3-2000

'Candidatus Xenohaliotis californiensis', a newly described pathogen of abalone, Haliotis spp., along the west coast of North America

CS Friedman

KB Andree

KA Beauchamp

JD Moore

TT Robbins

See next page for additional authors

Recommended Citation

Friedman, CS; Andree, KB; Beauchamp, KA; Moore, JD; Robbins, TT; Shields, Jeffrey D.; and Hendricks, RP, "'Candidatus Xenohaliotis californiensis', a newly described pathogen of abalone, Haliotis spp., along the west coast of North America" (2000). *VIMS Articles*. 1593.
https://scholarworks.wm.edu/vimsarticles/1593

Authors

CS Friedman, KB Andree, KA Beauchamp, JD Moore, TT Robbins, Jeffrey D. Shields, and RP Hendricks

International Journal of Systematic and Evolutionary Microbiology (2000), **50**, 847–855 Printed in Great Britain

'*Candidatus* Xenohaliotis californiensis', a newly described pathogen of abalone, *Haliotis* spp., along the west coast of North America

Carolyn S. Friedman,[1,2] Karl. B. Andree,[2] K. A. Beauchamp,[2]
James D. Moore,[3] Thea T. Robbins,[3] Jeffrey D. Shields[4]
and Ronald P. Hedrick[2]

Author for correspondence: Carolyn S. Friedman. Tel: +1 707 875 2067. Fax: +1 707 875 2009.
e-mail: csfriedman@ucdavis.edu

[1] California Department of Fish & Game, Bodega Marine Laboratory, PO Box 247, Bodega Bay, CA 94923, USA

[2] Department of Medicine & Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616, USA

[3] Bodega Marine Laboratory, PO Box 247, Bodega Bay, CA 94923, USA

[4] Virginia Institute of Marine Science, Gloucester Point, VA 23062, USA

Withering syndrome is a fatal disease of wild and cultured abalone, *Haliotis* spp., that inhabit the west coast of North America. The aetiological agent of withering syndrome has recently been identified as a member of the family *Rickettsiaceae* in the order *Rickettsiales*. Using a combination of morphological, serological, life history and genomic (16S rDNA) characterization, we have identified this bacterium as a unique taxon and propose the provisional status of '*Candidatus* Xenohaliotis californiensis'. The Gram-negative, obligate intracellular pleomorphic bacterium is found within membrane-bound vacuoles in the cytoplasm of abalone gastrointestinal epithelial cells. The bacterium is not cultivable on synthetic media or in fish cell lines (e.g. CHSE-214) and may be controlled by tetracyclines (oxytetracycline) but not by chloramphenicol, clarithromycin or sarafloxicin. Phylogenetic analysis based on the 16S rDNA of '*Candidatus* Xenohaliotis californiensis' places it in the α-subclass of the class *Proteobacteria* but not to the four recognized subtaxa of the *α-Proteobacteria* (α-1, α-2, α-3 and α-4). The bacterium can be detected in tissue squashes stained with propidium iodide, microscopic examination of stained tissue sections, PCR or *in situ* hybridization. '*Candidatus* Xenohaliotis californiensis' can be differentiated from other closely related *α-Proteobacteria* by its unique 16S rDNA sequence.

Keywords: *Haliotis*, pathogen, '*Candidatus* Xenohaliotis californiensis', new taxon, abalone

INTRODUCTION

A relatively new disease, withering syndrome (WS), has resulted in catastrophic declines in populations of black abalone, *Haliotis cracherodii* Leach, in California, USA (Haaker *et al.*, 1992; Steinbeck *et al.*, 1992; VanBlaricom *et al.*, 1993; Friedman *et al.*, 1997). This chronic disease is also responsible for heavy losses of red abalone, *Haliotis rufescens* Swainson, in California, USA, and Baja California, Mexico (Haaker *et al.*, 1995; Moore *et al.*, 2000). Withering syndrome is characterized by degeneration of the digestive gland of the host (atrophy and/or metaplasia) and depletion of glycogen reserves (Kismohandaka *et al.*, 1993; Gardner *et al.*, 1995). Animals cease feeding and catabolize foot muscle protein as an energy source that results in atrophy of the pedal muscle (Kismohandaka *et al.*, 1993), and, ultimately, death. The incubation period of WS is approximately 5–7 months in black abalone (Friedman *et al.*, 1997; Friedman & Fan, 1998) and approximately 7–8 months in red abalone at 18 °C (J. D. Moore & C. S. Friedman, unpublished results).

Until recently, an aetiological agent had not been identified. Gardner *et al.* (1995) suggested that a *Rickettsiales*-like prokaryote (RLP) that infects the mucosal epithelium of the gastrointestinal tract might be the causative agent of WS. Friedman & Fan (1998) who transmitted the disease between black abalone observed a significant difference in intensity of RLP

Abbreviations: WS, withering syndrome; RLP, *Rickettsiales*-like prokaryote; SP, Sobranes Point; VAFB, Vandenberg Airforce Base.

The GenBank accession number for the 16S rDNA gene sequence of '*Candidatus* Xenohaliotis californiensis' is AF133090.

infection between abalone with and without WS, but no correlation between intensity of infection with RLPs and condition of the abalone in the infected treatments. In contrast, significant correlations were observed between intensity of RLP infection and degree of WS in wild and farmed red abalone (Haaker *et al.*, 1995; Moore *et al.*, 2000). The differences in responses to RLP infections between the two species may account for this discrepancy. Black abalone respond predominantly with degenerative changes to the digestive gland, while red abalone display a metaplasia of the mucosal epithelium in this gland. The metaplasia results in more target tissue for the bacterium compared to a loss of the same tissues in black abalone. In another study, Friedman *et al.* (unpublished results) found that both the RLPs and WS were responsive to treatments with oxytetracycline, providing strong evidence that the RLP is the aetiological agent of WS. Collectively, these studies indicate that the RLP in abalone is the aetiological agent of WS and that the pathogenesis of WS is complex and differs between host species.

