

Lactococcus lactis subspecies *lactis* also causes white muscle disease in farmed giant freshwater prawns *Macrobrachium rosenbergii*

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ABSTRACT: From May to August 2001 in Taiwan, 27 farms for the giant freshwater prawn *Macrobrachium rosenbergii* experienced white tail disease outbreaks in animals approximately 3 to 5 mo old, with total lengths from 6 to 8 cm. Examination of the infected prawns revealed not only previously reported *Lactococcus garvieae* (16 farms) but also the novel *L. lactis* subsp. *lactis* (10 farms). One farm had shrimp infected with both bacteria. In the farms with *L. lactis* infections, the cumulative mortality was approximately 25 to 60%. Gross signs of disease were opaque and whitish muscles, while histopathology included 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. Eleven isolates from different farms were identified as *L. lactis* subsp. *lactis* using API 20 Strep and Rapid ID32 Strep tests and using PCR assays specific for the *L. lactis* subsp. *lactis* 16S rDNA gene (650 bp amplicon) and for the 16S to 23S rDNA interspacer region (380 bp amplicon). In addition, sequencing of the full 16S rDNA genes of 2 of the isolates (MR17 and MR26; GenBank Accession Numbers AF493058 and AF493057, respectively) revealed 99.9% identity between the isolates and 98.7% identity to several complete 16S rRNA sequences of *L. lactis* subsp. *lactis* at GenBank. Experimental infections with our isolates gave gross signs and histopathological changes similar to those seen in naturally infected prawns. The mean lethal dose of 4 isolates and the reference strain *L. lactis* subsp. *lactis* BCRC 10791 ranged from 4.2×10^6 to 2.5×10^7 colony-forming units prawn⁻¹, indicating virulence similar to that previously reported for *L. garvieae*. This is the first report confirming *L. lactis* subsp. *lactis* as a pathogen in juvenile and adult prawns from aquaculture.

KEY WORDS: *Lactococcus lactis* subsp. *lactis* · Giant freshwater prawn · *Macrobrachium rosenbergii* · PCR · 16S rDNA sequencing · 16S to 23S rDNA interspacer regions gene

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INTRODUCTION

There are 5 species of *Lactococcus*: *L. garvieae*, *L. piscium*, *L. lactis*, *L. plantarum* and *L. raffinolactis*, of which *L. lactis* comprises 2 subspecies: *lactis* and *cremoris*. Only 2 out of the 5 species of *Lactococcus* are pathogenic for fish, namely *L. garvieae* and *L. piscium* (Williams et al. 1990, Eldar et al. 1994, 1996), and only *L. garvieae* are pathogenic for giant freshwater prawn *Macrobrachium rosenbergii* (Chen et al. 2001).

The giant freshwater prawn *Macrobrachium rosenbergii* is commercially farmed throughout the world and intensively in Taiwan, where production decreased by 47 to 52% from 1992 to 1995 as result of disease (New 1995, Taiwan Fisheries Bureau 1996). Two diseases have been linked to production decline: a yeast infection that develops primarily in the cool season (October to March) and presents a yellow exoskeleton, a swollen hepatopancreas (HP), milky he-

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molymp and opaque or whitish muscles (Shu 1993, Chen et al. 2003), and a *Lactococcus garvieae* infection that occurs principally in the hot season (June to September, especially during phytoplankton blooms) and causes muscle necrosis (Cheng & Chen 1988a,b, Chen et al. 2001). *Streptococcus lactis*, recently reclassified as *Lactococcus lactis*, is not recognized as a common veterinary pathogen, but it has occasionally been reported in the etiology of cattle mastitis and in septic arthritis of the neonatal calf (Wichtel et al. 2003). *L. lactis* is considered to be a skin commensal, and cattle are natural hosts. Although rare, human infection with *L. lactis* was first reported by Ledger et al. (1974). It has since been reported as a cause of endocarditis (Mannion & Rothburn 1990, Clark & Burnie 1991), arthritis (Campbell et al. 1993) and septicemia in an immunocompromised patient (Durand et al. 1995). However, infection by *L. lactis* subsp. *lactis* in aquatic animals in aquaculture has not previously been reported. This study identified the causative bacteria of a serious disease in cultured *M. rosenbergii* in Taiwan as *L. lactis* subsp. *lactis* by Rapid ID 32 Strep, polymerase chain reaction (PCR) and 16S rDNA sequencing.

MATERIALS AND METHODS

Prawn samples. For histopathological and bacteriological analysis, 5 diseased prawns (body weight 3 to 5 g; length 6 to 8 cm) were collected from each of 27 prawn farms during outbreaks of lactococcosis from May to August 2001. Losses were as high as 60% in some ponds, with an average loss of approximately 35%. The prawns had been fed with commercial pellets.

