**Alteromonas gracilis** sp. nov., a marine polysaccharide-producing bacterium

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A novel exopolysaccharide-producing bacterium, designated strain 9a2T, was isolated from Pacific Ocean sediment. The strain was Gram-stain-negative, motile, strictly aerobic, oxidase- and catalase-positive, and required NaCl for growth. Its major isoprenoid quinone was ubiquinone-8 (Q-8), and its cellular fatty acid profile consisted mainly of C16:1ω7c, C18:1ω9c and C16:0. The DNA G+C content was 46.6 mol%. 16S rRNA gene sequence analysis suggested that strain 9a2T is a member of the genus *Alteromonas*. Strain 9a2T exhibited closest phylogenetic affinity to *Alteromonas macleodii* NBRC 102226T (99.3% 16S rRNA gene sequence similarity), *A. marina* SW-47T (99.3%), *A. litorea* TF-22T (99.0%), *A. australica* H17T (98.7%), *A. simiduii* BCRC 17572T (98.5%), *A. stellipolaris* LMG 21861T (98.3%) and *A. hispanica* F-32T (98.2%). The DNA–DNA reassociation values between strain 9a2T and *A. macleodii* JCM 20772T, *A. marina* JCM 11804T, *A. litorea* JCM 12188T, *A. australica* CIP 109921T, *A. simiduii* JCM 13896T, *A. stellipolaris* LMG 21861T and *A. hispanica* LMG 22958T were below 70%. Strain 9a2T contained phosphatidylethanolamine, phosphatidylglycerol and an unidentified polar lipid. Owing to differences in phenotypic and chemotaxonomic characteristics, phylogenetic analysis based on 16S rRNA gene sequences and DNA–DNA relatedness data, the isolate merits classification as a novel species, for which the name *Alteromonas gracilis* sp. nov. is proposed. The type strain of this species is 9a2T (=JCM 30236T=NCIMB 14947T).

The production of exopolysaccharides (EPSs) is found in many species of bacteria and has been the subject of numerous investigations. We have been trying to find new polysaccharide-producing bacteria from sediment of the ocean bottom (Matsuyama et al., 2006, 2013, 2014). Strain 9a2T was isolated as a polysaccharide producer and was considered to be a member of the genus *Alteromonas* based on 16S rRNA gene sequencing. The genus *Alteromonas* was originally described by Baumann et al. (1972) to accommodate marine, aerobic, Gram-negative, non-fermentative, polarly flagellated bacteria, and was later divided into two genera, *Alteromonas* and *Pseudoalteromonas*, on the basis of phylogenetic analysis (Gauthier et al., 1995). *Alteromonas macleodii* is the type species of the genus (Baumann et al., 1972). At the time of writing, the genus *Alteromonas* comprises 11 recognized species (Parte, 2014). Members of the genus *Alteromonas* are found in the marine environment, either in the open ocean or at the coast. Here, a novel *Alteromonas* strain isolated from sediment of the ocean bottom was studied using a polyphasic approach.

Sediment samples were collected during the R/V *Hakuho-Maru* cruise (KH-12-4) in 2012. Strain 9a2T was isolated by enrichment from a sediment sample taken from sediment of the Pacific Ocean at a water depth of 6310 m (47°00′N 170°35′E). The sediment sample was inoculated in 10 ml mineral salts medium containing 0.8 g peptone and 15 g glucose in one litre of seawater (pH 7.0). This medium was incubated at 25 °C with shaking at 150 r.p.m. After incubation for several days, a portion of the suspension was transferred into 10 ml of fresh medium and the medium was re-incubated. After three successive transfers, the suspension was plated onto marine agar 2216 (Difco).
to isolate pure cultures. The type strains of seven species of the genus *Alteromonas* were used as reference strains for phenotypic characterization, fatty acid analysis and DNA–DNA hybridization: *A. macleodii* JCM 20772<sup>T</sup> (Baumann et al., 1972, 1984), *A. marina* JCM 11804<sup>T</sup> (Yoon et al., 2003), *A. litorea* JCM 12188<sup>T</sup> (Yoon et al., 2004), *A. macleodii* JCM 20772<sup>T</sup> and *A. simiduii* JCM 13896<sup>T</sup> (Chiu et al., 2007) were obtained from Japan Collection of Microorganisms (JCM), *A. australica* CIP 109921<sup>T</sup> (Ivanova et al., 2013) was obtained from Collection de l’Institut Pasteur (CIP), and *A. stellipolaris* LMG 21861<sup>T</sup> (Van Trappen et al., 2004) and *A. hispanica* LMG 22958<sup>T</sup> (Martínez-Checa et al., 2005) were obtained from Belgian Coordinated Collections of Microorganisms/LMG Bacteria Collection (BCCM/ LMG). These bacteria were cultured in marine broth 2216 (MB; Difco) with reciprocal shaking (150 r.p.m.) at 30 °C until the early stationary phase of growth.

