Isolation and Characterization of *Bacillus* spp. as Potential Probiotics for Channel Catfish, *Ictalurus punctatus*

by

Chao Ran

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 3, 2013

Keywords: *Bacillus*, Probiotic, *E. ictaluri*, Channel Catfish,

Approved by

Mark R. Liles, Chair, Associate Professor of Biological Sciences
Jeffery S. Terhune, Associate Professor of Fisheries and Allied Aquacultures
Joseph W. Kloepper, Professor of Plant Pathology
Joseph C. Newton, Professor of Pathobiology
Paul A. Cobine, Associate Professor of Biological Sciences

Abstract

Enteric septicemia of catfish (ESC), caused by the bacterium *Edwardsiella ictaluri*, is considered the most important bacterial disease of cultured channel catfish *Ictalurus punctatus* and is estimated to cost the industry $40 to $60 million yearly in economic losses. In addition to ESC, the recent epidemic outbreak of motile *Aeromonas* septicemia (MAS) of catfish caused by a highly virulent and emerging strain of *Aeromonas hydrophila* is a major threat to the catfish industry in southeastern United States. Control of ESC and MAS has been elusive using available treatment agents. A promising alternative approach for controlling catfish diseases is the use of probiotics. *Bacillus* strains were isolated and tested as potential probiotics for channel catfish in this research.

Bacillus strains isolated from soil or the intestine of channel catfish were screened for their antagonism against *E. ictaluri* and *A. hydrophila* as well as their capacity to survive and persist in the intestine of channel catfish. *Bacillus* strains AP79, AP143, AP193, AP254 and AB01 that showed good antimicrobial activity and intestinal survival were incorporated into feed in spore form and fed to channel catfish for 14 days before they were challenged by *E. ictaluri* in replicates. They all conferred benefit in reducing catfish mortality. Four of the five *Bacillus* strains were tested in Vietnam and protection of striped catfish against *E. ictaluri* was also observed. The safety of the four strains exhibiting strongest *in vivo* protection activity was investigated in terms of the presence of plasmids or antibiotics resistance.

A long term feeding experiment was conducted to investigate the effect of selected *Bacillus* strains on growth and innate immune response of channel catfish. *Bacillus* strain AP79, AP143, AP193 and AB01 were supplemented in feed and given to fish for 10 weeks. All the *Bacillus* feeding groups showed improved weight gain and feed conversion ratio, however the difference was not significant. Similarly, a marginal increase in the serum lysozyme and respiratory burst activity were observed in the *Bacillus* fed groups. Fish were subjected to *E. ictaluri* challenge after the 10 weeks feeding regime, and the AP193 fed group had a 12% reduction in mortality compared with control ($P = 0.07$). As an important parameter, the effect of *Bacillus* dosage on the probiotic effect was also evaluated. Fish were fed with AP193 supplemented diets at doses from 4×10^6 CFU/g to 4×10^8 CFU/g for two weeks and challenged with *E. ictaluri*. Decreased mortality was observed as the quantity of AP193 increased in the diet.

The genome of *Bacillus* strain AP193 was determined by next-generation DNA sequencing. Genome analysis identified gene clusters for the production of polyketides (bacillaene, macrolactin and difficidin), lipopeptiedes (surfactin, bacillomycin D and fengycin) and a dipeptide antibiotic bacilycin. The recalcitrance of AP193 to uptake exogenous DNA made it very difficult to construct mutants deficient in the synthesis of specific antibiotics to determine their contribution to the biocontrol activity of AP193. Low efficiency transformation of AP193 was accomplished by isolation of plasmid from an *E. coli* ER2925 (*dam dcm*) strain followed by *in vitro* methylation of plasmid with cell–free extracts of AP193. A gene knock-out procedure was conducted that involved a temperature sensitive shuttle plasmid vector based on the pWV01 origin of replication, a two-step replacement recombination procedure and PCR screening of mutants in order to construct marker-free deletion mutants deficient in the biosynthesis of

specific antibiotic(s). Using an agar diffusion test, the major secondary metabolites responsible for *in vitro* antagonism of AP193 against *E. ictaluri* and *A. hydrophila* were identified as difficidin, one of the three polyketides.

A comparative genomic analysis was conducted on thirteen biocontrol strains from the *Bacillus subtilis* group. Phylogenetic analysis indicated that 6 of the *Bacillus* strains belong to *Bacillus amyloliquefaciens* subsp. *plantum*, a subspecies with *Bacillus amyloliquefaciens* FZB42 as the type strain, which was also included in the 13 strains. More than 11,000 pan-gene families comprising full sets of non-orthologous gene families were found within the genomes of the 13 strains, while the conserved core gene families were around 1,800. A pairwise BLASTp Matrix was generated to determine similarities between each two strains in terms of number and percentage of conserved gene families. The matrix revealed that the gene family similarity between any two *Bacillus* genomes ranges from 30% to 90%. Comparative analysis of the thirteen biocontrol strains with reference strains from *Bacillus subtilis* group with no biocontrol activity was carried out to identify unique genes present in the biocontrol strains that may be responsible for biocontrol activity. Fourteen unique genes were found present among all the 13 *Bacillus* strains. They express functions including signaling, transportation, secondary metabolite production, and carbon source utilization. Specifically, 70 unique genes were identified as shared by the 7 strains from *Bacillus amyloliquefaciens* subsp. *plantum*, including the complete difficidin biosynthetic pathway that was critical for the antibacterial activity of *Bacillus* strain AP193.

Acknowledgments

I would like to thank Dr. Mark R. Liles for the opportunity to conduct research under his guidance. His constant support and patience during the dissertation work is greatly appreciated. I am indebted to my advisory committee, Dr. Jeffery Terhune, Dr. Joseph Kloepper, Dr. Joseph Newton and Dr. Paul Cobine for always being available and willing to provide advice and support when requested. I am very grateful to Dr. Yolanda Brady for serving as University Reader. I would like to thank Dr. Abel Carrias and Dr. Mohammad Jahangir Hossain for their help and stimulating discussions throughout my dissertation work. I would also like to thank my colleagues in Dr. Liles lab including Nancy Capps, Shamima Nasrin, Malachi Williams, Dr. Larissa Parsley, Dr. Kavida Kakirde, Dr. Molli Newman, Joshua Stough, Jinglie Zhou, Dawei Sun and Charles Thurlow, for their constant support and encouragement. I am highly pleased and very thankful to my friends including Xingjiang Mu, Zhen Tao, Pengming Pan, Dong Chen and Ranzhi Li who made my graduate school experience pleasant. I am very thankful and indebted to my parents for their understanding and care during the five years of my Ph.D life in USA. Also, I want to thank my sisters for their constant encouragement.

Table of Contents

List of Tables

List of Figures

Chapter II

Chapter IV

Chapter V

Chapter I

Introduction and Literature Review

1. Introduction

Aquaculture farming of the channel catfish, *Ictalurus punctatus*, has been one of the most successful animal production industries in North America in the past 30 years and currently represents the largest aquaculture industry in the United States. Over 90% of all catfish produced in the U.S. are raised in Alabama, Arkansas, Louisiana, and Mississippi and are primarily grown in earthen ponds ranging in size from 2 to 10 ha (USDA, 2003a, 2003b).

Feed inputs associated with high-density fish culture stimulate the proliferation of opportunistic bacteria (Austin et al., 1995). The combination of high-density fish culture with rapidly changing water temperature and chemical composition of aquaculture ponds places stress on fish, and results in favorable conditions for the onset and spread of disease. Enteric septicemia of catfish (ESC), caused by the Gram negative bacterium *Edwardsiella ictaluri* (Hawke, 1979) is the most important endemic infectious disease in the channel catfish aquaculture industry (Hawke & Kahoo, 2004). Losses resulting from ESC were reported in over 78% of all operations with outbreaks being reported in 42% of catfish production ponds, with an economic loss between $20 and $30 million yearly (Wagner et al., 2002; USDA, 2003a, 2003b) . Another important pathogen in channel catfish is *Aeromonas hydrophila*, which is the primary

causative agent of motile aeromonad septicaemia (MAS) (Harikrishnan et al., 2003) and can infect multiple fish species including tilapia, catfish, goldfish, common carp, and eel (Pridgeon et al., 2011). In 2009 and 2010, *A. hydrophila* was identified as the etiologic agent of a disease epidemic in farmed channel catfish, resulting in higher mortality rates than typical for MAS with over five million pounds of catfish lost in the Alabama commercial catfish industry. Chemotherapy by oral administration of antibiotics in fish feeds is the most common treatment for bacterial diseases. However, the indiscriminant application of antibiotics may result in many problems including the spread of drug-resistant pathogens, environmental hazards and food safety problems. This has fostered an increased interest in alternatives to antibiotics. Also, the stress associated with vaccine administration to fish places a limitation on vaccination. Probiotics, which have various health-promoting properties and minor adverse side effects, are gaining an increasing scientific and commercial interest in aquaculture practice. The beneficial effects of probiotics involve improvement of feed utilization, modulation of intestinal microflora, enhancement of immune responses and antagonism to pathogens.

The main goal of this work was to investigate the probiotic effect of *Bacillus* strains in channel catfish, with an emphasis on the protection of catfish against ESC. We first targeted the isolation and *in vitro* screening of *Bacillus* strains for the ability to inhibit the growth of important fish pathogens, followed by evaluation of the protection efficiency of *Bacillus* strains against *E. ictaluri* infection of catfish in aquarium system. The effect of selected *Bacillus* strains on the growth and innate immune response of catfish was also assessed. To determine the contribution of antimicrobial compounds to the biocontrol efficacy of *Bacillus* strain AP193, its antimicrobial compounds were characterized at a genetic and biochemical level, and mutants deficient in antimicrobial synthesis were constructed. In addition to the top-performing strain

AP193, whole genome sequencing was conducted for five other candidate probiotic *Bacillus* strains from the Auburn collection, and together with six other biocontrol strains isolated in Korea a comparative genome analysis was conducted in order to identify the genetic loci that contribute to the biocontrol efficacy of these bacteria and to facilitate their future application.

2. Literature review

2.1 Channel catfish industry

The aquaculture of channel catfish *Ictalurus punctatus* (Rafinesque) experienced a huge growth in the 1980s, particularly in Mississippi (Mott & Brunson, 1997). Presently, the commercial catfish industry is the largest in the US aquaculture sector representing 46 % of its total value (USDA, 2006). The consumption of fish in the US is projected to increase and the per capita consumption of catfish (0.42 Kg) is the tenth highest of all consumed fish and sea food products (Muhammad & Jones, 2009).

Production and management schemes used for culturing catfish as well as other aquaculture organisms are designed to produce high numbers of market size fish in the least time possible and in the smallest water area/volume possible. In food fish production, catfish fingerlings (10-50 g) are stocked at high stocking densities (8,500 to 10,000 fish/hectare) and fed high feeding rates (114-136 Kg/ha/day) (Chapman, 2000) leading to stressful conditions in culture environments and a high propensity for disease outbreaks.

The catfish industry provides up to 14,000 on-farm jobs paying an estimated $168.7 million worth of salaries to workers in the states of Mississippi, Alabama, Arkansas, and Louisiana which are the major producers of catfish (USDA, 2006). Therefore, it is essential for

the industry to have better technology and become more efficient at managing losses from diseases as a means of remaining profitable.

2.2 Enteric septicemia of catfish

Enteric septicemia of catfish, a highly fatal systemic disease, is considered the most important disease of cultured channel catfish (Noga, 2000; Hawke, 2004). ESC affects catfish of all ages but predominantly affects young-of-the-year fingerlings (Francis-Floyd et al., 1987). The disease was first reported from pond-raised channel catfish in 1976 (Hawke, 1979), and can be found in most channel catfish culture environments (Hawke et al., 1998). ESC is a seasonal disease, with outbreaks commonly occurring during spring and fall when water temperatures range from 20 ºC to 28 ºC (Hawke et al., 1998). In the field *E. ictaluri* infected fish may become listless, may swim erratically and hang in the water column with their head up and tail down. Fish tend to go off-feed once infected, posing major problems for therapy efforts that involve administering treatment through feed (Blazer et al., 1985).

E. ictaluri, the causative agent of ESC, is a Gram-negative, facultative anaerobic rod belonging to the *Enterobacteriaceae* family (Hawke et al., 1998). *E. ictaluri* is closely related to *Edwardsiella tarda,* sharing 96 % identity based on 16S-23S rRNA intergenic spacer region (Panangala et al., 2005b). Additionally, it exhibits high homogeneity based on phenotypic (outer membrane protein profiles, antigenic determinants, and biochemical properties) and genetic characteristics (Panangala et al., 2005a).

Pathogenesis studies have shown that *E. ictaluri* can enter through the gastrointestinal tract, the nares (nasal openings), and possibly through the gills (Hawke et al., 1998). Two clinical forms of ESC occur in channel catfish: an acute form resulting in septicemia characterized by hemorrhage and necrosis of several organs, and a chronic form resulting in chronic encephalitis

(Johnson, 1989). It is postulated that when *E. ictaluri* enters the fish through the gut the acute form of ESC develops and when it enters through the nare the chronic form develops (Newton et al., 1989).

E. ictaluri is endemic but it is considered an obligate pathogen as it requires a proper host for overall survival (Plumb & Quinlan, 1986). However, the pathogen can remain viable in pond sediment for up to ninety five days at cool temperatures (Hawke et al., 1998; Plumb & Quinlan, 1986) Horizontal transmission of ESC may occur through the shedding of bacteria into the aquatic medium by infected fish, and by cannibalism of infected fish (Hawke et al., 1998; Tucker & Robinson, 1990). It is also postulated that birds, and contaminated equipment used at infected ponds, may also play a role in transmitting the disease from pond to pond or farm to farm (Hawke et al., 1998). In multiple batch production systems previously infected fish may serve as carriers of disease and infect newly introduced fish (Wagner et al., 2002).

2.3 *Aeromonas hydrophila*

Aeromonas hydrophila, a Gram-negative motile bacterium widely distributed in aquatic environments, is a causative agent of motile aeromonas septicaemia (MAS) (Harikrishnan et al., 2003). The symptoms of *A. hydrophila* infections include swelling of tissues, dropsy, red sores, necrosis, ulceration and haemorrhagic septicaemia (Karunasagar et al., 1989; Azad et al., 2001). Fish species affected by MAS include tilapia, catfish, goldfish, common carp, and eel (Pridgeon, 2011). Although usually considered as an opportunistic pathogen associated with disease outbreaks, *A. hydrophila* can also be a primary pathogen, causing outbreaks in fish farms with high mortality rates, resulting in severe economic losses to the aquaculture industry worldwide (Thore & Roberts, 1972; Nielsen et al., 2001; Fang et al., 2004).

Beginning in June of 2009, a disease outbreak occurred in 48 catfish farms in west Alabama, USA, causing an estimated loss of more than 3 million pounds (ca. 1339 metric tons) of food size channel catfish *Ictalurus punctatus* (Hemstreet, 2010). The disease produced a variety of symptoms that included sores on the skin, bulging eyes, ulcers, and bright red muscles and internal organs. Diseased catfish were collected by a fish health specialist at the Alabama Fish Farming Center, and bacteria were cultured, isolated, and later identified as *A. hydrophila* (Hemstreet, 2010). Based on both LD50 and LD95 values of intraperitoneal injection assays, the pathogenic strains from 2009 was at least 200-fold more virulent than an *Aeromonas hydrophila* stain isolated in 1998 (Pridgeon , 2011). In 2010, the *Aeromonas* disease came back again with vengeance (Hemstreet, 2010) and to date this epidemic has caused the loss of over 10 million pounds of catfish.

2.4 Traditional treatments of bacterial diseases

The use of antibiotics and vaccination are the two major options for controlling the disease at the present moment. In the US, few options are available for controlling fish diseases in general. There are only five drugs of which three are antibiotics that have been approved by the U.S. Food and Drug Administration (FDA) for use in aquaculture (Benbrook, 2002). In the catfish industry, only Romet® (ormethoprim plus sulfadimethoxine) and the recently approved Aquaflor® (florfenicol) are approved by the Federal Drug Administration (FDA) to specifically treat ESC. Aquaflor®; however, requires veterinary feed directive from a licensed veterinarian prior to its use by catfish farmers. Although this process may help regulate the use of antibiotics it may also delay treatment. The cost of developing and approving antibiotics, the public concerns of its misuse, coupled with the emergence of resistant strains of bacteria from its systematic use, poses problems for the approval process of additional antibiotics by the FDA

(Patrie-Hanson & Jerald Ainsworth, 1999). In 1994 the American Society of Microbiologists (ASM) antibiotic resistance task force reported that aquaculture represents “one of the biggest concerns” with regards to antibiotic use because aquaculture production is growing rapidly, and disease prevention and treatment practices are presently not standardized or regulated (Benbrook, 2002). In 2001-2002 the live-attenuated vaccine AQUAVAC-ESC® was licensed, and marketed by Intervet, Inc., for use against ESC (Shoemaker et al., 2002). Laboratory studies have demonstrated the safety and efficacy of AQUAVAC-ESC® for use in reducing mortality in channel catfish fry and fingerlings (Shoemaker et al., 2002, 2007; Lawrence & Banes, 2005; Wise, 2006). Field studies have reported larger fingerlings, and improved feed conversion, leading to improved return due to the use of the vaccine; however, data specifically showing similar mortality reduction due to the use of the vaccine as seen in laboratory studies are inconclusive in field studies (Intervet Inc., 2003; McNeely, 2006; Wise, 2006; Carrias et al., 2008; Shoemaker et al., 2009).

2.5 Probiotics in aquaculture

Fuller defined a probiotic as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.” (Fuller, 1989) In aquaculture, the skin and gill microflora of fish must be assumed also to contribute to disease prevention, also, the host and culture environment have more intensive interaction. Verschuere et al. gave a more specific definition of probiotics as “a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment”. (Verschuere et al., 2000)

Probiotic bacteria have been shown effective in controlling various infectious diseases in aquaculture including furunculosis caused by *A. salmonicida* in rainbow trout and Atlantic salmon (Smith & Davey, 1993; Nikoskelainen et al., 2001; Irianto & Austin, 2002), saprolegniosis by *Saprolegnia parasitica* in eel *Anguilla australis* Richardson(Lategan et al., 2004b) , edwardsiellosis by *Edwardsiella tarda* in cultured European eel, *Anguilla anguilla* L. (Chang & Liu, 2002) , lactococcosis by *Lactococcus garvieae* in rainbow trout (Brunt & Austin, 2005), enteric red mouth disease by *Yersinia ruckeri* in rainbow trout, (Raida et al., 2003), streptococcosis by *Streptococcus iniae* in rainbow trout and olive flouder (Brunt & Austin, 2005; Heo et al., 2013), vibriosis in rainbow trout, turbot larvae , prawn and Atlantic cod fry and large yellow croaker (Gatesoupe, 1994, 1997; Gildberg & Mikkelsen, 1998; Moriarty, 1998; Gram et. al, 1999; Ai et al., 2010). Most probiotics proposed as biological control agents in aquaculture belong to the lactic acid bacteria (*Lactobacillus*, *Carnobacterium*, etc.), to the genus *Bacillus*, *Pseudomonas*, and *Vibio* although other genera or species have also been mentioned. (Verschuere et al., 2000)

2.5.1 Larvae

In intensive cultivation of fish, mass mortality of larvae is a major complication for regular production of high quality juveniles. The mortality cannot be attributed to specific obligate pathogens, but rather to the proliferation of pathogenic bacteria (Silvi et al., 2008). Therefore, it is important to maintain a healthy microbial environment in the larval rearing tanks. During initial feeding, it is possible to induce an artificial dominance of a certain group of bacteria in the fish-associated microbiota by adding a strain to the rearing water (Strøm & Ringø, 1993) or to the culture medium of the live food (Gatesoupe, 1994). Gatesoupe introduced a lactic acid bacteria strain daily to the enrichment medium of rotifers used as live food for turbot larvae

(Gatesoupe, 1994). The added lactic acid bacteria could be retrieved in large amounts from the turbot larvae, and a significant reduction of larval mortality was observed when the larvae were challenged with a pathogenic *Vibrio* on day 9. Similarly, rotifer enrichment with a siderophore producing strain *Vibrio* type E improved the survival of the larval turbot after a 48-h challenge with the pathogenic *Vibrio* type P (Gatesoupe, 1997). Two bacterial strains *Lactobacillus fructivorans* (AS17B), isolated from adult sea bream (*Sparus aurata*) gut, and *Lactobacillus plantarum* (906), isolated from human faeces, were administered contemporaneously, during sea bream development using *Brachionus plicatilis* and/or *Artemia salina* and dry feed as vectors (Carnevali et al., 2004). Significantly higher level of *Lactobacillus fructivorans* was observed in the treatment group as compared with control at 90 days post-hatchery. Also, probiotic administration significantly decreased larval mortality. A further research demonstrated that administration of the two strains enhanced the tolerance of sea bream larvae to normal rearing conditions, as indicated by lower cortisol and HSP70 levels in the probiotic treated larval fish compared with control. Also, an improvement in tolerance to acute stress was observed in fry fed with probiotics (Rollo et al., 2006). Furthermore, early feeding of same probiotics exerted a significant increasing effect on the number of Ig^+ cells and acidophilic granulocytes in seabream gut-associated lymphoid tissue (GALT), indicating immunosystem enhancement effect of probiotics in larval fish (Simona et al., 2007). Matteo et al. applied a mixture of *Bacillus* strains to sea bream larvae. Although no significant difference in survival was observed, the *Bacillus* mixture significantly increased growth of sea bream in terms of standard length and body weight at both larval and juvenile stages. The morphometric analysis was supported by molecular results which evidenced higher expression of Insulin-like Growth Factor I and lower levels of myostatin in probiotics feeding groups. In addition, a better tolerance to farming conditions was also found

as indicated by lower expression of HSP70 and Glucocorticoid Receptor, suggesting beneficial effects of the supplied *Bacillus* mixture on larval fish welfare (Avella et al., 2010).

2.5.2 Fish Juveniles and Adults

Probiotic bacteria have been shown effective in promoting the resistance of fish against infection of various pathogens in challenge experiments (Smith & Davey, 1993; Gatesoupe, 1994; Gatesoupe, 1997; Gildberg & Mikkelsen, 1998; Moriarty, 1998; Gram et. al, 1999; Nikoskelainen et al., 2001; Chang & Liu, 2002; Irianto & Austin, 2002; Raida et al., 2003; Lategan et al., 2004b; Brunt & Austin, 2005; Ai et al., 2010; Heo et al., 2013). The representative studies are reviewed in detail next.

Irianto isolated four bacterial strains from intestinal contents of Atlantic salmon and rainbow trout with antagonistic activity against *Aeromonas salmonicida*. They belonged to *A. hydrophila*, *Vibrio fluvialis*, *Carnobacterium* sp. and an unidentified Gram-positive coccus. The probiotics were applied to dry rainbow trout feed separately or as an equal mixture at a dose of 10^7 -10^8 CFU/g. Groups of rainbow trout fingerlings and fry were fed with probiotic supplemented feed for 14 days and challenged with *Aeromonas salmonicida* by cohabitation or intraperitoneal (i.p.) injection for fingerlings and immersion for fry. In the i.p. injection challenge experiment with fingerlings, no mortality was recorded in the four groups fed with probiotics seven days after challenge, while the control group suffered 48% mortality. In the cohabitation challenge experiment, administration of each of the bacterial strains with the exception of the *Vibrio* strain resulted in a significant reduction in mortality. In terms of the mode of action, no serum or mucus antibodies to *A. salmonicida* were detected in fish fed with probiotics, however an increased number of erythrocytes, macrophages, lymphocytes and

leucocytes, and enhanced lysozyme activity were found, indicating a stimulation of cellular rather than humoral immunity (Irianto & Austin, 2002).

Chang tested two strains *Enterococcus faecium* SF68 and *Bacillus toyoi* isolated from commercial products for their inhibitory effects against *E. tarda* (Chang & Liu, 2002). Firstly, colonization property of the two strains in European eels *Anguilla anguilla* L. was tested by feeding eels with probiotic amended feed for 2 weeks with three eels randomly selected from each group for intestinal microbial flora analysis every second day. Results showed that *Enterococcus faecium* began to colonize eel intestines on day 4 post-inoculation and numbers reached 1.63×10^5 CFU /g on day 14, constituting 73% of the intestinal microflora, whereas *B. toyoi* cannot be isolated from the intestine of fish and total viable counts in intestines of fish fed with *B. toyoi* continuously decreased during the feeding period to 3.3×10^4 CFU/g, approximately 10% of the number at the beginning of the experiment. For the challenge experiment, the eels were fed with the probiotic supplemented feed for 2 weeks and then challenged with *E. tarda* by anal injection. Two weeks after challenge, the survival rates of eels fed on *E. faecium* SF68 supplement was significantly higher ($P < 0.05$) than those of control eels and those fed on *B. toyoi* supplement, whereas no significant difference was observed between the survival rates of the *B. toyoi* and control groups which was consistent with the colonization results.

Aly *et al.* evaluated the probiotic activity of two bacteria (*Bacillus subtilis* and *Lactobacillus acidophilus*) by their effect on the immune response of Nile tilapia, as well as their protective effect against challenge infections. The two probiotic strains were supplemented to commercial feed either separately at 10^7 CFU/g of feed or in combination at 5×10^6 CFU/g for each strain. Sampling for immunological analysis and challenge by *A. hydrophila, Peudomonas*

fluorescens or *Streptococcus iniae* were conducted after 1 and 2 months of feeding. Results showed that the hematocrit values and serum bactericidal activity were significantly higher in the group that received the mixture of *B. subtilis* and *L. acidophilus* compared with the control. The respiratory burst activity tested by NBT assay, neutrophil adherence and lysozyme activity showed a significant increase in all the probiotic-treated groups after 1 and 2 months of feeding compared with control. Similarly, the levels of mortality after challenge by the three pathogens after 1 and 2 months of feeding were significantly lower in all the probiotic groups compared with control, with group fed with the mixture giving the highest protection in most cases (Aly et al., 2008). Newaj-Fyzul *et al.* isolated a *Bacillus subtilis* strain (AB1) with inhibitory activity against pathogenic *Aeromonas* sp. from the intestine of rainbow trout and evaluated its effect in preventing disease in rainbow trout caused by the pathogen. *Bacillus subtilis* AB1 was administered for 14 days to rainbow trout in feed at a concentration of 10^7 cells/g either as viable, formalized or sonicated cells or as cell-free supernatant. While survival in control group was 5% to 15% after challenge by the *Aeromonas* sp., all the fish survived the challenge in each of the treatment groups. Also, results showed that AB1 stimulated immune parameters, specifically stimulating respiratory burst, serum and gut lysozyme, peroxidase, phagocytic killing, total and α_1–antiprotease and lymphocyte populations (Newaj-Fyzul et al., 2007).

In most of probiotic studies, a dose of 10^7-10^8 CFU/g of feed was adopted to test the probiotic effect (Gatesoupe, 1994; Robertson et al., 2000; Salinas et al., 2005; Kumar et al., 2008; Newaj-Fyzul et al., 2007; Aly et al., 2008; Ai et al., 2010; Sun et al., 2010; Heo et al., 2013). However, the reason for choosing this level of dose was generally not addressed. Brunt and Austin investigated the dose-effect relationship of a probiotic strain in rainbow trout. Protection of rainbow trout against both *Lactococcus garvieae* and *Streptococcus iniae* infection was

demonstrated in the probiotic feeding groups, with dosage levels of 10^7 CFU/g and 10^8 CFU/g conferring the best protection, while the higher dosage of 10^{10} CFU/g leading to less protection which was similar with the group of 10^3 CFU/g (Brunt & Austin, 2005). Similar tendency was demonstrated in another study testing effect of a *Lactobacillus rhamnosus* strain against *Aeromonas salmonicida* infection in rainbow trout, where fish fed with 10^{12} CFU/g probiotic supplemented feed gave higher mortality compared with the 10^9 CFU/g feeding (Nikoskelainen et al., 2001). In the research of *Bacillus subtilis* AB1, the effect of feeding varying concentrations of AB1 on the survival of rainbow trout after challenge with *Aeromonas* sp. was also investigated. A series of doses of the probiotic in feed from 10^4 to 10^9 CFU/g were tested. Similarly with above research, the 10^7 CFU/g group gave the highest survival (100% as compared with 15% of control). Survival of the 10^8 and 10^9 CFU/g groups was 68% and 65%, respectively, which were similar with the survival of 10^4 and 10^5 CFU/g groups (Newaj-Fyzul et al., 2007).

Duration of probiotic feeding is another important parameter to consider. Chang demonstrated that *E. faecium* SF68 suppressed the growth of *E. tarda in vitro*, only if its initial inoculum was much higher (about × 1000) than that of *E. tarda* and proposed that the oral application of *E. faecium* SF68 in aquaculture is more effective in disease prevention than treatment and ensuring the probiotic bacterium as the dominant microflora in the intestine before pathogen invasion may be the key to prevent disease. Accordingly, *E. faecium* SF68 amended feed was applied to fish 14 days before challenge by *E. tarda* (Chang & Liu, 2002). The effect of duration of the probiotic *Carnobacterium* sp. feeding on survival of Atlantic salmon challenged with different pathogens was evaluated. Feeding with probiotic for 7 days did not result in any protection following challenge with the fish pathogens, while after feeding with the probiotic for

14 days, an improvement in survival was consistently recorded following challenge with *A. salmonicida*, *V. ordalii*, and *Y. ruckeri* (Robertson et al., 2000). Furthermore, Sharifuzzaman and Austin. reported that a two-week feeding regime with probiotic *Kocuria* SM1 lead to the best defense against *V. anguillarum* challenge in rainbow trout, with a 16% mortality for fish challenged after two weeks probiotic feeding, as compared to mortalities of 62, 30 and 22% for one, three and four and four week feeding regimes, and 70–90% mortalities for the controls. In agreement with the mortality results, macrophage phagocytic activity and serum total protein levels were significantly higher after two weeks of probiotic feeding as compared to treatments with other feeding durations and controls. Moreover, in comparison with the controls, serum antiprotease and lysozyme activities were significantly enhanced at week two, which became non-significant by week three and four of the experiment (Sharifuzzaman & Austin, 2009).
The efficacy of protection effect of the probiotics against pathogen infection in challenges varies with different probiotic-host system. The protection effect can be measured by relative level of protection (RLP), which was calculated as RLP = [1 – (percent of mortality in treated group/percent of mortality in control group)] × 100 (Aly et al., 2008). For probiotics with high efficiency, a RLP over 90 was recorded, which equaled to around 100% survival in the probiotic treated fish group (Irianto & Austin, 2002; Brunt & Austin, 2005; Newaj-Fyzul et al, 2007). In most cases, a moderate protection effect was observed, which gave a RFP from 30 to 60 (Gram et al., 1999; Robertson et al., 2000; Nikoskelainen et al., 2001; Chang & Liu, 2002; Aly et al., 2008; Raida et al., 2003, Qinghui et al, 2011, Diaz-Rosales et al., 2009). Marginal reduction or only delay of the mortality of fish was also observed in some studies, with insignificant difference in mortality between the treatment and control groups (Gildberg & Mikkelsen, 1998; Heo et al., 2013). However, the marginal effect does not exclude the possibility that the

probiotics may confer considerable protection under normal rearing conditions in the presence of moderate levels of pathogenic bacteria. Consequently, a more reliable method for detection of probiotic effects would be to compare the mortality of fish given different feeds in large scale feeding trials, where the fish is not exposed to high concentrations of aggressive pathogens (Gilberg & Mikkelsen, 1998).

