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ISOLATION AND EVALUATION OF NEW PROBIOTIC BACTERIA FOR USE IN SHELLFISH HATCHERIES: I. ISOLATION AND SCREENING FOR BIOACTIVITY

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ABSTRACT Hatchery production of shellfish seed is necessary to supplement natural recruitment, which is constrained by various stresses, including habitat loss, pollutant contamination, overfishing, and climate change. Bacterial diseases are considered to be a major cause of mortality in hatchery shellfish larviculture; however, overuse of antimicrobials can result in development of resistant strains of bacterial pathogens. The use of probiotics for disease prevention and improved nutrition in aquaculture is becoming increasingly popular as the demand for environmentally-friendly aquaculture grows. The objective of this study was to isolate and evaluate the efficacy of new probiotic bacteria that, incorporated into functional foods for use in shellfish hatcheries, may significantly improve larval survival. First, 26 probiotic-candidate bacteria were isolated from oysters, scallops, and a mass culture of green algae. Fifteen of these isolates (8 oyster strains and 7 bay scallop strains) inhibited known scallop-pathogen bacterial strains B183 and B122 in disk-diffusion assays. Similar to control (unchallenged) oyster larvae, survival of oyster larvae exposed to these 15 probiotic candidates for 48 h was more than 90%. The probiotic candidates were then reisolated from challenged larvae and characterized by Gram stain, colony morphology on solid agar, and the Biolog Bacterial Identification System, finding only 7 distinct strains. Using 12-well microplate assays, 5-day challenges were performed to confirm positive effects of these 7 probiotic candidates on larval survival when challenged with pathogen B183. Oyster larvae exposed to probiotic candidate OY15 had the highest survival; furthermore, survival of pathogen-challenged larvae was significantly improved by the presence of OY15 compared with pathogen alone. In addition, probiotic candidate OY15 exhibited no toxic effects on the microalgal feed strain *Isochrysis* sp. (T-ISO) in the range of 10^2–10^4 cfu/mL. Future studies will confirm optimal dosage and positive effects of probiotic candidate OY15 on survival during long-term rearing of oyster larvae.

KEY WORDS: probiotic bacteria, shellfish larvae, oyster larviculture, larval survival

INTRODUCTION

Two key components to successful hatchery production of molluscan shellfish seed are disease prevention and nutrition. Disease outbreaks caused by pathogenic bacteria, commonly of the genus *Vibrio* (Estes et al. 2004), are considered be a major cause of mortality in shellfish larviculture and can result in financial losses for commercial growers. Bacterial infection in ligament and soft tissue of shellfish larvae appears to be the most significant mode of action, and cause of mortalities in metamorphosing and juvenile shellfish, the most critical phases of intensive shellfish larviculture (Elston 2009). Proper sanitation measures cannot always prevent disease outbreaks (Estes et al. 2004). Overuse of chemical antimicrobial agents for sanitation in shellfish hatcheries can result in increased emergence of resistant strains of bacterial pathogens that become more difficult to treat with standard antibiotics approved for use in aquaculture (World Health Organization 2002). As the need for environmentally-friendly aquaculture grows, the use of probiotic bacteria for disease prevention in commercial hatcheries is becoming increasingly popular as a sustainable alternative to conventional, chemical-agent methods.

Fuller (1989) defined a probiotic bacterium as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (p. 366). According to this author, probiotic bacteria can enhance nutrition of veliger larvae by providing early colonization of microflora in the gut to aid digestion. Along with other particles, shellfish can filter large amounts of bacteria from the surrounding culture water, causing a natural interaction between microbiota from the ambient environment and gut microflora (Verschuere et al. 2000). Given these findings, Verschuere et al. (2000) proposed a new definition of probiotic bacteria: "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" (p. 659). The ambient environment can also support the growth of pathogens, which can reach high densities depending on water conditions (Moriarity 1998). Probiotic bacteria can prevent damage to the host caused by a pathogenic bacterium by outcompeting the pathogen for nutrients, by producing an antimicrobial substance that inhibits the growth or attachment of the harmful bacteria, or by immune regulation (Moriarity 1998).