Bacteriology. Diseased prawns were placed in a sealable plastic bag and then on crushed ice for 20 min. The cuticles were surface sterilized by swabbing with 70% ethanol before being cut open to reveal underlying tissues. Swabs were then taken from muscles and from 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 for 3 d. Single colonies were streaked on the same media to obtain pure isolates. These isolates were then identified using the API 20 Strep, Rapid ID 32 Strep (Biomérieux Sa) and conventional bacteriological tests (Chen et al. 2001).

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 hematoxylin and eosin (H&E) and Gram stains and were viewed microscopically (Chen et al. 2001).

Bacterial strains. The following bacterial strains were used as controls for PCR assays: *Lactococcus garvieae* (American Type Culture Collection, ATCC 43921) and *L. garvieae* from rainbow trout (MZ9101 [KG-] from Dr. Terutoyo Yoshida, Miyazaki University, Japan). Additionally, the following strains were obtained from the Bioresource Collection Research Center, Taiwan: *Enterococcus faecalis* BCRC 10066, *E. durans* BCRC 10790, *L. lactis* subsp. *lactis* BCRC 10791, *L. lactis* subsp. *cremoris* BCRC 11067, *L. raffinolactis* BCRC 14039 and *E. avium* BCRC 10801. Eleven purified bacterial isolates derived from infected prawns were labeled MR13, MR14, MR16, MR17, MR19, MR20, MR26, MR27, MR28, MR29 and MR36.

DNA extraction. Bacteria were grown at 25°C in BHI broth and harvested by centrifugation at 7000 × *g* for 45 min at 4°C for nucleic acid extraction. The pelleted bacteria were then lysed with lysozyme at 30 mg ml⁻¹ for 1 h at 37°C, followed by the 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 was precipitated by addition of 0.5 vol of isopropanol and incubated for 30 min at -20°C, followed by centrifugation at 12 000 × *g* for 15 min at 4°C. The DNA pellets were washed with 70% ethanol, dried in a Speedvac and then resuspended in 20 µl of TE buffer.

PCR. Specific primers LLF (5'-GCAATTGCATCACTCAAAGA-3') and LLR (5'-ACAGAGAAGCTTATAGCTCCC-3') were designed from diagnostic regions of the *Lactococcus lactis* subsp. *lactis* 16S rRNA gene sequence (Accession Number M58837; Goyache et al. 2001). The PCR amplifications were performed in a 100 µl reaction volume containing 150 ng of each primer, 1 mM of each deoxynucleoside triphosphate, 1 U of *Taq* DNA polymerase (Takara) and 25 ng of template DNA in 1× reaction buffer. The following conditions were used for amplification: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation for 1 min at 92°C, annealing for 1.5 min at 50°C and extension for 2 min at 72°C, with final extension for 5 min at 72°C. All *L. lactis* subsp. *lactis* prawn isolates produced the expected PCR amplification product of 650 bp. No amplification was observed with any other *Lactococcus* species tested, indicating the specificity of the PCR assay.

Amplification of 16S to 23S rDNA spacer regions. Synthetic oligonucleotide primers developed by Jensen et al. (1993) (G1: 5'-GAAGTCGTAACAAGG-3'; L1: 5'-CAAGGCATCCACCGT-3') were employed to amplify the 16S to 23S rDNA intergenic spacer region. This produced a 430 bp amplicon for *Lactococcus garvieae* and a 380 bp amplicon for *L. lactis* subsp. *lac-*

tis (Blaiotta et al. 2002). Amplification conditions consisted of an initial denaturation step at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 3 min, with final extension at 72°C for 10 min. The 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 were visualized by ethidium-bromide staining. The DNA molecular weight marker comprised a 100 bp ladder (100 bp ET marker; Chenig Hsin Tang Chemical Company).

16S rDNA sequencing. Amplification of 16S rDNA was by PCR using purified DNA and a primer combination consisting of forward primer 5F (5'-TGGAGAGT-TTCCTGGCTCAG-3') and reverse primer 1540R (5'-AAGGAGGTGATCCARCCGCA-3'). The amplification protocol was initial denaturation for 10 min at 95°C, 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, with final extension for 10 min at 72°C. The PCR products were purified with a QIAquick PCR purification kit (Qiagen). Sequencing of the 16S rDNA PCR fragment from strains MR17 and MR26 was performed using a MicroSeq 16S rRNA Gene Kit (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 sequences of MR17 and MR28 were determined from overlapping sequence data using MicroSeq software (Applied Biosystems). The resultant 16S rDNA sequences were manually aligned with sequences of representative strains retrieved from GenBank, DDBJ and EMBL databases.