2.5.3 Selection of candidate probiotic bacteria

By definition, a probiotic need only benefit the host, and this could be either nutritional or a change in its immediate environment. Yet, screening to date has concentrated on the search for probiotics active against a pathogen. In screening for potential probiotics, most of this research employed identification of inhibitory activity in vitro (Bly et al., 1997; Sugita et al., 2002; Burgess et al., 1999; Spanggaard et al., 2001; Chythanya et al., 2002; Hjelm et al., 2004a,b). In some cases, initial in vitro screening was followed by small scale testing of short-listed candidates in vivo for either pathogenicity to the host (Makridis et al., 2000; Chythanya et al., 2002; Hjelm et al., 2004a) or host protection when challenged with a pathogen (Rengpipat et al., 1998; Robertson et al., 2000; Gram et al., 2001; Irianto & Austin, 2002; Lategan & Gibson, 2003; Vaseeharan et al., 2004; Lategan et al., 2004a, b). In other studies, *in vitro* short-listed probiotics have been tested further for properties such as bile resistance (Nikoskelainen et al., 2001; Chabrillón et al., 2006), adhesion capacity (Olsson et al., 1992; Nikoskelainen et al., 2001; Hjelm et al., 2004a), immunostimulation (Gullian et al., 2004; Rengpipat et al., 2000; Irianto & Austin, 2003), competition for adhesion sites (Vine et al., 2004; Chabrillón et al., 2006) and competition for nutrients (siderophore production) (Gram et al., 1999). In practice, these latter studies test whether or not a probiotic that produces diffusible inhibitory substances also possesses other modes of probiotic action. Screening for production of an inhibitory substance in

vitro and then taking likely candidates into the further testing stage limits the short list to those isolates which exhibit only one of the various modes of probiotic action, namely production of diffusible inhibitory substances. Although production of an inhibitory substance has been shown to work very well in probiotics and this screening method has identified very good probiotics in aquaculture (Irianto & Austin, 2002; Lategan & Gibson, 2003; Vaseeharan et al., 2004; Lategan et al., 2004a, b), there are two major limitations to this approach. The first is that other modes of probiotic activity (e.g. immunostimulation, digestive enzymes production, competition for attachment site, or nutrients) will not be expressed in the laboratory on an agar plate and, hence, a major source of potential beneficial action will be overlooked. The second drawback is that positive results in vitro cannot guarantee *in vivo* protiotic effect (Kesarcodi-Watson et al., 2008). Spanggaard tested the ability of several probiotic candidates to protect rainbow trout from vibriosis. Two *Carnobacterium* spp. antagonistic against *V. anguillarum* in vitro failed to protect rainbow trout challenged by the same pathogen (Spanggaard, 2001). Gram et al. (2001) tested the ability of *P. fluorescens* strain AH2 against salmon pathogen *A. salmonicida*. In vitro experiment showed strong antagonism of the probiotic against *A. salmonicida*. However, no protective effect was found when transferring the probiotic to an in vivo challenge experiment with salmon and the pathogen. The most likely reasons for the research approaches taken in the past are cost, ease of experimentation and lack of test animals and space (Kesarcodi-Watson et al., 2008). Setting up initial screening experiments involves very large numbers of candidates in order to screen as many isolates as possible in the hope of obtaining good probiotics. In reality, setting up this phase of experimentation with a suitable number of animals per replicate, a sufficient number of replicates per treatment, and screening even the modest number of 100 isolates, presents a huge demand for number of animals needed and also the space and resources

to carry out these experiments *in vivo*. In view of this, it is not surprising that a laboratory component is added to the screening before challenging animals with a substantially reduced short-list. Contrary to the in vitro approach of screening probiotics, Makridis et al. (2005) recently adopted a direct in vivo approach. Six bacteria isolated from healthy cultures of gilthead sea bream larvae food, *Artemia* sp. and rotifers were tested directly *in vivo* for their effect on the sea bream larvae. Results showed that addition of the bacteria significantly improved larval survival. Verschuere et al. (1999) investigated the effect of eighteen bacteria strains on *Artemia* juveniles by adding them directly to monoxenic cultures of *Artemia*. Nine out of eighteen strains increased the growth and survival of *Artemia* and were chosen for in vivo challenge experiments against *V. proteolyticus* CW8T2 (Verschuere et al., 2000b). All nine strains demonstrated a significant protective effect, indicating the high correlation of the *in vivo* screening criteria with the probiotic effect. The encouraging results from these two studies highlighted the benefit of including test animals at the initial stages of the screening process (Kesarcodi-Watson et al., 2008).

2.5.4 Colonization and persistence in intestine of fish

The colonization potential is a very important criterion to characterize probiotics. The process of colonization is characterized by attraction of bacteria to the mucosal surface, followed by association within the mucous gel or attachment to epithelial cells (Balcazar, 2006). Adhesion and colonization of the mucosal surfaces are possible protective mechanisms against pathogens through competition for binding sites and nutrients (Westerdahl et al., 1991), or immune modulation (Salminen et al., 1998). Also, probiotics can persist in the intestine for a long time if they have colonized the mucus of intestine, which is advantageous economically as repeated application of probiotics may be avoided.

In some cases, the probiotic bacteria do not truly colonize the gastrointestinal tract, but rather achieve a sustained transient state. (Fuller, 1992, Irianto & Austin, 2002) Jöborn et al. studied the ability of *Carnobacterium* strain K1 to colonize the intestinal tract of rainbow trout (*Oncorynchus mykiss*) (Joborn et al., 1997). *In vitro* experiment demonstrated that the strain was able to grow in extracts of intestine mucus and feces of rainbow trout. It can also adhere to intestinal mucus to the same degree as to the control surface BSA *in vitro*. Furthermore, 10^5 CFU/g *Carnobacterium* strain K1 were recovered from the faece of rainbow trout after oral administration of strains K1 amended feed for 6 days followed by 4 days of normal feeding, which indicated that the strain can survive and persist in the intestine of rainbow trout and suggested potential colonization ability of the strain. However, no *Carnobacterium* strain K1 were detected in the mucus of intestine after the four days of normal feeding, indicating the strain didn't really colonize the intestine of rainbow trout. This was consistent with the sharp decrease of the strain level in the feces after cessation of probiotic feeding. Negative colonization property of probiotic bacterium in the intestine of host was also demonstrated in another research on rainbow trout. Feed amended by a *Carnobacterium* spp. isolated from the intestine of Atlantic salmon was applied to rainbow trout for 28 days and the population of the probiotic strain in the digestive tract of the host was tested at weekly intervals. A constant increase of the number of probiotic cells in the digestive tract during feeding regimes was observed, with a maximal population (7×10^6 /g of intestine) achieved after feeding for 28 days. However, the level of probiotic bacterium declined rapidly after cessation of the probiotic feeding, such that *Carnobacterium* sp. could not be isolated from intestines 6 days later (Robertson et al., 2000). Although some bacteria may not be effective at colonizing their host, transient bacteria may be also efficient if the cells are introduced at high dose, continuously or semi-continuously

(Gournier-Chateau et al. 1994, Gatesoupe, 1999). In practice, it is therefore essential to evaluate the persistence of the probiotic in the gut, which can guide the frequency of probiotics application to aquatic animals.

Successful colonization of the intestine of aquatic animals by probiotic bacteria was also demonstrated in some research. Gildberg tested the probiotic effect of a *Carnobacterium divergens* strain on Atlantic salmon fry. After five weeks of feeding with the probiotic supplemented diet, intestinal microbioda was investigated. The gut contents were removed and the intestines were washed three times during sampling so that only adherent bacteria were collected. Spread plate results showed that high numbers of probiotic bacteria were recovered from the intestine of fish given probiotic supplemented feed, whereas much fewer bacteria and no probiotic bacteria were detected from the control fish, indicating the colonization of probiotic bacteria in the intestine of salmon fry. (Gildberg, 1995) Similarly, the colonization property of another *Carnobacterium divergens* strain isolated from cod was demonstrated in Atlantic cod fry. After six weeks feeding, microbial analysis of the intestine sample was conducted. Bacterial levels in both the intestine wall and gut content were analyzed by serial dilution followed by spread plate method. The results showed that while the intestine wall of control fish had a mixed microbiota, a monoculture of lactic acid bacteria was recovered from the intestine wall of fish fed with probiotic. As the gut content of fish fed with probiotic contained a mixed microbiota with considerable level of *Pseudomonas*, recovery of monoculture of lactic acid bacteria from the intestine wall indicated a true colonization of the probiotic strain, and that this bacteria has displaced other potential colonizers (Gildberg, 1997). Colonization of *Carnobacterium diĺergens* in Atlantic cod fry was further verified by immunostaining and light microscopy. The images indicated that most of the colonization occurred in the pyloric caeca (Gildberg & Mikkelsen,

1998). Although successful colonization was proved by recovery of the bacteria from washed intestine wall and by microscopy, the long term persistence of applied bacteria in the intestine of host, as expected for colonized probiotics, was not tested in above research. Andlid *et al.* reported the high colonization capacity of two yeast strains in rainbow trout. A single oral inoculation of the yeasts resulted in a continuous increase of yeast level in the intestine of host for 13 days and persistence of yeasts for at least 50 days (Andlid *et al.*, 1995). Further investigation indicated that the yeast strains can adhere to the intestinal mucus of rainbow trout (Vazquez-Juarez et al., 1997.), and the involvement of specific adhesins has been demonstrated (Vazquez-Juarez, 1996).

2.5.5 Possible Mode of action

An understanding of the mechanisms of the probiotic effects is important when designing a protocol for their selection. The common modes of action available to probionts as suggested by Fuller (1989) include the stimulation of the humoral and/or cellular immune response, modification of the metabolism of bacterial pathogens by changing their enzyme levels, and competitive exclusion either through production of inhibitory compounds that are antagonistic towards pathogens, or by competing for nutrients, attachment sites or oxygen. In human and agriculture application, probiotic research has enjoyed much more attention through history and several modes of action have been supported by unambiguous experimental data. (Fuller, 1989; Verschuere et al., 2000a) However, although many publications about probiotics in aquaculture have emerged during the last decade, the approach was generally empirical and the arguments with regard to the mode of action were often circumstantial. (Verschuere et al., 2000a)

Production of inhibitory compounds

Although the *in vitro* antagonistic activity against aquatic pathogens by the probiotics has been demonstrated in many literatures, the nature of the compounds responsible for the activity was rarely characterized, while knowledge of the nature of the compounds is a prerequisite to investigate the involvement of inhibitory compounds in the probiotic effect. Heo et al. (2012) isolated a *Lactococcus lactis* subsp. *lactis* strain antagonistic against *Strepococcus iniae*. The responsible compound was purified and characterized by HPLC-MS, with results showing that it belongs to bacteriocin Nisin Z. Many authors assign the inhibitory effects detected in *in vitro* antagonism tests to bacteriocins or antibiotics without looking for any other causes. It has been argued that observed growth inhibition can, in some cases, be accounted for by primary metabolites or simply be a decrease of the pH (Verschuere et al., 2000a). Vazquez investigated the inhibitory activity of lactic acid bacteria against *vibros* by comparison of the response of indicator pathogen to crude and dialyzed extracts (which retain the bacteriocins, but not the metabolites of low molecular mass). Results suggested that the inhibition is due to lactic and acetic acids produced by lactic acid bacteria rather than bacteriocin. (Vázquez et al., 2005) The *in vitro* production of inhibitory compounds toward known pathogens of the considered species has often been used in the selection of putative probiotic strains. However, it has not been demonstrated that production of such inhibitory compounds occurs under in vivo conditions, and the ecological relevance of the production of inhibitory compounds toward other bacteria is still unclear.

At this stage, the association between amensalistic activity and *in vivo* probiotic activity is very weak and circumstantial. Typically, a correlation is made between the *in vitro* ability of the probiotics to inhibit pathogens and the *in vivo* protection of the cultured aquatic species, but

in none of the studies published so far has it been shown unequivocally that the production of inhibitory compounds is the cause of the observed *in vivo* probiotic activity of the strains (Verschuere et al., 2000a). Similar research has already been performed in the field of plant disease suppression. Asaka (1996) investigated the suppression mechanism of *Bacillus subtilis* RB14 against damping-off of tomato seedlings caused by *Rhizoctonia solani*. Firstly, *in vivo* production of antibiotics surfactin and iturin A responsible for the *in vitro* inhibition was confirmed by recovery of them from the soils inoculated with RB14. The involvement of antibiotics production in disease control was further proved by comparison of disease suppression level of a RB14 mutant with the antibiotics production genes knocked out with the suppression level of wildtype RB14. Inability of the mutant to suppress the disease and recovery of suppression by complementation of the mutated genes elucidated that antibiotics production plays a major role in the suppression of damping-off caused by *Rhizoctonia solani*. Similar research for probiotics used in aquaculture is necessary for better understanding of the involvement of inhibitory compounds in the probiotic effect.

Competition for iron or other nutrients

Virtually all microorganisms require iron for growth. Siderophores are low-molecular weight, ferric ion-specific chelating agents which can dissolve precipitated iron and make it available for microbial growth. The ecological significance of siderophores resides in their capacity to scavenge an essential nutrient from the environment and deprive competitors of it. (Verschuere et al., 2000a) The ecological significance of siderophores in soils as important tools for iron acquisition by microorganisms and plants and their involvement in suppression of plant root pathogens have been established (Verschuere et al., 2000a). Harmless bacteria which can produce siderophores could be used as probiotics to outcompete pathogen. Gatesoupe (1997)

applied a siderophore-producing *Vibrio* strain E to the live food (rotifer) enrichment medium, which lead to a decrease in mortality of larval turbot challenged with a pathogenic strain *Vibrio* P. The role of iron availability in the interaction between the probiotic and pathogenic vibrios was further appraised by the fact that addition of the bacterial siderophore deferoxamine to live food (rotifers) also increased the resistance of turbot larvae challenged with the pathogenic *Vibrio* strain P, although to a less extent than the group enriched with *Vibrio* E. Pybus et al. tested thirty strains of *V. anguillarum* as potential probiotics against the salmon pathogen *V. ordalii* by antagonism test. Only one strain (*V. anguillarum* VL4335) inhibited strains of *V. ordalii in vitro*, and this effect was blocked when iron salts were added to the medium, indicating that the growth inhibition was linked to iron deficiency. Using the chrome-azurol sulfate assay to measure siderophore production, *V. anguillarum* VL4335 yielded significantly higher values than other *V. anguillarum* strains. Gram et al. examined *in vitro* antagonism of siderophore-producing *Pseudomonas fluorescens* AH2 against the fish-pathogenic bacterium *Vibrio anguillarum*. The results revealed that the growth of *V. anguillarum* was inhibited by the filter-sterilized supernatans from iron-limited cultures of *P. fluorescens* AH2 but not from iron-replete cultures. During co-culture, *P. fluorescens* AH2 inhibited the growth of *V. anguillarum* independently of the iron concentration, when the initial level of the siderophore producer was 100 to 1000 times greater than the level of the fish pathogen. Mortality of rainbow trout juveniles due to *V. anguillarum* infection was decreased by 46% when the culture was treated with *P. fluorescens* AH2, which was explained as a confirmation of the *in vitro* test (Gram et al., 1999). However, the correlation of the *in vitro* antagonism and *in vivo* effect is circumstantial, as no direct data proving the involvement of siderophore in the protection effect was available. In another study, *P. fluorescens* AH2 was tested as a probiont against *Aeromonas salmonicida*

infection (furunculosis) in salmon. Although the *in vitro* antagonism was strong and also depends on the availability of iron, indicating the involvement of siderophore, no protection of salmon against furunculosis was conferred by the *P. fluorescens* AH2 in vivo (Gram et al., 2001). The difference in the *in vivo* effect in the furunculosis-salmon system as compared with the vibriosis-rainbow trout system can either be attributed to a failure of siderophore to inhibit the *Aeromonas salmonicida* in the *in vivo* environment of salmon or it can be that the inhibition conferred in rainbow trout was not due to siderophore production but rather some other mechanisms which didn't occur in the furunculosis-salmon system. To further elucidate the involvement of siderophore in the probiotic effect, more detailed *in vivo* study is required.

Competition for nutrients can theoretically play an important role in the composition of the microbiota of the intestinal tract or ambient environment of cultured aquatic species, but to date there have been no comprehensive studies on this subject. The microbial ecosystem in aquaculture environments is generally dominated by heterotrophs competing for organic substrates as both carbon and energy sources. Specific knowledge of the factors governing the composition of the microbiota in aquaculture systems is required to manipulate it. This knowledge, however, is generally not available, and one must therefore rely on an empirical approach (Verschuere et al., 2000a). Nevertheless, Verschuere et al. selected several strains with a positive effect on the survival and growth of Artemia juveniles. The *in vitro* antagonism tests and filtrate experiments showed that no extracellular inhibitory compounds were involved in the protective action of these strains against *V. proteolytics* CW8T2 but that living cells were required to protect Artemia against the pathogen. It was suggested that the selected bacteria exerted their protective action by competing with the pathogen for chemicals and available energy. The competition of nutrients can be investigated *in vitro* by a coculture experiment. Sami

et al. examined the antagonism of a few lactic acid bacteria probiotic strains for human use against *A. salmonicida*. The probiotic strains showed significant inhibition of *A. salmonicida* in a co-culture expreiment while no secretion of inhibitory compounds was measured in the spent culture liquid of probiotic strains, indicating the mediation of growth inhibition by competition for nutrients (Nikoskelainen et al., 2001)

Enhancement of immune response

The enhancement of nonspecific immunity by probiotics has been demonstrated in many studies. Irianto & Austin (2002) showed that after feeding trout with probiotics for 2 weeks, stimulation of cellular immunity was detected with an increase in lysozyme activity and in the number of erythrocytes, macrophages and lymphocytes. Panigrahi fed trout a pellet containing the probiotic *Lactobacillus rhamnosus* JCM1136, resulting in an increased level of the serum lysozyme, complement activities and phagocytic activity of head kidney leucocytes (Panigrahi et al., 2004) Newaj-Fyzul isolated *Bacillus subtilis* AB1 from fish intestine and tested its ability to control *Aeromonas* infection in rainbow trout. When AB1 was administered for 14 days to rainbow trout in feed at a concentration of 10^7 cells per gram either as viable, formalized or sonicated cells or as cell-free supernatant, all the fish survived challenge with the pathogen while majority of the control group died. Study of the mode of action of the probiotic showed stimulated immune parameters, specifically stimulating respiratory burst, serum and gut lysozyme, peroxidase, phagocytic killing, total and a1-antiprotease and lymphocyte populations. (Newaj-Fyzul et al., 2007) Ai et al. (2011) investigated the effect of long term *B. subtilis* feeding on the immune response of juvenile large yellow croaker. Results showed that *B. subtil*is significantly enhanced serum lysozyme and serum SOD activity while the serum alternative complement pathway (ACP) activity and the respiratory burst activity of head kidney

macrophage were independent of dietary treatments. In all the above studies, conclusion of immune stimulation by the probiotic was based on some of immunological parameters with significant differences between treatments. The attribution of the probiotic effect to those stimulated parameters was circumstantial. More convincing data can be achieved by taking into account all the tested parameters to reflect the overall immune state of the aquatic animals. Gullian et al. (2004) tested immunostimulation by a live *Vibrio* sp. (P62) and *Bacillus* sp. (P64). An immune index was calculated by summing up standardized values of all the tested immunological parameters. The statistical conclusion was made from the index as either immunostimulatory or not. However, this method can only be adopted for well-studied aquatic animals, as historical data are required to standardize the value of the immune parameters. Although many studies have demonstrated stimulation of the immunity by probiotics, the duration of simulated immune response after cessation of the probiotic administration was rarely investigated. Salias fed *Lactobacillus delbrueckii* and *Bacillus subtilis* to gilthead seabream for three weeks followed by control feed for another week. Phagocytic activity and cytotoxicity of the head-kidney leucocytes were boosted during the probiotic feeding. However, after 1 week back on the control diet, the parameters in the experimental groups had recovered or even dropped below those recorded in the control group, suggesting that the bacteria did not persist in the seabream gut (Salinas et al., 2005). Similar results were also observed in study of rainbow trout, where the administered probiotic lactic acid bacterium disappeared from the intestine and the elevated immune parameters returned to the prefed level upon withdrawal of the probiotic diets after feeding for three weeks. (Panigrahi et al., 2005).

Competition for adhesion sites

Another possible mechanism is prevention of pathogen colonization on gut or other tissue surfaces through competition for adhesion sites. It is known that the ability to adhere to enteric mucus and wall surfaces is necessary for bacteria to become established in fish intestines. Since bacterial adhesion to tissue surface is important during the initial stages of pathogenic infection, competition for adhesion receptors with pathogens might be the first probiotic effect. In human medicine, adherent strains are key candidates for probiotic therapy. Adhesion can be nonspecific, based on physicochemical factors, or specific, involving adhesin molecules on the surface of adherent bacteria and receptor molecules on epithelial cells. Inhibition of the adhesion of pathogens to human or other mammal cells has already been demonstrated in vitro by several authors. Competitive exclusion resulting from preemptive colonization has been shown for the cecal walls of chickens, which kept their effect after the ceca were washed four times in buffered saline.

Adhesion capacity and growth on or in intestinal or external mucus has been demonstrated *in vitro* for candidate probiotics used in aquaculture. Olsson (1992) measured the *in vitro* capacity of nine lactic acid bacteria strains to adhere to and grow in turbot intestinal mucus in order to investigate their potential to colonize farmed turbot as a means of protesting the host from infection by *V anguillarum*. The intestinal isolates generally adhered much better to a film of turbot intestinal mucus, skin mucus, and bovine serum albumin than did *V. anguillarum*, indicating that they could compete effectively with the pathogen for adhesion sites on the mucosal intestinal surface. Also, some strains displayed enhanced adhesion to intestinal mucus than BSA control, suggesting specific adhesion. In another research, a few lactic acid bacteria strains intended for human use were tested for their adhesive ability to fish mucus and

competitive adhesion against pathogen. The results showed that most of the adhesive human probiotic bacteria also bound well to fish mucus. Also, the mucosal adhesion was specific, as shown by less adhesion to BSA and gelatin. However, no significant competitive adhesion effect was detected, probably due to low binding capacity of the tested fish pathogens (Nikoskelainen et al., 2001). Vine et al. demonstrated a competitive exclusion effect with five probiotics versus two pathogens on fish intestinal mucus. They found that the presence of one of the probiotics on the mucus inhibited the attachment of one of the pathogens tested. Interestingly, pre-colonization with the other probiotics encouraged attachment of the two pathogens. However, the general trend from their study showed that post treatment with the probiotics displaced the pathogen (Vine et al., 2004). More recently, the involvement of adhesion capacity in the protection of a probiotic strain against *E. ictaluri* infection was demonstrated unequivocally in a zebra fish larvae model. Pre-colonization of the protiotic strain *E. coli* MG1655 in the larvae provided a significant increase in survival upon *E. ictaluri* infection. No direct inhibition or a change in the zebrafish inflammatory response was detected, excluding the possibility of their involvement in the protection. Furthermore, a highly adherent derivative of MG1655 showed better colonization and protective effect against *E. ictaluri*, while deletion of type 1 fimbriae operon which is responsible for adhesion of *E. coli* to intestinal and epithelial cells deprived the probiotic strain of protective effect, indicating that adhesion capacity of the probiotic strain played the major role in protection against *E. ictaluri* infection. (Olaya, 2012)

Chapter II

Identification of *Bacillus* strains for biological control of catfish pathogens

1. Abstract

Bacillus strains isolated from soil or channel catfish intestine were screened for their antagonism against *Edwardsiella ictaluri* and *Aeromonas hydrophila*, the causative agents of enteric septicemia of catfish (ESC) and motile aeromonad septicaemia (MAS), respectively. Twenty one strains were selected and their antagonistic activity against other aquatic pathogens was also tested. Each of the top 21 strains expressed antagonistic activity against multiple aquatic bacterial pathogens including *Edwardsiella tarda*, *Streptococcus iniae*, *Yersinia ruckeri*, *Flavobacterium columnare*, and/or the oomycete *Saprolegnia ferax.* Survival of the 21 *Bacillus* strains in the intestine of catfish was determined as *Bacillus* CFU/g of intestinal tissue of catfish after feeding *Bacillus* spore-supplemented feed for seven days followed by normal feed for three days. Five *Bacillus* strains that showed good antimicrobial activity and intestinal survival were incorporated into feed in spore form at a dose of 8×10^7 CFU/g and fed to channel catfish for 14 days before they were challenged by *E. ictaluri* in replicate. Two *Bacillus subtilis* strains conferred significant benefit in reducing catfish mortality ($P < 0.05$). A similar challenge experiment conducted in Vietnam with four of the five *Bacillus* strains also showed protective effects against *E. ictaluri* in striped catfish. The three strains exhibiting the strongest biological

control *in vivo* did not contain plasmids or express resistance to clinically important antibiotics, and will be further evaluated for their potential as probiotics.

2. Introduction

The interest in probiotic for aquaculture use developed as a consequence of the continuous search for preventive strategies as an alternative to the use of chemotherapeutics and vaccination when fighting aquatic infectious diseases. The most commonly used probiotics in aquaculture are lactic acid bacteria and *Bacillus* spp. (Wang et al., 2008). *Bacillus* species have advantages as probiotics in that their spore-forming ability allows greater viability after pelleting and high survival rates after exposure to gastric acid (Casula & Cuting, 2002; Hong et al., 2005). *Bacillus* spp. have been reported to have various beneficial attributes when applied to fish, including improved growth performance, immunostimulation and enhanced resistance to disease challenge (Ai et al., 2008; Aly et al., 2008; Kumar et al., 2008; Nayak 2007, Newaj-Fyzul et al., 2007; Salinas et al., 2005, 2008; Sun et al., 2010).

Few studies have been conducted to investigate probiotic bacteria for mitigating infectious diseases in catfish. Queiroz and Boyd applied a commercial probiotic product, Biostart, which contained a few species of *Bacillus* spp., to channel catfish pond water and demonstrated that survival and net production of fish treated with *Bacillus spp.* were significantly greater than the control (Queiroz & Boyd, 1998). Shelby *et al.* tested commercially available probiotics as feed supplements for juvenile channel catfish. However, no growth promotion, immunostimulation, or enhanced resistance to disease challenge was detected (Shelby *et al.* 2007). In both of the research, the probiotic bacteria were directly tested *in vivo*

without any *in vitro* screening, which deviated from the standard procedure for development of probiotics, where a screening step was included before *in vivo* test. (Verschuere et al., 2000a).

A common way to screen the candidate probiotics is to perform *in vitro* antagonism tests, in which pathogens are exposed to the candidate probiotics or their extracellular products in liquid. (Verschuere et al., 2000a) Furthermore, the colonization potential is another important criterion to characterize probiotics (Gatesoupe, 1999). The survival and persistence of candidate probiotics in the intestine of host, although not used as selection criterion, was characterized in some studies as an initiation to investigate the colonization potential of those probiotics (Jöborn et al., 1997; Robertson et al., 2000). Thus, survival and persistence in the intestine of host may constitute another criterion during preselection of candidate probiotics. After the screening procedure, probiotic candidates with efficient antagonism and survival ability can be further tested by *in vivo* challenge experiment.

In this research an extensive collection of *Bacillus* strains (n=160) isolated from soil and strains from the intestine of channel catfish (n=17) was tested for *in vitro* antimicrobial activity against *E. ictaluri, A. hydrophila*, and other bacterial and fungal pathogens of channel catfish. *Bacillus* strains that showed effective antibiosis were further evaluated for their respective survival in the intestine of channel catfish. The protection effect of selected *Bacillus* strains against *E. ictaluri* infection in channel catfish and striped catfish was investigated by *in vivo* challenge experiments. The safety of selected *Bacillus* strains was also assessed in terms of the presence of plasmids and resistance to antibiotics.

3. Materials and methods

Bacterial strains

E. ictaluri strain S97-773 was used for the primary screening for *Bacillus* antibiosis and for ESC challenge experiments since this strain is highly pathogenic for channel catfish and has previously been used in challenge studies at the Southeastern Cooperative Fish Disease Laboratory (SCFDL), Auburn University. *E. ictaluri* strain R-4383, *E. ictaluri* strain Alg-08-200**,** *E. tarda*, *S. iniae*, *Y. ruckeri*, *F. columnare*, and *S. ferax* were from the collection of pathogenic isolates at the SCFDL. *E. ictaluri* NLF33 were isolated from diseased striped catfish in Vietnam. *A. hydrophila* ML09-119 was isolated from a diseased channel catfish with MAS in 2009. The collection of soil-derived *Bacillus* strains (n=160) was provided by the laboratory of Dr. Joseph Kloepper (Department of Entomology and Plant Pathology, Auburn University). *Bacillus subtilis* 1E17 was obtained from the *Bacillus* Genetic Stock Center (http://www.bgsc.org/).

Isolation of *Bacillus* spp. strains from the intestine of channel catfish and evaluation of antimicrobial activity

Healthy catfish (7-10 cm) were killed by administration of an overdose of MS-222, and the digestive tracts were removed in their entirety. Approximately 1.0 g was homogenized in 9.0 ml of sterile saline (0.9% w/v). Ten-fold serial dilutions were prepared to 10^{-6} in fresh 1x phosphate-buffered saline (PBS), and 0.1 ml was spread over the surface of triplicate plates of tryptone soy agar (TSA) with incubation at 28°C for 48 h (Irianto & Austin 2002). *Bacillus*-like colonies were picked at random, purified by streaking for isolated colonies on fresh media, and examined for inhibition against the growth of *E. ictaluri* using the double-layer soft agar method (Jack *et al.* 1996). For the soft agar overlay, the bacterial isolates were grown in 5 ml of tryptone soy broth (TSB) for 24 h at 30°C. A volume of 5 µl was then spotted onto triplicate

plates of TSA and incubated for a further 24 h. Soft agar (0.7% w/v agar) prepared with TSB was melted, cooled and seeded with an inoculum of log-phase *E. ictaluri* strain S97-773 to achieve slight turbidity (i.e., ~ 10^7 cells/ml). The bacterial cell suspension in soft agar was immediately poured over the TSA plates and incubated for 24 h at 30°C whereupon the presence of zones of clearing in the growth of the lawn of *E. ictaluri* were recorded (in mm) as evidence of growth inhibition. Cultures that were regarded as inhibitory to *E. ictaluri* were characterized by Gram staining and 16S rRNA gene sequencing using the 'universal bacteria' primer set 27F and 1492R (Weisburg *et al.* 1991). A consensus 16S rRNA sequence was produced using ChromasPro (Technelysium Pty Ltd., Queensland, Australia), and each sequence was compared to the GenBank non-redundant nucleotide database by BLASTn. *Bacillus* spp. strains were cryopreserved at -80^{o}C. The collection of soil-derived *Bacillus* strains (n=160) was tested for antimicrobial activity against *E. ictaluri* using the same method.

Fifty *Bacillus* strains with antagonistic activity against *E. ictaluri* S97-773 were tested for their inhibitory activity against other *E. ictaluri* strains (*E. ictaluri* R-483, *E. ictaluri* Alg-08-200). *Bacillus* strains that showed antimicrobial activity against all three *E. ictaluri* strains were evaluated further for their activity to inhibit the growth of *A. hydrophila* strain AL09-119. Twenty-one *Bacillus* strains that showed significant antimicrobial activity against both *E. ictaluri* and *A. hydrophila* were tested for their activity against several other channel catfish pathogens including *E. tarda, S. iniae, Y. ruckeri, S. ferax* with the soft agar overlay method described above.

The antimicrobial activity against *F. columnare* was tested by an agar well diffusion method. For the well diffusion assay, the *Bacillus* strains were grown in 5ml of TSB for 48h at 30°C. After centrifugation at 3,600 × g for 10 min, the culture supernatant was filtered through a

0.2 µm filter. Then 200 µl of the filter-sterilized supernatant was added to a round well (approx. 10 mm in diameter) made in a *F. columnare* growth medium (FCGM) agar plate (Farmer, 2004). After the supernatant was absorbed into the agar medium, a log-phase *F. columnare* culture grown in FCGM broth was spread thoroughly over the plate using a sterile cotton swab. The plates were incubated for 48 h at 30°C. The zones of clearing in the growth of the lawn of *F. columnare* were measured by the same method as in double-layer soft agar protocol.

Bacillus strains AP79, AP143, AP193, AP254, AB01 were sent to Vietnam and their *in vitro* antimicrobial activity was tested against *E. ictaluri* NLF33, the causative agent of BNP in striped catfish. A broth culture of *E. ictaluri* was adjusted to 10^6 CFU/mL and evenly swabbed onto TSA plates. Three wells were punched from the agar plate and 50 µL of a 10^8 CFU/mL of a *Bacillus* cell-free supernatant (48 h culture in TSB) was added into each well. Zones of inhibition were measured after 24 hours incubation at 30°C.

