Vol. 45: 45–52, 2001 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published May 4

Lactococcus garvieae infection in the giant freshwater prawn *Macrobranchium rosenbergii* confirmed by polymerase chain reaction and 16S rDNA sequencing

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ABSTRACT: An epizootic bacterial infection in the giant freshwater prawn *Macrobranchium rosenbergii* occurred in Taiwan from May to June 1999. The cumulative mortality was approximately 30 to 75%. The diseased prawns showed opaque and whitish muscles and were approximately 2 mo old with total lengths from 5 to 6 cm. Histopathologically, they showed marked edema and necrotic lesions with inflammation in the muscles and hepatopancreas. Bacteria isolated using brain heart infusion medium or tryptic soy agar were Gram-positive and ovoid. Three isolates from diseased prawns at different farms were tested using the API 20 Strepsystem and conventional tests and identified as *Lactococcus garvieae*. Experimental infections with these isolates gave gross signs and histopathological changes similar to those seen in the naturally infected prawns. The LD_{50} value of isolate MR1 was 6.6×10^5 colony forming units/prawn. Identification of MR1 was confirmed by a PCR assay for *L. garvieae* that gave the expected amplicon of 1100 bp. In addition, its 16S rDNA sequence (GenBank accession number AF283499) gave 99% sequence identity to *Enterococcus seriolicida* (synonym *L. garvieae*; GenBank accession number AF061005). This is the first report of confirmed *L. garvieae* infection in prawn aquaculture.

KEY WORDS: *Lactococcus garvieae* · Giant freshwater prawn · *Macrobranchium rosenbergii* · PCR · 16S rDNA sequencing

INTRODUCTION

Lactococcus garvieae, a Gram-positive coccus, is an emerging zoonotic pathogen. It has been isolated from cattle, from various species of fish and from humans (Eldar et al. 1996, James et al. 2000). With the development of intensive aquaculture, streptococcal infection of fish has become a major problem worldwide with reports from Japan (Kussuda et al. 1976, Kitao 1993), Taiwan (Tung et al. 1985), Italy (Ghittino & Prearo 1992, Eldar et al. 1996), Israel (Eldar et al. 1994, 1995), Spain (Barrera & Yortuero 1989, Nieto 1995), France (Nougaryrede 1995), and the United States (Rasheed & Plumb 1984, Eldar et al. 1995). Although similarity in pathology of fish infected by various Gram-positive cocci does not allow for rapid identification of the causative agents and although isolation and bacteriological analysis are complex and time-consuming, *L. garvieae* has been identified as one of the major Gram-positive coccus pathogens for fish. Streptococcosis caused by *Enterococcus*-like bacteria has been observed in rainbow trout *Oncorhynchus mykiss* (Ceschia et al. 1992, Ghittino & Prearo 1992, Palacios et al. 1993) and in turbot *Scophthalmus maximus* (Toranzo et al. 1994). An important disease of cultured yellowtail *Seri-*

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ola quinqueradiata in Japan was named *Enterococcus seriolicida* (Kusuda et al. 1991) after previously being classified as *Streptococcus* (Kusuda et al. 1976). However, taxonomic studies based on DNA–DNA hybridization (Eldar et al. 1996) and sequence analysis of 16S rRNA (Domenech et al. 1993) indicated *E. seriolicida* (Kusuda et al. 1991) was synonymous with *L. garvieae* (Eldar et al. 1996). In Spain, a serious septicemic disease of *Scopthalmus maximus* or large turbot (100 g to 3 kg) was accredited to an *Enterococcus*-like bacterium (Nieto et al. 1995) that was also found to match with *L. garvieae* (Eldar et al. 1996). Zlotkin et al. (1998) subsequently developed a polymerase chain reaction (PCR) assay based on unique regions of the *L. garvieae* 16S rRNA gene that allow for its specific identification.

