Aquimarina rubra sp. nov., isolated from sediment of a sea cucumber culture pond

Ji-Ru Han,1 Dong-Bo Fang,1 Hai-Feng Xia,1 Guan-Jun Chen1,2 and Zong-Jun Du1,2*

Abstract

A Gram-stain-negative, non-motile, rod-shaped, red-pigmented, facultatively anaerobic bacterium, designated SS2-9T, was isolated from sediment collected from a sea cucumber culture pond located in Rongcheng, Shandong province, China. Cells of strain SS2-9T were approximately 0.3–0.5 μm in width and 1.5–6.0 μm in length. The strain was able to grow at 10–37 °C, at pH 6.5–8.5 and in the presence of 0.5–6.0 % (w/v) NaCl. It grew optimally at 28 °C and in the presence of 2.0 % (w/v) NaCl. The DNA G+C content was 34.5 mol% and the sole respiratory quinone was menaquinone 6 (MK-6). The predominant cellular fatty acids were C15:0, iso-C15:0 3-OH, and C17:0 3-OH. The major polar lipids were phosphatidyethanolamine, an unidentified phospholipid, two unidentified aminolipids and four unidentified lipids. Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain SS2-9T was phylogenetically related to members of the genus Aquimarina and was closely related to Aquimarina amphilecti 92V (97.29 % similarity). On the basis of phenotypic, chemotaxonomic and phylogenetic data, strain SS2-9T was considered to represent a novel species of the genus Aquimarina, for which the name Aquimarina rubra sp. nov. is proposed. The type strain is SS2-9T (=KCTC 52274T=MCCC 1H00142T).

The genus Aquimarina, in the family Flavobacteriaceae, was proposed by Nedashkovskaya et al. [1] to accommodate Gram-stain-negative, aerobic, dark yellow or brownish-coloured bacteria, with production of flexirubin-type pigments. The genus Aquimarina was also characterized chemotaxonomically by having menaquinone 6 (MK-6) as the sole or major respiratory quinone [2]. At the time of writing, this genus comprises 18 recognized species, including the recently described species Aquimarina pacifica [3], A. megaterium [4], A. amphilecti [2], A. atlantica [5], A. agarivorans [6] and A. hainanensis [7]. Some members of the genus Aquimarina have been isolated from seawater [8], red alga [9], and the gut microflora of mussel [10] and marine sponge [11] specimens. Here, we report on the taxonomic characterization of one Aquimarina-like bacterial strain, SS2-9T, which was isolated from sediment of a sea cucumber culture pond located in Rongcheng, Shandong province, China (122° 14′ E 36° 54′ N).

A Gram-stain-negative, red-pigmented marine bacterium, designated strain SS2-9T, was isolated using a standard dilution plating technique at 28 °C on 2216E agar (Hopebio) and stored at −80 °C in sterile 1 % (w/v) saline supplemented with 15 % (v/v) glycerol. A. amphilecti DSM 25232T and Aquimarina muelleri KCTC 12285T were used as reference strains for determination of phenotypic characteristics and fatty acid analysis, which were obtained from the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and Korean Collection for Type Cultures (KCTC), respectively. They were cultured under the same conditions as for strain SS2-9T, unless otherwise specified.

The morphological, physiological and biochemical characteristics of strain SS2-9T were investigated after cultivation on 2216E agar at 28 °C for 3 days. The Gram reaction was determined by using the bioMérieux Gram-stain kit according to the manufacturer’s instructions. Cell size, morphology and motility were observed using light microscopy (E600; Nikon). Motility was assessed with the hanging-drop method. The presence of flagella on cells was tested by using a flagella staining kit (Solarbio). The presence of flexirubin-type pigments was investigated using 20 % (w/v) KOH solution [12].

The range and optimum of temperature for cellular growth were measured at 4–45 °C (4, 10, 15, 20, 25, 28, 30, 33, 37, 40, 42 and 45 °C) for up to 7 days. The pH range for growth was determined in 2216E liquid medium that was adjusted to various pH values (pH 4.5–9.5 at intervals of 0.5 pH units) by the addition of HCl or NaOH, with the addition of

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of Aquimarina rubra SS2-9T is KX262846.

Three supplementary figures and one supplementary table are available with the online Supplementary Material.
Strain SS2-9\(^T\) was red-pigmented, circular (1–2 mm in diameter) with entire margins on 2216E agar after culturing for 3 days at 28°C. Growth occurred at 10–37°C (optimal 28°C), at pH 6.5–8.5 (optimal pH 7.0–7.5) and in the presence of 0.5–6.0% (w/v) NaCl (optimal 2.0%). Nitrate was not reduced. Anaerobic growth did not occur on 2216E agar medium supplemented with or without nitrate. The strain was sensitive to vancomycin (30 µg), cefotaxime (30 µg), rifampicin (5 µg), lincomycin (2 µg), ampicillin (10 µg), penicillin G (10 µg), acetylsipramycin (30 µg), ceftiraxone (30 µg), chloromycetin (30 µg), ofloxacin (5 µg), clindamycin (30 µg) and erythromycin (15 µg), but not to nalidixic acid (30 µg), tetracycline (30 µg), norfloxacin (10 µg), streptomycin (10 µg), neomycin (30 µg), kanamycin (30 µg), gentamicin (10 µg) or tobramycin (10 µg).