Preparation of *Bacillus* spores and spore-amended feed

Bacillus spores were prepared by the method described by Kenny and Couch (1981) with some modifications. *Bacillus* strains were grown in TSB at 30°C overnight. The cell suspension was spread onto spore preparation agar (peptone 3.3 g/l, beef extract powder 1.0 g/l, NaCl 5.0 g/l, K_2HPO_4 2.0 g/l, KCl 1.0 g/l, $MgSO_4 \cdot 7H_2O$ 0.25 g/l, $MnSO_4$ 0.01 g/l, lactose 5 g/l, agar 15 g/l) using a sterile cotton swab and incubated at 28°C for 5 to 7 days. To collect the spores, 5 ml of sterile distilled water was added to the plate and the spores were suspended in water using an inoculation loop. The spore suspension was then incubated at 85°C for 15 min to kill the vegetative cells. The concentration of the spore suspension was determined by 10-fold serial dilution in 1x PBS and spreading onto TSA. The final concentration of the spore suspension was manipulated with sterile water to 1.25×10^{10} CFU/ml for the intestinal survival assay and 10^9

CFU/ml for the challenge study. To prepare spore-amended feed, 80 ml of the spore suspension was sprayed onto 1000 g commercially available slow-sinking pelleted fish feed (2 mm, 40 % protein, Zeigler, Gardners, PA) using a bleach- and ethanol-sterilized pump sprayer to achieve approximately 8% (v/w) spore suspension application. The feed was then mixed thoroughly with 30 ml fish oil. The control feed was ammended solely with fish oil.

Inoculation and quantification of *Bacillus* spp. in the intestine of channel catfish

Fingerling channel catfish (7-10cm) were distributed into twenty-three 60 L tanks each containing 15 L water and three fish. Fish were starved for one week prior to the experiment. Catfish feed was amended in separate batches with the 21 *Bacillus* strains that showed good antimicrobial activity against both *E. ictaluri* and *A. hydrophila* using the spore application method described above. Each unique *Bacillus* strain-amended feed (~10^9 CFU/g feed) was given to one aquarium tank. The fish were fed once daily with spore-amended feed or control feed for one week, and thereafter all fish received the control feed for three days. One tank was used as the control and received untreated fish feed for the duration of the experiment. Daily feeding rate was 3% of total body weight.

At the end of the experiment, all of the fish were killed by administration of an overdose of MS-222. The intestine was removed, weighed, and then homogenized in sterile saline (0.9% w/v). The final volume of the homogenized intestine sample was adjusted to 2 ml by sterile saline. Homogenized samples were then serially diluted in sterile saline and spread on TSA and incubated at 28°C for 48h. Three representative colonies with the same morphology as the applied *Bacillus* strain were randomly picked from the plate, purified on new plates and identified by 16S rRNA gene sequencing as described previously and compared with the known 16S rRNA gene sequence from each respective *Bacillus* strain. For the control and treatment

groups, only the unique colony morphology corresponding to that of the amended *Bacillus* strains was recorded. Cultured counts for each *Bacillus* strain recovered from the intestine were determined as CFU/g of intestine sample.

Aquarium challenge studies

In the first challenge, five *Bacillus* strains (AB01, AP143, AP193, AP254, and AP79) were selected for evaluation of their biological control of ESC in an aquarium challenge. Five *Bacillus* treatments and one control each with four replicate aquaria were included. Each replicate aquarium was stocked with 25 fingerling channel catfish weighing about 13 g. Fish were acclimated to commercial dry feed for one week. Fish from each treatment group were then fed with an experimental diet supplemented with spore of a *Bacillus* strain (8×10^7 CFU/g) at a daily feeding rate of 2.5 % fw/bw (feed weight / body weight) for two weeks. Fish in the control group received normal feed only.

Fish were challenged by immersion for 45 minutes in 10 L of water containing 4.5×10^6 CFU/ml *E. ictaluri* S97-773. All fish from the same group were immersed in a single container. The challenge condition for the control group was the same as other treatments except that BHI medium was added instead of *E. ictaluri* culture. Mortalities were monitored over a 21-day period, and dead fish were dissected and the presence of *E. ictaluri* confirmed by microbiological examination of kidney and liver swabs on TSA. The identity of the recovered *E. ictaluri* was confirmed by biochemical analysis.

Fish were reared in a recirculating system during the acclimation period. Upon initiation of *Bacillus* feeding and during the challenge phase, a static system was incorporated with a 20-30 min daily water exchange. Sponge biofilters and daily removal of uneaten/waste materials were incorporated to improve water quality. Water temperature was kept at 26 ± 2°C. During the static

phase, the central room heating system in conjunction with submersible aquarium water heaters was used to control the required water temperature, and a water heater system was used to control the temperature of the incoming water during water exchange.

In a second challenge trial using channel catfish, flow-through conditions were used to reduce catfish mortality. In this challenge experiment, four *Bacillus* treatments (AP79, AP143, AP193, AB01) and one control each with four replicate aquaria were included. Each aquarium was stocked with 20 fingerling channel catfish (~12 g). A lower dose of *E. ictaluri* S97-773 (8×10^5 CFU/ml) was used to challenge fish and starting immediately after challenge the aquaria were flushed for 5-8 hours a day. All other conditions in this challenge were the same as described above. Mortalities were monitored over a 21-day period after challenge, and presence of *E. ictaluri* in the dead fish was confirmed as previously described.

A third challenge trial was conducted to evaluate the protective effect of four *Bacillus* strains (AP79, AP193, AP254 and AB01) against *E. ictaluri* for striped catfish. Four *Bacillus* treatments and one control each with four replicate tanks were included in this study. Each tank was stocked with 18 striped catfish (~14g). Striped catfish were administered feed amended with *Bacillus* spores (~10^7 CFU/g feed) or control feed for two weeks and the fish were transferred to 80 L tanks for a bath challenge with *E. ictaluri* NLF33. Fish were immersed for 30 min in static, aerated aquaria at a dose of ~10^6 CFU/mL to target about 70% mortality in the control group. The control and test diets were offered throughout the challenge phase. The recording of mortality and confirmation of *E. ictaluri* in dead fish were conducted as above.

Plasmid analysis

Plasmid DNA was extracted from *Bacillus* strains AP79, AP143, AP193, and AB01 by the alkaline lysis method (Birnboim & Doly, 1979). *Bacillus subtilis* 1E17 containing plasmid pC194 was used as a positive control. The extracted DNA was analyzed by a Chef-DR II pulsed field electrophoresis system (Bio-Rad, Hercules, CA). Pulse time ranged from 1 to 15 seconds for 15 h at 6 V/cm. The gel was stained with ethidium bromide and visualized using an AlphaImager HP gel documentation system (ProteinSimple, Santa Clara, CA).

Antibiotic resistance analysis

The susceptibility of *Bacillus* strains AP79, AP143, AP193, and AB01 to carbenicillin, ampicillin, spectinomycin, oxacillin, vancomycin, cephalothin, novobiocin, sulfadiazine, amikacin, erythromycin, neomycin, penicillin, chloramphenicol, sulfamethoxazole, norfloxacin, gentamicin and ciprofloxacin was determined by disc diffusion test following procedures outlined by National Committee for Clinical Laboratory Standards (CLSI 2012). A log-phase culture of each strain was diluted to a concentration of approximately 1×10^8 to 2×10^8 CFU/ml (McFarland standard 0.5). The inoculum was then seeded onto a Mueller-Hinton agar plate using a cotton swab. Antibiotic-impregnated discs (BD Biosciences) were placed on seeded plates, and the diameter of the zone of growth inhibition was measured after 18 h of incubation at 37 °C. The experiments were repeated three times and the average diameter of inhibition zones was calculated.

Statistics

A completely randomized design was used in this research. Data were presented as mean ± standard error (SE). Challenge data were subjected to analysis of variance in SAS 9.2.

Differences between means were tested by Tukey's range test and were considered significant when probability (P) values < 0.05 were obtained.

4. Results

Characterization of *Bacillus* isolates

Each of the *Bacillus* strains isolated from soil or catfish intestine that exhibited inhibitory activity against both *E. ictaluri* and *A. hydrophila* was capable of endospore formation. Each pure *Bacillus* culture was ribotyped, indicating that most of the *Bacillus* strains were within the *B. subtilis* group (inclusive of *B. amyloliquefaciens*) (Table 1). All of the *Bacillus* strains that were subsequently determined to have *in vivo* biological control activity were within the *B. subtilis* group. This phylogenetic affiliation was also confirmed by comparison of genome sequences to the GenBank nr/nt database for strains AB01, AP79, AP143, and AP193, indicating > 80% average nucleotide identity to a previously sequenced genome within the *B. subtilis* group (data not shown). Two strains of *B. pumilus* (AP18 and AP280) were also within the collection. Strain AP76 was identified as *Bacillus cereus* and thus eliminated for further evaluation due to the potential for foodborne illness.

Antimicrobial activity of *Bacillus* strains

The *B. subtilis* strain AB01 isolated from the catfish intestine showed significant antimicrobial activity against *E. ictaluri*. From the collection of soil-derived *Bacillus* strains, 49 strains showed significant antagonism against *E. ictaluri.* All of the 50 *Bacillus* strains also showed inhibitory activity against *E. ictaluri* R-4383 and *E. ictaluri* Alg-08-200. A total of 21 *Bacilllus* strains showed potent antibiotic activity against both *E. ictaluri* and *A. hydrophila* (Table 1). The 21 *Bacillus* strains selected were tested for their activity against multiple

pathogens in aquaculture. All of the strains were antagonistic against multiple catfish pathogens, including Gram-negative and -positive bacteria, and the oomycete *Saprolegnia*. *Bacillus* strains AB01, AP219, and AP301 showed antimicrobial activity against all of the tested pathogens (Table 1). Also, all five of the *Bacillus* strains tested for biological control of BNP (AP79, AP143, AP193, AP254, AB01) showed significant antagonistic activity against *E. ictaluri* NLF33 (data not shown).

Survival and persistence of *Bacillus* strains in the intestine of channel catfish

Bacillus administered to channel catfish via feed for one week followed by three days of control feed was recovered from the catfish intestine. Over 10^7 CFU/g of introduced *Bacillus* was observed in the gut for strains AB01, AP76, AP77, AP79, AP143, and AP254 (Fig. 2). For strains AP18, AP280, and AP303, the counts of recovered bacteria were relatively low, and they were eliminated from further investigation. None of the 21 *Bacillus* strains were recovered from the control group. In all cases the 16S rRNA gene sequence determined from representative colonies matched the 16S rRNA gene sequence from the respective *Bacillus* strain that was added to catfish feed (data not shown).

Challenge study

In the first immersion challenge, the mean mortality of control group was 98.0 %, a very high level of mortality that was likely a consequence of the persistence of *E. ictaluri* within the aquaria water under static conditions. Treatment groups that were fed with feed amended with spores of *Bacillus* strains AP143 or AB01 showed significantly reduced mortality compared with the control ($P<0.05$), with 83.1% and 84.8% mortality, respectively. There was no significant difference in the mortality observed between the two strains. The treatment groups fed with

Bacillus strains AP79, AP193, or AP254 (with mortality 89.0%, 95.0%, and 93.7%, respectively) did not show significant differences compared with the control (Fig. 3A, Table 2).

For the second immersion challenge, flow-through conditions were adopted post-challenge to reduce catfish mortality, and this was successful as only 41.3% mortality was observed in the control group. However, under the flow-through conditions of this challenge the mortality in the treatment groups ranged from 35.0% to 46.3% with no significant differences observed between any of the treatment groups and the control (Fig. 3B, Table 2).

In the striped catfish challenge experiment, the catfish were again challenged by immersion and then maintained under static conditions similar to those used in the first channel catfish challenge. Under these conditions the control group had 70.8% mortality which is consistent with the higher mortality observed under static conditions when re-infection can occur (Fig. 3C, Table 2). The treatment group fed with feed amended with spores of strain AP79 had the lowest (9.7%) cumulative mortality and this was significantly different from the control ($P<0.05$). Catfish fed with spores of strains AP193, AP254 or AB01 had 30.6%, 54.2% and 56.9 % mortality, respectively.

Plasmid and antibiotic resistance study

An analysis of plasmid DNA extraction from *Bacillus* strains AP79, AP143, AP193, and AB01 was conducted by PFGE. We did not observe the presence of any plasmid within these three strains but the positive control did show the presence of plasmid pC194 (data not shown). Evaluation of antibiotic susceptibility determined that all of these strains were susceptible to all of the tested antibiotics to varying degrees. They were all highly susceptible to carbenicillin, cephalothin, sulfamethoxazone and ciprofloxacin (> 25mm diameter inhibition zone). Ampicillin, penicillin, vancomycin, novobiocin, amikacin, erythromycin, neomycin,

chloramphenicol, norfloxacin and gentamicin also inhibited their growth effectively (20 -25 mm zones of inhibition), whereas spectinomycin, oxacillin, sulfadiazine showed moderate inhibition (15-20 mm inhibition zones). These strains showed very similar antibiograms, with the variation in the diameter of inhibition zones less than 10% of the average diameter for each of the antibiotics tested.

5. Discussion

The results of this study indicate that specific strains within the *Bacillus subtilis* group showed promise for biological control of disease in catfish aquaculture. It provided another evidence of the efficacy of the *Bacillus* strains in boosting disease resistance of fish (Aly et al., 2008; Kumar et al., 2008; Nayak et al., 2007; Newaj-Fyzul et al., 2007; Panigrahi et al., 2007; Salinas et al., 2005).

Gatesoupe (1999) concluded that probiotics for aquaculture should be antagonistic to pathogens, colonize intestines, and increase resistance of the host to pathogens. Ideally probiotic bacteria should be selected by considering all three criteria. However, it is difficult to evaluate potential probiotic bacterial strains for the second and third criterion on a large number of candidate bacteria. Therefore, *in vitro* antimicrobial activity was the primary criteria by which a large number of strains were evaluated, with candidate bacterial strains that did not show antagonistic activity eliminated from further study. The primary objective of this research project was to identify bacterial strains that can be applied for the control of *E. ictaluri*, *A. hydrophila*, as well as other bacterial and oomycete pathogens of catfish. Since the bacterial pathogens *E. ictaluri* and *A. hydrophila* are responsible for the majority of the mortality observed currently in catfish aquaculture, the ability of a *Bacillus* strain to inhibit the growth of these two

pathogens was of paramount importance and only the strains capable of inhibiting both pathogens were selected for testing in aquarium disease challenges.

The ability of a probiotic bacterial strain to colonize and survive within or on its host is also an important criterion for strain selection. However, in many cases the probiotic bacteria may not permanently colonize the gastrointestinal tract but instead achieve a sustained transient state (Robertson et al., 2000; Irianto & Austin, 2002). Even transient bacteria may be efficient at mediating biological control of disease if the cells are introduced artificially via food either continuously or semi-continuously (Gournier-Chateau et al., 1994; Gatesoupe, 1999). High population levels of several *Bacillus* strains were recovered from catfish intestines three days post-feeding with *Bacillus*-spore amended feed. For *Bacillus* strains with high counts in the intestine, colonies with the same morphology as the applied *Bacillus* strain dominated the TSA plates, and the ribotype of the representative colonies confirmed their identity as the applied *Bacillus* strain. In a previous study of the persistence of an *E. ictaluri*-specific bacteriophage within the intestine of channel catfish, it was observed that 72 hours post-feeding the bacteriophage could not be detected within intestinal samples (Carrias, 2011). This implies that any inert particle would be cleared from the catfish intestine by 72 hours post-feeding and that bacterial strains detected after this time frame would have some degree of intestinal persistence. Considering that bacterial population levels in the intestine should decline after cessation of feeding with the spore-containing diets, the maximal level of *Bacillus* strain CFU/g of intestinal tissue reached during the feeding regime may be higher. The bacterial population levels here (10^6-10^7 CFU/g for most of the strains) are in general agreement with previous studies involving fish (Jöborn et al., 1997; Gildberg & Mikkelsen 1998; Robertson *et al.* 2000; Irianto & Austin 2002). These results demonstrate that some of the *Bacillus* strains evaluated in this study can

persist within the catfish gastrointestinal tract for at least three days. However, at this point the degree of persistence and ability to colonize the intestinal mucosa are unknown for each strain. A more detailed experiment evaluating the colonization and/or persistence of specific *Bacillus* strains within the catfish intestine will be conducted to help understand the mode of action of *Bacillus* strains and guide the duration and timing of *Bacillus* feeding. Future studies will also examine the impact of each *Bacillus* strain on the intestinal microbiota and the health and growth of the fish in the absence of aquaculture pathogens.

Three aquarium disease challenges were conducted in this study, two of which evaluated biological control of ESC in channel catfish. In the first channel catfish disease challenge, a very high mortality (98.0%) due to ESC was observed in the control group. Ideally, an aquarium disease challenge would result in a mortality of 60% -70%, which more accurately simulates the natural development of ESC in an aquaculture pond. The high mortality was probably the consequence of the incorporation of a static system after the immersion challenge, wherein the *E. ictaluri* persisted in the aquaria for an extended period of time and bacterial cells shed in feces could potentially infect other fish within the same aquarium. Despite the higher mortality observed in this first challenge, two *Bacillus* strains (AP143, AB01) provided significant protection to channel catfish. However, the degree of mortality reduction for the *Bacillus* treatment groups compared with control was lower in the first challenge compared to a later challenge with striped catfish. This discrepancy might be due to the much higher mortality in the first challenge, which could have reduced the biological control capacity of the *Bacillus* strains. Presumably the degree of biological control would be greater in an ESC challenge if lower mortality (~70%) was obtained in the control group. In addition, at the lower doses of *E. ictaluri*

that catfish are typically exposed to in an aquaculture pond the degree of biocontrol provided by *Bacillus* strains would presumably be of an even greater magnitude.

One solution to reduce channel catfish mortality during an aquarium disease challenge is to use a flow-through system, but we discovered that the use of a flow-through system also resulted in the loss of *Bacillus* biological control activity. The lack of a protective effect for *Bacillus* strains when catfish were maintained in flow-through conditions after immersion challenge is likely a consequence of the removal of the *Bacillus* cells from the aquarium water, thereby preventing the persistence of the *Bacillus* cells within the aquarium that would naturally occur within an aquaculture pond. This suggests that a more pond-like environment wherein the probiotic is maintained within the water, and potentially the skin and gills of the fish, may be more conducive for effective biological control of disease. Since a static aquarium system is a better model for evaluating the biological control of disease that would occur in an aquaculture pond, we considered the results of the first disease challenge more relevant to the evaluation of *Bacillus* strains for their future adoption in pond-scale aquaculture.

Considering the importance of striped catfish for the Vietnamese aquaculture industry, the best performing *Bacillus* strains were sent to collaborators in Vietnam to evaluate their protection activity for striped catfish against *E. ictaluri*. Significant reductions in mortality were obtained in this experiment, with an especially large reduction in mortality observed AP79 feeding group. The biological control activity observed for *Bacillus* strains in this striped catfish disease challenge was comparable with previous studies in other fish species (Aly et al., 2008; Kumar et al., 2008; Nayak et al., 2007; Newaj-Fyzul et al., 2007).

It is interesting that the *Bacillus* strains that showed a significant protective effect in the disease challenges were different for the two catfish species. This could reflect a biologically meaningful

difference in the interactions between *Bacillus* strains and their respective host. Also, there could be unique tripartite interactions between host, pathogen, and probiotic bacteria that could be influenced by environmental factors. Clearly more research is needed to understand the complex interplay between host, pathogen and probiotic *Bacillus* strains, and how to manipulate the environment to achieve the optimal biological control of disease. Further studies using an aquarium disease model with static conditions need to be conducted to optimize important parameters for challenge such as dosage and timing with the best performing *Bacillus* strains, with subsequent studies at a pond-scale to evaluate biological control efficacy within an aquaculture pond ecosystem.

One of the safety requirements for live bacteria directly consumed by humans is the absence of any acquired resistance to clinically important antibiotics (Sorokulova, 2008). Although the *Bacillus* strains used in this research were not for direct consumption by humans, they might be consumed inadvertently, as their hosts were cultured for food. Thus, it is important to analyze antibiotic resistance in probiotic strains and to distinguish the natural resistance, which is one of the phenotypic characteristics of a species, and acquired (i.e., transferable) resistance, which is associated with occurrence of plasmids. Also, pathogenicity and enterotoxin production are closely associated with plasmids (Pannucci et al., 2002). None of the four selected *Bacillus* strains carried any plasmids, and each of the strains was susceptible to a broad spectrum of antibiotics tested, which ensures their inability to conjugally transfer any plasmid that might confer antibiotic resistance.

Diffusible antimicrobial compounds were clearly involved in the *in vitro* antagonistic activity observed in soft agar overlay and in diffusion tests. The relative importance of secondary metabolites for *in vivo* biological control is unknown compared to other mechanisms

such as enhancement of immune response, competitive exclusion of pathogens and modulation of intestinal microbiota. Future studies will investigate the relative contribution of antibiotic compound(s) production to the biological control activity of the *Bacillus* strains by comparing the mortality reduction activity of transposon mutagenized *Bacillus* strains with no antagonism activity with the wild type strains in aquarium challenge experiments.

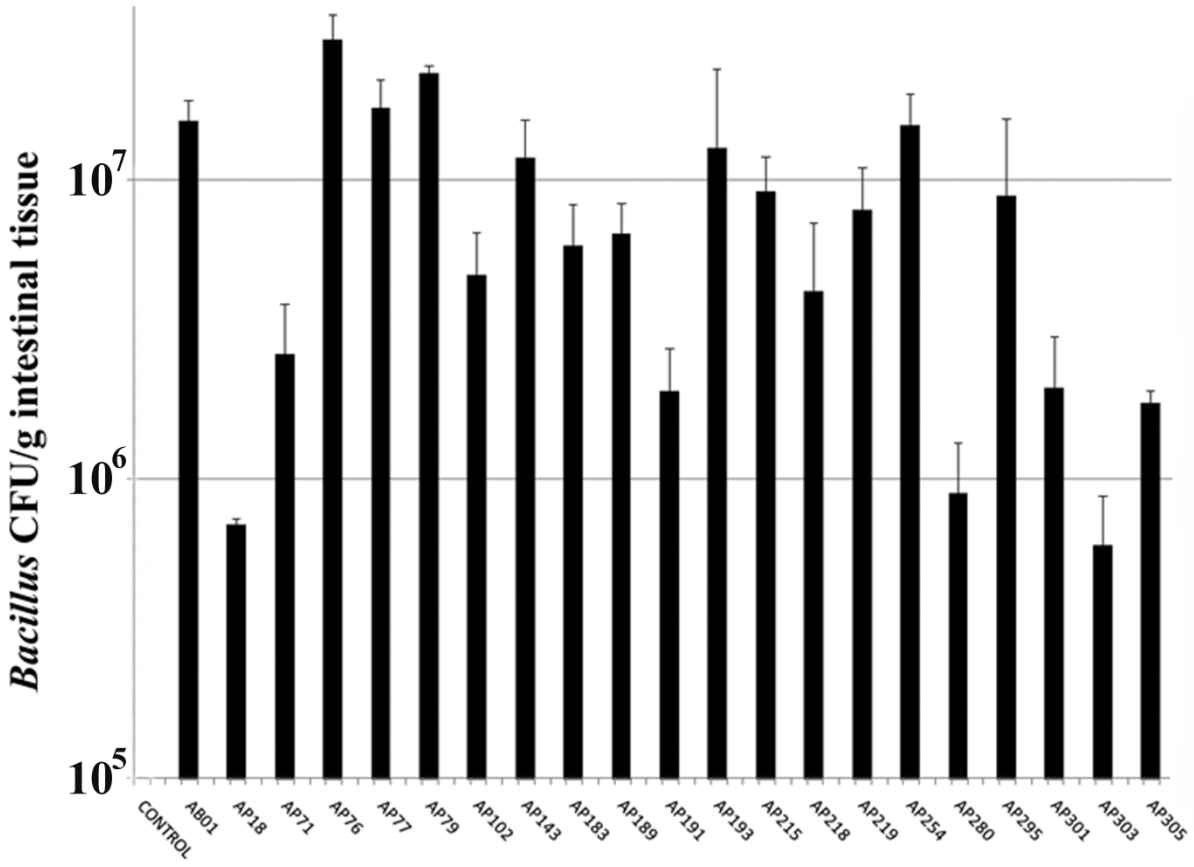
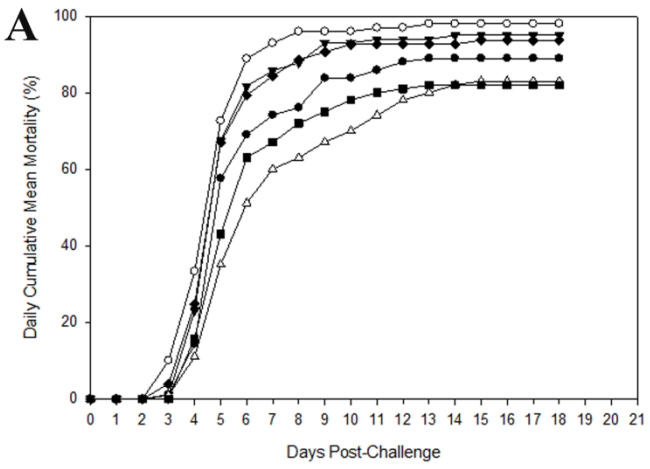
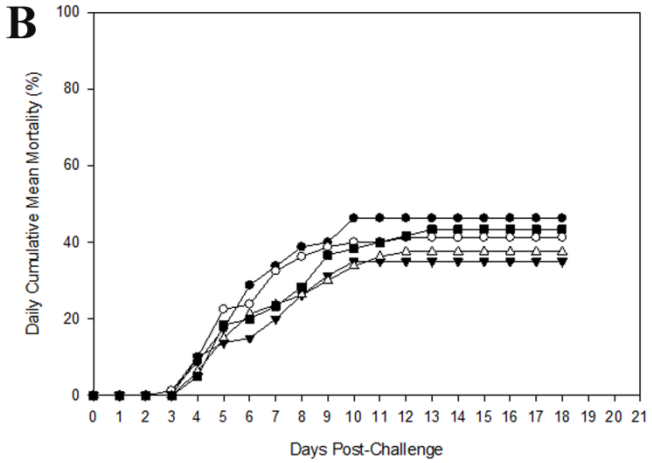


Figure 1. *Bacillus* strain CFUs/g of catfish intestine, after feeding with *Bacillus*-amended or non-amended feed (n=3 animals per *Bacillus* strain).


A
Daily Cumulative Mean Mortality (%)
Days Post-Challenge


B
Daily Cumulative Mean Mortality (%)
Days Post-Challenge

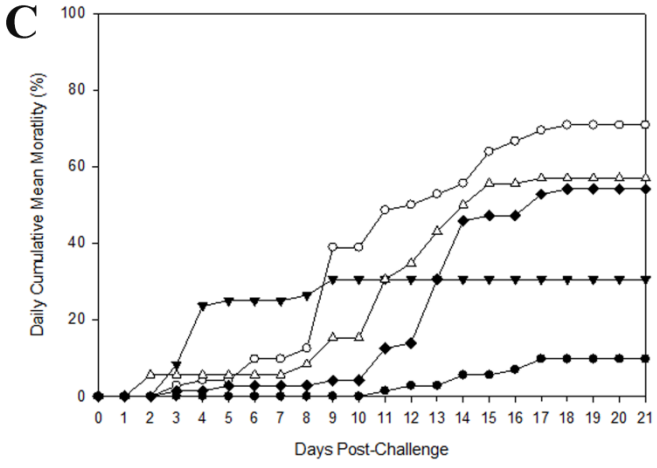

C
Daily Cumulative Mean Moratity (%)
Days Post-Challenge

Figure 2. Daily mean cumulative mortality of (A) channel catfish in static system with 20-30 min daily water exchange and (B) channel catfish with 5-7 h flow through water daily , or (C) striped catfish in static system with 20-30 min daily water exchange, fed with and without addition of *Bacillus* strains and challenged with *E. ictaluri*. All values are means of four replicates per treatment. Treatments: (○) Control, (●) AP79, (▼) AP193, (△) AB01, (■) AP143, and (◆) AP254.

Phylogeny	**Strain**	***Aeromonas hydrophila***	***E. ictaluri***	***E. tarda***	***F. columnare***	***Saprolegnia ferax***	***Streptococcus iniae***	***Yersinia ruckeri***
B. subtilis group	AB01	+	+	+	+++	+	++	++
B. pumilus	AP18	+	+	-	-	+	+	-
B. subtilis group	AP71	+	++	+	-	-	++	+
B. cereus	AP76	+	++	++	-	+	++	++
B. subtilis group	AP77	+	+++	++	-	+	+	++
B. subtilis group	AP79	++	++	+	-	-	++	++
B. subtilis group	AP102	+	++	++	++	+	-	++
B. subtilis group, *B.amyloliquefaciens*‡	AP143	++	++	++	-	+	+	++
B. subtilis group	AP183	+	++	++	-	-	++	+
B. subtilis group	AP189	++	+++	++	-	-	++	+
B. methylotrophicus	AP191	++	+++	+	+	+	++	-
B. subtilis group, *B.amyloliquefaciens*‡	AP193	++	++	++	++	+	+	-
B. subtilis group	AP215	+	+++	+	-	+	+	++
B. subtilis group	AP218	+	++	+	-	+	+	+
B. subtilis group	AP219	++	++	+	+	+	++	+
B. subtilis	AP254	+	++	+	++	-	-	-
B. pumilus	AP280	+	++	+	-	+	+	+
B. subtilis group	AP295	+	++	+	-	+	+	++
B. subtilis group	AP301	+	++	+	+++	+	++	+
B. subtilis group	AP303	++	++	++	-	+	+	++
B. subtilis group	AP305	++	++	++	-	-	+	++

Table 1. Antimicrobial activity of 22 *Bacillus* strains against multiple aquatic pathogens. Note that (+) indicates a zone of inhibition up to 5 mm, (++) indicates a zone of inhibition from 5 mm to 1cm, (+++) indicates a zone of inhibition greater than 1cm, and (-) indicates no observable zone of inhibition.

[ǂ]Genome sequences are available for these *Bacillus* strains, so the phylogenetic affiliation is inferred from a comparison of these *Bacillus* strain genome sequences with previously sequenced *Bacillus* genomes.

Treatment	Channel catfish challenge (Fig 3.A)	Channel catfish challenge (Fig 3.B)	Striped catfish challenge (Fig 3.C)
Control	98.0 ± 1.16^{a}	41.3 ± 5.91^{a}	70.8 ± 7.31^{a}
AB01	84.8 ± 1.95^{bc}	37.5 ± 9.46^{a}	56.9 ± 6.56^{ab}
AP143	83.1 ± 2.88^{bc}	43.3 ± 14.81^{a}	Not determined
AP193	95.0 ± 3.00^{ab}	35.0 ± 5.40^{a}	30.6 ± 23.73^{ab}
AP254	93.7 ± 2.79^{ab}	Not determined	54.2 ± 11.43^{ab}
AP79	89.0 ± 2.74^{abc}	46.3 ± 5.15^{a}	9.7 ± 6.56^{b}

Table 2. Mortality (%) (± SE) of groups of fish that received feed amended with different Bacillus strains or control feed and were challenged with E. ictaluri (n=4). Means in the same column sharing a common superscript letter were not significantly different ($P > 0.05$) as determined by Tukey's test.

Chapter III

Effect of *Bacillus* feeding on growth and the innate immune response of channel catfish

1. Abstract

Bacillus strains AP79, AP143, AP193 and AB01 were supplemented in feed and fed to fish for 10 weeks. All of the *Bacillus* fed groups showed improved weight gain and feed conversion ratios; however, the differences were not statistically significant. Similarly, a marginal increase in the serum lysozyme and respiratory burst activities were observed in the *Bacillus* fed groups. All of the fish were challenged with *E. ictaluri* after the 10 weeks feeding regime, and the AP193 group gave a 12% reduction in mortality compared with control ($P = 0.07$). The effect of *Bacillus* dosage on biocontrol efficacy was also evaluated. Fish were fed with AP193-supplemented diets at doses from 4×10^6 CFU/g to 4×10^8 CFU/g for two weeks and challenged with *E. ictaluri*. Although no significant difference was observed between different groups, mortality decreased gradually as the quantity of AP193 increased in the diet.

