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Alteromonas macleodii 0444 and *Neptunomonas* sp. 0536, two novel probiotics for hatchery-reared Greenshell™ mussel larvae, *Perna canaliculus*

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ABSTRACT

Antibiotic management of aquacultured animals, such as Greenshell™ mussel (GSM) larvae, *Perna canaliculus*, is undesirable because of health concerns and political pressures; hence, alternatives are needed. Herein, two novel probiotic bacteria were identified and trialled in a GSM larval rearing hatchery. Sequencing of the 16S rRNA gene and phylogenetic analysis identified the strains as *Alteromonas macleodii* 0444 and *Neptunomonas* sp. 0536. Both probiotics were evaluated separately at the Glenhaven Aquaculture Centre hatchery facility during routine larval rearing and when the larvae were challenged with both a high (10^7 and 10^6 CFU ml^{-1}) and low (10^6 and 10^5 CFU ml^{-1}) pathogenic dose of *Vibrio* sp. DO1 and *V. splendidus* respectively. In all experiments, probiotic application improved larval survival significantly when administered prior to pathogen exposure. Across all experiments, larvae that were exposed to the high and low dosages of pathogens averaged 14% and 36% survival respectively on the fourth day following pathogen exposure. The administration of probiotics prior to pathogen challenge resulted in larval survival of 50% and 66% respectively. Non-inoculated control larvae and larvae administered the probiotic alone demonstrated 67% and 79% survival respectively. *Neptunomonas* sp. 0536 appeared to suppress naturally occurring vibrios in the culture environment of healthy GSM larvae. This is the first report of *A. macleodii* and *Neptunomonas* sp. as probiotic bacteria in a large scale production facility.

1. Introduction

In New Zealand, the aquaculture production of Greenshell™ mussels (GSM), *Perna canaliculus*, is valued at US$145 million per annum and represents 65% and 90% of the total New Zealand aquaculture product by value and weight respectively (FAO, 2007). Currently, the farming practice involves on-growing wild-caught juvenile GSM until they reach market size. However, hatchery production of GSM is being developed to facilitate stock reliability and enable selective breeding programmes (King, 2005, 2008).

To date, the larval stages of GSM production intermittently encountered problems, which were often alleviated by administration of antibiotics. Isolation and demonstration of two pathogens of GSM larvae, *V. splendidus* and a *V. coralliilyticus*/*neptunius*-like isolate, implicated bacterial pathogenesis as a factor in these problems (Kesarcodi-Watson et al., 2009a). Because of the risks associated with antibiotic usage (Schwarz et al., 2001; Cabello, 2006), continued management of these pathogens with antibiotics was not the preferred method of choice, prompting investigation into probiotic alternatives. Following a screening bioassay and hatchery-pilot trials (Kesarcodi-Watson et al., 2009b), two potential probiotic isolates emerged as being worthy of full-scale hatchery experimentation.

In vivo trials have demonstrated the potential that probiotics hold for both disease prevention (Moriarty, 1998; Lategan et al., 2004a,b; Chabrillón et al., 2006) and growth enhancement (Gatesoupe, 2002; Lara-Flores et al., 2003; Macey and Coyne, 2005). Riquelme et al. (2001) examined probiotic use at a commercial scallop hatchery. Although they did not examine protection against a specific disease challenge, they demonstrated that a *Vibrio* sp., *Pseudomonas* sp. and *Bacillus* sp. allowed completion of the larval period without the need for antibiotics previously thought to be indispensable.

At the final stage in the development of any new treatment, controlled trials are required in the environment where an effect is intended. In the search for probiotics in aquaculture there have been few field trials under commercial aquaculture conditions. The aim of this study was to determine the benefits of two novel probiotics on GSM larvae reared under hatchery production conditions and exposed to two separate pathogen challenges. The experiments were designed to determine the practical potential of the tested probiotics for routine GSM larval rearing.

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2. Materials and methods

2.1. Experimental animals

Experiments were conducted at the Glenhaven Aquaculture Centre hatchery (GACL), Glenduan, Nelson, New Zealand. GSM larvae were obtained from the hatchery according to normal hatchery protocol (Kesarcodi-Watson et al., 2009a). Newly hatched D-veliger larvae were screened on nylon mesh size 45 μm and then placed into 2.5 l tanks at a density of 200 larvae ml^{-1} for use in experiments. Water flow was continuous at 80 ml min^{-1}, aeration was provided, water temperature was 19 °C and larvae were fed a 2:1 mix of *Chaetoceros calcitrans*:*Isochrysis galbana* to provide a final concentration in tanks of 40 algal cells μl^{-1}.

2.2. Culture and harvest of bacteria

Bacteria were grown and harvested in log phase prior to inoculation into test tanks. Initially, each bacterium was recovered from storage at −70 °C and revived in tryptone soy broth (Merck) made to 2% salinity using natural seawater (TSB-2%Sea). Test isolates were streaked to ensure purity and sub-cultured three times on tryptone soy agar (Merck) made to 2% salinity using natural seawater (TSA-2% Sea). Isolates were sub-cultured into separate 10 ml volumes of Marine Broth (MB, Difco) and incubated at 25 °C for 10 h. A 0.1 ml aliquot of each culture was further sub-cultured into another 10 ml MB and further incubated for 10 h at 25 °C. The final 10 ml volume of each culture was aseptically transferred into 500 ml MB and incubated for 20 h at 24 °C–27 °C on a New Brunswick G10 Gyrotory Shaker (N.J., U.S.A.) at 150 rpm.

