsis*, *Tetraselmis*, *Isochrysis*, *Dunaliella* and *Pavlova*. Combinations of live phytoplankton fed to Artemia cultures have demonstrated superior enrichment characteristics over feeding single phytoplankton species (D'Augostino, 1980). However, not all species of unicellular algae are appropriate for sustaining Artemia growth. For example, *Chlorella* and *Stichococcus* have a thick cell wall that cannot be digested by Artemia.

CONCLUSIONS

There are two dominant issues in shrimp aquaculture industry; 1) An increasing trend of seafood demand from the planet's growing population whereas the seafood supply is hugely affected by infectious diseases 2) The ban of large scale use of antibiotics in aquaculture sector and ineffectiveness of other traditional methods to ward off shrimp infections. Therefore, a major challenge in shrimp aquaculture is to develop new methods of infectious disease control in shrimp aquaculture. The main objective of this study is to conduct the proof of concept studies on developing marine paratransgenic approach as a sustainable, cheap and practical method of infectious disease control in marine shrimp farming. The aims and subaims of the study are listed in the following page.

MAJOR AIMS OF DISSERTATION RESEARCH

AIM 1: DEMONSTRATE THE CONCEPT OF PARATRANSGENESIS IN AQUACULTURE

Sub aim 1.1. Development of paratransgenic *Artemia*: A potential strategy for control of infectious diseases of commercial mariculture.

Sub aim 1.2. Develop the concept of biotransfer/bioamplification of recombinant proteins in marine paratransgenic approach.

AIM 2: USE OF ANTIMICROBIAL PEPTIDES (AMPS), EITHER INDIVIDUALLY OR IN COMBINATION AGAINST VIBRIO SPP.

Sub aim 2.1. Minimum inhibitory concentration (MIC)/ Minimum bactericidal concentration (MBC) experiments with single AMPs against pathogenic *Vibrio spp*. Determine whether AMPs in combination have synergistic actions and less probability of emergence of resistance mechanisms against peptides.

Sub aim 2.2. Cloning of AMPs into expression vectors and transformation into *Bacillus subtilis* strains. Characterization of the transgenic clone and AMP expression and anti-vibrio activity of these clones.

CHAPTER 2

DEVELOPMENT OF PARATRANSGENIC ARTEMIA

This chapter is based on the following published article:

Subhadra, B., Hurwitz, I., Fieck, A., Subba Rao, G., Subba Rao. D. V., Durvasula, R., 2009. Development of Paratransgenic *Artemia* as a Platform for Control of Infectious Diseases in Shrimp Mariculture. *Journal of Applied Microbiology* 106:831-840.

SUMMARY

Aim: To study the accumulation and retention of recombinant proteins in *Artemia* gut for optimizing paratransgenic disease control in shrimp aquaculture. **Materials and Results:** Transgenic *Escherichia coli* expressing fluorescent marker proteins and the transgenic cyanobacterium *Synechococcus bacillarus* expressing a functional murine single chain antibody, DB3, were fed to *Artemia franciscana*. Stable expression and retention of marker molecules up to 10 h after feeding with *E. coli* were evident within the gut of Artemia. Engineered strains of *S. bacillarus* expressing DB3 accumulated within the gut of Artemia, with detectable antibody activity for 8-10 h of feeding via ELISA, coincident with the time period of highest density of transgenic *S. bacillarus* in the Artemia gut. **Conclusions:** *Artemia* fed transgenic bacteria or algae accumulated recombinant proteins for up to 10 h, which retained biological activity. Co-delivery of multiple recombinant proteins simultaneously in the gut of *Artemia* was also demonstrated. **Significance and Impact of the study:** Expression of molecules which target infections agents of mariculture in shrimp via commonly deployed feed organisms such as Artemia could potentially offer powerful new tools in the ongoing global effort to increase food supply.

INTRODUCTION

Production loss to due to infectious diseases causes huge losses to shrimp farmers. Noval apporoaches against infectious disease control in shrimp is a pressing need. In this paper, we explore the application of paratransgenic strategies to target infectious diseases of shrimp aquaculture. To apply this strategy to farmed shrimp, we propose a modified paradigm that involves microbes that are frequently used as feed for marine animals (Durvasula et al., 2006). Probiotic bacteria such as *Bacillus subtilis* and marine

cyanobacteria are commonly deployed feed organisms in commercial mariculture settings. Though they are not true symbiotic organisms, they can serve as effective delivery vehicles for foreign proteins that disrupt transmission of pathogens. The continuous delivery of these feed organisms in commercial mariculture coupled with rapid assimilation of feed products by animals such as shrimp render this an attractive platform for paratransgenic interventions. Furthermore, bacteria can be delivered to farmed marine animals through intermediate mechanisms such as Artemia. Live food such as microalgae and Artemia nauplii are provided throughout development of shrimp larval stages to improve survival in hatcheries. We hypothesize that Artemia can be used as an effective delivery mechanism for recombinant proteins which impart immunity to shrimp against a variety of infectious pathogens. This paratransgenic method of delivering antibody-producing cyanobacteria via Artemia to shrimp larval stages utilizes the natural feeding behaviors of both Artemia and farmed shrimp. In this study, we conducted experiements to show the Artemia fed with recombinant proteins expressing cyanobacteria or *E.coli* will effectively accumulate functional proteins in their gut.

MATERIALS AND METHODS

Generating transgenic lines of *E. coli* and *S. bacillarus*

E. coli Nissle was provided by Dr Ulrich Sonnenborn, Ardypharm (Ardeypharm GmbH, Germany). Both *E. coli* DH5α and *E. coli* Nissle were transformed with pGFP which expresses Green Fluorescent Protein (Clontech Laboratories Inc., Mountain View, CA, USA) using a calcium chloride transformation protocol. The pGFP plasmid expresses Green Fluorescent Protein (GFP), a 27kDa that exhibits bright green fluorescence when exposed to blue light and first isolated from the jellyfish *Aequorea*

victoria (Prendergast and Mann, 1978). The DSRed plasmid was constructed by cloning the DSRed gene into the Xho1/Xba1 sites in pET 21 (Novagen-Merck KGaA, Darmstadt, Germany) and used to transform *E. coli* DH5α. The transformed bacterium expresses a red fluorescent protein (DsRed) and this protein was originally isolated from reef coral, *Discosoma sp.* (Dietrich and Maiss, 2002). Construction of the shuttle vector pRrMDWK6 was previously described (Durvasula et al., 1999). The pRrMDWK6 vector has the gene for rDB3 protein, which is a murine single-chain Vγ/Vκ antibody against progesterone (He et al., 1995). Wild type *S. bacillarus* (CCMP1333) were purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton and cultured in sterile seawater supplemented with *f/2* concentrate (Sigma-Aldrich, St Louis, MO, USA) trace metals, and vitamins (CCMP, Bigelow Laboratory for Ocean Sciences, West Boothway Harbor, ME, USA). *S. bacillarus* transformation with pRrMDKW6 was performed by adapting protocols for *E. coli*, and had been previously described (Durvasula et al., 2006).

Western Blot to detect DB3 protein from transgenic *S. bacillarus* lysates

Overnight cultures of wild type or transgenic *S. bacillarus* grown in *f*/2 media were centrifuged at 3000 ***g*** for 15 min. The cells were lysed in lysis buffer (100 mM Tris-Cl, 500 mM NaCl, 0.5 M EDTA, 0.1% Triton X-100, 0.1% Tween-20, 8% glycerol, 250 mM urea, 5 mM β-2 mercaptoethanol, 100 µg ml $^{-1}$ PMSF, 1 µg ml $^{-1}$ protease inhibitor cocktail, 50 µg ml $^{-1}$ lysozyme) and proteins were concentrated using 80% saturated ammonium sulfate precipitation followed by overnight dialysis against 25 mM Tris-HCl (pH 7.2). One hundred µg of total protein, as determined by Bradford assay, was electrophoresed on a 10% SDS-PAGE gel and transferred to PVDF membrane (BioRad,

Hercules, CA, USA). The blot was blocked with 5% BSA in TBST (Tris buffered saline containing 1% Tween 20) for 1 hour, and washed three times with TBST. The blot was then probed with alkaline phosphatase-linked goat anti-mouse kappa chain antibody (Southern Biotech, Birmingham, AL, USA) at a dilution of 1:5000 and developed using an alkaline phosphatase immunoblot detection kit. This reaction involves hydrolyzation of the primary substrate bromochloroindolylphosphate- ptoluidine by alkaline phosphatase to form an intermediate that undergoes dimerization to produce an indigo dye. The nitro-blue tetrazolium (NBT) is reduced to NBT-formazan (insoluble black-purple precipitate) by the two reducing equivalents generated by the dimerization (Chemicon Inc, Billerica, MA, USA).

Axenic hatching of Artemia

All experiments were performed with high quality hatching cysts of *Artemia franciscana* (INVE Aquaculture, Baasrode, Belgium). Sterile cysts were obtained via a de-capsulation protocol as adapted from the method of Marques et al. (2004). Cysts were hatched in a conical flask containing 1000 ml sterile filtered artificial seawater. To assure that nauplii were axenic, random Artemia were selected and homogenates were incubated on LB-seawater agar for 72 h at 37 °C.

Fluorescence microscopy to detect GFP and DS Red protein in the gut of paratransgenic Artemia

10^6 CFU ml $^{-1}$ of wild type or transgenic *E. coli* expressing fluorescent proteins (GFP or DSRed) were fed to Artemia (200 Artemia ml $^{-1}$). At intervals of 30 min, 2, 4, 6, 8 and 10h, 3-4 Artemia nauplii were anesthetized by immersion into a 30 mg l $^{-1}$ of tricaine methanesulfonate (Sigma-Aldrich, St. Louis, MO, USA) solution, and suspended

in a single drop of sterile PBS on a microscopic slide. Fluorescent microscopic images (10X objective magnification) were taken using a Zeiss Axioshop LSM 510 fluorescent microscope (Carl Zeiss Meditech Inc. Dublin, Ireland). All images were exported as tiff files and compiled in Slidebook software (Intelligent Imaging Innovations Inc., Denver, CO, USA).

Culture and algal feeding of Artemia

Fortyeight hours after hatching, 20000 Artemia nauplii were transferred to sterile 250-ml Erlenmeyer flasks containing 100 ml of filtered and autoclaved artificial seawater. Wild-type or transgenic *S. bacillarus* in *f*/2 media were washed in filtered seawater and added to the feeding vessels to a final density of 10^6 CFU ml $^{-1}$. Artemia were harvested at 2, 4, 6, 8, 10 and 20 h for further analysis.

Preparation of gut content from Artemia and quantification of kanamycin- resistant *S. bacillarus*

Gut contents of individual Artemia (n=100) were dissected out, disrupted using sterile needles, suspended in 0.1 ml of sterilized seawater and cultured on LB-seawater agar containing 50 µg ml $^{-1}$ of kanamycin. The total number of kanamycin-resistant colonies was counted and used to calculate the number of transgenic *S. bacillarus* accumulated in each Artemia. Representative colonies were screened by PCR for the DB3 gene.

PCR amplification for the detection of DB3 gene from Artemia gut extracts

The harvested Artemia were washed thoroughly 10-12 times with sterile seawater and viewed under a light microscope to assure that clumps of cyanobacteria were not adhering to the surface. The Artemia were gently homogenized in PBS buffer and 2 µl of extract was

used as template for PCR amplification of the 850bp gene as a doublet encoding DB3 using the oligonucleotide primer sets DB3F 5'-GCACCGCGGGAGC CCAGGTGAAACTGCTG-3' (forward) and DB3R 5'-CCTCGATTGCGGCCGCTTAAC-3' (reverse) (Durvasula et al., 1999). Thermal cycling reactions consisted of an initial denaturation at 94°C for 2 min followed by 32 cycles of denaturation at 94°C for 30s), annealing at 58°C for 30s, and extension at 72°C for 60s), with a single final extension at 74°C for 2 min.

Western blot to detect DB3 protein from Artemia gut extracts

Dissected guts from Artemia were resuspended in 1 ml lysis buffer and placed on ice for 20 min before 3 rounds of sonication (50-60 s duty cycles, 5-6 output). The lysate was centrifuged at 19000 ***g*** for 25 min, 4°C and the supernatant precipitated with saturated ammonium sulfate (Sigma-Aldrich, St. Louis, MO, USA). Protein was recovered by centrifugation at 19000 ***g*** for 25 min at 4°C. The protein pellet was dialyzed into 250 mM Tris-HCl (pH 7.2) with a 7 kDa Slide-A-lyzer (Pierce Biotechnology Inc., Rockford, IL, USA) overnight at 4°C. The dialyzed protein was then concentrated using Nanosep 30K spin columns (Pall Corporation, Ann Arbor, MI, USA). Equal amounts of protein as determined by Bradford assays were separated on 4-16% gradient SDS-PAGE gel (Bio-Rad, Hercules, CA, USA), and transferred to PVDF membrane (Bio-Rad, Hercules, CA, USA). Western blot analyses were performed as described above.

ELISA to detect DB3 protein progesterone binding activity from Artemia gut extracts

High-binding capacity 96-well polystyrene plates (Poly-Sorb, Nunc^{TM}) were coated with 6 µg Progesterone-CMO-BSA conjugate (Fitzgerald Inc., Concord, MA, USA) per well in a carbonate capture buffer (pH 9.5) at 37^{o}C overnight. After three

washes in TBST, the wells were blocked with 2.5% BSA (Fisher Scientific, Pittsburgh, PA, USA) for 1 h at 37°C. Positive control anti-progesterone IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or sample lysates were added in 100 µl TBS-T to triplicate wells and incubated at 37^{o}C for 2 h. The wells were washed as before and 100 µl of a 1:3000 dilution of goat-anti-mouse kappa chain IgG conjugated to Horshradishperoxidase was added (Southern Biotech, Birmingham, AL, USA) followed by incubation at 37^{o}C for 1 h. After a final set of three washes, 100 µl of tetramethylbenzidine peroxidase substrate (Bio-Rad, Hercules, CA, USA) was added to each well and color development to blue was allowed for 2 minutes. Color development was stopped with 100 µl 1mol l $^{-1}$ H_2SO_4 per well and the plates were analyzed on an ELISA plate reader (Molecular Devices, SupectraMAX 250, Sunnyvale, CA, USA) at 450nm.

Recombinant GFP protein dynamics in Artemia gut

The dynamics of a marker molecule within the gut of Artemia was determined in the following wash-out experiment. Artemia, at density of 20 Artemia ml^{-1}, were fed for 5 h with 10^6 CFUml^{-1} *E. coli* expressing GFP. Fifty Artemia were removed from the tank at 0.5, 1, 3, and 5 h for gut extract preparation as described above. The remaining Artemia in the tank was washed with sterile saline and transferred to a tank with no feed organism for another 5 h. Fifty Artemia were removed at 0.5, 1, 3, and 5h for sampling. In the control experiment, Artemia were fed continuously for 10 h with 10^6 CFUml^{-1} *E. coli* Nissle expressing EGFP. During this period, samples of 50 Artemia were removed at 0.5, 1, 3, 5, 8 and 10 h for gut extract preparation as described above. The experiments described were performed in triplicate. GFP was quantified as described below.

ELISA for quantifying rGFP

The rGFP was quantified using a commercial kit (GFP ELISA kit, Catalog #AKR 121, Cell Biolabs Inc, San Diego, USA) with few modifications in the kit protocol. The assay was optimized for the experiment using recombinant GFP spiked in Artemia extract to obtain a standard curve within the linear range of 1-25 ng of rGFP. Briefly, one hundred microlitre aliquots of serially diluted Artemia extracts were bound to the wells of antibody coated assay plates at 37°C for 3 h. After washing three times in wash buffer, the wells were blocked with 2.5% BSA in TBST for 1 h at 37°C. One hundred microlitres of a 1: 3000 dilution of primary anti-GFP antibody was added followed by incubation overnight at 4°C. Following incubation, the wells were washed as before and 100 µl of a 1: 5000 dilution of secondary antibody conjugated to horseradishperoxidase was added. Plates were allowed to incubate at 37°C for 1 h, and after a final set of three washes, 100 µl of tetramethylbenzidine peroxidase substrate solution was added to each well. Colour development was stopped after about 10 min with stop solution. Absorbance at 450 nm was determined on a SupectraMAX 250 Plate Reader.

Quantification of GFP$^+$ *E. coli* Nissle in Artemia gut extracts

Serially diluted Artemia homogenates were plated in triplicate onto LB-carbenicillin$^+$ plates and grown overnight at 37^{o}C. Colony counts were determined from each plate. Representative colonies of the last three LB- carbenicillin$^+$ dilution plates showing growth were isolated and was subjected to colony PCR to confirm *E. coli* Nissle strain using specific primers pMUT2-F (5′-GAC CAA GCG ATA ACC GGA TG-3′) and pMUT2-R (5′-GTG AGA TGA TGG CCA CGA TT-3′) (Blum-Oehler et al., 2003).

Presence of GFP expression from these representative colonies were confirmed by viewing under fluorescent microscope.

Statistical Analyses

The GFP concentrate and transgenic CFU count from the protein dynamics study were subjected to Students t test, and a $p \leq 0.05$ was considered significant. A statistical software GraphPad Prism was used (GraphPad Software, Inc., San Diego, CA) to perform this analysis.

RESULTS

GFP and DS Red protein in the gut of paratransgenic Artemia

Fig.2b shows by fluorescence microscopy that GFP-expressing *E. coli* accumulated in the gut of Artemia following 30 min of feeding; no fluorescence was observed in control Artemia that were fed on wild-type *E. coli* for up to 6 h (**Fig. 2a**). Fluorescence increased following 4 h and 10 h of feeding suggesting an accumulation of green fluorescent protein over time (**Fig. 2b, c, d**). Our data would suggest that recombinant proteins can accumulate in Artemia gut and retain biological activity. We then tested whether multiple protein products, expressed simultaneously by different bacterial populations, could accumulate in the gut of the Artemia via this method. For this, we fed Artemia with two strains of transgenic *E. coli* expressing GFP and DS Red. Fluorescent images taken at 6 h of feeding show that both GFP and DS Red accumulated in the Artemia gut (**Fig. 2f**).

DB3 protein from transgenic *S. bacillarus* lysates

S. bacillarus transformed with DB3 gene was used for our subsequent studies on retention and bioactivity of recombinant protein products in the gut of Artemia. Western

analysis revealed a 42 kDa protein of murine origin that was expressed by transformed *S. bacillarus*; wild-type *S. bacillarus* did not elaborate this protein (**Fig. 3**).

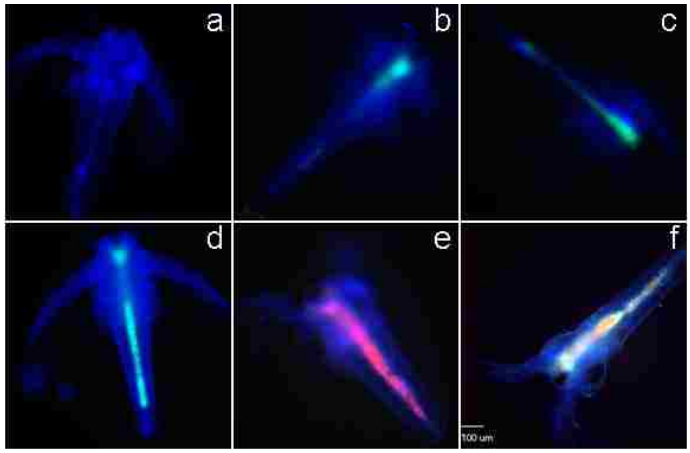

Figure 2. Fluorescent microscopy shows increased accumulation of recombinant proteins in a time dependent manner in 2nd instar nauplii of Artemia fed with fluorescent-protein expresssing *E. coli*. **a)** Artemia fed with wild type *E. coli* after 6 h of feeding **b)** Artemia fed with GFP-expressing bacteria after 30 min of feeding **c)** 4 h of feeding **d)** 10 h of feeding **e)** Artemia fed with DS Red expressing bacteria after 6 h of feeding **f)** Artemia fed simultaneously with both GFP- expressing bacteria and DS Red-expressing *E. coli* after 6 h of feeding.

Quantification of kanamycin-resistant *S. bacillarus*

The gut of second instar Artemia nauplii contained 4.2 x 10^4 CFU of recombinant *S. bacillarus* after 2 h of feeding. The number of CFU peaked to 1.5 x 10^5 at 8 h of feeding, followed by a slow decrease to 4.9 x 10^3 CFU after 20 hours of feeding (**Fig. 4**).

