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Parahaliea aestuarii sp. nov., isolated from the Asan Bay estuary

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Abstract

A Gram-stain-negative, strictly aerobic and moderate halotolerant bacterial strain, designated S2-26^T, was isolated from sediment of the Asan Bay estuary in South Korea. Cells were motile rods with two polar flagella showing oxidase and catalase activities. Growth of S2-26^T was observed at 15–45 °C (optimum, 25 °C) and pH 5.5–10.0 (optimum, pH 7.0–8.5) and in the presence of 0–8.0 % (w/v) NaCl (optimum, 2.0 %). S2-26^T contained $C_{17:1}\omega8c$, summed feature 8 (comprising $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), summed feature 3 (comprising $C_{16:1}$ $\omega7c/C_{16:1}$ $\omega6c$) and $C_{17:0}$ as the major fatty acids and ubiquinone-8 as the sole isoprenoid quinone. The polar lipids of S2-26^T consisted of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, an unknown phospholipid, an unknown aminolipid, an unknown glycolipid and an unknown lipid. The G+C content of the genomic DNA was 62.2 mol%. The results of phylogenetic analysis based on 16S rRNA gene sequences indicated that S2-26^T formed a tight phylogenetic lineage with *Parahaliea mediterranea* 7SM29^T with a 100 % bootstrap value. S2-26^T was most closely related to the type strain of *Parahaliea mediterranea*, with a 97.8 % 16S rRNA gene sequence similarity, and its DNA–DNA relatedness level was 45.2±2.2 %. On the basis of phenotypic, chemotaxonomic and molecular properties, it is clear that S2-26^T represents a novel species of the genus *Parahaliea*, for which the name *Parahaliea aestuarii* sp. nov. is proposed. The type strain is S2-26^T (=KACC 18801^T=JCM 31547^T).

The genus *Haliea* was first proposed by Urios *et al.* [1] with a single species, *Haliea salexigens*, isolated from the Mediterranean Sea and *Haliea rubra*, isolated from the same habitat, was the second species reported as a member of the genus *Haliea* [2]. However, *Haliea rubra* has been reclassified as the type species of a novel genus *Pseudohaliea* [3]. The genus *Parahaliea* in the family *Halieaceae* was also initially proposed by the reclassification of a member of the genus *Haliea*, *Haliea mediterranea*, isolated from seawater from Castellón in Spain [4, 5]. At the time of writing, the genus *Parahaliea* includes only one species, *Parahaliea mediterranea* [5]. In this study, a strain representing presumably a novel species belonging to the genus *Parahaliea* was isolated from a sample from an estuary and its taxonomic properties were characterized using a polyphasic approach.

Strain S2-26^T was isolated from the Asan Bay estuary of South Korea (36° 56′ 41.61″ N 26° 59′ 27.12″ E), using a previously described procedure [6] with some modifications. Briefly, an estuary sediment sample (sampled on January 18, 2015) was serially diluted with 0.85 % (w/v) saline solution and aliquots of each serial dilution were spread on marine agar 2216 (MA, BD) plates and incubated aerobically at 30 °C for 5 days. The 16S rRNA genes of colonies grown on MA were PCR-amplified using the universal primers, F1 and R13, and double-digested with *Hae*III and *Hha*I, and representative PCR amplicons of restriction fragment patterns were partially sequenced using the primer F1. The resulting 16S rRNA gene sequences were compared with those of validly reported type strains of species available in the EzTaxon-e server (http://eztaxone.ezbiocloud.net/; [7]). From the analysis, a putative novel strain of a member of the genus *Parahaliea*, designated S2-26^T, was selected for further phenotypic and phylogenetic analyses. S2-26^T was routinely incubated aerobically on MA at 25 °C for 3 days unless otherwise stated. S2-26^T was stored at −80 °C in marine broth (MB, BD) containing 15 % (v/v) glycerol for long-term preservation. *Parahaliea mediterranea* DSM 21924^T, purchased from DSMZ, was used as a reference strain for the comparison of phenotypic properties and fatty acid compositions and DNA–DNA hybridization.