Comparative 16S rRNA gene sequence analysis indicated that strain SS2-9\(^T\) was phylogenetically affiliated to the genus Aquimarina. Meanwhile, strain SS2-9\(^T\) was related most closely to A. amphilecti DSM 25232\(^T\) (97.29% 16S rRNA gene sequence similarity). Phylogenetic analysis based on the neighbour-joining algorithm (Fig. 1) showed that strain SS2-9\(^T\) formed a distinct branch within the genus Aquimarina. This topology was also supported by the maximum-likelihood and maximum-parsimony trees (Figs S1 and S2, available in the online Supplementary Material).

Cells of strain SS2-9\(^T\) were Gram-stain-negative, rod-shaped and non-gliding. Comparing strain SS2-9\(^T\) with the reference strains, A. amphilecti DSM 25232\(^T\) and A. muelleri KCTC 12285\(^T\), they all produced alkaline phosphatase, trypsin, leucine arylamidase, valine arylamidase and acid phosphatase. However, strain SS2-9\(^T\) produced acid from maltose, cellobiose, amygdalin and D-xylose, but not from starch or D-sorbitol, in contrast to A. amphilecti DSM 25232\(^T\) and A. muelleri KCTC 12285\(^T\). Strain SS2-9\(^T\) could also be distinguished from Aquimarina mytili PSC33\(^T\) [21], Aquimarina latercula LMG 1343\(^T\) [22] and Aquimarina addita JC2680\(^T\) [8], since it showed negative oxidase activity, and was unable to hydrolyse DNA, starch or Tween 80. Strain SS2-9\(^T\) was distinguishable from recognized Aquimarina species based on differences in several phenotypic characteristics (Table 1).

The cellular fatty acid composition of strain SS2-9\(^T\) is shown in Table S1 together with those of the two most closely related members of the genus Aquimarina. The major fatty acids (>10% of total) in strain SS2-9\(^T\) were iso-C\(_{15:1}\) G (11.0%), C\(_{15:0}\) (14.7%), iso-C\(_{17:0}\) 3-OH (15.9%) and iso-C\(_{15:0}\) (18.0%). Strain SS2-9\(^T\) had a cellular fatty acid composition similar to the reference strains with minor

Buffers [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) and CAPSO (pH 9.0 and 9.5) (Sangon)] at concentrations of 20 mM, and OD\(_{600}\) values of the cultures were measured after incubation for 3 days at 28°C. Tolerance to NaCl was tested using modified marine agar medium (1.80% agar, 0.50% peptone, 0.10% yeast extract, 0.32% MgSO\(_4\), 0.12% CaCl\(_2\), 0.07% KCl, 0.02% NaHCO\(_3\)), with the NaCl concentrations ranging from 0.0 to 10.0% (w/v) at intervals of 0.5% at 28°C. Susceptibility to antibiotics was assessed as follows: a cell suspension (McFarland standard 0.5) was swabbed over the surface of 2216E agar to create a uniform lawn before aseptic placement of antibiotic discs onto the agar surface, and inoculated plates were incubated at 28°C for 3 days. Growth under anaerobic (10% H\(_2\), 10% CO\(_2\) and 80% N\(_2\)) and microaerobic (5% O\(_2\), 10% CO\(_2\) and 85% N\(_2\)) conditions was determined after incubation for 14 days in an anaerobic jar with or without 0.1% (w/v) KNO\(_3\). Catalase and oxidase activities were determined as described by Cowan and Steel [13]. In addition, hydrolysis of agar, starch, cellulose, and Tween 20, 40, 60 and 80 were determined as described by Cowan and Steel [13]. DNase activity was examined by using DNase test agar with methyl green (Difco) supplemented with 2% (w/v) NaCl. Other physiological or biochemical characteristics were determined using the API 20E, API ZYM and API 50CHB identification system (bioMérieux) and the Biolog GEN III identification system, according to the manufacturers’ instructions (except for salinity, which was adjusted to 2%). The API 50CHB strips and Biolog GEN III were read every 24 h and cultured for 3 days at 28°C. Cellular fatty acids of strain SS2-9 were determined after incubation for 14 days at 28°C. The API 20E, API ZYM and API 50CHB identification systems (bioMérieux) and the Biolog GEN III identification system, according to the manufacturers’ instructions (except for salinity, which was adjusted to 2%).

