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Short Communication

Identification of Rickettsia-Like Organism (RLO) in the Oyster Crassostrea rivularis Gould

Yang Zhang[1#], Jing Fang[2#], Jingfeng Sun[3], and Xinzhong Wu[1,2*]

[1]South China Sea Institute of Oceanography, Chinese Academy of Sciences, China
[2]Ocean College, Qinzhou University, China
[3]Aquaculture College, Tianjin Agriculture University, China
[#]Yang Zhang and Jing Fang contributed equally to this work

*Corresponding author
Xinzhong Wu, South China Sea Institute of Oceanography, Chinese Academy of Sciences, China, Email: wuxzqinzhou@163.com

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- Rickettsia-like organism (RLO)
- 16S rDNA
- Phylogeny
- *In situ* hybridization

Abstract

The oyster *Crassostrea rivularis* Gould (also known as *Crassostrea ariakensis*) an important bivalve species cultured in southeastern China. Since 1992, these oysters have suffered high mortality during winter and spring. An intracellular rickettsia-like organism (RLOs) was proposed as the causative agent. In this study, RLOs were purified from the gill and digestive gland of dying oyster (*C. rivularis* Gould), and their 16S rDNA was amplified from the purified products. To eliminate non-specific bacterial 16S rDNA contamination, the cloned products of bacterial 16S rDNA from gill RLO were screened by the probes of bacterial 16S rDNA amplified from the digestive gland RLO. Finally, the five strongest hybridized dots were picked out and sequenced. The RLO's 16S rDNA sequence was reconfirmed and the pathogen was found only in epithelia cells by *in situ* hybridization (ISH) using specific probes. Sequence alignment and phylogenetic analysis indicated the RLO bacterium found in oyster *C. rivularis* Gould was most similar to *Piscirickettsia salmonis*, and might be classified into the family of gamma proteobacteria.

ABBREVIATIONS

RLP: Rickettsia-Like Prokaryote; RLOs: Rickettsia-Like Organisms; ISH: *in situ* Hybridization; PBS: Phosphate-Buffered Saline; min: minutes; h: hours; TEM: Transmission Electron Microscopy; s: seconds; PCR: Polymerase Chain Reaction; SSPE: Saline Sodium Phosphate Ethylenediaminetetraacetic Acid; AP: Alkaline Phosphatase; DIG: Digoxin

INTRODUCTION

The oyster, *Crassostrea rivularis* Gould, also known as *Crassostrea ariakensis*, is one of the most economically important cultured species in southeastern China, especially in the Guangxi, Guangdong and Fujian provinces. With the large expansion of culturing, mass mortalities have occurred persistently and caused great economic loss since 1992 in the Guangdong province of China. Recent studies suggested oyster culture suffered from severe mortality caused by the pathogen Rickettsia-like organism (RLO) [1,2].

Rickettsias are Gram-negative bacteria, generally described as obligate intracellular pathogens, that have been reported in various fishes, crustaceans [3-15], and mollusks [1,16-20]. Since the first report by Harshbarger et al. in *Mercenaria mercenaria*, 1977 [21], many mollusk species have been reported to be infected with RLO, causing mortality and dramatic economic losses, such as the scallop, *Pecten maximus* [22]; the oyster *Crassostrea virginia* and the hard clam *M. mercenaria* [23]; the pearl oyster *Pinctada fucata* and *Pinctada maximum* [18,24]; the oyster *C. rivularis* [1]; the abalone *Haliotis rufescens* [25]; and the scallop *Chlamys farreri* (unpublished results). Although RLOs have been recognized as an important pathogen to aquatic organisms, studies have been carried out mostly on a morphological and pathological level, while few studies have identified them on the molecular level.

In this paper, the RLO was purified from the infected oyster *C. ariakensis,* this bacterium might be classified as a member of gamma-proteobacteria by using 16S rDNA analysis and it is distantly related to *Piscirickettsia salmonis* by phylogenetic analysis. *In situ*-hybridization suggested that the pathogen is localized in oyster epithelia cells.