The 16S rRNA gene of S2-26^T was cloned into the pCR2.1 vector using a TOPO cloning kit (Invitrogen) according to the manufacturer's instructions and sequenced using the M13 reverse and T7 primers of the TOPO cloning kit at Macrogen (Seoul, Republic of Korea) to obtain a longer 16S rRNA gene sequence. The similarities of the resulting 16S rRNA gene sequence (1458 nucleotides) with those of type strains of related species with validly published names were determined using the Nucleotide Similarity Search program

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The GenBank accession number for the 16S rRNA gene sequence of strain S2-26^T is KX268609.
Three supplementary figures and one supplementary table are available with the online Supplementary Material.

[7]. The 16S rRNA gene sequences of S2-26^T and closely related type strains were aligned using the fast secondary-structure-aware Infernal aligner of the Ribosomal Database Project (RDP) [8]. Phylogenetic trees based on the neighbour-joining (NJ) and maximum-parsimony (MP) algorithms were reconstructed using the PHYLIP software (ver. 3.695, [9] and their tree topologies were evaluated through bootstrap analyses using a 1000 resampled dataset. Phylogenetic analysis based on the maximum-likelihood (ML) algorithm was performed using RAxML-HPC BlackBox (version 8.2.4) available in the Cyber-Infrastructure for Phylogenetic Research project (CIPRES, www.phylo.org; [10]). Genomic DNA relatedness between S2-26^T and the type strain of *Parahaliea mediterranea* (DSM 21924^T) was evaluated by DNA–DNA hybridization (DDH) using a genome-probing microarray method, as previously described [11]. The DDH experiments were conducted in triplicate and confirmed by reciprocally interchanging DDH between probe and target DNA. Signals produced by hybridization of probes to homologous target DNAs were taken to be 100 % and signal intensities by self-hybridization of the series of dilutions were used for the calculation of the levels of DNA relatedness between S2-26^T and DSM 21924^T.

The results of comparative analysis based on the 16S rRNA gene sequences revealed that S2-26^T was most closely related to *Parahaliea mediterranea* 7SM29^T, with a 97.8 % sequence similarity. Results of phylogenetic analysis using the NJ algorithm indicated that S2-26^T formed a tight phylogenetic lineage with *P. mediterranea* 7SM29^T with a 100 % bootstrap value within the family *Halieaceae* (Fig. 1). Phylogenetic trees reconstructed using the ML and MP algorithms also indicated that S2-26^T formed a phylogenetic lineage with *P. mediterranea* 7SM29^T (Fig. S1, available in the online Supplementary Material). The level of DNA–DNA relatedness between S2-26^T and the type strain of *P. mediterranea* (DSM 21924^T) was 45.2±2.2 %, which was clearly below the 70 % threshold generally accepted for species delineation [12]. These results clearly indicate that S2-26^T represents a novel species of the genus *Parahaliea*.

Growth of S2-26^T was tested at 25 °C for 3 days on MA, LB agar (BD), R2A agar (BD), nutrient agar (NA; BD) and tryptic soy agar (TSA; BD), which were supplemented with NaCl to give approximately 2 % (w/v) final NaCl concentrations. Growth of S2-26^T at different temperatures (10, 15, 20, 25, 30, 37, 40, 45 and 50 °C) was tested on MA for 3 days. Growth of S2-26^T at different pH values (5.0–10.5 at 0.5 pH unit intervals) was assessed in MB at 25 °C for 3 days. MB media with pH values below 8.0 or of 8.0–11.0 were prepared using the Na_2HPO_4–NaH_2PO_4 and Tris–HCl buffers, respectively, as previously described [13] and their pH values were adjusted again after sterilization (121 °C for 15 min). NaCl tolerance of S2-26^T was evaluated using MB media with different NaCl concentrations (0–10 % at 1 % intervals), which were prepared in the laboratory according to the BD formula. Gram-staining was tested using a Gram stain kit (bioMérieux) according to the manufacturer's instructions. Anaerobic growth was evaluated after 21 days of incubation on MA at 25 °C under anaerobic conditions (with 4–10 % CO_2) using the GasPak Plus system (BBL). Cell morphology and the presence of flagella were investigated using transmission electron microscopy (JEM-1010, JEOL) and phase-contract microscopy with cells grown on MA at 25 °C for 3 days. Motility of S2-26^T was determined using the hanging drop technique with *P. mediterranea* as a positive control, as described by Bernardet *et al.* [14]. Nitrate reduction was tested according to the method of Lányí [15]. Catalase and oxidase activities of S2-26^T were tested by the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution and the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck), respectively [16]. The following properties of 5S2-26^T and the reference strain (DSM 21924^T) were tested in parallel under the same conditions. Hydrolysis of starch, Tween 20, Tween 80, aesculin, casein, tyrosine, gelatin and urea was investigated on MA, according to the procedures described previously [15–17]. Additional enzymatic activities, biochemical features and oxidation of carbon sources were investigated using the API ZYM and API 20NE kits (bioMérieux) and the GN2 MicroPlate system (Biolog), respectively, according to the instructions of the manufacturers, except that resuspended cells in artificial seawater (20 g NaCl, 2.9 g $MgSO_4$, 4.53 g $MgCl_2.6H_2O$, 0.64 g KCl and 1.75 g $CaCl_2.2H_2O$ per litre) were used as inocula and the test strains were incubated at their optimal growth temperatures.