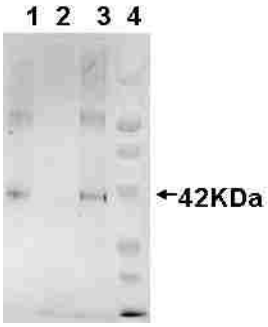

Figure 3. rDB3 expression in *S. bacillarus* transformed with pRrMDKW6. Lane 1, lysate from Transgenic *S. bacillarus* cells for DB3 expression; Lane 2, lysate from wild type *S. bacillarus* cells; Lane 3, recDB3 protein (purified from *E. coli* as positive control); Lane 4, Trichrom ranger TM Molecular marker.

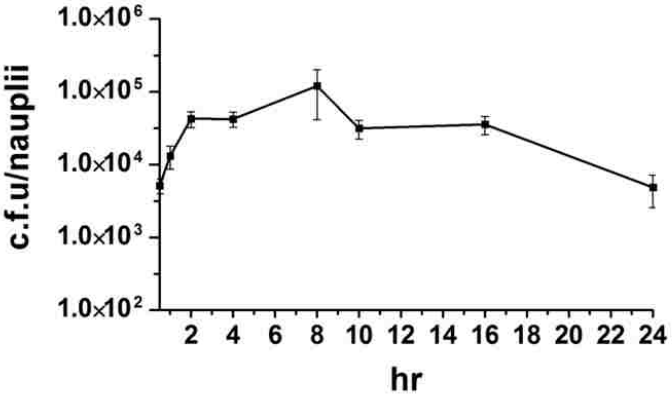

Figure 4. Total colony forming units (CFU) of transgenic *S. bacillarus* (kanamycin resistant colonies) from Artemia gut extracts fed with transgenic *S. bacillarus* at progressive time intervals. Values are means ± S.E. of three feeding tanks per treatment.

DB3 gene from Artemia gut extracts

PCR amplification was used to detect the 850 bp rDB3 murine Vγ/Vκ gene from Artemia fed with transgenic *S. bacillarus*. **Fig. 5** shows the presence of the DB3 gene from gut extracts of Artemia fed with transgenic *S. bacillarus*. The 850 bp product was absent from gut extracts of Artemia fed with wild type *S. bacillarus*.

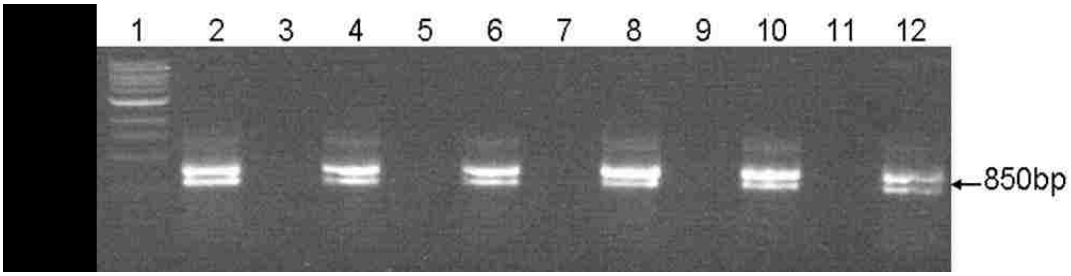

Figure 5. PCR amplification for the detection of DB3 gene from Artemia gut extracts. Lane 1, 1 Kb DNA ladder. Lane 2, positive control (pRrMDWK6). Lane 3, 5, 7, 9 and 11 represents PCR products from Artemia gut extracts fed with wild type *S. bacillarus* after 2, 4, 8, 10 and 20 h after feeding. Lane 4, 6, 8, 10 and 12 represents PCR products from Artemia gut extracts fed with transgenic *S. bacillarus* after 2, 4, 8, 10 and 20 h after feeding.

Detection of DB3 protein in gut of paratransgenic Artemia

Gut extracts were prepared from harvested Artemia samples to detect expression of the rDB3 antibody. Western blot analysis using a goat anti-mouse kappa antibody detected a band of 42 kDa in gut extracts of Artemia fed recombinant cyanobacteria following 2 h of feeding (**Fig. 6**). The size and murine origin of the protein in the paratransgenic Artemia confirms that it is recombinant DB3 antibody. Protein densitometric analysis of the blot also showed a progressive increase in amount of rDB3 antibody from 2 h to 8 h of feeding and a small decrease thereafter (not shown). Gut extracts of Artemia fed with wild type *S. bacillarus* did not display any signal with the anti-mouse antibody.

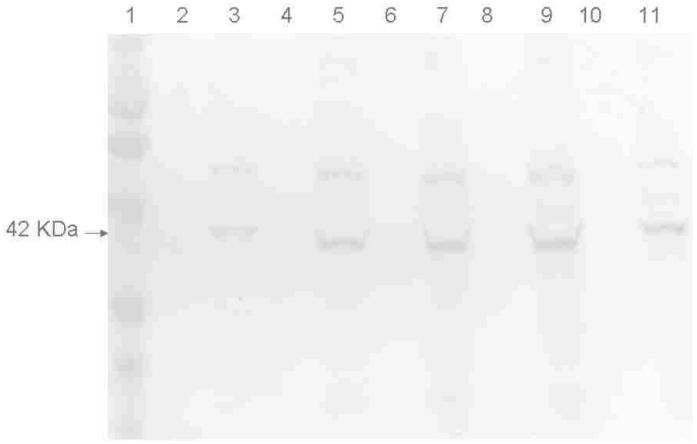

Figure 6. Western blots to detect DB3 protein from Artemia gut extracts. Lane 1, Trichrom ranger ™ Molecular marker. Lane 2, 4, 6, 8, and 10, Artemia fed wild type *S. bacillarus* and harvested 2, 4, 8, 10 and 20 h after the commencement of feeding. Lane 3, 5, 7, 9 and 11, Artemia fed transgenic *S. bacillarus* and harvested 2, 4, 8, 10 and 20 h after the commencement of feeding.

DB3 protein progesterone binding activity from the gut of paratransgenic Artemia

Artemia gut extracts were used for ELISA on plates coated with progesterone 3-(O carboxymethyl) oxime-BSA conjugate. Gut extracts of paratransgenic Artemia demonstrated binding to progesterone-BSA, with values increasing from 0.12 to 0.29

O.D. between 2 and 10 hours of feeding (**Fig. 7**). Gut extracts of Artemia that were fed wild type *S. bacillarus* yielded a background of ~0.07 O.D. which was not significantly different from extracts isolated from unfed Artemia (data not shown). Extracts from paratransgenic Artemia showed a twofold increase in progesterone binding activity from 2 h to 6 h of feeding and the binding activity continued to increase to at least 10 h of feeding. This suggests that breakdown of cyanobacteria in the gut of paratransgenic Artemia continued for several hours, with accumulation of functional rDB3 antibody for 10 h of feeding.

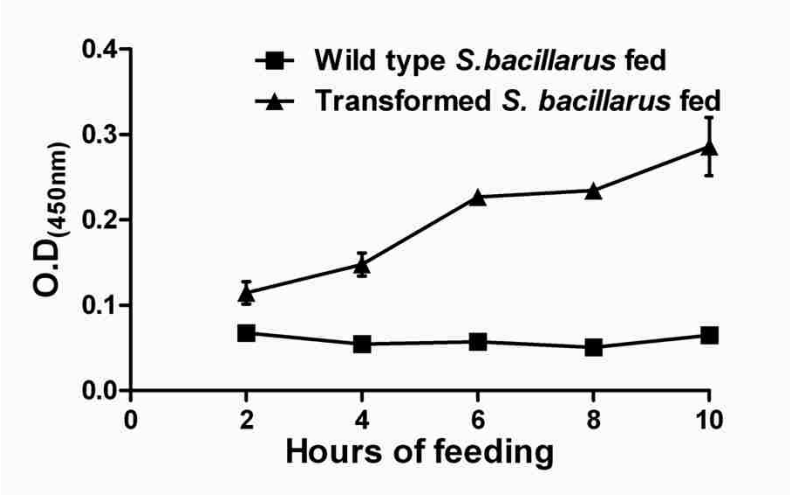

Figure 7. ELISA demonstrating progesterone-binding activity of gut extracts of Artemia fed with DB3-producing *S. bacillarus* or wildtype *S. bacillarus*. Values are means ± S.E. of three feeding tanks per treatment.

GFP protein dynamics in the Artemia gut

To study dynamics of a recombinant protein in Artemia gut we quantified the accumulation of rGFP via ELISA. In Artemia that were fed continuously for 10 h, gut concentration of the recombinant protein reached a peak value of 12.4 ± 0.3 ng rGFP g^{-1} after 8 hours. At 10 hours, a slight decrease was noted to 10.4 ± 0.3 ng rGFP g^{-1} (**Fig. 8A**). The transgenic bacteria accumulation in Artemia gut also peaked at 5 h in continous

feeding and thereafter the level remained more or less same (**Fig. 8B**). In the complementary trial in which Artemia were fed continuously for 5 h followed by a 5 h wash-out period, gut concentration of the recombinant protein reached a peak value of 9.2 ± 0.7 ng rGFP g^{-1} at 5h. After 30 minutes of transfer to the wash-out phase, recombinant protein concentration significantly decreased to 7.3 ± 0.6 ng rGFP g^{-1} followed by a sharp decrease to 3.7 ± 0.4 ng rGFP g^{-1} and 2.6 ± 0.4 ng rGFP g^{-1} at 8h and 10 h, respectively. (**Fig. 8A**). The transgenic bacteria accumulation in Artemia gut showed a sharp decline during the 5 h wash-out period (**Fig. 8B**).

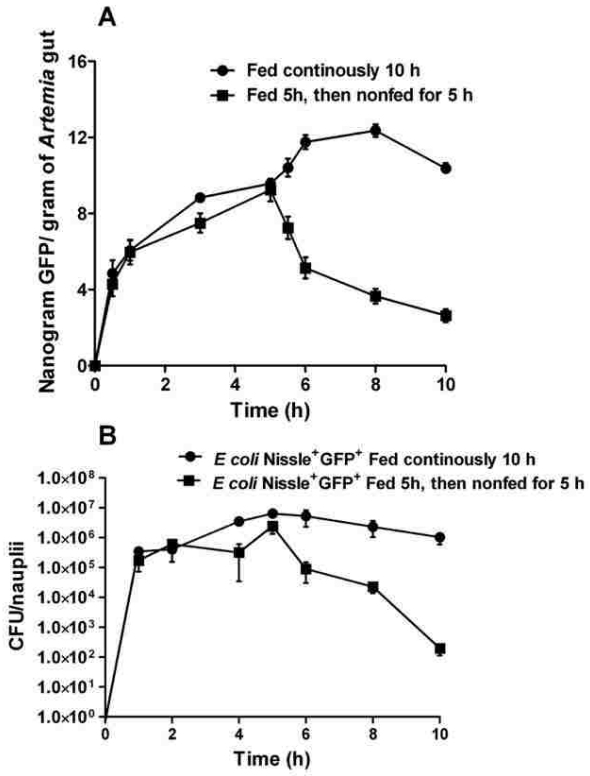

Figure 8. A) Recombinant GFP accumulation in Artemia gut. Animals were either fed continuously for 10 h or fed for 5 h followed by a 5 h washout period during which no feed organisms were given. **B)** Total colony forming units (CFU) of transgenic *E. coli* $^{+}$carbenicillin^{+} from Artemia gut fed continuously for 10 h or fed for 5 h followed by 5 h washout. Values are means ± S.E. of three feeding tanks per treatment.

DISCUSSION

Here we report a modified paratransgenic approach with the brine shrimp, *Artemia franciscana*. Using genetically transformed variants of *E. coli* Nissle and *S. bacillarus*, two feed organisms of shrimp, we have demonstrated delivery, retention and biological activity of recombinant proteins in the gut of Artemia. In this study, a non-

pathogenic *E. coli* is used as a platform for expression of fluorescent marker proteins in the gut of Artemia. Stable expression of these fluorescent markers, GFP and DS Red, throughout 10 hours of continuous feeding suggests that recombinant proteins can be delivered effectively to nauplii of Artemia and retain their biological activity. Furthermore, co-expression of multiple markers simultaneously in the gut of Artemia confirms that paratransgenic manipulation of these animals for delivery of a battery of molecules with activity against pathogens is possible. This strategy will be quite significant in settings of drug-resistant pathogens where concurrent delivery of molecules that act at different target sites of the pathogen would slow the emergence of resistant species.

Though the role of Artemia as a food source for larger commercially important animals such as shrimp has been evaluated (Chair et al., 1996; Dhont and Sorgeloos, 2002), no studies have considered engorging Artemia with recombinant protein-expressing bacteria or algae for delivery to shrimp. Our studies showed that Artemia nauplii fed to engorgement with antibody-producing cyanobacteria retained functional recombinant protein. Western blot analysis confirmed that the murine antibody, rDB3, was present in gut extracts of Artemia that engorged on genetically altered cyanobacteria after just two hours of feeding. The expected band at 42 kDa that was recognized by a goat anti-mouse kappa chain IgG represents the recombinant DB3 molecule. Additional bands seen at 70 kDa and above in the gut extracts of paratransgenic Artemia only could represent multimeric covalent assemblies of VH and kappa regions that were recognized by the anti-mouse kappa chain antibody (He et al., 1995; Durvasula et al., 1999). No proteins in the gut extracts of Artemia that were fed with wild-type cyanobacteria were

recognized by the anti-mouse kappa chain antibody, indicating that expression in paratransgenic Artemia was attributable to the engineered cyanobacteria.

Recombinant *S. bacillarus* reach a maximum of approximately 10^5 CFU in the gut of Artemia at 8 h of feeding suggesting that maximum engorgement occurs at this point. Several factors may account for the decrease in recombinant *S. bacillarus* CFU after this time point. A reduction in the bacterial load may be due to the degradative properties of Artemia gut. The absence of kanamycin selection in the Artemia tank raises the possibility of plasmid decay. Populations of recombinant *S. bacillarus*, carrying the pRrMDWK6 plasmid, could have reverted to the wild-type genotype resulting in steady decrease in CFU of recombinant cyanobacteria. However, replica plating of samples of Artemia gut taken beyond the 8 hour time point, did not reveal a significant shift from the transformed to wild-type cyanobacteria. Alternatively, depletion of the transgenic cyanobacteria from the Artemia tank could have occurred after 8 hours, though colony counts of cyanobacteria in the tank water remained stable, suggesting ongoing replication. Colony counts at 8 hours of feeding most likely represented the homeostatic threshold of these bacteria in Artemia gut. Despite the slight decrease in cyanobacterial CFU after 8 hours, maximum progesterone-binding activity from Artemia gut was noted at 10 hours of feeding, suggesting accumulation of the recombinant antibody.

The dynamics of recombinant protein expression and accumulation in the gut of Artemia were studied using a probiotic strain of *E. coli* Nissle (Grozdanov et al., 2004) transformed to express GFP. In this experiment we addressed the question of whether transformed feed bacteria and their recombinant protein products would be retained in the gut of Artemia in the absence of a continuous supply of feed organisms. Whereas

Artemia subjected to a 10 hour continuous feed with GFP-producing *E. coli* demonstrated accumulation of both bacteria and protein to a threshold value, animals that were transferred from a feed source to a new environment without transgenic feed bacteria exhibited very rapid loss of both GFP-producing bacteria and the protein product within 5 hours. Indeed, these results suggest that the Artemia gut is a conduit through which recombinant feed organism's transit. The majority of foreign protein production in this type of modified paratransgenic system is therefore related to molecules that are continuously synthesized by feed organisms that have been transformed with constitutive promoter elements. A small number of transgenic *E. coli* and their recombinant GFP products remained in the gut of Artemia 5 hours after the animals were transferred to an environment with no added feed. However, the relative role of feed bacteria that actually establish in the gut of these animals and produce molecules *in situ* appears to be minimal. Under commercial settings such as shrimp hatcheries, Artemia can readily be fed to engorgement with bacteria or algae, with subsequent delivery to tanks of shrimp within 6 to 8 hours. The rapid accumulation of foreign proteins over this time period indicates that this approach could have utility in mariculture operations.

Taken together, the data showing co-delivery of multiple populations of recombinant bacteria and establishment of paratransgenic Artemia capable of accumulating functional antibodies illustrate that a paratransgenic model can be developed to express foreign proteins in the gut of Artemia, through commonly deployed feed algae and bacteria. Our feeding trials were conducted under sterile and controlled culture conditions, which do not reflect hatchery settings for rearing Artemia. The competition from environmental and microbial flora of the Artemia itself could greatly

impact abundance of recombinant bacteria in the gut of paratransgenic brine shrimp, thus decreasing the accumulation of recombinant proteins. Effects of such competition should be studied further in controlled laboratory trials. In commercial hatcheries, feeding of Artemia to shrimp larval stages occurs when there is sufficient yolk sac associated with egg cysts to render superior nutritional quality to the shrimp. We used 2nd instar nauplii for our feeding trial with completely depleted yolk sac nutrients, which were thus forced to graze on cyanobacteria. The potential effects of these more mature paratransgenic Artemia on nutrition and growth of target shrimp larval stages should also be studied.

Expression of the rDB3 antibody that binds progesterone, serves as a proof-of-concept and has no immediate application toward infectious pathogens of mariculture. However, it is an important precursor to ongoing studies directed at expression of molecules with potent anti-viral and anti-Vibrio activity. Substitution of the DB3 gene with genes encoding anti-bacterial and anti-viral proteins, with subsequent delivery of paratransgenic Artemia to shrimp could offer new methods of defense against infections. The cecropins, penaeidin, mellitin and moricin are cationic antimicrobial peptides which exhibit very high lytic activity against gram negative bacteria (Destoumieux et al., 2000; Zasloff, 2002; Chiou et al., 2005). These molecules may eventually be expressed in shrimp probiotic bacteria (e.g. *Bacillus subtilis*) and algal feed organisms (e.g. *S. bacillarus* and *Dunaliella salina*) to control vibriosis in intensive shrimp aquaculture. Similarly, shrimp viral diseases might potentially be controlled via the modified paratransgenic approach. Identification and cloning of antiviral peptides such as PmAV (*Penaeus monodon* antiviral) have been reported from white spot virus-resistant *P. monodon* (Luo et al., 2007). A high affinity monoclonal antibody (MAb 216) was

developed against VP28, the anchor protein of white spot virus that facilitates viral entry to the cytoplasm (Yi et al., 2004). Studies showed 80% survival benefits in white spot virus-infected shrimp that were pre-treated with MAb 216 (Dante et al., 2007). Engineered lines of *S. bacillarus, D. salina* and *B. subtilis* expressing PmAV and anti-VP28 antibodies that are delivered to *Artemia* and, possibly, directly to *P. monodon* might be a viable strategy to protect commercial shrimp against White Spot Syndrome Virus infection.

Recombinant protein molecules and transgenic organisms might pose environmental risks. Our laboratory had been engaged in risk assessment studies of paratransgenic technologies for years. We have undertaken a comprehensive program to evaluate toxicities of recombinant bacteria and potential for horizontal gene transfer. These studies are requisites for approval by regulatory agencies and will facilitate eventual application of paratransgenic technologies (Durvasula et al., 2007).

Technology aimed at development of paratransgenic shrimp which are refractory to infectious pathogens under commercial hatchery and grow-out conditions is still at a very early stage of development. Nevertheless, the ease with which common feed organisms of farmed marine animals can be genetically engineered and delivered, either directly or via an Artemia intermediate, suggests that this approach could eventually be deployed in the commercial setting. Thus, the paratransgenic approach offers potential to reduce the global burden of infectious diseases in commercial mariculture and improve global food supply.

ACKNOWLEDGMENTS

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CHAPTER 3

DEVELOPMENT OF A PARATRANSGENIC SHRIMP (*LITOPENAEUS VANNAMEI*)

This chapter is based on the following article:

Subhadra, B., 2011. Development of a Paratransgenic Shrimp (*Litopenaus vannamei*). *Letter in Applied Microbiology (under preparation)*

SUMMARY

Novel paratransgenic approaches are being developed by our laboratory to control the crippling effects of infectious diseases on mariculture. In this method, Artemia engorged with recombinant protein expressing bacteria are fed to shrimp to accumulate biologically active anti-infectious molecules. As a continued effort to further develop this approach, we report results with recombinant protein marker molecules as a proof-of-concept, aimed at development of paratransgenic white shrimp, *Litopenaeus vannamei*, and a practical method of delivery of recombinant proteins to post-larval (PL) stages of *L. vannamei*. Three feeding protocols aimed at delivery of recombinant proteins to *L. vannamei* PL stages were conducted. Trial 1, which was designed to evaluate the accumulation of an engineered antibody in the gut of *L. vannamei*, consisted of an 18 day active feeding phase followed by 12 d wash-out phase. *L. vannamei* gut extracts showed a progressive increase in signal of the recombinant antibody, DB3, from day 2 of feeding to day 21. Rapid decrement in signal was noted on day 22; this reached undetectable levels on day 30. In trial 2 and 3, which were designed to determine the dynamics of recombinant GFP within the gut of paratransgenic *L. vannamei* fed with GFP expressing bacteria engorged Artemia or fed directly with GFP-expressing EcN bacteria, accumulation of GFP in shrimp was tightly coupled to periods of active feeding with rapid depletion of recombinant molecules during a non-feeding wash-out phase. We found no significant difference in the GFP levels in the shrimp fed with 20 or 40 Artemia per shrimp per day. However, during initial feeding hours, a higher level of recombinant GFP was seen in shrimp gut fed with Artemia engorged with transgenic bacteria expressing GFP when compared to shrimp fed with transgenic bacteria. Here, we report,

a practical paratransgenic delivery of bioactive recombinant molecules to the commercial white shrimp, *L. vannamei*. Delivery of neutralizing-antibodies, cationic peptides, and antivirals to shrimp by this approach has a great potential to control diseases in shrimp mariculture.