MATERIALS AND METHODS

Sample and processing

The oyster, *C. rivularis* Gould, 2-3 years old, were collected from Hailing Bay in Yang Xi county, GuangDong province, China, in October 2004 when oyster deaths occurred in the field. The dying oysters were picked out, the bodies cross-sectioned

into 5mm thick piece just above the ventricle, and fixed in 4% paraformaldehyde for pathogenic observation. The residual body was stored at -80 until used for RLO purification.

RLO purification

Infected gills and digestive glands were used for RLO purification using renografin density gradient centrifugation [26,27] as described previously with some modifications. Briefly, infected tissues were homogenized in phosphate-buffered saline (PBS, pH7.4: Na_2HPO_4, 53.9mM; KH_2PO_4, 12.8mM; NaCl, 72.6mM) and centrifuged at 11000×g for 40 minutes (min) at 4 to remove the fat, then, the pellets were re-suspended in PBS, centrifuged at 700×g for 20 min at 4 and 1100×g for 10 min to remove cell debris. Subsequently, supernatant fluids were collected and re-centrifuged at 11000×g for 40 min. The pellets were then used for density gradient centrifugation after being re-homogenized. About 300mg of the re-homogenized pellets were laid on the top layer of discontinuous renografin gradients (15%, 20%, 25%, 30%, 35% v/v from top to bottom in turn, each layer with about 5.5ml volume and 1.5 cm in depth) and centrifuged at 90,000×*g* for 2 hours (h) at 4 (Sorvall S80). The particles concentrated at the density interfaces of 20%-25% and 25%-30% were collected, diluted with 5 volumes of PBS, and re-centrifuged at 15000×*g* for 40 min. Finally, the pellets were diluted to a suitable suspension and stained with Uranyl Acetate and observed using JEOL transmission electron microscopes (TEM).

RLO DNA extraction and 16S rDNA PCR amplification

About 80mg granules purified from gills were used in DNA extraction, as well as granules purified from digestive glands. DNA was extracted according to the methods of Kellner-Cousin [28]. Briefly, purified RLOs were resuspended in TE buffer (Tris-HCl 10 mM, EDTA 1mM, pH 8.0) and incubated for 20 min at 37 with lysozyme (1mg/ml). Then, sodium dodecyl sulfate and proteinase K were added to a final concentration of 0.5% and 100µg/ml respectively and the suspension was incubated at 55 for 3 h. Samples were extracted with phenol-chloroform (twice) and chloroform (once). Nucleic acids were precipitated with 100% ethanol, washed with 70% ethanol (twice), air-dried and dissolved in sterile distilled water. The universal bacterial PCR primers were derived from the highly conserved bacterial 16S region. The forward primer sequence is 5'-gcttaacacatgcaagtcg-3' (*Escherichia coli* 16S rDNA positions 39-57), the reverse primer sequence is 5'- actaccgattccgacttca-3' (*E. coli* 16S rDNA positions 1322-1344). PCR was performed with 25µl reaction mixtures containing 1µl template DNA, 2.5µl 10×PCR buffer within Mg^{2+} (TaKaRa, Dalian, China), 1µl of 10mM each dNTPs, 0.8U Taq polymerase (TaKaRa), and 0.5µl each of 25mM universal bacterial 16S rDNA primers. The mixture was denatured at 94 for 2 min before amplification. The amplification profile consisted of 30 seconds (s) at 94, 30 s at 56 and 90 s at 72 cycled 30 times, with an additional 5 min at 72 following the final cycle using Thermal PX2 PCR amplifier (Thermal Ltd.). The PCR products were determined using 1.5% agarose gel electrophoresis and ethidium bromide staining. Expected PCR products (size ~1300bp) were collected from the agarose gel and cloned into a PMD-18T vector (Takara Inc.).

Eliminating unwanted bacterial contamination

To eliminate unwanted bacterial 16S contamination, the 16S fragment amplified from the oyster gill was screened using the probes from the fragments of digestive glands. Sixty-six of 16S fragments from the gill RLO were screened by probes from the digestive glands. The probes were labeled with biotin according to the instructions provided in DIG HIGH prime DNA labeling and Detection starter kit (Roche). The result was recorded by X-ray film (Koda).

