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A new bacterial strain, designated SHRIMP<sup>T</sup>, isolated from the haemolymph of the Pacific white shrimp, *Penaeus vannamei*, was serologically distinct from other spiroplasmas. Cells of this strain were helical in form and variable in length. Examination by electron microscopy revealed wall-less cells delineated by a single cytoplasmic membrane. The organisms grew well in M1D media supplemented with 2% NaCl. Strain SHRIMP<sup>T</sup> grew at temperatures of 20–37 °C, with optimum growth occurring at 28 °C. The strain catabolized glucose and hydrolysed arginine, but did not hydrolyse urea. The G + C content of the DNA was 29 ± 1 mol%. Strain SHRIMP<sup>T</sup> (= ATCC BAA-1082<sup>T</sup> = CAIM 1252<sup>T</sup>) is designated the type strain of a novel species, *Spiroplasma penaei* sp. nov., which represents a new subgroup (I-9) of the group I spiroplasmas.

In international trade, the most prominent product from aquaculture is marine shrimp, of which approximately 26% of the total product comes from pond-reared penaeid species (FAO, 2002). *Penaeus vannamei* (taxonomy according to Holthuis, 1980), the Pacific white shrimp, is one of the predominant species of farm-raised shrimp cultivated in both the eastern and the western hemispheres. As with any monoculture, raising shrimp in ponds in close proximity to each other increases the spread of diseases (Kautsky *et al.*, 2000), and disease has been attributed as the single largest source of economic loss in the aquaculture industry (Meyer, 1991). Cultured penaeid shrimp are susceptible to viral, rickettsial, bacterial, fungal, protozoan and metazoan infectious pathogens (Lightner, 1993). The most severe diseases of shrimp, causing the greatest economic losses to growers, are caused by viruses and bacteria (Lightner *et al.*, 1992).

In January 2002, severe mortalities of *P. vannamei* occurred in one pond at a Colombian shrimp farm located on the Caribbean coast. During the following stocking period in May–June 2002, two additional shrimp ponds at the same farm experienced high mortalities. The pathogenic spiroplasma identified as the causative agent spread during the

next grow-out cycle to a neighbouring farm, which suffered mortalities ranging from 10 to 90%.

Upon receipt of infected specimens from Colombia preserved in Davidson's alcohol–formalin–acetic acid, histological analysis was performed (Bell & Lightner, 1988). The presumptive diagnosis for the mortalities suffered was attributed to a severe bacterial infection. This diagnosis was further confirmed with the use of a universal digoxigenin-labelled bacterial probe and *in situ* hybridization assays (Nunan *et al.*, 2004). Tissue from frozen infected *P. vannamei* from the initial epizootic was sampled for DNA extraction. Using universal 16S rRNA primers and PCR, the 16S rRNA gene of the bacterium was sequenced (Nunan *et al.*, 2003). The sequence was submitted to GenBank and compared with other bacterial 16S rRNA genes. The results from a BLAST search revealed 16S rRNA gene sequence similarity of 99% to *Spiroplasma insolitum*.

The large genus *Spiroplasma* is a group of wall-less eubacteria displaying a helical cell morphology. Spiroplasmas are motile, even though they lack flagella (Davis & Worley, 1973; Daniels *et al.*, 1980; Trachtenberg, 1998). These bacteria have historically been associated with plants and arthropods, primarily insects and ticks (Clark, 1982; Tully *et al.*, 1982; Williamson *et al.*, 1989, 1998; Tully & Whitcomb, 1990), but more recently members of this genus have been discovered in the Chinese mitten crab (*Eriocheir sinensis*) (Wang *et al.*, 2003, 2004) and the deep-sea alvinocarid shrimp *Rimicaris exoculata* (Zbinden & Cambon-Bonavita, 2003). Spiroplasmas can be pathogenic

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**Abbreviations:** SPF, specific pathogen-free; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SHRIMP<sup>T</sup> is AY771927.

in plants and insects, and cause 'tremor disease' in freshwater mitten crabs (Wang *et al.*, 2003). The *Spiroplasma* species associated with the marine alvinocarid shrimp appears to be part of the normal gut flora. In this paper, we present the results of a taxonomic study of the causative organism of the *P. vannamei* epizootic in Colombia. We present supporting data for the basis of the proposed minimal requirements for assignment of binomial names to mollicutes. The strain investigated, designated SHRIMP<sup>T</sup>, is considered to be the type strain of a proposed novel species, *Spiroplasma penaei* sp. nov. *S. penaei* is the first pathogenic spiroplasma to be isolated from a marine crustacean.