INTRODUCTION

Recenly proposed marine paratransgenic approach to control shrimp diseases involves delivering anti-infectious molecules such as antibodies and antivirals to shrimp via Artemia and feed organisms such as algae and probiotic bacteria (Durvasula et al., 2006), Our initial studies demonstrated that Artemia carrying engineered lines of bacteria and algae accumulated recombinant proteins, which retained their biological activity for up to 10 h (Subhadra et al., 2010). As a continued effort to deliver recombinant proteins to shrimp for functional purposes, in the present study, we report results of three shrimp feeding trials aimed at (1) develoment of paratransgenic white shrimp, *Litopenaeus vannamei*, with marker molecules as a proof-of-concept (2) determining a practical method of delivery of recombinant proteins to post-larval (PL) stages of *L. vannamei.* Using the single chain antibody (DB3) (Subhadra et al., 2010), we demonstrate that the paratransgenic strategy can be used to accumulate biologically active molecules such as recombinant antibodies in the gut of *L. vannamei*. Furthermore, we show that recombinant Green Fluorescent Protein (GFP) can be delivered effectively to the gut of *L. vannamei* via direct feeding with engineered probiotic *Escherichia coli* Nissle (EcN) strain or via engorgement with paratransgenic Artemia that carry the recombinant bacteria. Ultimately, delivery of molecules with activity against infectious pathogens to farmed shrimp could offer new approaches to control of lethal outbreaks in this industry.

MATERIALS AND METHODS

Bacterial cultures, Artemia and Shrimp

Escherichia coli Nissle 1917 (EcN) is a nonpathogenic *E. coli* strain that has been characterized extensively at the phenotypic and molecular genetic level (Blum-Oehler et al., 2003; Grozdanov et al., 2004). EcN has evolved into one of the best characterized probiotics with its therapeutic efficacy and safety has convincingly been proven (Kruis, 2005; Henker et al., 2007). EcN also has remarkable colonization characteristics in the gut of diverse organisms (Westendorf et al., 2005). EcN was provided by Ardypharm (Ardeypharm GmbH, Germany). EcN expressing GFP and the progesterone binding single chain antibody, DB3, were generated as described in Chapter 2. All experiments were performed with premium grade eggs of *Artemia franciscana* (BrineShrimp Direct, Ogden, UT). *L. vannamei* PL (stages 10-12) were obtained from commercial shrimp seed hatcheries (Harlington Shrimp Farm, Las Fresco, TX or Earthcare Aquaculture Inc., Weston, FL).

Feeding trial designs

Three shrimp feeding protocols were performed. The shrimp were acclimated for 5 days before the feeding trials to ensure adequate health. Two hundred shrimp averaging 4.5±0.03g in mass were selected and stocked in each of three 20-l tanks. Artemia were hatched axenically and at the second naupliar stage were engorged with wild-type or transgenic EcN expressing DB3 in engorging flasks at densities of 10^6 CFU ml^{-1}. Trial 1 was a 30 d feeding trial designed to evaluate the tunable accumulation of an engineered antibody in the gut of *L. vannamei*. It consisted of two phases. In phase 1, shrimp were fed twice daily with paratransgenic Artemia that carried DB3-expressing EcN for 18 d.

Shrimp were fed with 20 Artemia per shrimp per day (total of 4,000 Artemia per tank per day). Phase 2 was a wash-out phase in which Artemia that carried wild-type EcN were fed to shrimp for days 19 to 30. On days 1-5, a total of 12-15 shrimp PL's were sampled; subsequently, 5-6 PL's were sampled on days 9, 12, 15, 19, 22, 25, 27, and 30. Dissected gut contents from Artemia and shrimp were suspended in 1 ml lysis buffer (100 mM Tris–Cl, 500 mM NaCl, 0.5 M EDTA, 0.1% TritonX-100, 0.1% Tween-20, 8% glycerol, 250 mM urea, 5 mM beta-2 mercaptoethanol, 100 µg ml $^{-1}$ PMSF, 1 µg ml^{-1} protease inhibitor cocktail, 50 µg ml^{-1} lysozyme) and placed on ice for 20 min before three rounds of sonication. The lysate was centrifuged at 19 000 g for 25 min at4ºC, and the supernatant precipitated with 80% saturated ammonium sulfate (Sigma-Aldrich). Protein was recovered by centrifugation at 19 000 g for 25 min at 4ºC. The protein pellet was dialyzed into 250 mM Tris–HCl (pH 7.2) with a 7 kDa Slide-A-lyzer (Pierce Biotechnology Inc., Rockford, IL, USA) overnight at 4ºC. The samples were flash frozen in liquid nitrogen and stored at -80 ºC for later use in ELISA. ELISA to detect DB3 and GFP quantification in gut extracts were conducted as described in Chapter 2.

Trial 2 was performed to determine the dynamics of recombinant GFP within the gut of paratransgenic *L. vannamei*. Shrimp were fed (20 or 40 Artemia/PL/d) engorged with GFP-expressing EcN for 7 days followed by a 5 day wash-out phase during which Artemia carrying wild-type EcN were used as feed. Shrimp PL's (n=4) were sampled on days 1, 3, 5, 6, 7, 8 and 12 and shrimp gut extracts were prepared.

Trial 3 was designed to determine the dynamics of a recombinant GFP within the gut of paratransgenic shrimp that were fed directly with GFP-expressing EcN at various

concentrations (10^4, 10^5, and 10^6 colony-forming units ml^{-1}) for 7 days followed by a 5 day wash-out phase during which wild-type EcN were used as feed.

Bacteriological assays

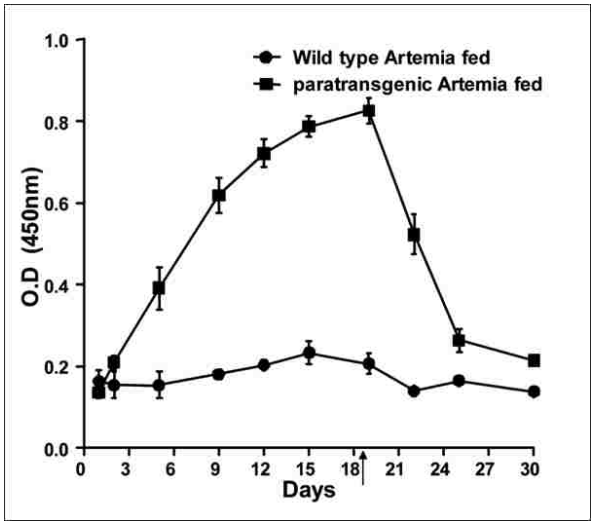
Shrimp samples were dissected using sterilized scissors to remove mid- and hind-gut and weighed. To avoid possible external contamination while removing organs, the surface of the shrimp was cleaned using 70% ethanol and allowed to dry. Shrimp gut tissues were suspended in 1 ml PBS and the tissue was disrupted using sterile needles before final re-suspension in 10 ml of sterile PBS. Bacteriological determination involved serial dilutions of this suspension in PBS followed by plating in triplicate on LB and LB supplemented with 50 µg ml^{-1} of kanamycin and carbenicillin to determine total cultivable heterotrophic bacteria and transgenic *E. coli*, respectively. After overnight incubation, colonies were counted and results were presented as CFU per shrimp. Representative colonies were subjected to colony PCR using specific primers for both DB3 forward primer:
5’-GCACCGCGGGAGC CCAGGTGAAACTGCTG-3’ and reverse primer: 5’-CCTCGATTGCGGCCGCTTAAC-3’) and EcN forward primer: 5’-GAC CAA GCG ATA ACC GGA TG-3’ and reverse primer: 5’-GTG AGA
TGA TGG CCA CGA TT-3’). For qualitative bacterial estimation, thirty to thirty-five random colonies were picked from bacterial plates and were gram stained. These clones were also subjected to PCR for GFP gene-specific primer set (GFP-Forward 5’TCTGTCAGTGGAGAGGGTGA-3’; GFP-Reverse, 5’TCCATGCCATGTGTAATCCC 3’) and EcN-specific primers sets.

Statistical analyses

To determine statistically significant differences in concentration of GFP in shrimp fed 20 or 40 Artemia per shrimp per day, we used the Students 't' test. Statistically significant differences in GFP concentration in shrimp directly fed with 10^4, 10^5 or 10^6 CFU ml^{-1} transgenic EcN were determined by one-way ANOVA test. When significant differences ($P \leq 0.05$) existed, the means were compared using the Newman-Keuls multiple comparison test. GraphPad Prism was used (GraphPad Software, Inc., San Diego, CA) to perform statistical analyses.

RESULTS

In Trial 1, in which *L. vannamei* were fed paratransgenic Artemia engorged with DB3-expressing EcN for 18 d, *L. vannamei* gut extracts showed a progressive increase in signal of the recombinant antibody, DB3, from day 2 of feeding to day 21. Rapid decrement in signal was noted on day 22, four days after the start of the washout period; this reached undetectable levels on day 30 (**Fig. 9**). Bacteriology of the same gut extracts



showed a progressive increase in CFU counts of DB3 producing- EcN during the active feeding phase with a sharp decrease during the wash-out phase. CFU counts of transgenic EcN reached a maximum in the shrimp gut on day 19 (3.8 x10^6 CFU $gram^{-1}$) in *L. vannamei* fed paratransgenic Artemia that were engorged with the transgenic bacteria (**Fig. 10A**). Gut extracts of *L. vannamei* fed Artemia that were engorged with wild-type EcN showed no change in CFU counts of transgenic bacteria (**Fig. 10B**).

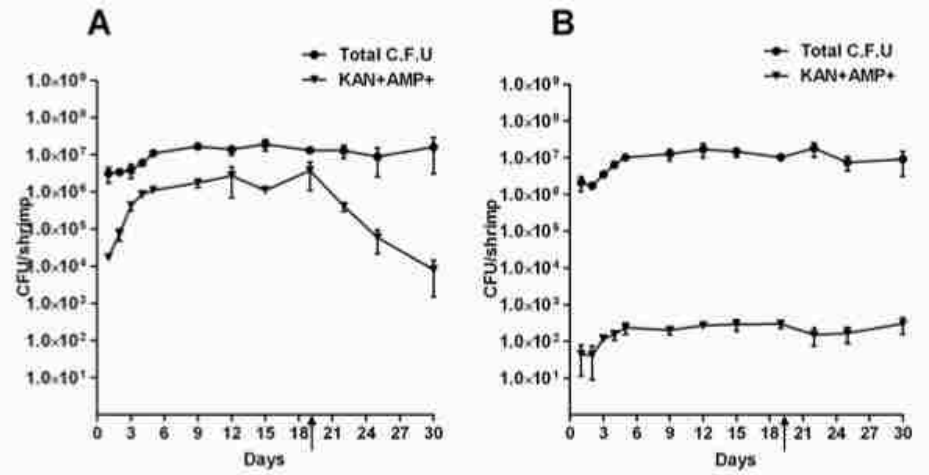


Figure 10. Total colony forming units from the shrimp gut fed with paratransgenic Artemia during 30 d feeding according to the trial 1 protocol. Total CFU and Kan^+Amp^+CFU in shrimp fed transgenic *E. coli* engorged Artemia (**A**). Total CFU and Kan^+Amp^+ CFU in shrimp fed wildytype *E. coli* engorged Artemia **(B**). The arrow (d 19) indicates the start of wash-out period.

To study the quantitative transfer and bioaccumulation of proteins in shrimp gut via Artemia, we conducted Trial 2 and quantified the accumulation of GFP-expressing EcN via ELISA in both *Artemia* and *L. vannamei* gut. Accumulation of GFP in Artemia gut was noted during the 10-hour feeding period. After 2 h, a concentration of 4.7 ± 1.2 ng GFP per gram of Artemia gut was achieved, which then reached a peak value of 12.5 ±

1.6 ng GFP g^{-1} after 8 hours. At 10 hours, a slight decrease was noted to 10.4 ± 0.3 ng GFP g^{-1} (**Fig. 11**).

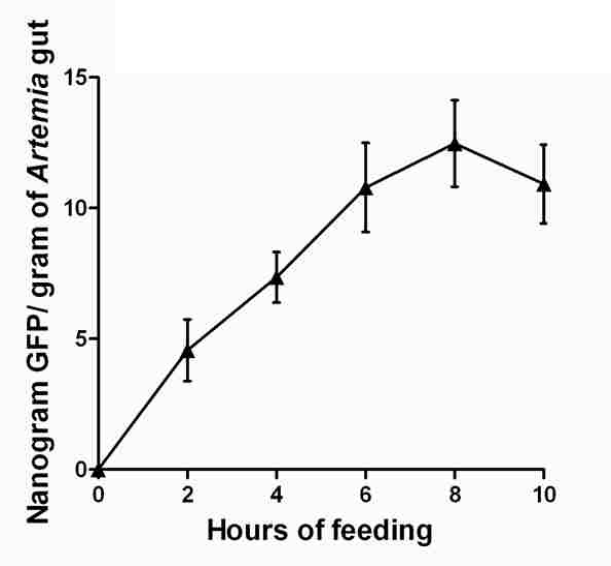


Figure 11. Quantity of GFP in the paratransgenic Artemia gut fed with transgenic GFP expressing *E. coli* fed at a rate of 10^6 CFU ml^{-1} for 10 h.

We then fed to shrimp the Artemia engorged with transgenic EcN (6-8 h of engorgement) at a rate of 20 or 40 Artemia per shrimp daily for 7 days followed by a 5 day wash-out. We quantified the GFP content per gram of shrimp gut by ELISA. Shrimp PL's fed with 20 Artemia per day accumulated 36.3 ± 2.8 ng GFP per gram of shrimp gut on day 5. Subsequently, GFP levels declined and at the last day of the wash-out phase (day 7), levels dropped to 10.2 ± 0.8 ng GFP g^{-1} (**Fig. 12**). *L. vannamei* PL's fed with 40 Artemia per day accumulated 41.6 ± 0.8 ng GFP g^{-1} on day 3 (**Fig. 12**). On the last day of the wash-out phase, levels decreased to 11.9 ± 0.9 ng GFP g^{-1}. Although our statistical analysis shows that GFP levels in shrimp fed 20 or 40 Artemia per day were not significantly different, the shrimp fed 40 Artemia per day accumulated 10-15% more GFP during the initial 3-4 h of feeding.

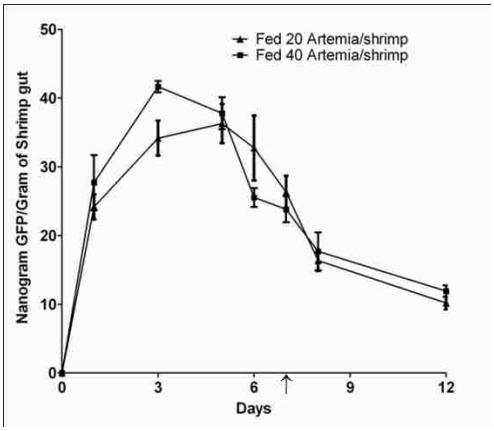


Figure 12. Quantity of GFP in the shrimp gut fed daily with paratransgenic Artemia (at a rate of 20 or 40 Artemia per day) engorged with GFP expressing *E. coli*. The data are averages ± SEM from two treatments. The arrow (d 7) indicates the start of wash-out period.

In Trial 3, we fed transgenic bacteria (10^4, 10^5 and 10^6 CFU ml^{-1}) directly to shrimp to quantify GFP accumulation. In shrimp fed 10^6 , 10^5 and 10^4 CFU ml^{-1}, the shrimp gut accumulated 32.2 ± 0.7, 19.2 ± 1.2 and 13.7 ± 1.6 ng GFP per gram shrimp gut, respectively. By day 6 and thereafter there was a slow decline in the GFP content to the last day of the wash-out (**Fig. 13**).

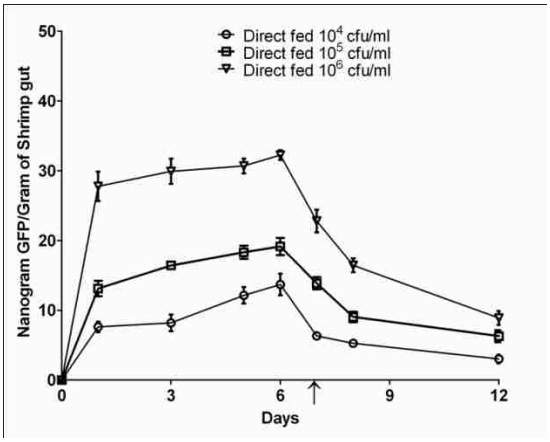


Figure 13. Quantity of GFP in the shrimp gut fed directly with GFP-expressing transgenic *E. coli* at 10^4, 10^5, or 10^6 CFUml^{-1}. The arrow indicates the beginning of wash-out phase. Values are represented mean ± SEM of three parallel treatments.

Pair wise comparison of GFP content from shrimp fed with10^4 CFU ml^{-1} and 10^5 CFU ml $^{-1}$was not significantly different at any timepoint. However, GFP accumulation in shrimp fed with 10^6 CFU ml^{-1} was significantly higher than in shrimp fed with 10^4 CFU ml^{-1} or 10^5 CFU ml^{-1}. However, during the intial feeding days (d 3), shrimp gut accumulated higher levels of rGFP (~40 ng GFP g^{-1}) via feeding through Artemia that

were engorged with rGFP-expresssing bacteria than feeding directly with transgenic bacteria at a rate of 10^6 CFU ml^{-1} (~30 ng GFP g^{-1}).

We also assessed the bacterial flora in shrimp before and during the period of feeding with transgenic bacteria. Our results show that almost 97% of *L. vannamei* gut bacteria after 6 d of active feeding were transgenic, with both genotypic and phenotypic evidence of carbenicillin resistance and GFP production (**Table 2**). Therefore, active feeding using transgenic bacteria can easily displace the inherent native flora in the shrimp gut. However, at the end of the washout phase, the transgenic bacteria represent only 35% of bacterial flora suggesting the interference of other bacterial flora in the shrimp gut.

Analysis Day (colonies analyzed)	**[1]EcN^+Car^+ GFP^+ (%)**
0 (30)	0
1 (35)	80
6 (35)	97
12 (34)	35

[1]Car^+ = colonies resistant to carbenicillin
EcN^+Car^+ GFP^+ represents the transgenic bacteria fed to shrimp

DISCUSSION

To optimize the delivery of recombinant proteins to shrimp larval stages, we conducted a long term feeding trial for 30 days in which first 18 days we fed Artemia that were engorged with transgenic EcN expressing DB3, a marker antibody followed by 12-day washout period. Our results showed that both transgenic bacteria and antibodies

accumulated in the shrimp gut during the active feeding phase. However, the recombinant proteins as well as transgenic bacteria depleted during the wash out phase suggesting a tunable recombinant accumulation system. Hence, we report a practical paratransgenic delivery of bioactive recombinant molecules to the commercial white shrimp, *L. vannamei*. Using a bioamplification pathway derived from natural aquatic food systems, we have demonstrated the accumulation of both a marker molecule and a functional antibody in the gut of *L. vannamei*, the site of transmission of many marine pathogens, such as Vibrios. This approach establishes a foundation for future applications that deploy anti-microbial peptides and anti-viral antibodies in settings of commercial mariculture.

As there was a possibility of directly using transgenic bacteria for shrimp feeding purpose, we also determined the protein accumulation in shrimp larval stages fed directly with transgenic bacteria compared to shrimp fed with Artemia engorged with the transgenic bacteria in short-term feeding trials. Our results show that shrimp larvae had higher recombinant protein accumulation that was fed with Artemia engorged with recombinant proteins during the initial 3-4 days of feeding. This trend was more obvious with shrimp that were fed with 40 Artemia per day. Since in commercial shrimp hatcheries the normal Artemia feeding rate is 10-50 Artemia per shrimp per day, the paratransgenic protein delivery using Artemia can be adapted to shrimp hatchery operation.

