# Probiotic Strains for Shellfish Aquaculture: Protection of Eastern Oyster, Crassostrea virginica, Larvae and Juveniles Againsl Bacterial Challenge

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# PROBIOTIC STRAINS FOR SHELLFISH AQUACULTURE: PROTECTION OF EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, LARVAE AND JUVENILES AGAINST BACTERIAL CHALLENGE

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***ABSTRACT*** Bacterial pathogens, including several *Vibrio* spp. and *Roseovarius crassostreae*, cause severe mortality of larval and juvenile eastern oysters. The introduction of beneficial bacterial isolates in oyster hatcheries and nurseries for the biocontrol of bacterial diseases is a good alternative to the use of antibiotics. The goal of this study was to screen and characterize marine bacterial isolates as potential agents to prevent larval and juvenile mortality by the oyster pathogens *Vibrio tubiashii* and *R. crassostreae*. Screening of bacterial isolates from Rhode Island marine organisms and environment using agar-based assay methods for detection of antimicrobial activity against oyster pathogens led to the isolation of candidate probionts *Phaeobacter* sp. S4 and *Bacillus pumilus* RI06-95. Pretreatment of larval and juvenile oysters for 24 h with $10^2$–$10^6$ cfu/mL *Phaeobacter* sp. S4 or *B. pumilus* RI06-95 protected larval oysters against mortality resulting from challenge with *R. crassostreae* and *V. tubiashii* (relative percent survival (RPS) range, 9%–56%). These probiotics also protected juvenile oysters against challenge with *V. tubiashii* (RPS, 37%–50%). Probiotic isolates had no negative impact on oyster survival. Protection conferred to larvae against bacterial challenge was short-lived, lasting for only 24 h after removal of the probiotics from the incubation water. These results suggest the potential of marine bacterial isolates *Phaeobacter* sp. S4 and *B. pumilus* RI06-95 to serve as biocontrol agents to reduce the impact of bacterial pathogens in the culture of *Crassostrea virginica*.

***KEY WORDS:*** oyster larvae, oyster juveniles, probiotic bacteria, *Roseovarius* oyster disease, shellfish hatchery, shellfish nursery, vibriosis, *Vibrio tubiashii*, *Crassostrea virginica*

## INTRODUCTION

The eastern oyster, *Crassostrea virginica* (Gmelin), a bivalve species of the Gulf of Mexico and Atlantic coasts of North America, has significant economic and ecological value (Kennedy et al. 1996). However, this species suffers from the impact of different bacterial and parasitic diseases that cause high mortalities in cultured and wild populations (Lee et al. 1996, Burreson & Ford 2004, Villalba et al. 2004). Bacterial infections are considered a major problem for the shellfish aquaculture industry and cause mass mortality, especially during the larval and juvenile stages (Paillard et al. 2004). The pathogens *Roseovarius crassostreae* and several *Vibrio* spp. are among the major causative agents of bacterial disease in the culture of the eastern oyster. As the causative agent of juvenile or *Roseovarius* oyster disease, *R. crassostreae* causes high seasonal mortalities of oyster juveniles in the Northeast Unites States (Boettcher et al. 2005, Maloy et al. 2007). Meanwhile, *Vibrio tubiashii* is a reemerging pathogen that causes vibriosis and severe losses of production in oysters during the larval stages (Tubiash et al. 1965, Elston et al. 2008).

Disease outbreaks in shellfish aquaculture are managed using methods such as disease avoidance, frequent water changes, good husbandry, and the use of immunostimulants and antibiotics (Elston & Ford 2011). Antibiotics have been used widely in aquaculture systems as a method for disease control. However, because of the emergence of antibiotic resistance and concerns about environmental pollution, alternatives to the use of antibiotics are needed (Austin 1985, Verschuere et al. 2000). One of these alternative methods is the use of nonpathogenic microorganisms called probiotics.

A probiotic is generally defined as a live microbial food supplement that, when administered in a sufficient amount, confers a health benefit on the host (Food and Agricultural Organization of the United States 2006). In aquaculture, probiotics can be administered either as a food supplement or as an additive to the water (Moriarty 1998). Probiotics in aquaculture have been proposed to have several modes of action: improvement of water quality, enhancement of immune responses of host species, enhancement of nutrition of host species through the production of supplemental digestive enzymes, competition for space with pathogenic bacteria, and production of antimicrobial compounds (Thompson et al. 1999, Verschuere et al. 2000, Kesarcodi-Watson et al. 2008). The potential for the beneficial impact of the use of probiotic bacteria on shellfish aquaculture has been shown for many different species, including oysters. Douillet and Langdon (1994) demonstrated that Pacific oyster larvae fed with algae and *Alteromonas* sp. show increased survival and growth compared with treatments fed with algae alone. They suggest that the bacteria may act as an essential nutrient to the larvae, which is not provided by the algae. Gibson et al. (1998) isolated successfully a bacterium producing bacteriocin-like inhibitory substances capable of inhibiting the growth of several pathogenic bacteria. This probiont, identified as *Aeromonas media* A199, inhibits significantly the growth of *Vibrio tubiashii* in the culture of Pacific oyster larvae. The addition of *Vibrio* sp. probiotic candidate OY15 provides a beneficial effect in the culture of *Crassostrea virginica* larvae with and without the presence of the shellfish

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pathogen *Vibrio* sp. B183 (Kapareiko et al. 2011). Most recently, the use of *Pseudoalteromonas* sp. D41 and *Phaeobacter gallaeciensis* was found to provide 50% and 40% improved survival, respectively, in Pacific oyster larvae after being challenged with *Vibrio coralliilyticus* (Kesarcodi-Watson et al. 2012). The introduction of selected beneficial bacterial isolates for biocontrol of *Roseovarius crassostreae* and vibriosis may help in combating diseases in the culture of eastern oysters.