### Spiroplasma strains

The method used initially to isolate strain SHRIMP<sup>T</sup> from the infected *P. vannamei* and the resultant mortalities observed experimentally have been described previously (Nunan *et al.*, 2004). Briefly, the strain was cultivated by injection of a tissue homogenate, produced from frozen, naturally infected *P. vannamei* from Colombia, into specific pathogen-free (SPF) (Wyban *et al.*, 1992; Pruder *et al.*, 1995) *P. vannamei*, which in turn became infected. Frozen tissue (1 g) from the infected shrimp was sampled and homogenized in 5 ml Tris/NaCl buffer. The homogenate was diluted 1:5 with sterile 2% saline and injected into the third abdominal segment of SPF *P. vannamei*. As shrimp became moribund, haemolymph was drawn by using a 25 g needle attached to a 1 ml syringe. The haemolymph was filtered through a 0.45 µm Acrodisc syringe filter (Pall Corporation) and added to M1D medium (Jones *et al.*, 1977; Whitcomb *et al.*, 1982) supplemented with 2% NaCl, and then incubated at 30 °C. Following growth in the medium, as visualized by the phenol red component of the medium changing first to orange and then to yellow, the spiroplasma was examined by transmission electron microscopy (TEM) (Nunan *et al.*, 2004). The cultured organism was triply cloned and used to infect additional SPF *P. vannamei*. The triply cloned spiroplasma culture (1 ml) was centrifuged for 3 min at 3000 g. The supernatant fluid was discarded and the bacterial pellet was resuspended in sterile 2% saline. This inoculum was injected into the SPF shrimp. As mortalities occurred, shrimp were sampled for histological assessment and haemolymph was drawn for culture and TEM, as described previously (Nunan *et al.*, 2004). Recovery of the organism and associated mortalities fulfilled Koch's postulates.

### Culture media and cultivation techniques

The primary culture from which strain SHRIMP<sup>T</sup> was isolated was grown at 30 °C in M1D liquid medium (Whitcomb, 1983) supplemented with 2% NaCl. After several passages, the isolate was triply cloned (Tully, 1983). This cloned isolate was designated SHRIMP<sup>T</sup> and was used in all subsequent characterization studies and also for the fulfilment of Koch's postulates. Temperature requirements for growth were assessed by the method of Konai *et al.* (1996). In addition, Serum Fraction (SF) medium,

supplemented with 2% NaCl, was used for all biochemical tests (Aluotto *et al.*, 1970).

### Morphological studies

Cells of strain SHRIMP<sup>T</sup> from cultures in the exponential phase of growth were examined by dark-field microscopy (magnification, ×1000). Material for electron microscopy was established by pelleting 1 ml from a 24 h triple-clone culture at 3000 g for 3 min. The M1D medium was removed and replaced immediately with 1 ml cold 4% buffered glutaraldehyde, prepared with 0.15 M Millonig's phosphate buffer (pH 7.0) and supplemented with 1% NaCl and 0.5% sucrose (Lightner, 1996). Following 1 h refrigeration (4 °C), the glutaraldehyde buffer was replaced with cold Millonig's phosphate buffer (0.15 M) and maintained at 4 °C until post-fixation. The pellet was then post-fixed with 1% phosphate-buffered osmium tetroxide, dehydrated in ethyl alcohol and embedded in Spurr's resin (Ladd Research Inc.). The embedded pellet was sectioned to 75–90 nm thickness, stained with lead citrate and uranyl acetate and viewed by using a JEOL JEM 100CXII electron microscope, operated at 60 kV (Fig. 1).