Experimental infections. Healthy prawns (*Macrobrachium rosenbergii*: body weight 3 to 4 g; length 6 to 8 g) were obtained from prawn farms in Pingtung, Taiwan, and held at a density of 100 prawns in continuously aerated 400 l aquaria containing 300 l of freshwater at approximately 27°C for 7 d until they were acclimatized to laboratory conditions. They were fed twice daily with commercial prawn pellets, and waste was removed daily. Subsequently, 280 prawns of uniform size were selected for challenge tests using 5 strains of *Lactococcus lactis* subsp. *lactis* (MR13, MR17, MR26, MR28 and BCRC 10791). For challenge with each strain, 56 prawns were subdivided into 8 groups of 7 prawns each. Bacterial suspensions at an optical density of 1.0 (absorbance at 610 nm), equal to a viable bacterial count of 10⁹ colony-forming units (cfu) per milliliter, were successively diluted 10-fold in sterile (121°C, 15 min) normal saline to obtain a range of 1.2 × 10⁹ to 1.2 × 10³ cfu ml⁻¹ (validated by standard

plate counts). For each of 7 dilutions for each strain, 0.1 ml bacterial solution was injected intramuscularly (IM) into 7 prawns. 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 for the acclimatization period. The prawns were continuously monitored for morbidity and mortality and were sampled for histopathological and bacteriological analyses.

RESULTS

Clinical signs, bacteriology and histopathology

All moribund prawns *Macrobrachium rosenbergii* from different prawn farms exhibited poor growth and a whitish body color. Gross pathological changes were whitish muscles and a swollen, yellowish HP. Smears from fresh HP, muscle tissue and tissues of other internal organs of diseased prawns showed the presence of numerous cocci ranging from 0.1 to 0.3 µm in diameter. Bacterial colonies from the muscle and hepatopancreas appeared 1 to 2 d after culture on the TSA, BA and BHI. Colonies were evident after 24 h incubation on BHA agar and were roughly the size of a pin head. They were semitransparent and grew larger (0.05 to 0.1 cm in diameter) after 48 h incubation. The bacteria were Gram positive and were typical cocci (0.1 to 0.3 µm).

Histopathologically, edematous fluid in moribund prawns accumulated between the cuticle and muscle, and large numbers of hemocytes infiltrated the subcuticular area, epidermis and dermis. Abdominal, pereopod and pleopod muscles showed substantial edema and liquefactive necrosis, with large numbers of bacterial cells. Diseased prawns showed marked edematous fluid accumulation between the cuticle and underlying muscle tissue, and there was fragmentation of muscle bundles with liquefactive necrosis. Large numbers of cocci were identified in muscles of the abdomen, pereopods and pleopods (Fig. 1). Necrotizing foci encapsulated by hemocytes (granulomas) also developed in the muscle. Fibrous tissues were also identified in the muscle. Sloughed HP tubular epithelial cells appeared in tubular lumens, and necrotizing foci with bacterial clumps and inflammation were found in interstitial spaces (Fig. 2). Sloughed HP tubular epithelial cells and bacteria formed mixed debris in tubule lumens of some prawns. Necrotic foci and bacterial clumps were also detected in the heart, stomach and other organs.

All 135 diseased prawns (i.e. 5 prawns collected from each of 27 farms) yielded bacterial colonies on agar