The giant freshwater prawn *Macrobranchium rosenbergii* is commercially cultured throughout the world and intensively so in Taiwan, where production, however, decreased by 47 to 52% from 1992 to 1995 (New 1995, Taiwan Fisheries Bureau 1996) due to disease problems. Two diseases have been linked to production decline. One is a yeast infection that occurs mostly in the cool season (October to March), with symptoms including a yellow exoskeleton, a swollen hepatopancreas (HP), milky hemolymph and opaque and whitish muscles (Shu 1993, Cheng & Chen 1998a). The other is an *Enteroccoccus*-like infection that occurs mostly in the hot season (June to September, especially during phytoplankton blooms) and causes muscle necrosis (Cheng & Chen 1988a,b). Recently studies have indicated that mortality in *M. rosenbergii* caused by this bacterium is exacerbated by environmental temperature and pH parameters that differ from those known to be optimal for prawn growth (Cheng & Chen 1999c). In this study, the causative bacterium was identified as *Lactococcus garvieae* by PCR assay and by 16S rDNA sequencing.

MATERIALS AND METHODS

Prawn samples. For histopathological and bacteriological analysis, diseased prawn samples (body weight 2 to 3 g and 5 to 6 cm in length) were collected from 3 prawn farms during outbreaks from May to June 1999. Losses were as high as 75% in some ponds, with average losses of approximately 50%. The prawns had been fed with commercial pellets.

Bacteriology. The cuticle of diseased prawns was surface sterilized by swabbing with 70% ethanol before being cut open to reveal underlying tissues. Swabs were then taken from muscles and the HP for streaking on tryptic soy agar (TSA), blood agar (BA = TSA+5% goat blood), brain heart infusion agar (BHI), and Lowenstein-Jensen medium (LJM). Plates were then incubated at 25°C. Single colonies were restreaked on the same media to obtain pure isolates. These isolates were then identified using the API Strepsystem and conventional bacteriological tests (Eldar et al. 1996).

Pathology. Muscle tissue, HP tissue and tissues of other internal organs with lesions were fixed in Davidson's fixative and processed for paraffin sectioning. Sections were stained using haematoxylin and eosin (H&E) and Gram stains and viewed with a light microscope.

Experimental infections. Healthy prawns (*Macrobranchium rosenbergii* of 2 to 3 g body weight) were obtained from a prawn farm in Pingtung, Taiwan, and held at a density of 100 prawns in continuously aerated 400 l aquaria containing 300 l of fresh water at approximately 27°C for 7 d until they had been acclimatized to laboratory conditions. They were fed twice daily with commercial prawn pellets and waste was removed daily. Subsequently, 56 prawns of uniform size were selected and subdivided into 8 groups of 7 prawns each. Serial 10-fold dilutions from 2.3×10^8 to 2.3×10^2 cfu (colony forming units) ml^{-1} were prepared in sterile normal saline from the purified bacterial isolate *Lactococcus garvieae* MR1. For each dilution, 0.1 ml was injected intramuscularly (IM) into 7 prawns each of treatment Groups 1 to 7. Sterile normal saline (0.1 ml) was injected IM into 7 prawns of Control Group 8. After injection, each group was incubated separately in an 80 l aquarium under the same conditions as described above for the acclimatization period. The prawns were continuously monitored for morbidity and mortality and sampled for histopathology and bacteriology.

Bacterial strains. The following bacterial strains were used as controls for PCR assays: *Lactococcus garvieae* (American Tissue Culture Center, ATCC 43921), *Enterococcus seriolicida* from rainbow trout (MZ 9101 [KG-] from Dr Terutoyo Yoshida, Miyazaki University, Japan). In addition, the following strains were obtained from the Culture Collection Research Center, Taiwan: *Enterococcus faecalis* CCRC 10066, *Enterococcus durans* CCRC 10790, *Lactococcus lactis* subspecies *lactis* CCRC10791, *Lactococcus lactis* subspecies *cremoris* CCRC 11067, *Lactococcus raffinolactis* CCRC14039, *Enterococcus avium* CCRC10801. Three purified bacterial isolates derived from infected prawns were labeled MR1, MR3 and MR4.

Extraction of DNA. For nucleic acid extraction, bacteria were grown at 25°C in BHI broth and harvested by centrifugation at $7000 \times g$ for 45 min at 4°C. The pelleted bacteria were then lysed with lysozyme at 30 mg ml^{-1} for 1 h at 37°C, followed by addition of SDS to 5%, w/v for 15 min at 37°C. Equal volumes of phenol/chloroform/isoamylalcohol (25:24:1) solution were added

to the lysate and gently mixed before centrifugation at 12 000 × *g* for 15 min at 4°C. The supernatant layer was then collected and DNA precipitated by addition of 0.5 volumes of isopropanol and incubation for 30 min at −20°C followed by centrifugation at 12 000 × *g* for 15 min at 4°C. The DNA pellet was washed with 70% ethanol, dried in a speedvac and then resuspended in 20 µl of TE buffer.