Rickettsia-like bacteria are commonly observed in many marine invertebrates (Sparks, 1985; Friedman *et al.*, 1989; LeGall *et al.*, 1988). Until recently, these intracellular bacteria were thought to be non-pathogenic commensal organisms, unlike their terrestrial relatives (Anderson *et al.*, 1992; Brenner *et al.*, 1993). Due to the current inability to culture RLPs that infect marine invertebrates, differentiation of these bacteria has relied upon morphological characteristics as observed by light and electron microscopy methods (Friedman *et al.*, 1989; VanBlaricom *et al.*, 1993; Gardner *et al.*, 1995; Bower *et al.*, 1996; Wu & Pan, 1999). Based on light and electron microscopy, the bacterium observed within membrane-bound cytoplasmic vacuoles of gastrointestinal epithelial cells of abalone resembled members of the tribe *Ehrlichieae* in the family *Rickettsiaceae*. In order to determine the relationship of the RLP in abalone to known members of the family *Rickettsiaceae*, we examined antigenic, physiological and life history characteristics and sequenced the 16S rDNA gene of the bacterium associated with WS in black abalone from California. The 16S rDNA has been exploited for developing primers for PCR (Andree *et al.*, 2000) and *in situ* hybridization tests (Antonio *et al.*, 2000) to detect the agent in abalone tissues.

METHODS

Animals. Adult black abalone that measured 76–127 mm were collected in July of 1996 from Vandenberg Airforce Base (VAFB), California, a location at which WS is found. Abalone free of WS that measured 51–76 mm were collected from Sobranes Point (SP), near Carmel, California in May of 1996.

Histology. In order to observe the morphology of RLP inclusions, selected tissues were placed in Invertebrate Davidson's solution (Shaw & Battle, 1957) for 24 h and processed for routine paraffin histology. Deparaffinized 5 µm sections were stained with haematoxylin and eosin or Brown and Brenn's Gram stain (Luna, 1968) and viewed by light microscopy.

Transmission electron microscopy. The post-oesophagus was excised from several black abalone and 1–2 mm^2 pieces were fixed in 4% glutaraldehyde, 0·15 M sodium cacodylate and 0·15 M sodium chloride, pH 7·3 for 2–18 h on ice, post-fixed in 1% OsO_4 for 1 h at 4 °C (Rosen *et al.*, 1978). Tissues were rinsed twice in buffer, dehydrated through a graded series of acetone, infiltrated and embedded in epoxy resin. Ultrathin sections (10–20 nm) were stained with uranyl acetate and lead citrate and examined with a Zeiss 10C transmission electron microscope at 80 kV.

Antigenic reactivity with *Chlamydiaceae*-specific antibodies. The heteromorphic appearance of individual bacteria within some inclusions is suggestive of the distinct developmental stages (e.g. elemental and reticulate bodies) of *Chlamydia* and *Chlamydophila* spp., the two genera that now comprise the family *Chlamydiaceae* (Everett *et al.*, 1999). Although other evidence suggested that the RLP is more closely related to members of the order *Rickettsiales* than to those in the order *Chlamydiales*, we tested the ability of two monoclonal antibodies that possess broad reactivity with species of *Chlamydiaceae* to recognize the RLP. The samples consisted of Invertebrate Davidson's-preserved deparaffinized tissue sections from two black abalone and one red abalone. Avian lung tissue infected with *Chlamydia psittaci* fixed in 10% neutral buffered formalin served as a positive control. The antibody (Mab AC-1; American Research Products) recognized a genus-specific epitope of the chlamydial lipopolysaccharide antigen. The second antibody we used (BIO29-65, *Chlamydia* Indirect IP KH, Bartels) recognizes all serovars of *Chlamydia trachomatis*, *Chlamydophila* (*Chlamydia*) *psittaci* and *Chlamydophila* (*Chlamydia*) *pneumoniae*. Both antibodies were used according to the manufacturer's protocols with indirect immunoperoxidase detection using 3-amino-9-ethylcarbazole as the chromogen. A section from each sample was incubated with normal mouse IgG in lieu of specific antibody as a negative control.

Antimicrobial susceptibility. Due to the inability to culture the RLP, antimicrobial susceptibility was evaluated *in vivo* using infected abalone. Both cultured red and wild black abalone infected with the RLP in the field were used as experimental animals. Abalone were administered the following antimicrobials in two trials: (1) daily intramuscular (IM) injections for 14 d: chloramphenicol (12 mg kg^{-1}, Sigma, $n = 8$–10), clarithromycin (1·96 mg kg^{-1}, Abbott Laboratories, $n = 6$–8), sarafloxicin (12·98 mg kg^{-1}, Abbott Laboratories, $n = 6$–8) and tetracycline (50 mg kg^{-1}, Sigma, $n = 8$–10). (2) In a separate trial, abalone were administered nine IM doses of oxytetracycline (21 mg kg^{-1}, Liquimycin-LA 200, Pfizer, $n = 9$–12; Table 1). Animals were sampled for histology as described above 1 d after administration of the final dose in the first trial and two weeks after the final dose in the second trial. The non-parametric Mann–Whitney Rank Sum test (SigmaStat version 2.03 1992–1997) was used to examine differences in prevalences of the bacterium between treated and untreated control groups.

Tissue selection and DNA extraction. A small piece of posterior oesophagus (post-oesophagus) was examined by phase-contrast microscopy to assess the degree of RLP infection in the VAFB animals and to assure that the SP abalone were RLP-free. The post-oesophagus from each abalone was individually rinsed 3 × in sterile 3% (w/v) saline/0·1% Tween 80 solution, followed by a rinse in sterile 3% saline to remove exogenous bacteria present in the

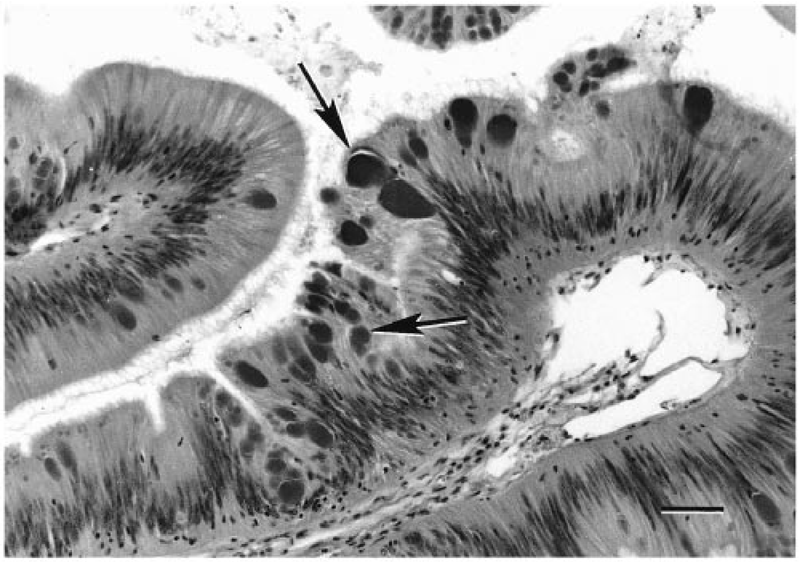

Fig. 1. RLPs (arrows) within posterior oesophagus of black abalone with withering syndrome. Note the homogeneous staining and apical location of the cytoplasmic vacuoles within host columnar epithelial cells. Haematoxylin and eosin. Bar, 50 μm.