Probiotic bacteria used as supplements to algal feed could be used as practical, functional foods that could benefit larval production in commercial oyster hatcheries. The objective of this study was to evaluate the safety and efficacy of naturally-occurring probiotic bacteria isolated from the digestive glands of the bay scallop *Argopecten irradians* (Lamarck 1819) and the Eastern oyster *Crassostrea virginica* (Gmelin 1791) to improve survival of oyster larvae from veliger to metamorphosis, the most critical phase of shellfish larviculture. This report also describes

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the effectiveness of a probiotic bacterium in improving survival of larvae when challenged with a known oyster-larvae pathogen.

MATERIALS AND METHODS

Isolation of Potential Probiotic Candidates

Whole digestive glands from 6 Eastern oysters (*C. virginica*) and five northern bay scallops (*A. irradians irradians*) of healthy broodstock obtained from waters local to the Milford Laboratory were dissected aseptically and transferred into sterile, nonselective Marine Broth 2216 medium (Becton Dickinson, Franklin Lakes, NJ) to enable growth of as many bacterial strains as possible. Broth cultures were incubated overnight at 28°C (Laboratory-Line Low Temperature B.O.D. Incubator, model 3550; Barnstead/Thermolyne, Melrose Park, IL). Bacterial growth from each broth culture was then streaked for isolation of bacterial colonies onto several types of nonselective, solid agar media in Petri dishes; marine agar (Becton Dickinson, Franklin Lakes, NJ), nutrient agar supplemented with 2.5% sodium chloride (Becton Dickinson, Franklin Lakes, NJ), trypticase soy agar supplemented with 2.5% sodium chloride (Sigma-Aldrich, St. Louis, MO), and seawater-based OZR agar. Thiosulfate citrate bile salt sucrose agar (Becton Dickinson, Franklin Lakes, NJ) was also used to select for *Vibrio* spp. bacteria.

Handling Practices for Bacterial Cultures

Pure cultures of individual probiotic candidates were cryopreserved in replicate at –80°C in a REVCO Ultra-Low freezer using suitable broth medium: Marine Broth or OZR broth, both supplemented with 10% glycerin. Upon startup of larval pathogen-challenge bioassays, cryopreserved isolates were revived, vortexed, and subsequently inoculated into the same broth medium (without glycerin) and incubated for 18 h at 23°C. Using sterile seawater, incubated broth cultures were washed 3 times, first by centrifugation at 4,000*g* for 20 min in a Beckman TJ-6 centrifuge to remove growth medium. Bacterial pellets were resuspended using 1 mL sterile seawater, and were then transferred into sterile Eppendorf tubes for two more washes using a high-speed Tomy MTX-150 microcentrifuge for 2 min at 4,000*g*. After the final wash, each pellet was resuspended in a 1-mL volume of sterile seawater by vortexing. Quantity of bacteria in an Eppendorf tube after final wash and resuspension in 1 mL sterile seawater was 2×10^9 cfu/mL.

Crassostrea virginica *Larvae*

Unselected native broodstock oysters were spawned at the Milford Laboratory using standard methods of temperature induction (FAO 1990). Fertilized eggs were transferred into a 400-L rearing tank, gently aerated to provide mixing throughout the tank, and held for 48 h until veliger larvae began to feed. The 48-h-old larvae were screened using 36-μm mesh, then were rinsed and resuspended in sterile seawater for startup of larval–pathogen–probiotic bioassays. Larvae were then distributed into 12-L buckets containing 8 L sterile seawater at a stocking density of 10 larvae/mL. Aeration provided mixing of larvae in the buckets during bioassays.

Pathogen Reactivation

Four Gram-negative *Vibrio* spp. bacteria pathogenic to shellfish larvae, designated as B39, B70, B122, and B183, were retrieved from liquid nitrogen storage and tested against readily available bay scallop larvae (*A. irradians irradians*) for virulence reactivation and pathogenicity. Isolate B39 is an ATCC culture of *Vibrio alginolyticus* (ATCC 17,749), B70 (*Vibrio* sp.) was previously isolated from Long Island Sound and was shown to be pathogenic to oyster larvae (*C. virginica)* (Tettlebach et al. 1984), B122 (*Vibrio* sp.) isolated from moribund bay scallop larvae at the Milford Laboratory in 1994 caused 92.6% mortality in a 48-h exposure at a dosage of 10^6 cfu/mL. B183, later identified as *Vibrio corallilyticus* (Schott et al. unpubl.) isolated from a 1998 bay-scallop larvae mortality incident at the Milford Laboratory, caused 99.7% mortality in a 4-day challenge at a dosage of 10^6 cfu/mL. In a 48-h challenge, 2-day old bay scallop larvae were exposed to each of these pathogens at a dosage of 10^6 cfu/mL (Tettlebach et al. 1984) utilizing a 12-well-microplate protocol developed by Estes et al. (2004). The most virulent of these 4 organisms was tested against oyster larvae for its ability to cause mortality, and then was used for Kirby-Bauer disk diffusion of probiotic candidates for their ability to inhibit a confirmed pathogen, and used in subsequent challenges to determine survival of oyster larvae, both with and without the addition of probiotic candidates.

