Aquimarina agarivorans sp. nov., a genome-sequenced member of the class Flavobacteria isolated from Gelidium amansii

Yan-Xia Zhou,¹ Chao Wang,¹ Zong-Jun Du¹,² and Guan-Jun Chen¹,²

¹College of Marine Science, Shandong University at Weihai, Weihai 264209, PR China
²State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, PR China

A novel Gram-stain-negative, facultatively anaerobic, rod-shaped, agar-digesting bacterial strain, designated HQM9ᵀ, was isolated from the surface of the marine red alga Gelidium amansii collected from the intertidal zone of Weihai, China. Cells of HQM9ᵀ were 3.0–4.0 μm long and 0.2–0.3 μm wide and lacked flagella. The new isolate grew optimally at 28–30 °C, at pH 7.0–7.5, and in the presence of 2.5–3.0 % NaCl. The predominant cellular fatty acids were iso-C₁₅ : 0 and iso-C₁₇ : 0 3-0H. The sole menaquinone was MK-6. The DNA G+C content was 33 mol%. The major polar lipids were comprised of phosphatidylethanolamine and four unknown polar lipids. Based on the 16S rRNA gene sequence, the closest relative was Aquimarina agarivorans ZC1ᵀ with 97.16 % sequence similarity, with which strain HQM9ᵀ formed a distinct cluster belonging to the genus Aquimarina in a phylogenetic tree. Moreover, average nucleotide identity and estimated DNA–DNA hybridization values between strains HQM9ᵀ and ZC1ᵀ were 78.7 % and 12.50 ± 2.95 %, respectively. On the basis of phenotypic, chemotaxonomic and phylogenetic analysis, strain HQM9ᵀ represents the type strain of a novel species within the genus Aquimarina in the family Flavobacteriaceae, phylum Bacteroidetes, for which the name Aquimarina agarivorans sp. nov. is proposed. The type strain is HQM9ᵀ (=ATCC BAA-2612ᵀ=CICC 10835ᵀ).

The genus Aquimarina was first proposed by Nedashkovskaya et al. (2005) to accommodate heterotrophic, Gram-stain-negative, aerobic, dark-yellow or brownish-coloured, gliding bacteria that produce flexirubin-type pigments. To date, the genus comprises 16 established species, all of which were isolated from marine habitats, such as Aquimarina gracilis (Park et al., 2013) and Aquimarina amphilecti (Kennedy et al., 2014) from marine animals, and Aquimarina longa (Yu et al., 2013), Aquimarina megaterium (Yu et al., 2014) and Aquimarina pacifica (Zhang et al., 2014) from seawater. Some species in the genus can degrade high-molecular-mass polysaccharides, which indicates that they play an important role in the recycling of carbon in marine environments. For example, Aquimarina agarivorans (Lin et al., 2012), Aquimarina latercula (Oh et al., 2010) and ‘Aquimarina litoralis’ (Miyazaki et al., 2010) can produce agarase. In this study, we report on the taxonomic characterization of another Aquimarina-like and agar-degrading bacterium from a marine environment whose draft genome, sequenced previously, provides some basis for the phylogenetic analysis (Du et al., 2011).

In the course of screening agar-degrading bacteria, a Gram-stain-negative, facultatively anaerobic, rod-shaped and agar-digesting strain, designated HQM9ᵀ, was isolated from a red alga (Gelidium amansii) collected from the intertidal zone of Weihai, China (122° 3’ 42.8”E 37° 31’ 59.2” N). The red algal cells were crushed in a mortar and diluted with sterile saline. The dilution was then inoculated on marine agar 2216 (MA; Hope Bio-Technology). After incubation at 28 °C for 3–5 days, strain HQM9ᵀ, which formed yellow colonies with obvious hollows on the plate, was purified by successive subculture on MA. The purified strain was routinely preserved in marine broth 2216 (MB) containing 20 % glycerol (v/v) at −80 °C. The reference strain, Aquimarina agarivorans ZC1ᵀ, was kindly provided by Professor Zhong Hu, Shantou University, Guangdong, China.

Strain HQM9ᵀ was cultivated in MB at 28 °C for 2 days. Genomic DNA extraction, PCR amplification, sequencing and similarity calculations for 16S rRNA gene sequence were carried out as described previously (Liu et al., 2014). A phylogenetic dendrogram of strain HQM9ᵀ and

Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HQM9ᵀ is HQ593612.

One supplementary table and one supplementary figure are available with the online Supplementary Material.
some closely related species, based on 16S rRNA gene sequence homology, was reconstructed by the neighbour-joining method with Kimura two-state parameter model analyses in the program MEGA version 6.0 (Tamura et al., 2013). The robustness of the tree topology was evaluated by bootstrap analyses based on 1000 resamplings.