lumen of the post-oesophagus. DNA was extracted according to the methods of Sambrook *et al.* (1989). Briefly, rinsed tissues were homogenized in lysis/proteinase K buffer. After 1 h at 55 °C the DNA was extracted in a phenol/chloroform solution. Isoamyl alcohol was added, mixed for 10 min and centrifuged. The top aqueous phase was removed and 0·1 vol. 3 M sodium acetate was added. Cold 100% ethanol was added to precipitate the DNA. The pellet was washed once in 70% ethanol, air-dried and resuspended in Tris-EDTA (TE) buffer. The DNA concentration was determined by spectrophotometry.

PCR amplification and sequencing. A sample containing 150 ng host and parasite DNA was used to amplify the RLP 16S rDNA sequence using EUB A and EUB B universal, eubacterial 16S rDNA primers (Gibco-BRL; Giovannoni, 1991). All the amplifications were performed in standard 50 μl reactions containing 10 mM Tris/HCl pH 8·3 (at 25 °C), 50 mM KCl, 1·5 mM $MgCl_2$, 0·001% (w/v) gelatin, 400 μM dNTPs, 5 μM tetramethyl ammonium chloride, 40 pmol each primer, and 2 U *Taq* polymerase. The PCR thermal cycler used was a model PTC-100 (MJ Research). The amplification entailed 37 cycles of 1 min at 94 °C, followed by 1 min at 56 °C, followed by 1 min at 72 °C. This was preceded by a denaturation step where samples were held at 95 °C for 5 min. The thermal cycler program finished with an extended elongation step where samples were held at 72 °C for 7 min. An aliquot of the amplified DNA was separated on a 1·5% (w/v) agarose gel, stained with ethidium bromide and examined using UV illumination. The entire RLP 16S rDNA gene (1525 bases) was cloned into pCR2.1 using a TOPO Cloning kit (Invitrogen) following the manufacturer's protocols. Clones were screened using PCR and primers that flanked the multi-cloning site of the vector. Clones were determined to be positive if they had the appropriate size insert in the plasmid (~1600 bp). The 16S rDNA gene from the clone designated p16RK3 was sequenced according to Andree *et al.* (1997) and aligned manually using Mac DNASIS software (Hitachi Software Engineering America).

Phylogenetic analysis. The sequence from clone p16RK3 was compared with data in GenBank using the BLAST algorithm (Altschul *et al.*, 1990). The organisms with the highest ranking were used in our initial taxonomic characterization together with extant *Rickettsiales*-like bacteria from various taxa. We applied the methods of Jukes & Cantor (1969) in performing evolutionary distance analysis with bootstrap support (500 replications) using MEGA software (Molecular Evolutionary Genetics Analysis version 1.01). This initial phylogenetic analysis used 1340 characters (or ~86%) of the 16S rDNA gene (data not shown). Positions in which no homology was observed between species were omitted from the analysis. A second evaluation included only those organisms that were most proximal to the abalone RLP in our first evaluation. For this second analysis we included most of the 16S rDNA sequence (1496 bases or 98% of the abalone RLP gene) from the 15 most proximal species used in our first analysis. We eliminated only the most distal ends of the 16S rDNA sequence such that the entire sequence of the shortest 16S rRNA gene deposited in GenBank served as the template for comparison. In addition, we deleted a large insertion of ~150 bases from the 16S rDNA gene of *Caedibacter caryophila* as this sequence was not shared with any of the other species in our study.

Accession numbers for nucleotide sequences. The 16S rDNA gene sequences of selected bacteria were electronically retrieved from GenBank. These included: *Anaplasma marginale*, M60313; *Caedibacter caryophila*, X71837; *Cowdria ruminantium*, X61659; *Coxiella burnetii*, M21291; *Ehrlichia sennetsu*, M73225; *Ehrlichia risticii*, M21290; *Ehrlichia phagocytophila*, M73224; *Ehrlichia bovis*, U03775; *Escherichia coli*, J01695; NHP bacterium, U65509; *Piscirickettsia salmonis*, X60783; *Rickettsia prowazekii*, M21789; *Rickettsia rickettsii*, M21293; and *Wolbachia pipientis*, X61768.

RESULTS

Histology

The RLP stained Gram-negative and was observed within cytoplasmic vacuoles of abalone gastrointestinal epithelial cells. The bacterial inclusions were usually situated apical to the nucleus of the host cells (Fig. 1). The bacterium was found in cells of the post-oesophagus, transport ducts of the digestive gland

