Aquimarina agarilytica sp. nov., an agarolytic species isolated from a red alga

Bokun Lin,† Guoyong Lu,† Yandan Zheng, Wei Xie, Shengkang Li and Zhong Hu

Department of Biology, Shantou University, Shantou 515063, PR China

A novel yellow-pigmented, agarolytic bacterial strain, designated ZC1T, was isolated from the surface of the marine red alga Porphyra haitanensis collected near Nan Ao Island, Guangdong province, China. The isolate was Gram-stain-negative, strictly aerobic and rod-shaped and displayed β-galactosidase, alkaline phosphatase, catalase and oxidase activities. The predominant cellular fatty acids were iso-C15:0 summed feature 3 (comprising C16:1ω7c and/or iso-C15:0 2-OH) and iso-C17:0 3-OH. The major menaquinone was menaquinone 6 (MK-6). The DNA G+C content was 32.8 mol%. Phylogenetic analysis of the 16S rRNA gene sequence revealed that strain ZC1T was closely related to members of the genus Aquimarina in the family Flavobacteriaceae, phylum Bacteroidetes. Based on phylogenetic and phenotypic evidence, strain ZC1T (=CCTCC AB 2010229T =NBRC 107695T) represents the type strain of a novel species in the genus Aquimarina, for which the name Aquimarina agarilytica sp. nov. is proposed.

The genus Aquimarina (family Flavobacteriaceae, phylum Bacteroidetes) was proposed by Nedashkovskaya et al. (2005). At the time of writing, the genus comprises seven established species, all of which have been isolated from marine environments: the type species Aquimarina muelleri (Nedashkovskaya et al., 2005) and Aquimarina intermedia (Nedashkovskaya et al., 2006), Aquimarina latercula (Nedashkovskaya et al., 2006), Aquimarina brevivitae (Nedashkovskaya et al., 2006; Yoon et al., 2006), Aquimarina macrocephali (Miyazaki et al., 2010), Aquimarina spongiae (Yoon et al., 2011) and Aquimarina addita (Yi & Chun, 2011). The name ‘Aquimarina litoralis’ (Oh et al., 2010) has not been validly published. This study reports the description of another Aquimarina-like bacterium that was also isolated from a marine environment.

In the course of a screening of marine environments for agar-degrading bacteria, a yellow-pigmented bacterium, designated strain ZC1T, was isolated from the surface of a red alga (Porphyra haitanensis) collected from shallow water near the coast of Nan Ao Island, located on the Tropic of Cancer, at 117° E, near the city of Shantou in Guangdong province, in south-eastern China. The red alga was crushed in a mortar and suspended in MB containing (w/v) 2.5 % NaCl, 0.63 % MgSO4 .7 H2O, 0.46 % MgCl2, 6H2O, 0.1 % CaCl2 and 0.07 % KCl. The plates were incubated at 25 °C for 3 days under aerobic conditions. Strain ZC1T, which formed colonies that sank into the agar and were each surrounded by a clear halo of liquid, was purified by successive streaking on MA. The purified strain was preserved at −80 °C in marine broth (MB) containing 15 % (v/v) glycerol.

For DNA extraction, strain ZC1T was cultivated aerobically in MB supplemented with 0.1 % starch at 25 °C for 24 h. Genomic DNA was extracted as described by Marmur (1961).

The 16S rDNA gene of the strain was amplified by PCR using a single colony as the template and the primers 27F and 1492R (Lane, 1991) before being sequenced twice. The almost-complete 16S rRNA gene sequence of strain ZC1T (1482 bp) was obtained. CLUSTAL X (Thompson et al., 1997) was used to align the novel sequence with the corresponding sequences of strains identified as close relatives using the EzTaxon server (Chun et al., 2007). The MEGA4 package (Tamura et al., 2007) was used to calculate distance matrices and reconstruct phylogenetic trees based on the neighbour-joining and maximum-parsimony algorithms. For the neighbour-joining analysis, distance matrices were calculated using Kimura’s two-parameter model (Kimura, 1980). The maximum-parsimony cladistic analysis employed the close neighbour interchange (CNI) algorithm (Nei & Kumar, 2000). The initial tree for the CNI search was created by random addition of 10 replications. The topology of each phylogenetic tree was evaluated by bootstrap analysis with 1000 replications.

16S rRNA gene sequence analysis indicated that strain ZC1T was closely related to the type strains of all seven
established Aquimarina species: A. addita JC2680T (95.8% sequence similarity), A. intermedia LMG 23204T (95.5%), A. macrocephali JAMB N27T (95.1%), A. latercula ATCC 23177T (94.7%), A. muelleri KMM 6020T (94.2%), A. spongiae A6T (94.0%) and A. brevitiae DSM 17196T (93.8%).