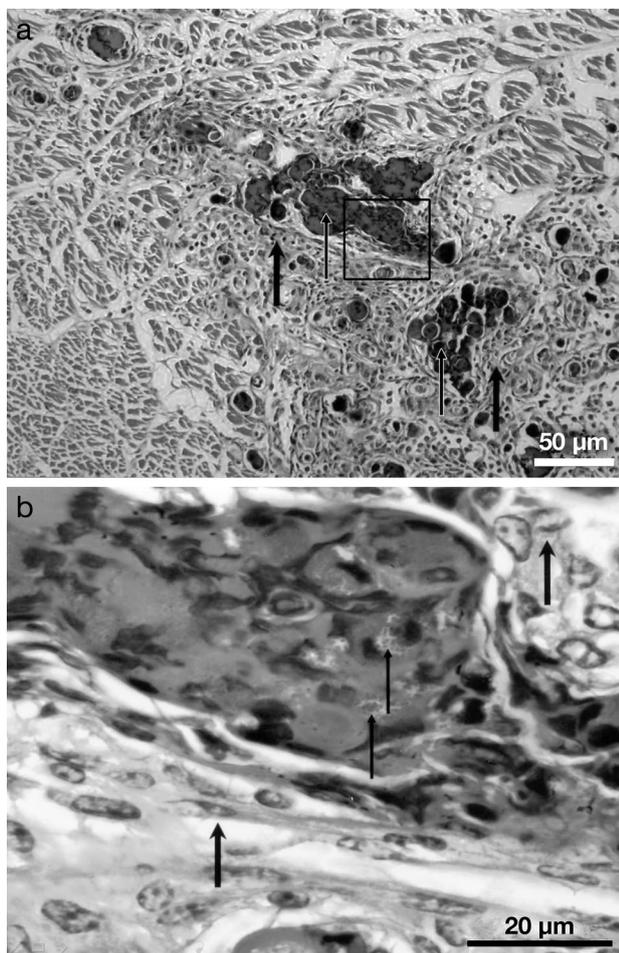


Fig. 1. *Macrobrachium rosenbergii*. (a) Giant freshwater prawn tissue section showing markedly necrotized foci with cellular debris (small arrows) surrounded by fibrous tissue and hemocytes (large arrows) as a granuloma in the muscle tissues. (b) Inset from (a), showing bacterial clumps (small arrows) and fibrous tissue (large arrows) in a granuloma (H&E)

plates. One colony was selected from each prawn for PCR followed by sequencing. This revealed that prawns from 10 farms (50 prawns) were infected with *Lactococcus lactis* subsp. *lactis*, while those from 16 farms (80 prawns) were infected with *L. garvieae*. At the remaining farm, 3 of the prawns were infected with *L. garvieae* and 2 with *L. lactis* subsp. *lactis*. Thus, the prevalence for *L. garvieae* and *L. lactis* subsp. *lactis* infections was 61.5% (83/135) and 38.5% (52/135), respectively. Since it was not possible to carry out detailed biochemical analysis of bacteria from all diseased prawns, 11 isolates of *L. lactis* subsp. *lactis* (1 prawn isolate from each farm labeled as MR13, MR14, MR16, MR17, MR19, MR20, MR26, MR27, MR28, MR29 and MR36) were selected for detailed biochemical and physiological tests and for comparison with reference strains of *L. lactis* subsp. *lactis* BCRC 10791, *L.*

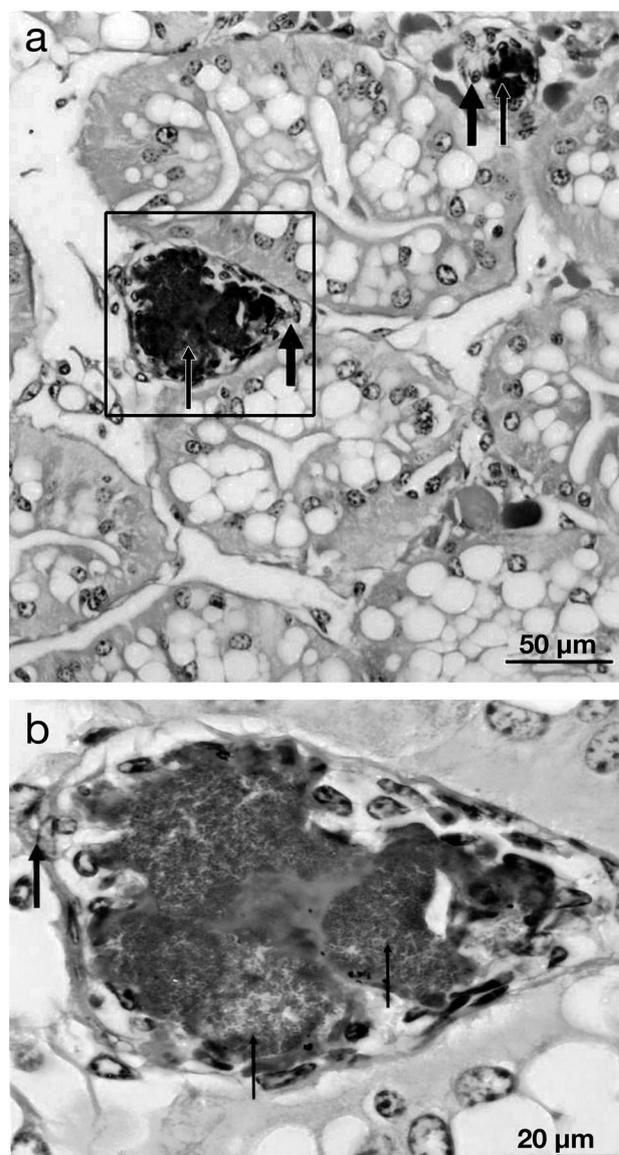


Fig. 2. *Macrobrachium rosenbergii*. (a) Giant freshwater prawn section showing necrotized foci in the hepatopancreas with cellular debris (small arrows) surrounded by hemocytes (arrows) (H&E). (b) Inset from (a), and showing bacterial clumps (small arrows) and hemocytes (large arrows) (Gram stain, 1000×)

lactis subsp. *cremoris* BCRC 11067 and *L. garvieae* ATCC 43921.