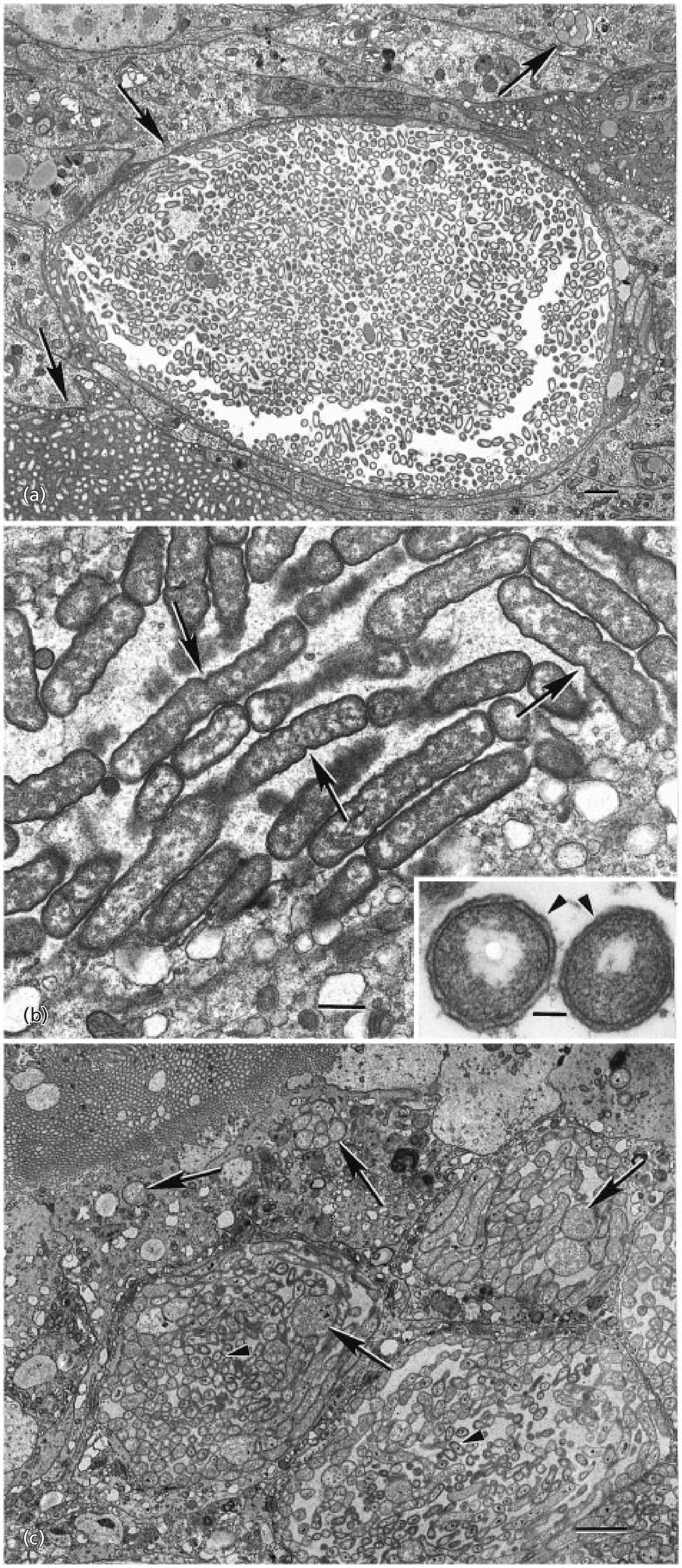

Fig. 2. (a) Transmission electron micrograph showing pleomorphic rod-shaped RLPs (arrows) within membrane-bound phagosomes of abalone gastrointestinal cells. Bar, 2 μm. (b) The bacterium divides by binary fission (arrows) and is emarginated by a trilaminar cell wall and lacks a visible peptidoglycan layer (inset: arrowhead). Ribosomes are located peripherally and fibrils of nucleoid material are centrally located. Bar, 500 nm, inset: Bar, 100 nm. (c) Inclusion containing heteromorphic bacteria. Arrows illustrate the larger spherical life stage that is predominantly observed in smaller phagosomes. The rod-shaped stage is denoted by an arrowhead. Bar, 2 μm.

Table 1. *In vivo* testing of the susceptibility of red and black abalone to several antimicrobials administered by 9–12 intramuscular injections

Antimicrobial Dose	Percentage of red abalone with RLP foci	Percentage of black abalone with RLP foci
Chloramphenicol		
None*	90 ($n = 10$)	100 ($n = 8$)
12 mg kg^{-1}	80 ($n = 10$)	87·5 ($n = 8$)
Clarithromycin		
None	100 ($n = 8$)	100 ($n = 6$)
1·96 mg kg^{-1}	100 ($n = 8$)	100 ($n = 6$)
Oxytetracycline (Liquimycin LA)		
None	100 ($n = 10$)	100 ($n = 8$)
21 mg kg^{-1}	**0**† ($n = 10$)	**0**† ($n = 9$)
Sarafloxacin		
None	100 ($n = 8$)	100 ($n = 6$)
12·98 mg kg^{-1}	100 ($n = 8$)	100 ($n = 6$)
Tetracycline		
None	90 ($n = 10$)	100 ($n = 8$)
50 mg kg^{-1}	**10**‡ ($n = 10$)	**0**† ($n = 8$)

* Control abalone were administered diluent (2 % sterile saline) only.

† Treatments with rickettsial infection prevalences significantly less than that of controls ($p < 0·001$), Mann–Whitney Rank Sum test.

‡ Treatments with rickettsial infection prevalences significantly less than that of controls ($p < 0·05$), Mann–Whitney Rank Sum test.

and, less frequently, the intestine. The predominantly observed inclusion was spherical to oblong in shape, measured 14·1–56·4 μm with a mean maximum dimension of 35·8 ± 13·0 μm, and contained homogeneous and densely packed bacteria that stained deeply purple with haematoxylin and eosin (Fig. 1). No morulae were observed.

Transmission electron microscopy

The bacterium was pleomorphic and was found dividing within cytoplasmic vacuoles of host cells that appear to be phagosomes or phagolysosomes (Fig. 2). The bacterium is predominantly a slender, rod-shaped organism with a pleomorphic spherical to coccobacillus form observed primarily in smaller and, presumably, younger phagosomes (Fig. 2a). The rod-shaped form replicates by binary fission and measures a mean of 332 nm × 1550 nm, with a range of 122–598 nm × 570–4460 nm (Fig. 2a, b). The spherical form measures a mean of 1405 nm in maximum dimension, with a range of 783–2190 nm (Fig. 2c). In addition, there appears to be an intermediate, coccobacillus size variant with a mean diameter of 620 nm (559–658 nm) and length of 1270 nm (Fig. 2c). The bacterium is emarginated by a trilaminar cell wall that consists of an electron-dense inner plasma membrane and an outer membrane of equal electron density that are separated by an electron-lucent layer without a visible peptidoglycan layer (Fig. 2b). The outer cell wall, although wavy, lacks the intense rippling observed in members of the genus *Ehrlichia*. The bacterium contains many ribosomes, located around the periphery of the cell, and centrally located fibrils of nucleoid material (Fig. 2b insert). The bacterium lacks a slime layer that is observed surrounding rickettsiae in the typhus and spotted fever groups (Weiss & Moulder, 1984a).

Antigenic reactivity with *Chlamydiaceae*-specific antibodies

No specific staining of the RLP inclusions was observed using either *Chlamydiaceae*-specific antibody or the preimmune mouse IgG. In contrast, the sections of avian lung infected with *Chlamydophila* (*Chlamydia*) *psittaci* had scattered positive (red) staining throughout the tissue (not shown).