These results, in which the 16S rRNA gene sequence of strain ZC1T was found to show no more than 95.8% similarity to the corresponding sequences of established Aquimarina species, indicated that strain ZC1T represented a novel species in the genus Aquimarina (Stackebrandt & Goebel, 1994). The close relationship between strain ZC1T and several Aquimarina species was evident in the neighbour-joining and maximum-parsimony phylogenetic trees (Fig. 1).

The type strains of all seven established species of Aquimarina (A. intermedia ICM 13506T, A. addita ICM 17106T, A. macrocephali ICM 15542T, A. latercula NBRC 15938T, A. muelleri DSM 19832T, A. spongiae DSM 22623T and A. brevitiae DSM 17196T) were obtained and used for reference in the phenotypic studies listed in Table 1. All the strains in this study were cultured routinely at 25°C except A. brevitiae DSM 17196T, which was incubated at 37°C.

Cell morphology was examined by scanning (JSM-6360LA; JEOL) and transmission (JEM-1400; JEOL) electron microscopy. Growth under anaerobic conditions was assessed on MA in an anaerobic chamber (YQX-II; Jintan Shenglan Ltd), in an atmosphere of 5% CO₂, 10% H₂ and 85% N₂. Gram staining and tests for oxidase and β-galactosidase activities, production of indole and H₂S, nitrate reduction, hydrolysis of DNA, casein, starch, gelatine and urea and acid and gas production on carbohydrates were performed as described by Harley (2005) except that MA or MB (pH 7.6) was used to grow the strains. Hydrolysis of Tween 80 (1%, w/v) was tested on MA and hydrolysis of CM-cellulose and crystalline cellulose was investigated as described by Cowan & Steel (1993). Alkaline and acid phosphatase activities were detected according to the methods described by Gerhardt et al. (1994). The method of Yaphe & Baxter (1955) was used to investigate the hydrolysis of κ-carrageenan. Catalase activity was revealed by the formation of bubbles after 3% (w/v) H₂O₂ solution was dropped onto a fresh colony. The presence of gliding motility and production of flexirubin-type pigments were investigated according to the methods described in the minimal standards for describing new taxa of the family Flavobacteriaceae (Bernardet et al., 2002). The ability to grow at 4, 10, 15, 20, 25, 30 and 35°C was assessed on MA. The pH range for growth was determined in MB supplemented with 0.2% (w/v) glucose, after the medium had been adjusted to pH 4.0–10.0 (at intervals of 1.0 pH unit) using 0.1 M citric acid/Na₂HPO₄ (pH 4.0–6.0), 0.1 M Na₂HPO₄/NaH₂PO₄ (pH 7.0–8.0) or 0.05 M glycine/NaOH (pH 9.0–10.0). The effect of NaCl concentration on growth was investigated in MB supplemented with 0.1% (w/v) agar and prepared with artificial seawater containing 0.5% or 1.0–6.0% (w/v) NaCl (at 1.0% intervals).

Utilization of substrates (at 1%, w/v) as sole carbon and energy sources was tested for 2 weeks in artificial seawater supplemented with 0.2% (w/v) NaNO₃. The ability of strain ZC1T to oxidize various carbon compounds was also examined by using a GN2 MicroPlate (Biolog) according to the manufacturer’s instructions, with the modification that sterile artificial seawater was used to prepare the bacterial
suspension for inoculation. The GN2 MicroPlate was incubated at 25 °C for 24 h.

Susceptibility to antibiotics was tested for 4 days in MB supplemented with the following antibiotics: kanamycin (100 μg), streptomycin (50 μg), neomycin (10 μg), gentamicin (10 μg), spectinomycin (50 μg), ampicillin (100 μg), penicillin (10 μg), chloramphenicol (17 μg), rifampicin (10 μg) and tetracycline (30 μg).

The phenotypic characteristics of strain ZC1T are given in the species description and in Table 1.

At the Yunnan Institute of Microbiology, menaquinones were isolated from the cell mass according to Minnikin et al. (1984) and separated by HPLC (Kroppenstedt, 1982).

As with all members of the family Flavobacteriaceae (Bernardet, 2011), menaquinone 6 (MK-6) was the major respiratory quinone (92.7 %) of strain ZC1T. A minor amount (7.3 %) of MK-4 was also present.

For fatty acid analysis, the strains were grown at 25 °C for 24 h in MB supplemented with 0.2 % (w/v) glucose. Extraction of the fatty acid methyl esters and their GLC separation were performed (at the Third Institute of Oceanography, State Oceanic Administration, China) according to the standard protocol of version 6 of the Sherlock Microbial Identification System (MIDI) and using the TSBA6 6.00 database. The dominant fatty acids of strain ZC1T were iso-C15 : 0 (21.6 %), summed feature 3 (comprising C16 : 1<sup>v7</sup>c and/or iso-C15 : 0 2-OH; 20.0 %) and iso-C<sub>17:0</sub>.
3-OH (15.4 %). As shown in Table 2, strain ZC1T and all of the type strains of established Aquimarina species used as reference strains had iso-C17:0 3-OH and iso-C15:0 as their major fatty acids, supporting the assignment of strain ZC1T to the genus Aquimarina. Compared with the reference strains, however, strain ZC1T had relatively large amounts of summed features 3 and 5.