In shrimp hatcheries, the infection due to Vibrio strikes at early stage PL's, 3-5 days post moulting (Brock and Lightner, 1990). The accumulation of recombinant anti-vibrio molecules or neutralizing antibodies in shrimp larvae against Vibrio in the first 3-4

days using Artemia delivery might be a viable and practical method to reduce the Vibrio infection in shrimp larval stages. In Artemia fed and in direct transgenic bacteria fed PL shrimp, the levels of recombinant proteins in shrimp gut tended to decrease before the wash-out phase began, suggesting a mechanism of degradation. The shrimp larval stages develop an active digestive system during the phase between PL15 to juvenile stage. These ontogenetic events are accompanied by significant changes in metabolic rates and digestive enzyme activities (Lovett and Felder, 1990; Lemos et al., 1999). The digestive system consists of highly active proteinase enzymes with proteolytic function for degrading food particles (Lemos et al., 2000). The depletion in recombinant protein level in shrimp gut during the 5^{th} day might be due to activitiy of shrimp digestive enzymes on recombinant proteins.

EcN is a well-studied probiotic bacterium and has been reported to colonize and establish itself in the human intestine not only by its ability to produce adhesions, microcins and sidereophores (Blum-Oehler et al., 1995, Boudeau et al., 2003), but also by adhesion to the epithelial cells and mucus via type-1 and FIC fimbriae. Also, EcN possesses unique systems for iron uptake, enhancing its vitality of fitness and competitive advantage over other microflora of host. We assessed the use of direct transfer recombinant proteins using EcN expressing GFP. Shrimp larval stages that were fed with 10^6 cfu/ml transgenic bacteria accumulated recombinant proteins in shrimp gut, supporting the direct use of transgenic bacteria for protein delivery. Further, even within 1 day of feeding almost 80% of shrimp bacteria were transgenic bacteria, demonstrating the efficacy of this EcN to displace the natural bacterial flora in shrimp gut. However, even though EcN is regarded as probiotic, the direct use of transgenic bacteria in culture

water might pose environmental issues related to the use of transgenic bacteria in aquaculture operations (Dunham, 2009). Hence, Artemia engorged with recombinant proteins might be better protein delivery systems to shrimp for paratransgenic approach.

Transfer of recombinant proteins through the food web has been studied in transgenic crops to evaluate the effect of a recombinant toxin on arthropod pests. Cry1Ab, a toxic protein expressed, in transgenic rice plants accumulated in the brown planthopper, *Nilaparvata lugens*, a common pest arthropod in rice fields. Subsequently, the protein was transferred to the wolf spider, *Pirata subpiraticus*, a predator of the planthopper, demonstrating that recombinant proteins from a primary source organism can accumulate at higher levels in the food chain (Chen et al., 2005, 2009). Because of the degradative nature of digestive systems, at least two criteria must be met for successful biotransfer of recombinant molecules. First, the feeding organisms should be prolific feeders. Second, the prey-predator feeding time interval should be very short. Both of these conditions are met in the food web comprised of bacteria (or algae), Artemia and shrimp larvae. Non-specific filter feeding by larval stages of Artemia and shrimp facilitates the biotransfer of proteins within the food chain. Furthermore, the larval digestive systems in the initial stages in both Artemia and shrimp are underdeveloped, thus reducing the deleterious effects of digestive enzymes on recombinant proteins.

Shrimp mariculture is a vital economic activity in many countries and involves intensive cultivation in confined environments that results in outbreaks of lethal viral and bacterial diseases. Prophylactic use of antibiotics in aquaculture is banned worldwide due to the emergence of antibiotic-resistant microbes. Hence, novel strategies to prevent

deadly outbreaks are needed to reduce the estimated $3 billion in annual losses due to shrimp diseases. Several approaches involve boosting the nutritional value of Artemia through bioencapsulation. Disease protection has been demonstrated using antibacterial agents like trimethoprim-sulfamethoxazole in Artemia nauplii (Chair et al., 1996). Furthermore, feeding of pre-formed molecules such as antibodies has proved efficacious. A high affinity monoclonal antibody (MAb 216) directed at VP28, the anchor protein of White Spot Syndrome Virus (WSSV) that facilitates viral entry in the cytoplasm of shrimp cells (Yi et al., 2004), resulted in an 80% survival benefit when fed to shrimp that were subsequently challenged with WSSV (Dante et al., 2007). Similarly, antiviral peptides such as PmAV (*Penaeus monodon* antiviral) have been reported from WSSV-resistant *P. monodon* (Luo et al., 2007). Vibrios, a group of bacteria with devastating impact on shrimp and shellfish industries worldwide. Engineered lines of bacteria and algae expressing PmAV, anti-VP28 single-chain antibodies and cationic peptides that are fed to Artemia and, possibly, directly to shrimp could be developed to protect commercial shrimp against White Spot Syndrome Virus infection and Vibriosis. Bioaccumulation of these protective molecules in shrimp such as *L. vannamei* via the paratransgenic approach holds great promise to address myriad diseases in shrimp mariculture.

ACKNOWLEDGMENTS

We acknowledge the excellent research support given by Harlington Shrimp Farm, TX or Earthcare Aquaculture Inc., FL by providing us with the shrimp post-larvae to conduct the feeding trials.

CHAPTER 4

ANTIMICROBIAL ACTIVITY OF CATIONIC PEPTIDES AGAINST PATHOGENIC MARINE *VIBRIO* SPP. OF FISH AND SHELLFISH

This chapter is based on the following article:

Subhadra, B., 2011. Antimicrobial Activity of Cationic Peptides against Pathogenic Marine *Vibrio* spp. of Fish and Shellfish. *Aquaculture (under preparation).*

SUMMARY

The activity of cationic peptides - apidaecin, cecropin, magainin, melittin and moricin- was tested against pathogenic *Vibrio* spp. and probiotic feed organisms of shrimp, to identify molecules for use in a paratransgenic control strategy against Vibriosis in commercial shrimp culture. Moricin was the most potent antimicrobial agent against *Vibrio* spp. with a minimum bactericidal concentration (MBC) in the 0.04-0.31 µM range. Cecropin and melittin killed Vibrios in the MBC range of 0.02-17.5 and 2.5-10.0 µM, respectively. Probiotic bacteria and algal feed organisms displayed high levels of resistance toward cecropin and melittin suggesting that they would be suitable for a paratransgenic approach to Vibriosis. A combination of 0.01 µM cecropin and 0.31 µM melittin was bactericidal against *Vibrio* spp. demonstrating synergistic activity. Independent clones of Vibrios evolved resistance to melittin over 80 passages that spanned 500-600 generations, but resistance did not develop in *Vibrio* strains exposed to a combination of cecropin and melittin at concentrations of 0.01 µM. Cecropin and melittin exerted potent and synergistic activity against pathogenic Vibrios with no toxicity toward probiotic or algal feed organisms. A combination of 1 µM cecropin and 1 µM melittin produced significant protection of Artemia against *Vibrio campbellii* in *in vivo* challenge studies. Paratransgenic delivery of cationic peptides that target Vibriosis in shrimp via feed organisms could offer powerful tools to increase protein food supply.
KEY WORDS: Antimicrobial peptides, Vibriosis, cecropin, melittin, paratransgenesis, shrimp diseases

INTRODUCTION

Vibriosis is a major shrimp disease which affects both hatchery and grow-out phase of production. The term Vibriosis describes a spectrum of diseases and includes oral and enteric Vibriosis, appendage and cuticular Vibriosis, localized Vibriosis of wounds, shell disease, systemic Vibriosis and septic hepatopancreatitis (Brock and Lightner, 1990). Outbreaks may occur when environmental factors trigger the rapid multiplication of bacteria usually tolerated at low levels within shrimp blood (Sizemore and Davis, 1985; Soto-Rodriguez et al., 2003), or by bacterial penetration of host barriers.

Here, we explore the application of paratransgenic strategies to vibriosis in shrimp. In this strategy, commensal or symbiotic bacteria found at mucosal sites of pathogen transmission are isolated and genetically altered to elaborate immune peptides or engineered single chain antibody fragments that neutralize infectious agents. The transgenic bacteria are then delivered back to mucosal sites where disease transmission occurs (Durvasula et al., 1997). To apply this paratransgenic strategy to farmed shrimp, we proposed a modified paradigm that involves microbes that are frequently used as feed for marine animals (Durvasula et al., 2006). This is particularly important as one of the main portals of entry for Vibrios is the midgut (Lovett and Felder, 1990). Oral acquisition of pathogenic Vibrios through infected food sources is an important factor in the pathogenesis of these bacteria (Lavilla-Pitago et al., 1998).

We recently demonstrated that Artemia fed transgenic lines of *Synechococcus bacillarus* and *Escherichia coli* Nissle accumulated recombinant proteins that retained biological activity for up to 10 h. These feed organsisms were used for paratransgenic delivery of bioactive recombinant molecules to the commercial white shrimp, *L.*

vannamei (results from Chapter 3). We hypothesize that such a system could be used to deliver to farmed shrimp and fish foreign proteins with vibriocidal activity. In the present study, we aim to identify specific peptides with potent bactericidal activity against marine vibrios as well as bacteria which can potentially be used as paratransgenic vehicle to express antibacterial peptides

Cationic peptides such as cecropin, apidaecin, magainin and melittin, which are part of natural innate immunity in many invertebrate organisms (Zasloff, 2002), could serve as effector molecules in a marine paratransgenic strategy to control Vibriosis. These antibacterial peptides are very potent, with their inhibitory concentration being at submicromolar or low micromolar range for neutralizing a variety of Gram- negative bacteria, including some antibiotic resistant strains (Stark et al., 2002). The majority of these peptides act through disintegrating the bacterial membrane or interfering with membrane assembly (Hancock, 1997), with the exception of apidaecin which deactivates a bacterial protein in a stereospecific manner (Otvos, 2000). We had previously demonstrated the effectiveness of cecropin A and other AMP's in controlling *Trypanosoma cruzi* via a paratransgenic approach (Durvasula et al., 1997; Fieck et al., 2010). This study examines the *in vitro* activity of five antimicrobial peptides against various *Vibrio* spp., as well as against bacteria and algae candidates for expressing these molecules as paratransgenic vectors. We also studied the evolution of resistance against single cationic peptide treatments and report here that a combination of cationic peptides can avoid emergence of resistance in *V. harveyi, V. penaeicida*, and *V. campbellii*, the three most important shellfish pathogens.

MATERIALS AND METHODS

Bacterial strains, Culture media and Growth conditions

The sources of bacterial and algal cultures and their culture conditions are described in **Table 3**.

Table 3. Sources and culture conditions of various bacterial and algal cultures used in this study.

Bacteria and Algae	***Strain, Source or Reference***	***Culture conditions***
Vibrio alginolyticus	ATCC[a]-19108	MB[e] at 30°C
V. alginolyticus	CAIM[b] 516	MB at 30°C
V. anguillarum	90-11-287 Dr Lone Gram (Skov et al., 2001)	MB at 30°C
V. campbellii	CAIM 519	MB at 28°C
V. ordalli	CAIM 608	MB at 30°C
V. harveyi	ATCC-BAA 1120	MB at 30°C
V. harveyi	CAIM 513	MB at 30°C
V. harveyi	BW106, Dr A. Parvathi, NIO, India	MB at 30°C
V. parahaemolyticus	CAIM 320	MB at 32°C
V. parahaemolyticus	BW108 Dr A. Parvathi, NIO, India	MB at 32°C
V. penaeicida	CAIM 285	MB at 28°C
V. furnissi	BW105 Dr A. Parvathi, NIO, India	MB at 32°C
Bacillus megaterium	WH320 (MolBioTech Inc., Germany)	BHI[f] at 30°C
B. subtilis	ATCC-128 Dr D Delfina (UTEP, Texas)	BHI at 30°C
B. subtilis	ATCC-6051 Dr D Delfina (UTEP, Texas)	BHI at 30°C
Brevibacterium linens	Durvasula lab- *Eg* 1, Hillesland et al. (2008)	LB[g] at 30°C
Synechococcus bacillarus	CCMP[c]-1333	f/2[h] at 25°C
Dunaliella salina	UTCC[d]- 197	f/2 at 25°C

[a] American Type Culture Collection (ATCC)
[b] Collection of Aquatically Important Microorganisms, CIAD, Mexico.
[c] Provasoli-Guillard National Center for Culture of Marine Phytoplankton, USA
[d] University of Toronto Culture Collection
[e] MB: Marine Broth (Sizemore and Stevenson, 1970), Difco 2216, BD Biosciences, Sparks, MD, USA
[f] Brain Heart Infusion Broth (Sambrook et al., 2000), EMD Chemicals, Gibbstown, NJ, USA
[g] Luria-Bertani Broth (Sambrook et al., 2000), EMD Chemicals, Gibbstown, NJ, USA
[h] f/2 media (Guillard, 1975), Sigma-Aldrich, St Louis, MO, USA.

Antimicrobial Peptides

Apidaecin (GNNRPVYIPQPRPPHPRL), cecropin A (KWKLFKKIEKVG-QNIRDGIIKAGPAVAVVGQATQIAL), magainin II (GIGKFLHSAKKFGKAF-

VGEIMNS), moricin (AKIPIKAIKTVGKAVGKGLRAINIASTANDVFNFPKPKKRK), and melittin (GIGAVLKVLTTGLPALISWIKRKRQQ) were chemically synthesized (Biosynthesis Inc. USA and GeneScript Corporation, USA). The peptides were dissolved as 1 mM stock solutions in phosphate buffered saline (137mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.2-7.4 at 25ºC) containing 0.01% BSA. The stock solutions were filter sterilized and stored at -80^{o}C.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determinations

The MIC and MBC were determined using a modified microbroth dilution protocol on 96-well plates (Wiegand et al., 2008). One row of a sterile 96 well plate was used for each bacterial isolate tested with up to 12 serial dilutions of the antibacterial peptide (column 1-12). Hundred micro-liters of sterile media were added to columns 2-12. The 1 mM stock solution of antimicrobial peptide was diluted 1:50 by adding 4 µl of the stock to 196 µL of LB broth in column-1 to obtain a final peptide concentration of 20 µM. A two-fold serial dilution of the peptide was achieved in columns 2 to 12 by adding 100µl to the next well of the microplate. Each well was then inoculated with 100 µl of test culture, suitably diluted to adjust the final cell density to 2 - 5 × 10^5 colony forming units (CFU) ml $^{-1}$. The plates were incubated for 18-24 h at the optimum temperature for each bacterial species (**Table 3**). For algal cultures, the plates were incubated at 72 h at 25°C at constant illumination. For this work, we defined minimum inhibitory concentration (MIC) to be the lowest AMP concentration that would reduce cell growth by more than 50% as determined by OD_{600} readings, while minimum bactericidal concentration (MBC) was defined as the lowest concentration of each peptide that

resulted in no growth on agar plates (24 h incubation) following treatment when compared to control (NCCLS, 2004). These experiments were performed in duplicate and repeated twice.

Antimicrobial Combination Studies

Two AMP combinations: cecropin + melittin (Cec + Mel) and melittin + magainin (Mel + Mag), were tested against *Vibrio campbellii* CAIM 519, *V. harveyi* CAIM 513, and *V. penaeicida* CAIM 285. Concentrations of 0.01, 0.03, 0.16, and 0.31 µM of the antimicrobials were used in the combination experiments. The selected concentrations of AMP's for combination studies were well below (10-50 fold less) the MBC's of the target organisms. The MBC and MIC of the Vibrio strains before and after selection in melittin were determined as described in the previous section.

Experimental Evolution of Resistance against Melittin or Peptide Combinations

This experiment was performed as a modified protocol of Perron et al., (2006). Here, we evaluated the ability of three virulent *Vibrio* spp., *Vibrio campbellii* CAIM 519, *V. harveyi* CAIM 513, and *V. penaeicida* CAIM 285, to grow in increasing concentrations of melittin. We established three independent clones of each test strain. Mid-log phase cells of each strain were diluted 1:200 in fresh marine broth (MB) in the absence of melittin on a 96 well plate and allowed to grow overnight at 30^{o}C. Cells from each well were maintained by daily passage, using the same dilution, into a fresh plate. At the 11th passage, a sub-MBC dose of melittin, 1.0 µM, was added to each well. This AMP concentration was maintained for 10 subsequent passages. Afterwards, we doubled the concentration of melittin at every tenth transfer. With each increase in melittin concentration, a 100 µl sample of the cells was inoculated in 25% glycerol in LB or MB

broth and stored at - 80°C. A total of 80 serial passages were performed, consisting of approximately 500-600 generations of *Vibrio* growth, to a highest melittin concentration of 128 µM. We selected 128 µM as the final concentration because melittin has a tendency to precipitate at higher concentrations in MB (Raghuraman and Chattopadhyay, 2007) and because 128 µM was 1-2 orders of magnitude higher than the MBC's of melittin against Vibrio strains. Cell growth was monitored daily by OD_{600} on a microtiter plate reader. The MBC and MIC of the glycerol cultures were determined as described previously. We then repeated the above experiment using a combination of two AMP's. Cells from independent colonies from each of the three *Vibrio* strains were grown in 0.01 µM of cecropin and 0.01 µM of melittin. Cells were passaged as described.

Axenic Hatching of Artemia

All experiments were performed with cysts of *A. franciscana* (BrineShrimp Direct, Ogden, UT). Sterile nauplii were hatched via the de-capsulation method. Briefly, two hundred milligrams of cysts were hydrated in 18 ml tap water for 1 h with gentle shaking and sterile aeration. Six hundred and sixty µl of freshly prepared NaOH (32%) and 10 ml of NaOCl (50%) were added to the hydrated cyst suspension, and the reaction was stopped after 2 min by adding 14 ml of $Na_2S_2O_3$ ($10g\ l^{-1}$). The de-capsulated cysts were washed twice with sterile seawater and re-suspended in a 50 ml tube containing 30 ml of sterile seawater prepared by dissolving 35 g ocean salt per liter distilled water. The cysts were then allowed to hatch at 28ºC with constant illumination for 24-28 h on a rotor. After hatching, groups of 20 nauplii were transferred to new sterile 50 ml tubes that contained 20 ml of sterile seawater. The axenic state of the Artemia was confirmed for each hatching trial by culturing a 3 ml sample of Artemia in fresh MB Broth for 2 days at

28ºC followed by observance of growth of any bacterial colonies. Nauplii from hatching trials with positive aerobic bacterial growth on MB were discarded.

Antimicrobial Immersion-treatment of Artemia

A cecropin and melittin combination was selected as the antimicrobial treatment based on our *in vitro* results. Combination treatments of cecropin and melittin at 5 μM and 1μM of each peptide in PBS were prepared. Single treatments of cecropin and melittin of 1 μM were also included as controls. Batches of 100-120 Artemia were immersed in peptide suspensions (treatment) or PBS (control) for 2 h prior to Vibrio challenge trials.

Vibrio Challenge Studies

All manipulations were performed under laminar flow to maintain the sterility of Artemia cysts and nauplii. Twenty healthy Artemia nauplii were selected from the immersion treatments and added to each of four 50 ml tanks. We selected *Vibrio campbellii* CAIM 372, a highly pathogenic strain to Artemia (Soto-Rodriguez et al. 2003), for the challenge studies. Our preliminary studies showed that *V. campbellii* CAIM 372 at a concentration of 10^7 ml^{-1} of culture water killed over 70-75% of Artemia in 12 h. Artemia in all tanks were exposed to *V. campbellii* CAIM 372 at a concentration of 10^7 CFU ml^{-1} for 12 h. A control tank was established with Artemia and no Vibrio inoculation. Since we will ultimately express antimicrobial petides via probiotic agents such as *Bacillus subtilis*, we added autoclaved *B. subtilis* 0179 (Lallemand Inc, Canada) once at the start of the experiments at a dose of 10^6 CFU ml^{-1}. Artemia tanks containing autoclaved *B. subtilis* with no prior antimicrobial peptide immersion were also used as controls. Mortality rates of the Artemia were recorded at 12 h post-challenge. The

experiment was repeated once. The quantitative *Vibrio* load in Artemia was determined by taking samples from the challenge trial tubes at 2, 4, 8 and 12 h. Artemia were suspended in 1 ml PBS and tissue was disrupted using sterile needles before final re-suspension in 10 ml of sterile PBS. Serial dilutions of this suspension were made followed by triplicate plating on LB agar. After overnight incubation colonies were counted. Results were presented as CFU per Artemia.

Statistical Tests

In AMP evolution of resistance studies the means of three clones before selection were the same, therefore we conducted a Wilcoxon signed rank test ($P \leq 0.05$) (GraphPad Software, Inc., San Diego, CA). In Vibrio challenge trials quadruplicate tubes served as the experimental units in all statistical analysis. The survival data from challenge trial were subjected to Students t test, and a $p \leq 0.05$ was considered significant. A statistical software GraphPad Prism was used (GraphPad Software, Inc., San Diego, CA) to perform this analysis.