Antimicrobial susceptibility

Microscopic examination of stained tissue sections from infected abalone treated with antimicrobials indicated that the RLP was only sensitive to the tetracyclines: oxytetracycline ($p < 0·001$, $n = 9–12$) and tetracycline ($p < 0·05$, $n = 8–10$, Table 1). Administration of nine IM doses of 21 mg oxytetracycline kg^{-1} effectively cured RLP infections in both red and black abalone (Table 1).

16S rDNA sequence analysis

A sequence alignment was made using the 16S rRNA sequences from p16RK3, and the 29 representative α- and γ-*Proteobacteria* listed above that were used in a similar analysis of the shrimp NHP bacterium (Loy *et al.*, 1996). The bacterial agent from abalone shares sequence motifs with the shrimp NHP bacterium but only 70·9 % sequence similarity overall. A BLAST search analysis determined that the 16S rDNA sequence of the abalone bacterium appears to be most closely related to those of *Anaplasma marginale* (77·3 % similarity), *Ehrlichia bovis* (75·8 %) and *Wolbachia pipientis* (74·1 %) when the entire 16S sequence was used in the analysis (data not shown).

The abalone RLP appears to be equally distant from the other bacteria used in this study (Table 2). The phylogenetic distance analysis suggests a slightly different evolutionary relationship, but groups the RLP similar to the sequence similarity results from the BLAST search (Fig. 3). In both phylogenetic analyses performed, the abalone RLP is clearly unique and is most closely related to *Wolbachia pipientis* followed by *Ehrlichia sennetsu* and *Ehrlichia risticii*. Parsimony analyses using PAUP (Phylogenetic Analysis Using Parsimony, PAUP version 3.1) provided tree topologies which also indicated that the abalone RLP grouped

Table 2. Genetic distance table illustrating the relationship of the WS bacterium and other *Proteobacteria* using MEGA

A total of 1496 nucleotides were included in the data set after missing information data and gaps were removed

Taxon	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. WFS	0·1920	0·2884	0·2661	0·2040	0·2402	0·2101	0·2050	0·2392	0·2705	0·2307	0·2339	0·2245	0·2861	0·2952
2. *Anaplasma marginale*		0·2060	0·1860	0·0787	0·1656	0·0379	0·0518	0·1637	0·1930	0·1762	0·1733	0·1346	0·2266	0·2255
3. *Coxiella burnettii*			0·2142	0·2224	0·2245	0·2131	0·2172	0·2224	0·2370	0·2101	0·2060	0·2203	0·1401	0·1791
4. *Caedibacter caryophila*				0·1900	0·2101	0·1870	0·1949	0·2142	0·1551	0·1685	0·1656	0·2152	0·2091	0·2328
5. *Cowdria ruminantium*					0·1752	0·0752	0·0855	0·1733	0·2224	0·1723	0·1714	0·1355	0·2328	0·2487
6. *Ehrlichia risticii*						0·1523	0·1598	0·0070	0·2091	0·1930	0·1900	0·1752	0·2339	0·2552
7. *Ehrlichia phagocytophila*							0·0306	0·1513	0·1949	0·1675	0·1656	0·1272	0·2193	0·2276
8. *Ehrlichia bovis*								0·1579	0·2010	0·1762	0·1762	0·1336	0·2318	0·2307
9. *Ehrlichia sennetsu*									0·2080	0·1900	0·1870	0·1752	0·2328	0·2552
10. NHP bacterium										0·1791	0·1752	0·2152	0·2392	0·2585
11. *Rickettsia prowazekii*											0·0101	0·1743	0·2091	0·2413
12. *Rickettsia rickettsii*												0·1733	0·2091	0·2434
13. *Wolbachia pipientis*													0·2286	0·2307
14. *Piscirickettsia salmonis*														0·1420
15. *Escherichia coli*														

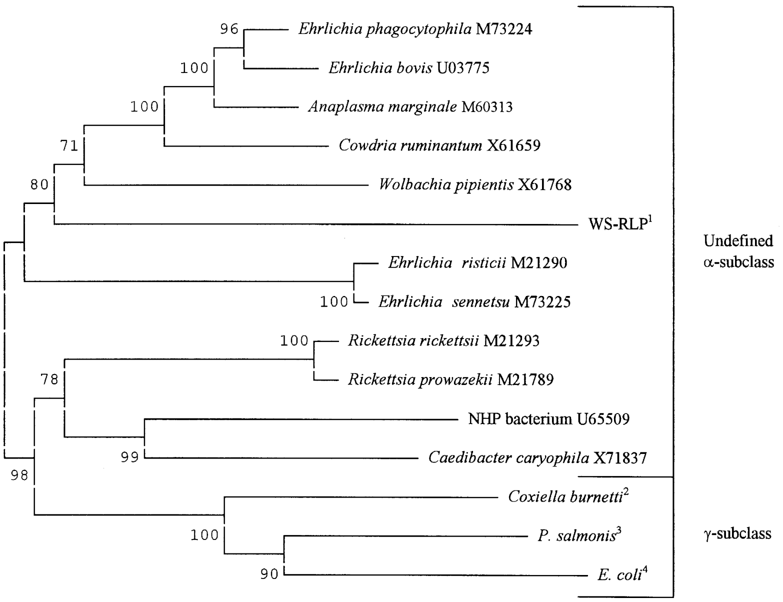

Fig. 3. Dendrogram showing evolutionary relationship of the abalone RLP (WS-RLP) to members of the families *Rickettsiaceae* and *Piscirickettsiaceae* with *Escherichia coli* as a representative genetically distant bacterium. The number at branch points represents the percentage occurrence of a given branch during 500 replicates of the bootstrap analysis. Each bar is approximately equal to a distance of 0·002668. Superscript numbers denote the following sequence accession numbers: 1, AF133090; 2, M21291; 3, X60783; 4, J01695.

most closely with *Ehrlichia risticii* and *Ehrlichia sennetsu* (data not shown). Collectively, these data illustrate the uniqueness of the 16S rDNA sequence of the abalone RLP.

