Aquimarina hainanensis sp. nov., isolated from diseased Pacific white shrimp *Litopenaeus vannamei* larvae

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One novel Gram-stain-negative, long rod-shaped, non-spore-forming, non-motile, non-flagellated and strictly aerobic strain, designated M124^T, was isolated from diseased Pacific white shrimp *Litopenaeus vannamei* larvae. Growth occurred at 16–37 °C (optimum 28 °C), in the presence of 2–5 % (w/v) NaCl (optimum 3 %) and at pH 7–8 (optimum pH 7). Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain M124^T belonged to the genus *Aquimarina* and showed highest sequence similarity to *Aquimarina penaei* P3-1^T (96.4 %). The dominant fatty acids of the isolate were iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH. The major polar lipids comprised phosphatidylethanolamine, one unknown aminolipid, three unknown phospholipids, two unknown glycolipids and one unknown polar lipid. The major respiratory quinone was menaquinone 6 (MK-6). The DNA G+C content of strain M124^T was 33.7 mol%. Based on the polyphasic analyses in this study, strain M124^T is considered to represent a novel species of the genus *Aquimarina*, for which the name *Aquimarina hainanensis* sp. nov. is proposed. The type strain is M124^T (=KCTC 42423^T=MCCC 1K00498^T).

The genus *Aquimarina*, belonging to the family *Flavobacteriaceae*, was first proposed by Nedashkovskaya *et al.* (2005). All species in the genus *Aquimarina* are Gram-negative, rod-shaped, heterotrophic and strictly aerobic. Most contain iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH as the major fatty acids, and menaquinone 6 (MK-6) is the major respiratory quinone of all species in the genus. The range of DNA G+C content is 31–38 mol%. At the time of writing, 17 species of the genus *Aquimarina* have been recognized or reclassified from other genera. All were isolated from the marine environment: seven from seawater, *Aquimarina muelleri* (Nedashkovskaya *et al.*, 2005), *A. latercula* (Lewin 1969; Nedashkovskaya *et al.*, 2006), *A. addita* (Yi & Chun, 2011), *A. longa* (Yu *et al.*, 2013), *A. megaterium* (Yu *et al.*, 2014), *A. atlantica* (Li *et al.*, 2014a) and *A. pacifica* (Zhang *et al.*, 2014); two from sediment, *A. brevivitae* (Yoon *et al.*, 2006; Nedashkovskaya *et al.*, 2006) and *A. macrocephali* (Miyazaki *et al.*, 2010); two from mussels, *A. mytili* (Park *et al.*, 2012) and *A. gracilis* (Park *et al.*, 2013); two from marine sponges, *A. spongiae* (Yoon *et al.*, 2011) and *A. amphilecti* (Kennedy *et al.*, 2014); one from sea urchin, *A. intermedia* (Nedashkovskaya *et al.*, 2006); one from the surface of the marine red alga *Porphyra haitanensis*, *A. agarilytica* (Lin *et al.*, 2012); one from saltpan, *A. salinaria* (Chen *et al.*, 2012); and one from Pacific white shrimp (*Penaeus vannamei*), *A. penaei* (Li *et al.*, 2014b). One novel bacterial strain, designated M124^T, was isolated from diseased Pacific white shrimp *Litopenaeus vannamei* larvae. The aim of the present study was to determine the exact taxonomic position of the isolate using a polyphasic approach.

Ten diseased Pacific white shrimp larvae obtained from a shrimp hatchery in Hainan, China, were sprayed with 75 % ethanol to remove surface microbes, then washed with sterile water immediately and homogenized with 1 ml of 0.85 % (w/v) sterile NaCl solution. The homogenate was serially diluted as required and 0.1 ml of each dilution was spread on marine agar 2216 (MA; Becton Dickinson) and incubated at 28 °C. Strain M124^T, which formed irregular, orange, convex and opaque colonies on MA after 2–3 days, was picked and purified by streaking three times on MA. Cultures were maintained on MA plates at 28 °C and stocks were preserved in sterile 0.85 % (w/v) saline supplemented with 15 % (v/v) glycerol at −80 °C. *A. muelleri* LMG 22569^T, *A. macrocephali* JCM 15542^T, *A. penaei* P3-1^T and *A. pacifica* SW150^T were used

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Aquimarina hainanensis* M124^T is KP200684.

Four supplementary figures are available with the online version of this paper.

as reference strains, which were cultured at the same conditions as strain M124^T [MA/marine broth 2216 (MB), 28 °C], unless otherwise specified.