Polymerase chain reaction (PCR). The sequences of specific PCR primers for identification of *Lactococcus garvieae* were obtained from Zlotkin et al. (1998) and comprised pLG-1 (5′-CAT AAC AAT GAG AAT CGC-3′) and pLG-2 (5′-GCA CCC TCG CGG GTT G-3′). These primers targeted a region of the 16S rRNA gene of *L. garvieae* (EMBL accession no. X54262; Zlotkin et al. 1998) and produced a 1100 bp amplicon. Typical PCRs were carried out in a final volume of 50 µl containing 1.25 U of recombinant Taq DNA polymerase (Takara, Japan) per 50 µl, 1× buffer (Takara, Japan), nucleotides (0.25 mM l^{-1} final concentration for each nucleotide) and primer (0.5 µM). Bacterial DNA (5 µl) template comprised extracted DNA (50 ng μl^{-1}) or whole cells obtained by touching colonies grown on BHI agar with a toothpick and then dipping the toothpick directly into the PCR mix. Amplification conditions consisted of an initial denaturation step at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1.5 min, except for the last cycle where extension was for 10 min. PCR products (5 µl) were subjected to electrophoresis (35 min, 100 V) in 2% agarose gel (SeaKem® LE agarose) with 1× TBE buffer (BDH Laboratory supplies) containing 90 mM Tris, 90 mM Borate and 2 mM EDTA (pH 8.3) and visualized by UV transillumination with ethidium bromide staining. The DNA molecular weight marker comprised a 100 bp DNA ladder (100 bp [electrophoretic types] ET marker, Chenig Hsin Tang Chemical Co. Ltd, Taiwan).

Sequencing of 16S rDNA. For sequencing, 16S rDNA was amplified by PCR using purified DNA and a primer combination consisting of forward primer 5F and reverse primer 1540R (MicroSeq™ 16S rRNA Gene Kit manufactured by Applied Biosystems). The following conditions were used for amplification: initial denaturation for 10 min at 95°C was followed by 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 60°C and extension for 45 s at 72°C. The amplification was completed by holding the reaction mixture for 10 min at 72°C to allow complete extension of the PCR product. The PCR product was purified with a QIAquick PCR purification kit (Qiagen). Sequencing of the 16S rDNA PCR fragment was carried out using an MicroSeq™ 16S rRNA Gene Kit manufactured by Applied Biosystems. The manufacturer's recommended protocols were followed. The purified sequencing reaction mixtures were automatically electrophoresed using an Applied Biosystems model 310 automatic DNA sequencer. The final sequence of the MR1 PCR fragment was determined from overlapping sequence data using Microseq™ software (Applied Biosystems). This final sequence was manually aligned with sequences of representative strains retrieved from GenBank, DDBJ and EMBL databases.