Table 1 shows the results of biochemical and physiological characteristics using API 20 Strep tests for our 11 isolates from diseased *Macrobrachium rosenbergii* (MR13, MR14, MR16, MR17, MR19, MR20, MR26, MR27, MR28, MR29 and MR36) and for the reference strains. Results for our 11 isolates are summarized in Column 1 of the table. All 11 isolates were Gram-positive cocci that showed γ hemolysis on blood agar, but did not grow in 6.5% NaCl. All grew over a wide tem-

Table 1. *Macrobrachium rosenbergii*. Biochemical characteristics of bacterial isolates from diseased *M. rosenbergii* compared to the characteristics of reference strains. Column 1 summarizes test results for our 11 test isolates, where a single symbol indicates a uniform positive (+) or negative (-) test result and fractions indicate the number of isolates that gave positive results over the number that gave negative results. ATCC: American Type Culture Center; BCRC: Bioresource Collection Research Center, Taiwan; LLSL: *Lactococcus lactis* subsp. *lactis* BCRC 10791; LLSC: *L. lactis* subsp. *cremoris* BCRC 11067; LR: *L. raffinolactis* BCRC 14039; LG1: *L. garvieae* from rainbow trout (MZ 9101 [KG-] from Dr. T. Yoshida, Japan); LG: *L. garvieae* ATCC 43921; EA: *Enterococcus avium* BCRC 10801; ED: *E. durans* BCRC 10790; EF: *E. faecalis* BCRC 10066

API 20 Strep test	Test	LLSL	LLSC	LR	LG1	LG	EA	ED	EF
Pyruvate	+	+	+	+	+	+	+	+	+
Hippurate	-	-	-	-	-	-	-	-	-
Esculin	+	+	+	+	+	+	+	+	+
Pyrrroldonyl 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	4/7	+	+	+	+	+	-	-	+
Arginine	9/2	+	+	-	+	+	-	+	+
Ribose	4/7	+	+	-	+	+	+	+	+
L-arabinose	-	-	-	-	-	-	+	-	-
Mannose	3/8	-	-	-	+	+	+	-	+
Sorbital	-	-	-	-	-	-	+	-	+
Lactose	3/8	-	-	+	-	-	+	+	+
Trehalose	8/3	+	+	+	+	+	+	-	+
Inulin	7/4	-	-	-	-	-	+	-	-
Raffinose	-	-	-	+	-	-	+	-	-
Starch	4/7	+	-	+	+	-	+	+	+
Glycogen	-	-	-	-	-	-	-	-	-
Hemolysis (goat RBC)	γ	γ	γ	γ	α	α	α	α	γ
pH 9.6	+	+	+	-	+	+	+	+	+
Tolerance at:									
6.0% NaCl	+	+	+	-	+	+	+	+	+
6.5% NaCl	-	-	+	-	+	+	+	-	+
8.0% NaCl	-	-	-	-	+	-	-	-	+
10% NaCl	-	-	-	-	-	-	-	-	-
Growth at:									
4°C	+	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+	+
45°C	5/6	-	-	-	+	+	+	+	+

perature range (4 to 40°C) and in media at pH 9.6 (25°C). All exhibited negative reactions for hippurate, β -bromo-2-naphthyl- α -D-galactopyranoside, naphthol-AS-BI- β -D-glucuronate, 2-naphthyl- β -D-D-galactopyranoside, 2-naphthyl phosphate, L-arabinose, sorbital, raffinose and glycogen, but a positive reaction for pyruvate, esculin and pyrroldonyl 2-naphthylamide. The characteristics of the 11 isolates were generally similar to those of *Lactococcus lactis* subsp. *lactis*. Exceptions were minor differences in some assimilation tests for some isolates. According to the morphology and the physiological and biochemical test results, the 11 strains isolated from *M. rosenbergii* would be classified in the genus *Lactococcus*. However, based on the biochemical tests results for API 20 Strep alone, it was difficult to conclude whether these isolates were conspecific with *L. lactis* subsp. *lactis*.

In further tests of our 11 isolates using a commercial ATB expression system (Rapid ID 32 Strep, bio-Merieux), scores of good to very good identification (96.3 to 99.8%) for *Lactococcus lactis* subsp. *lactis* were obtained (Table 2). With the same test system, excellent identification (99.9%) was obtained for reference strain BCRC 10791 (Table 2).