Molecular phylogenetic analysis

The nucleotide sequences of the RLO 16S rDNA DQ123914.1 and DQ118733.1 have been blasted within the RDP_SeqDescByOTU_tax_outline.txt. A total of 287 sequences from separate infected oysters with identity >=90% were selected. Then those sequences were further selected by OTU number, only those with OTU number >= 3 and with the best score were selected. The phylogenetic tree was made by MEGA 3.1 [29], with the Neighbor-Joining (NJ) Method.

In situ hybridization

Infected tissues were dissected and fixed with 4% paraformaldehyde in PBS (pH 7.4), dehydrated in an ascending ethanol series (50%, 70%, 80%, 90%, 95%, 100% v/v) for two times, followed by three washes in xylene, embedded in paraffin, sectioned at 5µm, mounted on APES-coated slides, and baked at 55 for 4 h. Then the section was deparaffinized in xylene and re-hydrated in a reverse ethanol series. The rehydrated slides used for *in situ* hybridization were digested in 30 µg ml^{-1} Proteinase K solution (pH 8.0) under 37 for about 20 min before hybridization. Slides were then neutralized in 2× saline sodium phosphate ethylenediaminetetraacetic acid (2×SSPE, pH 7.4) buffer for 10 min, and treated with prehybridization solution (0.5mg ml^{-1} salmon sperm DNA, 5×Denhardt's reagent, and 2×SSPE) in a moist chamber at 42 for 30 min. After the prehybridization, 100ng of the positive probes and the negative control (NC) probes were separately added onto two different slides and hybridized at 42 for about 16 h in the moist chamber. After hybridization, unbound probes were washed off with 2×SSPE, 1×SSPE, and 0.5×SSPE at 42. Finally, the slides were added into AP-conjugated (Alkaline phosphatase) anti-digoxin (DIG) antibody and detected by NBT/BCIP indication reagent.

The specificity of assumed probe sequences chosen from highly variable regions of the RLO 16S rDNA sequence (GenBank accession number DQ123914) were confirmed by retrieving the sequence within the databases DDBJ-EMBL-GenBank using the BLASTn service. The probe was monolabeled with DIG and the sequence is DIG-5'-aggtagtctgtgaataatgggctactg-3' at the position from 402-427 in DQ123914. A NC probe was also implemented to monitor the experimental conditions. The NC probe sequence was DIG-5'-gggatgtaggttaataccttgcatctt-3' with a 12 nucleotides mismatch to the RLO sequence, but less than 12 nucleotides mismatch to other bacterial 16S rDNA sequences by NCBI BLAST database.

Theory/calculation

In this study, it is reported that the RLO bacterium found

in oyster *Crassostrea rivularis* Gould was most similar to *Piscirickettsia salmonis* and could be classified into a new family of gamma proteobacteria but it necessary to develop more studies. It can provide a theoretical basis for analysis of the death of the oyster *C. rivularis* Gould and preventing or controlling the RLO.

RESULTS

RLO purification

The purified RLOs mainly existed at band 20%-25% and 25%-30%, which were coincident with the report by Li and Wu (Li and Wu, 2004). Under TEM observation, the purified products displayed particles containing not only the RLOs, but also some cell debris and contaminated bacteria (Figure 1).

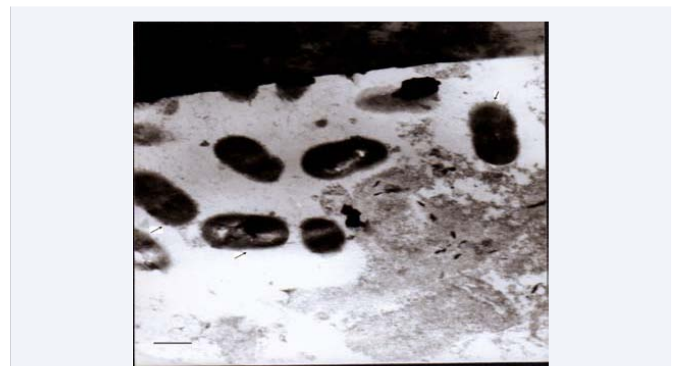

Figure 1 Electron micrograph of RLOs (arrows) from the oyster gill epithelial cell, with 2% uranyl acetate.