### Biological and biochemical properties

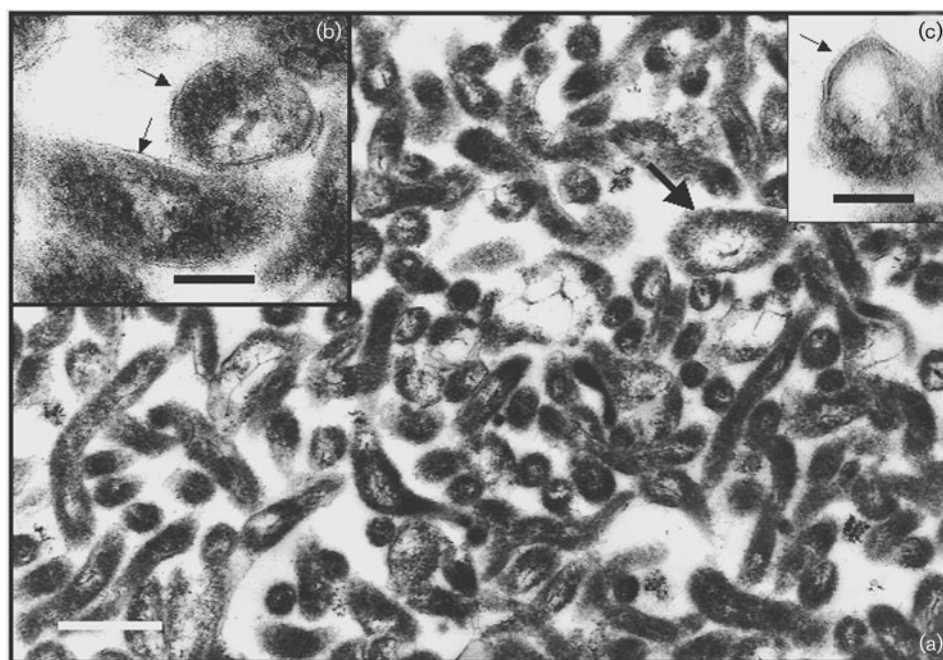
Procedures for determining carbohydrate fermentation and arginine and urea hydrolysis were as described by Aluotto *et al.* (1970). Filtration characteristics (Tully, 1983) were determined by using mid-logarithmic cultures grown in M1D medium.

### Serological tests

Anitserum to strain SHRIMP<sup>T</sup> was raised in rabbits as described by Williamson *et al.* (1979). The spiroplasma-deformation test (Williamson *et al.*, 1978) and metabolism-inhibition test were used to compare strain SHRIMP<sup>T</sup> serologically with the type strains from all recognized *Spiroplasma* groups and subgroups. Hyperimmune antisera to all recognized *Spiroplasma* groups and subgroups are maintained in the Towson University Spiroplasma Reference Collection. Reciprocal tests were performed with strains for which antisera reacted with the SHRIMP<sup>T</sup> strain.

### Genomic analysis

Extraction and purification of DNA from spiroplasmas was performed as described previously (Gasparich *et al.*, 1993). The DNA was diluted in 1 × SSC buffer to a final sodium concentration of 0.195 M. This sample was used (with a 1 × SSC buffer blank) in a Cary Varian thermal spectrophotometer equipped with software to determine the melting temperature, and the DNA G+C content was determined (Carle *et al.*, 1983). This process was repeated twice and a control of *Escherichia coli* DNA in 1 × SSC buffer was used to verify the results.



**Fig. 1.** Electron micrographs of the sectioned and stained pellet from strain SHRIMP<sup>T</sup>. The sections were stained with lead citrate and uranyl acetate. (a) The large arrow indicates a spiroplasma bleb. Bar, 0.5 µm. (b, c) The small arrows indicate the single cytoplasmic membrane. Bars, 0.1 µm.