Probiotic Candidate Selection

The Kirby-Bauer disk diffusion method, issued by the National Committee on Clinical Laboratory Standards (1997) for susceptibility testing, was adapted to test 26 probiotic candidates for the ability to inhibit 3 of the known shellfish larval pathogens described earlier: bacterial strains B70, B122, and B183. Pure cultures of each isolate were inoculated into suitable broth media—Marine Broth or OZR broth—and incubated for 18 h at 23°C. Inoculum densities of these pathogens were adjusted to 10^6 and 10^8 cfu/mL using sterile seawater. Mueller-Hinton agar plates were streaked individually with pathogens at two densities using sterile, Dacron polyester-tipped swabs dipped into the pathogen inoculum. The entire surface was streaked such that confluent bacterial growth would be produced on the agar after incubation. A pure culture of each probiotic candidate was inoculated aseptically into suitable broth media (Marine Broth or OZR broth) and incubated for 18 h at 23°C. Sterile paper disks were dipped into each of these probiotic suspensions and placed evenly onto the agar surface already streaked with pathogen, 5 disks per plate. In addition, a Neomycin sensitivity disk (dosage, 5 μg; Becton-Dickinson BBL Sensi-Disc, Franklin Lakes, NJ) was placed onto each inoculated plate as a positive control. A sterile disk was dipped into 0.22-μm filtered, sterile seawater and placed onto each inoculated plate as a negative control. Agar plates were incubated at 23°C within 15 min of disk application. After 18 h, plates were examined, and zones of complete inhibition were measured to the nearest millimeter. Zone diameters were then compared with zone diameter standards from CLSI Document M100-S17 (M2) and interpreted as resistant, intermediate, or susceptible to the probiotic candidates being screened (CLSI 2007). Probiotic candidates exhibiting total inhibition were later used in assays with oyster larvae to assess effects on larval survival and inhibition of pathogens.

Effects of Probiotic Candidates on Larvae Survival

In a 48-h bioassay, 2-day-old oyster larvae were challenged with 16 probiotic candidates individually to determine effects

on larval survival. Larvae were transferred by pipette into 1-L beakers containing 800 mL sterile filtered seawater for a concentration of approximately 10 larvae/mL. Each beaker (replicated 4 times) was dosed once with an individual probiotic candidate at a density of 1×10^3 cfu/mL (dosage based on a previous experiment on water quality condition, unpubl.). All beakers were incubated for 48 h at 23°C in a Laboratory-Line Low Temperature B.O.D. Incubator, model 3550 (Barnstead/Thermolyne, Dubuque, IA). Survival was determined by observation of internal structures of the larvae under 10× magnification with a compound microscope. Deterioration or retraction of internal organ structures, or empty shells, indicated moribund or dead larvae.

Effects of Probiotic Candidates on Microalgal Food: T-ISO

Cultures of the microalga T-ISO, *Isochrisis* sp., and three different bacterial dosages of probiotic candidate OY15 (10^2, 10^4, and 10^6 cfu/mL) were grown together in 10-mL test tube cultures using E medium (Ukeles 1973) for 16 days to determine the effects of probiotic candidate OY15 on the growth and survival of T-ISO. Optical densities of the microalgal cultures mixed with OY15 were measured at regular intervals for 16 days using a Bausch and Lomb Spectronic 20 Spectrophotometer and Colorimeter (Bausch and Lomb Corporation, Rochester, NY) set at a wavelength of 690-nm, to monitor growth of T-ISO. Optical density values were plotted over time, and the division rate (μ) of T-ISO was calculated for each of the treatments.

Preliminary Identification of Isolates

Gram stain and the Biolog MicroLog Microbial Identification System (Biolog Inc. 2001, Hayward, CA) were used to identify the 7 potential probiotic isolates to genus. Biolog technology uses 96-well plates that allow for simultaneous carbon-source utilization testing. The ability of a microbe to use a particular carbon source produces respiration, which reduces a tetrazolium redox dye and causes a color change in that well. The end result is a pattern of colored wells that is characteristic for that organism. This pattern is compared with a database to identify the organism (Biolog Inc. 2001).