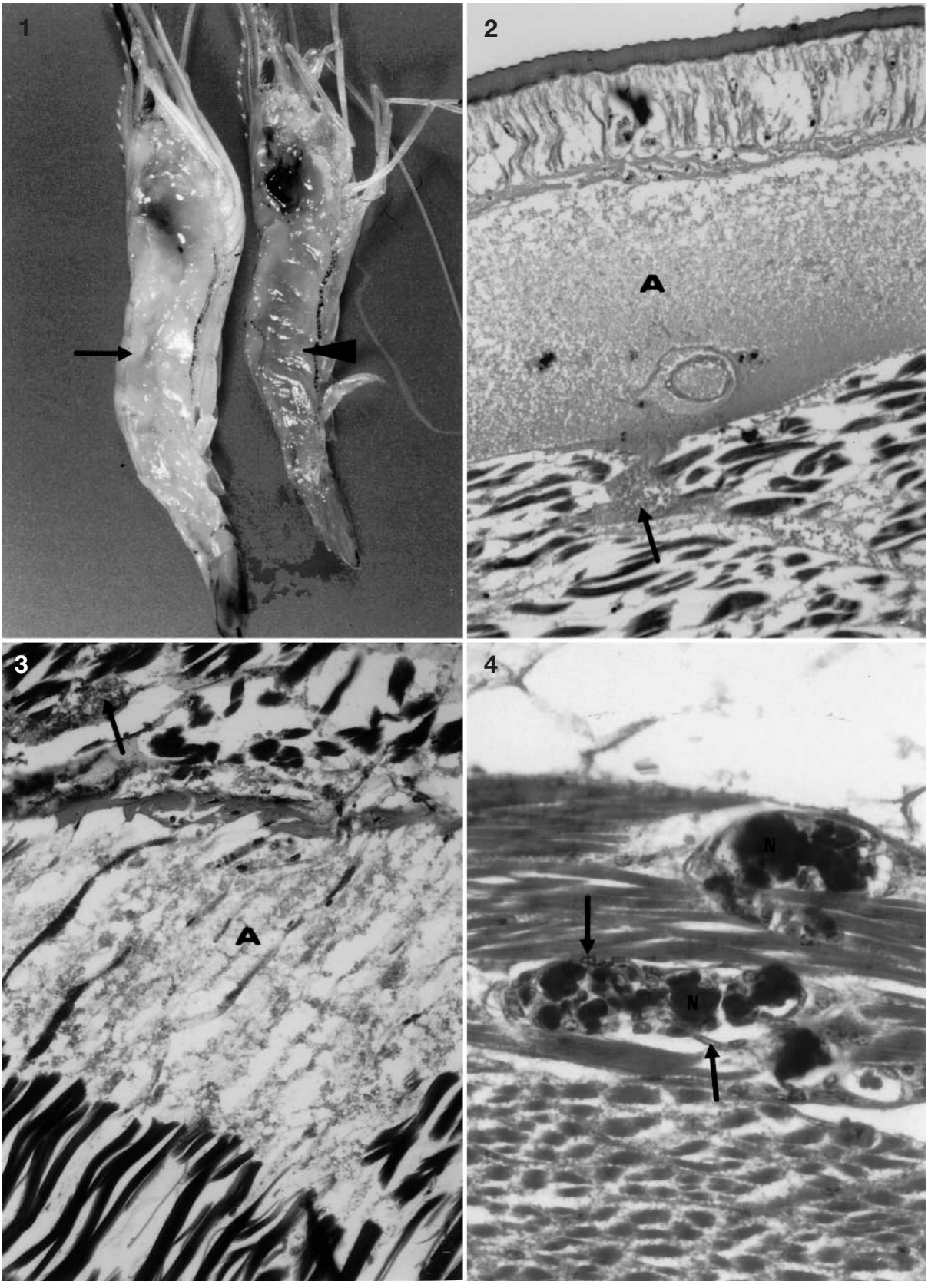
RESULTS AND DISCUSSION

Clinical signs, bacteriology and histopathology

Most moribund prawns exhibited anorexia, poor growth and a whitish body color. Gross pathological changes included whitish muscle and a swollen, yellowish HP (Fig. 1). Yellowish-white spots were seen on the muscle and varied in size from 0.5 to 2 cm. Smears from fresh HP, muscle tissue and tissues of other internal organs of diseased prawns showed the presence of numerous cocci ranging in size from 0.1 to 0.2 µm in diameter. Isolated bacterial colonies from the muscle and HP appeared 1 to 2 d after streaking on TSA, BA, and BHI.

Histopathologically, diseased prawns showed marked edematous fluid accumulation between the cuticle and underlying muscle tissue (Fig. 2), and there was fragmentation of muscle bundles with liquefaction necrosis. Large numbers of diplococci were detected in muscles of the abdomen, pereiopods and pleopods (Fig. 3). Necrotizing foci encapsulated by hemocytes (granulomas) also appeared in the muscle and HP (Fig. 4). Bacterial microcolonies were seen in the gill hemolymph (Fig. 5). In the HP, desquamated tubulular epithelial cells appeared in tubule lumens and necrotizing foci with bacterial clumps and inflammation were seen in the interstitial spaces (Fig. 6). Necrotic foci and bacterial clumps were also detected in the heart, stomach and other organs.