Eliminating bacterial contamination and molecular phylogenetic analysis

In density gradient centrifugation, only granules with the same density should be concentrated in the same layer, while particles with differing densities should be eliminated. Among the 66 dots in cross hybrid, the 5 most strongly reactive dots were picked out and sequenced (Figure 2). After being retrieved from the GenBank database, the sequences were found to belong to three known bacteria (*Vibrio ordalii*, *Pseudomonas putida*, *Serratia marcescens*) and one unknown bacterium. By comparing the morphological characters of the three known bacteria described in Bergey's manual of systematic bacteriology (second edition, 2004) with that of the RLO found in oyster, the morphological character could not fit well. So the unknown sequence was assumed to be the RLO sequence. 48 sequences (Showed in Table 1) were finally selected and used in alignment with two RLO sequences using the clustalX 1.83 software. By sequence alignment analysis, the sequences which are similar to the RLO 16S sequence all belong to the Gamma proteobacteria. Thus it can be inferred that the RLO is a type of gamma proteobactium. By phylogenetic analysis, the sequence was most similar to the 16S sequence of *Piscirickettsia salmonis* (Figure 3), but the similarity was not high enough to classify these two bacteria into one family. In this study, 1304 sequences of 16S rDNA of RLO were obtained as follows:

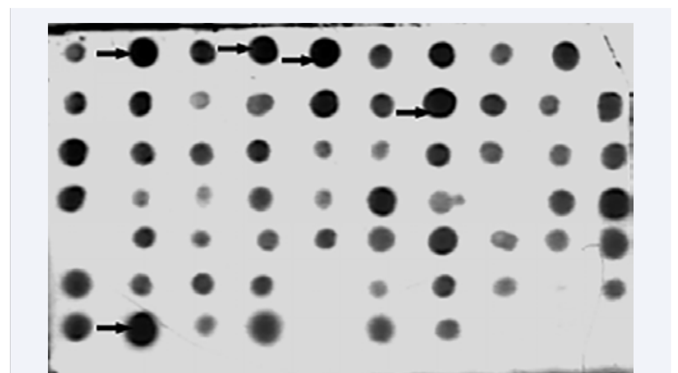

Figure 2 Sixty-six of 16S rDNA amplified fragment inserted were detected by probes synthesized from digestive gland purified products. Five of the strongest hybridized dots (indicated by arrows) were picked out and sequenced.