The cellular fatty acid composition of strain SS2-9\(^T\) was performed by organic extraction of freeze-dried material followed by TLC and HPLC analyses [17, 18]. Cultures for fatty acid analysis were incubated in 2216E liquid medium at 28°C for 3 days. Cellular fatty acids of strain SS2-9 were prepared and analysed on an Agilent 6890N gas chromatograph and identified using the Sherlock Microbial Identification System (MIDI) (version 4.5 and the TSBA40 database) [19]. Analyses of polar lipids were carried out by the Identification Service of the DSMZ. Total lipid material was detected using molybdate phosphoric acid and specific functional groups were detected using spray reagents specific for defined functional groups. Full details are given by Tindall [20].
The three strains differed in the proportions of C\textsubscript{15:0} and iso-C\textsubscript{15:1} G. The respiratory quinone of strain SS2-9\textsuperscript{T} was MK-6, which was in line with the genus Aquimarina. The total polar lipids of strain SS2-9\textsuperscript{T} were phosphatidylethanolamine, two unidentified phospholipids, three unidentified aminolipids and nine unidentified lipids (Fig. S3). The DNA G+C content of strain SS2-9\textsuperscript{T} was 34.5 mol%, which was similar to A. amphilecti DSM 25232\textsuperscript{T} (36.1 mol%) and A. muelleri KCTC 12285\textsuperscript{T} (31.6–32.5 mol%).

On the basis of the phylogenetic, chemotaxonomic and phenotypic analyses in this study, strain SS2-9\textsuperscript{T} is considered to represent a novel species of the genus Aquimarina, for which the name Aquimarina rubra sp. nov. is proposed.

**DESCRIPTION OF AQUIMARINA RUBRA SP. NOV.**

Aquimarina rubra (ru'bra. L. fem. adj. rubra red).

Cells are Gram-stain-negative, non-flagellated, facultatively anaerobic rods, approximately 0.3–0.5 µm in width and 1.5–6.0 µm in length. Motility is not detected. Flexirubin-type pigments are absent. Colonies on 2216E agar are circular, smooth, convex, red and 1–2 mm in diameter after incubation for 3 days at 28 °C. Na\textsuperscript{+} is required for growth; tolerates 0.5–6.0 % (w/v) NaCl; optimal growth is with 2.0 % (w/v) NaCl. Growth occurs at 10–37 °C, with an optimal temperature of 28 °C, and over a pH range of 6.5–8.5, with an optimum pH of 7.0–7.5. Nitrate is not reduced. Positive for catalase activity but negative for oxidase. Indole and H\textsubscript{2}S production are not detected. Growth does not occur under anaerobic conditions, but weak growth is observed under microaerobic conditions on 2216E agar with or without 0.1 % (w/v) KNO\textsubscript{3}. Tweens 20, 40, 60 and alginate are hydrolysed, but starch, CM-cellulose and agar are not. Cells are positive for Simmons’ citrate utilization, Voges–Proskauer reaction and gelatinase amylase, but negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease and tryptophan deaminase. Acid is produced from D-xylose, D-glucose, amygdalin, aesculin, cellobiose, maltose, trehalose, raffinose, glycogen, potassium 2-ketogluconate and potassium 5-ketogluconate. Alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, \(\alpha\)-chymotrypsin and acid phosphatase are produced, but esterase (C4),
Table 1. Differential phenotypic characteristics of some closely related Aquimarinia species

<table>
<thead>
<tr>
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<th>3</th>
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<tr>
<td>Nitrate reduction</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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<td>Oxidase</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Colour of cell mass</td>
<td>Red</td>
<td>Yellow-brown</td>
<td>Orange</td>
<td>Orange</td>
<td>Orange-red</td>
<td>Orange</td>
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<td>Gliding motility</td>
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<td>–</td>
<td>+</td>
<td>–</td>
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<td>Temperature range (°C)</td>
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<td>10–30</td>
<td>15–37</td>
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<td>4–30</td>
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<td>Tween 80</td>
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<td>Acid production from:</td>
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<td>–</td>
<td>–</td>
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<td>Cellobiose</td>
<td>+</td>
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<td>+</td>
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<td>Maltose</td>
<td>+</td>
<td>–</td>
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<td>Sucrose</td>
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<td>W</td>
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<tr>
<td>Trehalose</td>
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<td>+</td>
<td>–</td>
<td>+</td>
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<td>W</td>
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<td>d-Xylose</td>
<td>+</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>34.5</td>
<td>31.6–32.5</td>
<td>36.1</td>
<td>37.9</td>
<td>34.0</td>
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</table>

naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, N-acetyl-β-glucosaminidase, β-glucosidase, β-glucuronidase, lipase (C14) and α-mannosidase are not produced. In carbon source oxidation tests, positive results are obtained for maltose, cellobiose, N-acetyl-β-galactosamine, dextrin, raffinose, α-d-glucose, d-fructose, acetoacetic acid and acetic acid. The sole respiratory quinone is MK-6. The major fatty acids are C_{15:0} iso, C_{15:0} iso, C_{17:0} G and iso-C_{17:0} 3-OH. The major polar lipids are phosphatidylethanolamine, an unidentified phospholipid, two unidentified amino-lipids and four unidentified lipids.

The type strain, SS2-9^T (=KCTC 52274^T=MCCC 1H00142^T), was isolated from sediment collected from a pond for sea cucumber culture in Rongcheng, Shandong province, China (122° 14’ E, 36° 54’ N). The DNA G+C content of the type strain is 34.5 mol%.

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