The diffuse muscle opacity and softening of the exoskeleton described above for white syndrome in *Macrobranchium rosenbergii* displays some gross pathological changes similar to those for white muscle disease described by Brock (1983). White muscle necrosis or spontaneous necrosis yielding whitish muscle in decapod crustaceans is a condition caused or exacerbated by management and water quality parameters such as handling, crowding, salinity, temperature and low dissolved oxygen (Lakshmi et al. 1978, Brock 1983, Lightner 1983). This opaque and whitish musculature commences at the tail, and progresses to the head, which may then turn red (Lightner 1983). No bacterial cells are associated with this condition and it is reversible during the initial stages of development, if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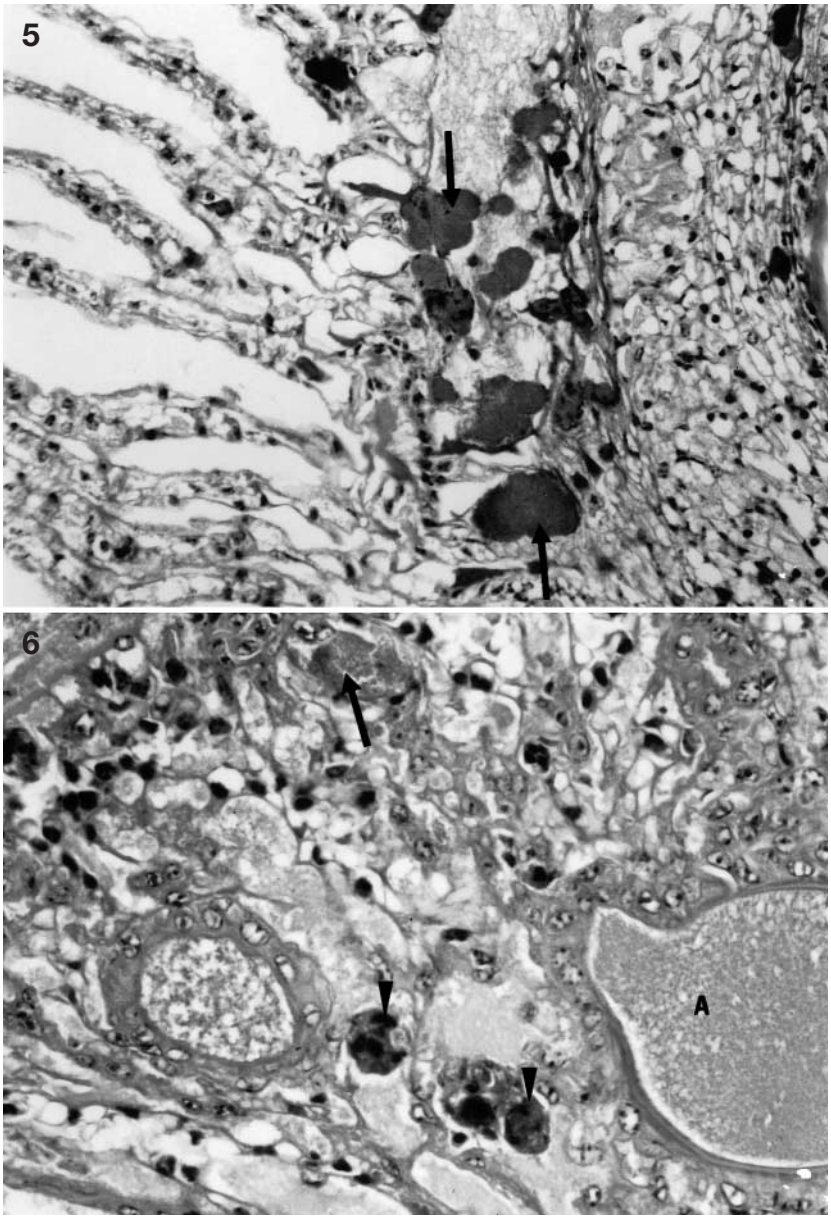
Figs. 1 to 4. *Lactococcus garvieae* infecting *Macrobranchium rosenbergii*. Fig. 1. Giant freshwater prawn showing marked opaque and white muscle lesions on the left (arrow) and compared to a normal prawn (arrowhead) on the right. Fig. 2. Giant freshwater prawn tissue section showing edematous fluid (A) accumulated between the cuticle and underlying muscle and muscle bundles (arrow). (H&E, ×200). Fig. 3. Giant freshwater prawn tissue section showing marked edematous fluid (A) and necrotizing foci in the muscle together with bacterial clumps (arrow) (H&E, ×400). Fig. 4. Necrotizing foci (N) surrounded by hemocytes (arrows) in muscle tissue. (H&E, ×400)