1 gcttaacaca tgcaagtcga gcggtaacag gaagagcttg ctctttgctg acgagcggcg

61 gacgggtgag taacgcgtag gaatctgact gtaagagggg gatagcccgg agaaatccgg

121 attaataccg cataacacct aagggtaaaa agaggcactt gtgctactgc ttacagagga

181 gcctgcgttg gattagctag ttggtggggt aaaggcttac caaggcgacg atccatagct

241 gctctgagag gatgatcagc cacactggga ctgagacacg gcccagactc ctacgggagg

301 cagcagtggg gaatattgca caatggggga aaccctgatg cagccatgcc gcgtgtgtga

361 agaaggcttt cgggttgtaa agcactttca gtggtgagga aaggtagtct gtgaataatg

421 ggctactgtg acgttagcca cagaagaagg accggcaaac tccgtgccag cagccgcggt

481 aatacggagg gtccgagcgt taatcggaat tactgggcgt aaagggtgcg taggcggata

541 tgtaagtggg tagtgaaaga cctgggctca acctgggagg tgctatccaa actgcataac

601 tagagtacag aagaggagtg tggaatttcc tgtgtagcgg tgaaatgcgt agatatagga

661 aggaacaccg gtggcgaagg cggcactctg gtctgatact gacgctgagg tacgaaagcg

721 tggggagcaa acaggattag ataccctggt agtccacgct gtaaacgctg tctactagtc

781 gttgggaact taaaagtttt tagtggcgaa gcaaacgcgc taagtagacc gcctggggag

841 tacggccgca aggttaaaac tcaaatgaat tgacgggggc ccgcacaagc ggtggagcat

901 gtggtttaat tcgacgcaac gcgaagaacc ttacctggtt ttgacatcct cggaatggcg

961 aagagatttg ccagtgcctt cgggagccga gtgacaggtg ctgcatggct gtcgtcagct

1021 cgtgtcgtga gatgttgggt taagtcccgc aacgagcgca acccttatcc

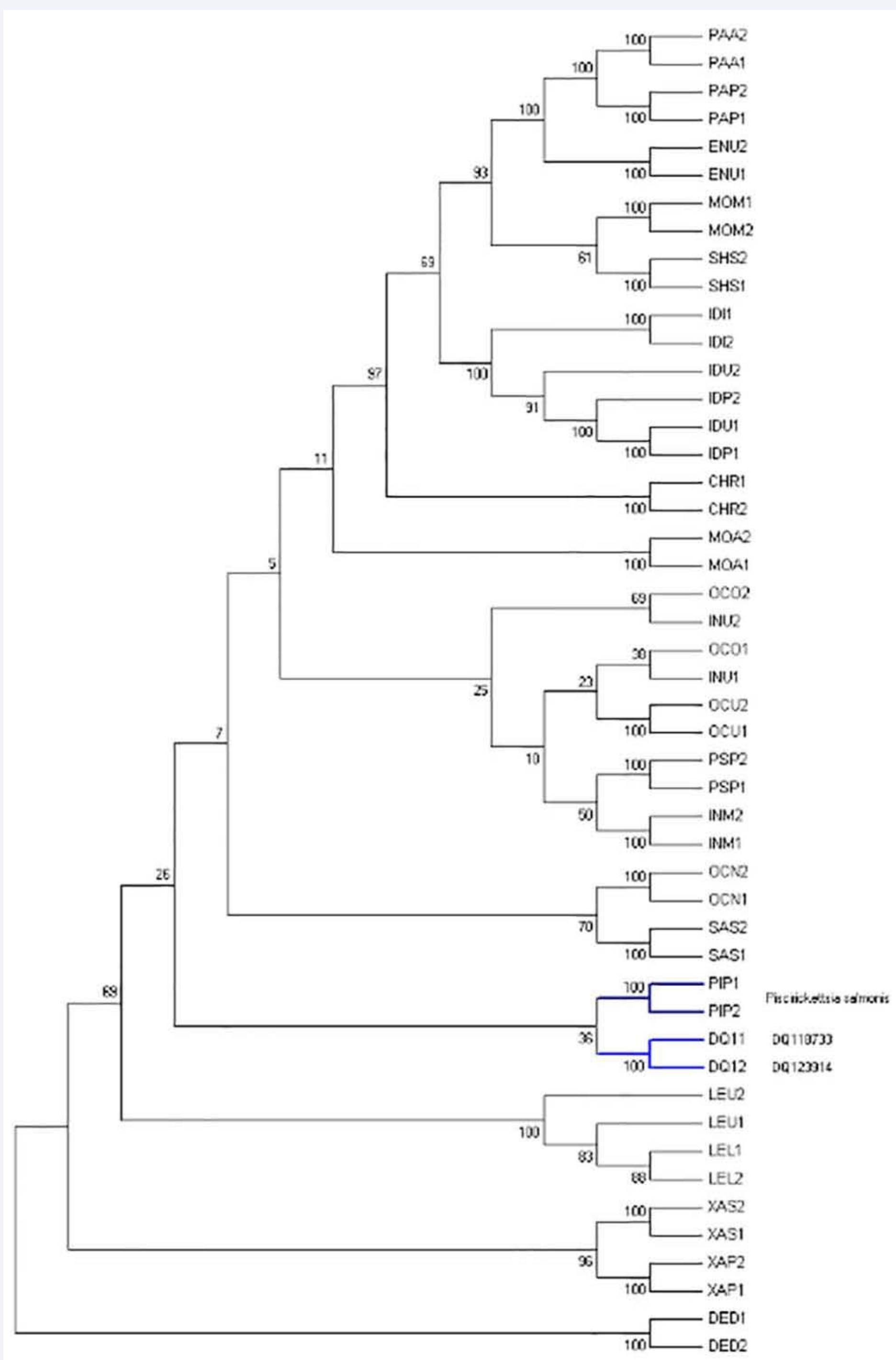

Figure 3 Phylogenetic analysis shows that the most similar sequence to RLO 16S rDNA is the 16S sequence of *Piscirickettsia Salmonis*.