For phenotypic characterization, MB was used as the basal medium. Production of EPSs was checked by adding three volumes of ethanol to the MB supernatant (Matsuyama et al., 2003). Acid production from carbohydrates was determined by the method of Leifson (1963). Growth at 5, 10, 15, 20, 25, 30, 35, 40, 43 and 45 °C was tested using MB. The basal medium (BM) for determination of the pH range for growth contained (per litre) 1 g peptone, 1 g yeast extract, 0.3 g KCl, 2.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g CaSO<sub>4</sub>, 2H<sub>2</sub>O and 20 g NaCl. Acetic acid (pH 4.5, 5.0), MES (pH 5.5, 6.0, 6.5), HEPES (pH 7.0, 7.5, 8.0), TAPS (pH 8.5, 9.0) and glycine (pH 9.5, 10.0, 10.5) were added to BM at a concentration of 50 mM, and 5 % (w/v) NaOH was used to adjust pH. NaCl requirement for growth was determined after 8 h and 1 day of cultivation at 25 °C in the following medium: 0.1 % (w/v) peptone, 0.1 % yeast extract, 0.03 % KCl, 0.25 % MgSO<sub>4</sub>·7H<sub>2</sub>O and 0.05 % CaSO<sub>4</sub>·2H<sub>2</sub>O, with 0–14 % NaCl. Other physiological and biochemical characteristics were examined according to the methods described by Barrow & Feltham (1993). Phenotypic characterization was also performed to compare characteristics with the reference species under the same conditions using API ZYM and API 20NE kits (bioMérieux), and Biolog GN2 microwell plates, according to the manufacturers’ instructions, but with minor modifications, i.e. cell suspensions for inoculation were prepared in seawater.

Cultural properties, cell morphology, motility and the results of some physiological tests of strain 9a2<sup>T</sup> are described in Table 1 and in the species description below. Cell morphology was examined via transmission electron microscopy according to the method described previously (Matsuyama et al., 2006; Fig. S1, available in the online Supplementary Material); cells were straight rods. Cells possessed a long flagellum. Motility was examined by using wet mounts. Cells were motile. Strain 9a2<sup>T</sup> produced about 1.82 g EPS l<sup>−1</sup> after incubation at 25 °C for 4 days and the sugar component of EPS consisted of mannose, galactose and glucose. *A. australica* CIP 109921<sup>T</sup> and *A. hispanica* LMG 22958<sup>T</sup> did not produce EPSs, in contrast to the other reference species. Growth occurred in media with 0.5–9 % (w/v) NaCl, with the optimum at 1.5–2 %. The temperature range for growth was 5–43 °C, with the optimum at 37 °C. The pH range for growth was 6.0–9.0, with the optimum at pH 7.0–8.0. Strain 9a2<sup>T</sup> could be differentiated readily from the phylogenetically closely related species *A. macleodii* JCM 20772<sup>T</sup>, *A. marina* JCM 11804<sup>T</sup>, *A. litorea* JCM 12188<sup>T</sup>, *A. australica* CIP 109921<sup>T</sup>, *A. simiduii* JCM 13896<sup>T</sup>, *A. stellipolaris* LMG 21861<sup>T</sup> and *A. hispanica* LMG 22958<sup>T</sup> based on several phenotypic properties as shown in Table 1. Strain 9a2<sup>T</sup> was positive for nitrate reduction and acid production from mannose, in contrast to *A. marina* JCM 11804<sup>T</sup> and *A. macleodii* JCM 20772<sup>T</sup>. Strain 9a2<sup>T</sup> was positive for assimilation of succinic acid, in contrast to all the other reference type strains.

