Aquimarina spongiae sp. nov., isolated from marine sponge *Halichondria oshoro*

Byoung-Jun Yoon,^{1,2} Han-Su You,² Dong-Heon Lee³
and Duck-Chul Oh^{1,2}

Correspondence

Duck-Chul Oh

duckoh@jejunu.ac.kr

¹The Research Institute for Basic Sciences, Jeju National University, Jeju 690-756, Republic of Korea

²Department of Biology, College of Natural Science, Jeju National University, Jeju 690-756, Republic of Korea

³Department of Environmental Education, College of Education, Suncheon National University, Suncheon 540-742, Republic of Korea

A Gram-stain-negative, yellow-pigmented, rod-shaped, strictly aerobic, non-flagellated, non-gliding and oxidase- and catalase-positive bacterium, designated A6^T, was isolated from a marine sponge, *Halichondria oshoro*, collected on the coast of Jeju Island, South Korea. Phylogenetic analysis based on the nearly complete 16S rRNA gene sequence revealed that strain A6^T was a member of the family *Flavobacteriaceae*. The closest relatives were *Aquimarina intermedia* LMG 23204^T, *A. latercula* ATCC 23177^T, *A. brevivitae* SMK-19^T and *A. muelleri* KMM 6020^T, with which strain A6^T shared 95.7, 95.1, 94.7 and 94.6% 16S rRNA gene sequence similarity, respectively. The dominant fatty acids of strain A6^T were iso-C_{15:0} (32.2%), iso-C_{17:0} 3-OH (20.0%), iso-C_{15:0} 3-OH (12.3%), iso-C_{15:1} G (7.2%) and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; 6.8%). The DNA G + C content of strain A6^T was 36.0 mol% and the major respiratory quinone was MK-6. On the basis of combined phenotypic and phylogenetic analyses, strain A6^T represents a novel species of the genus *Aquimarina*, for which the name *Aquimarina spongiae* sp. nov. is proposed. The type strain is A6^T (=KCTC 22663^T =DSM 22623^T).

The genus *Aquimarina* belongs to the family *Flavobacteriaceae* (Bernardet *et al.*, 2002; Bernardet & Nakagawa, 2006). The species *Aquimarina muelleri* was proposed by Nedashkovskaya *et al.* (2005) to accommodate three heterotrophic, Gram-negative, aerobic, dark-yellow or brownish bacterial strains from seawater that were motile by gliding and produced flexirubin-type pigments. Three other species of the genus *Aquimarina* with validly published names, also isolated from marine environments, have been subsequently described or reclassified from other genera: *A. intermedia* (Nedashkovskaya *et al.*, 2006), *A. brevivitae* (Nedashkovskaya *et al.*, 2006; Yoon *et al.*, 2006) and *A. latercula* (Lewin, 1969; Nedashkovskaya *et al.*, 2006). In the present study, we report on the taxonomic characterization of a strain, designated A6^T, that displayed the basic characteristics of the genus *Aquimarina*.

Strain A6^T was isolated from a marine sponge, *Halichondria oshoro* (class Demospongiae), collected from the Jeju coastal area, Republic of Korea. Serial dilutions of homogenized sponge tissues in sterile seawater were spread on marine agar

2216 (MA; Difco) and incubated at 30 °C for 7 days. Strain A6^T formed small, irregular, yellow-pigmented colonies on MA after 4 days. The strain was preserved at –80 °C in marine broth 2216 (MB; Difco) supplemented with 20% glycerol (v/v).

For 16S rRNA gene amplification, DNA was extracted from a single colony by a boiling method (Englen & Kelley, 2000). The crude extracts served as the template for PCR with *Taq* DNA polymerase (Bioneer) and primers 27F and 1492R (Lane, 1991). Amplification products were purified using the AccuPrep PCR Purification kit (Bioneer) and sequenced using an automatic sequencer (ABI 3730XL; Applied Biosystems) at Solgent, Daejeon, Korea. The 16S rRNA gene sequence (1420 bp) of strain A6^T was compared to those available in public databases using BLASTN (Altschul *et al.*, 1990) to determine an approximate phylogenetic affiliation and was then aligned with those of type strains of closely related species using CLUSTAL W (Thompson *et al.*, 1994). Gaps at the 5' and 3' ends of the alignment were omitted from further analysis. Sequence similarity values were computed using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Phylogenetic trees were reconstructed using the neighbour-joining,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A6^T is FJ348470.

maximum-likelihood and maximum-parsimony algorithms available in PHYLIP version 3.6 (Felsenstein, 2002). For the neighbour-joining method, evolutionary distance matrices were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1980) and a bootstrap analysis with 1000 replications was performed using PHYLIP to evaluate the stability of the phylogenetic tree.