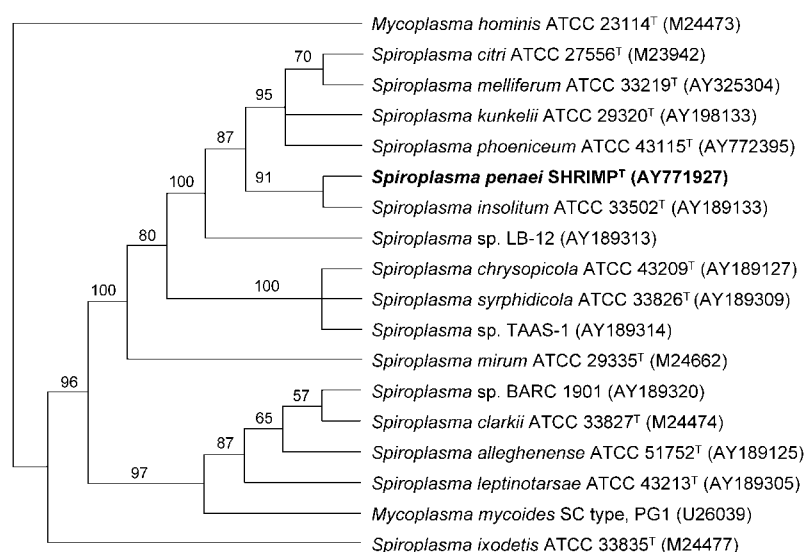
## Phylogenetic analysis

The 16S rRNA gene sequences used in this study with their GenBank accession numbers and ATCC culture collection accession numbers are indicated in Fig. 2. Sequences were aligned by using CLUSTAL W (Thompson *et al.*, 1994) and then aligned manually in MACCLADE (Maddison & Maddison, 1992). A 1000-replicate bootstrap analysis was performed by using a heuristic search and

the tree bisection–reconnection maximum-parsimony algorithm in PAUP (version 4.0b10; Swofford, 1998).

## Cultural and morphological properties

Strain SHRIMP<sup>T</sup> grew well in M1D broth and SF media (both supplemented with 2 % NaCl) and was resistant to 500 U penicillin ml<sup>-1</sup> in both media. Growth occurred over a temperature range of 20–37 °C, with optimal growth at



**Fig. 2.** Phylogenetic tree showing the position of *S. penaei* SHRIMP<sup>T</sup> among representatives of the genera *Spiroplasma* and *Mycoplasma*. *Mycoplasma hominis* and *Mycoplasma pulmonis* were used as out-group strains. A total of 1515 positions were used in the analysis, with the tree bisection–reconnection maximum-parsimony algorithm for branch swapping. The dataset was resampled 1000 times and bootstrap percentage values are given at the nodes. GenBank accession numbers are given in parentheses. All type strains were obtained from the ATCC. The 16S rRNA gene sequence of *S. penaei* SHRIMP<sup>T</sup> was included in a dataset described previously (Gasparich *et al.*, 2004).

28 °C. As determined by dark-field microscopy, logarithmic-phase cultures of strain SHRIMP<sup>T</sup> in M1D medium contained numerous long, helical, motile filaments. As determined by electron microscopy of logarithmic-phase cultures grown in M1D medium, cells of strain SHRIMP were filamentous with no evidence of a cell wall (Fig. 1b, c). The mean cell diameter was 195 nm ( $n=20$ ; range, 160–230 nm). Cells were surrounded by a single cytoplasmic membrane. Vesicular blebs are also displayed (Fig. 1a), very similar in appearance to those described previously from *Spiroplasma citri* (Razin *et al.*, 1973).

### Biochemical and biological properties

Strain SHRIMP<sup>T</sup> was able to grow in media containing glucose, glucose and arginine, and arginine alone, indicating abilities to ferment glucose with the production of acid and to catabolize arginine. A test was considered to be positive when the organism was able to grow in ten successive passages into the test medium. No growth was observed in unsupplemented SF medium or urea, and therefore urea hydrolysis was not tested. Cultures grew after passage through 220 nm filters, but not after passage through 100 nm filters.

