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Isolation and Evaluation of New Probiotic Bacteria for use in Shellfish Hatcheries: II. Effects of a *Vibrio* sp. Probiotic Candidate Upon Survival of Oyster Larvae (*Crassostrea virginica*) in Pilot-Scale Trials

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ISOLATION AND EVALUATION OF NEW PROBIOTIC BACTERIA FOR USE IN SHELLFISH HATCHERIES: II. EFFECTS OF A *VIBRIO* SP. PROBIOTIC CANDIDATE UPON SURVIVAL OF OYSTER LARVAE (*CRASSOSTREA VIRGINICA*) IN PILOT-SCALE TRIALS

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ABSTRACT Environmentally-friendly methods for controlling microbial pathogenesis in aquaculture with probiotic bacteria are becoming increasingly preferred over the use of chemical means, such as disinfectants or antibiotics. Previous research at the Milford Laboratory has shown that naturally-occurring bacteria isolated from the digestive glands of adult oysters (*Crassostrea virginica*) show promise as potential probiotic additives in oyster larviculture, based on bench-scale experiments. The previous, bench-scale challenge studies reported in the accompanying article (Lim et al. this volume) indicated that 48-h survival of 2-day-old oyster larvae supplemented with *Vibrio* sp. strain OY15 improved after challenge with pathogenic *Vibrio* sp. strain B183 compared with the pathogen alone. This study investigated further the effectiveness of probiotic candidate OY15 to improve survival of oyster larvae to metamorphosis under pilot-scale culture conditions, both with and without pathogen B183 challenge. The effective dosage of probiotic candidate OY15 that significantly improved larval survival was determined to be 10^3 cfu/mL. The LD_{50} calculated for pathogen B183 was 9.6×10^4 cfu/mL. Results from these bioassays indicated that addition of probiotic candidate OY15 significantly improved survival of oyster larvae to metamorphosis when challenged with pathogen B183 in pilot-scale trials. These studies can provide the basis for the development of functional foods for use in shellfish larviculture that incorporate a naturally-occurring, probiotic bacterial strain.

KEY WORDS: probiotic bacteria, shellfish larvae, oyster larviculture, larvae survival, *Vibrio*, *Crassostrea virginica*

INTRODUCTION

Environmentally friendly methods for controlling microbial pathogenesis in aquaculture with probiotic bacteria have gained considerable research interest and are becoming increasingly preferred as viable, alternative management practices for disease prevention. Bacterial diseases, commonly caused by *Vibrio* (Estes et al. 2004) and *Aeromonas* spp. (Kesarcodi-Watson et al. 2008), can result in major mortalities in bivalve hatcheries, and cause major financial losses for commercial shellfish growers. Chemical means, such as disinfectants and antimicrobial drugs, which can have obvious benefits to infected animals, have been overused for disease prevention or growth enhancement (Van den Bogaard & Stobberingh 2000). Prophylactic use of antimicrobial drugs has led to the emergence of antibiotic-resistant bacterial strains that have survived a course of treatment by antibiotics, and have the potential to transfer their resistance genes to other bacterial strains via horizontal gene transfer (Schwarz et al. 2001, Akinbowale et al. 2006). The emergence of antibiotic-resistant bacteria was most dramatically felt in the shrimp aquaculture industry; increased production, overstocking, and unregulated usage of antibiotics to control *Vibrio harveyi* (a main bacterial shrimp pathogen) caused significant production crashes in Asian countries (Karunasagar et al. 1994, Moriarty 1998). Shrimp production in the Philippines dropped 55% between 1995 and 1997 as a result of outbreaks of this pathogen, and Thailand's shrimp production dropped 40% between 1994 and 1997 because of *V. harveyi* as well as shrimp viruses (Moriarty 1998). Certain antibiotic-resistant bacteria of aquaculture farm origin have even been able to transfer resistance genes to human pathogens, causing a potential risk to human health (Van den Bogaard & Stobberingh 2000, Witte 2000, Schwarz et al. 2001). Tighter government regulations have been implemented in Asian countries that restrict antibiotic usage in animal production for human consumption. Although Thailand banned the use of chloramphenicol for disease prevention in shrimp aquaculture in 1999, trace levels were still being detected in exported product in 2004 (Heckman 2004).

Developing concerns regarding the unnecessary use of antimicrobial drugs in animal production for human consumption have raised awareness of the need for alternative, cost-effective methods, such as the use of probiotic bacteria, as microbial control agents in shellfish larviculture. Use of probiotic bacteria in shellfish larviculture may improve veliger larval survival to metamorphosis, the most critical phase of shellfish aquaculture when most mortality occurs (Loosanoff & Davis 1963). Desirable probiotic bacteria should benefit larval survival as well as benefit or not impair microalgae used as feed in culture systems (Kesarcodi-Watson et al. 2008). Supplementation of algal feeds with probiotic bacteria in shellfish larviculture has been shown to enhance the nutritional value of the algae to the larvae, and to provide early colonization of microflora in the gut to aid digestion (Verschuere et al. 2000). Probiotic strains have also been shown to speed development of, or stimulate, the innate immune response to potentially-pathogenic bacteria in shellfish (Vaughan et al. 2002).