2. Introduction

As defined by Verschuere et al. (2000a), multiple ways exist in which probiotics could be beneficial and they could act either singly or in combination for a probiotic. These include

inhibition of a pathogen via production of antagonistic compounds, competition for attachment sites, competition for nutrients, immunosimulatory functions, and nutritional benefits such as improvement of feed digestibility and feed utilization. With many studies demonstrating the *in vitro* antagonism of probiotics against aquatic pathogens and their protection of the considered aquatic animals challenged by corresponding pathogens, recently more research have investigated other beneficial effects of probiotics mainly focusing improved growth performance and enhanced nonspecific immune response of the cultured aquatic species.

Bairagi et al. (2004) reported that diets formulated with 40% Leucaena leaf meal inoculated with enzyme-producing fish intestinal bacteria *B. subtilis* resulted in enhanced feed efficiency ratio, protein efficiency ratio, apparent net protein utilization and growth response of rohu fingerlings. Ai et al. (2011) fed juvenile large yellow croaker with *Bacillus subtilis* supplemented diet for 10 weeks. The long term probiotic feeding lead to significantly enhanced specific growth rage and feed efficiency ratio. Serum nonspecific immunity was also stimulated in *Bacillus* feeding group, as indicated by enhanced serum lysozyme and SOD activity. Sun isolated two dominant *Bacillus* strains in the microbiota of fast growing grouper and tested their probiotic effect in a 60 days feeding study. Results showed that although no significant improvements of weight gain or specific growth rate were observed in the probiotic fed groups, a significant improvement of feed conversion ratio was conferred by both of the *Bacillus* strains. Also, *Bacillus* feeding enhanced the levels of serum lysozyme and complement C3 (Sun et al., 2010). Similarly, Heo et al. (2013) tested the effects of a potential probiotic strain Lactococcus lactis subsp. lactis I2 on the growth and immune response of olive flounder. After feeding 5 weeks, growth performance and non-specific immune parameters such as lysozyme,

antiprotease, serum peroxidase and blood respiratory burst activities were improved in the probiotic-diet group.

In most of probiotic studies, a dose of 10^7-10^8 CFU/g of feed was adopted to test the probiotic effect (Gatesoupe, 1994; Robertson et al., 2000, Newaj-Fyzul et al., 2007; Aly et al., 2008; Salinas et al., 2005; Sun et al., 2010; Ai et al., 2011; Kumar et al., 2008; Heo et al., 2013). However, the reason for choosing this level of dose was generally not addressed. Brunt and Austin (2005) investigated the dose-effect relationship of a probiotic strain in rainbow trout. Protection of rainbow trout against both *Lactococcus garvieae* and *Streptococcus iniae* infection was demonstrated in the probiotic feeding groups, with dosage levels of 10^7 CFU/g and 10^8 CFU/g conferring the best protection, while the higher dosage of 10^{10} CFU/g leading to less protection which was similar with the group of 10^3 CFU/g. Similar tendency was demonstrated in another study testing effect of a *L. rhamnosus* strain against *A. salmonicida* infection in rainbow trout, where fish fed with 10^{12} CFU/g probiotic supplemented feed gave higher mortality compared with the 10^9 CFU/g feeding (Nikoskelainen et al., 2001). Therefore, dosage of probiotics deserves consideration to avoid overdosing with resultant lower efficacy and unnecessary costs.

In chapter 2, a few *Bacillus* strains with antagonism against multiple aquatic pathogens *in vitro* were tested in a two weeks feeding experiment followed by challenge of channel catfish with *E. ictaluri*. Protection of the challenged catfish was conferred by several strains as indicated by a reduction in mortality. To further evaluate the selected strains as potential probiotics, their effects on growth performance and non-specific immunity of channel catfish were studied in a ten-week feeding experiment. The safety of those *Bacillus* strains to fish was also evaluated.

Additionally, the protective effect of different doses of *Bacillus subtilis* AP193 against ESC was investigated in a disease challenge.

3. Materials and Methods

Bacteria Strains

Bacillus strains AP79, AP143, AP193 and AB01 were selected based on their previously demonstrated protective effect against *E. ictaluri* in aquarium studies. AP79, AP143 and AP193 were provided by the laboratory of Dr. Joseph Kloepper (Department of Entomology and Plant Pathology, Auburn University). AB01 was isolated from the intestine of channel catfish in North Auburn, Alabama. *E. ictaluri* strain S97-773 was used for challenge.

Spore and feed preparation

Bacillus spores were prepared by the method described in chapter 1. Concentration of the spore suspension was calculated by 10-fold serial dilution and CFU counting. Spore-supplemented feed was prepared by spray-coating finfish slow-sinking starter pellets (2mm, 40% protein, Zeigler, Gardners, PA) with a spore suspension manipulated as 10^9 CFU/ml, at a rate of 8% (w/v) of the dry/lyophilized weight of feed. Control feed was prepared the same way by applying 8% sterile water to dry feed. The feed in all batches was immediately coated with 5% menhaden fish oil at a rate of 3% of the dry feed weight. For the dose effect study, spore preparation of *Bacillus* strain AP193 was applied to dry feed the same way as mentioned above. Three doses of AP193 (4×10^6 CFU/g, 4×10^7 CFU/g and 4×10^8 CFU/g) were achieved by

manipulating the concentration of the spore suspension applied to the dry feed. Prepared feed stocks were stored at 4 ºC.

Experimental design and rearing of fish

At the initiation of the experiment, channel catfish fingerlings of similar sizes were randomly distributed to 25 aquaria each containing 45 L of de-chlorinated Auburn city tap water supplied through a flow thru system. Each aquarium was stocked with 30 fish and each diet was randomly assigned to five aquaria. Water was aerated continuously by using an air blower. Water flow rate was maintained at approximately 2 L/h. Photoperiod was 12h light/ 12h dark. Water temperature was maintained at (26 ± 1 ºC) by using a central water heater system.

Growth parameters

Fish were acclimated by feeding with standard catfish fingerling feed for seven days. After the acclimation, they were fed once daily with corresponding experimental diet at 3% of the total biomass. The feeding trial lasted for 10 weeks. After the acclimation and biweekly during the feeding trial, fish from each unit were counted and batch-weighed. The amount of feed applied to each tank was adjusted every two weeks by the updated weight information. The body weight gain and FCR were calculated as:

Weight gain = (Wf – Wi) / Wi

FCR = F / (Wf –Wi)

Where Wf and Wi were final and initial mean weight (g) per fish in a tank. F is the cumulative amount of feed (g) given to one fish in a tank.

Sample collection

At the termination of the experiment, blood samples were collected from the caudal vein of five fish per tank and pooled together. Portions of the blood were drawn directly into vials containing heparin to prevent coagulation. For serum preparation, another portion of the blood sample was allowed to clot at room temperature for 2 h and 5 ºC overnight. Serum was collected by centrifugation (1000 × g, 10 min, 4 °C) and frozen at −80 °C until use.

Respiratory burst activity

The respiratory burst activity of the phagocytes was measured by nitroblue tetrazolium (NBT) assay (Kumar 2007). Fifty microliters of blood was placed into the wells of 'U' bottom microtitre plates and incubated at 37 °C for 1 h to facilitate adhesion of cells. Then the supernatant was removed and the adhered cells were washed three times in PBS (pH 7.2). After washing, 50 ml of 0.2% NBT was added and the cells were incubated for a further 1 h. The cells were then fixed with 100% methanol for 2-3 min and again washed thrice with 30% methanol. The plates were then air dried and 60 ml of 2 N potassium hydroxide (KOH) and 70 ml dimethyl sulphoxide were added into each well to dissolve the formazan blue precipitate. The optical density was then read in an ELISA reader at 650 nm.

Serum lysozyme activity

A turbidometric assay using lyophilized Micrococcus lysodeikticus was used to determine lysozyme activity in serum (Ellis, 1990). 200 µl of *M. lysodeikticus* at a concentration of 0.2 mg ml/m1 (w/v) in 0.05 M sodium phosphate buffer (SPB), pH 6.2 was added to 10µl of serum sample. As a negative control, SPB was used instead of serum. The decrease in OD was recorded at 530 nm at 1 min and 6 min at room temperature. A unit of lysozyme activity was

defined as the amount causing a reduction in absorbance of 0.001 units per min. Results were expressed in units of lysozyme ml^{-1} serum.

Serum bactericidal activity

Bacterial cultures of *E. ictaluri* were centrifuged, and the pellet was washed and suspended in phosphate buffered saline (PBS). The optical density of the suspension was adjusted to 0.5 at 546 nm. This bacterial suspension was serially diluted (1:10) with PBS five times. The serum bactericidal activity was determined by incubating 2 µl of the diluted bacterial suspension with 20 µl of the serum in a micro-vial for 1 h at 37°C. PBS replaced the serum in the control group. The number of viable bacteria was determined by counting the colonies after culturing on trypticase soya agar (TSA) plates for 24 h at 30°C.

ESC Challenge

At the end of feeding trial, fish were challenged to evaluate the protection effect of *Bacillus* strains against *E. ictaluri* infection. At this time, with the exception of the control which had only 4 replicates remaining, each of the other treatment groups consisted of five replicates each containing 16 fish. Fish were challenged by immersion for 1 hour in 10 L of water containing 5.2×10^6 CFU/ml *E. ictaluri* strain S97-773. *Bacillus* feeding was continued for only one day post-challenge due to the cessation of feeding by the fish. Mortalities were monitored over a 21-day period, and dead fish were dissected and the presence of *E. ictaluri* confirmed by microbiological examination of kidney and liver swabs on TSA. The identity of the recovered *E. ictaluri* was confirmed by biochemical analysis.

Dosage experiment

Channel catfish fingerlings were randomly distributed to 24 tanks and each tank was stocked with 25 fish. The 24 tanks belong to two parallel systems with 12 tanks in each. Control feed and strain AP193 supplemented feed at doses of 4×10^6 CFU/g, 4×10^7 CFU/g or 4×10^8 CFU/g were formulated as described before. Each diet was assigned to 3 tanks in each system. Therefore, each treatment consists of six replicates in two blocks. After feeding for 14 days, Fish were challenged by immersion for 1 hour in 10 L of water containing 4.5×10^6 CFU/ml *E. ictaluri* S97-773. Mortality recording and identification of *E. ictaluri* from dead fish were same as described before.

Statistics

Data were presented as mean ± standard error (SE) and subjected to analysis of variance in SAS 9.2. Differences between means were tested by Tukey's range test and were considered significant when probability (P) values < 0.05 were obtained. Power analysis was conducted before the dose-effect experiment. Sample size for detecting the mortality difference between any two treatments as large as 10% with a power of 0.8 was calculated. Standard deviation of the mortality was assumed as 5% based on experience from challenges before.

4. Results

Growth performance

As shown in Table, dietary supplementation of *Bacillus* did not confer significantly enhanced growth performance in channel catfish as measured by weight gain and FCR.

However, all of the *Bacillus* feeding groups showed slightly better parameters compared with the control. The best performance parameters were observed in AP193 group, with weight gain and FCR as 6.78 ± 0.15 and 1.04 ± 0.03, respectively (Table 1).

Innate immune response

The serum lysozyme activity in fish fed with *Bacillus* AP143, AP193 and AB01 supplemented diets was higher compared with control fish (P >0.05). Similarly, higher respiratory burst activity was observed in all *Bacillus* feeding groups compared with control (P >0.05). *Bacillus* AB01 group showed highest lysozyme (212.0 ± 24.3 units ml^{-1}) and respiratory burst activity (0.067 ± 0.0041) (Table 2.). Also, serum bactericidal activity was not significantly affected by the *Bacillus* feeding, as shown in Table 3.

ESC Challenge

Cumulative mortality of each treatment after *E. ictaluri* challenge is presented in Table 4. Treatment AP193 gave the lowest mean mortality at the end of the study (49.3 ± 2.5), which was around 12% reduction in mortality compared with control (P = 0.07).

Dose effect

The cumulative mortality of channel catfish fed with different doses of AP193 is shown in Table 5. Mortality decreased gradually as the quantity of AP193 was increased in the diet. Cumulative mortality in the control group was 84.1%, whereas supplementation of AP193 decreased the mortality to 81.2%, 74.0% and 72.4% for doses of 4×10^6, 4 × 107, and 4×10^8 CFU/g of feed, respectively.

5. Discussion

The slightly enhanced weight gain and feed conversion ratio in the four *Bacillus* feeding groups suggests the potential for growth promoting properties of the *Bacillus* strains. More significant results were observed in studies on Indian major carp (Kumar et al., 2006), tilapia (Aly et al., 2008), white shrimp (Balcázar et al., 2007), large yellow croaker (Ai et al, 2011), koi carp (He et al., 2011), and sea cucumber (Zhang et al., 2010). Such an increase in the growth performance in fish fed with probiotic-supplemented diets, could be attributed to the improved digestive activity by enhancing the synthesis of vitamins, cofactors and enzymatic activity (Aly et al., 2008), with a consequent improvement of the digestion, nutrient absorption and weight gain. A positive correlation of the growth promotion efficiency and dose of *Bacillus subtilis* has been observed, where a dose of ~0.5×10^7 CFU/g gave marginal enhancement of growth performance, while an increase to ~1.5×10^7 CFU/g of the *Bacillus* feeding lead to significant difference in growth of fish compared with the control (Ai et al., 2011). A similar tendency was also reported by Kumar, within a dose range of 0.5×10^7 CFU/g to 1.5×10^7 CFU/g (Kumar et al., 2006). Although the dose we adopted (8×10^7 CFU/g) was already higher, a further increase of the dose or length of the feeding trial might lead to significant growth promotion by the *Bacillus* strains in channel catfish. However, beneficial effects on growth and feed utilization of dietary *B. subtilis* were not always observed. Günther and Jiménez-Montealegre (2004) reported a deteriorated effect of *Bacillus* feeding on the specific growth rate (SGR) and feed conversion ratio (FCR) in both tilapia and fresh water prawn under laboratory conditions.

Lysozyme is an important humoral innate defence parameter, and is widely distributed in invertebrates and vertebrates (Magnadóttir et al., 2005). It has bactericidal activity and can be an opsonin that activates the complement system and phagocytes (Jolles, 1984).Significantly

enhanced serum lysozyme activity in fish fed with probiotics was demonstrated in several studies (Irianto & Austin, 2002; Newaj-Fyzul et al., 2007; Kumar et al., 2008; Aly et al., 2008; Ai et al., 2011) As an important parameter of cellular innate immunity, respiratory burst activity reflects the level of highly microbiocidal reactive oxygen species (ROS), comprising superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl free radicals (OH^-), generated by fish phagocytic cells, i.e. monocytes, macrophages and neutrophils, after activation (Sharifuzzaman & Austin, 2009). Increased oxidative killing mechanism, as observed with blood superoxide anion and head kidney macrophage peroxidase activities, has been correlated with enhanced pathogen killing capacity of phagocytes in fish (Sharp & Secombes, 1993). Several studies have reported a stimulatory effect of some probiotics on respiratory burst activity and correlated it with the improvement of disease resistance (Brunt & Austin 2005, Aly et al., 2008, Kumar et al., 2008, Newaj-Fyzul et al., 2007). However, findings of respiratory burst activity following probiotics treatment in fish are often contradictory (Nayak, 2010). Also, an increase of the respiratory burst is not essential to enhance disease resistance, as indicated in research by Diaz-Rosales, where a probiotic strain with no significant increase effect on respiratory burst activity in fish conferred significantly better protection against *Photobacterium damselae* challenge compared with another probiotic strain which significantly enhanced the respiratory burst activity (Díaz-Rosales et al., 2009).

In our research, both the lysozyme and respiratory burst activity in *Bacillus* feeding groups were higher than control after 10 week feeding of the four *Bacillus* strains. However, the difference was not significant, indicating marginal stimulation of the two immunology parameters by *Bacillus* feeding, which might be attributed to dose and feeding duration. Alternatively, the lack of sensitivity of these assays and/or the degree of variability among fish

may not be sufficient to observe immunostimulatory effects. The correlation of immunostimulatory activities of probiotics with dose was demonstrated in several studies (Kumar et al., 2008; Panigrahi et al., 2004; Ai et al., 2011). Specifically, Panigrahi et al. (2004) reported that serum lysozyme and complement activities were significantly enhanced in rainbow trout fed with feed supplemented with *Lactobacillus rhamnosus* at 10^{11} CFU/g, while the group fed with the probiotic at 10^{9} CFU/g only showed marginal, insignificant increase. The dose of our *Bacillus* strains during the 10 weeks feeding (8×10^{7} CFU/g) was much lower than in Panigrahi's research, indicating a potential to increase the immune stimulation capacity of our *Bacillus* strains by trying higher doses. However, a high dose of 10^{11} CFU/g would not make economic sense for application of the probiotic in practice. Also, a dose at 10^{7} of some *Bacillus* probiotics strains showed stronger stimulation of the immune parameters in fish (Newaj-Fyzul et al., 2007; Ai et al., 2011), compared with the stimulation by the *Lactobacillus* strain at the dose of 10^{11} in Panigrahi's study (Panigrahi et al., 2004). This discrepancy might be attributed to different hosts or probiotic-host interaction. And the dose-performance characteristic reflects the efficiency of a candidate probiotic strain.

Stimulation of immune parameters may depend on duration of feeding. Sharifuzzaman and Austin (2009) reported that a two-week feeding regime led to more significant enhancement of cellular and humoral immune response and maximum mortality reduction in rainbow trout, as compared with three or four weeks of feeding. Similar tendency was also observed in other studies, where two to three weeks of feeding lead to most significant stimulation of innate immune parameters (Salinas et al., 2005; Panigrahi et al., 2005). Therefore, the marginal enhancement of the immune parameters in our study might be due to the long duration of feeding. However, in another experiment, three weeks feeding of AP79 and AP193 to channel

catfish didn't make any difference in terms of the enhancement of lysozyme and respiratory burst activity compared with control (data not shown).

Collectively, these results indicate that the protective effect of *Bacillus* strains against *E. ictaluri* infection is not primarily due to immune stimulation. For example, strain AB01 induced the highest lysozyme and respiratory activity while the observed mortality reduction was lowest in the fish fed with this strain. This suggests that the marginal stimulation of the two immune parameters by the *Bacillus* strains was not sufficient to trigger a considerable protection of channel catfish against pathogens and that the protective effect attributed to them, if there was any, was counteracted by other factors. This suggests that the primary mechanism of biocontrol by these probiotic *Bacillus* strains involves modulation of some other immune parameters or alternative modes of action rather than host immune stimulation.

The importance of dose was discussed above in terms of its effect on the efficiency of probiotics in growth promotion and immune stimulation. In this study, the dose effect was only investigated in terms of the protection efficacy of *Bacillus* strains against *E. ictaluri* infection in channel catfish. A lower dose at 10^6 CFU/g feed barely conferred any protection effect. The dose at 10^7 CFU/g gave a similar result as the experiments before, wherein a ~10% reduction in mortality was observed. A higher dose of 10^8 CFU/g led to a slightly lower mortality than the 10^7 CFU/g group, which was an almost negligible difference. This indicates that further increasing the bacterial dose from 10^7 CFU/g only gave marginal improvement in the protective effect, under the conditions evaluated. Considering the cost:benefit issue of probiotics, 10^7 CFU/g might be the optimal dose for our *Bacillus* strains.

A power analysis was conducted before the dose experiment in order to determine the number of replicates required to evaluate the biocontrol efficacy by the tested *Bacillus* strains. With the standard deviation of mortality assumed as 5%, a 10% mortality difference between groups should be detected with a power around 0.8 for the sample size of 6. However, the actual variance in mortality was much higher in the dosage experiment, as indicated by the pooled standard deviation (10%). Therefore, the mortality reduction conferred by the two higher levels of *Bacillus* doses was not detected statistically. By increasing the number of fish per tank which theoretically decreases the variance and using a larger sample size, a significant reduction of mortality by the *Bacillus* strain may be observed. In future studies, field trials are necessary to further evaluate the efficiency of selected *Bacillus* strains.

	Weight Gain	FCR
Control	6.25 ± 0.17^{a}	1.09 ± 0.01^{a}
AB01	6.64 ± 0.36^{a}	1.07 ± 0.04^{a}
AP193	6.78 ± 0.15^{a}	1.04 ± 0.03^{a}
AP143	6.32 ± 0.25^{a}	1.09 ± 0.02^{a}
AP79	6.58 ± 0.18^{a}	1.04 ± 0.01^{a}

Table 1. Growth performance (means ± SE) of channel catfish fed with different experimental diets for ten weeks. Means in the same column sharing a common superscript letter were not significantly different ($P > 0.05$).

Treatment	Lysozyme (units ml^{-1})	Respiratory burst (OD_{650})
Control	166.7 ± 18.3^{a}	0.057 ± 0.008^{a}
AP79	165.3 ± 6.5^{a}	0.063 ± 0.0054^{a}
AP143	184.0 ± 10.9^{a}	0.062 ± 0.0027^{a}
AP193	169.3 ± 10.0^{a}	0.064 ± 0.0025^{a}
AB01	212.0 ± 24.3^{a}	0.067 ± 0.0041^{a}

Table 2. Immune response (means ± SE) of channel catfish fed with different experimental diets for ten weeks. Means in the same column sharing a common superscript letter were not significantly different ($P > 0.05$)

Treatment	No. of bacterial colonies
Control	489 ± 23.6^{a}
AP79	496 ± 19.5^{a}
AP143	486 ± 20.4^{a}
AP193	478 ± 23.7^{a}
AB01	487 ± 21.5^{a}
PBS (control)	600 ± 25.0

Table 3. Effect of *Bacillus* feeding on serum bactericidal activity of channel catfish

Treatment	Mortality (%)
Control	62.1 ± 9.0^{a}
AP79	56.2 ± 4.8^{a}
AP143	65.2 ± 4.8^{a}
AP193	49.3 ± 2.5^{a}
AB01	69.3 ± 3.6^{a}

Table 4. Cumulative mortality of channel catfish fed with different diets for 10 weeks and challenged by *E. ictaluri*. Means in the same column sharing a common superscript letter were not significantly different ($P > 0.05$).

Treatment	Control	AP193_4 × 10^{6}	AP193_4 × 10^{7}	AP193_4 × 10^{8}
Mortality (%)	84.1 ± 3.1^{a}	81.2 ± 4.3^{a}	74.0 ± 4.4^{a}	72.4 ± 4.8^{a}

Table 5. Cumulative mortality of channel catfish fed with control feed or feed supplemented with three doses of AP193 for two weeks and challenged by *E. ictaluri*. Means in the same row sharing the common superscript letter were not significantly different ($P > 0.05$)

Chapter IV

Gene clusters for synthesis of secondary metabolites in AP193 and compounds responsible for the *in vitro* antagonism of AP193 against pathogens

1. Abstract

The genome of AP193 was generated using next-generation sequencing. Genome analysis identified gene clusters for the production of polyketides (bacillaene, macrolactin and difficidin), lipopeptiedes (surfactin, bacillomycin D and fengycin) and a dipeptide antibiotic bacilycin. The recalcitrance of AP193 to uptake exogenous DNA made it difficult to construct mutants deficient in synthesis of specific antibiotics to determine their relative importance in the biocontrol efficacy of AP193 against pathogens. Low efficiency transformation of AP193 was accomplished by isolation of plasmid from an *E. coli* ER2925 (*dam dcm*) strain followed by *in vitro* methylation of plasmid with cell–free extracts of AP193. A gene knock-out procedure was used that involved a temperature-sensitive shuttle plasmid vector based on pWV01 origin of replication, a two-step replacement recombination procedure and PCR screening of mutants to construct marker-free deletion mutants deficient in production of specific antibiotic(s). By agar diffusion assay of the mutants, the secondary metabolite responsible for much of the *in vitro* antagonism of AP193 against *E. ictaluri* and *A. hydrophila* was identified as the polyketide

difficidin. This research has laid the foundation for future studies to understand the contribution of secondary metabolites production to the *in vivo* biocontrol effect of strain AP193.

2. Introduction

The *in vitro* antagonism against pathogens of the considered aquatic animals has often been used in the selection of putative probiotic strains. Typically, a correlation is made between the *in vitro* ability of the probiotics to inhibit pathogens and the *in vivo* protection of the cultured aquatic species. However, in none of the studies published so far has it been shown unequivocally that the production of inhibitory compounds is the cause of the observed *in vivo* probiotic activity of the strains (Verschuere et al., 2000a). In the field of biocontrol of plant diseases, the contribution of specific secondary metabolite(s) to their biocontrol efficacy has been determined. This was accomplished by 1) characterizing the nature of the compound(s) responsible for *in vitro* antagonism, followed by 2) construction of mutants deficient in production of those responsible metabolite(s), and lastly 3) comparing the *in vivo* biocontrol effect of the mutants with wild-type strains (Asaka & Shoda, 1996; Chen et al., 2009a).

Bacillus sp. AP193 showed protection effect against *E. ictaluri* in channel catfish and *A. hydrophila* in tilapia (data not shown) and was selected for further characterization as a potential probiotic in aquaculture. Whole genome sequencing was conducted on strain AP193 using 454 pyrosequencing. Genome analysis revealed that AP193 is closely related to the plant-associated strain *Bacillus amyloliquefaciens* FZB42, which can stimulate plant growth and suppress soil-borne plant pathogens as a result of its ability to produce antifungal and antibacterial metabolites (e.g., lipopeptides and polyketides) (Chen, 2007).

Construction of mutants deficient in the production of specific secondary metabolites is essential in order to determine their relative contribution to the *in vitro* antagonism and *in vivo* protection effect of the probiotic strain. Genetic transformation is the crucial step for genetic manipulation of bacteria. Although some *B. amyloliquefaciens* strains like FZB42 can develop natural competence as found in *B. subtilis* (Coukoulis & Campbell, 1971; Chen et al., 2007), allowing automatic incorporation of DNA and subsequent integration into their chromosomes at a high frequency, many other strains from this species are not naturally competent and some *B. amyloliquefaciens* strains are extremely recalcitrant to exogenous DNA (Zhang et al., 2011; He et al., 2012). *Bacillus* sp. AP193 is unfortunately not naturally competent. Electroporation was tried repeatedly to introduce plasmids into AP193, and every attempt to increase the electroporation efficacy of AP193 was unsuccessful. The low transformation efficiency makes it impossible to construct mutants by introducing suicide vectors or linearized DNA into the bacterial cells. To circumvent the limitation of low transformation efficiency, we used a gene knock-out procedure that involves a temperature sensitive shuttle plasmid vector based on the pWV01 origin of replication, a two-step replacement recombination procedure and PCR screening of the resulting clones for the desired mutation.

In this chapter, the gene clusters in AP193 responsible for the synthesis of secondary metabolites were analyzed. Mutants deficient in production of secondary metabolites were constructed. The contribution of these secondary metabolites to the *in vitro* antagonism of AP193 against *E. ictaluri* and *A. hydrophila* were investigated agar diffusion test of the mutants and wildtype strain. The work in this chapter will enable future studies to understand the contribution of secondary metabolites production to the *in vivo* biocontrol effect of AP193.

3. Methods and materials

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *dam, dcm Escherichia coli* K12 ER2925 (New England Biolabs) was used to prepare unmethylated pladmids. *E. coli and Bacillus* cells were routinely grown in Luria-Bertani (LB) medium. For electro-competent cell preparation, *Bacillus* was grown in NCM medium (17.4 g K_2HPO_4, 11.6 g NaCl, 5 g glucose, 5 g tryptone, 1 g yeast extract, 0.3 g trisodium citrate, 0.05 g $MgSO_4 \cdot 7H_2O$ and 91.1 g sorbitol in 1 L deionized water, pH 7.2). For production of secondary metabolites, *Bacillus* cultures were grown for 48h at 30 °C in Tryptic Soy broth (TSB). When required, ampicillin (100 µg/ml), chloramphenicol (12.5 µg/ml) or erythromycin (200 µg/ml for *E. coli* or 5µg/ml for *Bacillus*) were also added to the medium. Solid medium was obtained by adding 15g/l agar to the liquid medium.

Genome sequencing and prediction of gene clusters devoting to synthesis of secondary metabolites

The genome of *Bacillus* strain AP193 was sequenced by bar-coded 454 pyrosequencing. Genomic DNA was isolated by the use of lysozyme treatment followed by alkaline lysis and isopropanol precipitation, and the genomic DNA was sent to the Lucigen Corporation (Middleton, WI) for bar-coded genomic sub-library construction. 454 pyrosequencing was conducted at Engencore using the Genome Sequencer FLX. The reads were trimmed for quality at a setting of 0.01 and then *de novo* assembled using CLCBio Genomics Workbench (version 4.9). Contigs were then exported as a FASTA format. Gene clusters encoding secondary metabolites were located by using the secondary metabolite identification tool antiSMASH (http://antismash.secondarymetabolites.org/). PCR amplification as well as primer-walking was

used to fill the gaps between contigs containing gene clusters encoding secondary metabolite biosynhtesis. Gene prediction and annotation were carried out by GeneMark (Lukashin & Borodovsky, 1998) and BLASTx (NCBI), respectively.

DNA manipulation and plasmid construction

Chromosomal DNA was isolated with E.Z.N.A. Bacterial DNA Isolation Kit (Omega Biotek, Atlanta, GA). All plasmids were isolated with E.Z.N.A. Plasmids Mini Kit II (Omega Biotek). All of the primers used are listed in Table 2. The gene knock-out construct was assembled by two rounds of PCR using *Ex Taq* polymerase (Takara). In the first round of PCR, the upstream and downstream fragments of the targeted deletion sequence were PCR amplified and both reactions were purified using the Gel/PCR DNA Fragments Extraction Kit (IBI). The forward primer for the downstream fragment contained a 20 bp sequence complementary to the 3' end of the upstream fragment (Table 2). The two outer primers contained appropriate restriction sites for cloning. The two fragments were assembled as the gene knock-out construct in the second round PCR in a 50 μl system (0.5 μl cleaned product of the first round PCR for each fragment, the two outer primers at 0.4 μM, 1 × *Ex Taq* Buffer, 0.2mM dNTPs, 1.25 U Ex Taq polymerase). The assembled product was gel purified with Gel/PCR DNA Fragments Extraction Kit (IBI), digested with appropriate restriction enzymes and cloned to pNZT1 to construct the delivery plasmids for gene replacement.

***In vitro* methylation of plasmids**

To methylate plasmids before transformation of AP193, the method developed for *Lactobacillus plantarum* (Alegre et al., 2004) was used, with some modifications. Cells from 100 ml of an overnight culture of *Bacillus* sp. AP193 (OD_{600} = 1.3-1.5) were pelleted by

centrifugation (8000*g*), washed with 100 ml of chilled PENP buffer (10 mM potassium phosphate, 10 mM EDTA, 50 mM NaCl and 0.2 mM PMSF, pH 7.0), and then re-suspended in the same buffer to a final volume of 4 ml. The cells were disrupted by performing two bursts of 5 min (amplitude 50, pluse 3, 25-30 W) in a Vibra-Cell sonicator with a pause of 2 min. The sample was maintained on ice during disruption to prevent overheating. Cell debris was pelleted by centrifugation (8000*g*) at 4 °C. The extract was decanted and 3 ml aliquots of extract were mixed with 3 ml of glycerol (100% v/v) and 0.6 ml of BSA (1 mg/ml), and stored at -20°C.

The DNA modification assay included in a final volume of 100 µl of the following: 53 µl TNE buffer [50 mM Tris (pH 7.5), 50 mM NaCl, 10 mM EDTA]; 10 µl Sadenosylmethionine (0.8 mM); 2 µl BSA (5 mg ml_1); 25 µl extract; and 10 µl plasmid DNA extracted from *E. coli* K12 ER2925 (0.5-1 µg/ µl). The mixture was incubated at 37°C for 16 h. Methylated DNA was extracted with a Zymo Research DNA Clean & Concentrator Kit, re-suspended in water and stored at -20°C.