Protective Effect of Probiotic Candidate OY15 on Survival of Oyster Larvae Challenged with Pathogen B183

In a 5-day bioassay utilizing 12-well microplates, 2-day-old oyster larvae were challenged with pathogen B183 at a dosage of 10^5 cfu/mL while in the presence of probiotic OY15 (10^3 cfu/mL), or without, to test for possible beneficial effects of OY15 with pathogen challenge. Each well contained 4 mL filtered sterile seawater, 60 2-day-old larvae (15 larvae/mL seawater), and appropriate dosages of each bacterial isolate by treatment. Treatments consisted of a larval control (no bacteria), a pathogen control comprised of larvae and pathogen B183 only, a probiotic control comprised of larvae and probiotic candidate OY15 only, and a combination treatment comprised of larvae and both probiotic and pathogen. All treatments were fed the microalga T-ISO daily (10^4 cells/mL) and were replicated 4 times. Larvae were maintained at 25°C for the duration of the bioassay.

Statistical Analysis

Larval percent survival values (presented as frequency) for all assays were first transformed by taking the square root of the frequency and then normalized by arcsine transformation (Zar 1996). Analysis of variance (Statgraphics Plus 5.1; Statpoint Technologies, Warrenton, VA) was used to test the transformed, normally distributed data, followed by the least significant difference multiple-comparison test (Statistix 9; Analytical Software, Tallahassee, FL).

RESULTS

Isolation of Potential Probiotic Candidates

Culture of the digestive glands from broodstock oysters and bay scallops on solid agar media yielded 26 morphologically different colonies. These isolates subsequently were screened for use as potential probiotic candidates using the Kirby-Bauer disk diffusion method. Isolates that exhibited an inhibitory effect against a known *Vibrio* sp., shellfish-larval pathogen (B183) on Mueller Hinton agar, seen as clear zones of inhibition surrounding disks saturated with individual probiotic candidates, were considered to be potential probiotic candidates. During this screening process, 15 of the 26 isolates were shown to inhibit growth of the pathogen and, therefore, could possibly confer a protective effect on oyster larval survival when challenged with the B183 pathogen.

Pathogen Reactivation

Results (Fig. 1) indicated that pathogen B183 was the most virulent of the four isolates, causing significantly the highest mortality (98.6%) of bay scallop larvae (94% mortality in oyster larvae at the same dosage). B122 was the next virulent, causing mortality of 94.2%. In addition, bay scallop larvae challenged with pathogen B70 had significantly higher mortality than those challenged with B39 or control larvae with no bacteria ($P < 0.05$). The latter two isolates could have lost virulence after long-term storage in liquid nitrogen. Mortality of larvae challenged with B39 was similar to that of the control larvae ($P > 0.05$). Hence, use of isolates B183, B122, and B70 as pathogens for disk diffusion testing of probiotic candidates and subsequent oyster larvae challenges was indicated.

Probiotic Candidate Selection

Competitive exclusion (Gause's Law) states that two species of bacteria competing for the same nutritional resources cannot

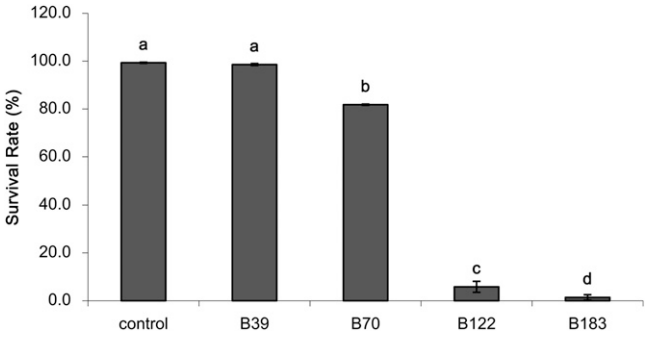

Figure 1. Forty-eight-hour pathogen reactivation challenge. Percent survival of bay scallop larvae (*Argopecten irradians*) exposed to four *Vibrio* spp. bacteria potentially pathogenic to shellfish larvae. Pathogen treatments with different letters were statistically significant ($P < 0.05$).