Gram-staining and flagellum staining were investigated using standard methods (Beveridge *et al.*, 2007). Cell morphology was observed by transmission electron microscopy (JEM-1200EX; JEOL) after cells had been negatively stained with 1 % (w/v) phosphotungstic acid. The presence of gliding motility and the production of flexirubin-type pigments were investigated using the methods described by Bernardet *et al.* (2002) after cultures were grown on MA at 28 °C for 1 day. To test for anaerobic growth, bacterial strains were cultured on MA with resazurin (0.02 %, w/v) as an indicator of anaerobic conditions in an anaerobic jar filled with nitrogen and a packet of AneroPack-Anaero (Mitsubishi Gas Chemical) at 28 °C for 1 month. The growth temperature range was determined on MA by incubating cultures at 10–42 °C (10, 16, 20, 24, 28, 32, 37 and 42 °C) for 5 days, and at 0 and 4 °C on MA for 30 days. Salinity and pH ranges for growth were investigated in 96-well microplates by measuring the optical densities (wavelength 590 nm) of cultures. In the salinity experiment, distilled water was used for preparation of synthetic marine ZoBell broth (per litre: 5 g Bacto peptone, 1 g yeast extract and 0.01 g $FePO_4$); NaCl concentration was adjusted to 0–15 % (w/v, at intervals of 1 %). The pH range for growth was determined at pH 2–10 (at 1 pH unit intervals) using the following buffer systems: H_3PO_4/KH_2PO_4 (pH 2), NaAc/HAc (pH 3–6), KH_2PO_4/NaOH (pH 7–8) and Na_2CO_3/$NaHCO_3$ (pH 9–10). The physiological and biochemical characteristics of strain M124^T and the four reference strains were investigated according to standard protocols (Tindall *et al.*, 2007), including catalase and oxidase activities, and hydrolysis of casein, starch, gelatin, CM-cellulose and Tweens 20, 40 and 80. DNase activity was examined by using DNase agar (Qingdao Hope Bio-technology) according to the manufacturer's instructions. Degradation of chitin was examined on chitin agar with sterile seawater (Hsu & Lockwood, 1975). Activities of constitutive enzymes and other physiological properties were determined after growth on MA at 28 °C for 2 days by using API 20E, API 20NE, API 50CH and API ZYM strips (bioMérieux) and Gram-negative MicroPlates kit (Biolog) according to the manufacturers' instructions except that artificial seawater was used to prepare the inocula. The detailed morphological, physiological and biochemical characteristics of strain

Table 1. Differential characteristics between strain M124^T and other members of the genus *Aquimarina*

Strains: 1, M124^T; 2, *A. penaei* P3-1^T; 3, *A. pacifica* SW150^T; 4, *A. macrocephali* JCM 15542^T; 5, *A. muelleri* LMG 22569^T. All data are from this study and Zhang *et al.* (2014) except for DNA G+C content and cell size, which are from the original species descriptions. +, Positive reaction; −, negative reaction; w, weakly positive reaction.

Characteristic	1	2	3	4	5
Colony colour	Orange	Orange	Orange	Orange	Yellow–brown
Cell characteristics					
Width (μm)	0.5	0.2–0.4	0.2	0.4–0.6	0.3–0.5
Length (μm)	3.0–5.7	4–10	2.5–4.5	3.0–25.5	5–7
Range for growth					
Temperature (°C)	16–37	15–37	8–30	8–30	4–34
NaCl (%)	2–5	2–7	2–4	1–4	1–8
Nitrate reduction	+	−	−	−	−
Hydrolysis of:					
Casein	−	+	−	+	+
DNA	+	+	+	−	−*
Starch	−	+	−	+	+
Tween 20	+	−	w	−	+
Tween 40	+	−	−	−	+
Tween 80	+	−	w	−	+
Utilization of:					
D-Mannose	+	−	−	+	−
Leucine	−	+	−	−	+
API ZYM results					
α-Chymotrypsin	−	+	−	w	w
α-Glucosidase	−	+	+	w	−
Naphthol-AS-BI-phosphohydrolase	+	+	+	w	w
Trypsin	+	+	w	−	w
DNA G+C content (mol%)	33.7	33.3	33.8	33.1	31–33

*Data different from the original reference (Nedashkovskaya *et al.*, 2005).

M124^T are given in Table 1, Fig. S1 (available in the online Supplementary Material) and the species description.