In a review article, Verschuere et al. (2000) listed properties that a safe, desirable, and effective probiotic should possess:

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1. It should not be harmful to the host.
2. It should be accepted by the host through ingestion and potential colonization and replication within the host's digestive system.
3. It should reach the location where the desired probiotic effect is required to take place.
4. It should work *in vivo* as opposed to *in vitro*.
5. It should not contain virulence resistance genes or antibiotic resistance genes.

Recently, we isolated and evaluated the safety and efficacy of naturally-occurring probiotic bacteria from the digestive glands of the bay scallop *Argopecten irradians* (Lamarck 1819) and the Eastern oyster *Crassostrea virginica* (Gmelin 1791), and described the effectiveness of one *Vibrio* sp. probiotic candidate (OY15) in improving survival of oyster larvae when challenged with a known *Vibrio* sp. shellfish-larvae pathogen in bench-scale experiments. This stepwise examination of probiotic candidate OY15 has confirmed that it is indeed safe for use during coculture of oyster larvae and the microalgal feed T-ISO (*Isochrysis galbana*), and is effective in improving survival of oyster veligers when challenged with the *Vibrio* sp. shellfish-larvae pathogen in short-term *in vivo* microplate bioassays (Lim et al. 2011).

This article describes the ability of probiotic candidate OY15 to improve survival of veliger oyster larvae to metamorphosis when challenged with the same *Vibrio* sp. shellfish-larval pathogen in pilot-scale *in vivo* trials. In addition, this article also presents results of *in vitro* antibiotic sensitivity testing of OY15, three other probiotic candidate bacteria isolates (S1, S2, and S7), and pathogen B183 against a panel of antibiotic disks. The goal of screening these isolates for the presence of antibiotic resistance genes is to guard against transmission of such genes to other animal or human pathogens (Decamp & Moriarty 2006). Last, this study investigated the ability of the larvae to assimilate probiotic candidate OY15 by ingestion. Results can provide the basis for the development of a naturally-occurring amendment to aquaculture feed that can safely and significantly improve survival of oyster larvae to metamorphosis, improve digestion of algal feed, and confer protection against pathogenic bacteria.

METHODS

Preliminary Molecular Identification of Isolates

Of six initial, potential probiotic candidates, five were identified by the Biolog Microbial Identification System (Biolog MicroLog System, release 4.2, Biolog Inc., Hayward, CA) as being "similar to *Vibrio* spp.," and one remained unknown. These five potential probiotic candidates, plus B183, a known shellfish-larval pathogen, were further characterized by 16S rRNA gene sequencing using the methods of Marchesi et al. (1998), Thompson et al. (2005), and Thompson et al. (2007) at the University of Maryland Center for Environmental Science at the Institute of Marine and Environmental Technology in Baltimore, MD.

LD_{50} Calculation of Pathogen B183

During a 48-h exposure, 2-day-old oyster larvae were challenged with five individual dosages of pathogen B183 (10^6, 10^5, 10^4, 10^3, and 10^2 cfu/mL; $n = 4$ per treatment) using 12-well microplates (Estes et al. 2004) held at 25°C in an Ambi-Hi-Low Incubator (Laboratory-Line Instruments). Each well contained 4 mL sterile filtered seawater, 60 2-day-old larvae (15 larvae/mL seawater), and the indicated dosage of pathogen. After 48 h of incubation, larvae were preserved with Lugol's solution and formaldehyde, and larval counts were completed by light microscopy to determine survival. LC_{50} for this organism was calculated using the following equation (Reed & Muench 1938):

$$\text{Log LD}_{50} = \frac{(\log D_n + 50) - \%\text{ of death at } D_n}{(\%\text{ of death at } D_v - \%\text{ of death at } D_n) \times \log(\text{dilutionfactor})}$$

where D_n is the dilution when the percent of death is immediately less than 50%, D_v is the dilution when the percent of death is immediately greater than 50%, and log (dilution factor) is log of $10 = 1$, based on the 10-fold serial dilution of pathogenic dosages.

LD_{50} for pathogen B183 was determined to be 9.6×10^4 cfu/mL. In addition, this value was confirmed using the trimmed Spearman-Karber method (Hamilton et al. 1977) for estimating median lethal dose. The U.S. Environmental Protection Agency (2006) provides a program that calculates the LC_{50} based on this method. Hence, effective dosage of pathogen B183 was 9.6×10^4 cfu/mL (see Results) for all larval–pathogen bioassays conducted during this study.

Effective Dosage of Probiotic Candidate OY15

The effective dosage of probiotic candidate OY15 that would significantly improve survival of oyster larvae was determined by a 21-day bioassay during which 2-day-old oyster larvae were supplemented with three doses of probiotic candidate OY15 (10^2, 10^3, and 10^4 cfu/mL) and one dose of pathogen B183 (10^5 cfu/mL; Table 1). Three control treatments were incorporated into the design of this experiment: a larvae control treatment comprised of oyster larvae with no bacteria, a probiotic control treatment comprised of oyster larvae supplemented only with 10^3 cfu/mL probiotic candidate OY15, and a pathogen control treatment comprised of oyster larvae challenged with 10^5 cfu/mL pathogen B183. All treatments were fed the microalga *Isochrysis* sp. T-ISO daily and replicated 4 times. Larval counts were 10 oyster larvae/mL in 800 mL sterile seawater contained in a 1-L beaker held at 25°C for approximately 3 wk or to pediveliger stage. Seawater changes in beakers and bacterial dosing were done every other day for the duration of the challenge. Effective dosage of probiotic candidate OY15 was 10^3 cfu/mL (Table 2, see Results).