According to data from the ongoing whole-genome sequencing of strain ZC1T (unpublished results), the DNA G+C content of the novel strain is 32.8 mol%. This value is within the range reported for established Aquimarina species (Table 1).

Based on the phenotypic and phylogenetic evidence, strain ZC1T represents a novel species in the genus Aquimarina, for which the name Aquimarina agarilytica sp. nov. is proposed.

**Description of Aquimarina agarilytica sp. nov.**

Aquimarina agarilytica [a.gar.ily'ti.ca. N.L. n. agarum agaragar, algal polysaccharide; N.L. adj. **agarilytica** -a -um (from Gr. adj. lutikos -ê-on) able to loosen, able to dissolve; N.L. fem. adj. **agarilytica** agar-dissolving].

Cells are Gram-stain-negative, strictly aerobic, non-motile rods, approximately 0.5–0.6 μm in diameter and 1.8–2.0 μm long. Colonies are light yellow, circular with regular edges, sunken into the agar and surrounded by a clear halo of softened agar. Grows with 1–4 % NaCl (optimum 2–3 %), at pH 6–9 (optimum pH 7–8) and at 4–30 °C (optimum 22–26 °C). Flexirubin-type pigments are not produced. Positive for catalase, oxidase, β-galactosidase and alkaline phosphatase activities but negative for acid phosphatase activity, indole production, H2S production and nitrate reduction. Agar, DNA, casein, Tween 80 and starch are hydrolysed but gelatin, κ-carrageenan, urea, CM-cellulose and crystalline cellulose are not. Acid is produced from maltose, glucose and galactose, but not from lactose or sucrose. Gas is not produced from maltose, glucose, galactose, lactose or sucrose. In the GN2 MicroPlate, D-galactose, x-D-glucose, lactose, maltose, x-ketobutyric acid, dextrin and glycogen are strongly utilized, D-mannose, Tween 40, cellobiose and L-glutamic acid are weakly utilized and the other substrates are not utilized. Resistant to kanamycin (100 μg), streptomycin (50 μg), neomycin (10 μg), gentamicin (10 μg) and spectinomycin (50 μg) but susceptible to ampicillin (100 μg), penicillin (10 μg), chloramphenicol (17 μg), rifampicin.

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**Table 2. Cellular fatty acids of strain ZC1T and the type strains of established Aquimarina species**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<th>8</th>
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<tr>
<td>iso-C13:0</td>
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<td>tr</td>
<td>3.8</td>
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<td>2.4</td>
<td>tr</td>
<td>tr</td>
<td>ND</td>
</tr>
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<td>1.3</td>
<td>tr</td>
<td>1.4</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>ND</td>
</tr>
<tr>
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<td>tr</td>
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<td>1.5</td>
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<td>27.0</td>
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<td>39.9</td>
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<tr>
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<td>3.8</td>
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<td>5.4</td>
<td>4.9</td>
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<td>6.6</td>
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<td>1.1</td>
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<tr>
<td>C17:0 2:OH</td>
<td>1.9</td>
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<td>tr</td>
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<tr>
<td>C18:0 9c</td>
<td>1.4</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Summed features**

1  ND  ND  ND  ND  ND  ND  ND  4.6
3  20.0| 1.8 | 2.4 | 5.4 | 4.6 | 2.3 | 3.1 | 4.8 |
5  5.8 | ND  | tr  | ND  | ND  | ND  | tr  | ND  |
8  1.5 | 2.6 | tr  | tr  | tr  | tr  | tr  | ND  |
9  2.8 | 16.0| 8.5 | 5.6 | 9.8 | 8.0 | 3.7 | 13.9|

*Summed features represent groups of two or three fatty acids that could not be separated by GLC using the MIDI system. Summed feature 1 comprised C13:0 3:OH and/or iso-C15:1 H. Summed feature 3 comprised iso-C15:0 2:OH and/or C16:1 o7c. Summed feature 5 comprised C18:0 9c and/or anteiso-C18:0. Summed feature 8 comprised C18:2 o6c and/or C18:1 o7c. Summed feature 9 comprised 10-methyl C16:0 and/or iso-C17:1 9c.
(10 µg) and tetracycline (30 µg). Requires NaCl for growth. The major cellular fatty acids are iso-C₁₅:₀, summed feature 3 (comprising C₁₆:₁ω₇c and/or iso-C₁₅:₀ 2-OH) and iso-C₁₇:₀ 3-OH. The major respiratory quinone is MK-6.

The type strain ZC1T (≡ CCTCC AB 2010229T = NBRC 107695T) was isolated from the surface of a marine red alga (Porphyra haitanensis) collected near Nan Ao Island, Guangdong province, China. The genomic DNA G+C content of the type strain is 32.8 mol%.

Acknowledgements

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References