ttatttgcca

1081 gcatgtaaag atgggaactc taaggagact gccggtgaca agccggagga aggtggggac

1141 gacgtcaagt catcatggcc cttacgacca gggctacaca cgtgctacaa tggggcgtac

1201 aaagggaagc gaagcggtga cgtggagcca aacctatcaa agcgcctcgt agtccggatc

1261 gcagtctgca actcgactgc gtgaagtcgg aatcggtagt aatc

In situ hybridization

The specificity of the bacterium probe was identified by comparing the hybridization signal produced using the RLO specific probe and non-specific probe under the same condition. The bacteria were recognized clearly when hybridized with the RLO specific probes, while no signals presented when hybridized with negative control probes (Figure 4A,4B). Meanwhile, ISH

Table 1: Sequence name used in alignment and phylogenetic analysis.			
AccessionId	**AlignI**	**taxonomy**	**DESC**
AJ704694.1	DED1	Bacteria; ProteoBacteria; DeltaproteoBacteria; Desulfobacterales; Desulfobacteraceae; Desulfobacula	AJ704694.1 marine sediment clone HMMVBeg-47
AY177803.1	DED2	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobacteraceae; Desulfobacula	AY177803.1 Antarctic sediment clone SB4_98
AY465366.1	PAA1	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Actinobacillus	AY465366.1 Actinobacillus rossii str. JF2073
AY465368.1	PAA2	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Actinobacillus	AY465368.1 Actinobacillus rossii str. P.. 12
AF139582.1	PAP1	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Pasteurella	AF139582.1 Pasteurella aerogenes str. JF2039
AY465358.1	PAP2	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Pasteurella	AY465358.1 Pasteurella aerogenes str. 4-97; JF2420
EU341176.1	MOA1	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter	EU341176.1 Evaluation Rapid Technologies Estimate Microbial Burden and Commercial Airline Cabin Air commercial aircraft cabin air clone AV_4R-S-C13
DQ834360.1	MOA2	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter	DQ834360.1 Acinetobacter sp. str. BYC2
AY486375.1	PSP1	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	AY486375.1 Pseudomonas sp. str. AU2390
AY486377.1	PSP2	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	AY486377.1 Pseudomonas sp. str. AU4899
AY498633.1	PIP1	Bacteria;Proteobacteria;Gammaproteobacteria;Thiotrichales;Piscir ickettsiaceae;Piscirickettsia	AY498633.1 Piscirickettsia salmonis IRE-91A
AY498636.1	PIP2	Bacteria; Proteobacteria; Gammaproteobacteria; Thiotrichales; Piscirickettsiaceae; Piscirickettsia	AY498636.1 Piscirickettsia salmonis SCO-95A
EU250940.1	XAP1	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Pseudoxanthomonas	EU250940.1 Pseudoxanthomonas sp. str. NFC7-F12
EU177791.1	XAP2	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Pseudoxanthomonas	EU177791.1 Pseudoxanthomonas sp. str. Ca7-1J03
AB218877.1	XAS1	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Schineria	AB218877.1 Koukoulia aurantiaca str. IAM 15137
EF608545.1	XAS2	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Schineria	EF608545.1 predatory Poecilus chalcites their response lab rearing and antibiotic treatment digestive tract ground beetle clone PCD-40
DQ337031.1	IDI1	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Idiomarinaceae; Idiomarina	DQ337031.1 subsurface water clone EV818EB5CPSAJJ20
DQ235576.1	IDI2	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Idiomarinaceae; Idiomarina	DQ235576.1 biofilm population water pipeline biofilms steel pipelines Gulf Mexico clone 100
DQ899878.1	IDP1	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Idiomarinaceae; Pseudidiomarina	DQ899878.1 structure receiving long-term augmentations chromium contaminated wastes landfill sediments Gorwa industrial estate Cr(VI) contamination clone G1DMC-174
DQ234155.2	IDP2	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Idiomarinaceae; Pseudidiomarina	DQ234155.2 determined library mangrove clone DS071
DQ899898.1	IDU1	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Idiomarinaceae; unclassified_Idiomarinaceae	DQ899898.1 structure receiving long-term augmentations chromium contaminated wastes landfill sediments Gorwa industrial estate Cr(VI) contamination clone G2DMC-116
AY345388.1	IDU2	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Idiomarinaceae; unclassified_Idiomarinaceae	AY345388.1 Loihi submarine volcano isolate str. JB11
AY532642.1	INU1	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Incertae; unclassified_Incertae	AY532642.1 Bugula simplex symbiont
DQ351747.1	INU2	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Incertae;unclassified_Incertae	DQ351747.1 Microbial Adherent Sediment Particles Heavy Metal Contaminated North Sea Surface Sediments marine sediments clone Belgica2005/10-120-16
EU399549.1	INM1	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Incertae; Marinobacter; Unclassified	EU399549.1 Marinobacter sp. str. BR-13