Phylogenetic analysis based on the nearly complete 16S rRNA gene sequence revealed that strain A6^T was a member of the genus *Aquimarina* (Fig. 1). The distinct phylogenetic position of strain A6^T was confirmed in the maximum-likelihood and maximum-parsimony trees (data not shown). The closest relatives of strain A6^T were *A. intermedia* LMG 23204^T, *A. latercula* ATCC 23177^T, *A. brevivitae* SMK-19^T and *A. muelleri* KMM 6020^T, with which the isolate shared 95.7, 95.1, 94.7 and 94.6 % 16S rRNA gene sequence similarity, respectively.

Cell morphology and the presence of flagella for strain A6^T were assessed by light and transmission electron microscopy (JEM100; JEOL) using cells grown on MA at 30 °C for 4 days. The following tests were performed on strain A6^T, *A. muelleri* KMM 6020^T, *A. intermedia* KMM 6258^T, *A. latercula* ATCC 23177^T and *A. brevivitae* SMK-19^T using the same culture conditions and methods. Gliding motility was assessed on 0.1 × MA (10 % marine broth and 1 % agar; Bowman, 2000) and by the hanging-drop method (Bernardet *et al.*, 2002). Gram-staining was performed using the bioMérieux Gram stain kit, according to the

manufacturer's instructions. Growth at 5–40 °C (at intervals of 5 °C) was assessed on MA. Growth at pH 3–5 (at intervals of one pH unit) and 5.5–11 (at intervals of 0.5 pH unit) and with 1–4 % (at intervals of 0.5 %), 5, 6 and 7 % (w/v, final concentration) NaCl was assessed in MB. Tolerance to sea salts was determined on trypticase soy agar (TSA; Difco) supplemented with 0–12 % (w/v) sea salts (Sigma). Growth with NaCl as the sole salt was studied on TSA supplemented with 0–12 % (w/v) NaCl. Anaerobic growth was assessed on MA incubated in a GasPak anaerobic system (BBL) at 30 °C for 15 days. The presence of catalase activity was revealed by the production of oxygen bubbles from cells mixed with 3 % (v/v) H₂O₂ on a glass slide. The presence of oxidase activity was determined by oxidation of 1 % (w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine solution. DNase activity was examined by using DNase test agar with methyl green (Difco) supplemented with 2 % (w/v) sea salt (Sigma). Hydrolysis of casein, starch and Tweens 20, 40 and 80 was tested on MA, using the substrate concentrations given by Cowan & Steel (1965). Hydrolysis of agar and CM-cellulose was investigated as described by Cowan & Steel (1993). Hydrolysis of aesculin, gelatin and urea and reduction of nitrate were investigated as described by Lányi (1987) using media supplemented with 2 % (w/v) sea salts. H₂S production was tested as described by Bruns *et al.* (2001). Other tests were performed using the API 20 NE, API 20 E and API ZYM systems (bioMérieux), according to the manufacturer's instructions. Strips were inoculated with a heavy bacterial suspension in artificial seawater (Yoon

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain A6^T and related taxa in the family Flavobacteriaceae. Bootstrap values (>70 %) based on 1000 replications are shown at branch nodes. *Brumimicrobium glaciale* IC156^T (AF521195) was used as an outgroup (not shown). Bar, 0.01 changes per nucleotide position.

et al., 2002) and results were recorded after 4 days at 30 °C. Susceptibility to antibiotics was determined using the disc-diffusion method on MA using commercial antibiotic-impregnated discs (BBL Becton Dickinson); results were interpreted according to the guidelines set forth by the CLSI (2003) after 4 days at 30 °C on MA. The physiological, biochemical and morphological characteristics of strain A6^T are given in the species description and Table 1.