For cellular fatty acid analysis, cells of strain M124^T and the four reference strains were obtained from MA plates incubated at 28 °C until they reached the late logarithmic stage of growth. Fatty acids in whole cells were saponified, methylated and extracted according to the standard protocol of MIDI (Sherlock Microbial Identification System, version 6.0) (Sasser, 1990) and identified via the TSBA 6.0 database of the Microbial Identification System (Sasser 1990). For analyses of respiratory quinones and polar lipids, cells were harvested from MB after shaking at 28 °C for 4–5 days and freeze-dried. The major respiratory quinones were extracted with chloroform/methanol (2 : 1, v/v), separated by TLC and identified by HPLC. Polar lipids were extracted according to Minnikin *et al.* (1984), and separated by two-dimensional TLC on silica gel 60 F254 plates (Merck) using chloroform/methanol/water (65 : 25 : 4, by vol.) for the first dimension and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.) for the second dimension (Collins & Shah, 1984). Extracted lipids were identified by spraying the plates with appropriate detection reagents (Komagata & Suzuki, 1987). In brief, 5 % ethanolic molybdophosphoric acid was used to detect total lipids. Ninhydrin spray was applied to determine most of the lipids with free amino groups, while spraying the plate with the lipid phosphate reagent of Dittmer and Lester after the ninhydrin spray revealed the presence of phospholipids. Spraying with α-naphthol-sulphuric acid determined the presence of glycolipids. Quaternary nitrogen compounds, such as phosphatidylcholine, appeared pinkish orange after spraying with Dragendorff's reagent. The periodate–Schiff reagent spray was applied to reveal lipids with a terminal CH_2OH group, such as glycerol. The DNA G+C content was determined according to the methods described by Mesbah & Whitman (1989) using reversed-phase HPLC.

The cellular fatty acid profiles of strain M124^T and the four reference strains are given in Table 2. The dominant fatty acids of strain M124^T were iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH, which were similar to the four reference strains, but there were some differences in the fatty acid composition and proportion of some components. Strain M124^T contained less iso-$C_{15:0}$ than *A. penaei* P3-1^T, more iso-$C_{15:0}$ and less iso-$C_{15:1}$ G than *A. pacifica* SW150^T, less $C_{16:0}$ and more iso-$C_{17:0}$ 3-OH than *A. macrocephali* JCM 15542^T, and less 10-methyl $C_{16:0}$ and/or iso-$C_{17:1}\omega9c$ (summed feature 9) than *A. muelleri* LMG 22569^T. The polar lipid profile of strain M124^T comprised phosphatidylethanolamine, one unknown aminolipid, three unknown phospholipids, two unknown glycolipids and one unknown polar lipid (Fig. S2). Phosphatidylethanolamine and glycolipids were found in all species of the genus *Aquimarina*, whereas the other components were variously present in the different species. In accordance with other members of the family *Flavobacteriaceae*, strain M124^T contained MK-6 as the major respiratory quinone. The DNA G+C content of strain M124^T was 33.7 mol%, which was within the range reported for the genus *Aquimarina* (31–38 mol%).

Table 2. Cellular fatty acid compositions of strain M124^T and related species of the genus *Aquimarina*

Strains: 1, M124^T; 2, *A. penaei* P3-1^T; 3, *A. pacifica* SW150^T; 4, *A. macrocephali* JCM 15542^T; 5, *A. muelleri* LMG 22569^T. All data are from this study. TR, Trace amount (<1 %); −, not detected. Values are percentages of the total fatty acids. Fatty acids amounting to <1 % of the total in all strains are not shown. Bold type indicates major fatty acids (>10 %).

Fatty acid	1	2	3	4	5
Straight-chain					
$C_{14:0}$	TR	TR	TR	1.0	TR
$C_{16:0}$	5.8	4.7	1.3	**10.7**	5.7
$C_{18:0}$	2.0	TR	TR	3.2	2.9
Unsaturated					
$C_{15:1}\omega6c$	TR	TR	5.5	TR	TR
$C_{17:1}\omega8c$	TR	TR	2.3	TR	TR
Branched					
iso-$C_{13:0}$	TR	TR	4.5	TR	1.3
iso-$C_{15:0}$	**27.3**	**32.2**	**18.5**	**24.4**	**25.8**
iso-$C_{15:1}$ G	8.4	8.4	**11.6**	8.1	6.1
iso-$C_{16:0}$	2.0	TR	1.7	3.0	1.1
iso-$C_{16:1}$ G	TR	−	1.6	1.1	−
Hydroxy					
$C_{15:0}$ 3-OH	−	−	1.2	−	−
iso-$C_{15:0}$ 3-OH	7.1	5.2	6.7	5.2	6.3
$C_{16:0}$ 3-OH	3.4	TR	1.4	3.7	1.3
$C_{17:0}$ 3-OH	−	TR	2.0	TR	−
iso-$C_{17:0}$ 3-OH	**23.5**	**22.7**	**24.1**	**18.7**	**22.9**
Summed features*					
3	9.2	9.3	6.6	**10.1**	8.8
8	−	1.4	TR	−	−
9	5.3	5.4	5.1	5.5	**12.2**

*Summed features represent groups of two or three fatty acids that are treated together for the purpose of evaluation in the MIDI system and include both peaks with discrete equivalent chain-lengths (ECLs) as well as those where the ECLs are not reported separately (Montero-Calasanz *et al.*, 2013). Summed feature 3 contained $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$; summed feature 8 contained $C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$; summed feature 9 contained 10-methyl $C_{16:0}$ and/or iso-$C_{17:1}\omega9c$.