RESULTS

Activity of Antimicrobial Peptides against Vibrio* spp. *and Probiotic bacteria

Moricin was the most potent anti-bacterial agent against all *Vibrio* spp. tested, with MBC and MIC ranging from 0.04-0.313 µM and 0.005-0.01 µM, respectively (**Table 4**). The MBC of cecropin ranged from 0.02-17.5 µM. Different strains of a similar *Vibrio* spp. demonstrated significant differences in susceptibility toward the peptides. For example, *Vibrio harveyi* ATCC-BAA-1120 was highly sensitive to cecropin

Table 4. Minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of different cationic peptides against bacterial and algal species (µM).

AMP	**Apidaecin**		**Cecropin A**		**Magainin**		**Melittin**		**Moricin**	
Bacteria /Alga tested	***MBC***	***MIC***	***MBC***	***MIC***	***MBC***	***MIC***	***MBC***	***MIC***	***MBC***	***MIC***
***Vibrio* spp.**										
V. alginolyticus ATCC-19108	>20	10	1.3	0.15	10	2.5	2.5	0.01	0.31	0.01
V. alginolyticus CAIM 516	>20	5	2.5	0.31	15	5	5	1.25	0.31	0.01
V. anguillarum 90-11-287	>20	10	5	0.02	>20	2.5	5	1.25	0.31	0.01
V. campbellii CAIM 519	>20	5	2.5	0.31	7.5	2.5	2.5	0.63	0.04	0.01
V. campbellii CAIM 372	-	-	-	0.63	-	-	1.3	0.31	-	-
V. ordalli CAIM 608	>20	10	10	5	10	5	10	2.50	0.04	0.01
V. harveyi ATCC-BAA 1120	>20	5	0.02	0.01	7.5	2.5	5	0.01	0.01	<0.01
V. harveyi CAIM 513	>20	5	1.3	0.04	>20	2.5	5	0.01	0.31	0.01
V. harveyi BW106	>20	5	2.5	0.31	>20	5	10	2.5	0.63	0.31
V.parahaemolyticus CAIM320	>20	5	2.5	0.31	>20	2.5	10	1.25	0.31	0.01
V. parahaemolyticus BW108	>20	10	10	2.5	>20	2.5	10	2.5	0.63	0.04
V. penaeicida CAIM 285	>20	5	17.5	12.5	>20	2.5	10	2.5	0.31	0.01
V. furnissi BW105	>20	10	5	1.3	7.5	1.3	10	2.5	0.31	0.01
Probiotic bacteria										
Bacillus megaterium WH320	>20	10	1.3	0.01	2.5	0.31	0.31	0.01	0.04	0.01
B. subtilis 168	>20	15	10	5	>20	5	>20	5.0	2.5	0.63
B. subtilis 6051	>20	10	>20	10	>20	5	>20	5.0	2.5	0.63
Brevibacterium linens Eg	>20	>20	>20	15	>20	>20	>20	10.0	5	1.3
Algal species			>20							
Synechococcus bacillarus	>20	10		12.5	>20	15	>20	5.0	2.5	0.63
Dunaliella salina	>20	10	>20	>20	>20	>20	>20	>20	10	2.5

with an MBC of 0.02 µM. However, *V. harveyi* CAIM 513, a strain isolated from diseased fish and shellfish, had an MBC that was approximately 63-fold higher. The bactericidal concentrations of melittin against all the *Vibrio* spp. tested were rather consistent, with most strains succumbing within the range of 5-10 µM. *V. penaeicida,* another strain that was isolated from diseased shrimp, appeared to be resistant to all the anti-microbial peptides tested. None of the *Vibrio* spp. appeared susceptible to even 20 µM apidaecin.

Apidaecin, cecropin, magainin, and melittin had minimal effect on *Bacillus subtilis 6051* and *Brevibacterium linens* indicating that these strains might be used to express cecropin and melittin against Vibrios in a paratransgenic approach. The MBC for all the antimicrobial agents tested against these two microbes was greater than 20 µM. Again, the tolerance to each AMP was strain dependent. The MBC for cecropin was 10 µM in *B. subtilis* 168. Moricin was the only peptide that was bactericidal (5 µM) against *Brevibacterium linens,* and *B. megaterium* WH320, a non-spore forming species, had very low MBC and MIC values for all the cationic peptides used in these experiments (**Table 4**).

Activity of Antimicrobial Peptides against Marine Algal Species

Moricin was the only peptide with algicidal activity against Synechococcus bacillarus and *Dunaliella salina* with an MIC of 0.63 and 2.5 µM, respectively (**Table 4**). These two algal species appeared to be resistant to the other antimicrobial peptides tested again indicating that these algal strains may be used to express cecropin and melittin in a paratransgenic approach.

Antimicrobial Combination Studies against Vibrio spp.

The MBC's of melittin and cecropin for *Vibrio harveyi* CAIM 513 were 1.3 and 5 µM, respectively (**Table 4**). However, when used in combination, concentrations as low as 0.01 µM cecropin and 0.16 µM melittin exerted bactericidal effect against *V. harveyi* CAIM 513 and *V. campbelli* CAIM 519. Similarly, a combination of 0.01 µM cecropin and 0.31 µM melittin was bactericidal against *V. penaeicida* CAIM 285 (**Table 5**). Magainin and melittin combination studies showed that a combination of 0.31 µM of each peptide was bactericidal against the three strains tested. These results suggest that combinations of AMP's are highly synergistic against *Vibrio* spp. The concentrations of AMP's required for vibriocidal activity against all three *Vibrio* spp. were well below the MBC values of cecropin and melittin individually (**Table 5**).

Table 5. The lowest concentration of antimicrobial peptides in combination that demonstrated bactericidal effect against pathogenic *Vibrio* strains.

***Vibrio* spp.**	**Cecropin/Melittin mixture** (µM, each)	**Melittin/Magainin mixture** (µM, each)
Vibrio campbellii CAIM 519	0.01/0.16	0.31/0.16
Vibrio harveyi CAIM 513	0.01/0.16	0.31/0.31
Vibrio penaeicida CAIM 285	0.01/0.31	0.31/0.31

Experimental Evolution of Resistance in V. campbellii, V. harveyi, and V. penaeicida Toward Melittin

Progressive selection in increasing concentration of melittin for 500-600 generations of the pathogenic Vibrios resulted in cells that were significantly more resistant to this AMP when compared to the progenitor culture, suggesting the emergence of resistance. While no change in the MBC was observed, a 200-fold increase in MIC was noted in two of the melittin-selected *Vibrio campbellii* lines. All three melittin-

selected *V. harveyi* lines had significant increase in both MBC and MIC. Similarly the melittin-selected *V. penaeicida* lines also demonstrated a 3-5 fold increase in the MBC

Table 6. Development of resistance to AMPs. Minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of *Vibrio penaeicida* CAIM 285, *Vibrio harveyi* CAIM 513 and *Vibrio campbellii* CAIM 519 clones before and after selection for experimental evolution of antimicrobial resistance against melittin.

***Vibrio* spp.**	***Melittin before selection***		***Melittin after selection***	
	***MBC** (uM)*	***MIC** (uM)*	***MBC** (uM)*	***MIC** (uM)*
Vibrio campbellii, CAIM 519				
Clone 1	2.5	0.63	10.0	2.5
Clone 2	2.5	0.63	5.0	1.0
Clone 3	2.5	0.63	5.0	1.0
Average	*2.5 ± 0.0*	*0.63 ± 0.0*	*6.7 ± 1.7*	*1.5 ± 0.5****
Vibrio harveyi, CAIM 513				
Clone 1	5.0	0.005	60.0	40.0
Clone 2	5.0	0.005	30.0	20.0
Clone 3	5.0	0.005	30.0	15.0
Average	*5.0 ± 0.0*	*0.005 ± 0.0*	*40.0 ± 10.0**	*25.0 ± 7.6**
Vibrio penaeicida, CAIM 285				
Clone 1	10.0	2.5	32.0	15.0
Clone 2	10.0	2.5	16.0	10.0
Clone 3	10.0	2.5	50.0	20.0
Average	*10.0 ± 0.0*	*2.5 ± 0.0*	*32.7 ± 9.8*	*15.0 ± 2.8**

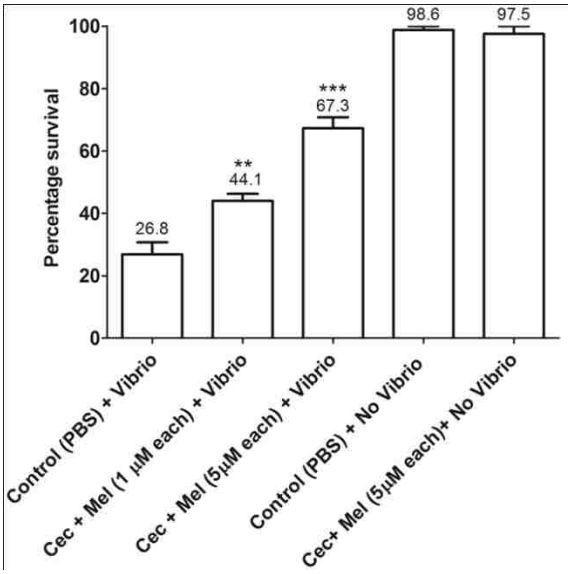
* significant difference compared to values before selection. Variances are SEM.

values (**Table 6**). We were unable to generate any double-AMP- resistant cells of *Vibrio campbellii* CAIM 519, *V. harveyi* CAIM 513 or *V. penaeicida* CAIM 285 to cecropin and melittin. All three species died within three passages of being cultured in 0.01 μM of cecropin and melittin, suggesting that combination therapy can prevent simultaneous emergence of resistance against the two AMP's (data not shown).

Artemia Challenge Trials

Artemia treated with cecropin plus melittin at 1 μM or 5 μM and then challenged with *Vibrio campbellii* 372 had significantly higher survival than Artemia treated with PBS (**Fig. 14**). Artemia that were given either cecropin or melittin alone at 1 μM failed to

enhance survival rates (data not shown). The control group of Artemia without Vibrio challenge had 98.8 ± 1.2 percent survival which was similar, to the survival of Artemia treated with cecropin plus melittin (5 μM) without Vibrio challenge (**Fig. 14**) indicating absence of toxicity due to the AMPs. In the control group of Artemia that was challenged



with *Vibrio campbellii* without prior antimicrobial treatment, the average *V. campbellii* bacterial level reached 1.4 x 10^7CFU per Artemia 2 h post challenge. Thereafter, average *V. campbellii* number per Artemia remained in the range of 2.2-5.3 x 10^7 up to 12 h post challenge (**Fig. 15**). Amongst Artemia treated with Cecropin and Melittin (5 μM) and challenged with *V. campbellii*, the average *V. campbellii* bacterial level reached 3.0 x 10^4 CFU/Artemia at 2 h post challenge and remained in the range of 1.9-3.9 x 10^4 CFU/Artemia up to 12 h post challenge. Amongst Artemia treated with Cec + Mel (1μM) and challenged with *V. campbellii*, the Vibrio load during the 2-8 h period post challenge was approximately 2 logs lower than the control group; however in the final 4 h post challenge the Vibrio load approximated that of controls (**Fig. 15**).

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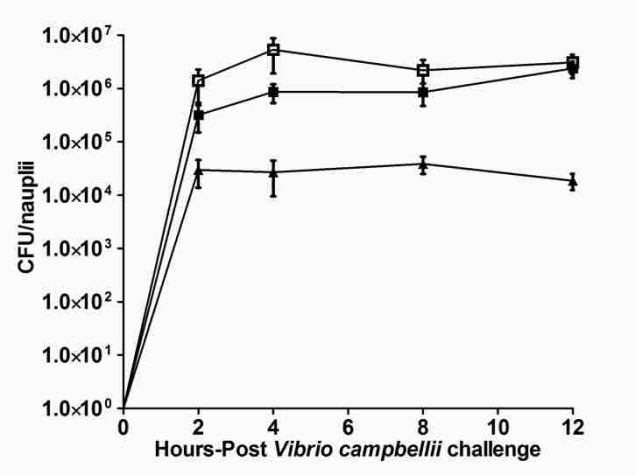

Figure 15. *V. campbellii* (CAIM 372) colonization of Artemia treated with Cec + Mel (cecropin and melittin) (5.0 µM) (filled triangles) or Cec + Mel (1.0 µM) (filled rectangles) or control (Phosphate buffered saline) (clear rectangles) at 0, 2, 4, 8 and 12 h post-challenge with *V. campbellii*.

DISCUSSION

In our assessment of the action of peptides against a variety of *Vibrio* strains and larval feed organisms, moricin was the most potent antimicrobial peptide with MBC values in the nanomolar range. However, moricin was lethal to *Bacillus* spp., *Brevibacterium* spp. and algal species, all feed organisms that would be required as a platform for expression of anti-pathogen molecules. We have therefore excluded moricin from further consideration in the marine paratransgenic strategy. Similar to this finding Hara et al. (1994) earlier reported that moricin at a concentration range of 0.27-0.53 µM had bactericidal activity against Gram negative and Gram positive bacteria such as *E. coli, Acinetobacter spp., Pseudomonas spp.* and *Staphylococcus aureus*.

Cecropin and melittin were active against *Vibrio* spp. with bactericidal concentrations in the micromolar range (5-10 µM). The use of these two peptides for control of fish pathogens has been proposed previously (Kelly et al., 1990). Continuous

delivery of CEME, a cecropin-melittin hybrid peptide, via an implanted mini-osmotic pump in the peritoneal cavity of Coho salmon significantly reduced mortality in *Vibrio anguillarum*-infected fish (Jia et al., 2000). However, the high cost of these synthetic peptides, especially when delivered by continuous infusion, may be prohibitive.

Bacillus subtilis, *Brevibacterium linens*, *Synechococcus bacillarus* and *Dunaliella salina* were resistant to cecropin and melittin with MBC values in the range of 10 to greater than 20µM. Gram positive bacteria are generally more resistant to antimicrobial peptides than Gram negative bacteria (Brogden, 2005). However, the *Bacillus megaterium* strain we used in this study was highly susceptible to all the peptides tested. We used *B. megaterium* WH320 strain which is asporogenic and was primarily developed for bacterial transformation protocols (Rygus and Hillen, 1991). We did not establish whether the asporogenic nature of this strain might have affected its susceptibilities toward various peptides. Nevertheless, the susceptibility of this organism to antimicrobial peptides eliminates it from further consideration in a paratransgenic strategy. Magainin has been reported to be active against Gram negative and Gram positive bacteria (Vaara, 2009) but we tested a version of this peptide that lacks amide modifications and exhibited little activity. We, therefore, eliminate this peptide from further paratransgenic systems. Apidaecin exhibited no activity (MBC >20 µM) against all the bacterial and algal species tested. Our results contradict earlier reports that apidaecin is very active against Gram-negative bacteria, especially *Enterobacteriaceae* (Czihal and Hoffmann, 2009). The antimicrobial activity of apidaecin is primarily via steric hindrance of bacterial proteins such as membrane transporters, thereby causing disruption of key cellular functions (Castle et al., 1999). Possibly, the target proteins of

apidaecin in *Vibrio* spp. are structurally different from those of other *Enterobacteriaceae* rendering this peptide inactive against this genus. Though further studies into the mechanisms of apidaecin are required, our initial screening shows that apidaeicin and magainin are not ideal candidates for a paratransgenic approach.

The *Vibrio* strains used in our study exhibit a difference in susceptibility toward the various antimicrobial peptides tested. Further, different isolates of each species show evidence of varying susceptibility toward a given peptide. Antimicrobial peptides bind to the surface of a cell membrane in a carpet like manner, thereby increasing permeability to ions and other metabolites via pore formation (Brogden, 2005). It is possible that, though related, the different *Vibrio* spp. we tested might have subtle changes in membrane characteristics. Adaptation of bacterial membranes to changing environments using amino-acylated phospholipids as a resistance mechanism to cationic antimicrobial peptides has been described (Roy et al., 2009). Dramatic differences in MIC were also observed in *Pseudomonas aeruginosa* on increasing one positive charge via charged amino acid residue on the polar lipid bilayer (Hancock and Farmer, 1993). The Vibrio strains used in this study had been isolated from diverse geographic areas and were likely subjected to different environmental effects.

The pathogenic strains of *Vibrio harveyi* CAIM 513 and *V. alginolyticus* CAIM 516 which were isolated from diseased fish and shellfish had approximately 10-fold higher resistance to antimicrobial peptides than the commercially available *V. harveyi* ATCC-BAA 1120. *Vibrio* spp., *V. harveyi* in particular, isolated from shrimp farms are more resistant to antibiotics compared to isolates from other environments (Vaseeharan et al., 2005). Similarly Teo et al., (2002) identified several antibiotic resistance genes and

plasmids from *V. harveyi* W3B, a marine isolate from a shrimp farm. Possibly, the *V. harveyi* CAIM 513 strain used in this study used inherited antibiotic resistance genes and gene products such as efflux pumps to partially eliminate antimicrobial peptides (Okusu et al., 1996). This explanation might also hold true for *V. penaeicida*, which was one of the most resistant *Vibrio* spp. in our study.

Emergence of resistant mechanisms in bacteria because of the pervasive and indiscriminate use of antibiotics in aquafeed to prevent infections and promote growth has been a major public health issue related to the sustainable production of animal foods. Molecular mechanisms of resistance, which has been identified as a necessary component of pathogenesis of certain bacteria, to antibacterial peptides have been identified in several groups of bacteria (Yeaman and Yount, 2005). It has been reported that most cationic peptides do not induce resistance mutants in vitro and enhance antimicrobial activity of classical antibiotics thus serving as an anti-resistant compounds (Hancock, 1997). Since our paratransgenic approach uses antimicrobial peptides, concerns exist about the public health consequence of drug resistance in the targeted microbes. Therefore, we studied the evolution of resistance of *Vibrio* spp. in the presence of sub-lethal concentrations of melittin over several generations. Our results showed that the MIC and MBC of *Vibrio harveyi* and *V. penaeicida* clones after exposure to low concentrations of peptides were significantly higher than those of the parent clones, confirming experimental evolution of resistance. Similarly, *Escherichia coli* and *Pseudomonas fluorescens* acquired heritable resistance against pexiganan, an analogue of magainin, when propagated with this antimicrobial peptide for 600-700 generations (Perron et al., 2006). Likewise, *V. anguillarum*, *V.vulnificus* and *Yersinia ruckeri*

exhibited an inducible resistance to cecropin B with reversible changes in their ultra structure (Sallum and Chen, 2008).

The use of antimicrobial agents in combination is an effective method to slow development of resistance in target organisms. None of the tested *Vibrio* isolates in this study developed resistance against combination treatment with melittin and cecropin. This might be due to several reasons. Cecropin and melittin have multiple mechanisms of action such as disruption of the outer cell membrane, the cytoplasmic membrane and direct targeting of nucleic acids (Zhang et al., 2000). When used in combination, 0.01 µM cecropin and 0.16 µM melittin exerted full vibriocidal effect toward *Vibrio harveyi* CAIM 513. A similar effect was noted with these two peptides against *V. penaeicida* CAIM 285 and *V. campbellii* CAIM 519. Such synergism has been described in shellfish as a natural phenomenon. Two peptides from *Crassostrea gigas*, a proline-rich peptide and a defensin, are expressed exponentially and act synergistically to ward off pathogens in the event of infection (Gueguen et al., 2009). Delivery of peptides with vibriocidal activity in combination will permit synergistic killing of pathogenic Vibrios in the gut of farmed marine animals while reducing the evolution of drug-resistant microbes. Indeed, the very low concentrations of cecropin and melittin needed to kill otherwise drug-resistant isolates such as *V. harveyi* CAIM 513 and *V. penaeicida* CAIM 285 in this study suggest that paratransgenic expression and delivery of these peptides in the gut of animals such as shrimp and fish is possible and merits further study under field conditions.

To test the protective efficacy of these peptides in vivo, we conducted Vibrio challenge trials on Artemia. A mixture of cecropin and melittin at a concentration as low

as 1 μM partially protected Artemia against the highly pathogenic *Vibrio campbellii* CAIM 372. Toxicity of the peptides toward Artemia was not observed, even at concentrations of 5 μM. The protective effect of the peptides appears to be due to low colonization of Vibrio as indicated by the significantly lower *V. campbellii* load in Artemia samples treated with peptides during the initial 2-4 h after bacterial challenge.

Paratransgenic systems involve the differential activity of peptides toward the targeted infectious agent and host bacteria that serve as a platform for expression of the peptides (Durvasula et al., 1997). We have demonstrated concurrent delivery of multiple populations of recombinant bacteria to the brine shrimp, *Artemia franciscana* and *L. vannamei* (Chapter 2 and Chapter 3). Cecropin and melittin can be considered ideal for a paratransgenic approach because of their lethal effects on *Vibrio* spp. and minimal activity toward feed organisms of shrimp. *In vitro* experiments using these AMP's demonstrate significant protective effects against pathogenic Vibrios, suggesting that synergistic activities of very low concentrations of cecropin and melittin in the gut of commercial marine animals, delivered via feed bacteria and algae either directly or through Artemia, could offer a new tool in the control of infectious diseases in commercial mariculture.