The presence of flexirubin-type pigments was investigated using the KOH test as described by Bernardet *et al.* (2002). Flexirubin-type pigments were absent. For pigment characterization, strain A6^T, *A. muelleri* KMM 6020^T, *A. intermedia* KMM 6258^T, *A. latercula* ATCC 23177^T and *A. brevivittae* SMK-19^T were grown on MA at 30 °C for 4 days and pigments were extracted with acetone/methanol (7:2, v/v). The absorption spectrum of pigments was determined between 300 and 700 nm with a UV-visible spectrophotometer (Evolution 300; Thermo Scientific;

Nedashkovskaya *et al.*, 2006). Cells of strain A6^T produced yellow carotenoid pigments with maximum absorption at 456 nm.

Cellular fatty acid methyl esters of strain A6^T, *A. muelleri* KMM 6020^T, *A. intermedia* KMM 6258^T, *A. latercula* ATCC 23177^T and *A. brevivittae* SMK-19^T were prepared from cultures grown on MA at 30 °C for 4 days and determined using the Sherlock Microbial Identification System (MIDI), according to the manufacturer's protocol. DNA of strain A6^T was isolated by the method of Marmur (1961) and the G + C content was determined using thermal denaturation (Marmur & Doty, 1962). Isoprenoid quinones were analysed using HPLC (ZQ 4000; Waters) with a reversed-phase column (300 × 3.9 mm, Nova-Pak C18; Waters) as described by Komagata & Suzuki (1987). The dominant fatty acids of strain A6^T were iso-C_{15:0} (32.2 %), iso-C_{17:0} 3-OH (20.0 %), iso-C_{15:0} 3-OH (12.3 %), iso-C_{15:1} G (7.2 %) and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or

Table 1. Differential characteristics of strain A6^T and other members of the genus *Aquimarina*

Strains: 1, *Aquimarina spongiae* sp. nov. A6^T; 2, *A. intermedia* KMM 6258^T; 3, *A. brevivittae* SMK-19^T; 4, *A. latercula* ATCC 23177^T; 5, *A. muelleri* KMM 6020^T. All data were taken from this study unless otherwise stated. All strains are positive for oxidase and alkaline phosphatase, hydrolysis of casein, gelatin, starch and Tween 80 and susceptibility to ampicillin, lincomycin and oleandomycin. All strains are negative for nitrate reduction, urease, production of acetoin, indole and H₂S and susceptibility to gentamicin, kanamycin, neomycin and polymyxin B. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5
Flexirubin-type pigments	–	+	–	–(+)*	+
Gliding motility	–	+	+	–	+
Catalase	+	+	+	–	+
β-Galactosidase	–	+	–	+	–
Range for growth					
Temperature (°C)	10–35	4–35	10–40	4–35	4–35
NaCl (%)	1–5	1–10	1–10	1–5	1–8
Hydrolysis of:					
Agar	–	–	–	+	–
CM-cellulose	+	–	–	w(–)*	–
DNA	+	+	–	+	+
Tween 20	–	+	+	+	+
Tween 40	–	+	+	+	+
Utilization of:					
L-Arabinose	–	+	–	–	–
D-Glucose	+	+	+	–	–
D-Mannose	–	+	–	–	–
Sucrose	–	+	–	–	–
H ₂ S production	–	+	–	+	–
Susceptibility to:					
Benzylpenicillin	+	+	+	+	–
Carbenicillin	+	+	+	–	+
Streptomycin	–	–	+	+	–
Tetracycline	+	+	–	–	+
Maximum absorption of pigment (nm)	456.0	470.0	475.0	464.5	445.0
DNA G + C content (mol%)†	36.0	37.1	36.0	34.0	31–33

*Data in parentheses were taken from Nedashkovskaya *et al.* (2006).

†Data for columns 2–5 were taken from Lewin (1969), Nedashkovskaya *et al.* (2005, 2006) and Yoon *et al.* (2006).

C_{16:1}ω7c; 6.8 %). The detailed fatty acid profile of strain A6^T is compared with those of the reference strains in Table 2. The fatty acid compositions of the five strains were very similar, with only slight differences in the proportions of some components, and the main distinguishing characteristic of strain A6^T was the absence of iso-C_{17:1}ω9c. The DNA G + C content of strain A6^T was 36.0 mol%, which was within the range reported for *Aquimarina* species (Nedashkovskaya *et al.*, 2006), and the major respiratory quinone was menaquinone 6 (MK-6), which is in accordance with that described for members of the family *Flavobacteriaceae* (Bernardet & Nakagawa, 2006).