coexist stably. One of the competitors will have an advantage over the other and will render the inferior competitor extinct (Gause 1934). Competitive exclusion may be the mechanism by which a naturally-occurring probiotic bacterium can inhibit colonization by a pathogen (Jeffrey 1999) and hence provide protection against disease in larval cultures. Probiotic candidate selection for this study was based on the ability of these isolates to inhibit pure cultures of known shellfish larval pathogens (bacterial strains B70, B122, and B183) preinoculated onto Mueller Hinton agar. Table 1 shows the zone diameters for 15 of 26 probiotic candidates that were able to inhibit growth of the three pathogenic strains. Four probiotic candidates had no inhibitory effect. Results (Table 1) indicated that pathogen B183 (identified subsequently by 16S ribosomal RNA sequences as a *V. corallilyticus*-like organism, Schott et al. unpublished data) was susceptible to most of the probiotic candidates (12 of 15). This *Vibrio* sp. pathogen also showed intermediate susceptibility to one probiotic candidate (S6) and was resistant to two others (S1 and S2). Pathogen B70 (*Vibrio* sp.) was susceptible to eight of the probiotic candidates, showed intermediate susceptibility to five other probiotic candidates, and was resistant to two (S5 and S6). Last, pathogen B122 (*Vibrio* sp.) showed the least susceptibility to the probiotic candidates tested, being susceptible to 7, and showing intermediate susceptibility to 5 and resistance to 3 probiotic candidates (S1, S2, and S6). Probiotic candidates OY5, OY6, OY11, OY15, and S7, based upon their ability to inhibit all three pathogens, were regarded superior probiotic candidates most likely to confer a protective effect on larvae challenged with a known shellfish pathogen.

TABLE 1.

Kirby-Bauer disk diffusion testing: zone diameters for 15 probiotic candidates able to inhibit three shellfish pathogens.

	Inhibition zone diameter interpretive table by pathogen								
	B70			B122			B183		
Probiotic candidates	Res	Int	Sus	Res	Int	Sus	Res	Int	Sus
OY2			○		○				○
OY3		○			○				○
OY4		○			○				○
OY5			○			○			○
OY6			○			○			○
OY9			○		○				○
OY11			○			○			○
OY15			○			○			○
S1		○		○			○		
S2			○	○			○		
S5	○				○				○
S6	○			○				○	
S7			○			○			○
S8		○				○			○
S9		○				○			○

Zone diameters based on standards from CLSI Document M100-S17 (M2): Disc Diffusion Supplemental Tables, Performance Standards for Antimicrobial Susceptibility Testing (National Committee for Clinical Laboratory Standards 1997).

Int, intermediate (zone diameter, 13.7–16.7 mm); Res, resistant (zone diameter, ≤12.4 mm); Sus, susceptible (zone diameter, ≥17.5 mm).

Effects of Probiotic Candidates on Larvae Survival

A short-term exposure (48 h) of 2-day-old oyster larvae to single doses of 10^3 cfu/mL of the 16 probiotic candidates showed survival statistically similar to control larvae with no added bacteria (ANOVA, $P > 0.05$), indicating no harmful effects on larval survival and confirming safe usage of these probiotic candidates as additive supplemental feeds (Fig. 2). Survival of control larvae was 95%. Based on these results, as well as its ability to inhibit pathogen B183 completely, probiotic isolate OY15 was selected for further screening for its protective effects in larval–probiotic–pathogen bioassays.

Effects of Probiotic Candidate OY15 on Microalgal Food: T-ISO

In Figure 3, growth curves for T-ISO indicated no significant differences in growth between the control treatment (T-ISO with no bacteria) and T-ISO grown with the 10^2 and 10^4 dosages of probiotic candidate OY15. Growth of T-ISO given the 10^6 dose of OY15, however, although showing a steady increase initially, was slowed or inhibited after day 4 of the experiment. Oyster larvae in all subsequent bioassays were fed with 5×10^4 cells/mL T-ISO, with volume added and adjusted according to algal culture cell density.

Preliminary Identification of Isolates

The number of potential probiotic candidates was reduced from 16 to 6 based on redundancies suggested by the Biolog Microbial Identification System. Five were identified as similar to *Vibrio* spp. isolates, whereas one remained unknown.