In this study, 2 potential probionts were isolated from 2 different local sources in Rhode Island. A Gram-negative *Phaeobacter* sp. S4 was isolated from the inner shell of oysters and a Gram-positive *Bacillus pumilus* RI06-95 was isolated from a marine sponge from Narrow River, a tidal estuary in Narragansett, Rhode Island. Both of these candidate probionts showed promising results during *in vitro* screening of antibiotic activity against oyster and fish pathogens, and protected larvae and juveniles during *in vivo* challenge experiments with 2 bacterial pathogens (*Vibrio tubiashii* RE22 and *Roseovarius crassostreae* CV919-312$^T$). We also describe the length of the protection conferred by the probiotic treatment.

## MATERIALS AND METHODS

### *Bacterial Strains*

Bacterial strains *Vibrio tubiashii* RE22 (Hasegawa et al. 2008) and *Roseovarius crassostreae* CV919-312$^T$ (Boettcher et al. 2005) were kindly supplied by H. Hasegawa (Department of Biomedical Sciences, Oregon State University) and K. Boettcher (formerly at the University of Maine), respectively. Strain *Vibrio harveyi* BB120 (Bassler et al. 1997) was obtained from B. Bassler (Princeton University). The marine bacteria *Phaeobacter* sp. S4 and *Bacillus pumilus* RI06–95 were identified as potential probiotics using the *in vitro* plate assays described later. The isolates were characterized to the level of species using 16S rDNA sequence analysis (Gauger & Gómez-Chiarri 2002) (GenBank accession nos. KC625490 and KC625491). All the isolates were maintained and stored in 50% glycerol stocks at –80°C. Probiotic candidates and pathogens were grown routinely overnight in yeast peptone with 3% NaCl (YP3) broth (5 g/L peptone, 1 g/L yeast extract, 30 g/L ocean salt, Instant Ocean) at 28°C (*V. tubiashii, V. harveyi,* and *R. crassostreae*) or 25°C (*B. pumilus* RI06-95) with shaking.

### In Vitro *Screening of Probiotic Candidates*

A bacterium–bacterium competition assay described by Teasdale et al. (2009) was used in this assay with several modifications. In the colony-on-top assay, 5 mL 0.8% of YP3 soft agar containing 50 µL of approximately $10^8$ cfu/mL of the pathogen from an overnight culture was poured over YP3 agar plates. After the agar cooled, 2 µL of a solution of about $10^8$ cfu/mL of the candidate probiotic from an overnight culture was spotted onto the plate and incubated at 30°C for 12–16 h before the inhibition zones were measured. For the membrane overlay assay, an aliquot of 2 µL of a solution of approximately $10^8$ cfu/mL of the candidate probiotic was spotted onto YP3 agar plates and incubated at 23°C for 48 h. After incubation, a sterile 12–14 kDa molecular-weight cutoff dialysis membrane (Spectra/Por; Spectrum Medical Industries, Inc., Houston, TX) was laid atop the colonies and covered with 6 mL 0.8% YP3 agar containing 60 µL of approximately $10^8$ cfu/mL of pathogen from an overnight culture. Plates were incubated at 30°C for 12–16 h after agar solidification, and the diameter of the clear (inhibitory) zones around the probiont colonies was measured using a ruler.

### *Characterization of* Phaeobacter *sp. S4 Growth and Morphology*

Single colonies of *Phaeobacter* sp. S4 were inoculated onto YP3 media, grown for 48 h at 27°C with shaking, and then back-diluted into fresh YP + 2% NaCl (YP2) or YP + 3% NaCl (YP3) media at a 1:1,000 dilution. Cultures were incubated at 27°C with shaking for up to 72 h, and aliquots were taken at selected time points to determine bacterial concentration (measured in colony-forming units per milliliter) by plating of serial dilutions. Aliquots of bacterial cells taken from cultures grown to late exponential (36 h) and stationary (48 h) phases were placed on glass coverslips and examined by phase-contrast microscopy at the Rhode Island Genomics and Sequencing Center at the University of Rhode Island with a Zeiss Axio Imager 2 microscope using phase-contrast optics. Biofilm-containing samples were grown in static culture conditions for 48 h at 27°C and scraped from the walls of the glass culture tubes (15 × 150 mm) before being placed on glass slides and observed by phase-contrast microscopy.