the causative stress is eliminated. By contrast, the muscle whitening studied herein was previously reported by Cheng & Chen (1998a) to be a different type of opaque and whitish muscle tissue that developed first in the cephalothorax and then progressed towards the abdomen in association with an *Enterococcus*-like bacterium that was also found in the HP. Clearly, the 2 conditions are very different.

In earlier publications, we described *Enterococcus*-like infections from *Macrobranchium rosenbergii* (Cheng & Chen 1998a,b) with similar histopathology to those described herein. However, there were also differences. For example, in the current study, marked necrotic areas with edematous fluid, bacterial clumps and granulomas were detected in several organs such as the gills, heart, stomach, and intestine in addition to the muscles and HP, the only organs described as infected in the earlier study by Cheng & Chen (1998a). The reasons for these differences are unclear but may relate to differences in bacterial strains or rearing conditions.

Biochemical and physiological characteristics of 3 strains of bacteria purified from diseased *Macrobranchium rosenbergii* (MR1, MR3 and MR4) are shown in Table 1. All 3 were Gram-positive cocci that showed α hemolysis on BA but did not grow in 6.5% NaCl. All grew over a wide temperature range (4 to 40°C) and in media at pH 9.6 (25°C). They are compared to reference strains of *Enterococcus* and *Lactococcus* in Table 1. All 3 exhibited negative reactions for catalase, oxidase, mannose, sorbital, lactose, trehalose, inulin, raffinose and starch, but a positive reaction for pyruvate. In general, the characteristics of the disease isolates were similar to



Figs. 5 & 6. *Lactococcus garvieae* infecting *Macrobranchium rosenbergii*. Fig. 5. Infected giant freshwater prawn section showing bacterial microcolonies (arrows) in the gill. (H&E, ×200). Fig. 6. Infected giant freshwater prawn tissue section showing bacterial microcolonies (arrow) in the hemal sinus of the hepatopancreas. Also seen are necrotizing foci (arrowheads), edematous fluid (A) and inflammatory cells. (H&E, ×400)

those of *L. garvieae* and *E. seriolicida*, but there were some differences. These included major differences for all the isolates in lack of ability to grow at 45°C and on media with NaCl at 6% or more. There were also minor differences in some assimilation tests for some isolates only. Tests for hemolysis, for hippurate, for salt-tolerance (6.5%), for the heat tolerance (45°C) and for growth at pH 9.6 are important criteria for streptococcus differentiation (Facklam & Carey 1985, Schleifer & Kilpper-Balz 1987). According to morphology and the physiological and biochemical tests, the 3 strains isolated from *M. rosenbergii* would be classified in the genus *Lactococcus*. However, based on the biochemical tests alone, it was difficult to conclude whether or not the isolates were conspecific with *L. garviae*.

PCR

PCR assays with DNA extracts of MR1, MR3, MR4, *Lactococcus garvieae* ATCC43921 and *Enterococcus seriolicida* (MZ9102) resulted in the amplification of a fragment of 1100 bp (Fig. 7). MR4 data is not shown. PCR assays with DNA extracts from other bacteria including *E. faecalis* CCRC 10066, *E. durans* CCRC 10790, *L. lactis* subspecies *lactis* CCRC10791, *L. lactis* subspecies *cremoris* CCRC 11067, *L. raffinolactis* CCRC14039 and *E. avium* CCRC10801 gave no amplicons. When the PCR assay was carried out with 10-fold dilutions of *L. garvieae* MR1, it gave positive results for the 1100 bp amplicon down to the dilution corresponding to 20 bacterial cells (20 cfu).