Electrotransformation

An overnight cell culture grown in TSB medium was diluted 100-fold in NCM medium for preparation of electro-competent cells. The culture was grown at 37°C on a rotary shaker until OD_{600} reached 0.7. The cell culture was cooled on ice for 15 min, and collected by centrifugation at 4 °C, 8000*g* for 5min. After washing four times with ice cold ETM buffer (0.5 M sorbitol, 0.5 M mannitol, and 10% glycerol), the electro-competent cells were re-suspended in 1/100 vol of the original culture (Zhang et al., 2011). During electrotransformation, the cells were mixed with 100 ng of plasmid DNA in an ice-cold electroporation cuvette (1 mm electrode gap). After 1 min incubation, the cells were exposed to a single 21 kV/cm pulse generated by

Gene-Pulser (Bio-Rad Laboratories), with the resistance and capacitance set as 200 Ω and 3μF, respectively. The cells were immediately diluted into 1 ml of recovery medium (NCM plus 0.38M mannitol) (Zhang et al., 2011) and shaken gently at 37°C or 30°C for 3 h to allow expression of the antibiotic resistant genes. Aliquots of the recovery culture were then spread onto LB agar plates supplemented with appropriate antibiotics. Transformation efficiencies were calculated by counting the colonies on plates and designated as CFU/μg DNA.

Two-step replacement recombination procedure

The two-step replacement recombination procedure was performed as previously described (Zakataeva et al., 2010) with some modifications. In the first step, *Bacillus* sp. AP193 clone bearing a delivery plasmid that contained the gene knock-out construct was cultivated in LB at 37°C (a nonpermissive temperature for plasmid replication) for 24h. This was done to initiate integration of the entire plasmid into the chromosome via a single crossover between the target gene and a homologous sequence on the plasmid. The culture was then serially diluted, plated on LB with erythromycin and incubated at 37°C. Clones were screened by colony PCR using two sets of primers. Each set of primers combines primers located on one of the homology fragments and on the chromosome region just outside of the other homology fragment (Table 2). PCR product with reduced size for either primer sets indicates successful integration of the plasmid into the chromosome. In the second step, a separate clone of the integrant was cultivated with aeration in LB at 30°C for 24 - 48 h in order to initiate the second single-crossover event and the excision of the plasmid, which yielded erythromycin sensitive (EmS) clones with either a parental or a mutant allele on the chromosome (Figure 3.B). Colony PCR analysis was used to examine the presence of desired mutation by primer sets flanking the deleted sequence (Table 2). Deletions were detected by size reduction in the PCR products.

Colony PCR analysis

A small volume of cells was scraped from a colony and introduced into a 25 µl PCR reaction mixture (1 × *Taq* Mastermix (Lucigen), 0.4 µM primers). The amplification program consisted of 4 min at 94 °C, 35 cycles of 20 s at 94 °C, 30 s at an appropriate annealing temperature, 1 min at 72 °C for every 1 kb of amplified product, followed by a 5 min stage at 72 °C. The PCR product was analyzed by electrophoresis in a 0.8% (*w/v*) agarose gel.

Construction of mutants defective in secondary metabolites synthesis

A summary of all the mutant strains and plasmids used in this study is given in Table 1. To generate *sfp* mutant 93M1 (Δ*sfp*), DNA fragments corresponding to positions -781 to +29 with respect to the *sfp* translation initiation site and +95 to + 935 with respect to *sfp* translation termination site were PCR amplified using AP193 chromosome DNA as template, assembled by fusion PCR, digested with HindIII and PstI and cloned to pNZT1 to construct pNZ-sfp. *In vitro* modified pNZ-sfp was transformed into AP193. Mutant 93M1 was obtained by the two-step replacement recombination procedure as described above.

Mutant 93M2 (Δ*srfAA*) was obtained as follows. DNA fragments corresponding to positions +5375 to +6091 and +6627 to +7366 with respect to *srfAA* translation initiation site were PCR-amplified, fused by fusion PCR, digested with HindIII and PstI and cloned into pNZT1 as pNZ-srf. A frameshift mutation was introduced during the fusion of the upstream and downstream fragments of the target deletion sequence to make sure a complete disruption of the gene function. *In vitro* modified pNZ-srf was transformed into AP193. Mutant 93M2 was generated by a two-step replacement recombination procedure as above described.

Gene disruption of the *dfnD* gene was achieved as follows. DNA fragments corresponding to positions -867 to +247 and +643 to +1570 with respect to the *dfnD* translation initiation site were PCR amplified using AP193 chromosome DNA as template. The two fragments were assembled by fusion PCR. Similarly, a frameshift mutation was introduced during the fusion to make sure a complete disruption of the gene function. The assembled knock-out construct was digested with XhoI and SpeI and cloned to pNZT1, yielding pNZ-dif. The plasmid was introduced into AP193 followed by the recombination procedure to generate mutant 93M3 (Δ*dfnD*)

Agar diffusion test

Pathogens *E. ictaluri* S97 or *A. hydrophila* M-09-119 were grown in TSB till the OD_{600} reached 1.0. Soft agar (1% *w/v* agar) prepared with TSB were melted, cooled, and seeded with the pathogen broth culture as 1:40 dilution. The bacteria suspension in soft agar was poured immediately on an OmniTray plate (Thermo Scientific, NY). The supernatants of the *Bacillus* culture were loaded in round wells (approx. 10mm in diameter) made on the agar and dried at room temperature. The plates were incubated overnight at 30 °C. Zones of inhibition were compared between mutants and wildtype strain.

4. Results

Gene clusters devoting to secondary metabolites

De novo assembly of AP193 sequences gave 38 contigs larger than 1 kb, with the summed length as 3990509 bp. Gaps between contigs 5 and 6, contigs 33 and 38, as well as

contigs 27 and 28 were filled. Analysis of the DNA sequence data using the antiSMASH secondary metabolite prediction program suggests that AP193 encoded gene clusters responsible for synthesis of three polyketides, which were bacillaene, macrolactin, and difficidin. Each of the three gene clusters in AP193 was collinear to its counterpart in *Bacillus amyloliquefaciens* FZB42 and the identity on amino acid level ranged between 98% and 100% as compared with FZB42 (Figure 2; Table 7, 8, 9) . Large gene clusters homologous to the gene clusters involved in nonribosomal synthesis of cyclic lipopeptides surfactin, fengycin and bacillomycin D, and of antimicrobial dipeptide bacilysin in FZB42 were also detected in the AP193 genome (Figure 1). Identity on amino acid level was from 98% to100% (Table 3, 4, 5, 6). Collinear organization was also observed between these gene clusters in AP193 and FZB42. However, the *ycxB, ycxC* and *ycxD* genes downstream of the surfactin producing *srf* operon were missing in AP193. Thus, the *sfp* and *yczE* genes, which are essential genes for non-ribosomal synthesis of lipopeptides and polyketides, were directly adjacent to the *srfAD* and *att* gene (Figure 1.a).

Efficiency of electroporation

Electrocompetent cells of AP193 were electroporated with modified or non-modified pMK4 isolated from *E. coli* DH5a or *E. coli* K12 ER2925. Non-modified pMK4 isolated from *E. coli* did not generate transformants in AP193. Transformants could not be obtained by *in vitro* modified plasmid from *E. coli* DH5a. *In vitro* modified pMK4 from *E. coli* ER2925 gave rise to transformants at an efficiency of 5×10^2 transformants per microgram of plasmid.

Two-step replacement recombination procedure

pNZT1 (Zakataeva et al., 2010), the shuttle vector we used to deliver target modifications, is based on the pG^+host replicon, a replication-thermosensitive derivative of the

rolling-circle plasmid pWV01 (Maguin et al., 1996). The plasmid replicates in both *E. coli* and *Bacillus* strains at temperatures at or below 30°C, permits plasmid selection on erythromycin in both types of bacteria, and contains a muticloning site for cloning of DNA fragments (Figure 3.A). The plasmid is replication-thermosensitive and readily lost at temperatures of ≥ 37°C, making it well suited as a delivery vector to aid knock-out of genes in *Bacillus* strains with low transformation efficiency (Zakataeva et al., 2010)

Replacement recombination proceeded in the plasmid-bearing strain via two subsequent single crossovers (Figure 3.B). The frequency of integration of the entire plasmid into the chromosome was at 10^{-2} level. The frequency of the second single crossover, which was estimated as percentage of EmS cells, was from 20% to 70%. The resulting plasmid-less clones were screened for the desired mutation using colony PCR analysis. Size reduction in the PCR products, indicating successful deletion of the target sequence, was detected in all the mutant strains as compared with the wild type (Figure 4).

In vitro antagonism of AP193 and mutants against *E. ictaluri* and *A. hydrophila*

The following mutants were used to investigate the contribution of specific compounds to the *in vitro* antagonism of AP193 against *E. ictaluri* and *A. hydrophila*: 93M2 (Δ*srfAA*), deficient in surfactin production, 93M3 (Δ*dfnD*), deficient in the production of polyketide difficidin and 93M1 (Δ*sfp*), unable to produce any polyketide or lipopepetide due to a deletion introduced into the gene encoding 4'-phosphopantetheinyl transferase, SfP, an enzyme catalysing a necessary processing step in non-ribosomal synthesis of lipopeptides and polyketides. The mutant 93M1 (Δ*sfp*) was completely devoid of inhibition effect against both pathogens, underlining the contribution of lipopeptides and polyketides to the antagonism of AP193. This

also indicated that the dipeptide bacilysin, whose synthesis was independent of Sfp, was not involved in the antagonistic activity observed here. Mutant 93M2 (Δ*srfAA*) led to reduced inhibition effect compared with the wildtype for both *E. ictaluri* and *A. hydrophila*, indicating surfactin as a minor contribution to the antibacterial activity of AP193. Difficidin acted as the major antibiotic in the antagonism of AP193 against both *E. ictaluri* and *A. hydropila*, as proved by the drastic reduction of the inhibition effect in mutant 93M3. Unexpectedly, the inhibitory effect of 93M3 against *E. ictaluri* was completely lost (Figure 5), which was surprising given that surfactin also contributed to the antagonism of AP193 against this pathogen, as indicated above by the reduced activity of mutant 93M2. This may suggest a synergistic action of the two compounds against the growth of *E. ictaluri*, where surfactin functions as a facilitator to the activity of difficidin.

5. Discussion

Multiple mechanisms including production of inhibitory compounds, competition for iron and other nutrients, enhancement of immune response and competition of adhesion sites have been suggested to contribute to the protection effect of probiotics for aquatic animals against pathogens (Verschuere et al., 2000a). Typically, the *in vitro* production of inhibitory compounds against specific pathogens has been used as a criterion for selection of putative probiotics. However, the contribution of inhibitory compounds production to the *in vivo* protection effect of the probiotics has never been elucidated. Even the nature of the responsible compounds for the *in vitro* antagonism of the probiotics against pathogens of aquatic animals has rarely been clarified. In this study, as an initiative to elucidate the contribution of inhibitory compounds to the

protection effect of probiotics, the contribution of specific secondary metabolites expressed by the probiotic candidate *Bacillus* AP193 against catfish pathogens *E. ictaluri* and *A. hydrophila* were uncovered by genome sequence analysis, construction of mutants deficient in production of specific secondary metabolites and comparison of the *in vitro* antagonism of the mutants with the wildtype strain.

Genetic transformation is the crucial step for genetic manipulation of bacteria. However, for many *Bacillus* species, specifically for some *B. amyloliquefaciens* strains, plasmid transfer remains a limiting factor for genetic manipulations. Electroporation protocols combining high osmolarity and high voltage have been reported to be efficient in introducing syngeneic plasmids into *Bacillus* strains (Xue et al., 1999). Electroporation of AP193 with pC194 isolated from *Bacillus subtilis* 168 by the high osmolarity method yielded transformants at an efficiency around 10^4 CFU/ ug of plasmid. However, integration and shuttle plasmids are usually constructed in *E. coli*. The restriction enzyme in some *Bacillus* strains may digest and degrade xenogeneic DNA from *E. coli*. Specifically, the transformation efficiency of plasmids into *Bacillus* depends on the methylation capacity of the *E. coli* strain used for plasmid construction. Plasmids isolated from Dam^- Dcm^- *E. coli* strain transform *Bacillus* more efficiently or exclusively, as opposed to Dam^+ Dcm^+ *E. coli* strains. (Marrero & Welkos, 1995; Macaluso & Mettus, 1991). However, unmethylated plasmids extracted from *E. coli* ER2925 (*dam dcm*) were not able to transform AP193 in our study. *In vitro* methylation of plasmids by cell-free extract of the host was an efficient method to circumvent the restriction system during transformation (John et al., 2000; Alegre et al, 2004). By *in vitro* modification of plasmids isolated from *dam dcm E.coli* strain, we successfully electroporated large shuttle plasmids into AP193. However, the efficiency was relatively low (5×10^2 transformants per ug DNA), which was probably due

to the low efficiency of methylation by the cell-free extract. The efficiency may be improved by *in vitro* modification of plasmids using specific DNA methyltransferases or *in vivo* methylation in *E. coli* host expressing corresponding DNA methyltransferases (Yasui et al, 2009), which requires thorough knowledge of the restriction modification system of the host, on which we may continue to work. We found that electroporation with high voltage at the capacitance of 25 μF was very sensitive to the salinity of the electrocompetent cell suspension, making the transformation efficiency highly variable for different batches of cells, and no transformant was obtainable when the time constant was below 4.2. Interestingly, electroporation at low capacitance of 3 μF gives comparable and more constant efficiency, making it well suited for cases where high transformation efficiency was not required.

Construction of mutants deficient in the production of specific secondary metabolites is essential for validation of their contribution to the *in vitro* antagonism and *in vivo* protection effect of the probiotic strain. Gene knock-out methods for naturally transformable *B. subtilis* and *B. amyloliquefaciens* strains have been well established (Fabret et al., 2002; Zhang et al., 2006; Yan et al., 2008; Liu et al., 2008; Koumoutsi et al., 2004). These methods typically utilize linearized plasmids or PCR fragments to deliver a target modification to the chromosome via double homologous recombination, which requires high transformation efficiencies. Relatively fewer publications are devoted to developing methods for the genetic manipulation of naturally nontransformable *Bacillus* strains (Zakataeva et al., 2010; Shatalin & Neyfakh, 2005). Thermosensitive plasmids based on the thermosentive-derivative of rolling cycle plasmid pWV01 were exploited in both studies. The use of such replicons allows one to separate the steps of plasmid introduction and chromosome recombination, thereby overcoming the relatively low efficiencies of genetic transfer in naturally nontransformable *Bacillus* strains. Furthermore, after

integration of the plasmid into chromosome though the first homologous recombination and incubation of the integrant at 30°C (the temperature supportive for replication) the frequency of the second homologous recombination and the excision of the plasmid increases by two to three orders of magnitude. This leads to a high ratio of clones with the desired mutation (0.1 to 0.5), which may avoid the incorporation of a counterselectable marker and allows screening of the mutants directly by PCR. pNZT1 (Zakataeva et al., 2010), the plasmid we used as delivery vector for deletion of the target gene sequence through replacement recombination, proved to be effective in AP193. The only problem we encountered was that curing of the free pNZT1 from AP193 cells was not complete during the screening of the first single crossover event at 37°C, the nonpermissive temperature for plasmid replication. Complete curing of pWV01-derived thermosensitive plasmids at 37°C was reported in a former study (Zakataeva et al., 2010; Shatalin & Neyfakh, 2005). Nevertheless, clones with successful integration of the whole plasmid into the chromosome were screened by colony PCR using flanking and internal primer sets as described above. The frequency of positive integration in the clones on the LB plate containing erythromycin after incubation at 37°C was from 1% to 10%. Further increasing of the incubation temperature might increase the efficiency of plasmid curing. Consistent with results in former study, the frequency of the second crossover event at 30°C was very high, giving rise to around 50% of mutants for some loci. ×

The collinearity and high amino acid identity of gene clusters devoting to secondary metabolite production in AP193 as compared with the type strain FZB42 indicated high similarity of the two strains. However, a gene loss event was detected in AP193. Three genes downstream of the surfactin producing *srf* operon with unknown function, *ycxB*, *ycxC* and *ycxD*, were completely missing in AP193. Those genes were considered as members of the gene cluster

for surfactin synthesis (Chen et al., 2009b). As production of surfactin and other secondary metabolites were observed in AP193, the three genes can be considered as non-essential in the secondary metabolite synthesis.

Difficidin, first detected in culture broth of two *B. subtilis* strains, are highly unsaturated 22-membered macrocylic polyene lactone phosphate esters with broad-spectrum antibacterial activity (Zimmerman et al., 1987). Its molecular structure has been resolved (Wilson et al., 1987) and the *dif* gene sequences reflect a reasonable synthesis pathway (Chen et al., 2006). Difficidin was demonstrated as one of the major compounds responsible for biocontrol activity of FZB42 against fire blight disease of orchard trees caused by *Erwinia amylovora* (Chen et al., 2009a). Consistently, the major part of antagonism activity of AP193 against both *E. ictaluri* and *A. hydrophila* was also attributed to difficidin in our study, underlining its importance in the antibacterial activity of these closely related *Bacillus amyloliquefaciens* strains. As mentioned above, Sfp is a substrate-unspecific PPTase which plays an essential role in priming polyketide synthases, NRPS, and siderophore synthetases by covalently converting serine residues in acyl carrier protein (ACP), peptidyl carrier protein (PCP), or aryl carrier protein (ArCP) domains from inactive apo forms to active holo forms. The deletion of *sfp* gene in FZB42 led to complete loss of lipopeptides and polyketides production (Chen et al., 2006). However, slight antibacterial activity was still observed in FZB42 Δ*sfp* mutant (Chen et al., 2006), which was attributed to the dipeptide bacilysin (Chen et al., 2007). Besides difficidin, bacilysin was demonstrated as another major compound responsible for the biocontrol activity of FZB42 against fire blight disease of orchard trees caused by *E. amylovora* (Chen et al., 2009a). However, in our study, the AP193 Δ*sfp* mutant was completely devoid of any antibacterial activity against both *E. ictaluri* and *A. hydrophila*, suggesting that bacilysin does not contribute to the antagonism of AP193 *in vitro*.

The production of bacilysin by AP193 in TSB can be proved by slight inhibition activity exhibited by AP193 Δ*sfp* mutant when *B. megaterium* was used as the indicator bacterium in the agar diffusion test (data not shown). Therefore, the complete loss of activity in AP193 Δ*sfp* mutant against *E. ictaluri* and *A. hydrophila* can either be due to the unsusceptibility of the two pathogens for bacilysin or that the amount of bacilysin produced in our condition was too low to be detected in the agar diffusion assay. The influence of medium on the amount and profiles of isomers for lipopeptide production has been described (Eric et al., 2001). Specifically, bacilysin production was more efficient in GA-medium compared with Landy medium, the medium for lipopeptides and polyketides production (Chen et al., 2009a). The contribution of bacilysin to the antagonism of AP193 against *E. ictaluri* and *A. hydrophila* may be observed in other medium. Surfactin is a heptapeptide with an LLDLLDL chiral sequence linked by a β-hydroxy fatty acid consisting of 13-15 carbon atoms to form a cyclic lactone ring structure. Surfactin is surface active and acts hemolytic, antimicrobial and antiviral by altering membrane integrity (Peypoux et al, 1999). A slight contribution of surfactin to the antibacterial activity of FZB42 was described (Chen et al., 2006). Consistent with the former research, AP193 mutant deficient in surfactin synthesis showed reduced inhibition against *E. ictaluri* and *A. hydrophila*. However, to our surprise, the surfactin activity against *E. ictaluri* was not observed in AP193 Δ*dfnD* mutant. This might be due to a synergistic action of surfactin and difficidin in the repression of *E. ictaluri*, where surfactin facilitates the antibacterial activity of difficidin, and does not have the capability to inhibit growth of *E. ictaluri* by itself. Synergy of multiple secondary metabolites is prevalent in *Streptomyces* species and has been interpreted as driving forces for the evolution of multiple secondary metabolites production (Challis & Hopwood, 2003). As an uncommon phenomenon in *Bacillus* species, synergistic action of fengycin and bacillomycin D was

observed in FZB42 against *Fusarium oxysporum* (Koumoutsi et al., 2004). However, further studies are required to validate our hypothesis.

Notably, the relative importance of each antibacterial compound in the biocontrol activity of AP193 against the pathogens may not be concluded from the *in vitro* test. As mentioned above, the medium and conditions can influence the amount of secondary metabolites. The *in vivo* environment may present different conditions that can change the profile of secondary metabolite production of the biocontrol strains. As an example, the contribution of bacilysin to the *in vitro* repression of FZB42 against *E. amylovora* was incomparable with difficidin, even for cultures in the GA-medium, which was specifically selected for bacilysin synthesis. However, a mutant strain blocked in the production of difficidin inhibited growth of *E. amylovora* and suppressed fire blight disease nearly in the same range as the wild type, indicating the importance of bacilysin for the biocontrol activity (Chen et al., 2009a).

Research in this study established a strong foundation to investigate the contribution of secondary metabolite(s) for the biocontrol activity of AP193 in channel catfish. The comparison of protection effect of AP193 mutants devoid in the synthesis of secondary metabolite(s) and the wildtype on channel catfish challenged with a pathogen may answer this question. Moreover, the specific compounds responsible for the *in vivo* protection effect can be identified, which may facilitate strategies to improve the biocontrol activity of probiotic strains such as *Bacillus* AP193.

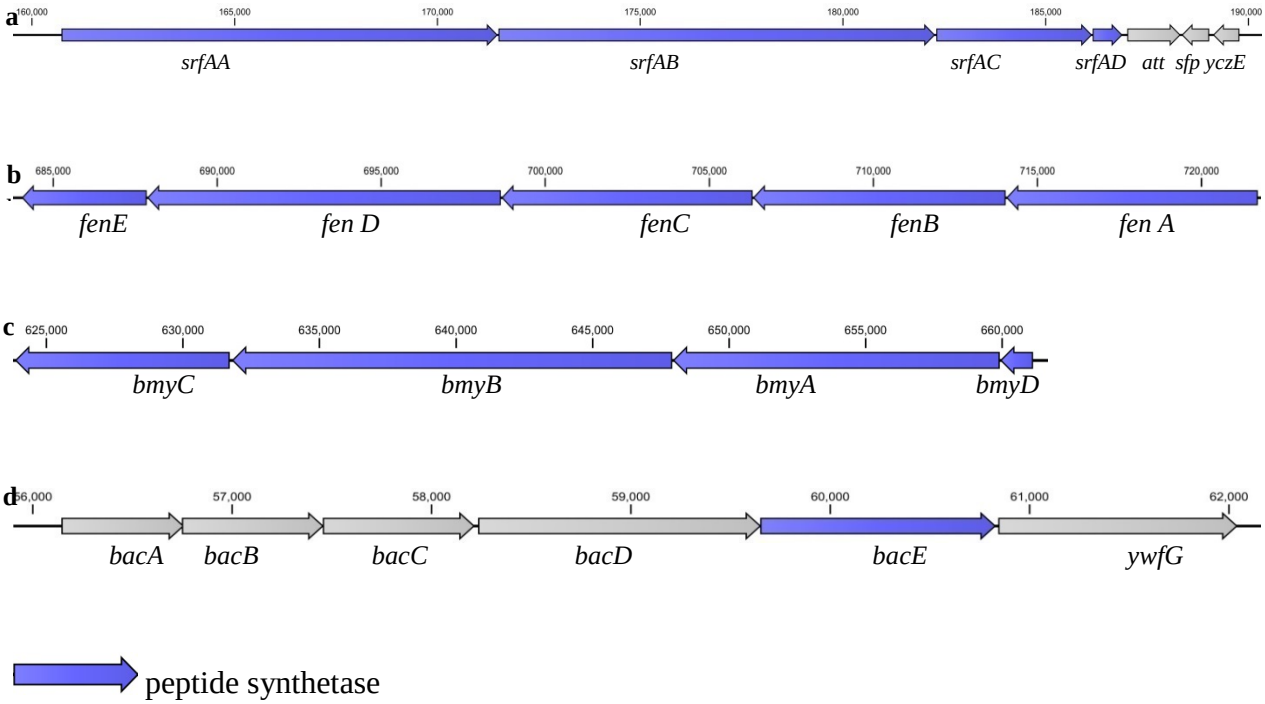


Figure 1. Gene clusters in AP193 involved in the non-ribosomal synthesis of surfactin (a), fengycin (b), bacillomycin D (c) and bacilysin (d).

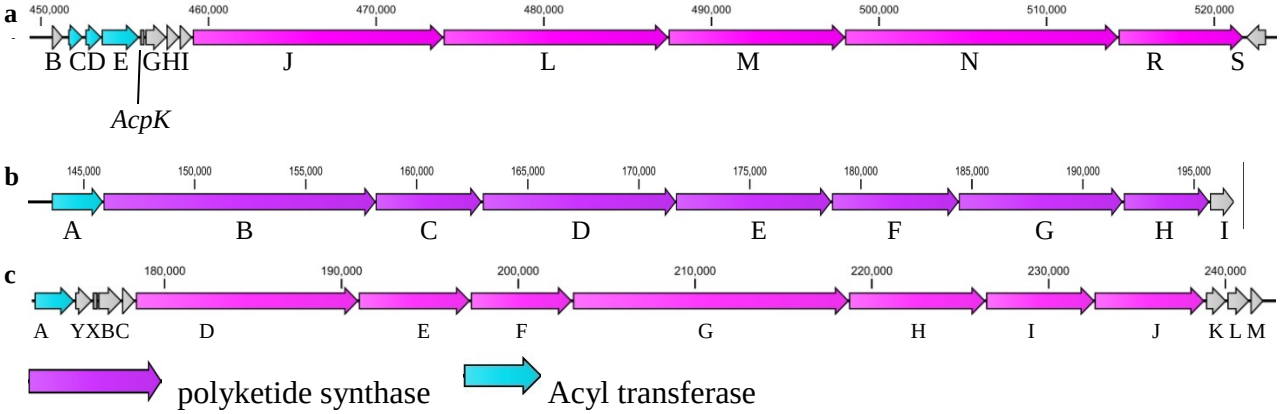


Figure 2.Gene clusters in AP193 involved in the synthesis of polyketides bacillaene (a), macrolactin (b) and difficidin (c).

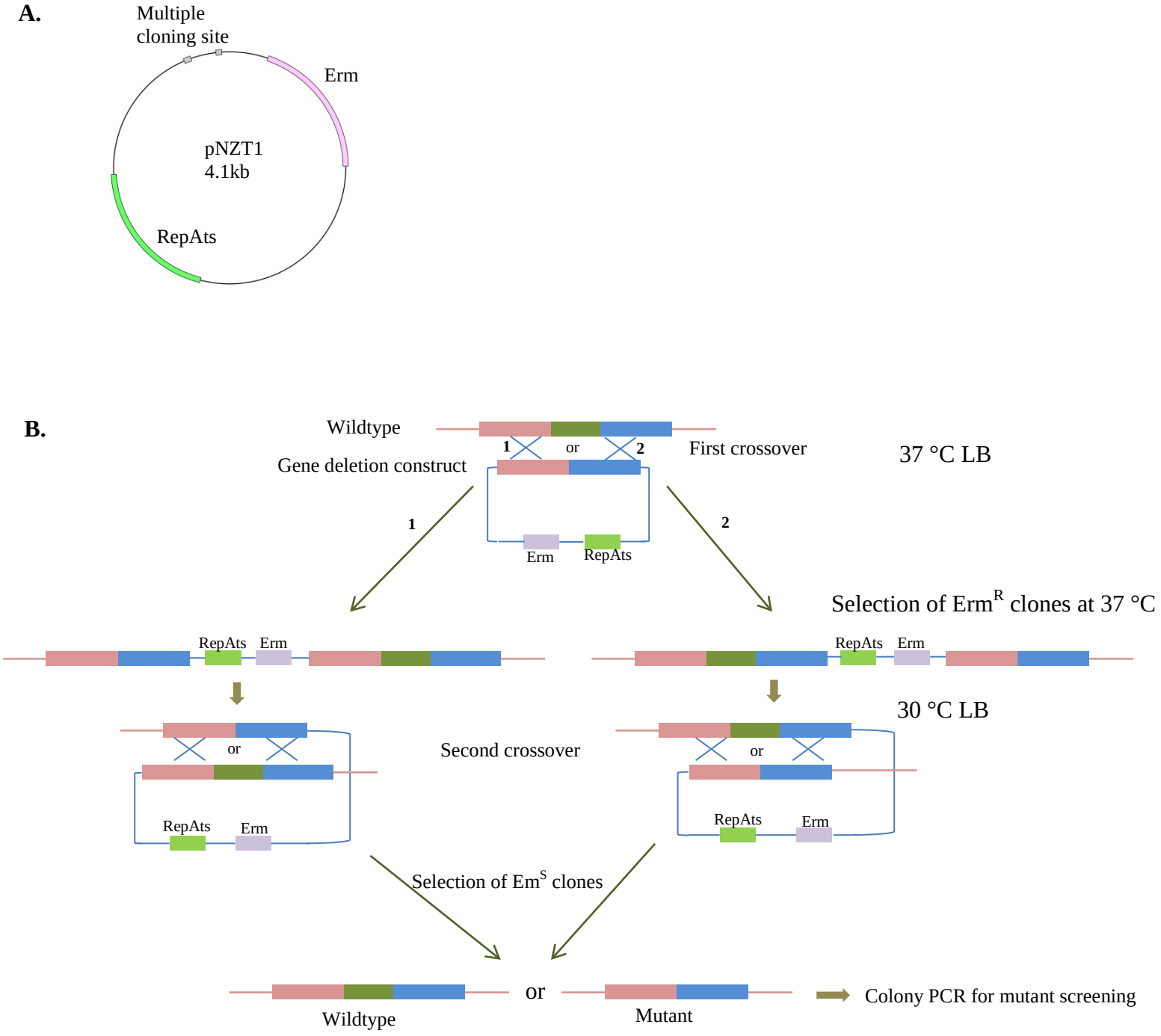


Figure 3. Schematic representation of pNZT1 (A) and the two-step replacement recombination procedure (B).

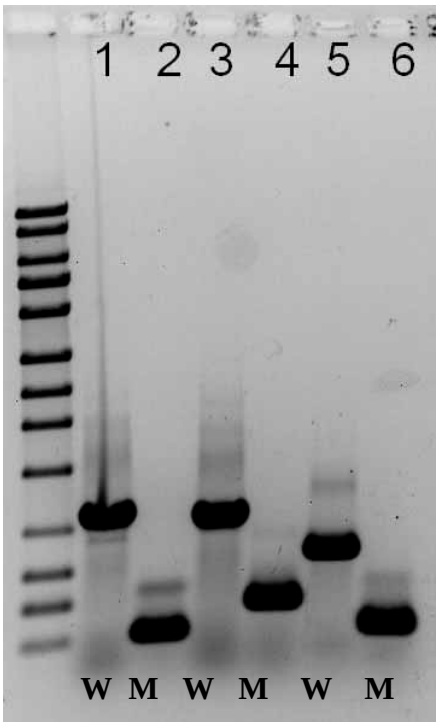


Figure 4. Products of the colony PCR analysis in screening of the mutants. *Lane 1* and *Lane 2*: products of the wildtype and mutant 93M1 (Δ*sfp*) by primer set *sfpDL* and *sfpDR*; *Lane 3* and *Lane 4*: products of the wildtype and mutant 93M2 (Δ*srfAA*) by primer set *srfDL* and *srfDR*. *Lane 5* and *Lane 6*: product of the wildtype and mutant 93M3 (Δ*dfnD*) by primer set *difDL* and *difDR*.