### *Preparation of Bacterial Isolates for Challenge*

Candidate probiotics and pathogens were cultured overnight with shaking in 10 mL YP3 broth. Overnight cultures were transferred to 50-mL sterile Falcon tubes and centrifuged at 2,300*g* for 10 min to harvest the cells. Cells were washed twice with 10 mL filtered sterile seawater (FSSW) and the cell pellet was resuspended in 10 mL FSSW and mixed using a vortex mixer. The bacterial density was determined by measuring optical density at 550 nm using a spectrophotometer (Synergy HT; BioTek) and assuming that an optical density of 1.000 corresponds to $1.2 \times 10^9$ cfu/mL according to the McFarland standard (BioMerieux, Marcyl'Etoile, France). After the concentration of the bacteria was determined, the bacterial suspension was diluted to the target concentration in FSSW. The final target concentration was confirmed by plating serial dilutions of the bacterial cultures for each treatment on the appropriate agar plates and counting colony forming units after overnight incubation at 25°C or 28°C. The commercial probiotic mix (Sanolife MIC; INVE Aquaculture, Belgium) was mixed by adding 0.1 g Sanolife to 50 mL FSSW following the manufacturer's protocol. The solution was then adjusted to a stock concentration of $5 \times 10^6$ cfu/mL and used at a target concentration of $10^4$ cfu/mL.

### *Larval Oyster Bacterial Challenges*

Experimental challenges were performed as described previously (Gómez-León et al. 2008) with minor modifications. Larvae of eastern oysters, *Crassostrea virginica* (age, 12–20 days; size, 50–150 µm) were obtained from the Blount Shellfish Hatchery at Roger William University (Bristol, RI). Oysters (25–30 larvae) were placed in each well of a 6-well plate containing 5 mL FSSW at 28 psu. The candidate probiotics isolates S4 and RI06-95 were added to the wells at final concentrations ranging from $10^2$–$10^6$ cfu/mL. The commercial probiotic Sanolife MIC was used at a final concentration of

$10^4$ cfu/mL. Larval oysters were fed with commercial algal paste (20,000 cells/mL; Reed Mariculture Inc., San Jose, CA) to promote ingestion of the probiotics. Plates were incubated at 22–23°C for 24 h with gentle rocking. Water in the wells was then changed to remove the probiotics. Either *Vibrio tubiashii* RE22 or *Roseovarius crassostreae* CV919-312$^T$ was added to 5 mL FSSW containing the larvae to achieve the target concentration of pathogen ($10^5$ cfu/mL or $10^6$ cfu/mL). Control wells included untreated larvae (with and without pathogen) and larvae incubated with probiotics but not with the pathogen. Each treatment was run in triplicate. Larval survival was determined 24 h after addition of the pathogen by adding 200 µL neutral red to each well to a final concentration of 0.53 mg/L and incubating for 2 h before counting living and dead oysters. The neutral red staining technique distinguishes between live (stained) and dead (unstained) larvae (Fig. 1) (Gómez-León et al. 2008). The survival rate was calculated by using the formula

$$\text{Survival rate } (\%) = 100 \times \left(\frac{\text{No. of live larvae}}{\text{Total no. of larvae}}\right).$$

The relative percent survival (RPS) (Amend 1981) conferred by the probiont (treatment) with respect to the challenged larvae (control) was calculated by using the formula

$$\text{RPS} = \left[1 - \left(\frac{\%\ \text{Mortality treatment}}{\%\ \text{Mortality control}}\right)\right] \times 100.$$

These experiments were run at least 3 times in triplicate for the candidate probionts S4 and RI06–95, and once for the commercial probiont Sanolife MIC.

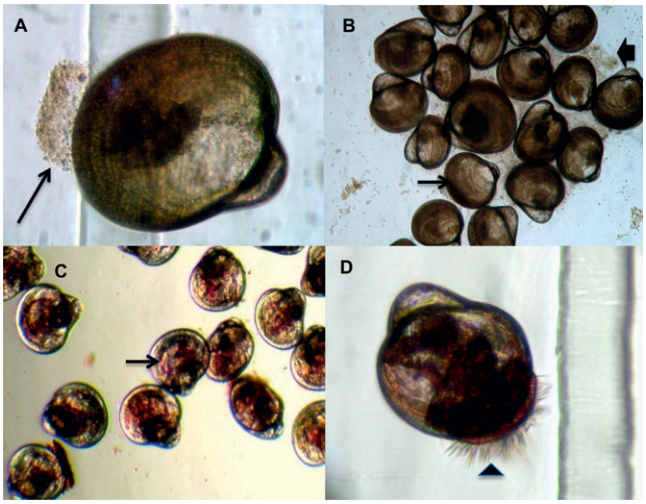


**Figure 1. Effect of preincubation with candidate probiont *Phaeobacter* sp. S4 on the morphology of larval oysters 24 h after challenge with the bacterial pathogen *Vibrio tubiashii* RE22. The candidate probionts were introduced 24 h before pathogen challenge. (A) Larva challenged with RE22 show clumping of cilia (arrow). (B) Group of larva challenged with RE22 show cell debris (thick arrow) and dead larvae as indicated by empty shells not stained with neutral red (arrow). (C, D) Larvae preincubated with S4 and challenged with RE22 were viable and show staining with neutral red (arrow) and normal cilia (arrowhead).**