TABLE 1.

Dosage concentrations of probiotic candidate OY15 and pathogen B183 used as treatments for larval–probiotic–pathogen bioassays to determine the optimal dosage of probiotic candidate OY15 that would promote significantly higher survival of oyster larvae.

	Dosage (cfu/mL)	
	OY15	**B183**
High OY15 + B183	10^4	10^5
Med OY15 + B183	10^3	10^5
Low OY15 + B183	10^2	10^5
Control OY15	10^3	0
B183 control	0	10^5
Larvae only	No added bacteria	

TABLE 2.

Results of least significant difference multiple-comparison test (Statistix 9, 2008) of treatments from the larval–probiotic–pathogen bioassay to determine optimal dosage of probiotic candidate OY15 that would promote significantly higher survival of larvae.

Significant Comparisons by Treatment	*P* Value
Day 7	
Medium OY15 + B183 vs.	
Larvae only	0.05
B183 control	<0.01**
Low OY15 + B183	<0.01**
Day 14	
OY15 probiotic control vs.	
B183 control	0.02*
Low OY15 + B183	0.02*
Hi OY15 + B183	0.02*
Med OY15 + B183 vs.	
B183 control	0.01**
Low OY15 + B183	0.02*
Hi OY15 + B183	0.01*
Day 21	
No significant differences	

See Table 1 for low, medium, and high dosage concentrations.
* Statistically significant *P* value.
** Highly significant *P* value.

Pilot-Scale Trial

Pilot-scale (12-L bucket) trials were conducted to confirm results from 12-well-microplate and 1-L-beaker studies. Two-day-old oyster larvae were supplemented with 10^3 cfu/mL probiotic candidate OY15 and fed with the microalga T-ISO. After a 3-day, pre-exposure time with probiotic candidate OY15, larvae were challenged with pathogen B183 at a dosage of 10^5 cfu/mL. Treatments included a larvae survival control (no added bacteria), a mortality control comprised of larvae challenged by pathogen B183 only, a probiotic larvae survival control comprised of larvae supplemented with probiotic candidate OY15, and a combination treatment comprised of larvae challenged with pathogen B183 in the presence of probiotic OY15. Larvae were cultured in 12-L buckets containing 8 L sterile-filtered seawater, and maintained at 25°C to pediveliger stage (16 days). Although the larvae were on a daily feeding regime with the microalga T-ISO, bacterial dosing occurred every other day, concurrent with water changes.

Probiotic Strain Resistance to Antibiotics Specific for Gram-Negative Organisms

Mueller-Hinton agar plates of uniform thickness were streaked individually with suspensions of 4 probiotic candidates (OY15, S1, S2, and S7) and pathogen B183 using sterile swabs to produce a confluent lawn of bacterial growth on the surface of the agar on incubation. Fourteen antibiotic sensitivity disks (Becton-Dickinson Sensi-Disk Susceptibility Tests) (Table 3), selected specifically against Gram-negative organisms, were placed evenly onto the agar surface using sterile forceps, 5 disks per 100-mm Petri dish. Blank, sterile disks were dipped aseptically into sterile seawater and placed onto the center of each inoculated plate as negative controls. Within 15 min after disk application, plates were inverted within plastic sleeves and incubated at 23°C. After 18 h, plates were examined, and zones of complete inhibition were measured to the nearest millimeter (NCCLS 1999). Zones of complete inhibition were compared with zone diameter standards from CLSI Document 100-S17 (M2): Disk Diffusion

TABLE 3.

Antimicrobial susceptibility testing: zone diameters for 4 probiotic candidates and pathogen B183 against 14 antibiotic sensitivity disks.

Strain (10^6 cfu/mL)			S1			S2			S7			OY15			B183(Pathogen)		
Antibiotic disc	Initials	Dosage (μg)	Res	Int	Sus	Res	Int	Sus	Res	Int	Sus	Res	Int	Sus	Res	Int	Sus
Ampicillin	AM	10	*				**		*			*				**	
Ceftazidime	CAZ	30	*					***			***			***			***
Cefuroxime	CXM	30	*					***		**				***			***
Cephalothin	CF	30		**			**			**	*	*					***
Chloramphenicol	C	5			***			***			***			***			***
Ciprofloxacin	CIP	5			***			***			***			***			***
Gentamycin	GM	10			***			***			***			***			***
Imipinem	IPM	10			***			***			***			***			***
Neomycin	N	5			***		**		*				**			**	
Oxolinic acid	OA	2			***			***			***			***			***
Oxytetracycline	T	30			***			***			***			***			***
Sulfamethoxine–trimethoprim	SXT	25			***			***			***			***			***
Tetracycline	Te	5			***			***			***			***			***
Trimethoprim	TMP	5	*					***		**			**				***

Gentamycin results based on Res = 6 mm, Int = 7–9 mm, and Sus = 10 mm.
Oxolinic acid results based on Res ≤ 10 mm and Sus ≥ 11 mm.
Int, intermediate (zone diameter, 13.7–16.7mm); Res, resistant (zone diameter, ≤12.4mm); Sus, susceptible (zone diameter, ≥17.5).
Standards from CLSI document M100-S17 (M2): Disc Diffusion Supplemental Tables, Performance Standards for Antimicrobial susceptibility testing, from Clinical and Laboratory Standards Institute, Wayne, PA (CLSI 2007).