On the basis of 16S rRNA gene sequence analysis, strain HQM9\(^T\) showed highest similarity to \textit{A. agarilytica} ZC1\(^T\) (97.16\%), followed by \textit{Aquimarina addita} JC2680\(^T\) (95.50\%), \textit{A. latercula} LMG 1343\(^T\) (95.43\%), \textit{Aquimarina intermedia} KMM 6258\(^T\) (95.22\%), \textit{Aquimarina macrocephali} JAMB N27\(^T\) (95.15\%), ‘\textit{A. litoralis}’ CNURIC011 (95.07\%) and \textit{Aquimarina amphilecti} 92\(^T\) (95.03\%). Levels of 16S rRNA gene sequence similarity with other taxa were below 95.0\%.

Strain HQM9\(^T\), \textit{A. agarilytica} ZC1\(^T\) and \textit{A. addita} JC2680\(^T\) formed a distinct lineage in the genus \textit{Aquimarina} (Fig. 1), which suggested that the new isolate belongs to the genus \textit{Aquimarina}.

Average nucleotide identity (ANI) was calculated online using the ExGenome Web service (http://www.ezbiocloud.net/ezgenome/ani; Kim et al., 2012). DNA–DNA hybridization (DDH) values were analysed using the genome-to-genome distance calculator (GGDC2.0) (Auch et al., 2010; Lai et al., 2014). The ANI and DDH values between strain HQM9\(^T\) and \textit{A. agarilytica} ZC1\(^T\) were 78.7 % and 12.50 ± 2.95 \%, respectively. Both values were below cut-off values for species differentiation (Richter & Rossello-Móra, 2009; Wayne et al., 1987; Bai et al., 2014). These results indicate that strain HQM9\(^T\) represents a novel species in the genus \textit{Aquimarina}.

Media for morphological and biochemical characterization were based on MA. Phenotypic features of the isolate and reference strain (i.e. colony morphology, pigment production and agar hydrolysis) were determined by cultivating the isolate on MA at 28 °C. The type of flagellum was observed under an electron microscope (Jem-1200; JEOL). Temperature range and optimum for cellular growth were

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**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S RNA gene sequences showing the phylogenetic positions of strain HQM9\(^T\), the type strains of other species of the genus \textit{Aquimarina} and representatives of some other related members of the family \textit{Flavobacteriaceae}. \textit{Olleya marilimosa} was used as an outgroup. Tree topology from neighbour-joining was evaluated by bootstrap analyses based on 1000 resamplings. Percentage bootstrap values above 70 \% are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.
was tested by using the bioMérieux oxidase reagent kit. All of the tests were performed in duplicate. Oxidase activity was measured at 4–45 °C (4, 10, 15, 20, 25, 28, 30, 33, 35, 40 and 45 °C) for up to 7 days. The pH range for growth tested was pH 5.5–9.0 at 0.5 pH unit intervals, by adding MES (pH 5.5 and 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), Tricine (pH 8.5) or CAPSO (pH 9.0 and 9.5) to MB at concentrations of 20 mM. The effect of NaCl on growth was determined as described previously (Du et al., 2014). Flexirubin-type pigment was detected by the KOH test (Bernardet et al., 2002). Gliding motility was observed by oil-immersion phase-contrast microscopy (AX70; Olympus) according to the method described by Bowman (2000). Growth under anaerobic conditions was determined after incubation on MA in an anaerobic chamber for 2 weeks at 28 °C. Antibiotic sensitivity was investigated on MA plates by using discs (Tianhe) containing different antibiotics for 2 days at 28 °C.

Physiological and biochemical characteristics were determined with API 20E and API ZYM kits (bioMérieux) according to the manufacturer’s instructions, except that the suspension medium was replaced by 3.0 % (w/v) NaCl solution. Acid production from carbohydrates was assessed according to the API 50CH fermentation kit (bioMérieux) according to the manufacturer’s instructions. The salinity of the 50CH medium was adjusted by addition of autoclaved 30.0 % (w/v) NaCl solution before inoculation. The API 50CH strips were read after 3 days of incubation at 28 °C. Carbon source utilization was tested using Biolog GEN III microplates according to the manufacturer’s instructions. All of the tests were performed in duplicate. Oxidase activity was tested by using the bioMérieux oxidase reagent kit according to the manufacturer’s instructions. In addition, tests for the hydrolysis of agar, chitin, starch and cellulose were performed using MA containing 1.0 % (w/v) substrate. The phenotypic characteristics of strain HQM9T are given in the species description and Table 1.

For analysis of fatty acids, strains were grown in MB supplemented with 0.5 % (w/v) glucose, with incubation at 28 °C for 3 days. Extraction, methylation and analysis of the fatty acids were according to the standard MIDI (Microbial Identification) system (Sasser, 1990). Respiratory quinones were analysed by HPLC as described by Hiraishi et al. (1996). Polar lipid analysis was performed by the Identification Service of the DSMZ, Braunschweig, Germany. DNA G+C data were from the draft genome sequence of strain HQM9T.