### *Length of Protection Conferred by Candidate Probionts*

Larval oysters were placed in 6-well plates containing 5 mL FSSW; candidate probionts were introduced to a final concentration of $10^4$ cfu/mL. Plates were incubated at 22–23°C for 24 h with gentle rocking. At 24 h of incubation, FSSW was removed from the wells and exchanged with 5 mL FSSW without the probiotics. The pathogen *Vibrio tubiashii* RE22 (final concentration, $10^5$ cfu/mL) was applied to the wells 24 h, 72 h, or 120 h after addition of the candidate probionts (equivalent to 0 h, 48 h, or 96 h after removal of the probiont). After 24 h of incubation with the pathogen, larval oyster survival and RPS were determined as described earlier. Larval oysters were fed daily with commercial algal paste (20,000 cells/mL). This assay was run only once with each treatment tested in triplicate.

### *Juvenile Oyster Bacterial Challenges*

Ten juvenile oysters (shell height, 8–15 mm) per container were placed in 500-mL buckets containing 200 mL FSSW, and each container was provided with continuous aeration via air stones. Candidate probionts were applied at a final concentration of $10^5$ cfu/mL and containers were incubated at 22–23°C for the length of the experiment. After 24 h of incubation with the probiont, *Vibrio tubiashii* RE22 was applied to a final concentration of $10^5$ cfu/mL. Mortalities were recorded every 2–3 days for 13 days, and cumulative percent survival was calculated. Water was exchanged every 2–3 days and the oysters were fed daily with commercial algal paste (20,000 cells/mL). This experiment was performed once using duplicate containers per treatment.

### *Statistical Analysis*

Survival and cumulative mortality data were analyzed using 1- or 2-way analysis of variance (ANOVA), and multiple comparison tests (Tukey's test) was used to determine significance among groups. Data collected as a percentage were arcsine of the square root-transformed before analysis. Results were considered significant at a 95% level of confidence ($P < 0.05$). All statistics were run using SigmaStat 3.1 software (Systat).

## RESULTS

### *Antibiotic Activity Against Bacterial Pathogens*

In this study, among 64 bacteria strains isolated from the inner shell of healthy oysters, only *Phaeobacter* sp. S4 was found to have antibiotic activity against *Vibrio harveyi* BB120 by using 2 different plates assays. In the membrane overlay assay, the use of the membrane prevents direct contact between probiont and pathogen, only allowing chemicals with a molecular mass less than 12–14 kDa to go through. This method allows observation of chemical interactions between probiont and pathogen. Meanwhile, the colony-on-top assay allows for direct bacterial interaction between probiont and pathogen. The probiont candidate *Bacillus pumilus* RI06-95 inhibited the growth of pathogens *V. harveyi* BB120 at 28°C and *Roseovarius crassostreae* CV919-312$^T$ at 20°C and 28°C using both the colony-on-top and the membrane overlay assays (Table 1). This isolate, however, showed no growth-inhibiting activity against

**TABLE 1.**

**Antibiotic activity of candidate probionts *Phaeobacter* sp. S4 and *Bacillus pumilus* RI06-95 against selected bacterial pathogens of finfish (*Vibrio harveyi* BB120) and shellfish (*Vibrio tubiashii* RE22 and *Roseovarius crassostreae* CV919-312$^T$) as determined by 2 plate diffusion assays at 2 temperatures.**

| Probiotic, temperature | Colony on top (mm) | | | Membrane overlay (mm) | | |
|---|---|---|---|---|---|---|
| | BB120 | RE22 | CV919-312$^T$ | BB120 | RE22 | CV919-312$^T$ |
| RI06-95, 20°C | — | 0 | 11 ± 1 | — | 0 | 4 ± 1 |
| RI06-95, 28°C | 10 ± 1 | 0 | 13 ± 1 | 7 ± 1 | 0 | 5 ± 1 |
| S4 20°C | — | 0 | 16 ± 0 | — | 6 ± 1 | 15 ± 1 |
| S4, 28°C | 6 ± 1 | 7 | 13 ± 1 | 6 ± 1 | 6 ± 1 | 13 ± 2 |

The antibiotic activity is reported as the diameter of the inhibition zone in millimeters ± SEM, including the size of the colony for the candidate probiont (3 mm). —, not tested

*Vibrio tubiashii* RE22. The candidate probiont *Phaeobacter* sp. S4 inhibited the growth of all pathogens with the exception of *V. tubiashii* RE22 at 20°C in the colony-on-top assay (Table 1). Differences in the pattern of inhibition between the 2 assays for this probiont are probably the result of differences in the length of the incubation times of the probiotic with the pathogen (12–16 h for the colony on top and 48 h for the membrane overlay).