The genomic DNA of strain M124^T was extracted and purified using standard methods (Ausubel *et al.*, 1995). The 16S rRNA gene was amplified by PCR with two universal primers (B8F: 5′-AGAGTTTGATCCTGGCTCAG-3′; and B1510R: 5′-GGTTACCTTGTTACGACTT-3′). The PCR product was purified with a TIANgel Midi Purification kit (Tiangen Biotech), ligated into the pUCm-T vector (TaKaRa), cloned to *Escherichia coli* JM109^T and sequenced at BGI. Two nearly complete 16S rRNA gene sequences (both 1488 nt) of strain M124^T were determined in this study. Levels of 16S rRNA gene sequence similarity

between the isolate and other strains were calculated using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). The 16S rRNA gene sequences of related strains were aligned using the CLUSTAL X program (Thompson *et al.*, 1997). Phylogenetic trees based on the neighbour-joining (Fig. 1), maximum-likelihood (Fig. S3) and maximum-parsimony (Fig. S4) algorithms were reconstructed with the program MEGA version 5.0 (Tamura *et al.*, 2011). The genetic distance matrices of the first two trees were estimated by Kimura's two-state parameter model (Kimura, 1980). In each case, bootstrap values were calculated based on 1000 replicates.

Pairwise alignment of the 16S rRNA gene sequence of strain M124^T revealed highest similarity with *A. penaei* P3-1^T (96.4 %), followed by *A. pacifica* SW150^T (95.3 %), *A. macrocephali* JAMB N27^T (94.4 %) and *A. latercula* DSM 2041^T (94.3 %). The phylogenetic trees obtained with the neighbour-joining, maximum-likelihood and maximum-parsimony methods revealed that strain M124^T formed a distinct cluster with *A. penaei* P3-1^T within the genus *Aquimarina* (Figs. 1, S3 and S4). However, the relatively low sequence similarity to the type strains of recognized species in the genus *Aquimarina* (93.2–96.4 %) implied that it may represent a novel species (Stackebrandt & Goebel, 1994).

The major characteristics of strain M124^T, including cell morphology, hydrolysis of starch, citrate and aesculin, catalase and oxidase activity, DNA G+C content and major respiratory quinone, were consistent with the type species of the genus *Aquimarina*. However, some unique features distinguish strain M124^T from other recognized species in the genus *Aquimarina*, including the temperature and NaCl ranges that support growth, nitrate reduction, hydrolysis of casein and Tweens 20, 40 and 80 (Table 1), the proportion of some fatty acids (Table 2) and the profile of polar lipids. On the basis of phenotypic characteristics and phylogenetic inference, strain M124^T is considered to represent a novel species of the genus *Aquimarina*, for which the name *Aquimarina hainanensis* sp. nov. is proposed.