### Serological studies

Metabolism-inhibition and spiroplasma-deformation tests indicated that strain SHRIMP<sup>T</sup> was unrelated serologically to representatives of recognized *Spiroplasma* groups or species. Antigens and antisera of strain SHRIMP<sup>T</sup> were tested in all combinations against all recognized and putative groups and subgroups. Strains R8A2 (I-1), E275 (I-3), 277F (I-4), LB-12 (I-5) and P40 (I-8) showed only minimal serological cross-reactivity at a 20-fold dilution titre, but at no higher titres. All other reactions were negative. Reaction of strain SHRIMP<sup>T</sup> antiserum against strain SHRIMP<sup>T</sup> culture was to a dilution of 5120.

### DNA base composition

The mean  $T_m$  value for strain SHRIMP<sup>T</sup> DNA was 81.8 °C, resulting in a base composition (G+C content) of  $29 \pm 1$  mol%. The *E. coli* standard measurements averaged  $45 \pm 1$  mol%.

### Phylogenetic analysis

Fig. 2 shows the phylogenetic tree constructed by using maximum parsimony. The dataset was resampled 1000 times and bootstrap percentage values are indicated for each branch. Strain SHRIMP<sup>T</sup> groups consistently with the group I spiroplasma cluster and is associated most closely with *S. insolitum*, which is the type species for subgroup 6 of the group I spiroplasmas (Hackett *et al.*, 1993). All strains within the group I cluster are pathogenic to plants, insects or now shrimp. This is also consistent with findings from the serological analyses, in which there was a very minor reaction with the subgroup I-6 antisera.

### Habitat and pathogenicity

Strain SHRIMP<sup>T</sup> was isolated from the haemolymph of the Pacific white shrimp, *P. vannamei*. This pathogenic strain was first recognized in February 2002 in two shrimp ponds in Colombia. The marine shrimp were being cultured in a low-salinity (2–10 parts per million) region. The initial outbreak occurred during a period of unusually high temperatures, with the pond water reaching 37 °C for more than 3 h each day for over 2 weeks. Mortalities from the initial epizootic were highly variable and ranged from 20–30 to 60–70 %. To examine the pathogenicity of the spiroplasma, infectivity studies were initially performed by injecting a tissue homogenate from the infected Colombian shrimp into SPF *P. vannamei* (Nunan *et al.*, 2004). As shrimp began to die and were moribund, haemolymph was extracted, filtered and added to M1D medium. The medium-cultured spiroplasma was then injected into SPF *P. vannamei*. To complete Koch's postulates, haemolymph was again sampled from moribund shrimp, filtered, added to M1D medium and examined by TEM, following 3 days growth, for the presence of the spiroplasma.

The properties described here for strain SHRIMP<sup>T</sup> fulfil the proposed criteria (ICSB, 1995) for species of the class *Mollicutes*, including the absence of a cell wall, filterability and penicillin resistance. The helicity and motility of cells of this organism, as well as its inability to hydrolyse urea, indicate that it is a member of the family *Spiroplasmataceae* in the order *Entomoplasmatales* (Tully *et al.*, 1993). Serological comparisons of strain SHRIMP<sup>T</sup> with representatives of all of the major *Spiroplasma* species, groups and subgroups demonstrated the uniqueness of the strain. We therefore propose the name *Spiroplasma penaei* sp. nov. for this organism.

### Description of *Spiroplasma penaei* sp. nov.

*Spiroplasma penaei* (pe.nae'i. N.L. n. *Penaeus* a genus of penaeid shrimp; N.L. gen. n. *penaei* of *Penaeus vannamei*, from which the organism was isolated).

Cells are helical and motile filaments with a mean diameter of 195 nm. Cells lack true cell walls. Acid is produced from glucose. Temperature range for growth is 20–37 °C and optimum growth occurs at 28 °C in M1D broth. Serologically distinct from previously characterized *Spiroplasma* species. Pathogenicity is indicated by injection into *P. vannamei*. The G+C content of the DNA is  $29 \pm 1$  mol%.

The type strain, SHRIMP<sup>T</sup> (= ATCC BAA-1082<sup>T</sup> = CAIM 1252<sup>T</sup>), was isolated from the haemolymph of the Pacific white shrimp, *P. vannamei*.

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