Supplemental Tables, Performance Standards for Antimicrobial Susceptibility testing (Table 3) to determine resistance, intermediate susceptibility and susceptibility (CLSI 2007).

Ingestion of Probiotic Candidate OY15 by Larvae

Oyster larvae 11 days postfertilization were exposed for 20 min to fluorescently labeled (BacLite, Invitrogen) probiotic candidate OY15 in sterile seawater. Fluorescence microscopy (Zeiss Axioskop 2 mot *plus* microscope, emission BP 515-565) was used to visualize the fluorescent-green-stained probiotic isolate within the esophagus and stomach of the larvae after ingestion.

Statistical Analysis

Larval survival values (presented as square root of the frequency) for all bioassays were arcsine-transformed to normalize variance (Zar 1996). Analysis of variance (Statgraphics Plus 5.1, 2001; Statpoint Technologies, Warrenton, VA) was used to test the transformed, normally distributed data, followed by the least significant difference multiple comparison test (Statistix 9, 2008; Tallahassee, FL).

RESULTS

Preliminary Molecular Identification of Isolates

Based on sequence identities of the six potential probiotic candidates, this number was reduced to two distinct species. OY15 was identified as a *Vibrio* species with affinities to the *V. parahaemolyticus*/*V. harveyi* group, and S1 was identified as a *Bacillus cereus*-like isolate. Ribosomal RNA gene sequence analysis also identified pathogen B183 as a *Vibrio coralliilyticus*-like organism. Additional molecular tools (multilocus analysis) (Marchesi et al. 1998, Thompson et al. 2005, Thompson et al. 2007) will be used in the near future to refine the identification of probiotic OY15 and pathogen B183, as well as to identify the presence or absence of virulence resistance genes and antibiotic resistance genes in these two organisms.

LD_{50} Calculation of Pathogen B183

The Reed equation calculated the LD_{50} for pathogen B183 to be 9.6×10^4 cfu/mL. This LD_{50} result was also confirmed using the trimmed Spearman-Karber method (Hamilton et al. 1977) for estimating median lethal concentration.

Effective Dosage of Probiotic Candidate OY15

After 7 days, percent survival of larvae treated with the medium dose (10^3 cfu/mL) of OY15 + pathogen B183 was significantly higher than that of larvae treated with the low dose (10^2 cfu/ mL) of OY15 + B183, as well as B183 control treatment and larvae only. At day 14, mean percent survival of larvae given the medium dose (10^3 cfu/mL) OY15 + B183 and the OY15 probiotic control treatment (10^3 cfu/mL) were significantly higher than the high dose (10^4 cfu/mL) as well as the low dose (10^2 cfu/ mL) of OY15 + B183 and the B183 pathogen control (10^5 cfu/ mL) treatment. No significant differences (ANOVA) were observed for any of the treatments at day 21 when larvae were observed to be setting on the walls of the culture buckets, thus terminating the experiment.

Throughout weekly sampling during the course of this 21-day bioassay, no significant differences (ANOVA) were evident in percent survival of larvae supplemented with the medium dose of probiotic candidate OY15 and challenged with pathogen B183 or the probiotic control with no pathogen added. These results suggest that the medium dosage of probiotic candidate OY15 (10^3 cfu/mL) protected larvae against pathogen B183, significantly improving larvae survival by approximately 20% (Figs. 1, 2, 3 and Table 2).

Pilot-Scale Trial

This pilot-scale trial used a 3-day pre-exposure time of oyster larvae to the probiotic candidate OY15 before challenge with pathogen B183 so that OY15 could be ingested by larvae and possibly establish residency in the larval culture buckets. At day 3, before pathogen challenge, no significant differences were observed between the control larvae and larvae supplemented with probiotic OY15 ($P < 0.3883$; Fig. 4), indicating no adverse effects from OY15 on larval survival. Larvae were challenged with pathogen B183 on day 3 after sampling larvae, water change, feeding, and dosing with OY15. Effects from the pathogen could be seen at day 5 (Fig. 4), when mortalities were observed for both the pathogen treatment and the combination pathogen and probiotic treatment. After the initial "hit" from the pathogen occurred, however, larvae survival was significantly improved by the presence of probiotic OY15, especially at day 9 ($P < 0.0180$) and day 12 ($P < 0.0022$). By day 16, metamorphosis occurred and larvae were beginning to set onto the bucket walls and Mylar strips suspended in the seawater.