Protective Effect of Probiotic Candidate OY15 on Survival of Oyster Larvae Challenged with Pathogen B183

Two-day-old oyster larvae were supplemented individually with 10^3 cfu/mL doses of probiotic candidate OY15 (4 replicates) and challenged with one dose of pathogen B183 (10^5 cfu/mL). Results (Fig. 4) indicated that, at 5 days, percent survival of larvae treated with OY15 was significantly higher (95%) than larvae of the control group (10^4 cells/mL T-ISO supplement without pathogen and probiotic candidate). Oyster larvae challenged with pathogen B183 alone had the lowest survival (62%). In addition, OY15 improved the survival of larvae exposed to the pathogen compared with pathogen alone, suggesting that probiotic candidate OY15 provided a protective effect against pathogen B183 and improved larval survival significantly.

DISCUSSION

Molluscan aquaculture accounts for 24.9% of the total world aquaculture product and 65% of total mollusc product when capture fisheries are considered (FAO 2010). Climate changes attributed to global climate change and pollution of fisheries habitats have caused reductions in natural shellfish seed and, with that, the increased need for farmed seed production. Diseases, mainly caused by pathogenic bacteria such as *Vibrio* and *Aeromonas* spp., can cause mass mortalities and significantly affect production of farmed shellfish (Kesarcodi-Watson et al. 2008). Traditionally, antimicrobial drugs approved for use in aquaculture have been used to treat bacterial diseases in farmed-raised finfish. Use of antimicrobial drugs, however, can lead to the emergence of antibiotic-resistant bacteria and, furthermore, can threaten human health. In the European Union, a third of all

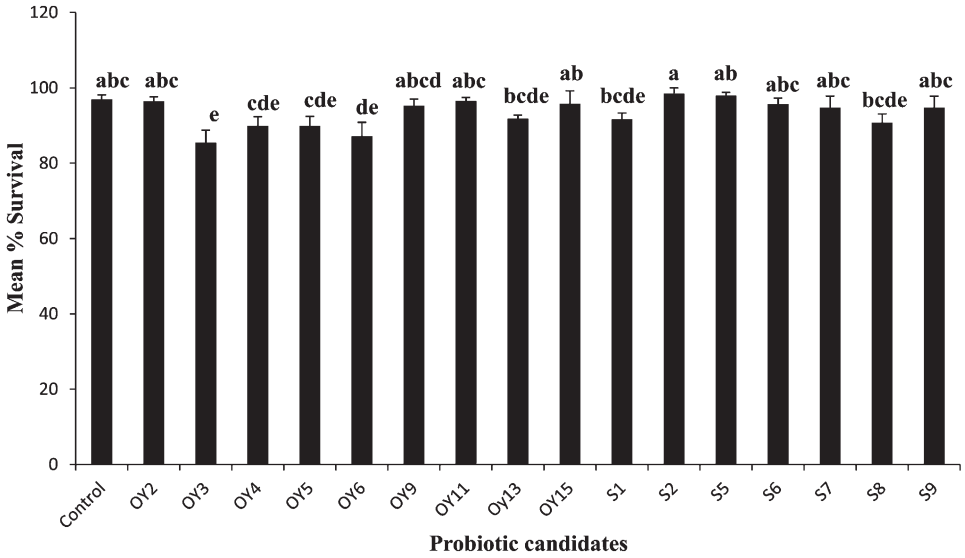

Figure 2. Two-day-old oyster larvae were challenged with 16 probiotic candidates for 48 h to determine effects on survival. Overall, significant differences were observed (ANOVA, $P < 0.02$) between survival and the probiotic candidate strain used. Five homogeneous groups were indicated by using the least significant difference multiple-comparison tests and are indicated on the graph by letters a through e.

antimicrobials used for veterinary purposes are administered as antimicrobial growth promoters, which have beneficial effects on feed-animal growth (Ungemach 2000). Use of antimicrobial growth promoters, however, has been shown to increase the emergence of antibiotic-resistant strains of bacteria (Goossens 1998, MAFF 1998), which could be transmitted to humans from feed animals. In an effort to minimize this threat, the European Union has implemented a ban on the use of all non-therapeutic antimicrobials in animal production (Delsol et al. 2005). In Korea, the Aquatic Animal Disease Control Act and Enforcement Regulation of 2008 enforces the surveillance, monitoring, quarantine, disease control, and jurisdiction for fisheries disease control, including antimicrobial drugs. The U.S. Food and Drug Administration's Center for Veterinary Medicine works to ensure that safe and effective drugs are available for use to treat diseases of farmed aquatic animals. In accordance with the U.S. National Environmental Policy Act of 1969, the U.S. Food and Drug Administration's Center for Veterinary Medicine works with other government agencies to conduct studies and prepare environmental impact statements to assess effects that any proposed drug may have on the environment before issuing approval for use in aquaculture. In addition, aquaculture drugs for food fish must meet human food safety standards before being approved (U.S. Food and Drug Administration's Center for Veterinary Medicine 2010). Use of vaccines and selective breeding programs can decrease or prevent bacterial disease. Because invertebrates have only innate immune functions, use of vaccines to prevent disease is not possible in growing larval shellfish. Selective breeding and supplementation with probiotic bacteria are, therefore, recommended methods of disease prevention in shellfish larviculture (Maroni 2000).