DQ015835.1	INM2	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Incertae; Marinobacter; Unclassified	DQ015835.1 Antarctic lake water clone ELB19-223
AY394860.1	MOM1	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Moritellaceae; Moritella	AY394860.1 Moritella sp. str. 762 G
AY380781.1	MOM2	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Moritellaceae; Moritella	AY380781.1 Moritella viscosa str. 2002/09/1069-1
AB003190.1	SHS1	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Shewanellaceae; Shewanella	AB003190.1 Shewanella sp. str. SC2A
AF132875.1	SHS2	Bacteria;Proteobacteria; Gammaproteobacteria; Alteromonadales; Shewanellaceae; Shewanella	AF132875.1 Shewanella frigidimarina str. ACAM 533
AY241547.1	CHR1	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae; Rheinheimera	AY241547.1 aggregates water column German Wadden Sea part North Sea isolate str. HP1 HP1
AJ441080.1	CHR2	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae; Rheinheimera	AJ441080.1 Rheinheimera baltica str. OSBAC1
AY136145.1	ENU1	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; unclassified_Enterobacteriaceae	AY136145.1 Cacopsylla pyri symbiont
AY136162.1	ENU2	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; unclassified_Enterobacteriaceae	AY136162.1 Uroleucon nigrotuberculatum symbiont
EU134750.1	LEL1	Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales; Legionellaceae; Legionella	EU134750.1 evolutionary between rare and abundant members communty tallgrass soil undisturbed mixed grass prairie preserve clone FFCH14647
EU250248.1	LEL2	Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales; Legionellaceae; Legionella	EU250248.1 acid mine drainage clone GXDC-34
AY536230.1	LEU1	Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales; Legionellaceae; unclassified_Legionellaceae	AY536230.1 host gut clone LAgut--P18
EU134792.1	LEU2	Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales; Legionellaceae; unclassified_Legionellaceae	EU134792.1 evolutionary between rare and abundant members communty tallgrass soil undisturbed mixed grass prairie preserve clone FFCH4066
EF202341.1	OCU1	Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales; Oceanospirillaceae; unclassified_Oceanospirillaceae	EF202341.1 Matching and function marine one cell time Boothbay Harbor 1m depth clone MS024-3A
EF516584.1	OCU2	Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales; Oceanospirillaceae; unclassified_Oceanospirillaceae	EF516584.1 grassland soil clone FCPP727
AY922202.1	OCN1	Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales; Oceanospirillaceae; Nitrincola	AY922202.1 whalefall clone 131636
AY567473.1	OCN2	Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales; Oceanospirillaceae; Nitrincola	AY567473.1 Nitrumincola lacisaponis str. 4CA
AJ315984.1	SAS1	Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales; Saccharospirillaceae; Saccharospirillum	AJ315984.1 Arhodomonas sp. str. EL-201
AJ315983.1	SAS2	Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales; Saccharospirillaceae; Saccharospirillum	AJ315983.1 Saccharospirillum impatiens str. EL-105 = DSM 12546
DQ123914.1	DQ12	Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales; unclassified_Oceanospirillales	DQ123914.1 Oceanrickettsia ariakensis
DQ334644.1	OCO1	Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales; unclassified_Oceanospirillales	DQ334644.1 Impact metals on sediments heavy metal polluted marine sediment clone HB2-9-21
AY344367.1	OCO2	Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales; unclassified_Oceanospirillales	AY344367.1 Lake Kauhako 30 m clone K2-30-25

positive signals were often found in the epithelia cells of gill and mantle, and occasionally found in digestive gland cells, but were not observed in hemocytes, muscles or pericardium. The morphology of RLO inclusions were also identified by HE staining method (Figure 5A,5B).