ACKNOWLEDGMENTS

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CHAPTER 5

EXPRESSION OF MELITTIN IN *BACILLUS SUBTILIS* FOR MARINE PARATRANSGENIC DISEASE CONTROL

SUMMARY

The marine paratransgenic approach involves the expression of anti-infectious molecules in feed algae or probiotic bacteria. Previous studies have shown that melittin has antibacterial activity against pathogenic vibrios. The main objective of the present study was to optimize melittin expression in *B. subtilis* as a step towards developing a paratransgenic disease control strategy. The melittin gene was cloned into pHT01and transformed into *B. subtilis* to generate *B.subtilis*- pHT01-Mel strains.-Growth of transgenic *B. subtilis* strains was similar to the wild type strain. The pHT01-based plasmids were stable in *B. subtilis* with neglible plasmid loss during the 7 day study without antibiotic selection. Transgenic *B. subtilis* pHT-Mel exhibited a low level of melittin expression based on ELISA. Several strategies will be proposed in Chapter 6 for further optimization of melittin expression in *B. subtilis* as well as algal species for ultimate use as disease control paratrangenic agents.

INTRODUCTION

Recently, we explored the application of paratransgenic strategies to target Vibriosis, one of the most devastating shrimp diseases caused by at least 14 species of *Vibrio* (Brock and Lightner, 1990). To apply the paratransgenic strategy to farmed shrimp, we proposed a modified paradigm that involves microbes that are frequently used as live feed organisms for marine animals (Durvasula et al., 2006). We demonstrated that *Artemia* -a common live feed organism- fed transgenic lines of *Synechococcus bacillarus* and *Escherichia coli* Nissle accumulated recombinant proteins (GFP and marker single chain antibodies) which retained biological activity for up to 10 h inside the gut (Subhadra et al., 2010). This delivery system was also tunable in that accumulation of

recombinant proteins in *Artemia* occurred only during the active feeding period with rapid decrement in protein levels during a washout period (Subhadra et al., 2010). We also screened potential anti-vibrio molecules and found that melittin, magainin and cecropin, naturally occurring antimicrobial peptides that form part of innate immunity in insects, have potential applications as anti-Vibrio agents as parts of a paratransgenic strategy. These AMPs exhibited selective toxicity toward diverse strains of Vibrios with minimal effect on feed bacteria and algae. Furthermore, when used in combinations, they exhibit marked synergistic activity toward *Vibrio* spp. and appear to reduce evolution of target bacterial resistance. As a next step in developing marine paratransgenic approach, the present study was aimed at cloning, melittin -a potent AMP- into paratransgenic host organisms (algae, *Bacillus* spp, cyanobacteria).

Melittin is the principal toxic component in the venom of the European honeybee, *Apis mellifera* (Mackler and Kreil, 1977). It is a cationic, linear peptide composed of 26 amino acid residues (NH_2- GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$) in which the amino-terminal region is predominantly hydrophobic whereas the carboxy-terminal region is hydrophilic due to the presence of a stretch of positively charged amino acids (Mackler and Kreil, 1977). Though melittin has a high proportion of non-polar amino acids, it is highly soluble in water (>250 mg/ml) (Raghuraman and Chattopadhyay, 2007). Melittin is monomeric at low concentration and adopts essentially a random coil conformation in aqueous solution. Aggregation of monomeric melittin to a tetramer is promoted by high salt, high melittin concentration, and high pH.

Our earlier studies have shown that melittin was toxic against pathogenic *Vibrio* spp. while selected strains of *B. subtilis* were resistant up to a concentration of 20 µM.

Hence, we selected melittin as the anti-vibrio agent and *B. subtilis* as the host bacteria for anti-vibrio expression platform for further developing the concept of marine paratransgenesis. There are several advantages of using *B. subtilis* as host bacteria for themarine paratransgenic approach. (i) *B. subtilis* is a well-known host for industrial enzyme production (Ferreira et al., 2005); (ii) *B. subtilis* is nonpathogenic and regarded as 'generally recognized as safe' (GRAS); (iii) the strains have a capacity for secreting recombinant proteins such as neutralizing antibodies in biologically active forms and can serve as an oral vaccine carriers (Gat et al., 2003); (iv) it can grow in simple and inexpensive media at fast rates for large-scale fermentation (Fu et al., 2008); (v) it is highly amenable to genetic manipulations such as transformation and expression of recombinant proteins (Nguyen et al., 2003); (vi) it has been extensively used in aquaculture for better nutrient assimiliation, bioremediateion of nutrients in aquaculture operations and for probiotic infectious disease control (Verschuere et al., 2001; Nakayama et al., 2009). The main objective of the present study is to optimize melittin production from *B. subtilis* to further develop the concept of the marine paratransgenic infectious disease-control approach.

MATERIALS AND METHODS

Bacteria and culture media

*Bacillus subtilis*1012 (wild type: *leuA8 metB5 trpC2 hsdRM1*) was obtained from Bacillus Genetic Stock Center (BGSC), and *B.subtilis* ATCC168 was a generous gift from Dr D. Delfina (University of Texas, El Paso). Penessay broth 2X (1% peptone, 0.15% yeast extract, 0.15% beef extract, 600 mM sodium chloride, 0.1% glucose, 0.05

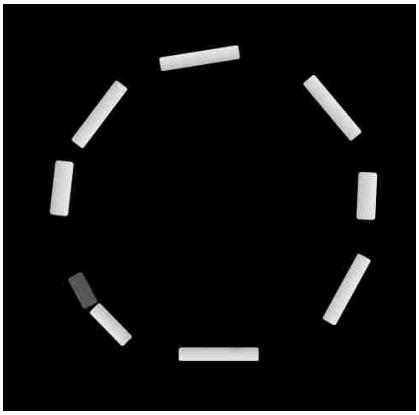
mM potassium phosphate, pH 7.0) and 2x SMM (1M sucrose, 40 mM maleic acid, 40 mM $MgCl_2$, pH 6.5) were sterilize by autoclaving for 12 minutes. SMMP (equal volumes of 2x SMM and 2x Penessay broth) was prepared freshly before use.

Plasmid vector contruction and bacterial transformation

We selected pHT01 (MolBiotec, Germany) as the expression plasmid for melittin expression. pHT01 is a high protein-expressing inducible vector with *B.subtilis* origin of replication and chloramphenicol selection (Nguyen et al., 2005). For constructing pHT01-GFP vector, GFP gene from pGFP was digested using Xba1 and ligated into Xba1 site in pHT01 vector (**Fig. 16**). One hundred µl of the transformation

mixture were plated onto LB-carbenicillin agar selection plates and positive clones were selected and streaked for isolation. The clones were screened by PCR and DNA from selected clones was isolated and sequenced with an upstream primer (pHT-seq primer: 5'-GAATGATGTAAGCGTG-3') to confirm the orientation of the gene insert. Once the gene sequence was confirmed, a clone was used to prepare a large-scale plasmid DNA preparation (Qiagen). For constructing pHT01-Mel (mellitin) vector, the melittin gene was amplified from pRr-Mel DNA vector using the forward (MelATG) and reverse (T3)

primers. This gene fragment was ligated into Sma1 site of pHT01 to give pHT01-Mel (**Fig. 17**) and XL-1 competant cells were transformed in SMMP medium using the ligated



DNA. We initially screened 18 carbenicillin resistant clones (C1-C18) for the gene insert. Among these clones, 3 clones (C3, C15 and C18) showed a specific DNA band by PCR using HT and T3 primer. The plasmid DNA was prepared from these three clones. Sequencing confirmed that C-15 and C-18 had the gene in the correct orientation in the plasmid. The transformed clones were screened by PCR and DNA from selected clones were isolated and sequenced with upstream primer (pHT-seqprimer 5'-GAATGATGTA-AGCGTG-3') to confirm the orientation of the gene insert.

***Bacillus subtilis* protoplast preparation**

Overnight culture of *B. subtilis* (1012 or 168) in LB were inoculated in 200 ml Penessay broth and grown at 37ºC to midlog phase ($O.D_{600}$ = 0.6). The cells were centrifuged and resuspended in 20 ml SMMP containing 100 µg/ml lysozyme and incubate with gentle shaking at 37ºC for 30 min. The cells were centrifuged, washed and re-suspended in 10 ml cold SMMP. The protoplasts were aliquoted as 500 µl into cryotubes and stored at -80°C until used.

Bacillus subtilis protoplast transformation

For each transformation, a 500µl protoplast preparation was mixed with 5 µg of plasmid DNA in a 15 ml tube. 1.5 ml of 40% polyethylene glycol was immediately added and incubated for 2 minutes at room temperature. Protoplasts were diluted with 5 ml SMMP and harvested by gentle centrifugation and removal of supernatant. After addition of 500 µl SMMP cells were incubated overnight at 37 °C with gentle shaking. Then 50 to 200 µl cells were added to a prewarmed plate of LB containing the chloramphenicol and incubated overnight at 37 °C. Controls without DNA or with empty plasmid (pHT01) without insert were also prepared.

GFP and melittin protein induction studies

A single colony was selected from a freshly streaked LB-chloramphenicol (transgenic *B.subtilis* pHT01-GFP clone or transgenic *B.subtilis* pHT01-Mel clone) and put into 2 ml of LB- chloramphenicol broth for 16h. 500 µl of this culture was used as inoculum to a 250 ml $LBCm^R$ broth and allowed to grow until the $O.D_{600}$ reaches 0.4-0.5. The culture was then induced with 1mM IPTG for various time periods. Uninduced cells were used as the negative control. The induced cells at different time intervals were centrifuged at 3000 ***g*** for 15 min. The cells were placed in lysis buffer (100 mM Tris-Cl, 500 mM NaCl, 0.5 M EDTA, 0.1% Triton X-100, 0.1% Tween-20, 8% glycerol, 250 mM urea, 5 mM β-2 mercaptoethanol, 100 µg ml^{-1} PMSF, 1 µg ml^{-1} protease inhibitor cocktail, 50 µg ml^{-1} lysozyme). The protein extracts from transgenic clones were dialyzed overnight against 25 mM Tris-HCl (pH 7.2) with a 2 KDa (melittin extract) or

10 kDa (GFP extract) Slide-A-lyzer (Pierce Biotechnology Inc., Rockford, IL, USA) at 4°C.

Western blot for GFP expression in transgenic *Bacillus subtilis*

One hundred µg of total protein, as determined by Bradford assay (BioRad, QuickBradford kit, Hercules, CA, USA), were mixed with Lane Marker Reducing Sample Buffer (Pierce Biotechnology Inc, Rockford, IL) and heated for 5 minutes in 80°C. The sample was electrophoresed on a 10% SDS-PAGE gel and transferred to a PVDF membrane (BioRad, Hercules, CA, USA). The blot was blocked with 5% BSA in tris buffered saline containing 1% tween-20 (TBST) for 1 hour, and washed three times with TBST. It was then probed with a mouse anti-GFP antibody (Sigma-Aldrich) for 1 hour and washed three times with TBST, then probed with alkaline phosphatase-linked goat anti-mouse antibody (Sigma Aldrich) at a dilution of 1:5000 and developed using an alkaline phosphatase immunoblot detection system (Chemicon Inc, Billerica, MA, USA).

Growth characteristics and plasmid stability studies of the transgenic *B.subtilis* strains

A single colony was selected from a freshly streaked LB (wild type *B. subtilis* 1012) or LB-chloramphenicol (transgenic *B. subitlis*-pHT01-Mel) plate to LB or LB-chloramphenicol broth. The $O.D_{600}$ values were determined at progressive time intervals to track the growth curve of the wildtype and transgenic strain. The plasmid stability studies were conducted as a modified protocol of Fleming et al. (1988). Briefly, LB was inoculated from a single chloramphenicol-resistant colony. The culture was grown to an $O.D_{600}$ of 0.3-0.4 and then an aliquot of culture was serially diluted and plated in LB or LB-chloramphenicol agar. After 24 h incubation, the colonies were counted. Another

colony from LB-chloramphenicol agar was taken and inoculated into LB medium and allowed to grow to an $O.D_{600}$ of 0.2-0.3. This process was repeated daily for 7 days. The number of colonies growing on the LB-chloramphenicol and on the LB was determined.

Western Blot for melittin expression in transgenic *Bacillus subtilis*

The samples were mixed with tricine sample buffer (Bio-Rad, Hercules, CA, catalog # 161-0739) and boiled to at 80°C for 5 min. Equal amounts of protein as determined by Bradford assays were separated on 4-16% Bis-Tris (NuPage catalog#NP0322BOX) or 4-16% Tricine gels and transferred to 0.2 µm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Polypeptide marker (Biomarker) and melittin control (2µl – 10 µM) was run simultaneously in separate lanes. The blot was blocked with 5% BSA in TBST for 1 hour, and washed three times with TBST before adding rabbit anti-melittin antiserum (Biosynthesis Inc., Lewisville, TX) at 1: 2000 and incubated overnight at 4°C. The blot was then washed thrice, probed with an alkaline phosphatase-linked goat anti-rabbit IgG antibody (Sigma-Aldrich) at a dilution of 1:4000 and developed using an ECL chemiluminescent detection system (Chemicon Inc, Billerica, MA, USA).

ELISA for melittin detection

Purified *B. subtilis* bacterial extracts were diluted to 500µl in a carbonate buffer (pH 9.5), and 1 µg total protein was incubated per well of a polystyrene 96-well plate (high-binding capacity, Poly-Sorb, NuncTM) at 37°C for 90 min. After three washes in TBST, the wells were further blocked with 2.5% BSA for 1 h at room temperature. The wells were washed as before, and 100 µl of a 1:500 dilution of rabbit anti-melittin antiserum was added (Biosynthesis Inc., Lewisville, TX) followed by incubation at 4°C

overnight. The wells were washed, and 100 µl of a 1:2500 dilution of anti-rabbit IgG conjugated to horseradish peroxidase (Sigma-Aldrich, MO) was added. After a final set of three washes, 100 µl of tetramethylbenzidine peroxidase substrate (BioRad, Hercules, CA, USA) was added to each well and allowed to react for 10 min to develop color. Color development was stopped with 100 µl 1µM H_2SO_4 per well and the absorbance was read on ELISA plate reader (Molecular Devices, SupectraMAX 250, Sunnyvale, CA, USA) at 450 nm. The background signal from *B.subtilis* control extract was subtracted from samples. The melittin concentration in the extracts was estimated based on a standard curve generated with synthetic pure melittin (Biosynthesis Inc., Lewisville, TX) spiked into *Bacillus subtilis* extracts.

Statistical analyses

The means of the ELISA data from uninduced control and 2h-induced sample were subjected to Students t test, and a $p \leq 0.05$ was considered significant. A statistical software GraphPad Prism was used (GraphPad Software, Inc., San Diego, CA) to perform this analysis.

RESULTS AND DISCUSSION

GFP expression by pHT01-GFP transformed *B. subtilis* strains

We selected pHT01 vector for expressing recombinant protein in *B. subtilis*. pHT01 is an inducible vector which contains *B.subtilis* origin of replication. The promoter, Pgrac, drives protein production with Cm^R as selection marker (Nguyen et al., 2005). Although previous studies have shown that pHT01 can attain high recombinant protein expression in *B. subtilis* (Phan et al., 2006), we wanted to confirm this. To that end, we cloned the GFP gene downstream of the promoter to create pHT01-GFP and

transformed two strains of *B.subtilis* (1012 and 168). We initially confirmed the GFP expression in transgenic *B.subtilis* pHT01-GFP via fluorescence microscopy. Cells induced with IPTG were glowing in fluorescent light from 30 min (**Fig. 18B**) post induction, whereas no glowing cells were observed in uninduced sample (**Fig. 18A**).

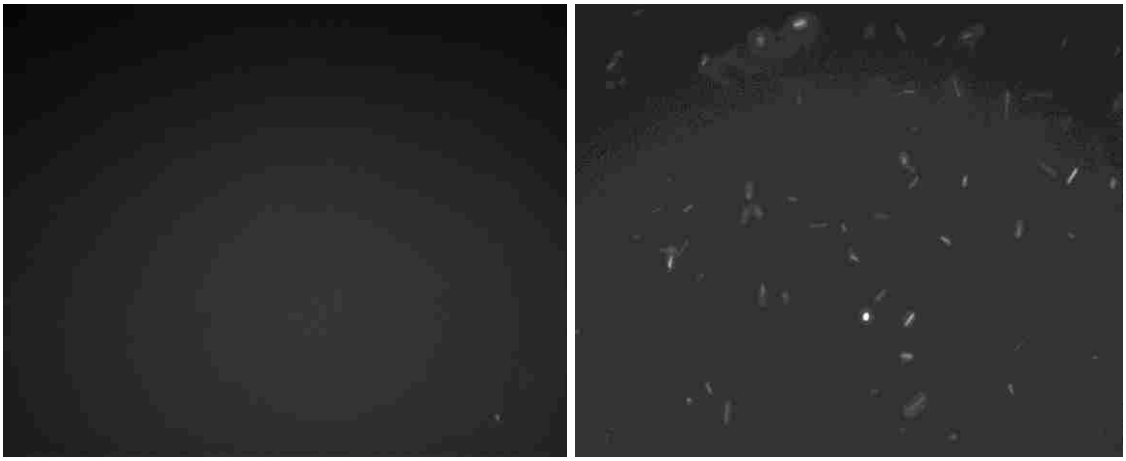


Figure 18. GFP expression in *B. subtilis* 1012 transformed with pHT01-GFP. **A)** Uninduced control **B)** Induced with IPTG 1 h.

We then biochemically demonstrated GFP expression via Western Blot. *B. subtilis* 1012 transformed with pHT01-GFP showed protein expression from 0.5 to 3 h, whereas no GFP expression was observed in the uninduced samples (**Fig. 19**). To determine whether there is strain-specific difference in recombinant protein expression, we also tested the GFP expression in-*B.subtilis* strain 168. The same expression trend as in *B.subtilis* 1012 was seen with *B.subtilis* 168 transformed with pHT01-GFP (data not shown). These studies confirmed that pHT01 can effectively express recombinant proteins in several *B.subtilis* strains.

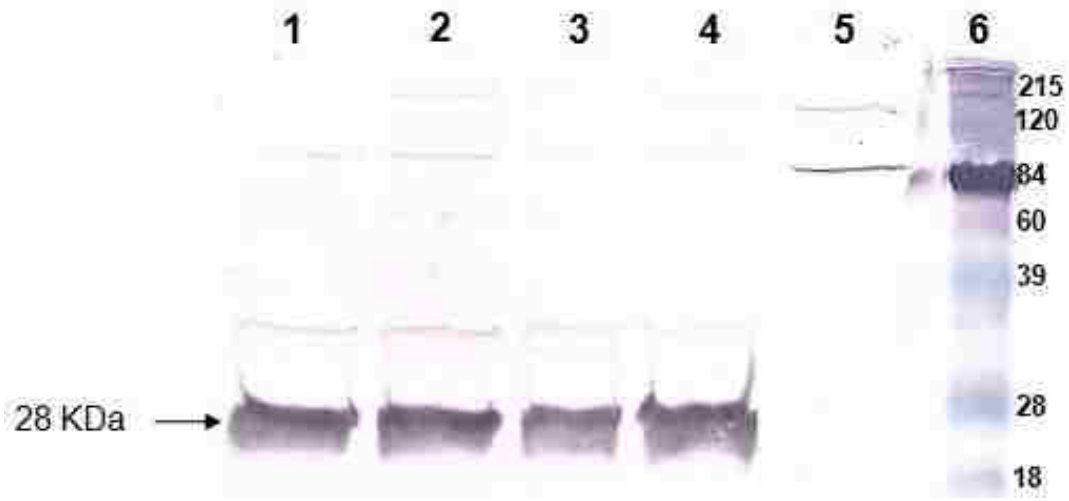


Figure 19. Western Blot showing GFP expression in *B subtilis* 1012 transformed with pHT01-GFP. *B.subtilis* induced with IPTG for 3 hr (lane 1), 2 hr (lane 2), 1 hr (lane 3) and 0.5 hr (lane 4). Lane 5 is uninduced control; lane 6 is the protein marker.

Cloning melittin gene into pHT01 and molecular transformation of Bacillus subtilis

We cloned melittin gene upstream the promoter and confirmed the sequence and orientation of the insert via PCR and sequencing. We then transformed *B.subtilis* 1012 and *B.subtilis* 168 with pHT01 (empty vector) and pHT01-Mel plasmid. The transformation efficiency was low with ~ 15-20 CFU/5µg of plasmid DNA used. DNA was purified from selected clones and subjected to PCR for confirming the presence of melittin gene insert. PCR using specific primers showed DNA bands from both *B.subtilis* 168 and *B.subtilis* 1012 transformed with pHT01-Mel (**Fig. 20**) which was similar in size compared to the positive control, pHT01-Mel plasmid. No PCR amplification was observed in the negative control.