Strain Resistance to Antibiotics Specific for Gram-Negative Organisms

Phenotypic screening of probiotic candidates OY15, S1, S2, and S7, and pathogen B183 for antibiotic sensitivity using disk diffusion against a panel of 14 antibiotic disks effective against Gram-negative organisms confirmed that the probiotic candidates were either susceptible or had intermediate susceptibility to most of the antibiotics tested. Probiotic candidate OY15 was

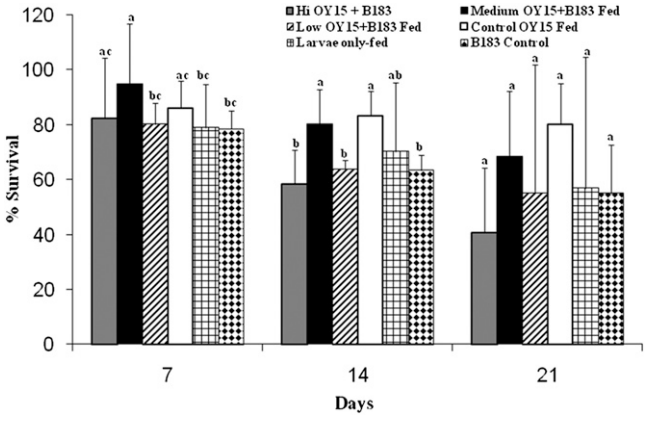

Figure 1. Optimal dose of probiotic candidate OY15. Bars indicate percent survival of oyster larvae at 7, 14, and 21 days of exposure to 3 doses of probiotic candidate OY15 and challenged with pathogen B183 to determine the optimal probiotic dose that would confer protection against pathogen challenge and significantly improve larval survival to metamorphosis. Dosage amounts for treatments included in Figures 1, 2, and 3 are indicated in Table 1. Treatments with different letters were significantly different from each other (ANOVA, $P < 0.05$).

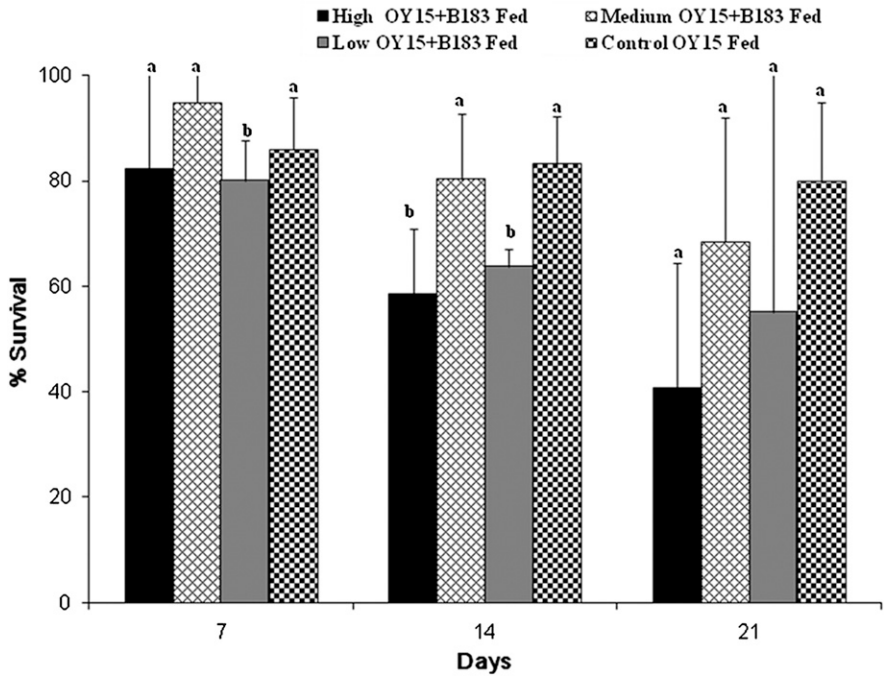

Figure 2. Bars indicate percent survival of 2-day-old oyster larvae at 7, 14, and 21 days of exposure to 3 doses of probiotic candidate OY15 and challenged with pathogen B183 compared with probiotic OY15-only treatment as the control. Percent survival for fed oyster larvae supplemented with only probiotic candidate OY15 (10^3 cfu/mL) remained relatively constant at 7, 14, and 21 days of exposure. At 7 days, percent survival of larvae treated with the medium dose of OY15 + B183 pathogen was significantly higher than those treated with the low dose of OY15. At day 14, percent survival of larvae for both the medium-dose OY15 + B183 and the OY15 probiotic control treatment was significantly higher than both the high-dose OY15 + B183 as well as the low-dose OY15 + B183.

susceptible to 10 of the antibiotic disks, showing intermediate susceptibility to neomycin (5 μg) and trimethoprim (5 μg), and exhibiting resistance to ampicillin (10 μg) as well as cephalothin (30 μg). S1 was susceptible to nine of the antibiotic disks, showing intermediate susceptibility to cephalothin (30 μg), and exhibiting resistance to ampicillin (10 μg) as well as ceftazidime (30 μg), cefuroxime (30 μg), and trimethoprim (5 μg). S2 was susceptible to 11 of the antibiotic disks, showing intermediate susceptibility to ampicillin (10 μg), cephalothin (30 μg), and neomycin (5 μg), and was not resistant to any. S7 was susceptible to nine of the antibiotic disks, showing intermediate susceptibility to cefuroxime (30 μg), cephalothin (30 μg), and trimethoprim (5 μg), and exhibiting resistance to ampicillin (10 μg) as well as neomycin (5 μg). Pathogen B183 was susceptible to 12 of the antibiotic disks, showing intermediate susceptibility to ampicillin (10 μg) and neomycin (5 μg), and was not resistant to any. Future molecular studies are necessary to verify genetic determinants (Kastner et al. 2006) of OY15 antibiotic resistance to ampicillin and cephalothin.