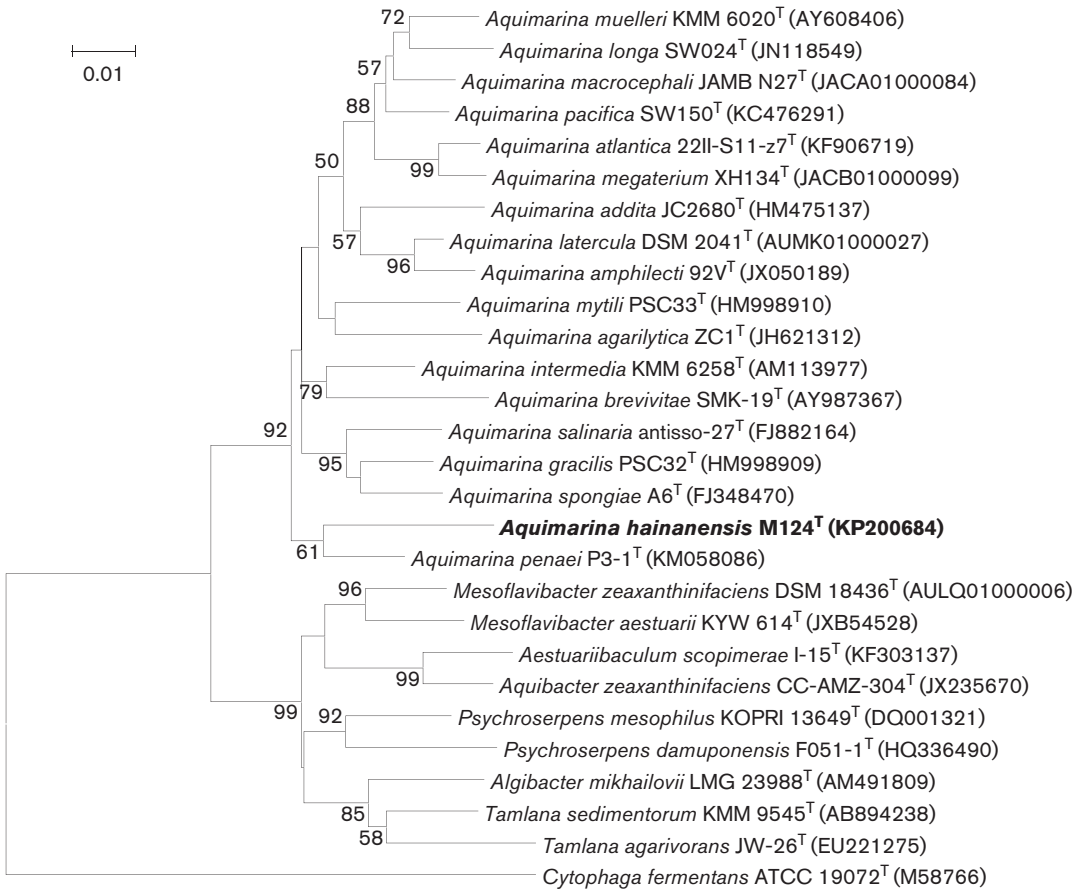

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain M124^T, the type strains of recognized *Aquimarina* species and representatives of other genera in the family *Flavobacteriaceae*. Bootstrap values ≥50 % (1000 replicates) are shown at branch nodes. *Cytophaga fermentans* ATCC 19072^T (M58766) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Description of *Aquimarina hainanensis* sp. nov.

Aquimarina hainanensis (hai.nan.en′sis. N.L. fem. adj. *hainanensis* pertaining to Hainan, a province of south China, from where the type strain was isolated).

Cells are Gram-stain-negative, strictly aerobic, long rod-shaped (approx. 3.0–5.7 μm in length and 0.5 μm in width), non-flagellated and non-motile. Flexirubin-type pigments are absent. Colonies on MA are orange, opaque, and convex with irregular margins. Growth is poor in MB. Growth occurs at 16–37 °C (optimum 28 °C), in the presence of 2–5 % (w/v) NaCl (optimum 3 %) and at pH 7–8 (optimum pH 7). Oxidase and catalase are positive. DNA, chitin, CM-cellulose and Tweens 20, 40 and 80 can be hydrolysed, but casein, alginate and starch cannot. In API 20E/20NE strips, there are positive results for gelatin hydrolysis, reduction of nitrate to nitrite and aesculin hydrolysis, but negative results for other characteristics. In the API ZYM strip, alkaline phosphatase, esterase (C4), trypsin, valine arylamidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, acid phosphatase and *N*-acetyl-β-glucosaminidase activities are present, but esterase lipase (C8), lipase (C14), cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, α-mannosidase and α-fucosidase activities are absent. Acid is produced from aesculin and 5-ketogluconate, but not from other compounds in the API 50CH system. There are positive reactions in the Biolog GN2 MicroPlate system for *N*-acetyl-D-glucosamine, cellobiose, D-fructose, D-mannose, α-D-glucose, acetic acid, D-galacturonic acid, α-ketobutyric acid, α-ketovaleric acid, DL-lactic acid, propionic acid, succinic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, hydroxy-L-proline, L-ornithine, L-proline, D-serine, L-serine, L-threonine, inosine, uridine, thymidine, α-D-glucose 1-phosphate and D-glucose 6-phosphate. The dominant fatty acids are iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH. The major respiratory quinone is MK-6. The major polar lipids comprise phosphatidylethanolamine, one unknown aminolipid, three unknown phospholipids, two unknown glycolipids and one unknown polar lipid.

The type strain, M124^T (=KCTC 42423^T=MCCC 1K00498^T), was isolated from diseased Pacific white shrimp obtained from a hatchery in Hainan, China. The DNA G+C content of the type strain is 33.7 mol%.

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