Although research results have indicated promise, development of probiotics applicable to commercial use in aquaculture is a multistep process that requires fundamental research and full-scale trials (Verschuere et al. 2000). Gomez-Gil et al. (2000)

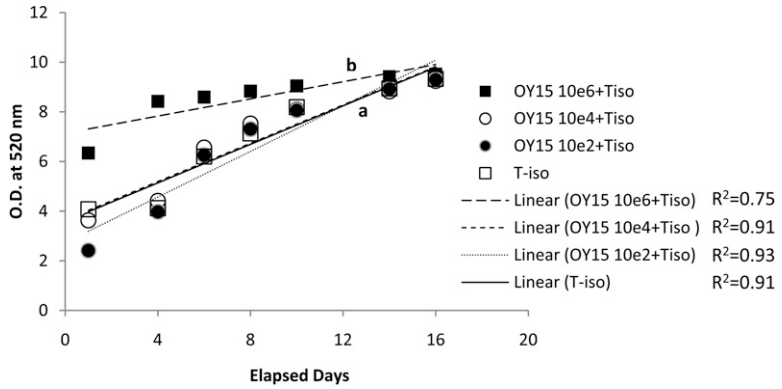

Figure 3. Safety testing of 3 doses of probiotic candidate OY15 to determine bacterial effects on growth and survival of T-ISO. The figure indicates that the 10^2 and 10^4 dosages of probiotic candidate OY15 had no effect on the growth of T-ISO microalgae. However, at the 10^6 dose, growth inhibition of T-ISO is evident. The mean division rate (μ) of T-ISO for each of the treatments is plotted in the figure.

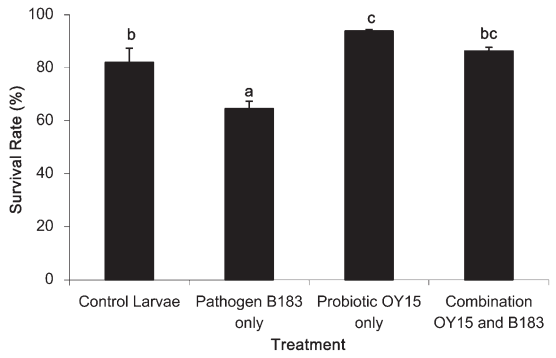

Figure 4. Effects of probiotic candidate OY15 (dosage, 10^3 cfu/mL) on survival of oyster larvae challenged with 10^5 cfu/mL pathogen B183 (5-day exposure in 12-well microtiter plates). Treatments that were statistically significant from each other ($P < 0.05$) are designated with different letters (a–c).

described the methods to select probiotic bacteria for use in the larviculture of aquatic animals:

1. Collection of background information.
2. Acquisition of potential probiotics.
3. Evaluation of the ability of potential probiotics to outcompete pathogenic strains.
4. Assessment of the pathogenicity of the potential probiotics.
5. Evaluation of the effect of potential probiotics in larvae.
6. An economic cost–benefit analysis.

This screening model is the first stage in the development of a naturally-occurring, safe, and effective probiotic bacterium. In the current study, such bacteria were isolated from the digestive glands of the Eastern oyster and northern bay scallop, but expectation that these isolates would have beneficial effects on the survival of metamorphosing oyster larvae in culture systems required several steps. The initial phase allowed for: (1) the screening of naturally-occurring isolates for use as probiotic candidates acting by any mode of probiotic activity (Kesarcodi-Watson et al. 2008) and (2) identifying the safety and efficacy of a *Vibrio* sp. isolate with potential use as a probiotic for shellfish larviculture. Consistent with the findings of Verschuere et al. (2000), *in vivo* bioassays using 2-day-old oyster larvae were used throughout this study. Evidence of possible modes of action of the potential probiotic candidates needs further investigation.