Ingestion of Probiotic Candidate OY15 by Larvae

Within 20 min of feeding (in sterile seawater), oyster larvae ingested fluorescently-labeled probiotic candidate OY15. Viable, fluorescent bacteria are observed in the esophagus and stomach of the larva in Figure 5, confirming acceptance by ingestion (Verschuere et al. 2000).

DISCUSSION

Aquatic animals have a close, interactive relationship with their external environment. The microbial communities in the digestive tracts of bivalve larvae are reflective of microbes that live and proliferate in the surrounding environment, and can influence larvae health and survival (Cahill 1990). These microbes can live independently of the host animal (Hansen & Olafsen 1999, Verschuere et al. 2000) and are constantly being taking up by bivalve larvae during feeding and osmoregulation. The ambient environment of farmed shellfish also supports the growth of bacteria, both benign and pathogenic, that can reach high densities depending on temperature and water quality. The digestive tract of a filter feeder is a prime niche for disease when high densities of pathogen are present in the culture water (Harris 1993), causing widespread mortality in a culture system. The prophylactic use of naturally-occurring, probiotic bacteria as biological control agents is considered an environmentally friendly method for disease prevention in bivalve hatchery culture.

Recently, we (Lim et al. 2011) conducted a stepwise evaluation (Verschuere et al. 2000) of the safety and efficacy of new probiotic bacteria for use in shellfish hatcheries. This study showed that naturally-occurring bacteria isolated from the digestive glands of adult Eastern oysters, *Crassostrea virginica*, improved survival of oyster veliger larvae in miniature bioassay tests. The Kirby-Bauer disk diffusion method was used as the selection process for probiotic candidates, screening 26 isolates for competitive exclusion or diffusible inhibitory substances against a known, *Vibrio* sp. shellfish-larval pathogen (B183). Sixteen of these probiotic candidates exhibited either partial or total inhibition of pathogen B183 and were further screened for their safe use in coculture of oyster larvae and their microalgal feed T-ISO (*Isochrysis* sp.). A desirable probiotic bacterium should be safe and beneficial to coculture with oyster larvae and microalgal feed (Kesarcodi-Watson et al. 2008). Oyster larvae

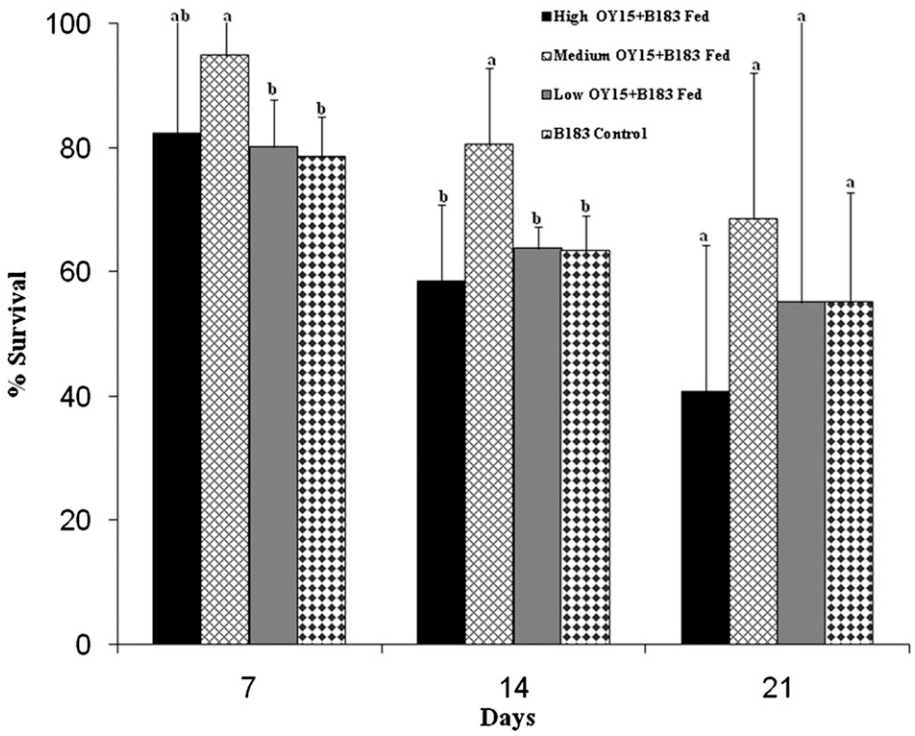

Figure 3. Bars indicate percent survival of 2-day-old oyster larvae at 7, 14, and 21 days of exposure to 3 doses of probiotic candidate OY15 compared with pathogen B183 control treatment. At 7 days, percent survival of larvae treated with the medium dose of OY15 + B183 pathogen was significantly higher than larvae treated with the low dose of OY15 + B183 as well as the B183 control treatment. At day 14, percent survival of larvae for the medium-dose OY15 + B183 treatment was significantly higher than the high-dose OY15 + B183 treatment, the low-dose OY15 + B183 treatment, and the B183 pathogen control.

exposed to monoxenic cultures of each of these 15 (1 candidate could not be cultured further) probiotic candidates for 48 h exhibited low mortality (10%), similar to control (no bacteria) larvae. Based on its strong ability to inhibit pathogen B183 in disk diffusion assays, as well as the beneficial effects on survival of oyster larvae, probiotic candidate OY15, a *Vibrio* sp. bacterium, was selected for further screening for its protective effects in larger-scale larvae cultures. Probiotic candidate OY15 did not impair growth of the microalgal feed strain *Isochrysis* sp. (T-ISO) at a dosage of 10^4 cfu/mL, confirming compatibility with the larvae and their feed. In addition, preliminary, 5-day bioassays in 12-well microplates confirmed no harmful effects on the larvae; survival was similar (ANOVA, $P < 0.3883$) to that of unchallenged, control larvae (no bacteria). In addition, probiotic candidate OY15 significantly improved survival (ANOVA, $P < 0.0141$) of oyster larvae when challenged with pathogen B183 (10^5 cfu/mL) compared with the pathogen alone.