### *Characterization of* Phaeobacter *sp. S4 Growth and Morphology*

We characterized *Phaeobacter* sp. S4 with regard to some basic properties that might affect its ability to serve as a probiotic organism in marine aquaculture—namely, growth curves in marine media and the ability to form biofilms. Briefly, S4 grew well in YP2 or YP3 at temperatures from 18–30°C (not shown). Cells were unable to grow at 37°C. At 27°C, there was no difference in the growth rate of S4 when cells were grown in either YP2 or YP3 (Fig. 2). The fastest doubling times for the cells in YP2 and YP3 were 2.2 h and 1.9 h, respectively. The average doubling time for each condition was 3.1 h for YP2 and 3.2 h for YP3. The final density of S4 in either YP2 or YP3 was more than $2.0 \times 10^9$cfu/mL.

Although growth in YP2 and YP3 produces virtually identical growth rates and final cell densities, these 2 conditions resulted in 2 different morphologies for *Phaeobacter* sp. S4 (Fig. 3). Growth in YP3 resulted in small, ovoid, motile cells (Fig. 3A) that, when entering the stationary phase, form rosettes. Cells grown in YP2 elongate to spindle-shaped cells during the late stationary phase, lose motility, and form rosettes (Fig. 3B, C). If grown in static culture, the cells formed a thick biofilm on glass surfaces (Fig. 3D). Plastic surfaces (polycarbonate, polystyrene, and polypropylene) did not support the formation of a biofilm by S4 (not shown).

### *Effect of Pretreatment with Probiotics on Larval Oyster Survival of Bacterial Challenge*

Candidate probionts were not pathogenic to the host because the survival of oyster larval treated with the candidate probionts was not significantly different from the control (Fig. 4). Rapid deaths of larval oysters were seen after exposure to pathogens *Vibrio tubiashii* RE22 and *Roseovarius crassostreae* CV919-312$^T$ for 24 h, with survival ranging from 14%–31%, depending on the pathogen and dose (>80% for unchallenged controls). Survival of oysters pretreated with candidate probionts for 24 h and then exposed to the bacterial pathogens were significantly greater than those larvae that had not been exposed to the probiont, increasing from a survival of 14%–31% for untreated larvae to 32%–64% for probiotic-treated larvae (Fig. 4).

The level of protection was different depending on the relative concentrations of candidate probionts and pathogen added (Table 2). Candidate probiont *Phaeobacter* sp. S4 was found to protect larval oysters more effectively against *Vibrio tubiashii* RE22 than against *Roseovarius crassostreae* CV919-312$^T$. This study also demonstrated *Phaeobacter* sp. S4 gave greater levels of protection against both pathogens than *Bacillus pumilus* RI06-95. The optimal concentration for probionts

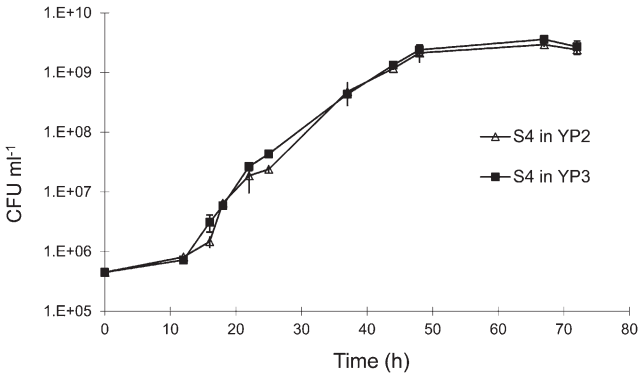


**Figure 2. Growth curve of *Phaeobacter* sp. S4 in YP2 and YP3 at 27°C. Cells were grown for 48 h in YP3 and then back-diluted into fresh YP2 (△) or YP3 (■) at a 1:1,000 dilution. Samples were taken at the indicated times and the cell density was determined by serial dilution and plating onto YP3.**

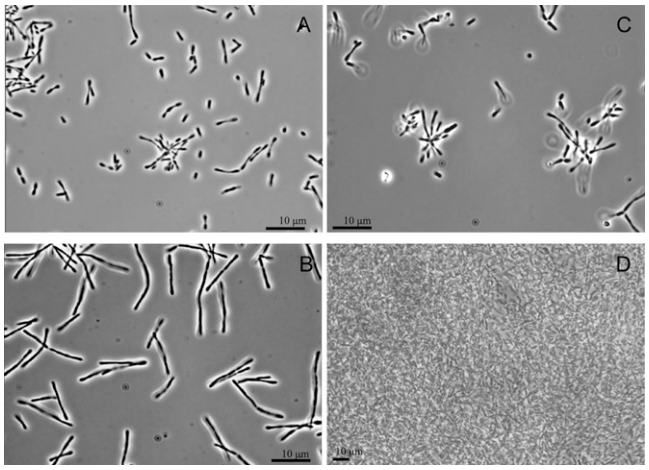


**Figure 3. Phase contrast micrographs showing the morphology of *Phaeobacter* sp. S4 in different growth phases. (A) Late-exponential phase cells grown in YP3. (B) Late-exponential phase cells grown in YP2. (C) YP2-grown cells in rosettes. (D) S4 cells grown in YP2 in a biofilm. Size bar = 10 µm.**