S2-26^T grew well and formed white colonies on MA, but grew slowly on NA agar, LB agar, TSA and R2A agar containing 2 % NaCl. Cells were Gram-stain-negative motile rods with two polar flagella (1.6–2.1 µm in length and 0.4–0.6 µm in width) (Fig. S2). Anaerobic growth was not observed after 21 days of incubation at 25 °C. S2-26^T in the GN2 MicroPlate system oxidized Tween 40, Tween 80, pyruvic acid, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, α-ketovaleric acid, propionic acid, succinic acid, L-aspartic acid, L-glutamic acid, glycyl L-glutamic acid, xylitol, D-galacturonic acid, D-glucuronic acid, α-ketobutyric acid, quinic acid, L-alaninamide, L-alanine, L-alanyl glycine, glycyl L-aspartic acid, hydroxy-L-proline, L-leucine, L-phenylalanine, L-proline, L-serine, L-threonine, γ-aminobutyric acid, putrescine, turanose, γ-hydroxybutyric acid, D-glucose 6-phosphate, cellobiose, D-mannose and succinic acid monomethyl ester, but did not oxidize adonitol, L-arabinose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, lactulose, maltose, D-mannitol, melibiose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, formic acid, *p*-hydroxyphenlyacetic acid, itaconic acid, malonic acid, sebacic acid, bromosuccinic acid, glucuronamide, L-histidine, inosine, uridine, thymidine, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, dextrin, D-arabitol, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, D-saccharic acid, L-asparagine, α-cyclodextrin, glycogen, *myo*-inositol, α-D-lactose, methyl β-D-glucoside, D-psicose, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-gluconic acid, D-glucosaminic acid, α-ketoglutaric acid, DL-lactic acid, succinamic acid, D-alanine, L-ornithine, L-pyroglutamic acid,

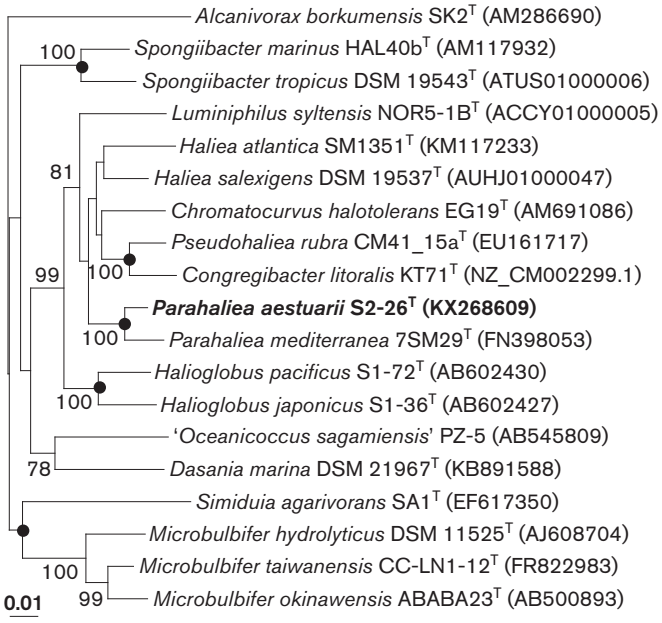

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences indicating the phylogenetic relationships between S2-26^T and related taxa. Bootstrap values are shown at nodes as percentages of 1000 replicates; when only values over 70 % are indicated. Filled circles (●) indicate that the corresponding nodes were also recovered by the ML and MP algorithms. *Alcanivorax borkumensis* SK2^T (AM296690) was used as the outgroup. Bar, 0.01 changes per nucleotide position.