DISCUSSION

The Oyster, *Crassostrea rivularis* Gould (also known as *Crassostrea ariakensis*), mainly distributed in estuary areas, is a major farmed mollusk species in the Hailing Bay area of Guang Dong province, China. Since 1992, farmed oysters have suffered from high mortality during winter and spring of every year. An intracellular rickettsia-like prokaryotic parasite was tentatively identified to be the causative agent using histological and ultra structural characteristics. The morphology of individual RLOs consist mostly of a round shape, with occasional short and rod-shaped morphologies, ranging from approximately 0.58 to 1.20 µm in size and with a smooth trilaminar cell wall [1]. Some observations reported that RLOs could form basophilic inclusions [17,30] under H&E staining, while other studies reported that they could form eosinophilic inclusions [1,24], or even two types of inclusions can be observed in the same mollusk [23,31,32]. In

Figure 4 Positive signals were detected using specific probes under *in situ* hybridization (ISH) staining method. (A) Arrows indicated the positive RLO inclusions found in oyster epithelia. Bar=20um. (B) No signals were detected using none specific probes ISH under the same conditions.

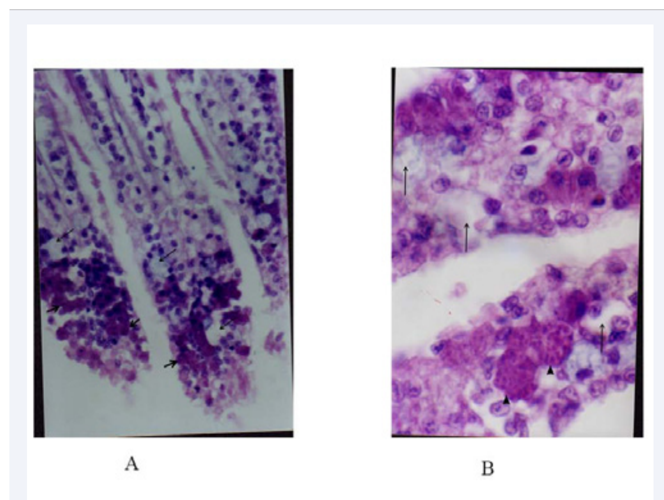

Figure 5 (A) Numerous intracytoplasmic inclusions (Bold arrows) of RLO parasitizing the epithelial cells of gill of Crassostrea rivularis Gould by HE staining method. Note the lytic hollows (small arrows) of the gill epithelial cells by RLO infection (H&E, 500×). (B) Inclusions (arrowheads) in the gill epithelial cells. Note the lytic hollows (arrows) of the gill epithelial cells by RLO infection (H&E, 1250×).

this paper, the pathogen was ound only in epithelia cells by *in situ* hybridization.

The RLOs pathogenicity were also different, some studies revealed that RLOs could cause diseases and are responsible for the death of marine mollusks [17,22], whereas other reports suggested that RLOs only exhibit benign infection [4,30,33,34]. By now, 16S sequence analysis has been widely used in bacteria classification as it has both a highly conserved region that can be used in alignment between dissimilar microorganism and a variable region that can allow sequences to be distinguished. In this study, sequence alignment analysis and phylogenetic analysis indicate the RLOs found in oyster is a new kind of bacterium and can be classified into the gamma subfamily, proteobacteria. It is most similar to *Piscirickettsia salmonis*, but the similarity is not high enough to support classification of these two kinds of bacteria into the same family. It is representative of a new family in the order of Rickettsiales and we propose the name *Oceanrickettisa ariakensis* to *C. ariakensis* RLP. "Oceanrickettisia" is pertaining to the RLO bacterium found in the sea and "ariakensis" is pertaining to this bacterium found in oyster "*Crassostrea ariakensis*".

CONCLUSION

In conclusion, the final assumed RLOs 16S rDNA sequence was reconfirmed and the pathogen was found only in epithelia cells by *in situ* hybridization (ISH) using the specific probes. Sequence alignment and phylogenetic analysis indicated the assumed RLO bacterium found in oyster *C. rivularis* Gould was most similar to *Piscirickettsia salmonis*, and could be classified into a family of gamma proteobacteria.

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