Polymerase chain reaction assay results

PCR assay for *Lactococcus lactis* subsp. *lactis* revealed that all 11 of our isolates and the reference strain *L. lactis* subsp. *lactis* BCRC 10791 gave the expected amplicon of 650 bp for the *L. lactis* subsp. *lactis* 16S rDNA gene (Goyache et al. 2001) (Fig. 3). PCR assays with DNA extracts from other bacteria,

including *L. lactis* subsp. *cremoris* BCRC 11067, *Enterococcus durans* BCRC 10790, *L. raffinolactis* BCRC 14039 and *E. avium* BCRC 10801, *L. garvieae* ATCC 43921 and *L. garvieae* MZ9101 gave no amplicons (data not shown for the latter 2 strains). When the PCR assay was performed with 10-fold dilutions of isolate MR26, the 650 bp amplicon was obtained with a dilution corresponding to 20 bacterial cells (20 cfu, data not shown).

Primers G1 and L1 specific for the 16S to 23S rDNA intergenic spacer region of *Lactococcus lactis* subsp. *lactis* gave the expected 380 bp amplicon, with all 11 of our isolates and the reference strain *L. lactis* subsp. *lactis* BCRC 10791. No amplicons were obtained using *L. lactis* subsp. *cremoris* BCRC 11067, *L. raffinolactis* BCRC 14039, *Enterococcus avium* BCRC 10801, or *E. durans* BCRC 10790 (Fig. 4).

Table 2. *Macrobrachium rosenbergii*. Rapid ID 32 Strep system characteristics of the *Lactococcus lactis* strains isolated from diseased *M. rosenbergii* in comparison to the reference strains of the *Lactococcus* group. Column 1 summarizes test results for our 11 test isolates, where a single symbol indicates a uniform positive (+) or negative (-) test result and fractions indicate the number of isolates that gave positive results over the number that gave negative results. See Table 1 legend for reference isolate names

Rapid ID 32 Strep test	Test	LLSL	LLSC	LG
Arginine dihydrolase	+	+	+	+
β -glucosidase	+	+	+	+
β -galactosidase	-	-	-	-
β -glucuronidase	-	-	-	-
α -galactosidase	-	-	-	-
Alkaline phosphatase	-	-	-	-
Ribose	-	+	-	-
Mannitol	+	-	+	-
Sorbitol	-	-	-	-
Lactose	+	+	+	-
Trehalose	+	+	+	+
Raffinose	-	-	-	-
Saccharose	+	+	+	-
L-arabinose	+	-	+	-
D-arabitol	6/5	-	+	-
Cyclodextrin	+	+	-	-
Acetoin production	3/8	+	-	+
Anyl-phenylalanyl-proline arylamidase	3/8	+	-	+
β -galactosidase	6/5	-	+	-
Pyroglutamic acid arylamidase	-	-	-	-
N-acetyl- β -glucosaminidase	-	-	-	-
Glycyl-tryptophan arylamidase	9/2	-	+	-
Hydrolysis of hippurate	6/5	+	-	-
Glycogen	-	-	-	-
Pullulane	-	-	+	-
Maltose	+	+	+	+
Melibiose	-	-	-	-
Melezitose	-	-	+	-
Methyl- β -D-glucopyranoside	-	-	-	+
Tagatose	+	-	+	-
β -mannosidase	+	-	+	-
Urease	-	-	-	-

Sequencing of the 16S rDNA PCR amplicon

Two of our isolates (MR17 and MR26) were randomly selected for sequencing of the PCR amplicon of the complete 16S rDNA gene (1542 bp). These sequences were 99.9% identical (100% coverage) and were deposited at GenBank under Accession Numbers AF493058 and AF493057, respectively. A BLAST search against all bacteria gave top hits for 6 complete 16S rRNA sequences of *Lactococcus lactis* (AE006456, AE006454, AE006447, AE006443, X64887 and AY675242), all with 99% coverage and identity ranging from 98.6 to 98.7%. Also included was 1 record for the subspecies *cremoris* (CP000425), with 99% coverage and 98.6% identity. By contrast, a BLAST search against complete 16S rRNA sequences for *L. garvieae* (3 available; L32813, AF283499 and X54262) gave coverages ranging from 90 to 94% only and identities ranging from 92.5 to 95%. One of these (AF283499), with 94% coverage and 94.8% identity, was for *L. garvieae* previously isolated by our group from diseased giant freshwater prawns in Taiwan (Chen et al. 2001). One partial 16S rRNA sequence record (DQ010113) gave 98.6% identity to our sequence, but with only 92% coverage. When this sequence itself was used in a BLAST search against all bacteria, it yielded top hits for complete 16S rRNA sequences of *L. lactis*, with 100% coverage and 100% identity. By contrast, a BLAST search restricted to complete sequences of 16S rRNA genes of *L. garvieae* yielded identities ranging from 92.1 to 94.6%, with coverage of only 94 to 96%. The results indicated that our isolates were *L. lactis* and not *L. garvieae* and that the record for DQ010113 should probably be labeled *L. lactis* rather than *L. garvieae*.