DISCUSSION

Wayne *et al.* (1987) recommended that bacterial nomenclature should reflect genomic relationships. Confirmatory phenotypic data should also be sought prior to the introduction of a new name (Nelson *et al.*, 1984). Recent bacterial taxonomy has increasingly relied on both phenotypic and genomic information including a new species in the genus *Rickettsia*, *Rickettsia japonica* (Uchida *et al.*, 1992) and in the genus *Nocardia*, *Nocardia crassostreae* (Friedman *et al.*, 1998). Those bacteria that cannot be cultured provide fewer phenotypic characters and therefore phylogenetic analyses become central to taxonomic placement. Based on proposals by Murray & Schleifer (1994) and Murray & Stackebrandt (1995; the provisional status of *Candidatus* was created for unculturable organisms and poorly characterized organisms, e.g. '*Candidatus* Arsenophonus tristoninarum', Hypsa & Dale, 1997). Despite this, several species of *Ehrlichia* have been described based principally upon genomic sequences such as *Ehrlichia chaffeensis* (Anderson *et al.*, 1991), *Ehrlichia ewingii* (Anderson *et al.*, 1992) and *Ehrlichia muris* (Wen *et al.*, 1995). *Ehrlichia chaffeensis* was differentiated largely on its unique 16S rRNA sequence. Phylogenetic analyses using 16S sequence analysis have been used to unify two genera: *Bartonella* and *Rochalimea* in the family *Bartonellaceae*, and to remove the family *Bartonellaceae* from the order *Rickettsiales* (Brenner *et al.*, 1993). We used a combination of morphological, tinctorial, physiological, pathological and genomic traits to identify the bacterium associated with WS in abalone as a previously undescribed bacterium in the family *Rickettsiaceae* and propose inclusion in the provisional status, *Candidatus* with the name of '*Candidatus* Xenohaliotis californiensis'.

The RLP that infects abalone is a small, Gram-negative, pleomorphic bacterium that forms large intracytoplasmic inclusions within gastrointestinal epithelial cells. These inclusions lack the morphology of morulae (Fig. 1), characteristic of the genus *Ehrlichia*. The bacterium is an obligate intracellular parasite as evidenced by unsuccessful culture on artificial media (C. S. Friedman & R. P. Hedrick, unpublished results). Collectively, these traits confirm placement of the abalone RLP in the order *Rickettsiales*. Tissue-specificity of the abalone RLP resembles that of *Rickettsia* spp. or *Anaplasma marginale* which often infect the midgut of arthropod vectors (Ribeiro *et al*., 1996). *Anaplasma marginale* also infects blood cells of its mammalian host, as do members of the genus *Ehrlichia* (Anderson *et al*., 1991; Ribeiro *et al*., 1996; Ristic & Huxsoll, 1984). *Wolbachi pipientis* infects germinal cells and epithelia of the insect vector (mosquito), while *Wolbachi melophagi* is observed in the lumina of the host (sheep) gastrointestinal tract (Weiss *et al*., 1984). Host-specificity and transmission of the abalone RLP is unique as this type of bacterium has not previously been described in a marine gastropod and transmission is direct (Friedman *et al*., 1997) as suggested for *Piscirickettsia salmonis*, a marine agent that infects salmon (Fryer *et al*., 1992). The abalone bacterium is pleomorphic with multiple size variants as observed in some genera of rickettsiae. For example, the life cycle of *Coxiella burnetii* and *Cowdria ruminantium* includes a vegetative and relatively electron-lucent large cell variant and a small cell variant that is quite electron-dense (Weiss & Moulder, 1984b; Jongejan *et al*., 1991). Bonami & Pappalardo (1980) observed a dimorphic, membrane-bound, intracytoplasmic rickettsia which is pathogenic to the crab, *Carcinus mediterraneus*. The life cycle of this crab pathogen also included an electron-dense small-cell variant and a larger and more electron-lucent cell variant. The progression of size variants suggests that this is a single species with a dimorphic life history as observed in other members of the family *Rickettsiaceae*. In addition, the rickettsia found in abalone lacks a visible peptidoglycan layer that is often lacking in rickettsial bacteria (e.g. *Ehrlichia* spp.; Kawahara *et al*., 1993). However, the outer cell wall of the abalone bacterium lacks the intense rippling observed in members of the genus *Ehrlichia*. The RLP from abalone lacked a slime layer found in *Rickettsia* spp. in the typhus and spotted fever groups (Weiss & Moulder, 1984a). In addition, pan-*Chlamydia* antibodies did not recognize the abalone RLP. These traits illustrate the unique morphology and life history of the RLP that infects abalone relative to described members of the family *Rickettsiaceae*.

Using rRNA sequence analyses, Weisburg *et al*. (1989) determined that the family *Rickettsiaceae* is polyphyletic and is comprised three tribes: *Rickettsiaea*, *Ehrlichieae* and *Wolbachieae*. Our study indicates that the abalone RLP shares similarities with members of all three tribes but is phylogenetically distinct from each tribe. Evolutionary relationships to other rickettsiae shown in the distance table (Table 2) and dendrogram (Fig. 3) illustrate that the 16S rDNA of the abalone RLP is a member of the α-subclass of the class *Proteobacteria*. Like other members of the family *Rickettsiaceae* in the α-subclass, the bacterium does not belong to the four recognized subtaxa of the α-*Proteobacteria* (α-1, α-2, α-3 and α-4; Fig. 3). This information clearly differentiates the abalone RLP from the γ-subclass of the class *Proteobacteria* that includes genera such as *Coxiella* and *Piscirickettsia*. In addition, members of the genus *Rickettsia* form a monophyletic group within the α-subclass that is distinct from the abalone RLP and four genera (*Ehrlichia*, *Wolbachia*, *Cowdria* and *Anaplasma*) with which it forms a polyphyletic group. As illustrated in Fig. 3, the 16S rDNA sequence of the abalone RLP represents a lineage that is distinct from the other genera within this group. As shown in our study, vanVliet *et al*. (1992) also found that despite differences in morphology and host and target cell preferences, *Cowdria ruminantium* and *Ehrlichia canis* and *Ehrlichia chaffeensis* form a closely related cluster upon analysis of 16S gene sequences. Collectively, these traits in conjunction with a lack of the full complement of phenotypic data required for validation of a new genus and species, we propose the name, '*Candidatus* Xenohaliotis californiensis' (Xe′no.ha.li.o.tis′ L. fem. n. *xeno* foreign; *Haliotis* generic name of its abalone host; N.L. fem. n. *Xenohaliotis* a foreign organism in abalone; cal′i.for.ni.en.sis. N.L. fem. adj. *californiensis* regarding California, USA, the area where the organism was initially identified).