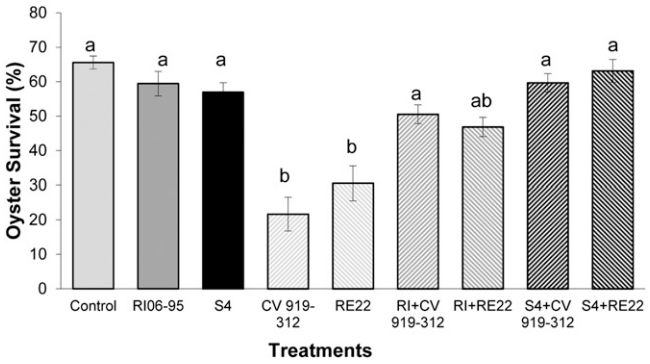


**Figure 4. Effect of preincubation of larval oysters with candidate probionts RI06-95 and S4 at $10^4$ cfu/mL on survival (% ± SEM) 24 h after challenge with bacterial pathogens *Roseovarius crassostreae* CV919-312$^T$ and *Vibrio tubiashii* RE22 at $10^5$ cfu/mL. The candidate probionts were introduced 24 h before larvae were challenged. Representative of at least 3 experiments; different letters indicate statistical significance among groups (1-way ANOVA, $P < 0.05$).**

*Phaeobacter* sp. S4 and *B. pumilus* RI06-95 was $10^4$ cfu/mL. At this concentration, both probiotics were able to confer significant survival against *V. tubiashii* RE22 ($P < 0.05$) and *R. crassostreae* CV919-312$^T$ (Table 2). On the other hand, no protection effect was found in larval oysters treated with commercial probiotic Sanolife MIC after challenge with *V. tubiashii* RE22 (survival of challenged larvae pretreated with Sanolife MIC of 2 ± 2% compared with 98 ± 2% for unchallenged larvae pretreated with Sanolife MIC; Table 2).

### *Length of Protection Conferred by Probiotics*

To determine the duration of protection provided by a 24-h exposure of larval oysters to the candidate probionts, we determined the survival of larval oysters challenged at different time points after exposure to the probionts (0 h, 48 h, and 96 h after removal of the candidate probionts). As observed earlier, larval oysters incubated with probionts for 24 h were protected significantly against a 24-h bacterial challenge with *Vibrio tubiashii* when the pathogen was added immediately after the removal of the probiont (Fig. 5). However, no significant protection was obtained when the larvae were challenged 48 h and 96 h after removal of the probionts. The RPS of larval oysters exposed to *Phaeobacter* sp. S4 for 24 h decreased significantly, from 78% when oysters were challenged immediately after removal of the probiont to 14% and 13% when oysters were challenged 48 h and 96 h, respectively, after removal of the probiont. The RPS of larval oysters exposed to *Bacillus pumilus* RI06-95 decreased from 44% when oysters were challenged right after removal of the probiont to 1% (challenged at 48 h) and 4% (challenged at 96 h).

### *Effect of Pretreatment with Probionts on Juvenile Oyster Survival of Bacterial Challenge*

We wanted to determine whether exposure to the probiotic bacteria would protect juvenile oysters from *Vibrio tubiashii* in a manner similar to what was observed for larval oysters. Although juvenile oysters showed a sharp increase in mortalities on day 6 after challenge with *V. tubiashii* RE22, oysters in containers to which probiotic strains were added 24 h before the challenge showed relatively low levels of mortality (<15%) until day 8 after challenge (Fig. 6). At the end of the assay (13 days), exposure to the probionts reduced significantly juvenile oyster mortalities after challenge with *V. tubiashii* ($P < 0.05$; RPS: *B. pumilus* RI06-95, 60 ± 0%; *Phaeobacter* sp. S4, 67 ± 0%). Co-incubation of juvenile oysters with both S4 and RI06-95 did not confer added levels of protection compared with preincubation with either one of the probiotics alone ($P > 0.05$).

## DISCUSSION

This study identified successfully 2 potential bacterial candidates to be used as probiotics for disease management control in oyster aquaculture. Bacterial strains *Phaeobacter* sp. S4 and *Bacillus pumilus* RI06-95 were selected as candidate probionts because of their antagonistic properties against the oyster

**TABLE 2.**

**Effect of preincubation with candidate probionts *Bacillus pumilus* RI06-95, *Phaeobacter* sp. S4, and commercial probiotic mix Sanolife MIC (INVE Aquaculture, Belgium) on larval oyster survival 24 h after challenge with bacterial pathogens *Roseovarius crassostreae* CV919-312$^T$ and *Vibrio tubiashii* RE22.**

| | | | Relative percent survival* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Probiotics and concentration (cfu/ml) | | | | | | |
| | | | RI06-95 | | | S4 | | | INVE |
| Bacterial pathogens and concentration (cfu/mL) | | | $10^6$ | $10^4$ | $10^2$ | $10^6$ | $10^4$ | $10^2$ | $10^6$ |
| Oyster | RE22 | $10^6$ | 31 ± 2$^a$ | — | — | 53 ± 3$^b$ | — | — | — |
| | | $10^5$ | 29 ± 3$^a$ | 29 ± 3$^a$ | — | 44 ± 3$^b$ | 55 ± 2$^b$ | — | 0 |
| | CV919–312$^T$ | $10^6$ | 11 ± 2$^x$ | — | — | 14 ± 3$^x$ | — | — | — |
| | | $10^5$ | 22 ± 2$^y$ | 42 ± 3$^z$ | 20 ± 1$^{xy}$ | 43 ± 5$^z$ | 49 ± 3$^z$ | 29 ± 2$^y$ | — |

The candidate probionts were introduced 24 h before larvae were challenged. Data are expressed as relative percent survival (±SEM) of challenged oysters pretreated with probiotic to control challenged oysters. Different letters in superscript indicate statistical differences among treatments for each probiont (1-way ANOVA, $P < 0.05$).