Bacterial dosages used in 12-well-microplate bioassays were based on past experimental larval challenge data (unpubl.). Larvae were supplemented with probiotic candidates at a dosage of 10^3 cfu/mL (dosage based on a previous experiment on water-quality condition, unpubl.), and the pathogen dosage (10^5 cfu/mL) was based on previous virulence and pathogenicity data (Lim et al. 2011). The current study reports the effective dosage of probiotic OY15 to be 10^3 cfu/mL (Table 2). In addition, calculation of the LD_{50} for pathogen B183 established a consistent, stable pathogen dosage for use in all larvae-pathogen bioassays. Confirmation of the effective probiotic and pathogen dosages against 2-day-old oyster larvae allowed for consistency of controlled conditions for pilot-scale oyster larvae–probiotic–pathogen trials, as well as for possible future probiotic applications in commercial-scale hatchery field trials.

Douillet and Langdon (1991) found that bacteria may be used directly as food by oyster larvae. Hence, further investigation into the development of a safe, effective probiotic feed component required the probiotic bacteria to be accepted by the host animal through ingestion (Riquelme et al. 2000, Verschuere et al. 2000). Larval ingestion of probiotic OY15 was confirmed in the current study. Once in the gut, OY15 may exert its probiotic effects through improved digestion, competitive exclusion of the pathogen, or immune regulation. Further studies are necessary to assess which of these mechanisms may be involved.

Even though *in vitro* screening was used to select probiotic candidates on the basis of pathogen inhibition, *in vivo* larval bioassays allow for the examination of direct effects of probiotic bacteria on the host animal, by any mode of action (Kesarcodi-Watson et al. 2008). Lim et al. (2011) confirmed, in small-scale bioassays (12-well microplates and 1-L beakers), that probiotic candidate OY15 protected oyster veligers from pathogen B183. The current study confirmed that benefits of probiotic OY15 on survival of oyster larvae in 12-well-plate and 1-L beaker bioassays also occurred in larger-scale larviculture conditions. Riquelme et al. (2000) found that a pre-exposure time of 6 h was required for scallop larvae to ingest probiotic strains dosed at 10^6 cfu/mL so that competitive exclusion of the pathogen by the probiotic could occur. In our study, a pre-exposure time of 3 days for the probiotic was used before pathogen B183 was introduced into the larval culture buckets, allowing time for larvae to ingest the probiotic, and for it to establish itself in the culture system. Initially, larval mortalities did occur in both pathogen

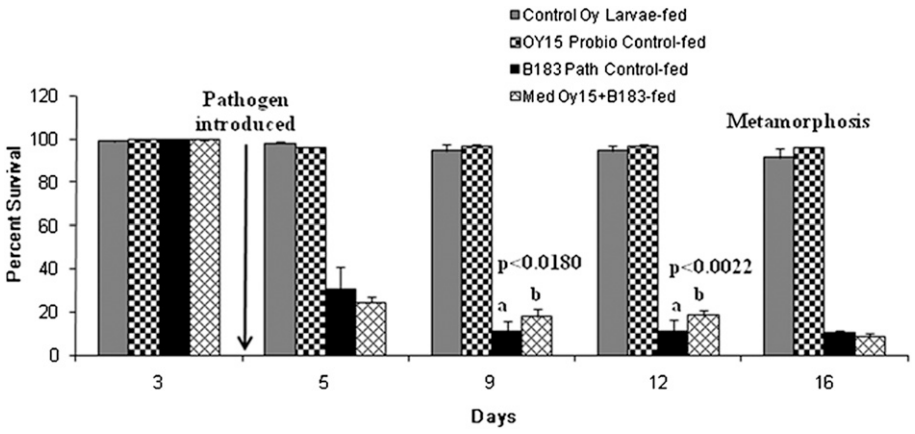

Figure 4. Percent survival of 2-day-old oyster larvae fed the microalgae T-ISO and challenged with a 10^5 cfu/mL dosage of pathogen B183, both in the presence of a 10^3 cfu/mL dose probiotic candidate OY15 and without. This pilot-scale trial was conducted in 12-L buckets held at 25°C to pediveliger stage (16 days). Larvae were pre-exposed to probiotic candidate OY15 for 3 days prior to challenge with pathogen B183. Survival of larvae supplemented with probiotic candidate OY15 (checked bars) was similar to control larvae (solid gray bars), indicating no harmful effects on larvae survival for every sampling day. Pathogen-challenged larval survival was significantly improved by the presence of probiotic OY15 (cross-hatched bars) at day 9 ($P < 0.0180$) and day 12 ($P < 0.0022$) compared with pathogen-challenged larvae only (solid black bars).