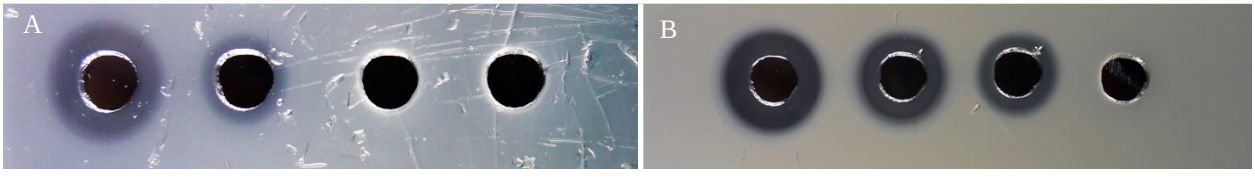


Figure 5. Agar diffusion test with indicator pathogen *E. ictaluri* S97 (A) and *A. hydrophila* M-09-119 (B).

Strains or plasmids	Relevant characteristics	Source or reference
E. coli K12 ER2925	*dcm-6 dam13::Tn9*	New England Biolabs
Bacillus sp. AP193	Wild type	This work
93M1	Δ*sfp*, deficient in lipopeptides and polyketides	pNZ-sfp →AP193, This work
93M2	Δ*srfAA*, deficient in surfactin production	pNZ-srf →AP193, This work
93M3	Δ*dfnD*, deficient in difficidin production	pNZ-dif → AP193, This work
Bacillus amyloliquefaciens FZB42	Wild type	(Chen et al., 2007)
pMK4	*E. coli-Bacillus* shuttle pladmid, rolling circle replicative, Cm^R	BGSC
pNZT1	Replication thermosensitive derivative of the rolling-circle pladmid Pwv01 (pG^+ replicon, Em^R) (Figure 3.A)	Xiaozhou Zhang, Virginia Tech
pNZ-sfp	pNZT1 with upstream and downstream sequences of gene *sfp*	This study
pNZ-srf	pNZT1 with knock-out construct of *srfAA*	This study
pNZ-dif	pNZT1 with knock-out construct of *dfn*D	This study

Table 1. Bacteria and plasmids used in this study.

Name	Sequence (5' to 3')
HindIII*sfpLL*	ATCAAAGCTTATACGCTGCTTCTGCCTGAT
SfpLR	CAGATCCGCGATGTGTTCTT
SfpRL	**AAGAACACATCGCGGATCTG**CGGTCCATATATACTCCGT
PstI*sfpRR*	ATCCTGCAGTGGCGGTTATGCTACAATGA
SfpUp	CGCTTTAACACACGGACTGA
SfpDn	TTTGTAGGAGCGGGAGAAGA
SfpDL	AAAGAGAGGAATCGGGACGA
SfpDR	TGTTTTGACGGGGCTGAT
HindIII*SrfLL*	ATCCAAGCTTATATGTACGGTCCGTCGGAA
SrfLR	GTTCCATTTGCAGCACTTCA
SrfRL	**TGAAGTGCTGCAAATGGAAC**ACTGGTCAAGCTGGCTGAAC
PstI*SrfRR*	ATCCCTGCAGGGTGCTTCAGCTCAATTCCT
SrfUp	GCGAAAGAGCGTCTGTAGAA
SrfDn	AGCCGTCATTGTCAGGTCAA
SrfDL	TCGGTCACAGGGAAATCTCT
SrfDR	CTGCTTGCGGTACTGCTCT
XhoI*DifLL*	TCAACTCGAGGGCGATTCTCGGTTTATCTC
DifLR	GATGGAGGATGCCGGTTAC
DifRL	**GTAACCGGCATCCTCCATC**CAAGAACGCTTTCGGGATT
SpeI*DifRR*	ATCCACTAGTGCCATATCAGATACCGCAGA
DifUp	TGGCTGATAAGCACCTACGA
DifDn	AAATCCGATTACAGGCGAGA

DifDL	ATAAGAAACCCGGTTCGGA
DifDR	TGGCGTGACGTCTCTCATC

Table 2. Primers used in this study. Restriction sites are underlined. Bold sequences in the 'RL' primers are complementary to corresponding 'LR' primer. For each group of primers, 'LL' and 'LR', 'RL' and 'RR' were used in the first round of PCR to amplify the upstream and downstream fragments of the target sequence for deletion, respectively. 'LL' and 'RR' were used in the second round fusion PCR to assemble the two fragments. The two primers both have restriction sites for cloning of the fused gene knock-out construct into pNZT1. Primer sets 'Up' and 'DR', 'Dn' and 'DL' were used for screening the first single crossover by colony PCR. Primer sets 'DL' and 'DR' were used for screening the mutant.

ORF ID	Putative functions	Nucleotides Position*	Gene	Top BLASTx hit	% Identity	E-value
ORF1	Surfactin synthetase A	160730.. 171484	*srfAA*	*Bacillus amyloliquefaciens* FZB42	99%	0.0
ORF2	Surfactin synthetase B	171506.. 182269	*srfAB*	*Bacillus amyloliquefaciens* FZB42	99%	0.0
ORF3	Surfactin synthetase C	182304.. 186140	*srfAC*	*Bacillus amyloliquefaciens* FZB42	99%	0.0
ORF4	Surfactin synthetase D	186159.. 186890	*srfAD*	*Bacillus amyloliquefaciens* FZB42	99%	1E-136
ORF5	Amino transferase	187012.. 188322	*att*	*Bacillus amyloliquefaciens* FZB42	100%	0.0
ORF6	Phosphopantetheinyl transferase	189032.. 188358	*sfp*	*Bacillus subtilis*	99%	5E-165
ORF7	Hypothetical protein	189777.. 189130	*yczE*	*Bacillus* sp. 5B6	100%	9E-129

Table 3. Summary of ORFs in the gene cluster for production of surfactin.

ORF ID	Putative functions	Nucleotides Position*	Gene	Top BLASTx hit	% Identity	E-value
ORF1	Bacilysin synthetase A	56144.. 56758	*bacA*	*Bacillus amyloliquefaciens* FZB42	99%	1E-112
ORF2	Isomerase component of bacilysin synthetase	56748.. 57458	*bacB*	*Bacillus amyloliquefaciens* FZB42	100%	6E-131
ORF3	Bacilysin biosynthesis oxidoreductase	57455.. 58216	*bacC*	*Bacillus amyloliquefaciens* FZB42	100%	7E-141
ORF4	Alanine-anticapsin ligase	58234.. 59652	*bacD*	*Bacillus amyloliquefaciens* FZB42	99%	0.0
ORF5	Bacilysin exporter	59649.. 60830	*bacE*	*Bacillus amyloliquefaciens* FZB42	99%	0.0
ORF6	Transaminase	60843.. 62042	*ywfG*	*Bacillus Amyloliquefaciens* subsp. *plantarum* AS43.3	99%	0.0

Table 4. Summary of ORFs involved in the synthesis of dipeptide bacilysin.

ORF ID	Putative functions	Nucleotides Position*	Gene	Top BLASTx hit	% Identity	E-value
ORF1	Fengycin synthetase A	721715.. 714057	*fenA*	*Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5036	99%	0.0
ORF2	Fengycin synthetase B	714031.. 706335	*fenB*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF3	Fengycin synthetase C	706319.. 698671	*fenC*	*Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5036	99%	0.0
ORF4	Fengycin synthetase D	698645.. 687870	*fenD*	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99%	0.0
ORF5	Fengycin synthetase E	687851.. 684048	*fenE*	*Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* DC-12	99%	0.0

Table 5. Summary of ORFs in the gene cluster for fengycin production.

ORF ID	Putative functions	Nucleotides Position*	Gene	Top BLASTx hit	% Identity	E-value
ORF1	Malonyl-CoA transacylase	661135.. 659933	*bmyD*	*Bacillus subtilis*	98%	0.0
ORF2	Bacillomycin D synthetase A	659913.. 647945	*bmyA*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF3	Bacillomycin D synthetase B	647920.. 631796	*bmyB*	*Bacillus subtilis*	98%	0.0
ORF4	Bacillomycin D synthetase C	631712.. 623853	*bmyC*	*Bacillus amyloliquefaciens* FZB42	98%	0.0

Table 6. Summary of ORFs in the gene cluster for synthesis of Bacillomycin D.

ORF ID	Putative functions	Nucleotides Position*	Gene	Top BLASTx hit	% Identity	E-value
ORF1	Hydroxyacylglutathione hydrolase	450647.. 451324	*baeB*	*Bacillus amyloliquefaciens* FZB42	99%	3E-130
ORF2	Malonyl-CoA-acyltransferase	451639.. 452508	*baeC*	*Bacillus amyloliquefaciens* FZB42	98%	1E-153
ORF3	Acyltransferase	452645.. 453619	*baeD*	*Bacillus amyloliquefaciens* FZB42	99%	0.0
ORF4	Malonyl CoA-acyl carrier Protein transacylase	453621.. 455861	*baeE*	*Bacillus amyloliquefaciens* FZB42	99%	0.0
ORF5	Acyl carrier protein	455927.. 456175	*acpK*	*Bacillus amyloliquefaciens* FZB42	100%	1E-36
ORF6	3-hydroxy-3-methylglutaryl CoA synthase	456227.. 457489	*baeG*	*Bacillus amyloliquefaciens* FZB42	99%	0.0
ORF7	Enoyl-CoA hydratase	457486.. 458259	*baeH*	*Bacillus amyloliquefaciens* FZB42	98%	2E-143
ORF8	Enoyl-CoA hydratase	458269.. 459018	*baeI*	*Bacillus amyloliquefaciens* FZB42	100%	8E-139
ORF9	Hybrid NRPS/PKS protein	459058.. 474009	*baeJ*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF10	Polyketide synthase of type I	474011.. 487426	*baeL*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF11	Polyketide synthase of type I	487444.. 497982	*baeM*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF12	Hybrid NRPS/PKS protein	497972.. 514273	*baeN*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF13	Polyketide synthase of type I	514287.. 521744	*baeR*	*Bacillus amyloliquefaciens* FZB42	97%	0.0
ORF14	Hypothetical protein	523092.. 521881	*baeS*	*Bacillus* sp. 916	99%	0.0

Table 7. Summary of ORFs in the gene cluster for bacillaene synthesis.

ORF ID	Putative functions	Nucleotides Position*	Gene	Top BLASTx hit	% Identity	E-value
ORF1	Malonyl CoA [acyl-carrier-protein] transacylase	143562.. 145868	*mlnA*	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99%	0.0
ORF2	Polyketide synthase type I	145890.. 158150	*mlnB*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF3	Polyketide synthase type I	158150.. 162922	*mlnC*	*Bacillus amyloliquefaciens* FZB42	97%	0.0
ORF4	Polyketide synthase type I	162970.. 171678	*mlnD*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF5	Polyketide synthase type I	171671.. 178675	*mlnE*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF6	Polyketide synthase type I	178699.. 184410	*mlnF*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF7	Polyketide synthase type I	184410.. 191789	*mlnG*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF8	Polyketide synthase type I	191840.. 195691	*mlnH*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF9	MlnI	195724.. 196815	*mlnI*	*Bacillus* sp. *916*	99%	0.0

Table 8. Summary of ORFs involved in the synthesis of macrolactin.

ORF ID	Putative functions	Nucleotides Position*	Gene	Top BLASTx hit	% Identity	E-value
ORF1	Malony-CoA [acyl-carrier-protein] transacylase	172633.. 174891	*dfnA*	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99%	0.0
ORF2	putative long-chain fatty acid CoA ligase	174932.. 175890	*dfnY*	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99%	0.0
ORF3	Putative acyl carrier protein	175937.. 176209	*dfnX*	*Bacillus amyloliquefaciens* FZB42	100%	5E-44
ORF4	O-succinylbenzoic acid-CoA ligase	176239.. 177570	*dfnB*	*Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5036	99%	0.0
ORF5	3-oxoacyl-(acyl carrier protein) reductase	177585.. 178322	*dfnC*	*Bacillus* sp. 5B6	99%	9E-154
ORF6	Polyketide synthase type I	178362.. 190955	*dfnD*	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	98%	0.0
ORF7	Polyketide synthase type I	190974.. 197269	*dfnE*	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	97%	0.0
ORF8	Polyketide synthase type I	197309.. 203034	*dfnF*	*Bacillus* sp. 916	99%	0.0
ORF9	Polyketide synthase type I	203086.. 218702	*dfnG*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF10	Polyketide synthase type I	218706.. 226424	*dfnH*	*Bacillus amyloliquefaciens* FZB42	99%	0.0
ORF11	Polyketide synthase type I	226458.. 232598	*dfnI*	*Bacillus amyloliquefaciens* FZB42	99%	0.0
ORF12	Polyketide synthase type I	232595.. 238810	*dfnJ*	*Bacillus amyloliquefaciens* FZB42	99%	0.0
ORF13	Putative cytochrome P450 monoxygenase	238892.. 240046	*dfnK*	*Bacillus amyloliquefaciens* Y2	99%	0.0
ORF14	Putative 3-hydroxy-3-methylglutaryl	240104.. 241351	*dfnL*	*Bacillus amyloliquefaciens*	99%	0.0

	coenzyme A synthase			FZB42		
ORF15	Enoyl-CoA hydratase	241411..242157	*dfnM*	*Bacillus amyloliquefaciens* FZB42	99%	8E-137
ORF ID	Putative functions	Nucleotides Position*	Gene	Top BLASTx hit	% Identity	E-value
ORF1	Malonyl-CoA transacylase	661135..659933	*bmyD*	*Bacillus subtilis*	98%	0.0
ORF2	Bacillomycin D synthetase A	659913..647945	*bmyA*	*Bacillus amyloliquefaciens* FZB42	98%	0.0
ORF3	Bacillomycin D synthetase B	647920..631796	*bmyB*	*Bacillus subtilis*	98%	0.0
ORF4	Bacillomycin D synthetase C	631712..623853	*bmyC*	*Bacillus amyloliquefaciens* FZB42	98%	0.0

Table 9. Summary of ORFs in the gene cluster responsible for production of difficidin.

Chapter V

Comparative genomics of plant associated *Bacillus* strains

1. Abstract

A comparative genomic analysis was conducted on thirteen biocontrol strains from the *Bacillus subtilis* group. Phylogenetic analysis indicated that 6 of the *Bacillus* strains belong to *Bacillus amyloliquefaciens* subsp. *plantum*, a subspecies with *Bacillus amyloliquefaciens* FZB42 as the type strain (also included in the 13 strains). More than 11,000 non-orthologous gene families were found within the genomes of the 13 strains, while the conserved core gene families present within them were around 1,800. A pairwise BLASTp Matrix was generated to determine similarities between each two strains in terms of number and percentage of conserved gene families. The matrix revealed that the average gene family similarity between any two *Bacillus* genomes ranges from 30% to 90%. Comparative analysis of the thirteen biocontrol strains with reference strains from the *B. subtilis* group with no (reported) biocontrol activity was carried out to identify unique genes present in the biocontrol strains that may be contribute to biocontrol activity. Fourteen unique genes were found present among all the 13 *Bacillus* strains. They express functions including signaling, transportation, secondary metabolite production, and carbon source utilization. Specifically, 70 unique genes were identified as shared by the 7 strains from *Bacillus amyloliquefaciens* subsp. *plantum*, including the difficidin biosynthetic pathway that was determined to be critical for the antibacterial activity of *Bacillus* strain AP193.

2. Introduction

A group of Gram positive aerobic endospore-forming bacteria (AEFB), called in the vernacular the "*Bacillus subtilis*" group, is traditionally of outstanding importance in basic and applied microbiology (Fritze, 2004). All members of the group, which originally consisted of *B. subtilis*, *Bacillus licheniformis*, and *Bacillus pumilus*, are placed in 16S rRNA/DNA group 1. The first novel species added to this group was *B. amyloliquefaciens*. Other species in the *B. subtilis* group that have been validly published within the last 20 years include *Bacillus atrophaeus* (Nakamura, 1989), *Bacillus mojavensis* (Roberts et al., 1994), *Bacillus vallismortis* (Roberts et al., 1996), *B. sonorensis* (Palmisano et al., 2001), and *B. tequilensis* (Gaton et al., 2006). Bacteria that are associated with plant roots and exert beneficial effects on plant development are referred to as plant growth–promoting rhizobacteria (PGPR) strains (Kloepper et al., 2004). They competitively colonize plant roots and can simultaneously act as biofertilizers and as antagonists (biopesticides) of recognized root pathogens, including bacteria, fungi and nematodes. Bacterial strains belonging to different species of *Bacillus subtilis* group have been identified as plant-growth promoting bacteria for their ability to stimulate plant growth and suppress pathogens in plant rizosphere (Kloepper et al., 2004; Jeong et al., 2011; Pengfei et al., 2012). Specifically, plant-associated *Bacillus* strains closely related to *B. amyloliquefaciens* are widely distributed (Idriss et al., 2002). Reva *et al*. reported that seven out of 17 *Bacillus* strains isolated from plants and soil, formed a cluster distinct from *B. amyloliquefaciens* type strain DSM7^{T} (Reva et al., 2004). These strains were generally better adapted to colonization of the rhizosphere than other members of the *B. subtilis* group and were considered as a distinct ecotype of *B. amyloliquefaciens*. Furthermore, plant-associated *B. amyloliquefaciens* strains

related to FZB42^T was grouped as a novel subspecies designated as *B. amyloliquefaciens* subsp. *plantum* based on DNA genome sequence comparisons, diversity in molecular marker genes, different capabilities in the nonribosomal synthesis of lipopeptides and polyketides and life-styles as compared with the industrially important strain *B. amyloliquefaciens* DSM7^T, the representative of another proposed subspecies, *B. amyloliquefaciens* subsp. *amyloliquefaciens* (Borris et al., 2011).

We sequenced the whole genome of six *Bacillus* strains belonging to *B. subtilis* group used as potential probiotics in channel catfish. Five of them were from a collection of PGPR *Bacillus* strains as described in chapter II. Also, draft genome sequences of six other PGPR *Bacillus* strains within the *B. subtilis* group were kindly provided by Dr. Choong-Min Ryu, at the Korea Research Institute of Bioscience & Biotechnology, Daejeon, Korea. The twelve strains belong to different *Bacillus* species, i.e., *B. subtilis*, *B. pumilus*, *B. amyloliquefaciens*, *B. majavensis*, *B.* siamensis, *B. sonorensis* and *B. tequilensis*, which makes a good representation of the species diversity in the *B. subtilis* group. The type plant-associated strain *B. amyloliquefaciens* FZB42 was also included in this group of PGPR *Bacillus* strains. Comparative genomic analysis was conducted between these PGPR *Bacillus* strains and representative type strains in *Bacillus subtilis* group that do not have PGP activity. Unique genes only present in the genome of PGPR strains were retrieved for each strain. Our purpose was to find the cohesive unique genes amongst the PGPR strains within *B. subtilis* group. Also, as five out of six our potential catfish probiotic strains and one of the Korean strains were identified as members of *B. amyloliquefaciens* subsp. *plantum*, comparative analysis of their unique genes was conducted, which helps to identify the genes responsible for key functions of this ecotype.

3. Materials and methods

Sequencing, assembly and annotation

Barcoded Illumina libraries were prepared from strains AP71, AP79 and AB01 using a Nextera DNA Sample Prep Kit (Illumina, San Diego, CA). In addition to Illumina sequencing, strain AP143, AP193 and AP254 were subjected to 454 pyrosequencing. A bar-coded phage DNA sublibrary was prepared at the Lucigen Corporation (Middleton, WI) and sequencing was conducted at Engencore (Univ. of South Carolina). The reads from Illumina and 454 were *de novo* assembled using CLCBio Genomics Workbench (version 4.9). Gene prediction and annotation were carried out using GeneMark (Lukashin & Borodovsky, 1998) and the RAST annotation server (Aziz *et al.*, 2008), respectively.

Phylogenetic analysis

The *gyrB* gene sequence for each strain was retrieved from the respective genome sequence. *B. amyloliquefaciens* strain AS43.3, FZB42, YAU B9601-Y2, CAU B946 and *Bacillus* sp. 5B6 were included in the phylogenetic analysis as reference strains of *B. amyloliquefaciens* subsp. *plantum*. *B. amyloliquefaciens* strain DSM7, LL3 and TA208 were used as reference for *B. amyloliquefaciens* subsp. *amyloliquefaciens*. The model strains used for reference mapping, i.e. *B. subtilis* subsp. *subtilis* str. 168, *B. pumilus* SFR032, *B. atrophaeus* 1942 and *B. licheniformis* DSM13 were also included in the analysis. A phylogenetic tree was constructed with MEGA5.05 using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 1911 positions in the final dataset.

Identification of gene conservation and definition of gene families

The set of gene families for genomes of the 13 PGPR *Bacillus* strains including the six strains from our lab, six strains from Korea and type strain FZB42 were found by BLASTp algorithm of all proteins in each genome against all proteins in the query genome using the microbial pan-genomics tool (Snipen & Ussery, 2010). A hit was considered significant if the alignment covered at least 50% of both sequences, and contained at least 50% identities (Binnewies et al., 2005). A pair of genes having significant hits was considered as belonging to the same gene family. Two gene families were merged into one if any one gene in a given family would meet the similarity criterion in an alignment against any member of another gene family (Binnewies et al., 2005; Ussery et al., 2009). This also included aligning proteins within each proteome, and while self-hits were ignored, hits to other homologous genes within the same genome were recorded.

BLAST matrix

The BLAST matrix algorithm was used for pairwise comparison of the proteomes of the 13 PGPR *Bacillus* strains according to the methods described elsewhere (Friis et al., 2010).The matrix determines the percent similarities between each two strains by measuring the ratio of the number of conserved gene families shared between the two strains to the total number of gene families of the two strains. The absolute number of shared and combined gene families for each two strains was also displayed on the matrix. In addition, the matrix shows the results from aligning proteins within each proteomes against each other.

Pan- and core- genome analysis

Conservation data across various genomes were also visualized by an accumulative plot showing the changes to the number of gene families in the core and pan-genomes as more

sequential genomes were added to the dataset (Tettelin et al., 2005). The number of novel gene families for each added genome is also depicted. Any novel gene family encountered is automatically added to the pan-genome unless it is found to be conserved in the previously considered genomes.

Identification of unique genes in PGPR strains

Genome sequences of *Bacillus* strains in the *B. subtilis* group with no recorded biocontrol abilities, *B. pumilus* SFR032, *B. licheniformis* DSM13, *B. atrophaeus* 1942, *B. subtilis* subsp. *subtilis* str. 168 and *B. amyloliquefaciens* DSM7 were downloaded from NCBI. Trimmed, paired-end sequence reads of AP71, AP79, AP143, AP193, AP254 and AB01 were assembled using *B. pumilus SFR032* as a scaffold by CLC Bio Genomic Workbench (v 4.9). The parameters that were used for each reference mapping was as follows: mismatch cost =2, insertion cost =3, deletion cost =3, length fraction =0.5 and similarity =0.5. The un-mapped (paired) reads from this reference mapping were then reference mapped against the genome sequence of *B. licheniformis* DSM13, and this process was repeated with *B. atrophaeus* 1942, *B. subtilis* subsp. *subtilis* str. 168 and *B. amyloliquefaciens* DSM7. The un-mapped sequence reads that did not match with any of model strains were considered as unique sequences for each of the six *Bacillus* strains. The un-mapped sequence reads were *de novo* assembled by CLC Bio Genomic Workbench (v 4.9). For each contig, gene prediction was carried out by GeneMark (Lukashin & Borodovsky, 1998). The predicted ORFs were pooled together for each strain, and considered as unique ORFs. A local BLASTx algorithm was conducted with unique ORFs of AP193 as the query against unique ORFs of AB01. A hit with E-value lower than 0.001 was considered as significant. A pair of genes having a significant hit was considered as orthologous. Unique ORFs of AP193 with significant hits were extracted and considered as the common unique ORFs

between AP193 and AB01. The common ORFs were then compared with unique ORFs of AP143 by local BLASTx. ORFs of AP193 with significant hits were extracted and considered as common unique ORFs among AP193, AB01 and AP143. The same process was repeated with unique ORFs of AP71, AP79 and AP254. The final common unique ORFs among the six strains, as a subset of unique ORFs of AP193, were aligned against the contigs of *B. pumilus* INR7 by BLASTx. ORFs with significant hits were extracted and aligned against the contigs of *B.* siamensis KCTC 13613. The same process was repeated with *B. sonorensis* KCTC 13918, *B. tequilensis* KCTC 13622, *B. subtilis* GB03 and *B. majavensis* KCTC 3706. The final common unique ORFs among all the 12 PGPR strains, as a subset of unique ORFs of AP193, were retrieved and their functions were predicted by BLASTx in NCBI.

The common unique ORFs of the *B. amyloliquefaciens* subsp. *plantum* strains in our collection, namely AP71, AP79, AB01, AP143, AP193 and *Bacillus subtilis* GB03, as well as the type strain FZB42, were identified by the algorithm described above. The only difference in the process was that the common unique ORFs of the five strains in our collection were aligned with ORFs of the whole genome of FZB42. Genes in the query with significant hits were retrieved and considered as the common unique ORFs of the six *B. amyloliquefaciens* subsp. *plantum* strains. Their functions were predicted by BLASTx in NCBI.

4. Results

Phylogenetic analysis

A phylogenetic analysis based on *gyrB* gene sequences demonstrated sufficient resolution among the different strains and species. Strains AP71, AP79, AP143, AP193, AB01 and *B. subtilis* GB03 grouped together with the reference strains of *B. amyloliquefaciens subsp. plantum*

with high bootstrap support, indicating that they are affiliated together as members of this subspecies. The three reference strains of *B. amyloliquefaciens* subsp. *amyloliquefaciens* clustered as a single clade separated from strains in subsp. *plantum*, supporting the division of two subspecies in *B. amyloliquefaciens* (Borris et al., 2011). Strain AP254 grouped with *B. subtilis* subsp. *subtilis* str. 168 as a single clade with strong bootstrap support, which suggests it affiliates with *B. subtilis* (Figure 1).

BLAST matrix

A comparison of all genomes was undertaken, based on a matrix of an all-against-all BLAST analysis. This provides an overview of how similar any genome is to any other genome, in terms of the ratio of the number of conserved gene families shared between the two strains to the total number of gene families of the two strains. A high diversity among the 13 PGPR *Bacillus* strains was indicated by the matrix, with gene family similarity between any two PGP *Bacillus* strains genomes ranging from 32% to 90% (Figure 2). Consistent with the phylogenetic analysis, high similarity was found among strains AP71, AP79, AP193, AB01, *B. subtilis* GB03 and FZB42, with the ratio ranging from 70% to 90%.

Pan- and core- genome analysis

More than 11,000 gene families were found within the pan-genome of the 13 strains, while the conserved core gene families present within these strains were around 1,800. The vast number of pan-gene families as compared to the core gene families reflected the diversity of the 13 PGPR strains.

Common unique genes for the thirteen plant-growth promoting strains

Fourteen unique genes were identified as common among the 13 *Bacillus* PGPR strains. Their functions included transportation (4 genes), regulation (3 genes), carbohydrate degradation or synthesis (3 genes) and secondary metabolite production (2 genes) (Table 1). Specifically, genes in the gene clusters responsible for the synthesis of polyketides difficidin and macrolactin, *dfnC* and *mlnD* respectively, were found as common among the 13 strains. However, the complete gene clusters for synthesis of the two polyketides were only present in *B. amyloliquefaciens* subsp. *plantum* strains.

Comparative analysis of unique genes from strains in *B. amyloliquefaciens* subsp. *plantum*

Different numbers of unique genes were found in the strains from *B. amyloliquefaciens* subsp. *plantum*, indicating the diversity among strains (Table 2). A total of 76 common genes among them were obtained. BLASTx results showed that multiple ORFs devoted to the functions of MlnA, MlnB, DfnD, DfnF and FenB, suggesting the incorrectness of the original ORF prediction of these genes by GeneMark. The original contigs containing these pseudo ORFs were aligned with the corresponding genes of FZB42 to identify the correct ORF for each gene, which reduced the total number of genes from 76 to 70 (Table 3). Functions expressed by the 70 genes included transportation (6 genes), regulation (7 genes), signaling (3 genes), carbon degradation or synthesis (8 genes) and synthesis of secondary metabolites (25 genes) (Figure 4). Many of them are likely to be involved in interactions with plants and the rhizosphere competence of the *plantum* strains. Interestingly, the complete gene clusters for biosynthesis of polyketides difficidin and macrolactin were found as prevalent in all the *plantum* strains (Table 3), suggesting their importance for the plant-growth promoting and/or biocontrol activities of these strains.

5. Discussion

It has been recognized that species in *B. subtilis* group cannot be differentiated on the basis of their phenotypic characteristics and 16S rRNA gene nucleotide sequences alone (Borris et al., 2011), which explains the lack of exact species information for some important *Bacillus* strains, such as *Bacillus* sp. 5B6 and *Bacills* sp. 916. In our study, a phylogenetic analysis based on *gyrB* sequence provided sufficient differentiation of our 12 PGPR strains, as well as the reference strains from the *B. subtilis* group. Six strains were identified as members of the recently proposed taxa *B. amyloliquefaciens* subsp. *plantum*, including the Korean strain GB03, formerly designated as *B. subtilis*. Strain AB01 was isolated from the intestine of channel catfish, and its identity as a plant-associated strain was unexpected. This suggests AB01 was a transient bacterium in the channel catfish intestine, or that members of *B. amyloliquefaciens* subsp. *plantum* are not exclusively plant-associated. The efficacy of AB01 as well as other plant-associated strains as probiotics in channel catfish suggests some common functions in the biocontrol of pathogens of plants and aquatic animals.

With rapid advances in sequencing technologies and the subsequent explosion in the amount of available sequence data, it is now possible to describe microbial genomes not only as individual entities, but also to analyze their collective pan and core genomes (Medini et al., 2008). A core genome was first defined by Lan and Reeves (2000) as comprising those genes present in almost all individuals of a species. The pan-genome was introduced as comprising core genome containing genes present in all strains, and a dispensable genome containing genes present in two or more strains and genes unique to single strains (Medini et al., 2005). As an extension of the original definition, the pan- and core- genome analysis of the genus *Campylobacter* was tentatively described based on genomes of representative strain(s) of each

Campylobacter species (Friis et al., 2010). In our study, pan- and core- genome analysis was conducted on plant-growth promoting strains from different species of the *Bacillus subtilis* group, a collection of strains representing both the taxa of *Bacillus subtilis* group and the subset of this group characterized by plant-growth promoting function, as only limited number of strain(s) for each species was included in the analysis. The core genome of the thirteen strains, which comprises around 1750 genes, may tentatively estimate the actual size of the core genome of the *Bacillus subtilis* group based on the fact that the number of core genes for a species would reach a minimum after including a number of strains (8 in the case of *S. agalactiae*) and remain relatively constant even as many more genomes are added (Tettelin et al., 2005). The number of core genes of the plant-growth promoting subset of the *B. subtilis* group may also be estimated by the core genome of the 13 strains, due to their dual representation. Over 10,000 pan genes were found in the 13 strains. Mathematical modeling based on eight genomes of *S. agalactiae* showed that unique genes will continue to emerge even after hundreds or thousands of genomes are sequenced, and the pan-genome of a bacterial species might be orders of magnitude larger than any single genome (Medini et al., 2005). Therefore, the vast number of pan genes of the 13 *Bacillus* strains as compared with the core genes is not surprising. Given the different environments that members of the *B. subtilis* group may colonize, their competence in multiple ways of exchanging genetic material (transformation, transduction, conjugation) and the larger taxa level we investigated (multiple species vs. single species), the actual pan-genome for either the *B. subtilis* group and the plant-growth promoting subgroup should be much larger than the number we obtained from the 13 representative strains.