Table 1. Biochemical characteristics of bacterial isolates from diseased *Macrobranchium rosenbergii* compared to the characteristics of reference strains. MR1, MR3 and MR4: strains isolated from *M. rosenbergii*; ES: *Enterococcus seriolicida* from rainbow trout (MZ 9101, KG-, from Dr T. Yoshida, Japan); LG: *Lactococcus garvieae* ATCC 43921; LLSC: *L. lactis* subspecies *cremoris* CCRC 11067; LLSL: *L. lactis* subspecies *lactis* CCRC10791; LR: *L. raffinolactis* CCRC14039; EA: *E. avium* CCRC10801; ED: *E. durans* CCRC 10790; EF: *E. faecalis* CCRC 10066. RBC: red blood cells; ATCC: American Tissue Culture Center: CCRC: Culture Collection Research Center, Taiwan

API strepsystem	MR1	MR3	MR4	ES	LG	LLSC	LLSL	LR	EA	ED	EF
Pyruvate	+	+	+	+	+	+	+	+	+	+	+
Hippurate	–	–	–	–	–	–	–	–	–	–	–
Esculin	–	+	–	+	+	+	+	+	+	+	+
Pyrrolldonyl 2-naphthylamide	+	+	–	+	+	–	+	–	+	+	+
β-bromo-2-naphthyl-α-D galactopyranoside	–	–	–	–	–	–	–	+	–	+	–
Naphthol-AS-BI β-D glucuronate	–	–	–	–	–	–	–	–	–	–	–
2.naphthyl-β-D D galactopyranoside	–	–	–	–	–	+	–	–	–	+	+
2.naphthyl phosphate	–	–	–	–	–	–	–	–	–	–	–
L-leucine-2-naphthylamide	+	–	–	+	+	+	+	+	–	–	+
Arginine	–	+	–	+	+	+	+	–	–	+	+
Ribose	–	–	–	+	+	+	+	–	+	+	+
L-arabinose	–	–	–	–	–	–	–	–	+	–	–
Mannose	–	–	–	+	+	–	–	–	+	–	+
Sorbitol	–	–	–	–	–	–	–	–	+	–	+
Lactose	–	–	–	–	–	–	–	+	+	+	+
Trehalose	–	–	–	+	+	+	+	+	+	–	+
Inulin	–	–	–	–	–	–	–	–	+	–	–
Raffinose	–	–	–	–	–	–	–	+	+	–	–
Starch	–	–	–	+	–	–	+	+	+	+	+
Glycogen	–	–	–	–	–	–	–	–	–	–	–
Hemolysis (goat RBC)	α	α	α	α	α	γ	γ	γ	α	α	γ
pH 9.6	+	+	+	+	+	+	+	–	+	+	+
Tolerance at 6.0% NaCl	–	–	–	+	+	+	+	–	+	+	+
6.5% NaCl	–	–	–	+	+	+	–	–	+	–	+
8.0% NaCl	–	–	–	+	–	–	–	–	–	–	+
10% NaCl	–	–	–	–	–	–	–	–	–	–	–
Growth at 4°C	+	+	+	+	+	+	+	+	+	+	+
10°C	+	+	+	+	+	+	+	+	+	+	+
15°C	+	+	+	+	+	+	+	+	+	+	+
25°C	+	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+	+	+	+
45°C	–	–	–	+	+	–	–	–	+	+	+