D-serine, DL-carnitine, urocanic acid, phenylethylamine, 2-aminoethanol and α-D-glucose 1-phosphate. The phenotypic characteristics of S2-26^T are presented in the species description and compared with those of *P. mediterranea* in Tables 1 and S1. Many characteristics, including catalase and oxidase activities, motility, nitrate reduction and temperature ranges, were in agreement with those of *P. mediterranea*, whereas other properties such as colony colour, NaCl range and hydrolysis of Tween 20, starch and casein allowed the differentiation of S2-26^T from *Parahaliea mediterranea* (Table 1).

Isoprenoid quinones of S2-26^T were analyzed with a HPLC (model LC-20A, Shimadzu) system equipped with a reversed-phase column (250×4.6 mm, Kromasil, Akzo Nobel) and a diode array detector (SPD-M20A, Shimadzu) using methanol/isopropanol (2 : 1, v/v) as an eluent (1 ml min^{-1}), as described previously [18]. S2-26^T and *P. mediterranea* DSM 21924^T were cultivated in MB at 25 °C and microbial cells were harvested at the same growth stage (exponential phase, OD_{600}=0.8) for cellular fatty acid analysis. The cellular fatty acids of microbial cells were saponified, methylated and extracted using the standard MIDI protocol. The fatty acid methyl esters were analyzed by gas chromatography (model 6890, Hewlett Packard) and identified by using the TSBA6 database of the Microbial Identification System (Sherlock ver. 6.0B; [19]). The polar lipids of S2-26^T were analyzed by TLC using cells harvested during the exponential growth phase, as described by Minnikin *et al.* [20]. The DNA G+C content of S2-26^T was determined by a fluorometric method [21], using SYBR green I and a real-time PCR thermocycler (Bio-Rad).

The only respiratory quinone of S2-26^T detected was ubiquinone-8 (Q-8). The major cellular fatty acids (>10 % of the total fatty acids) of S2-26^T were $C_{17:1}\omega8c$ (26.7 %), summed feature 8 (comprising $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$, 15.5 %), summed feature 3 (comprising $C_{16:1}\omega7c$/$C_{16:1}\omega6c$, 14.8 %) and $C_{17:0}$ (10.9 %). The overall fatty acid profile of S2-26^T was almost identical with that of *P. mediterranea* although there were some differences in the respective proportions of some components (Table 2). Phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, an unknown phospholipid, an unknown aminolipid, an unknown glycolipid and an unknown lipid were detected as the polar lipids from S2-26^T (Fig. S3), which was, in general, consistent with those of *Parahaliea mediterranea* [4]. The DNA G+C content of S2-26^T was 62.2 mol%, which was almost identical to that of the type strain of *Parahaliea mediterranea* [4]. In conclusion, the phylogenetic inference and the physiological and biochemical properties of S2-26^T support its classification as representing a novel species of the genus *Parahaliea*, for which the name *Parahaliea aestuarii* sp. nov. is proposed.

DESCRIPTION OF *PARAHALIEA AESTUARII* SP. NOV.

Parahaliea aestuarii (ae.stu.a'ri.i. L. gen. n. *aestuarii* of an estuary, isolated from an estuary).

Table 1. Comparisons of phenotypic characteristics of S2-26^T and the type strain of *Parahaliea mediterranea*

Taxa: 1, S2-26^T (this study); 2, *P. mediterranea* DSM 21924^T [4, 5]. All strains are positive for the following characteristics: hydrolysis* of aesculin and gelatin, nitrate reduction, motility, oxidase and catalase activities, activity* of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arlyamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase and assimilation of D-glucose and phenylacetic acid. All strains are negative for the following characteristics: Gram-staining, indole production, hydrolysis* of tyrosine, urea and Tween 80, activity* of lipase (C14), trypsin, α-chymotrysin, β-glucuronidase, α-glucosidase, α-mannosidase, arginine dihydrolase and α-fucosidase and assimilation* of L-arabinose, D-mannitol, potassium gluconate, capric acid and adipic acid. +, Positive; –, negative; NA, not available.