Description of '*Candidatus* Xenohaliotis californiensis'

Gram-negative, periodic acid–Schiff base stain-negative, pleomorphic coccobacillus, ~332 × 1550 nm with an irregular spherical cell stage ~1450 nm in maximum dimensions. Stains basophilic with haematoxylin and eosin. Non-motile. Gram-negative cell wall that lacks visible peptidoglycan and slime layers, ribosome rich with fibrilar nucleoid. Replicates within spherical to oblong, intracytoplasmic membrane-bound vacuoles of abalone gastrointestinal transport or absorptive epithelium. Bacteria-filled vacuoles are generally located distal to host nucleus. Not cultivable on synthetic media or in fish cell lines (e.g. CHSE-214, American Type Culture Collection). Sensitive to tetracyclines but not to chloramphenicol, clarithromycin or sarafloxicin. Based on 16S rDNA sequence analysis, the bacterium is a member of the undefined α-subclass of the class *Proteobacteria*. The bacterium can be diagnosed with tissue squashes stained with propidium iodide, microscopic examination of stain tissue sections and can be differentiated from other closely related α-*Proteobacteria* by its unique 16S rDNA sequence.

ACKNOWLEDGEMENTS

This work was supported by the Saltonstall-Kennedy Program of the National Oceanic and Atmospheric Administration, US Department of Commerce under grant number NA76FD0046. Additional support was provided by the California State Resources Agency, California Department of Fish and Game and the Aquaculture and Fisheries Program, University of California, Davis.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Meyers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Anderson, B. E., Dawson, J. E., Jones, D. C. & Wilson, K. H. (1991). *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. *J Clin Microbiol* **29**, 2838–2842.

Anderson, B. E., Greene, C. E., Jones, D. C. & Dawson, J. E. (1992). *Ehrlichia ewingii* sp. nov., the etiological agent of canine granulocytic erhlichiosis. *Int J Syst Bacteriol* **42**, 299–302.

Andree, K. B., Gresoviac, S. J. & Hedrick, R. P. (1997). Small subunit ribosomal RNA sequences unite alternate actinosporean and myxosporean stages of *Myxobolus cerebralis*, the causative agent of whirling disease in salmonid fish. *J Eukaryot Microbiol* **44**, 208–215.

Andree, K. B., Friedman, C. S., Moore, J. D. & Hedrick, R. P. (2000). A polymerase chain reaction for detection of genomic DNA of a Rickettsiales-like prokaryote associated with Withering Syndrome in Black Abalone (*Haliotis cracherodii*). *J Shellfish Res* (in press).

Antonio, D. B., Andree, K. B., Moore, J. D., Friedman, C. S. & Hedrick, R. P. (2000). Detection of Rickettsiales-like prokaryotes (RLPs) by *in situ* hybridization in black abalone *Haliotis cracherodii* with Withering Syndrome. *J Invertebr Pathol* **75** (in press).

Bonami, J. R. & Pappalardo, R. (1980). Rickettsial infection in marine crustacea. *Experientia* **36**, 180–181.

Bower, S. M., Meyer, G. R. & Boutillier, J. A. (1996). Stained prawn disease (SPD) of *Pandalus platyceros* in British Columbia, Canada, caused by a rickettsial infection. *Dis Aquat Org* **24**, 41–54.

Brenner, D., O'Connor, S. P., Winkler, H. K. & Steigerwalt, A. G. (1993). Proposals to unify the genera *Bartonella* and *Rochalimea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and to remove the family *Bartonellaceae* from the order *Rickettsiales*. *Int J Syst Bacteriol* **43**, 777–786.

Brock, J. A., Nakagawa, L. K., Hayashi, T., Teruya, S. & VanCampen, H. (1986). Hepatopancreatic rickettsial infection of the penaeid shrimp, *Penaeus marginatus* (Randall), from Hawaii. *J Fish Dis* **9**, 73–77.

Everett, K. D. E., Bush, R. M. & Andersen, A. A. (1999). Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol* **49**, 415–440.

Friedman, C. S. & Fan, T. W.-M. (1998). Withering Syndrome of black abalone: Causes and physiological alterations. In *California Sea Grant Reports of Completed Projects* 1994–97, pp. 101–106. La Jolla, CA: Sea Grant College System.

Friedman, C. S., McDowell, T., Groff, J. M., Hollibaugh, J. T., Manzer, D. & Hedrick, R. P. (1989). Presence of *Bonamia ostreae* among populations of the European flat oyster, *Ostrea edulis* Linné, in California, USA. *J Shellfish Res* **8**, 133–137.

Friedman, C. S., Thomson, M., Chun, C., Haaker, P. L. & Hedrick, R. P. (1997). Withering syndrome of the black abalone, *Haliotis cracherodii* (Leach): water temperature, food availability, and parasites as possible causes. *J Shellfish Res* **16**, 403–411.

Friedman, C. S., Beaman, B. L., Chun, J., Goodfellow, M., Gee, A. & Hedrick, R. P. (1998). *Nocardia crassostreae* sp. nov., the causal agent of nocardiosis in Pacific oysters. *Int J Syst Bacteriol* **48**, 237–246.

Fryer, J. L., Lannan, C. N., Giovannoni, S. J. & Wood, N. D. (1992). *Piscirickettsia salmonis* gen. nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes. *Int J Syst Bacteriol* **42**, 120–126.

Gardner, G. R., Harshbarger, J. C., Lake, J. L., Sawyer, T. K., Price, K. L., Stephenson, M. D., Haaker, P. L. & Togstad, H. A. (1995). Association of procaryotes with symptomatic appearance of withering syndrome in black abalone, *Haliotis cracherodii*. *J Invertebr Pathol* **66**, 111–120.

Giovannoni, S. (1991). The polymerase chain reaction. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 177–201. Edited by E. Stackebrandt & M. Goodfellow. New York: Wiley.

Haaker, P. L., Richards, D. V., Friedman, C. S., Davis, G. E., Parker, D. O. & Togstad, H. (1992). Mass mortality and withering syndrome in black abalone *Haliotis cracherodii* in California. In *Abalone of the World*, pp. 214–224. Edited by S. A. Shephard, M. J. Tegner & S. A. Guzman del Proo. Oxford: Blackwell.

Haaker, P. L., Harris, S. L., Taniguchi, I. K. & Friedman, C. S. (1995). Withering syndrome in California: does it occur in subtidal populations? *J Shellfish Res* **14**, 267.

Hypsa, V. & Dale, C. (1997). In vitro culture and phylogenetic analysis of "*Candidatus* Arsenophonus triatominarum", an intracellular bacterium from the triatomine bug, *Triatoma infestans*. *Int J Syst Bacteriol* **47**, 1140–1144.