The cell concentration of final broth cultures was determined via optical density at 600 nm using a PharmaSpec UV-1700 spectrophotometer (Shimadzu). Broth cultures were then centrifuged (3700 rpm, 10 min, 15 °C, Beckman J2-21M/E) and washed twice in sterile seawater. Supernatant-free cells were re-suspended in sterile seawater to their original concentrations and transported to the GACL (approximately 10 min drive) for use in experiments. Prior to use in the experiments, 10-fold dilutions of the washed cultures were made and then surface-spread plated for enumeration on TSA-2%Sea to verify experimental concentrations.

2.3. Phenotypic profiling of probiotic strains

Two potential probiotics, strains 0444 and 0536, were selected for hatchery trials, based on previous pilot studies conducted at GACL (Kesarcodi-Watson et al., 2009b). Each was profiled using both phenotypic and genotypic characterisation. Gram stain, oxidase reaction, motility and catalase tests were carried out initially, followed by inoculation of API 20NE (BioMérieux). The strains were further profiled for phenotypic characteristics in order to develop selective growth conditions that could be used to monitor their presence both in the larvae and the hatchery water. These included temperature growth range (17–50 °C), salt tolerance (0–200 ppt) and antibiotic susceptibility/resistance patterns (Etest®; AB BIODISK, Sweden). Prior to Etests, antibiotic susceptibility patterns to a variety of antibiotics were conducted using the disc diffusion method in order to determine which antibiotics would be suitable (Kesarcodi-Watson, 2009). Ampicillin and aztreonam were found suitable for strain 0444, with erythromycin, trimethoprim and aztreonam suitable for strain 0536. Etests followed to yield the required MIC for the strains under test. *E. coli* (ATCC 25922) was used as a control of known susceptibility for ampicillin and aztreonam, while *Staphylococcus aureus* (ATCC 29213) was a control for erythromycin and trimethoprim. Etests for 0444 and 0536 were carried out on TSA-2%Sea, with 24 h incubation at 30 °C, a temperature recommended by the Etest manufacturer and at which all organisms grew well. *E. coli* tests were performed on Mueller–Hinton (MH) agar (Oxoid) and those of *S. aureus* were performed on MH agar + 2% NaCl, according to the manufacturers' recommendations.

2.4. Genotypic profiling

Genotypic characterization of strains entailed the amplification and sequencing of the 16S rRNA gene. Chromosomal DNA was extracted from bacterial cultures using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) following the manufacturer's instructions. The universal bacterial PCR primer pair used to amplify the 16 S rRNA gene was (Suzuki and Giovannoni, 1996):

forward: EUBB-F (5′-AGAGTTTGATCMTGGCTCAG-3′),
reverse: EUB-A-R (5′-AAGGAGGTGATCCANCCRCA-3′).

Final amplification reaction mixtures consisted of: 20 μl 2.5× HotMasterMix (Eppendorf, Hamburg, Germany), 2.0 μl DNA template, primers (final concentration 0.8 μM) and water to final volume of 50.0 μl. Amplification reactions were performed using an iCycler (Bio-Rad, Carlsbad, USA) with the following thermocycling conditions: 94 °C/2 min, 52 °C/1 min, 65 °C/1 min, 1 cycle; 94 °C/30 s, 55 °C/30 s, 65 °C/2 min, 35 cycles; 65 °C/7 min, 1 cycle. Amplification products were electrophoresed through 1.0% (w/v) agarose gels stained with ethidium bromide and visualized under UV light. Bands of the expected size (1.5 kb) were purified (QIAquick gel extraction kit, Qiagen) and used as templates for sequencing by an external contractor (Environmental Science & Research, Porirua, New Zealand). Assembled sequences were used as query strings for interrogation of the following databases: Ribosomal Database Project II (RDP, http://www.rdp.cme.msu.edu/) and GenBank (http://www.ncbi.nlm.nih.gov/). The 16S rRNA sequences obtained in this study were deposited into GenBank with accession numbers FJ463597 and FJ463598.

2.5. Phylogenetic analysis

The 16S rRNA gene sequences of strains 0444 and 0536 and top matches from GenBank and RDP were aligned in BioEdit (Hall, 1999) using ClustalW. *Nitrosomonas europaea* (accession number M96399), a β-proteobacterium, was used as an outgroup. Aligned sequences were transported in Nexus format to MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) to construct phylogenetic trees. MrBayes analysis comprised two simultaneous runs for three million generations, with four chains each. The trees were sampled every 100 generations, with the burn-in set at 20,000.

2.6. Experimental design

Pathogens, *Vibrio* sp. DO1 and *V. splendidus* (GenBank accession nos. EU358784 and EU358783, respectively), as prepared by Kesarcodi-Watson et al. (2009a), were used to challenge the larvae after administration of strains 0444 and 0536. Challenge experiments were conducted on four separate occasions; each probiotic/pathogen combination being assigned a separate experiment. At a later stage, each probiotic/pathogen combination trial was repeated.

During each experiment, larvae were inoculated daily with the test probiotic at 10^8 CFU ml^{-1} (final tank concentration), beginning on the first larval day post-hatching. At this stage, water flow was stopped and kept static for a two-hour period. Pathogen challenge of the larvae occurred on the third day following hatching (as described in Kesarcodi-Watson et al., 2009c). Pathogens were inoculated at doses previously determined to cause infection (Kesarcodi-Watson et al., 2009c). A higher dosage, one order of magnitude higher than the minimum effective dose, was also included in order to establish the extent of benefit afforded by the probiotic strains. These concentrations were represented by 10^5 and 10^6 CFU ml^{-1} for *V. splendidus*, and