* RPS $= \left[1 - \left(\frac{\%\ \text{Mortality treatment}}{\%\ \text{Mortality control}}\right)\right] \times 100$. —, not tested.

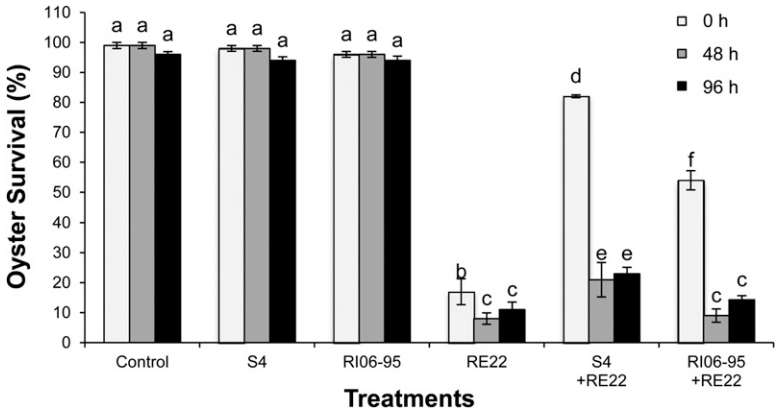


**Figure 5. Length of protection to bacterial challenge provided by preincubation of larval oysters with candidate probionts RI06–95 and S4. Larval oysters were preincubated for 24 with $10^4$ cfu/mL probiont, washed, placed in filtered sterile seawater, and then challenged by adding $10^5$ cfu/mL of the pathogen *Vibrio tubiashii* RE22 0 h, 48 h, or 96 h after removal of the probionts. Different letters indicate statistical significance among treatments and times (2-way ANOVA, $P < 0.05$).**

pathogens *Roseovarius crassostreae* and *Vibrio tubiashii*, and also the marine finfish and shellfish pathogen *Vibrio harveyi*. We demonstrate here that they also conferred significant protection to larval oysters against experimental bacterial challenge. Furthermore, preincubation of juvenile oysters with these probionts led to significantly improved survival of juvenile oysters 13 days after challenge with *V. tubiashii*.

These 2 candidate probionts were able to protect oyster larvae and juveniles against the severe bacterial challenges used in our research, and show the potential to provide protection when used prophylactically in hatcheries, where the levels of pathogenic bacteria in seawater sometimes approach levels similar to the challenge doses used in our experiments (Elston et al. 2008). The bacterial pathogens *Vibrio tubiashii* and *Roseovarius crassostreae* caused rapid mortalities in larval oysters in our experiments; oysters stopped swimming and most of the tissue was digested completely, leaving an empty shell after 24 h of exposure to the pathogens. This is consistent with previous research on these pathogens (Gibson et al. 1998, Elston et al. 2008, Gómez-León et al. 2008).

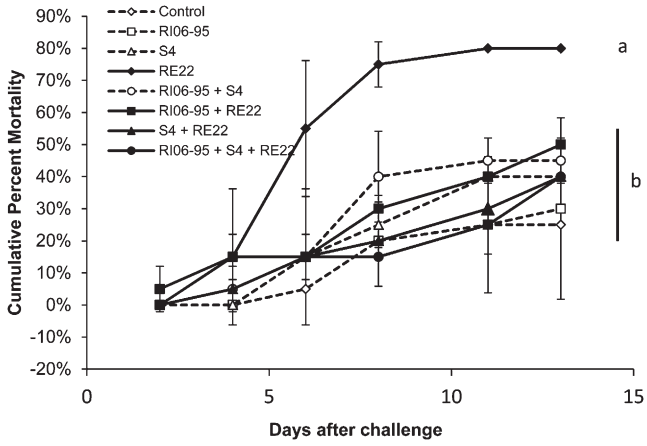


**Figure 6. Effect of preincubation of juvenile oysters with candidate probionts on oyster survival after bacterial challenge. Oysters were preincubated with $10^4$ cfu/mL of probionts *Bacillus pumilus* RI06-95 or *Phaeobacter* sp. S4 for 24 h. Then, $10^5$ cfu/mL of the pathogen *Vibrio tubiashii* RE22 was added to the incubation seawater; survival was determined every 2–3 days for 13 days. Different letters indicate statistical significance among treatments (1-way ANOVA, $P < 0$).**