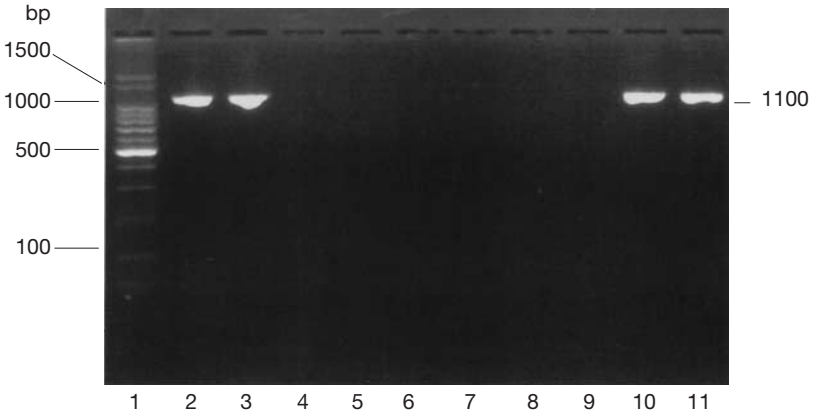


Fig. 7. Specificity of the *Lactococcus garvieae* PCR assay. Agarose gel (2%) of PCR products from bacterial DNA templates stained with ethidium bromide. Lane 1: DNA molecular weight marker; Lane 2: *L. garvieae* ATCC 43921; Lane 3: *Enterococcus seriolicida* from rainbow trout; Lane 4: *E. faecalis* CCRC 10066; Lane 5: *E. durans* CCRC 10790; Lane 6: *L. lactis* subspecies *lactis* CCRC10791; Lane 7: *L. lactis* subspecies *cremoris* CCRC 11067; Lane 8: *E. avium* CCRC10801; Lane 9: *L. raffinolactis* CCRC14039; Lane 10: isolate MR1; Lane 11: isolate MR3

Zlotkin et al. (1998) reported the PCR assay for an amplicon of 1100 bp from 35 strains of *Lactococcus garvieae*, including *L. garvieae* ATCC 43921 and *Enterococcus seriolicida* ATCC 49156 reference strains. If that PCR assay is considered definitive for *L. garvieae*, then the bacterial isolates obtained from diseased *Macrobranchium rosenbergii* would have to be considered strains of *L. garvieae*. In further support of this argument, we found that isolates MR1 to MR4 were resistant to clidamycin, a feature used to distinguish *L. garvieae* from susceptible *L. lactis* by Elliott & Facklam (1996). According to those authors, *L. lactis* and *L. garvieae* are phenotypically so similar that the only methods for distinction other than clindamycin susceptibility include comparisons of whole-cell protein patterns, restriction fragment length polymorphism data and nucleic acid sequences (Elliot & Facklam 1996). To be more confident that the MR isolates corresponded to *L. garvieae*, the PCR product from MR1 was sequenced and compared to sequences for *L. garvieae* in public databases.

Sequence of the 16S rDNA PCR amplicon

The sequence of the 1540 bp PCR amplicon of 16S rDNA from isolate MR1 was determined and deposited in the GenBank database under accession number AF283499. This sequence showed 99% identity with *Enterococcus seriolicida* (GenBank accession number AF061005), 98% identity to *E. seriolicida* (GenBank accession number L32813), 98% identity to *Lactococcus garvieae* (DBJ accession number AB012306), 96% identity to *L. garvieae* (DDBJ accession number AB018211) and 95% identity to *L. garviae* (EMBL, accession number X54262). The sequence comparison results give strong support to the argument that MR1, *L. garvieae* ATCC43921 and *E. seriolicida* should be assigned to the single species *L. garvieae*. Eldar et al. (1996) also argue that *E. seriolicida* is a synonym of *L. garvieae*. The reasons for differences (10 gaps, and 1 base difference) between the sequences for *L. garvieae* MR1 and *E. seriolicida* (GenBank accession number AF061005), are unclear but may relate to differences in methodology used, in hosts or in bacterial strains.

Experimental infections

All prawns injected with 2.3×10^6 to 10^7 of bacterial strain MR1 died within 2 d. White muscle and histopathological changes similar to those in naturally infected prawns were seen. The calculated LD_{50} dose was 6.6×10^5 cfu ml^{-1}. Pure cultures of bacteria were re-isolated from the HP and muscle of moribund prawns after bacterial challenge and no lesions formed in the control group. Altogether, the results constitute the first confirmation that *Lactococcus garvieae* can cause disease outbreaks in the giant freshwater prawn *Macrobranchium rosenbergii*.

Acknowledgements. The authors thank Dr Terutoyo Yoshida, Miyazaki University, Miyazaki, Japan, for supplying the bacterial isolate *Enterococcus seriolicida* MZ9101. This study was supported by the National Science Council, Taiwan, ROC (grants NSC89-2313-B020-029).

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Editorial responsibility: Timothy Flegel, Bangkok, Thailand, and Carey Cunningham, Aberdeen, Scotland, UK

Submitted: September 4, 2000; Accepted: January 14, 2001
Proofs received from author(s): April 18, 2001