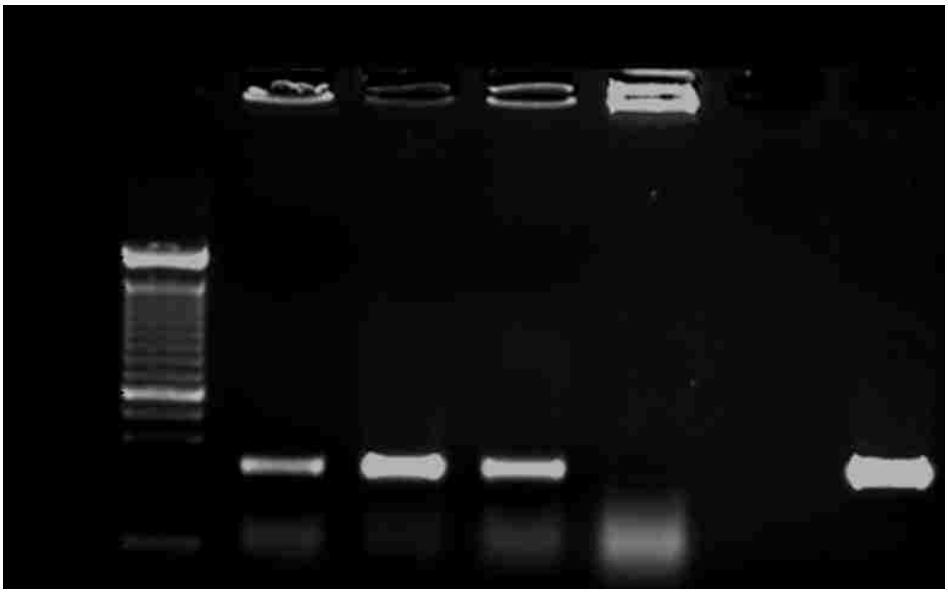


Figure 20. Detection of the melittin gene in *B subtilis* transformed with pHT01-Mel by PCR. 100bp DNA marker (lane 1); *B.subtilis* 1012 pHT01-Mel (lane 2); *B.subtilis* 168 pHT01-Mel (lane 3); pHT01-Mel DNA (lane 4, + control); *B.subtilis*-pHT01- (lane 5,- control); PCR-Blank (lane 6, no DNA); pHT01-Mel DNA, colony (lane 7, + control).

Growth characteristics and plasmid stability of the transgenic strains

The growth of *B.subtilis* 1012-pHT01-Mel was similar to that of the non-transformed clone. Similarly, *B.subtilis* 168-pHT01-Mel showed similar growth as *B.subtilis* 168 without the plasmid (**Fig. 21A and B**). This suggests that the transformation using pHT-based plasmids did not alter the growth properties of *B.subtilis*. The pHT01-Mel plasmid was stable in the *B. subtilis* 1012 clones for 7 d (**Fig. 22**) consistent with previous studies in *B.subtilis* (Phan et al., 2006).

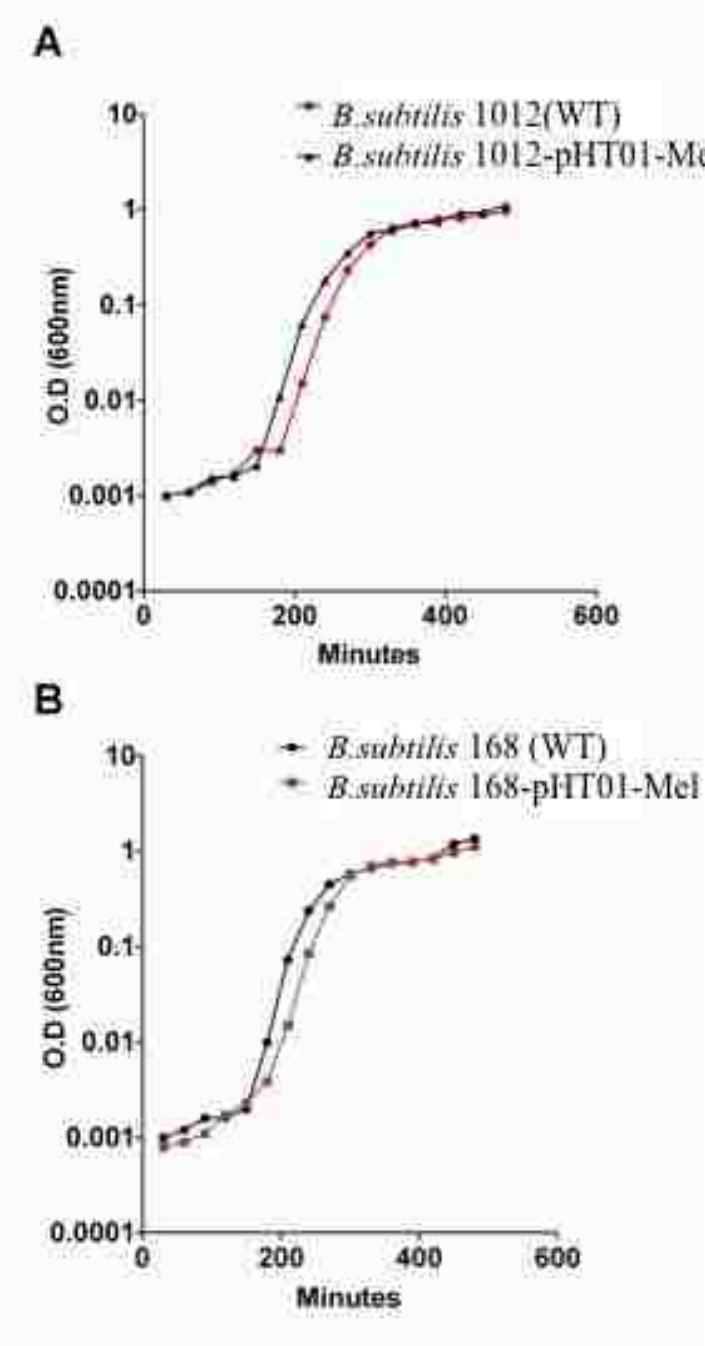


Figure 21. Growth characteristics of wildtype and transgenic *B. subtilis* lines.
A)Transgenic and non-transformed (WT) *B.subtilis* strain 1012 **B)** Transgenic and non-transformed (WT) *B.subtilis* strain 168.

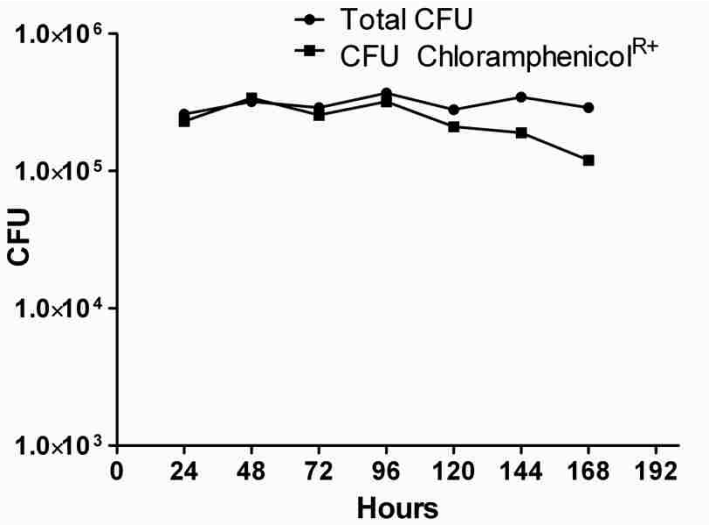


Figure 22. Plasmid stability of transgenic *B. subtilis*-pHT01-Mel clone. Colony forming units (CFU) were measured over the course of 7 days in the presence (which requires the pHT01 plasmid) or absence of chloramphenicol.

Standardization of Western Blot

Extract of wild type *B. subtilis* 1012 was spiked with different concentrations of synthetic melittin to determine the detection limit of our assay. We used two different gel systems to resolve the peptides; Tricine gel system (Novax) and Bis-Tris (Novagen). We blotted the gel into 0.2 micron nitrocellulose membranes and probed with anti-melittin serum (1:2000 dilution). The tricine gel clear melittin-specific band in *B. subtilis* extract spiked with as low as 44 ng melittin (**Fig. 23**). High molecular weight bands (16KDa)

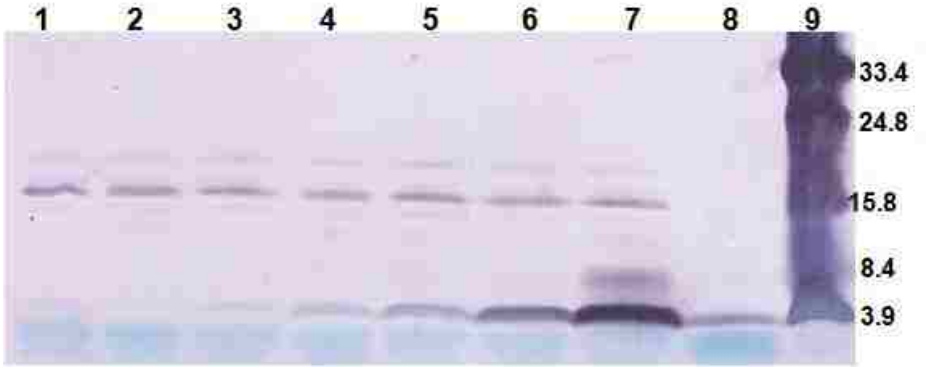


Figure 23. Detection limit by Western blot of melittin spiked after extract preparation. Melittin spiked *B.subtilis* Ext- 43.8 ng (lane 1); 87.5 ng (lane 2); 175 ng (lane 3); 350 ng (lane 4); 700 ng (lane 5); 1400 ng (lane 6); 2800 ng (lane 7); positive control synthetic melittin in PBS (lane 8); polypeptide marker (lane 9).

were observed in the blot suggesting cross reactivity of antiserum with the *B.subtilis* extract. The bis-tris gel system and subsequent blot gave melittin specific band with 175 ng melittin (not shown). There was more background in bis-tris based blot, hence all our subsequent experiments we used only tricine gel system. We then asked whether the extract preparation and dialysis process reduced the melittin signal by spiking-the *B.subtilis* extract during lysis step (dring the first vortexing after adding the lysis buffer) with increasing concentration of melittin. Our Western Blot showed specific bands at minimum of 350 ng mellittin as compared to 44 ng when melittin was added after dialysis, indicating that there was an 80% loss due to dialysis (**Fig. 24**).

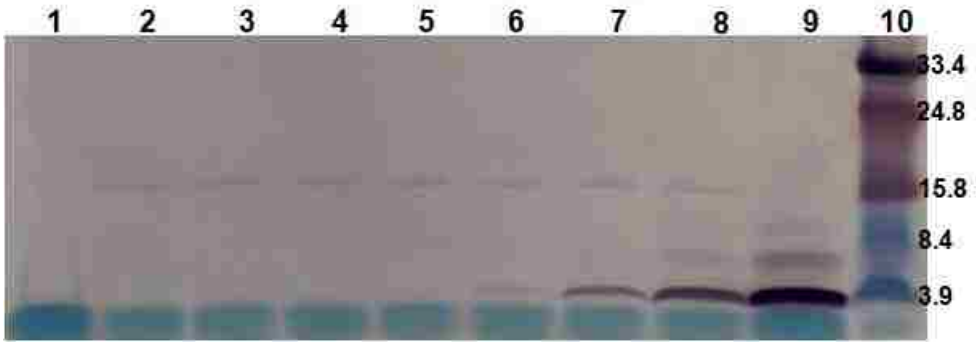


Figure 24. Detection limit by Western blot of melittin spiked before dialysis of extract preparation. No sample- loading buffer only (lane 1); Melittin spiked *B.subtilis* Ext- 43.8 ng (lane 2); 87.5 ng (lane 3); 175 ng (lane 4); 350 ng (lane 5); 700 ng (lane 6); 1400 ng (lane 7); 2800 ng (lane 8); positive control synthetic melittin in PBS (lane 9); polypeptide marker (lane 10).

Melittin detection using Western Blot and ELISA

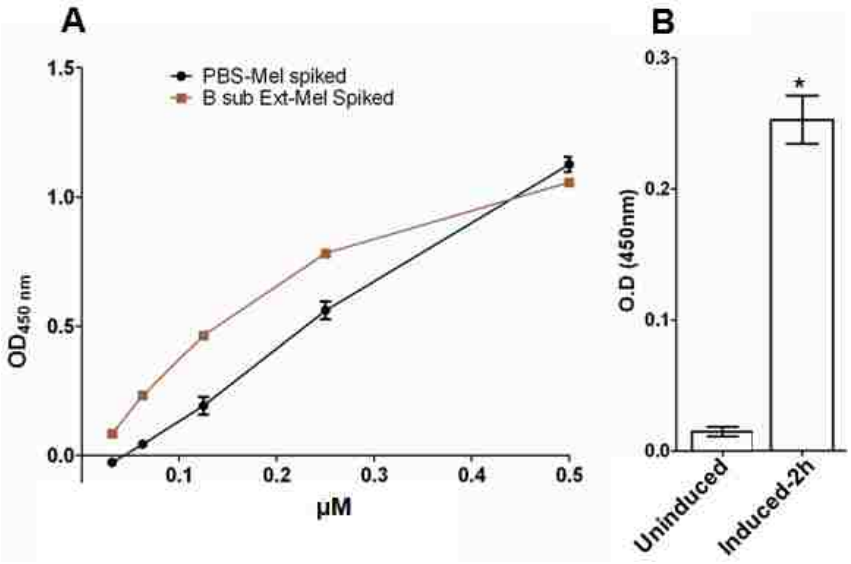
We conducted a series of melittin induction studies using two transgenic *B.subtilis* strains (*B.subtilis*168- and *B.subtilis* 1012) carrying pHT01-Mel plasmid. We varied the induction temperature or induction time in these trials. The first three studies (Study1-3), we used *B.subtilis*168-pHT01-Mel and the next four studies, we used *B.subtilis* 1012-

pHT01-Mel (**Table 7**). None of the protein extracts prepared from these studies showed any melittin expression in our Western Blot assay (**Fig. 25**). We also failed to detect melittin expression in insoluble fractions and media supernatant from induced samples (not shown). The Western Blot from various induction studies suggested that either the clones are not expressing melittin or it was below the detection limit (300-400ng).

Figure 25. Western blot to detect melittin (Study 4). No melittin specific band was observed from the induced samples. pHT-Mel 3 h Ind (lane 1); pHT-Mel 2h Ind (lane 2); pHT-Mel 1.0 h Ind (lane 3); pHT-Mel Uninduced (lane 4); Syn Mel- positive Control (lane 5); Polypeptide marker (lane 6).

In order to have a more sensitive assay for melittin, we employed ELISA and spiked melittin into *B.subtilis* extracts for a standard curve. The standard curve gave a dose-dependent melittin specific signal range of 0.03 to 0.5-µM (**Fig. 26A)**. The extracts from IPTG induced *B. subtilis* 168-pHT01-Mel clones showed a 12-fold increase in melittin signal in 2 h induced samples compared to uninduced control samples (**Fig. 26B**). A similar trend of melittin expression was observed from the induced extracts from *B. subtilis*-1012-pHT01-Mel clones (not shown). An estimate of melittin expression in transgenic *B.subtilis* were calculated using the melittin-spiked *B.subiltis* extract standard curve and was in the range of 200-400 ng per 10 million cells (2 - 4 x 10^{-5}ng/cell).

Studies have shown that transgenic strains of *B.subtilis* can produce 5-8µg protease and 2-3µg single-chain antibody from 10 million cells (Schumann, 2007). Compared to this efficiency, the level of recombinant melittin production in the present study was low.



[redacted]

There might be several reasons for this low-level melittin expression. The stability of short peptides in a cellular protease-rich environment might be low due to peptide degradation. The peptides might aggregate into high molecular structures in the cellular environment. Further, melittin could interact with intracellular targets, reducing expression. Although our previous studies have shown that *B. subtilis* is resistant to exogenous peptides, there might be deleterious effects to the host cell expressing the peptides. We also inserted a methionine codon to initiate translation, which was

Table 7. Summary of melittin induction studies *using B.subtilis* 168-pHT01-Mel and *B.subtilis* 1012-pHT01-Mel[1]

Study #	***B.subtilis* strain**	**Growth temperature**	**Induction temperature**	**Induction time**	**Lysis method**	**Melittin detection by method**	
						WB	**ELISA**
Study 1	168	30ºC	25ºC	1h, 2h, 3h	CLB[2]+vortex	-	+
Study 2	168	30ºC	30ºC	1h, 2h, 3h	CLB+vortex	-	+
Study 3	168	30ºC	25ºC	30m, 45m, 90m	CLB+vortex	-	nd
Study 4	1012	30ºC	25ºC	1h, 2h, 3h	CLB+vortex	-	+
Study 5	1012	30ºC	30ºC	1h, 2h, 3h	CLB+vortex	-	+
Study 6	1012	30ºC	25ºC	30m, 45m, 90m	CLB+vortex	-	nd
Study 7	1012	30ºC	25ºC	1h, 2h, 3h	CLB+sonication	-	+

[1]Abbreviations: CLB, complex lysis buffer; NB40, NB40 lysis buffer; WB-, not detected by Western blot; ELISA+, low level detection by enzyme linked immunosorbent assay; nd, not done

not part of the native peptide, and this might have contributed to the low expression.

For functional anti-vibrio activity for paratransgenic approaches it would be ideal to increase the melittin expression at least an order of magnitude in the range of 1-2 $x10^{-4}$ ng/cell. Several strategies for accomplishing this are proposed in Chapter 6.

CHAPTER 6

GENERAL SUMMARY AND FUTURE DIRECTIONS

GENERAL SUMMARY

Although infectious disease is a major threat in shrimp mariculture throughout the world, there are not many sustainable methods available to combat these disease problems (Hill, 2005). However, shrimp aquaculture is the fastest growing food sector globally and holds great promise for closing the nutritional gap for much of the world's population (Smith et al., 2010). The need to devise novel and sustainable methods against various infectious agents is pressing as traditional methods such as antibiotics are banned and other methods are not effective. We seek to develop a practical and viable infectious disease control system for shrimp producers based on a novel approach called paratransgenesis. The main aim of this dissertation is to develop a marine paratransagenic approach, a disease control method initially developed to control vector-borne-diseases, as a way to control shrimp diseases. In the classical paratransgenic strategy, commensal or symbiotic bacteria found at mucosal sites of pathogen transmission are isolated and genetically altered to elaborate immune peptides or engineered single-chain antibody fragments (scFv) that neutralize infectious agents. The transgenic bacteria are then delivered back to mucosal sites where disease transmission occurs. In large-scale shrimp rearing, larval stages are fed with *Artemia* -an aquatic crustacean feed organism to boost nutrition and improve survival in hatcheries. *Artemia,* which are reared separately, are fed with microalgae and other probiotic bacteria. Artemia is a nearly ideal feed organism: economical, hardy, and readily available worldwide. As with classical paratransgenesis, there are four elements associated with the shrimp culture system; the Artemia, which is the arthropod intermediate, the microalgae feed organisms, the disease-causing bacteria

such as Vibrio, Aeromonas etc, and finally the host shrimp, which succumbs to the disease.

To apply this paratransgenic strategy to diseases of farmed shrimp, we proposed a modified paratransgenic approach that involves organisms used as feed for marine animals (Durvasula et al., 2006; Durvasula et al., 2009). We hypothesized that Artemia can be used as an effective delivery for recombinant proteins that impart passive immunity to shrimp against a variety of infectious pathogens.