Chemotaxonomy analysis indicated that the predominant fatty acids of strain HQM9T (>10 %) were iso-C15:0 and iso-C17:0 3-OH, which essentially corresponded to the reference strain (Table S1, available in the online Supplementary Material) and other type strains of the genus Aquimarina, except for the proportions of major components and the types of minor components. The novel strain HQM9T contained MK-6 as the sole respiratory quinone. MK-6 is the sole or major respiratory quinone in all species of the genus Aquimarina and all members of the family Flavobacteriaceae (Park et al., 2013). The total polar lipids were phosphatidylethanolamine, glycolipid (GL) and eight unknown lipids (Fig. S1), which differed from those of other species of the genus Aquimarina by the presence of GL and at least four additional unknown polar lipids.

Table 1. Different characteristics of strain HQM9T and type strains of its close relatives in the genus Aquimarina

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tbody>
<tr>
<td>Colony colour</td>
<td>Yellow</td>
<td>Light yellow</td>
<td>Orange</td>
<td>Orange-red</td>
<td>Reddish</td>
<td>Orange</td>
<td>Red</td>
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<td>Gliding motility</td>
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<td>Growth at 33 °C</td>
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<td>Growth with 5 % (w/v) NaCl</td>
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<td>+</td>
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<tr>
<td>D-Galactose</td>
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<td>+</td>
<td>w</td>
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<td>-</td>
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<td>+</td>
<td>w</td>
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<td>Resistance to streptomycin</td>
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<td>-</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>33</td>
<td>33*</td>
<td>35</td>
<td>34</td>
<td>37</td>
<td>33</td>
<td>36</td>
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</table>

*Data from Lin et al. (2012).
and by the absence of aminolipid. The DNA G + C content of the novel strain was 33 mol%, a value in the range of those for recognized species of the genus *Aquimarina* (Table 1).

Despite the common chemotaxonomic features, there were some different phenotypic characteristics between strain HQM9\(^T\) and closely related known species of the genus *Aquimarina* (Table 1). Strain HQM9\(^T\) differed from its closest phylogenetic neighbour, *A. agarilytica* ZC1\(^T\), in that it was able to grow at 33 °C and in the presence of 5.0 % (w/v) NaCl; it was negative for hydrolysis of starch and resistance to streptomycin; and HQM9\(^T\) produced acid from sucrose but not D-galactose. Strain HQM9\(^T\) was also distinguishable from its second closest phylogenetic neighbour, *A. addita* JC2680\(^T\), by its ability to hydrolyse agar but not starch or Tween 80. In view of the low values of sequence similarity, ANI and DDH, and the phenotypic, chemotaxonomic and phylogenetic analyses, strain HQM9\(^T\) represents a novel species within the genus *Aquimarina*, for which the name *Aquimarina agarivorans* sp. nov. is proposed.

**Description of *Aquimarina agarivorans* sp. nov.**


Cells are Gram-stain-negative, non-motile, facultatively anaerobic, 3.0–4.0 μm long and 0.2–0.3 μm wide, and lack flagella. Colonies are yellow, circular with entire edges, convex and butyrous with a diameter of 1.0–1.5 mm after growing on MA for 2 days. Growth occurs at 10–33 °C (optimum, 28–30 °C) and at pH 6.0–8.5 (optimum, pH 7.0–7.5). Cells require sea salts for growth (range, 1.0–5.0%; optimum, 2.5–3.0%). Flexirubin-type pigment is not formed (KOH-test-negative). Positive for catalase and oxidase, negative for H₂S production, indole production and nitrate reduction. Agar is degraded; casein, chitin, gelatin, starch, cellulose, urea and Tween 80 are not degraded. Acids are produced from glycerol, D-xylene, D-glucose, maltose, D-fructose, inositol, D-mannitol, D-sorbitol, methyl α-D-glucopyranoside, aesculin, sucrose, trehalose, melezitose, raffinose, turanose, D-arabitol and potassium gluconate, but not from other compounds in the API 50CH system. In the API ZYM system, alkaline and acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase and naphthol-AS-Bl-phosphohydrolase activities are present, but lipase (C14), cystine arylamidase, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and β-fucosidase activities are absent. The type strain is resistant to trimethoprim, gentamicin, kanamycin, polymyxin B, neomycin, tobramycin and amikacin, but sensitive to penicillin G, cefuroxime, cefalotin, cefamandole, midecamycin, clindamycin, lincomycin, carbencillin and ceftobiprole. The major fatty acids are iso-C₁₅:₀ and iso-C₁₇:₀ 3-ΟΗ. The sole menaquinone is MK-6. The DNA G + C content is 33 mol%. The polar lipids comprise phosphatidylethanolamine, glycolipid and eight unknown lipids.

The type strain, HQM9\(^T\) (=ATCC BAA-2612\(^T\)=CICC 10835\(^T\)), was isolated from the surface of the marine red alga *Gelidium amansii*, collected from the intertidal zone of Weihai (Shandong Province, China).

**Acknowledgements**

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**References**