In 1980, Yasuda and Taga published their findings on the use of bacteria as food for mass culture of *Artemia salina*, as well as biological control agents of pathogens in aquaculture. Moriarity (1998) described the advantages of using *Bacillus* strains as biological agents to control the growth of pathogenic, luminous *Vibrio* strains and to improve survival in penaeid shrimp aquaculture ponds.

A probiotic strain of *Vibrio* sp. bacteria fed with algae could prevent mortality of Pacific oyster larvae (*Crassostrea gigas*) when challenged *in vivo* with *Vibrio tubiashii* (Gibson et al. 1998). The probiotic *Vibrio* sp. caused a marked decrease of the pathogenic strain in larvae compared with those in the larvae that were treated with *V. tubiashii* only. A strain of *Alteromonas haloplanktis*, a bacterium isolated from the gonad of the Chilean scallop (*Argopecten purpuratus*) displayed *in vitro* inhibitory activity against the known pathogens *Vibrio ordalii, Vibrio parahaemolyticus, Vibrio anguillarum, Vibrio alginolyticus,* and *Aeromonas hydrophila* (Riquelme et al. 1997). Our pathogens B183 (*V. coralliilyticus*) and B122 (*Vibrio* sp.), virulent to Eastern oyster larvae causing mortalities of 98.6% and 94.2%, respectively, should be characterized as organisms for surveillance in oyster hatcheries.

Probiotic bacteria can be antagonistic to pathogens by means of production of antimicrobial substances, such as antibiotics, antimicrobial peptides, or siderophore substances (Sugita et al. 1998). Consistent with the findings of Sugita et al. (1998), the Kirby-Bauer disk diffusion method (Table 1) was used in this study, and which demonstrated that 15 of 26 isolates were able to inhibit growth of shellfish larvae pathogens B70, B122 or B183. These 15 isolates were screened further to determine the safety and efficacy of their use as probiotic candidates. In addition, the screening and development of potential probiotic candidates suitable for use in commercial aquaculture requires research into the safety of the probiotic candidate for application to the host animal as well as the algal feed. A 48-h *in vivo* bioassay of 2-day-old oyster larvae dosed with 1-time individual doses of 10^3 cfu/mL of each of the 16 probiotic candidates demonstrated positive effects on survival with no apparent mortalities for 15 of them, suggesting safe use of these 15 isolates for oyster larviculture (Fig. 2). Probiotic candidates OY3 and OY6 had significantly lower survival than the control larvae with no bacteria, and could not be considered. However, with survival similar to control larvae, and its ability to inhibit pathogen B183 completely, probiotic isolate OY15 was further screened for its protective effects in larvae–probiotic–pathogen bioassays.

Bacteria antagonistic toward algal feed would be undesirable in larviculture fed by unicellular algae (Verschuere et al. 2000). A probiotic bacterium that could be cocultured with both larvae and their microalgal feed would be optimal. Results of a 16-day test tube bioassay testing the safety of 3 bacterial dosages (10^2, 10^4, and 10^6 cfu/mL) of probiotic OY15 on the growth and survival of the unicellular microalga *Isochrisis* sp. (T-ISO) used as feed for growing oyster larvae demonstrated no difference in growth between the control T-ISO (no bacteria) and T-ISO supplemented with 10^2 cfu/mL or 10^4 cfu/mL probiotic OY15. Growth of T-ISO given 10^6 cfu/mL OY15 was inhibited, but only after 4 days of steady growth (Fig. 3). In this study, we accomplished several steps in probiotic development: (1) isolation and selection (using the Kirby-Bauer disk diffusion method) of naturally-occurring bacteria from the digestive glands of Eastern oysters and northern bay scallops for use as probiotic candidates, (2) positive effects of probiotic candidates on survival of veliger oyster larvae (short term), (3) threshold effects of probiotic candidates on growth of phytoplankton used as larval feed, and (4) positive effects on survival during larviculture conditions (phytoplankton, shellfish larvae, and pathogen). As we progress in understanding the role of probiotic candidates in shellfish larviculture, this study confirms that use of probiotic candidates confers protection to veliger oyster larvae against a known shellfish larvae pathogen, and hence improves survival. These results can be used as a guideline for isolation and screening of potential probiotics candidates for similar aquaculture applications.

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