First, we wanted to study the accumulation and retention of recombinant proteins in Artemia gut for a specific period of time for optimizing paratransgenic disease control in shrimp aquaculture. Transgenic *Escherichia coli* expressing fluorescent marker proteins and the transgenic cyanobacterium *Synechococcus bacillarus* expressing a functional murine single-chain antibody, DB3, were fed to Artemia. Stable expression and retention of several marker molecules (e.g. GFP, DS Red and DB3) up to 10 h after feeding with *E. coli* were evident within the gut of Artemia. Engineered strains of *S. bacillarus* expressing DB3 accumulated within the gut of Artemia, with detectable antibody activity for 8-10 h of feeding via ELISA, coincident with the time period of highest density of transgenic *S. bacillarus* in the Artemia gut, suggesting that recombinant proteins can be delivered effectively to nauplii of Artemia and retain their biological activity (Subhadra et al., 2010). Co-delivery of two recombinant proteins simultaneously in the gut of Artemia was also demonstrated. During these first sets of studies for developing a marine paratransgenic approach, we have demonstrated delivery, retention and biological activity of recombinant proteins in the gut of Artemia. Furthermore, co-expression of multiple markers in the gut of Artemia confirms that

paratransgenic manipulation of these animals for delivery of a battery of molecules with activity against pathogens is possible. This method of delivering recombinant protein-producing microbes via Artemia to shrimp larval stages utilizes the natural feeding behaviors of both Artemia and farmed shrimp and may permit-bioamplification of the functional antibodies through the food chain (Durvasula et al., 2009). This strategy could be quite significant in settings of drug-resistant pathogens where concurrent delivery of molecules that act at different target sites of the pathogen would slow the emergence of resistant species. Expression of molecules which target infections agents of mariculture in shrimp via commonly deployed feed organisms such as Artemia could potentially offer powerful new tools in the ongoing global effort to increase food supply.

To begin to develop paratransgenic shrimp, we studied the delivery of transgenic proteins to the shrimp, *Litopenaeus vannmei* via direct feeding of transgenic bacteria or via feeding through transgenic-bacteria engorged Artemia. We conducted three shrimp feeding protocols. Trial 1 was designed to evaluate the accumulation of an engineered antibody in the gut of *L. vannamei* consisted of an 18 day active feeding phase followed by 12 d wash-out phase. In trial 2 and 3, which were designed to determine the dynamics of recombinant GFP within the gut of paratransgenic *L. vannamei* fed with GFP expressing bacteria engorged Artemia or fed directly with GFP-expressing EcN bacteria. Results from the feeding protocols showed that shrimp gut fed with paratransgeinc Artemia and transgenic bacteria showed an increase in the recombinant protein during the active feeding phase and a decrease in recombinant protein during the wash-out phase. The biotransfer of recombinant GFP via Artemia to shrimp caused accumulation of 10-15% more protein in the gut of shrimp fed 20 or 40 Artemia per shrimp per day than via

direct feeding of transgenic bacteria (10^6 $CFUml^{-1}$). Through these studies we demonstrated that recombinant functional proteins can be delivered to shrimp via Artemia in a paratransgenic approach. Therefore, bioaccumulation of anti-infectious protein molecules such as antibodies, cationic peptides, and antivirals to shrimp via a paratransgenic approach holds promise for sustainable disease –mitigation in shrimp mariculture.

We selected Vibriosis as the target disease for proof of concept studies. Vibriosis is one of the most devastating diseases caused by at least 14 species of *Vibrio* (Brock and Lightner, 1990). Highly virulent strains such as *Vibrio harveyi*, *V. campbellii*, *V. penaeicida,* and *V. nigripulchritudo* can cause up to 90% mortality within a day of infection in *Penaeus monodon, P. japonicus* and *L. vannamei* (Lavilla-Pitago et al., 1998). The lack of an adaptive immune response limits the use of Vibrio-specific vaccination strategies in shrimp (Hill, 2005). Use of antibiotics in aquaculture is banned in many countries due to emergence of drug-resistant microbes (FAO-WHO, 2006).

The first task was to screen antivibriocidal molecules, which can be used in the marine paratransgenic approach. Cationic peptides such as cecropin, apidaecin, magainin and melittin, which are part of natural innate immunity (Zasloff, 2002), could serve as effector molecules in a marine paratransgenic strategy to control Vibriosis. These peptides neutralize a variety of Gram- negative bacteria at submicromolar to low micromolar concentrations, including some antibiotic resistant strains (Stark et al., 2002). This study examines the *in vitro* activity of 5 different antimicrobial peptides against different *Vibrio* spp., probiotic bacteria and algae, the last two being potential candidates to express these molecules in paratransgenic control. We also studied the evolution of

resistance against single cationic peptide treatments and report here that a combination of cationic peptides can avoid emergence of resistance in *V. harveyi*, *V. penaeicida*, and *V. campbellii,* the three most important shellfish pathogens.

Moricin was the most potent antimicrobial agent against *Vibrio* spp. with a minimum bactericidal concentration (MBC) in the 0.04-0.31 µM range. Cecropin and melittin killed Vibrios in the MBC range of 0.02-17.5 and 2.5-10.0 µM, respectively. Probiotic bacteria and algal feed organisms (e.g. *Bacillus subtilis*, *Brevibacterium linens, Dunaliella salina* and *Synechococcus bacillarus*) displayed high levels of resistance toward cecropin and melittin suggesting that they would be suitable for a paratransgenic approach to Vibriosis in shrimp. A mixture of 0.01 µM cecropin and 0.1 µM melittin was bactericidal against *Vibrio* spp. demonstrating synergistic activity. A mixture of 0.01 µM cecropin and 0.01 µM melittin prevented evolution of bacterial resistance, suggesting that simultaneous expression of these peptides could offer new tools in a paratransgenic approach to Vibriosis in shrimp aquaculture. To test the protective efficacy of these peptides *in vivo*, we conducted Vibrio challenge trials on Artemia. A mixture of cecropin and melittin at a concentration as low as 1 µM partially protected Artemia against the highly pathogenic *Vibrio campbellii* CAIM 372. Toxicity of the peptides toward *Artemia* was not observed, even at concentrations as high as 5 µM. The protective effect of the peptides appears to be due to low colonization of *Vibrio* as indicated by the significantly lower *V. campbellii* load in Artemia samples treated with peptides during the initial 2-4 h after bacterial challenge. In summary, this study indicates that melittin and cecropin, two naturally occurring antimicrobial peptides, have potential applications as anti-Vibrio agents in paratransgenic strategy for control of pathogenic Vibrios. Both agents exhibit

selective toxicity toward diverse strains of Vibrios with minimal effect on feed bacteria and algae. Furthermore, when delivered concurrently, they exhibit marked synergistic activity toward *Vibrio* spp. and appear to slow evolution of target bacterial resistance.

Paratrangenic systems make use of transgenic host bacteria that expresses anti-infectious molecules. Since melittin and magainin have antibacterial activity against pathogenic vibrios and no activity against *B. subtilis*, we cloned melittin and magainin gene into pHT01, a *B. subtilis*-specific inducible expression vector, for the production of melittin and magainin. The growth patterns of transgenic strains were similar to the wild type strain. The pHT-based plasmids were stable in *B. subtilis* with neglible plasmid loss during 7 d study without antibiotic selection. Our ELISA data showed that transgenic *B. subtilis* pHT-Mel exhibited low but detectable levels of melittin expression. However, *in vitro* and *in vivo* anti-vibrio studies indicated that this level of recombinant peptide expression was insufficient to cause detectable anti-bacterial activity.

FUTURE RESEARCH DIRECTIONS

The following are key research areas for further developing marine paratransgenic system as an environmentally sustainable disease mitigation strategy in the aquaculture sector.

1. Optimization of melittin expression in Bacillus subtilis

There might be several reasons for the low level expression of melittin. The stability of peptides such as melittin in a cellular protease-rich environment might be low (Jenssen et al., 2006). The cationic peptides such as melittin can also aggregate into high molecular structure in cellular environment (Raghuraman and Chattopadhyay, 2007). Further, melittin has some intracellular targets (e.g. it can affect intracellular signaling,

calcium channels, cell-wall synthesis, and ribosome stoichiometry (Mix et al., 1984; Kataoka et al., 1989)), which might have negatively interacted with the protein synthesis machinery and reduced the expression (Jenssen et al., 2006). Although we showed that *B. subtilis* is resistant to exogenous antimicrobial peptides (Chapter 4), there might be deleterious effects to the host if the peptide is expressed intracellularly. We also inserted a translation start codon for methionine amino acid upstream of the melittin gene. The change in the structural nature of the peptide due to this addition might have interfered with the level of melittin expression.

Various strategies can be devised to optimize the expression of AMP in *B.subtilis*. Inserting an inactive pre-pro gene segment upstream of coding region of melittin gene may enhance the expression of AMP. A similar strategy naturally occurs with certain *B.subtilis* strains for the production of subtilin. Subtilin is a short cationic peptide of 3.4 kDa and has broad-spectrum bactericidal activity against both Gram positive and Gram negative bacteria. The subtilin is expressed as a propeptide which is secreted outside the cell. Multiple of proteins are associated with the production, post translational modification and secretion of subtilin (**Fig. 27**). The inactive pro-subtilin secreted outside the cells is cleaved to active subtilin by nonspecific serine proteases (Abriouel et al., 2001).

Similar to this natural mechanism, melittin can also be expressed as an inactive propeptide with a signal sequence to affect secretion outside the cell. Such a pro-peptide can be cleaved to its active form using the dipeptidases in Artemia or shrimp digestive system (**Fig. 28**). A comparable approach using another AMP, bovine lactoferricin

(LFB), has been recently described by Li et al. (2009) for the control of *Vibrio parahaemolyticus* infection in fish.

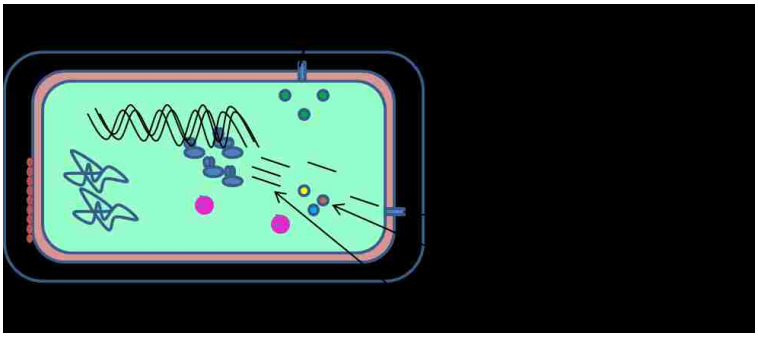


Figure 27. Mechanisms of natural expression of AMP in *B.subtilis*. Subtilin is expressed as a propeptide in the cell prior to secretion. Multiple proteins are associated with the production, post-translational modification and secretion of subtilin. The inactive secreted pro-subtilin is cleaved to active subtilin by nonspecific serine proteases (Abriouel et al., 2001).

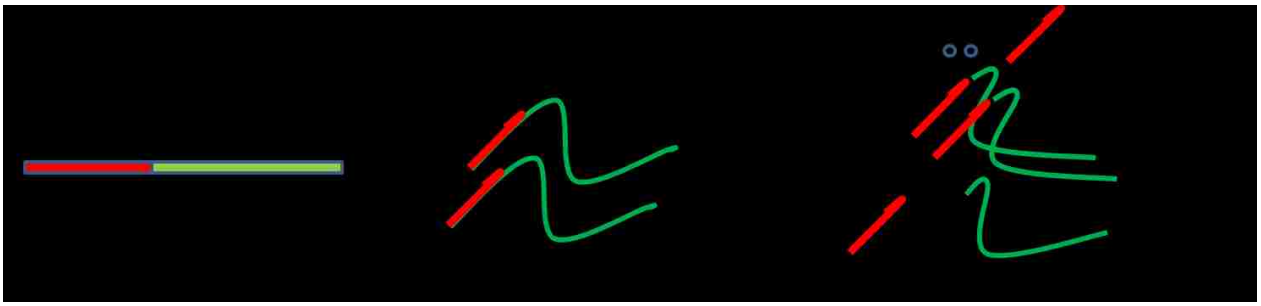


Figure 28. Possible strategy to express melittin in *B.subtilis* for paratransgenesis. Melittin can be expressed as a pro-peptide by cloning a signal peptide gene upstream of the melittin gene. The inactive pre-melittin could be cleaved by shrimp gut proteases to release active melittin.

They transformed *Nannochloropsis oculata,* a larval feed alga, to express LFB as an inactive propeptide. The propeptide was designed in such as way that the fish digestive enzymes cleaved off the active peptide. The medaka fish (*Oryzias latipes*) were fed with the transgenic algae followed by *Vibrio parahaemolyticus* infection challenge. For fish

fed transgenic algae, the average survival rate after a 24-h period of infection was much higher than that of medaka fed with wild-type algae suggesting that the LFB-containing transgenic microalgae had bactericidal action against *V. parahaemolyticus* in medaka fish digestive tract.

Recently, for the recombinant extracellular expression of cecropin AD, a chimeric AMP which has the first 11 amino acid residues from cecropin A and the last 26 residues from cecropin D, was achieved in *B. subtilis* (Chen et al., 2009). They fused the cecropin AD gene with a small ubiquitin-like modifier gene (SUMO) and a signal peptide, SacB, and a *B.subtilis* expression system was constructed by the introduction of an operon including an isopropyl-beta-D-thiogalactopyranoside (IPTG)-inducible Spac promoter, a signal peptide of amyQ, and the SUMO protease gene (**Fig. 29**). High level recombinant cecropin AD production was obtained using this expression system (30.6 mg of recombinant cecropin AD was purified from one liter of culture supernatant).

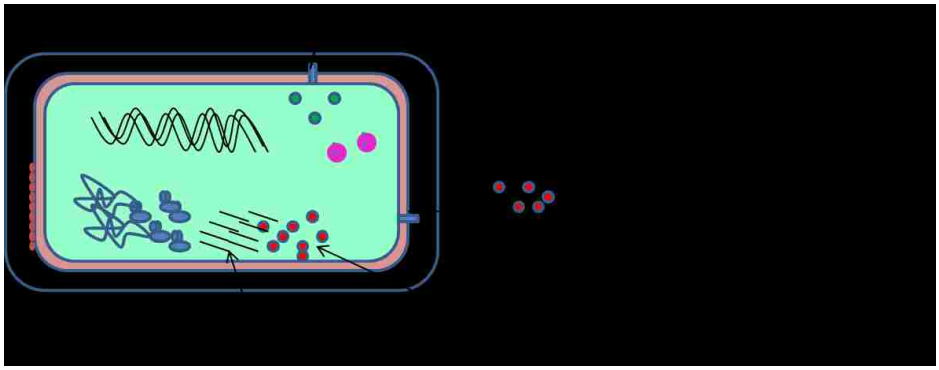


Figure 29. Recombinant cecropin AD expression in *B.subtilis*. *B.subtilis* was transformed with plasmid to express cecropin AD as a fusion protein with SUMO protein (cecropin AD+SUMO protein). The signal peptide in the plasmid, SacB, promotes the extracellular secretion of cecropin AD+Sumo protein. The plasmid also expresses and extracellularly secretes SUMO protease, which specifically cleaves SUMO protein from the fusion protein to release the active cecropin AD (Chen et al., 2009).

The SUMO (small ubiquitnin-like modifier) protein component of the protein has a chaperone characteristic, which enhanced protein expression, enhanced stability, reduced proteolytic action, and enhanced solubility/folding. A similar approach for the melittin expression can also be devised. Plasmid can be designed to expression melittin fused to SUMO protein with concurrent expression of SUMO protease from the same plasmid. This will give melittin high stability and the peptide can be cleaved via the expressed SUMO protease into its active form as depicted in **Fig. 30**.

2. Transformation of algal species to express anti-infectious agents

Algal strains such as *Dunaliella salina*, *Phaeodactylum tricornutum*, *Nannochloropsis* are extensively used in aquaculture larval nutrition. These strains are ideal because of the high nutritious components such as long chain fatty acids and vitamin contents. Genetic transformations of these strains to express recombinant proteins are also described (Apt et al., 1996; Mayfield et al., 2003).

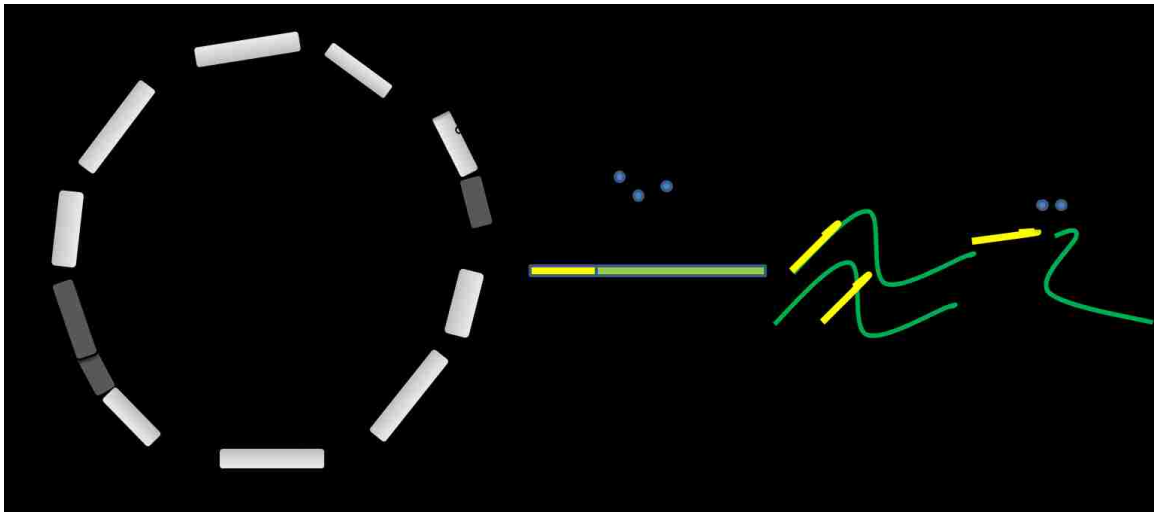


Figure 30. Possible strategy to express melittin in *B.subtilis* for paratransgenesis. *B.subtilis* can be transformed with plasmid to express melittin as a fusion protein with SUMO protein (melittin+SUMO protein). The plasmid also expresses and extracellularly secretes SUMO protease, which specifically cleaves SUMO protein from the fusion protein to release the active melittin.

Moreover, our studies have found that algal species such as *Dunaliella* and *Synechococcus* are more resistant to AMPs. As algal species are ideal larval feed, another future direction in marine paratransgenic approach is the development of strains of algae which express anti-infectous molecules against marine pathogenic virusus and bacteria.

3. Developing specific anti-infectious molecules in a paratrangenic approach

Anti-VP28 single chain antibody, a White spot syndrome virus viral entry-blocking molecule, can be used against WSSV. Similary, PmAV is antiviral protein expressed in WSSV-resistant shrimp, can also be used for paratransgenic approach. Engineering single chain antibodies with high neutralizing capacity against marine infectious virus (WSSV) and bacteria (*Vibrio* spp, *Aeromonas* spp) may have considerable value. These molecules are preferred over AMPs for the following reasons:

1) They are larger proteins: Antibodies are high molecular weight proteins and chemically more stable. The expression of these molecules for functional purposes in shrimp gut may undergo less degradation compared to small peptide molecules.

2) There should be less host cellular effects: The expression of antimicrobial peptides in host bacteria for paratransgenic application may interfere with the physiology of host bacteria. However, target-specific antibodies should not have deleterious effects on host bacteria.

3) They target specific pathogens: Specific antibodies can be engineered for pathogens so there should be no non-specific effect on other bacterial flora in the shrimp gut.

4) There is less probability of antibiotic resistance: Use of antimicrobial peptides poses the risk of emergence of peptide-resistant bacteria. The use of target-specific antibodies against an essential bacterial epitope will not have this negative implication.

Similarly, broad-spectrum antiviral proteins such as cyanovirin (Bewley et al., 1998), griffithsin (Mori et al., 2005), and PmAV (Luo et al., 2003) can also be explored for controlling viral diseases of mariculture via marine paratrangenic approach.

4. Expression of quorum-sensing molecules in paratransgenic host bacteria

Quorum-sensing blocking molecules are a new strategy for disease control against infectious diseases. The term "quorum sensing" describes the ability of a microorganism to perceive and respond to microbial population density, usually relying on the production and subsequent response to diffusible signal molecules (Miller and Bassler, 2001). A significant number of gram-negative bacteria produce acylated homoserine lactones as signal molecules that function in quorum sensing. This is particulary important in controlling Vibros because most of the Vibrio species rely on quorum sensing molecules for growth, proliferation and pathogenicity (Miller and Bassler, 2001). Research groups have already proposed the scope of disruption of quorum sensing against *Vibrio harveyi* infection in aquaculture (Defoirdt et al., 2007; Liu et al., 2010). Recently, Duan and March (2010) investigated the possibility of using commensal bacteria as signal mediators for inhibiting cholera. Their study found that pretreatment of mice for 8 hours with *Escherichia coli* Nissle expressing cholera autoinducer 1, a quorum sensing blocking molecule, significantly increased mouse survival from ingestion of *V. cholera*. Similar to this, engineering algae and bacteria to express quorum-sensing blocking molecules against marine pathogenic vibrios holds promise for sustainable and specific disease control strategies in mariculture.

In conclusion, the dissertation research has demonstrated the feasibility of a paratransgenic approach for delivering proteins to Artemia and shrimp for disease

control. Further laboratory-based studies for the refinement of this approach for various bacterial and viral diseases are needed for the ultimate goal of farm-level applications.

CHAPTER 7

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