The candidate probionts we have tested here are commensals of marine organisms and were proved in our experiments to be safe to larval and juvenile oysters because they had no significant effect on larval or juvenile survival at the concentrations tested (up to $10^6$ cfu/mL). Although we have not tested the effect of these probionts on algal cultures directly, previous research on another *Phaeobacter* sp. (*Phaeobacter gallaeciensis*) with probiotic activity on cod larval cultures showed no negative effects of this probiont on the survival of the microalgae *Tetraselmis suecica,* a species used commonly in aquaculture hatcheries (D'Alvise et al. 2012). Our experiments also showed that significant levels of protection were obtained with a dose of probiotic of $10^4$ cfu/mL, a dose achieved easily even in the large culture tanks used at commercial hatcheries. The length of protection conferred to larval oysters by exposure to the probionts, however, is short term (24 h), suggesting that these probiotics may need to be supplied to larvae in the hatcheries daily to maintain their effectiveness. This is not uncommon for other probiotics, which are usually provided daily with the feed to host organisms to provide maximum benefits (Verschuere et al. 2000, Kesarcodi-Watson et al. 2008). Interestingly, a single dose of probiotics added to the culture water of juvenile oysters 24 h prior to bacterial challenge provided significant levels of protection for at least 13 days, suggesting that the probionts may persist longer in juvenile oysters compared with larval oysters, or that additional mechanisms of protection are involved in juvenile oysters. More research should be done to determine the effectiveness and mechanisms of action of these probiotic bacterial strains during different developmental stages of oysters and different growing conditions.

Our study showed that lack of growth-inhibiting activity *in vitro* toward a particular pathogen is not necessarily predictive of how a candidate probiont would perform *in vivo*. Candidate probiont *Bacillus pumilus* RI06-95 was not able to inhibit the growth of *Vibrio tubiashii in vitro*, but it showed a protective effect toward larval and juvenile oysters during *in vivo* challenge, suggesting that protection conferred by *B. pumilus* RI06-95

against *V. tubiashii* may not be a result of antibiotic activity or that the *in vitro* assays used in the screening process do not predict the production of the antibiotic *in vivo*. Probiotics are able to improve survival of the hosts by different mechanisms (Verschuere et al. 2000, Kesarcodi-Watson et al. 2008). Beside secretion of antibiotic compounds, it is known that probiotics are capable of various other modes of action that give benefits to the host. In previous research, *Bacillus* sp. S11 has been reported to improve health by stimulating the immunity of the host organism (Rengpipat et al. 2000). This may be one of potential mechanism provided by *B. pumilus* RI06-95 to protect the oysters against *V. tubiashii* in our *in vivo* assay. This probiotic may also promote enhanced digestion in oysters. Research by Olmos et al. (2011) demonstrated the ability of *Bacillus subtilis* to enhance carbohydrate digestion and improve the health of shrimp (*Litopenaeus vannamei*). Furthermore, Sun et al. (2010) demonstrated that grouper (*Epinephelus coioides*) consumed dietary nutrients better after supplementing the feed with *B. pumilus* or *Bacillus clausii*.

In contrast, the results from the *in vitro* tests with *Phaeobacter* sp. S4 showed growth-inhibiting activity against the 2 oyster pathogens, and this coincided with increased protection seen in the *in vivo* assays. Research performed by Porsby et al. (2008) showed that members of the *Roseobacter* clade such as *Phaeobacter gallaeciensis* and *Phaeobacter inhibens* produce an antibiotic compound named tropodithietic acid, which is capable of inhibiting the growth of the bacterial pathogens *Vibrio anguillarum, Vibrio splendidus, Vibrio cholerae, Bacillus subtilis*, and *Halomonas* spp. Furthermore, the application of bacterial cultures or cell extracts of *Phaeobacter* spp. improve survival of fish larvae (Makridis et al. 2005, Planas et al. 2006) and shellfish (Ruiz-Ponte et al. 1999, Balcázar et al. 2006) in rearing tanks. Recently, D'Alvise et al. (2012) demonstrated the ability of *P. gallaeciensis* to protect cod larvae from vibriosis. Besides producing tropodithietic acid, *Phaeobacter* spp. are known as primary colonizers of various inorganic and organic marine surfaces, including marine algae and dinoflagellates (Dang & Lovell 2002, Mayali et al. 2008). Our observations of this bacterium confirm that *Phaeobacter* sp. S4 forms rosettes and biofilms avidly on inorganic surfaces, such as glass. Furthermore, our results, which show the ability of a *Phaeobacter* sp. isolated from the inner side of an adult oyster shell (and probably a member of the natural oyster microbiome) to protect larval oysters from bacterial challenge, provide further evidence of the potential of *Phaeobacter* spp. as probiotic species.

In conclusion, these studies successfully isolated 2 candidate probionts for disease management in oyster hatcheries. The probiont *Phaeobacter* sp. S4 is a good probiont candidate that showed clear antibiotic activity *in vitro* and protection *in vivo*. The relationship between probiotic activity *in vivo* and antibiotic activity *in vitro*, however, is not so strong in the case of protection of larval oysters against *Vibrio tubiashii* conferred by *Bacillus pumilus* RI06-95, suggesting that other mechanisms contribute to probiotic activity. Thus, in addition to good candidates for use in shellfish aquaculture, these candidate probionts will be useful in evaluating the relationship between antibiotic and probiotic activities to help establish rational strategies for the screening for potential probiotics.

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