treatments within 48 h; however, by days 9 through 12 of the bioassay, larval survival was significantly improved (ANOVA, $P < 0.0180$ at day 9, and $P < 0.0022$ at day 12) in the pathogen treatment that was supplemented with 10^3 cfu/mL OY15. Supplementation of growing oyster larvae with 10^3 cfu/mL probiotic OY15 conferred protection against the challenge with 10^5 cfu/mL pathogen B183, significantly improving larval survival by 20% in this pilot scale trial (Fig. 4). Douillet and Langdon (1993) reported variations in growth and survival of *Crassostrea gigas* larvae based on different broodstock cohorts in a growing season. Similarly, our findings confirmed variations in survival of *Crassostrea virginica* larvae that were supplemented with probiotic candidate OY15. Although survival of pathogen-challenged larvae was improved by 20% in this pilot-scale trial, survival of early-season larvae was improved by up to 35% (no figure shown).

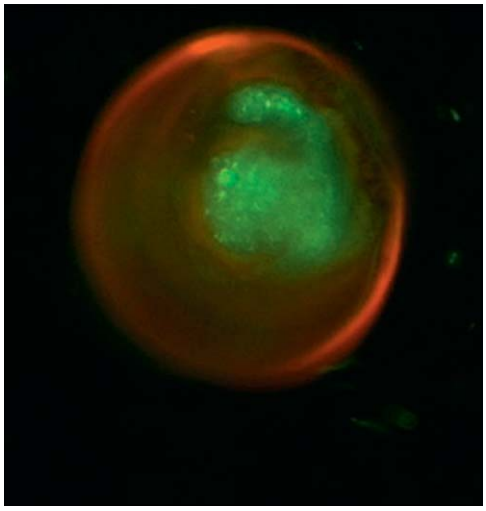

Figure 5. Fluorescently-labeled (BacLite) probiotic bacterial strain OY15 is ingested and concentrated in the esophagus and stomach of Eastern oyster larva (11 days postfertilization) after 20 min; shell edge is seen as red, refracted light (scale bar = 100 μm).

Regulated use of antimicrobials in aquaculture is strictly enforced in countries in North America and Europe. Yet globally, a large part of aquaculture takes place in countries that have little or no regulations in place for authorized use of antimicrobial agents in feed animals (World Health Organization 2006). This use and overuse of antimicrobials in aquaculture can result in the emergence of antibiotic-resistant bacteria within reservoirs of farmed food fish, shellfish, and their culture water (Sorum 2006). Fish pathogens such as *Aeromonas salmonicida*, *Vibrio anguillarum*, and *Vibrio salmonicida,* among others (Sorum 2006), as well as *V. harveyi*, a known shrimp pathogen (Karunasagar et al. 1994), have been shown to have developed resistance as a result of prophylactic use of antimicrobial agents. In addition, some fish pathogens can also cause disease in humans, and are a likely avenue of spreading antimicrobial resistance from aquaculture to humans (Heuer et al. 2009).

Transfer of resistance genes between aquatic bacteria and other ecological environments, such as aquaculture and the human environment, has been well documented (Kruse & Sorum 1994, Akinbowale et al. 2006). These antibiotic-resistant bacteria can exchange resistance genes with human pathogens via horizontal gene transfer. This exchange can occur either in the aquaculture environment, in the food chain, or in the human intestinal tract, and poses a potential human health risk (Kruse & Sorum 1994, Neela et al. 2008, Heuer et al. 2009). In addition, plasmids from aquatic bacteria that carry resistance factors to antimicrobial agents cannot only be exchanged between other bacteria within the same genus, but also to *Escherichia coli* as well, increasing the probability that this human pathogen can become resistant to standard antibiotics used as treatment in humans (Kruse & Sorum 1994, Akinbowale et al. 2007).

Consistent with Verschuere et al.'s (2000) guidelines for the development of a safe, effective probiotic product, antibiotic-sensitivity disk diffusion testing was completed to confirm the lack of antibiotic-resistance genes in probiotic candidate OY15. Probiotic candidate OY15 was susceptible to 12 of 14 antibiotics tested (Table 3), indicating that the strain is unlikely to contribute

antibiotic resistance genes to either an aquaculture or human environment. OY15 did show apparent resistance to ampicillin (10 μg) and cephalothin (30 μg) on Mueller Hinton agar during sensitivity testing. Further molecular testing must be conducted to investigate the genetic basis for this resistance.

In aquaculture environments, continuous change occurs within the microbial community in the culture water. Unless the host has been exposed to a limited range of microorganisms during development, a single, one-time dose of a probiotic is not expected to result in long-term colonization (Verschuere et al. 2000). Confirmation of the effective dosage of probiotic candidate OY15 (10^3 cfu/mL) was supplied on a regular basis (every 2 days). The consistent, beneficial effects of probiotic OY15 on survival of metamorphosing oyster larvae during larviculture of oyster larvae, both with and without the presence of pathogen B183, in any size culture vessel, demonstrated the reliability of OY15's probiotic effect, even in the presence of the resident microflora associated with the larvae and culture water.

Future studies will help to elucidate the mechanisms of OY15's probiotic effect on metamorphosing oyster larvae. In addition, using gene-specific molecular tools, we can develop a better understanding how probiotics affect the microbial ecology of the larvae of Eastern oysters in hatchery culture.

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