Only 14 unique genes were found in common among the 13 PGPR strains, which cannot explain all of their respective plant-growth promoting functions. This suggests that different

genes expressing diverse functions among different species of PGPR strains may contribute to their plant-growth promoting activity. As opposed to PGPR strains from different species, the closely related strains from *B. amyloliquefaciens* subsp. *plantum* are more likely to utilize common gene functions for plant-growth promoting and/or biocontrol activities. Therefore, we then focused on the strains in *B. amyloliquefaciens* subsp. *plantum* and intended to find the unique genes that they share in common that are likely to be key contributors to their plant-growth promoting and/or biocontrol. A total of seventy core genes were found among these strains. The two complete gene clusters for polyketide synthesis suggests the robustness of our analysis. The fact that both gene clusters belongs to a genomic island in FZB42 that is absent in *B. amyloliquefaciens* DSM7 (Rückert et al., 2011) suggests that the common ancestor of these closely related *plantum* strains acquired these gene clusters via lateral gene transfer. Two genes from the gene cluster required for fengycin synthesis, *fenB* and *fenD*, were also found in the pool of core unique genes, which is consistent with the fact that the genes *fenB-fenD* are missing in *B. amyloliquefaciens* DSM7 (Rückert et al., 2011), the strain used as a reference genome with no PGPR activity in our comparative analysis. The diversity of these *plantum* strains was reflected by the number of singletons in each strain and the large number of pan-unique-genes. This was not surprising, as the rhizosphere, the environment these strains colonize, is a dynamic, complex system with vast and diverse populations of bacteria (Gans et al., 2005), bacteriophages (Hendrix, 2003) and a continuous exchange of genetic materials between them (Medini et al., 2005).

For defining the core and pan-genome, genes are considered members of a gene family when they share significant homology, using specified criteria. Gene families were used for the BLAST Matrix construction and pan- and core- genome analysis in our study. However, when

we were searching the common unique genes for both the 13 strains and the *plantum* strains, the idea of gene family was not exploited. Rather, the original list of genes was used for local BLASTx analysis and unique genes from AP193 were always used as the query. Probably the final number of unique genes in common for the *plantum* strains will be different if using other strains as query, as the number of unique genes with duplicated functions may be different for each query strain. However, as a detailed search for a particular subset of genes this bias is acceptable. Actually, the use of an original list of genes as query for comparison may provide advantages, as some genes with duplicated or similar functions may belong to different genetic machineries which contribute to closely related but different functions. For example, the genes *fenD* and *bmyB* in the gene clusters of AP193 for synthesis of fengycin and bacillomycin D, respectively, showed high similarity in BLASTx. Also, the three polyketide synthesis clusters in FZB42 displayed high degree of homology (Chen et al., 2006). These genes would have been combined as a single gene family by the criteria described above, which may lose some valuable information for further analysis, while comparison analysis with the original list of genes as the query would provide a more detailed description of functions.

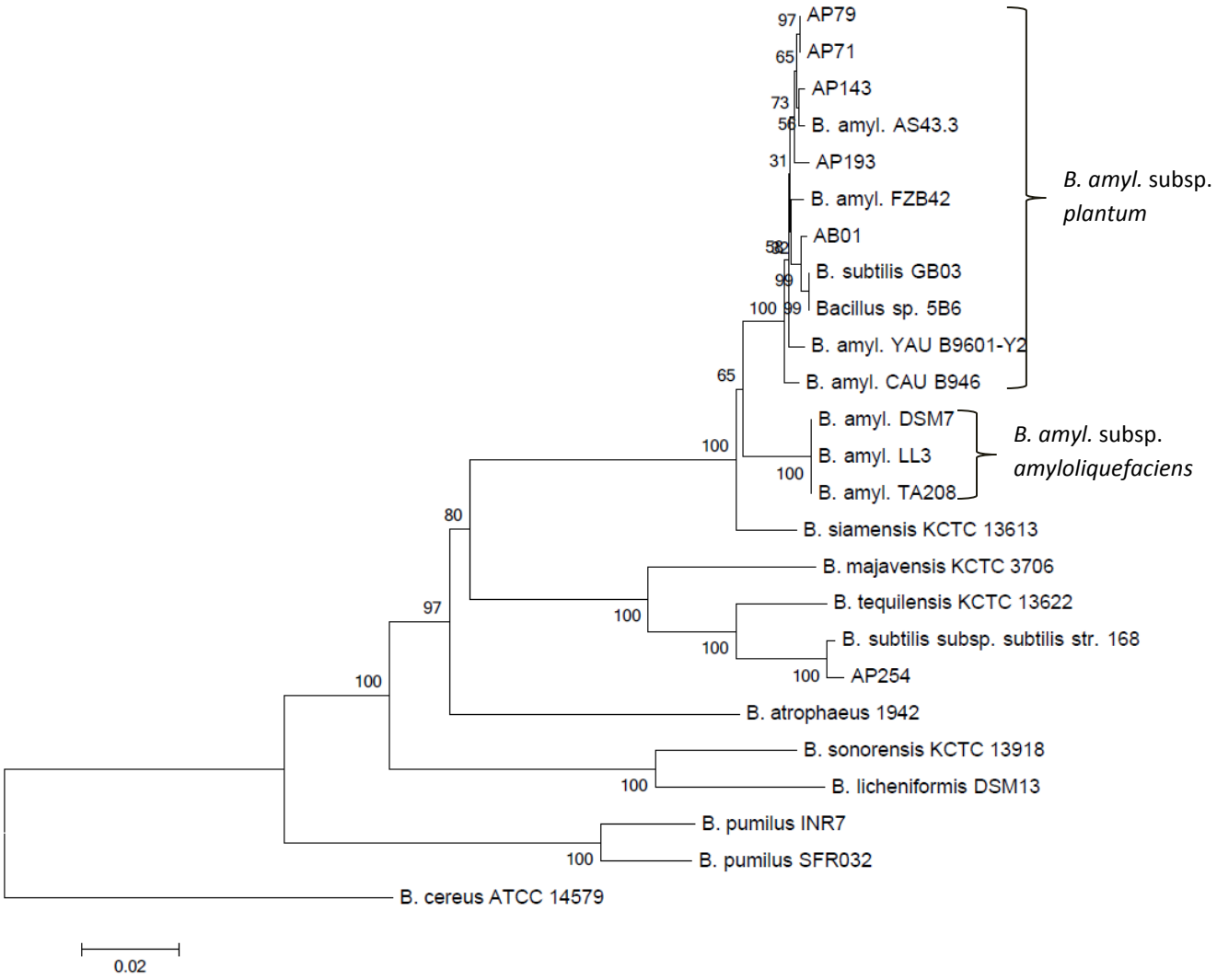


Figure 1. Neighbor joining phylogenetic tree based on *gyrB* sequences using *B. cereus* ATCC 14579^{T} as an outgroup. The two clusters belonging to *B. amyloliquefaciens* subsp. *plantum* and *B. amyloliquefaciens* subsp. *amyloliquefaciens* were indicated.

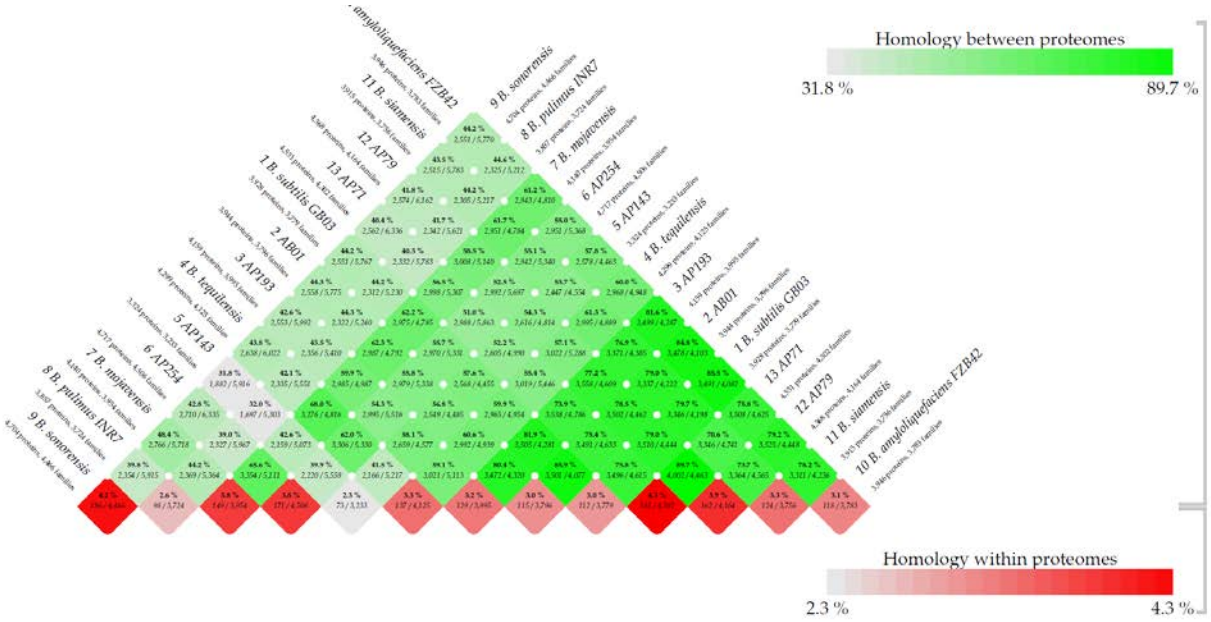


Figure 2. Blast matrix of the 13 PGP *Bacillus* strains. This matrix showed the output from pairwise comparison of proteomes of the 13 strains. It presents the absolute number of gene families preserved between any two strains along with the total number of families between them. The ratio of preserved gene family number over the total number is also given and used as a basis for the color intensity. The green color represents the % homology between proteomes and the red color represents % homology within proteomes.

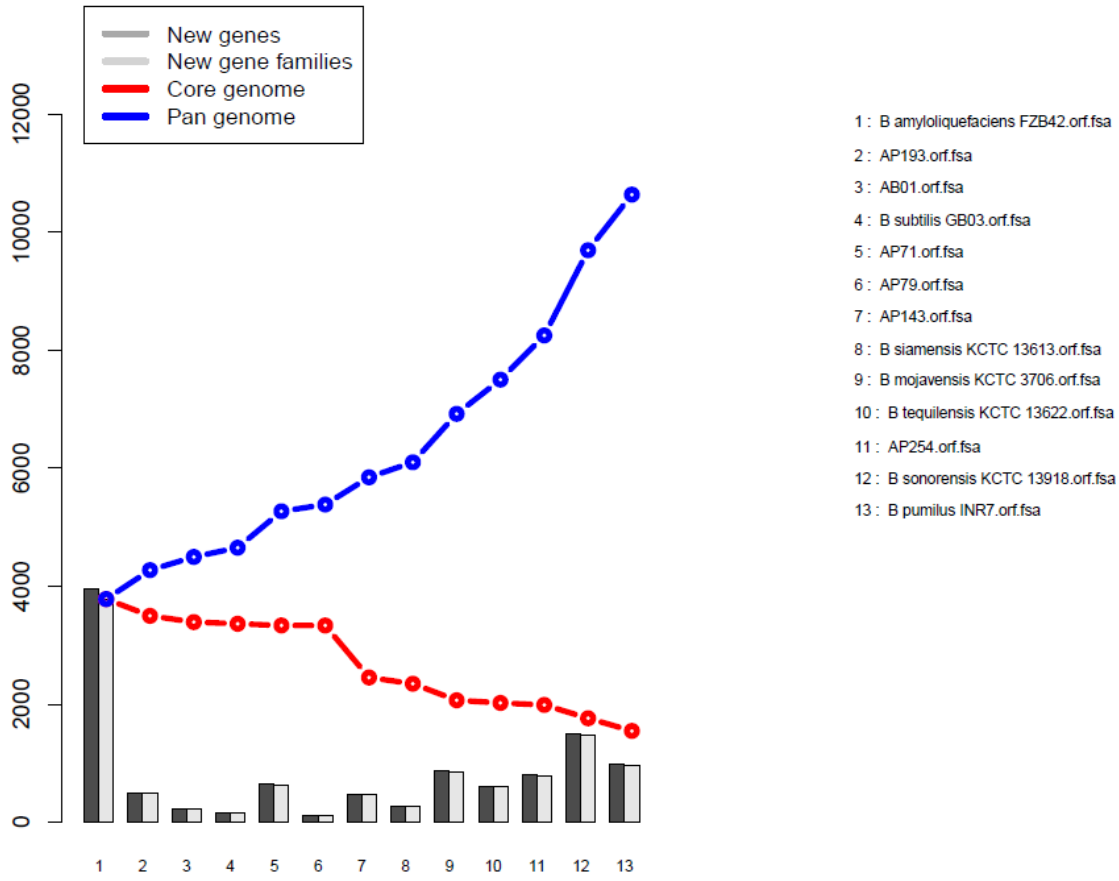


Figure 3. Pan and core-genome plot of the 13 PGP *Bacillus* strains. The columns give the number of new gene families (and genes) introduced with the addition of each new genome. The red and blue lines indicate the number of gene families in the core- and pan-genomes, respectively.

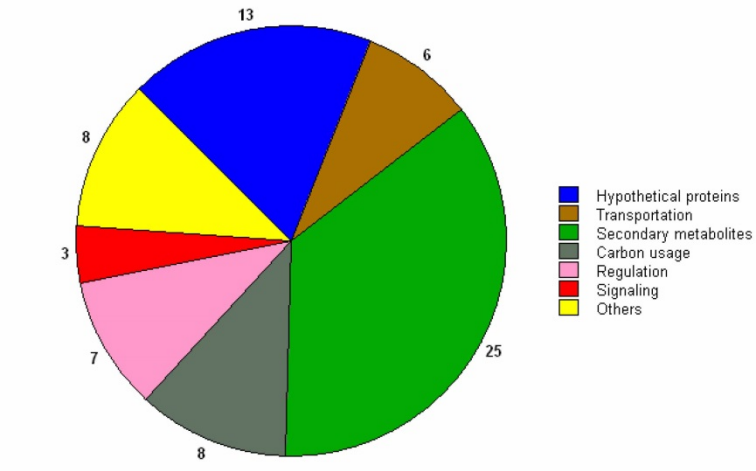


Figure 4. Categories of functions expressed by the 70 common unique genes shared by the 7 strains from *B. amyloliquefaciens* subsp. *plantum*. The number beside each subgroup of the pie figure represents the number of genes encoding the function.

ORF ID	Putative function	Top BLASTx hit	% Identity
ORF1	ABC transporter permease	*Bacillus cereus*	99%
ORF2	3-hydroxybutyrate dehydrogenase	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99%
ORF3	Macrolactin synthase MlnD	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99%
ORF4	Difficidin synthase DfnC	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99%
ORF5	Hypothetical protein	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99%
ORF6	Hexuronate transporter	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99%
ORF7	LacI family transcriptional regulator	*Bacillus amyloliquefaciens* FZB42	99%
ORF8	D-mannonate oxidoreductase	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99%
ORF9	Two component response regulator	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99%
ORF10	Spermidine/putrescine import ATP-binding protein potA	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99%
ORF11	Response regulator uvrY	*Bacillus amyloliquefaciens* FZB42	100%
ORF12	ABC transporter ATP-binding protein	*Bacillus vallismortis*	87%
ORF13	Putative ribose/galactose/methyl galactoside import ATP-binding protein	*Bacillus amyloliquefaciens* subsp. *plantarum* YAU B9601-Y2	99%
ORF14	Glycosyl transferase, minor teichoic acid biosynthesis protein ggaA	*Bacillus amyloliquefaciens* FZB42	99%

Table 1. Common unique genes among the 13 PGP *Bacillus* strains

	AP193	AB01	AP143	AP71	AP79	GB03	FZB42
Unique CDSs	422	203	387	489	490	n.a.[a]	n.a.
Common CDSs[b]	n.a.	115	95	80	80	77	76
New CDSs[c]	n.a.	91	128	307	94	n.a.	n.a.
Pan-unique-CDSs[d]	422	513	641	948	1042	n.a.	n.a.

Table 2. Comparative analysis of the unique genes of strains in *B. amyloliquefaciens* subsp. *plantum*

[a.] not applicable

[b.] Numbers in each column indicates common unique genes present among the present strain and the strain(s) in all the left columns

[c.] Numbers in each column indicates new unique genes in the strain not occurring in all the strain(s) in the left columns.

[d.] Numbers in each column indicates the sum of all unique genes present in the present strains combining all the strain(s) in the left columns.

Query		Description (E-value)	% Identity
ORF1	hypothetical protein	*Bacillus amyloliquefaciens* FZB42	99
ORF2	MlnI	*Bacillus* sp. 916	99
ORF3	MlnH	*Bacillus amyloliquefaciens* FZB42	99
ORF4	MlnG	*Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5036	99
ORF5	MlnF	*Bacillus amyloliquefaciens* FZB42	99
ORF6	MlnE	*Bacillus amyloliquefaciens* FZB42	99
ORF7	MlnD	*Bacillus amyloliquefaciens* FZB42	99
ORF8	MlnC	*Bacillus* sp. 916	98
ORF9	MlnB	*Bacillus* sp. 916	99
ORF10	MlnA	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99
ORF11	DfnA	*Bacillus amyloliquefaciens* FZB42	99
ORF12	DfnY	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	100
ORF13	DfnX	*Bacillus amyloliquefaciens* FZB42	100
ORF14	DfnC	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	100

ORF15	DfnD	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99
ORF16	DfnE	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99
ORF17	DfnF	*Bacillus* sp. 916	98
ORF18	DfnH	*Bacillus amyloliquefaciens* FZB42	99
ORF19	DfnI	*Bacillus amyloliquefaciens* FZB42	100
ORF20	DfnJ	*Bacillus amyloliquefaciens* FZB42	99
ORF21	Putative cytochrome P450, DfnK	*Bacillus amyloliquefaciens* Y2	99
ORF22	DfnM	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	100
ORF23	hexuronate transporter	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	100
ORF24	D-mannonate oxidoreductase	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99
ORF25	L-asparaginase	*Bacillus* sp. 916	99
ORF26	FenB	*Bacillus amyloliquefaciens* FZB42	99
ORF27	FenD	*Bacillus amyloliquefaciens* FZB42	99
ORF28	spermidine/putrescine import ATP-binding protein potA	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99
ORF29	hypothetical protein	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99
ORF30	2-keto-3-deoxygluconokinase	*Bacillus amyloliquefaciens* FZB42	99
ORF31	sensor histidine kinase YdfH	*Bacillus* sp. 916	98
ORF32	phosphoenolpyruvate synthase	*Bacillus* sp. 916	99
ORF33	galactokinase GalK1	*Bacillus* sp. 916	99
ORF34	UDP-glucose 4-epimerase GalE1	*Bacillus amyloliquefaciens* FZB42	100
ORF35	permease	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	100
ORF36	Molybdenum cofactor synthesis protein 3	*Bacillus amyloliquefaciens* subsp. *plantarum* YAU B9601-Y2	99
ORF37	LacI family transcription regulator KdgR	*Bacillus amyloliquefaciens* FZB42	99
ORF38	LysR family transcriptional regulator	*Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5036	99
ORF39	minor teichoic acid biosynthesis protein	*Bacillus amyloliquefaciens* FZB42	99

	ggaA glycosyl transferase		
ORF40	putative ribose/galactose/methyl galactoside import ATP-binding protein 2	*Bacillus amyloliquefaciens* subsp. *plantarum* YAU B9601-Y2	100
ORF41	HTH-type transcriptional regulator	*Bacillus amyloliquefaciens* FZB42	100
ORF42	Histone arginine demethylase JMJD6	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	100
ORF43	3-hydroxybutyrate dehydrogenase	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	100
ORF44	NAD(P)-binding domain / putative oxidoreductase / FMN reductase	*Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5036	99
ORF45	chemotaxis protein CheY	*Bacillus amyloliquefaciens* FZB42	100
ORF46	alcohol dehydrogenase YogA	*Bacillus amyloliquefaciens* FZB42	98
ORF47	transcription antiterminator NusG	*Bacillus amyloliquefaciens* FZB42	100
ORF48	hypothetical protein B938_19340	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	99
ORF49	hypothetical protein	*Bacillus amyloliquefaciens* FZB42	98
ORF50	Rrf2 family transcriptional regulator	*Bacillus* sp. 5B6	99
ORF51	hypothetical protein	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	100
ORF52	Mannose-6-phosphate isomerase	*Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5036	99
ORF53	transcriptional regulator	*Bacillus amyloliquefaciens* FZB42	100
ORF54	hypothetical protein	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	97
ORF55	hypothetical protein	*Bacillus amyloliquefaciens* FZB42	100
ORF56	LysR family transcriptional regulator YybE	*Bacillus amyloliquefaciens* FZB42	99
ORF57	Two-component response regulator	*Bacillus amyloliquefaciens* FZB42	99
ORF58	NarL family DNA-binding response regulator	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	100

ORF59	ABC transporter permease	*Bacillus cereus*	99
ORF60	hypothetical protein	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	100
ORF61	hypothetical protein	*Bacillus amyloliquefaciens* Y2	93
ORF62	hypothetical protein	*Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5036	97
ORF63	ABC transporter ATP-binding protein	*Bacillus vallismortis*	87
ORF64	Predicted HTH-type Transcriptional regulator	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3	100
ORF65	hypothetical protein	*Bacillus amyloliquefaciens* FZB42	94
ORF66	hypothetical protein	*Bacillus amyloliquefaciens subsp. plantarum CAU B946*	74
ORF67	4-carboxymuconolactone decarboxylase	*Bacillus amyloliquefaciens subsp. plantarum AS43.3*	98
ORF68	hypothetical protein	*Bacillus sp. 5B6*	99
ORF69	Non-ribosomal peptide synthase	*Bacillus cereus* HuB1-1	97
ORF70	Non-ribosomal peptide synthase	*Bacillus cereus* HuB1-1	97

Table 3. Common unique genes of the seven strains belonging to *B. amyloliquefaciens* subsp. *plantum*.

Chapter VI

Summary and conclusions

This study initially selected four *Bacillus* strains as potential probiotics for channel catfish, namely AP79, AP143, AP193 and AB01, based on their *in vitro* antagonism against aquatic pathogens specifically *E. ictaluri* and *A. hydrophila*, as well as the survival and persistence ability in the intestine of channel catfish. All the four strains showed different levels of protection effect on channel catfish challenged with *E. ictaluri* in aquarium system. Also, safety test indicated that they were all susceptible to clinically important antibiotics, and didn't contain plasmids with putative virulence factors.

The four strains showed marginal promotion effect on the growth performance of channel catfish based on a 10 week feeding experiment. They can also marginally enhance the innate immune response of the channel catfish indicated by improved lysozyme and respiratory burst activity. Based on the performance in all the tests, AP193 was selected from the four strains for further study. The investigation of dosage effect on the probiotic efficiency of AP193 suggested that 10^7 CFU/g of feed provided the best cost/effect ratio.

The genome of *Bacillus* strain AP193 was determined by next-generation DNA sequencing. Genome analysis identified gene clusters for the production of polyketides (bacillaene, macrolactin and difficidin), lipopeptiedes (surfactin, bacillomycin D and fengycin) and a dipeptide antibiotic bacilycin. Low efficiency transformation of AP193 was accomplished by isolation of plasmid from an *E. coli* ER2925 (*dam dcm*) strain followed by *in vitro*

methylation of plasmid with cell–free extracts of AP193. A gene knock-out procedure was conducted that involved a temperature sensitive shuttle plasmid vector based on the pWV01 origin of replication, a two-step replacement recombination procedure and PCR screening of mutants in order to construct marker-free deletion mutants deficient in the biosynthesis of specific antibiotic(s). Using an agar diffusion test, the secondary metabolites responsible for most of *in vitro* antagonism of AP193 against *E. ictaluri* and *A. hydrophila* were identified as difficidin, one of the three polyketides.

A comparative genomic analysis was conducted on thirteen biocontrol strains from the *Bacillus subtilis* group. Phylogenetic analysis indicated that 6 of the *Bacillus* strains belong to *Bacillus amyloliquefaciens* subsp. *plantum*, a subspecies with *Bacillus amyloliquefaciens* FZB42 as the type strain, which was also included in the 13 strains. Comparative analysis of the thirteen biocontrol strains with reference strains from *Bacillus subtilis* group with no biocontrol activity was carried out to identify unique genes present in the biocontrol strains that may be responsible for biocontrol activity. Fourteen unique genes were found present among all the 13 *Bacillus* strains. They express functions including signaling, transportation, secondary metabolite production, and carbon source utilization. Specifically, 70 unique genes were identified as shared by the 7 strains from *Bacillus amyloliquefaciens* subsp. *plantum*, including the complete difficidin biosynthetic pathway that was critical for the antibacterial activity of *Bacillus* strain AP193.

Investigation of the efficiency of AP193 as a probiotic to protect channel catfish against ESC in pond scale is underway. The results will be analyzed in an economic cost-benefit way to evaluate the feasibility to develop probiotic products from AP193. Research in this study established a strong foundation to investigate the contribution of secondary metabolite(s) to the

protection effect of AP193 on channel catfish. Aquarium challenge study comparing the protection effect of mutants deficient in secondary metabolite(s) synthesis and the wildtype on channel catfish will be started soon, which will answer the question and present significant advance in our knowledge of the mechanisms of probiotic effect in aquatic animals. Genomic analysis facilitates our understanding of the probiotic and/or biocontrol activity of the *Bacillus* strains and will provide target gene(s) for investigation of other putative mechanisms involved in the probiotic/biocontrol effect, which may finally facilitate strategies to improve the efficiency of the probiotic/biocontrol strains.

Comprehensive Bibliography

Ai, Q.H., Xu, H.G., Mai, K.S., Xu, W., Wang, J., Zhang, W., 2011. Effects of dietary supplementation of *Bacillus subtilis* and fructooligosaccharide on growth performance, survival, non-specific immune response and disease resistance of juvenile large yellow croaker, *Larimichthys crocea*. Aquaculture 317, 155–161.

Alegre, M.T., Rodríguez, M.C., Mesas, J.M., 2004. Transformation of *Lactobacillus plantarum* by electroporation with in vitro modified plasmid DNA. FEMS Microbiol. Lett. 241, 73-77.

Aly, S.M., Ahmed, Y.A., Ghareeb, A.A., Mohamed, M.F., 2008. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. Fish Shellfish Immunol. 25, 128–136.

Andlid, T., Vazquez-Juarez, R., Gustafsson, L., 1995. Yeast colonizing the intestine of rainbow trout (*Salmo gairdneri*) and turbot (*Scophthalmus maximus*). Microb. Ecol. 30, 321–334.

Asaka, O. and Shoda, M., 1996. Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. Appl. Environ. Microbiol. 62, 4081-4085.

Austin, B., Stuckey, L.F., Robertson, P., Effendi, I., Griffithc, D., 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida,Vibrio anguillarum and Vibrio ordalii*. J. Fish Dis. 18: 93-96.

Avella, M.A., Gioacchini, G., Decamp, O., Makridis, P., Bracciatelli, C., Carnevali, O., 2010. Application of multi-species of *Bacillus* in sea bream larviculture. Aquaculture 305, 12-19.

Azad, I.S., Rajendran, K.V., Rajan, J.J.S., Vijayan, K.K. and Santiago, T.C., 2001. Virulence and histopathology of *Aeromonas hydrophila* (Sah 93) in experimentally infected tilapia, *Oreochromis mossambicus* (L.). J. Aquac. Trop. 16, 265–275.

Aziz, R., Bartels, D., Best, A., DeJongh, M., Disz, T., Edwards, R., Formsma, K., Gerdes, S., Glass, E..et al., 2008. The RAST Server: Rapid Annotations using Subsystems Technology. BMC Genomics 9, 75.

Bairagi, A., Sarkar Ghosh, K., Sen, S.K., Ray, A.K., 2004. Evaluation of the nutritive value of *Leucaena leucocephala* leaf meal, inoculated with fish intestinal bacteria *Bacillus subtilis* and *Bacillus circulans* in formulated diets for rohu, *Labeo rohita* (Hamilton) fingerlings. Aquac. Res. 35, 436–446.

Balcázar, J.L., Rojas-Luna, T., Cunningham, D.P., 2007. Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*. J. Invertebr. Pathol. 96, 147–150.

Benbrook, C.M., 2002. Antibiotic drug use in US aquaculture. Institute for Agriculture and Trade Policy Report.

Birnboim, H.C. and Doly, J., 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513–1523.

Binnewies, T.T., Hallin, P.F., Staerfeldt, H.H., Ussery, D.W., 2005. Genome Update: proteome comparisons. Microbiology 151, 1–4.

Blazer, V.S., Shotts, E.B., Waltman, W.D., 1985. Pathology associated with Edwardsiella ictaluri in catfish, Ictalurus punctatus Rafinesque, and Danio devario (Hamilton-Buchanan, 1822). J. Fish Biol. 27, 167-175.

Bly, J.E., Quiniou, S.M.-A., Lawson, L.A., Clem, L.W., 1997. Inhibition of Saprolegnia pathogenic for fish by Pseudomonas fluorescens. J. Fish Dis. 20, 35–40.

Borris, R., Chen, X.H., Rueckert, C., Blom, J., Becker, A., Baumgarth, B., Fan, B., Pukall, R., Schumann, P., Spröer, C., Junge, H., Vater, J., Pühler, A., Klenk, H.P., 2011. Relationship of *Bacillus amyloliquefaciens* clades associated with strains DSM 7^T and FZB42^T: a proposal for *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* subsp. nov. and *Bacillus amyloliquefaciens* subsp. *plantarum* subsp. nov. based on complete genome sequence comparisons. Int. J. Syst. Evol. Microbiol. 61, 1786-1801.

Brunt, J. and Austin, B., 2005. Use of a probiotic to control lactococcosis and streptococcosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. 28: 693-701.

Burgess, J.G., Jordan, E.M., Bregu, M., Mearns-Spragg, A., Boyd, K.G., 1999. Microbial antagonism: a neglected avenue of natural products research. J. Biotechnol. 70, 27–32.

Carnevali, O., Zamponi, M.C., Sulpizio, R., Rollo, A., Nardi, M., Orpianesi, C., Silvi, S., Caggiano, M., Polzonetti, A.M., Cresci, A., 2004. Administration of probiotic strain to improve sea bream wellness during development. Aquacult. Int. 12, 377–386.

Carrias, A.A., Terhune, J.S., Sayles, C.A., Chappell, J.A., 2008. Effects of an extended hatchery phase and vaccination against enteric septicemia of catfish on the production of channel catfish, Ictalurus punctatus, fingerlings. J. World Aquacult. Soc 39, 259-266.

Casula, G., Cutting, S.M., 2002. *Bacillus* probiotics: spore germination in the gastrointestinal tract. Appl. Environ. Microbiol. 68, 2344–2352.

Chabrillón,M.,Arijo, S.,Díaz-Rosales, P.,Balebonz, M.C.,Moriñigo,M.A., 2006. Interference of *Listonella anguillarumwith* potential probioticmicroorganisms isolated from farmed gilthead seabream (*Sparus aurata*, L.). Aquac. Res. 37, 78–86.

Chang, C.I. and Liu, W.Y., 2002. An evaluation of two probiotic bacterial strains, *Enterococcus faecium* SF68 and *Bacillus toyoi*, for reducing edwardsiellosis in cultured European eel, *Anguilla anguilla* L. J. Fish Dis. 25: 311-315.

Challis, G.L., and Hopwood, D. A., 2003. Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. Proc. Natl. Acad. Sci. USA 100(Suppl. 2)**:**14555–14561.

Chapman, F.A., 2000. Farm-raised channel catfish.

Chen, X.H., Vater, J., Piel, J., Franke, P., Sholz, R., Schneider, K., Koumoutsi, A., Hitzeroth, G., Grammel, N., Strittmatter, A.W., Gottschalk, G., Süssmuth, R.D., Borris, R., 2006. Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB42. J. Bacteriol. 188, 4024-4036.

Chen, X.H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I., Morgenstern, B., Voss, B., Hess, W.R., Reva, O., Junge, H., Voigt, B., Jungblut, P.J., Vater, J., Süssmuth, R., Liesegang, H., Strittmatter, A., Gottschalk, G., Borriss, R., 2007. Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nat. Biotechnol. 25, 1007–1014.

Chen, X.H., Scholz, R., Borriss, M., Junge, H., Mögel, G., Kunz, S., Borriss, R., 2009a. Difficidin and bacilysin produced by plant-associated *Bacillus amyloliquefaciens* are efficient in controlling fire blight disease. J. Biotechnol. 140, 38-44.