Experimental infections

All prawns injected with 1.2×10^8 cfu of *Lactococcus lactis* subsp. *lactis* isolates MR13, MR17 and MR28 died within 2 d. All prawns injected with 1.2×10^8 of bacterial strain *L. lactis* subsp. *lactis* BCRC 10791 and our isolate MR26 died within 3 d. The LD₅₀ values for our *L. lactis* subsp. *lactis* isolates from diseased prawns and the

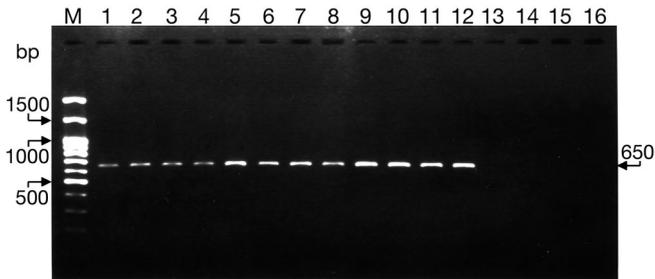


Fig. 3. Agarose gel of PCR products illustrating the specificity of the *Lactococcus lactis* subsp. *lactis* PCR assay. Lane M: DNA molecular weight marker; Lanes 1 to 11: test isolates MR13, MR14, MR16, MR17, MR19, MR20, MR26, MR27, MR28, MR29 and MR36, respectively; Lane 12: *L. lactis* subsp. *lactis* BCRC 10791; Lane 13: *L. lactis* subsp. *cremoris* BCRC 11067; Lane 14: *L. raffinolactis* BCRC 14039; Lane 15: *Enterococcus avium* BCRC 10801; Lane 16: *E. durans* BCRC 10790

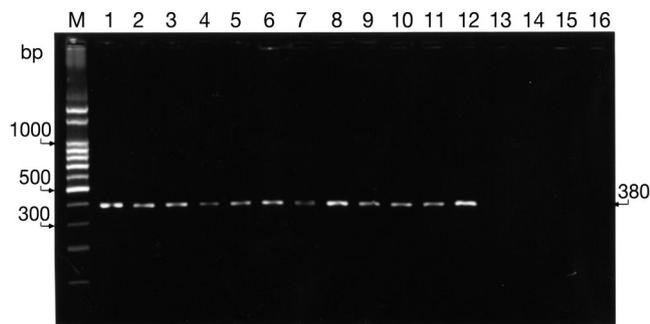


Fig. 4. Agarose gel of PCR products illustrating the specificity of the PCR assay for the 16S to 23S rDNA intergenic spacer region for *Lactococcus lactis* subsp. *lactis*. Lane identities as in Fig. 3 legend

reference strain ranged from 10^6 to 10^7 cfu g^{-1} body weight. The LD₅₀ values of isolates MR13, MR17, MR26 and MR28, and the reference strain *L. lactis* subsp. *lactis* BCRC 10791, were 4.2×10^6 , 2.1×10^6 , 1.2×10^7 , 4.8×10^6 and 2.5×10^7 cfu prawn⁻¹, respectively. White muscle and histopathological changes similar to those in naturally infected prawns were observed. Pure cultures of bacteria were re-isolated from the HP and muscle of moribund prawns after bacterial challenge, and no lesions developed in the control group.

DISCUSSION

Our previous reports described *Enterococcus*-like (Cheng & Chen 1998a) infections and *Lactococcus garvieae* (Chen et al. 2001) infections from *Macrobrachium rosenbergii* with similar histopathology to those described herein. However, there were also some differences. In the current study and in our previous studies with *L. garvieae* (Chen et al. 2001), 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 in the muscles and HP. However, the only organs described as infected in the study by Cheng & Chen (1998a) were the muscles and HP. The reasons for the differences are unclear, but may relate to differences in bacterial strains or rearing conditions. *L. lactis* subsp. *lactis* also caused chronic granulomatous lesions in the giant freshwater prawn similar to those seen with *L. garvieae* infection in the grey mullet (Chen et al. 2002).