Jongejan, F., Zandbergen, T. A., van de Wiel, P. A., de Groot, M. & Uilenberg, G. (1991). The tick-borne rickettsia *Cowdria ruminantium* has *Chlamydia*-like developmental cycle. *Onderstepoort J Vet Res* **58**, 227–237.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Kawahara, M., Suto, C., Rikihisa, Y., Yamamoto, S. & Tsuboi, Y. (1993). Characterization of ehrlichial organisms isolated from a wild mouse. *J Clin Microbiol* **31**, 89–96.

Kismohandaka, G., Friedman, C. S., Roberts, W., Hedrick, R. P. & Crosby, M. P. (1993). Investigation of physiological parameters of black abalone with withering syndrome. *J Shellfish Res* **12**,131–132.

LeGall, G., Chagot, D., Mialhe, E. & Grizel, H. (1988). Branchial rickettsiales-like infection associated with a mass mortality of sea scallop *Pecten maximus*. *Dis Aquat Org* **4**, 229–232.

Loy, J. K., Dewhirst, F. E., Weber, W., Frelier, P. F., Garbar, T. L., Serban, I. T. & Templeton, J. W. (1996). Molecular phylogeny and in situ detection of the etiological agent of necrotizing helpatopancreatitis in shrimp. *Appl Environ Microbiol* **62**, 3439–3445.

Luna, L. G. (editor) (1968). *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*, 3rd edn, pp. 38–39. New York: McGraw-Hill.

Moore, J. D., Robbins, T. T. & Friedman, C. S. (2000). Withering syndrome in farmed red abalone, *Haliotis rufescens*: thermal induction and association with a gastrointestinal *Rickettsiales*-like prokaryote. *J Aquat Anim Health* **12**, 26–34.

Murray, R. G. E. & Schleifer, K. H. (1994). Taxonomic notes: a proposal for recording the properties of putative taxa of procaryotes. *Int J Syst Bacteriol* **44**, 174–176.

Murray, R. G. E. & Stackebrandt, E. (1995). Taxonomic note: implementation of the provisional status *Candidatus* for incompletely described procaryotes. *Int J Syst Bacteriol* **45**, 186–187.

Nelson, D. C., Waterbury, J. B. & Jannasch, H. W. (1984). DNA base composition and genome size of the prokaryotic symbiont in *Riftia pachyptila* (Pogonophora). *FEMS Microbiol Lett* **24**, 267–271.

Ribeiro, M. F. B., Lima, J. D. & Salcedo, J. H. P. (1996). Attempted transmission of *Anaplasma marginale* by infected *Boophilus microplus*. *Arq Bras Med Vet Zootec* **48**, 397–402.

Ristic, M. & Huxsoll, D. L. (1984). Tribe II. *Ehrlichiae* Philip 1957, 948^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 704–711. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Rosen, M. D., Stasek, C. R. & Herman, C. O. (1978). The ultrastructure and evolutionary significance of the cerebral ocelli of *Mytilus edulis*, the bay mussel. *The Veliger* **21**, 10–18.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Shaw, B. L. & Battle, H. I. (1957). The gross and microscopic anatomy of the digestive tract of the oyster, *Crassostrea virginica* (Gmelin). *Can J Zool* **35**, 325–347.

Sparks, A. K. (1985). Rickettsiae, chlamydiae, and mycoplasmas of invertebrates. In *Synopsis of Invertebrate Pathology Exclusive of Insects*, pp. 165–180. New York: Elsevier.

Steinbeck, J. R., Groff, J. M., Friedman, C. S., McDowell, T. & Hedrick, R. P. (1992). Investigation into a coccidian-like protozoan from the California abalone, *Haliotis cracherodii*. In *Abalone of the World*, pp. 203–213. Edited by S. A. Shephard, M. Tegner & S. Guzman del Proo. Oxford: Blackwell.

Sudhir, K., Tamura, K. & Nei, M. (1993). MEGA: Molecular Evolutionary Genetic Analysis, version 1.01. Pennsylvania State University, University Park, PA 16802, USA.

Uchida, T., Uchiyama, T., Kumano, K. & Walker, D. H. (1992). *Rickettsia japonica* sp. nov., the etiological agent of spotted fever group rickettsiosis in Japan. *Int J Syst Bacteriol* **42**, 303–305.

VanBlaricom, G. R., Ruediger, J. L., Friedman, C. S., Woodard, D. D. & Hedrick, R. P. (1993). Discovery of withering syndrome among black abalone *Haliotis cracherodii* Leach, 1814 populations at San Nicolas island, California. *J Shellfish Res* **12**, 185–188.

vanVliet, A. H. M., Jongejan, F. & van der Zeijst, B. A. M. (1992). Phylogenetic position of *Cowdria ruminatum* (*Rickettsiales*) determined by analysis of amplified 16S ribosomal DNA sequences. *Int J Syst Bacteriol* **42**, 494–498.

Wayne, L. G., Brenner, D. J., Cowell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Weisburg, W. G., Dobson, M. E., Samuel, J. E. & 7 other authors (1989). Phylogenetic diversity of the rickettsiae. *J Bacteriol* **171**, 4202–4206.

Weiss, E. & Moulder, J. W. (1984a). Genus I. *Rickettsia* da Rocha-Lima 1916, 567^{AL} (Nom. Gen. Cons. Opin. 19, Jud. Comm. 1958, 158). In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 688–698. Edited by N. R. Kreig & J. G. Holt. Baltimore: Williams & Wilkins.

Weiss, E. & Moulder, J. W. (1984b). Genus III. *Coxiella* (Philip 1943) Philip 1948, 58^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 701–704. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Weiss, E., Dasch, G. A. & Chang, K.-P. (1984). Tribe III. *Wolbachieae* Philip, 1956, 266^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 711–713. Edited by N. R. Kreig & J. G. Holt. Baltimore: Williams & Wilkins.

Wen, B., Rikihisa, Y., Mott, J., Fuerst, P. A., Kawahara, M. & Suto, C. (1995). *Ehrlichia muris* sp. nov., identified on the basis of 16S rRNA base sequences and serological, morphological, and biochemical characteristics. *Int J Syst Bacteriol* **45**, 250–254.

Wu, X. & Pan, J. (1999). Studies on *Rickettsia*-like organism disease of the tropical marine pearl oyster. I. The fine structure and morphogenesis of *Pinctada maxima* pathogen *Rickettsia*-like organism. *J Invertebr Pathol* **73**, 162–171.