On the basis of 16S rRNA gene sequence analysis and phenotypic and chemotaxonomic features, we conclude that strain A6^T represents a novel species within the genus *Aquimarina*, for which the name *Aquimarina spongiae* sp. nov. is proposed.

Description of *Aquimarina spongiae* sp. nov.

Aquimarina spongiae (spon'gi.æ. L. gen. n. *spongiae* of a sponge, pertaining to the source of the type strain).

Cells are strictly aerobic, Gram-stain-negative rods (0.2–0.4 µm wide and 3.5–6.0 µm long). On MA, colonies are yellow, convex and circular with slightly irregular margins (1.0–1.5 mm in diameter). Produces non-diffusible

Table 2. Cellular fatty acid compositions of strain A6^T and other members of the genus *Aquimarina*

Strains: 1, *Aquimarina spongiae* sp. nov. A6^T; 2, *A. intermedia* KMM 6258^T; 3, *A. brevivita* SMK-19^T; 4, *A. latercula* ATCC 23177^T; 5, *A. muelleri* KMM 6020^T. All data were taken from this study. Fatty acids representing <1 % of the total in all strains are not shown.

Fatty acid (%)	1	2	3	4	5
iso-C _{13:0}	—	—	—	—	2.1
iso-C _{14:0}	—	—	1.6	—	—
C _{15:0}	1.6	2.6	3.2	6.1	—
C _{15:0} 2-OH	—	—	—	1.6	—
iso-C _{15:0}	32.2	18.6	22.1	17.6	14.6
iso-C _{15:1}	—	—	5.2	7.3	—
iso-C _{15:1} G	7.2	16.2	9.8	11.8	11.2
iso-C _{15:0} 3-OH	12.3	7.5	6.9	7.4	10.2
C _{16:0}	—	—	1.5	—	—
C _{16:0} 3-OH	—	—	1.5	2.8	2.2
iso-C _{16:0}	2.0	2.0	3.0	3.9	—
iso-C _{16:1} H	—	—	2.3	1.4	—
iso-C _{16:0} 3-OH	1.8	2.3	1.9	3.4	2.5
C _{17:1} ω6c	2.0	1.8	2.1	2.8	—
iso-C _{17:1} ω9c	—	7.7	4.0	4.9	9.2
iso-C _{17:0} 3-OH	20.0	24.3	19.2	21.7	25.3
Summed feature 3*	6.8	5.7	8.0	5.4	6.7

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

carotenoid pigments with maximum absorption at 456 nm. No flagella or gliding motility. Grows at 10–35 °C (optimum 30–35 °C) and at pH 6–11 (optimum pH 7–8). NaCl alone does not support growth; requires sea salts or artificial seawater for growth. Grows in MB with 0–1.5 % (w/v) NaCl and on TSA with 3–5 % (w/v) sea salts. Aesculin, casein, CM-cellulose, DNA, gelatin, starch and Tween 80 are hydrolysed, but agar, Tweens 20 and 40 and urea are not. Acetoin, indole and H₂S are not produced. Nitrate is not reduced. Lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are absent. Acid is not produced from arabinose, melibiose, rhamnose, sucrose, turanose, amygdalin, inositol, mannitol or sorbitol. Arabinose, glucose, maltose, mannose, mannitol, gluconate, malate, *N*-acetylglucosamine, adipic acid, capric acid, trisodium citrate and phenylacetic acid are not assimilated. With API ZYM, acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), cystine arylamidase, leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin and naphthol-AS-BI-phosphohydrolase are present and lipase (C14), α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase and α-mannosidase are absent. Susceptible to (µg per disc unless otherwise stated) ampicillin (10), benzylpenicillin (10 U), carbenicillin (100), lincomycin (15), oleandomycin (15) and tetracycline (30); resistant to gentamicin (10), kanamycin (30), neomycin (30), polymyxin B (30) and streptomycin (10). The major fatty acids (>12 %) are iso-C_{15:0}, iso-C_{17:0} 3-OH and iso-C_{15:0} 3-OH. The DNA G + C content of the type strain is 36.0 mol%.

The type strain, A6^T (=KCTC 22663^T =DSM 22623^T), was isolated from a marine sponge, *Halichondria oshoro*, collected on the coast of Jeju Island, Republic of Korea.

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