The characteristics of the bacterial isolates from diseased prawns were generally similar to those of *Lactococcus lactis* subsp. *lactis*, with the exception of minor differences in some assimilation tests for some isolates only. According to morphology and physiological and biochemical test results, the 11 strains isolated from *Macrobrachium rosenbergii* would be classified in the genus *Lactococcus*. The biochemical and physiological characteristics of our 11 isolates were similar to those previously reported for *L. lactis* subsp. *lactis* by Facklam & Elliott (1995). For instance, the exhibited 100% positive results for trehalose and 100% negative results for hippurate, sorbitol and raffinose. However, based on the biochemical tests of the API 20 Strep kit alone, it was difficult to conclude whether the isolates were conspecific with *L. lactis* subsp. *lactis*. Because this API 20 Strep system is widely used in many clinical microbiology laboratories for the identification of Gram-positive cocci, this system should be updated or modified to improve its proficiency in identifying this as well as other emerging pathogens. On the other hand, our isolates were correctly identified as *L. lactis* subsp. *lactis* with the Rapid ID 32 Strep kit (bio-Merieux).

Until recently, *Lactococcus garvieae* and *L. lactis* subsp. *lactis* were considered opportunistic pathogens. They are often misidentified as enterococci or streptococci, and elucidation of their clinical significance has likely been hindered by the difficulties in correct identification. However, the number of clinical cases associated with infections by *L. lactis* and *L. garvieae* has increased in the last decade in both humans and animals (Clark & Burnie 1991, Campell et al. 1993, James et al. 2000, Wichtel et al. 2003).

Lactococcus lactis subsp. *lactis*, *L. piscium* and *L. garvieae* are recognized as the species with clinical significance for human and veterinary medicine. In humans, *L. garvieae* and *L. lactis* subsp. *lactis* have been identified in clinical samples of blood (Wang et al. 2007), skin lesions and urine. *L. garvieae* and *L. piscium* are pathogenic for various fish species. However, infection by *L. lactis* subsp. *lactis* in cultured prawns has not previously been reported.

Although physiological tests, differences in antimicrobial susceptibility, whole-cell protein, and DNA

or RNA analysis have been proposed to distinguish between *Lactococcus lactis* subsp. *lactis* and *L. garvieae* (Elliott et al. 1991, Elliott & Facklam 1996), some of these techniques are not reliable and may be too time consuming, limiting their use for routine identification. We found that rapid PCR amplification of the 16S to 23S rDNA spacer region could differentiate well between *L. garvieae* (430 bp amplicon) and our *L. lactis* subsp. *lactis* isolates (380 bp amplicon) (Jensen et al. 1993). PCR amplification, sequencing and sequence comparison of the complete 16S rDNA from 2 of our isolates gave strong support to the argument that they and the reference strain BCRC 10791 are isolates of a single subspecies, *L. lactis* subsp. *lactis*.

BLAST searches using our AF493057 and AF493058 sequences revealed 1 anomalous identity match of 98% with DQ010113 (92% coverage) that is listed at GenBank as a partial sequence of 16S rRNA of *Lactococcus garvieae* isolated from flounder. However, using DQ010113 as the query for a BLAST search against all bacteria, yielded only *L. lactis* records with 100% identity and 100% coverage, while a search limited to *L. garvieae* gave a maximum identity of 94.8% with only 94% coverage (L32813). Higher coverage (96%) for record X54262 yielded a lower identity of 92.1%. Altogether, the results suggest that DQ010113 is a sequence from the rRNA gene of *L. lactis* and that the originating bacterium isolated from flounder was mis-identified as *L. garvieae*.

White muscle disease is a major problem for giant freshwater prawn *Macrobrachium rosenbergii* farmers in the summer in Taiwan. Our results from both field specimens and experimental infections provide the first confirmation that *Lactococcus lactis* subsp. *lactis* infection can cause such disease outbreaks in addition to *L. garvieae*. Challenge tests with our field isolates and the reference strain BCRC 10791 revealed that *L. lactis* subsp. *lactis* has virulence for the freshwater prawn similar to that of *L. garvieae* (Chen et al. 2001).

Epidemiological studies suggest that white muscle disease caused by bacteria is most prevalent during the summer, peaking in June to August, and that it does not occur during the winter. No differences have been observed in water quality between ponds with diseased and healthy prawns. However, a surprisingly strong positive correlation exists between the percentage of infected prawns and high water temperature. Since we isolated only *Lactococcus lactis* subsp. *lactis* from disease outbreaks at 10 farms and only *L. garvieae* from disease outbreaks at 16 other farms, we may conclude that both bacterial species are significant causes of white muscle disease. Thus, in areas with a history of outbreaks of white muscle disease, frequent monitoring of prawns for these 2 bacterial species would be prudent during the summer.

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