Chen, X.H., Koumoutsi, A., Scholz, R., Borris, R., 2009b. More than anticipated – production of antibiotics and other secondary metabolites by *Bacillus amyloliquefaciens* FZB42. J Mol Microbiol. Biothenonol. 16, 14-24.

Chythanya, R., Karunasagar, I., Karunasagar, I., 2002. Inhibition of shrimp pathogenic vibrios by a marine *Pseudomonas* I-2 strain. Aquaculture 208, 1–10.

Coukoulis, H. and Campbell, L.L., 1971. Transformation in *Bacillus amyloliquefaciens*. J. Bacteriol. 105, 319–322.

Díaz-Rosales, P., Arijo, S., Chabrillón, M., Alarcón, F.J., Tapia-Paniagua, S.T., Martínez-Manzanares, E., Balebona, M.C., Moriñigo, M.A., 2009. Effects of two closely related probiotics on respiratory burst activity of Senegalese sole (Solea senegalensis, Kaup) phagocytes, and protection against Photobacterium damselae subsp. piscicida. Aquaculture 293, 16–21.

Donahue, J.P., Israel, D.A., Peek JR, R.M., Blaser, M.J., Miller, G.G., 2000. Overcoming the restriction barrier to plasmid transformation of *Helicobacter pylori*. Mol. Microbiol. 37, 1066-1074.

Ellis, A.E., 1990. Lysozyme assays. In: Stolen, J.S., Fletcher, T.C., Anderson, D.P., Kaattari, S.L., Rowley, A.F. (Eds.), Techniques in Fish Immunology. SOS Publications, Fair Haven, NJ, pp. 101–103.

Fabret, C., Ehrlich, S.D., Noirot, P., 2002. A new mutation delivery system for genome-scale approaches in *Bacillus subtilis*. Mol. Microbiol. 46, 25–36.

Fang, H.M., Ge, R. and Sin, Y.M. (2004) Cloning, characterization and expression of *Aeromonas hydrophila* major adhesin. Fish Shellfish Immunol. 16, 645–658.

Farmer, B., 2004. Improved methods for the isolation and characterization of *Flavobacterium columnare*. Thesis. Baton Rouge, LA: Department of Pathobiological Sciences, Louisiana State University.

Francis-Floyd, R., Beleau, M.H., Waterstrat, P.R., Bowser, P.R., 1987. Effect of water temperature on the clinical outcome of infection with Edwardsiella ictaluri in channel catfish. J. Am. Vet. Med. Assoc. 191, 1413.

Friis, C., Wassenaar, T.M., Javed, M.A., Snipen, L., Lagesen, K., Hallin, P.F., Newell, D.G., Toszeghy, M., Ridley, A. et al., 2010. Genomic characterization of *Campylobacter jejuni* strain M1. PLoS One 5, e12253.

Fritze, D., 2004. Taxonomy of the genus *Bacillus* and related genera: the aerobic endospore-forming bacteria. Phytopathology 94, 1245–1248.

Fuller, R., 1989. A review, probiotics in man and animals. J. Appl. Bacteriol. 66, 365–378.

Fuller, R., 1992. Probiotics. The Scientific Basis. Chapman and Hall, London.

Gans, J., Wolinsky, M., Dunbar, J., 2005. Computational improvements reveal great bacterial diversity and high metal toxicity in soil. Science 309, 1387-1390.

Gatesoupe, F.J., 1994. Lactic acid bacteria increase the resistance of turbot larvae, Scophthalmus maximus, against pathogenic vibrio. Aquat. Living Resour. 7, 277-282.

Gatesoupe, F.J., 1997. Siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae, *Scophthalmus maximus*. Aquat. Living Resour. 10, 239-246.

Gatesoupe, F.J., 1999. The use of probiotics in aquaculture. Aquaculture 180, 147-165.

Gildberg, A., Johansen, A., BØgwald, J., 1995. Growth and survival of Atlantic salmon *(Salmo salar)* fry given diets supplemented with fish protein hydrolysate and lactic acid bacteria during a

challenge trial with *Aeromonas salmonicida*. Aquaculture 138, 23-24.

Gildberg, A., Mikkelsen, H., Sandaker, E., RingØ, E., 1997. Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod (*Gadus morhua*). Hydrobiologia 352, 279-285.

Gildberg, A. and Mikkelsen, H., 1998. Effects of supplementing the feed to Atlantic cod (*Gadus morhua*) fry with lactic acid bacteria and immuno-stimulating peptides during a challenge trial with *Vibrio anguillarum*. Aquaculture 167, 103-113.

Gournier-Chateau, N., Larpent, J.P., Castellanos, I., Larpent, J.L., 1994. Les Probiotiques en Alimentation Animale et Humaine. Technique et Documentation Lavoisier, Paris, 192 pp.

Gram, L., Melchiorsen, J., Spanggaard, B., Huber, I., Nielsen, T.F., 1999. Inhibition of *Vibrio anguillarum* by*Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. Appl. Environ. Microbiol. 65, 969-973.

Gram, L., Løvold, T., Nielsen, J., Melchiorsen, J., Spanggaard, B., 2001. *In vitro* antagonism of the probiont *Pseudomonas fluorescens* strain AH2 against *Aeromonas salmonicida* does not confer protection of salmon against furnculosis. Aquaculture 199, 1–11.

Gullian, M., Thompson, F., Rodriguez, J., 2004. Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. Aquaculture 233, 1–14.

Günther, J. and Jiménez-Montealegre, R., 2004. Effect of the probiotic Bacillus subtilis on the growth and food utilization of tilapia (Oreochromis niloticus) and prawn (Macrobrachium rosenbergii) under laboratory conditions. Rev. Biol. Trop. 52 (4), 937–943.

Harikrishnan R, Nisha Rani M, Balasundaram C (2003) Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. Aquaculture 221: 41-50.

Hawke JP (1979) A bacterium associated with disease of pond cultured channel catfish, *Ictalurus punctatus*. J. Fish Res. Board Can. 36: 1508–1512.

Hawke, J.P., Durborow, R.M., Thune, R.L., Camus, A.C., 1998. Enteric Septicemia of Catfish. SRAC Publication 477.

Hawke, J.P. and Khoo, L.H., 2004. Infectious diseases. In: Tucker CS, Hargreaves JA, editors. Biology and Culture of Channel Catfish. Amsterdam, The Netherlands: Elsevier. pp. 387-443.

He, P., Hao, K., Blom, J., Rückert, C., Vater, J., Mao, Z., Wu, Y., Hou, M., He, P., He, Y., Borriss, R., 2012. Genome sequence of the plant growth promoting strain *Bacillus amyloliquefaciens* subsp. *plantarum* B9601-Y2 and expression of mersacidin and other secondary metabolites. J. Biotechnol. 164, 281-291.

He, S., Liu, W., Zhou, Z., Mao, W., Ren, P., Marubashi, T., RingØ, E., 2011. Evaluation of probiotic strain *Bacillus subtilis* C-3102 as a feed supplement for koi carp (*Cyprinus carpio*). Aquaculture Research & Development

Hemstreet, B. (2010) An update on *Aeromonas hydrophila* from a fish health specialist for summer 2010. Catfish J. 24, 4.

Hendrix, R.W., 2003. Bacteriophage genomics. Curr. Opin. Microbiol. 6, 506-511.

Heo, W.S., Kim, Y.R., Kim, E.Y., Bai, S., Kong, I.S., 2013. Effects of dietary probiotic, *Lactococcus lactis* subsp. *lactis* I2, supplementation on the growth and immune response of olive flounder (*Paralichthys olivaceus*). Aquaculture 20-24, 376-379.

Hjelm, M., Bergh, Ø., Riaza, A., Nielsen, J., Melchiorsen, J., Jensen, S., Duncan, H., Ahrens, P., Birbeck, H., Gram, L., 2004a. Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. Syst. Appl. Microbiol. 27, 360–371.

Hjelm, M., Riaza, A., Formoso, F., Melchiorsen, J., Gram, L., 2004b. Seasonal incidence of autochthonous antagonistic *Roseobacter* spp. and *Vibrionaceae* strains in a turbot larva (Scophthalmus maximus) rearing system. Appl. Environ. Microbiol. 70, 7288–7294.

Hong, H.A., Duc, L.H., Cutting, S.M., 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. 29, 813–835.

Idriss, E.E., Makarewicz, O., Farouk, A., Rosner, K., Greiner, R., Bochow, H., Richter, T., Borriss, R., 2002. Extracellular phytase activity of Bacillus amyloliquefaciens FZB45 contributes to its plant-growth-promoting effect. Microbiology 148, 2097–2109.

Intervet Inc., 2003. AQUAVAC® Technical Bulletin-Control of Enteric Septicemia of Catfish, Millsboro, Delaware, USA.

Irianto, A. and Austin, B., 2002. Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. 25: 333-342.

Irianto, A., Austin, B., 2003. Use of dead probiotic cells to control furunculosis in rainbow trout, Oncorhynchus mykiss (Walbaum). J. Fish Dis. 26, 59–62.

Jack, R.W., Wan, J., Gordon, J., Harmark, K., Davidson, B.E., Hiller, A.J., Wettenhall, R.E., Hicky, M.W., Coventry, M.J., 1996. Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. Appl Environ Microbiol 62: 2897–2903.

Jeong, H., Jeong, D.E., Kim, S.H., Song, G.C., Park, S.Y., Ryu, C.M., Park, S.H., Choi, S.K., 2012. Draft genome sequence of the plant growth-promoting bacterium *Bacillus siamensis* KCTC 13613^{T}. J. Bacteriol. 194, 4148.

Johnson, M.R., 1989. Culture methods and diseases of channel catfish, Ictalurus punctatus, pp. 86-100.

Jöborn, A., Olsson, J.C., Westerdahl, A., Conway, P.L., Kjelleberg, S., 1997. Colonization in the fish intestinal tract and production of inhibitory substances in intestinal mucus and faecal extracts by *Carnobacterium* sp. strain K1. J. Fish Dis. 20, 383–392.

Jolles, P. and Jolles, J., 1984. What's new in lysozyme research? Always a model system, today as yesterday. Mol. Cell. Biochem. 63, 165-189.

Karunasagar, I., Rosalind, G.M., Karunasagar, I. and Gopal Rao, K., 1989. *Aeromonas hydrophila* septicaemia of Indian major carps in some commercial fish farms of West Godavari District, Andhra Pradesh. Curr Sci 58, 1044–1045.

Kenney, D.S. and Couch, T.L., 1981. Mass production of biological agents for plant disease, weed and insect control. In: Papavizas GC, editors. Biological Control in Crop Production BARC Symposium No. 5. Totowa, NJ: Allenheld and Osmum. 143–150.

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

Kloepper, J.W., Ryu, C.M., Zhang, S., 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94, 1259–1266.

Koumoutsi, A., Chen, X.H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., Vater, J., Borriss, R., 2004. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J. Bacteriol. 186, 1084-1096.

Kumar, R., Mukherjee, S.C., Prasas, K.P., Pal, A.K., 2006. Evaluation of *Bacillus subtilis* as a probiotic to Indian major carp *Labeo rohita* (Ham.). Aquac. Res. 37, 1215-1221.

Kumar, R., Mukherjee, S.C., Ranjan, R., Nayak, S.K., 2008. Enhanced innate immune parameters in Labeo rohita (Ham.) following oral administration of Bacillus subtilis. Fish Shellfish Immunol. 24, 168–172.

Lan, R. and Reeves, P.R., 2000. Intraspecies variation in bacterial genomes: the need for a species genome concept. Trends Microbiol 8, 396–401.

Lategan, M.J., Gibson, L.F., 2003. Antagonistic activity of *Aeromonas media* strain A199 against *Saprolegnia* sp., an opportunistic pathogen of the eel, *Anguilla australis* Richardson. J. Fish Dis. 26, 147–153.

Lategan, M.J., Torpy, F.R., Gibson, L.F., 2004a. Biocontrol of saprolegniosis in silver perch *Bidyanus bidyanus* (Mitchell) by *Aeromonas media* strain A199. Aquaculture 235, 77–88.

Lategan, M.J., Torpy, F.R., Gibson, L.F., 2004b. Control of saprolegniosis in the eel *Anguilla australis* Richardson, by *Aeromonas media* strain A199. Aquaculture 240, 19-27.

Lawrence, M.L., Banes, M.M., 2005. Tissue persistence and vaccine efficacy of an O polysaccharide mutant strain of Edwardsiella ictaluri. J. Aquat. Anim. Health 17, 228-232.

Liu, S., Endo, K., Ara, K., Ozaki, K., Ogasawara, N., 2008. Introduction of marker-free deletions in *Bacillus subtilis* using the AraR repressor and the ara promoter. Microbiology 154, 2562–2570.

Lukashin, A. V. and Borodovsky, M., 1998. GeneMark.hmm: New solutions for gene finding. Nucleic Acids Res. 26, 1107-1115.

Macaluso, A. and Mettus, A.M., 1991. Efficient transformation of *Bacillus thuringiensis* requires nonmethylated plasmid DNA. J. Bacteriol. 173, 1353-1356.
Magnadóttir, B., Lange, S., Gudmundsdottir, S., Bøgwald, J., Dalmo, R.A., 2005. Ontogeny of humoral immune parameters in fish. Fish Shellfish Immunol. 19, 429–439.

Maguin, E., Prevost, H., Ehrlich, S.D., Gruss, A., 1996. Efficient insertional mutagenesis in lactococci and other Gram-positive bacteria. J. Bacteriol. 178, 931–935.

Makridis, P., Fjellheim, A.J., Skjermo, J., Vadstein, O., 2000. Colonization of the gut in first feeding turbot by bacterial strains added to the water or bioencapsulated in rotifers. Aquacult. Int. 8, 367–380.

Makridis, P., Martins, S., Vercauteren, T., Van Driessche, K., Decamp, O., Dinis, M.T., 2005. Evaluation of candidate probiotic strains for gilthead sea bream larvae (*Sparus aurata*) using an *in vivo* approach. Lett. Appl. Microbiol. 40, 274–277.

Marrero, R. and Welkos, S.L., 1995. The transformation frequency of plasmids into *Bacillus anthracis* is affected by adenine methylation. Gene 152, 75-78.

McNeely, J., 2006. Evaluation of an Aquavac-ESC® booster on production of food-size channel catfish Ictalurus punctatus in earthern ponds.

Medini, D., Donati, C., Tettelin, H., Masignani, V., Rappuoli, R., 2005. The microbial pan-genome. Curr. Opin. Genet. Dev. 15, 589–594.

Medini, D., Serruto, D., Parkhill, J., Relman, D.A., Donati, C., et al., 2008. Microbiology in the post-genomic era. Nat. Rev. Microbiol. 6: 419–430.

Moriarty, D.J.W., 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164, 351-358.

Mott, D.F., Brunson, M.W., 1997. A historical perspective of catfish production in the

southeast in relation to avian predation, pp. 23-30.

Muhammad, A., Jones, K.G., 2009. An Assessment of Dynamic Behavior in the US Catfish Market: An Application of the Generalized Dynamic Rotterdam Model. J. Appl. Agric. Econ. 41.

Nakamura, L. K., 1989. Taxonomic relationship of black-pigmented *Bacillus subtilis* strains and a proposal for *Bacillus atrophaeus* sp. nov. Int. J. Syst. Bacteriol. 39, 295–300.

National Committee for Clinical Laboratory Standards, 2012. Performance standards for antimicrobial disk susceptibility test; Approved Standard-Ninth Edition. Wayne, PA: Clinical and Laboratory Standards Institute.

Nayak, S.K., Swain, P., Mukherjee, S.C., 2007. Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, *Labeo rohita* (Ham.). Fish shellfish Immunol. 4, 892-896.

Nayak, S.K., 2010. Probiotics and immunity: a fish perspective. Fish Shellfish Immunol. 29, 2–14.

Newaj-Fyzul, A., Adesiyun, A.A., Mutani, A., Ramsubhag, A., Brunt, J., Austin, B., 2007. *Bacillus subtilis* AB1 controls *Aeromonas* infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum). J. Appl. Microbiol. 103, 1699–1706.

Newton, J.C., Wolfe, L.G., Grizzle, J.M., Plumb, J.A., 1989. Pathology of experimental enteric septicemia in channel catfish, *Ictalurus punctatus*, following immersion exposure to *Edwardsiella ictaluri*. J. Fish Dis. 12, 335-347.

Nikoskelainen, S., Ouwehand, A., Salminen, S., Bylund, G., 2001. Protection of rainbow trout (*Oncorhynchus mykiss*) from furunculosis by *Lactobacillus rhamnosus*. Aquaculture 198, 29-236.

Nielsen, M.E., Høi, L., Schmidt, A.S., Qian, D., Shimada, T., Shen, J.Y. and Larsen, J.L. (2001) Is *Aeromonas hydrophila* the dominant motile *Aeromonas* species that causes disease outbreaks in aquaculture production in the Zhejiang Province of China? Dis. Aquat. Organ 46, 23–29.

Noga, E.J., 2000. Fish disease: diagnosis and treatment. Iowa State University.

Olsson, J.C., Westerdahl, A., Conway, P.L., Kjelleberg, S., 1992. Intestinal colonization potential of turbot (*Scophthalmus maximus*)-and dab (*Limanda limanda*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. Appl. Environ. Microbiol. 58, 551-556.

Palmisano, M.M., Nakamura, L.K., Duncan, K.E., Istock, C.A., Cohan, F.M., 2001. *Bacillus sonorensis* sp. nov., a close relative of *Bacillus licheniformis*, isolated from soil in the Sonoran Desert, Arizona. Int. J. Syst. Evol. Microbiol. 51, 1671–1679.

Panangala, V., Shoemaker, C., Klesius, P., 2005a. Phenotypic and genotypic comparisons of *Edwardsiella ictaluri* and *Edwardsiella tarda* isolates from fish. Aquaculture America Conference

Panangala, V.S., Santen, V.L., Shoemaker, C.A., Klesius, P.H., 2005b. Analysis of 16S–23S intergenic spacer regions of the rRNA operons in *Edwardsiella ictaluri* and *Edwardsiella tarda* isolates from fish. J. Appl. Microbiol. 99, 657-669.

Panigrahi, A., Kiron, V., Kobayashi, T., Puangkaew, J., Satoh, S., Sugita, H., 2004. Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. Vet. Immunol. Immunopathol. 102, 379-388.

Panigrahi, A., Kiron, V., Puangkaew, J., Kobayashi, T., Satoh, S., Sugita, H., 2005. The viabiligy of probiotic bacteria as a factor influencing the immune response in rainbow trout *Oncorhynchus mykiss*. Aquaculture 243, 241-254.

Panigrahi, A., Kiron, V., Satoh, S., Hirono, I., Kobayashi, T., Sugita, H., Puangkaew, J., Aoki, T., 2007. Immune modulation and expression of cytokine genes in rainbow trout *Oncorhynchus mykiss* upon probiotic feeding. Dev. Comp. Immunol. 31, 372–382.

Pannucci, J., Okinaka, R.T., Sabin, R., Kuske, C.R., 2002. *Bacillus anthracis* pXO1 plasmid sequence conservation among closely related bacterial species. J. Bacteriol. 184: 134–141.

Patrie-Hanson, L., Jerald Ainsworth, A., 1999. Humoral immune responses of channel catfish (*Ictalurus punctatus*) fry and fingerlings exposed to *Edwardsiella ictaluri*. Fish Shellfish Immunol. 9, 579-589.

Peypoux, F., Bonmatin, J.M., Wallach, J., 1999. Recent trends in the biochemistry of surfactin. Appl. Microbiol. Biotechnol. 51, 553–563.

Plumb, J.A., Quinlan, E.E., 1986. Survival of Edwardsiella ictaluri in pond water and bottom mud. Prog. Fish-Cult. 48, 212-214.

Pridgeon, J.W., Klesius, P.H., Mu, X., Song, L., 2011. An *in vitro* screening method to evaluate chemicals as potential chemotherapeutants to control *Aeromonas hydrophila* infection in channel catfish. J. Appl. Microbiol. 111, 114-124.

Queiroz, J.F. and Boyd, C.E., 1998. Effects of bacterial inoculum in channel catfish ponds. J. World Aquacult. Soc 29, 67-73.

Raida, M.K., Larsen, J.L., Nielsen, M.E., Buchmann, K., 2003. Enhanced resistance of rainbow trout, *Onchorhynchus mykiss* (Walbaum), against *Yersinia ruckeri* challenge following oral administration of Bacillus subtilis and B. licheniformis (BioPlus2B). J. Fish Dis. 26, 495-498.

Rendueles, O., Ferrières, L., Frétaud, M., Bégaud, E., Herbomel, P., Levraud, J.P., Ghigo, J.M., 2012. A new zebrafish model of oro-intestinal pathogen colonization reveals a key role for adhesion in protection by probiotic bacteria. PLoS Pathog 8(7): e1002815. doi:10.1371/journal.ppat.1002815

Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., Menasveta, P., 1998. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture 167, 301–313.

Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., Menasaveta, P., 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). Aquaculture 191, 271–288.

Reva, O.N., Dixelius, C., Meijer, J., Priest, F.G., 2004. Taxonomic characterization and plant colonizing abilities of some bacteria related to *Bacillus amyloliquefaciens* and *Bacillus subtilis*. FEMS Microbiol. Ecol. 48, 249–259.

Roberts, M.S., Nakamura, L.K., Cohan, F.M., 1994. *Bacillus mojavensis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. Int. J. Syst. Bacteriol. 44, 256–264.

Roberts, M. S., Nakamura, L. K., Cohan, F. M., 1996. *Bacillus vallismortis* sp. nov., a close relative of *Bacillus subtilis*, isolated from soil in Death Valley, California. Int. J. Syst. Bacteriol. 46, 470–475.

Robertson, P.A.W., O'Dowd, C., Burrells, C., Williams, P., Austin, B., 2000. Use of *Carnobacterium* sp. as a probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). Aquaculture 185, 235-243.

Rollo, A., Sulpizio, R., Nardi, M., Silvi, S., Orpianesi, C., Caggiano, M., Cresci, A., Carnevali, O., 2006. Live microbial feed supplement in aquaculture for improvement of stress tolerance. Fish Physiol. Biochem. 32, 167–177.

Rückert, C., Blom, J., Chen, X.H., Reva, O., Borris, R., 2011. Genome sequence of *B. amyloliquefaciens* type strain DSM7^{T} reveals differences to plant-associated *B. amyloliquefaciens* FZB42. J. Biotechnol. 155, 78-85.

Salinas, I., Cuesta, A., Esteban, M.Á., Meseguer, J., 2005. Dietary administration of Lactobacillus delbrüeckii and Bacillus subtilis, single or combined, on gilthead seabream cellular innate immune responses. Fish Shellfish Immunol. 19, 67–77.

Salinas, I., Abelli, L., Bertoni, F., Picchietti, S., Roque, A., Furones, D., Cuesta, A., Meseguer, J., Esteban, M.Á., 2008. Monospecies and multispecies probiotic formulations produce different systemic and local immunostimulatory effects in the gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 25, 114–123.

Sharifuzzaman, S.M. and Austin, B., 2009. Influence of probiotic feeding duration on disease resistance and immune parameters in rainbow trout. Fish Shellfish Immunol. 27, 440-445.

Sharp, G.J.E., Secombes, C.J., 1993. The role of reactive oxygen species in the killing of the bacterial fish pathogen *Aeromonas salmonicida* by rainbow trout macrophages. Fish Shellfish Immunol. 3,119–129.

Shatalin, K.Y. and Neyfakh, A.A., 2005. Efficient gene inactivation in *Bacillus anthracis*. FEMS Microbiol. Lett. 245, 315-319.

Shoemaker, C.A., Klesius, P.H., Evans, J.J., 2002. In ovo methods for utilizing the modified live Edwardsiella ictaluri vaccine against enteric septicemia in channel catfish. Elsevier, pp. 221-227.

Shoemaker, C.A., Klesius, P.H., Evans, J.J., 2007. Immunization of eyed channel catfish, Ictalurus punctatus, eggs with monovalent Flavobacterium columnare vaccine and bivalent F. columnare and Edwardsiella ictaluri vaccine. Vaccine 25, 1126-1131.
Shoemaker, C.A., Klesius, P.H., Evans, J.J., Arias, C.R., 2009. Use of modified live vaccines in aquaculture. J. World Aquacult. Soc. 40, 573-585.

Silvi, S., Nardi, M., Sulpizio, R., Orpianesi, C., Caggiano, M., Carnevali, O., Cresci, A., 2008. Effect of the addition of *Lactobacillus delbrueckii* subsp. *delbrueckii* on the gut microbiota composition and contribution to the well-being of European sea bass (*Dicentrarchus labrax*, L.). Microb. Ecol. .Health D. 20, 53-59.

Simona, P., Massimo, M., Anna, R.T., Raffaella, R., Anna, M.F., Victoriano, M., Oliana, C., 2007. Effects of administration of probiotic strains on GALT of larval gilthead seabream: Immunohistochemical and ultrastructural studies. Fish Shellfish Immunol. 22, 57-67.

Smith, P. and Davey, S., 1993. Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent pseudomonad. J. Fish Dis, 16, 521-524.

Snipen, L. and Ussery, D. W., 2010. Standard operating procedure for computing pangenome trees. Stand. Genomic Sci. 2, 135-141.

Sorokulova, I., 2008. Preclinical testing in the development of probiotics: a regulatory perspective with *Bacillus* strains as an example. Clin. Infect. Dis. 46: SS92–95.

Spanggaard, B., Huber, I., Nielsen, J., Sick, E.B., Pipper, C.B., Martinussen, T., Slierendrecht,W.J., Gram, L., 2001. The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. Environ. Microbiol. 3, 755–765.

Strøm, E. and Ringø, E., 1993. Changes in bacterial flora in developing cod, *Gadus morhua* (L.), larvae after inoculation of *Lactobacillus plantarum* in the water. In: Walther, B., Fyhn, H.J.

(Eds.), Physiological and Biochemical Aspects of Fish Larval Development. University of Bergen, Norway, pp. 226–228.

Sugita, H., Okano, R., Suzuki, Y., Iwai, D., Mizukami, M., Akiyama, N., Matsuura, S., 2002. Antibacterial abilities of intestinal bacteria from larval and juvenile Japanese flounder against fish pathogens. Fisheries Sci. 68, 1004–1011.

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

Tettelin, H., Masignani, V., Cieslewicz, M.J., Donati, C., Medini, D., et al., 2005. Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial ‘‘pan-genome’’. Proc. Natl. Acad. Sci. U SA 102, 13950–13955.

Thore, J.E. and Roberts, R.J. (1972) Aeromonad epidemic in brown trout (*Salmo trutta* (L.) J. Fish Biol. 4, 441–541.

Tucker, C.C., Robinson, E., 1990. Channel catfish farming handbook. Kluwer Academic Pub.

USDA, 2003a. Part I: Reference of Fingerling Catfish Health and Production Practices in the United States. Fort Collins, CO, USA: National Health Monitoring System.

USDA, 2003b. Part II: Reference of Foodsize Catfish Health and Production Practices in the United States. Fort Collins, CO, USA: National Health Monitoring System.

USDA, 2006. 2005 Census of Aquaculture. National Agricultural Statistics Service (NASS), United States Department of Agriculture

Ussery, D.W., Wassenaar, T.M., Borini, S., 2009. Computing for comparative microbial genomics: bioinformatics for microbiologists: Springer.

Vaseeharan, B., Lin, J., Ramasamy, P., 2004. Effect of probiotics, antibiotic sensitivity, pathogenicity, and plasmid profiles of *Listonella anguillarum*-like bacteria isolated from *Penaeus monodon* culture systems. Aquaculture 241, 77–91.

Vázquez, J.V., González, M.P., Murado, M.A., 2005. Effects of lactic acid bacteria cultures on pathogenic microbiota from fish. Aquaculture 245, 149-161

Vazquez-Juarez, R., 1996. Factors involved in the colonization of fish intestine by yeasts. Fil. Dr. thesis, Göteborg University, Sweden, 134 pp.

Vazquez-Juarez, R., Andlid, T., Gustafsson, L., 1997. Adhesion of yeast isolated from fish gut to crude intestinal mucus of rainbow trout, *Salmo gairdneri*. Molec. Mar. Biol. Biotechnol. 6, 64–71.

Verschuere, L., Rombaut, G., Huys, G., Dhont, J., Sorgeloos, P., Verstraete, W., 1999. Microbial control of the culture of *Artemia* juveniles through preemptive colonization by selected bacterial strains. Appl. Environ. Microbiol. 65, 2527–2533.

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

Verschuere, L., Heang, H., Criel, G., Sorgeloos, P., Verstraete,W., 2000b. Selected bacterial strains protect Artemia spp. from the pathogenic effects of *Vibrio* proteolyticus CW8T2. Appl. Environ. Microbiol. 66 (3), 1139–1146.

Vine, N.G., Leukes, W.D., Kaiser, H., Daya, S., Baxter, J., Hecht, T., 2004. Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. J. Fish Dis. 27, 319–326.

Wagner, B.A., Wise, D.J., Khoo, L.H., Terhune, J.S., 2002. The epidemiology of bacterial diseases in food-size channel catfish. J. Aquat. Anim. Health 14, 263-272.
Wang, Y.B., Li, J.R., Lin, J., 2008. Probiotics in aquaculture: challenges and outlook. Aquaculture 281, 1–4.

Wilson, K.E., Flor, J.F., Schwartz, R.E., Joshua, H., Smith, J.L., Pelak, B.A., Liesch, J.M., Hensens, O.D., 1987. Difficidin and oxydifficidin: novel broad spectrum antibacterial antibiotics produced by *Bacillus subtilis* . 2. Isolation and physicochemical characterization. J. Antibiot. 40, 1682–1691.

Wise, D.J., 2006. ESC vaccination shown to improve production efficiencies. Catfish J., 6–7.

Xue, G.P., Johnson, J.S., Dalrymple, B.P., 1999. High osmolarity improves the electro-transformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus Licheniformis*. J. Microbiol. Meth. 34, 183-191.

Yan, X., Yu, H.J., Hong, Q., Li, S.P., 2008. Cre/lox system and PCR-based genome engineering in *Bacillus subtilis*. Appl. Environ. Microbiol. 74, 5556–5562.

Yasui, K., Kano, Y., Tanaka, K., Watanabe, K., Shimizu-Kadota, M., Yoshikawa, H., Suzuki, T., 2009. Improvement of bacterial transformation efficiency using plasmid artificial modification. Nucleic Acids Res. 37 (1): e3. doi: 10.1093/nar/gkn884

Zakataeva, N.P., Nikitina, O.V., Gronskiy, S.V., Romanenkov, D.V., Livshits, V.A., 2010. A simple method to introduce marker-free genetic modifications into the chromosome of naturally nontransformable *Bacillus amyloliquefaciens* strains. Appl. Microbiol. Biothenol. 85, 1201-1209.

Zhang, G.Q., Bao, P., Zhang, Y., Deng, A.H., Chen, N., Wen, T.Y., 2011. Enhancing electro-transformation competency of recalcitrant *Bacillus amyloliquefaciens* by combining cell-wall weakening and cell-membrane fluidity disturbing. Anal. Biochem. 409, 130-137.

Zhang, Q., Ma, H., Mai, K., Zhang, W., Liufu, Z., Xu, W., 2010. Interaction of dietary *Bacillus subtilis* and fructooligosaccharide on the growth performance, non-specific immunity of sea cucumber, *Apostichopus japonicas*. Fish shellfish Immunol. 29, 204-211.

Zhang, X.Z., Yan, X., Cui, Z.L., Hong, Q., Li, S.P., 2006. mazF, a novel counter-selectable marker for unmarked chromosomal manipulation in *Bacillus subtilis*. Nucleic Acids Res. 34:e71

Zimmerman, S.B., Schwartz, C.D., Monaghan, R.L., Pelak, B.A., Weissberger, B., Gilfilan, E.C., Mochales, S., Hernandez, S., Currie, S.A., Tejera, E., Stapley, E.O., 1987. Difficidin and oxydifficidin: novel broad spectrum antibacterial antibiotics produced by *Bacillus subtilis.* 1. Production, taxonomy and antibacterial activity. J. Antibiot. 40, 1677–1681.