Characteristic	1*	2
Colony colour	White	Slightly yellow
Growth at:		
Temperature (°C)	15–45	15–40
NaCl (%)	0–8	0.35–15
pH (optimum)*	5.5–10 (7–8.5)	5.5–10 (8–9)
Hydrolysis* of:		
Tween 20, starch	+	–
Casein	–	+
Enzyme activity (API ZYM)* of:		
β-Glucosidase	+	–
α-Galactosidase, β-galactosidase	–	+
Assimilation (API 20NE)* of:		
Malic acid	+	–
D-Mannose, maltose, *N*-acetyl-glucosamine, trisodium citrate	–	+
DNA G+C content (mol%)	62.2	62.1

*These analyses were conducted under the same conditions in this study.

Table 2. Cellular fatty acid compositions (percentages) of S2-26^T and the type strain of *Parahaliea mediterranea*

Taxa: 1, S2-26^T; 2, *P. mediterranea* DSM 21924^T. All data are from this study. Data are expressed as percentages of the total fatty acids and fatty acids amounting to less than 1.0 % in both strains are not shown. Major components (>10.0 %) are highlighted in bold type; TR, trace amount (<1.0 %).

Fatty acid	1	2
Saturated:		
$C_{11:0}$	2.0	1.8
$C_{13:0}$	1.6	1.8
$C_{14:0}$	1.2	1.8
$C_{16:0}$	7.8	**10.2**
$C_{17:0}$	**10.9**	8.8
Unsaturated:		
$C_{15:1}\omega6c$	1.6	TR
$C_{15:1}\omega8c$	3.5	3.2
$C_{17:1}\omega6c$	4.8	3.8
$C_{17:1}\omega8c$	**26.7**	**22.9**
Hydroxyl:		
$C_{11:0}$ 3-OH	3.7	2.7
Summed feature*:		
3	**14.8**	**15.5**
8	**15.5**	**19.9**

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed features 3 and 8 comprise $C_{16:1}\omega7c/C_{16:1}\omega6c$ and $C_{18:1}\omega7c/C_{18:1}\omega6c$, respectively.

Cells are Gram-stain-negative, strictly aerobic and moderately halotolerant rods with two polar flagella. Colonies on MA are white, irregular, raised and smooth. Growth occurs at 15–45 °C (optimum, 25 °C), at pH 5.5–10.0 (optimum, pH 7.0–8.5) and in the presence of 0–8.0 % (w/v) NaCl (optimum, 2.0 %). Oxidase- and catalase-positive. Tween 20, aesculin, starch and gelatin are hydrolyzed but casein, Tween 80, tyrosine and urea are not. Nitrate is reduced to nitrite but does not produce nitrogen gas. Indole production is negative. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arlyamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase, β-glucosidase and N-acetyl-β-glucosaminidase activities are positive, but lipase (C14), α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase, α-fucosidase, trypsin and arginine dihydrolase activities are negative. Assimilation of D-glucose, malic acid and phenylacetic acid is positive, but assimilation of L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, maltose, potassium gluconate, capric acid, adipic acid and trisodium citrate is negative. The polar lipids consist of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, an unknown phospholipid, an unknown aminolipid, an unknown glycolipid and an unknown lipid. The major cellular fatty acids are $C_{17:1}\omega8c$, summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), summed feature 3 ($C_{16:1}$ $\omega7c/C_{16:1}$ $\omega6c$) and $C_{17:0}$. The only respiratory isoprenoid quinone is ubiquinone-8.

The type strain is S2-26^T (=KACC 18801^T=JCM 31547^T), isolated from the Asan Bay estuary, South Korea. The DNA G+C content of the type strain is 62.2 mol%.

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Conflicts of interest
The authors declare no competing financial conflicts of interests.

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