The 16S rRNA gene was amplified by PCR using primers 9F and 1510R as described previously (Matsuyama et al., 2014). The resulting PCR product was purified with the QIAquick PCR purification kit (Qiagen) and sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (PRISM 3100; Applied Biosystems) with a Big Dye termination RR mix version 3.1 (Applied Biosystems) according to the manufacturer’s instructions. Multiple alignments of the sequences were performed using the program SILVA (Quast et al., 2013). A phylogenetic tree was reconstructed by the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Guindon & Gascuel, 2003; Fig. 1) and minimum-evolution (Rzhetsky & Nei, 1993; Fig. S2) methods in MEGA 6 (Tamura et al., 2013). Distances were calculated according to Kimura’s two-parameter model (Kimura, 1980). The similarity between sequences was calculated using the EzTaxon-e server (Kim et al., 2012). The 16S rRNA gene sequence (1444 bp) of strain 9a2<sup>T</sup> was obtained and compared with the type strains of related species. Strain 9a2<sup>T</sup> was related most closely to *A. macleodii* NBRC 102226<sup>T</sup>(99.3 % 16S rRNA gene sequence similarity), *A. marina* SW-47<sup>T</sup> (99.3 %), *A. litorea* TF-22<sup>T</sup> (99.0 %), *A. australica* H17<sup>T</sup> (98.7 %), *A. simiduii* BCRC 17572<sup>T</sup> (98.5 %), *A. stellipolaris* LMG 21861<sup>T</sup> (98.3 %) and *A. hispanica* F-32<sup>T</sup> (98.2 %).

For polar lipid analysis, cells of strain 9a2<sup>T</sup> and *A. macleodii* JCM 20772<sup>T</sup> (the type species of the genus *Alteromonas*) were grown in MB at 25 °C for 2 days. The polar lipid profiles were analysed by extracting polar lipids with 24 ml chloroform/methanol (1 : 2, by vol.) from 100 mg of freeze-dried cells. Separation of lipids was achieved by two-dimensional chromatography on a silica gel 60 TLC plate (Merck) using chloroform/methanol/water (75 : 32 : 4) in the first dimension and chloroform/methanol/acetic acid/water (86 : 16 : 15 : 4) in the second dimension. Total lipids were detected by spraying with primulin reagent and further characterized by spraying with ninhydrin (specific for amino groups), and Dittmer and Lester reagent (Dittmer & Lester, 1964) (specific for phosphates). The polar lipid profile is shown in Fig. S3. Strain 9a2<sup>T</sup> and *A. macleodii* JCM 20772<sup>T</sup> contained phosphatidylethanolamine, phosphatidylglycerol and an unidentified polar lipid. Analysis of isoprenoid quinones was carried out by TechnoSuruga. For the
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<td>Growth at 5 °C</td>
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<td>NaCl concentration for growth (%)</td>
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<td>Reduction of nitrate to nitrite</td>
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Hydrolysis of:
- Gelatin: –
- Casein: –
- Starch: +

Production of:
- H2S: +
- Lipase (C14): +
- Trypsin: –
- α-Galactosidase: –
- β-Galactosidase: –
- α-Glucosidase: –

Acid production from:
- Trehalose: +
- Fructose: +
- Melibiose: +
- Mannose: +
- Raffinose: –
- Xylose: –
- Arabinose: –

Assimilation of:
- α-Cyclodextrin: +
- Dextrin: +
- Glycogen: W
- Tween 40: +
- Tween 80: +
- l-Arabinose: –
- d-Galactose: +
- 2-Lactose: +
- D-Mannose: –
- Melibiose: +
- Acetic acid: –
- cis-Aconitic acid: –
- DL-Lactic acid: –
- Succinic acid: +

DNA G+C content (mol%)*: 46.6, 44.9–46.4, 45, 46.0, 43.4, 45.3, 43–45, 46.3

*Data for A. macleodii ATCC 27126T, A. marina SW-47T, A. litorea TF-22T, A. australica H 17T, A. simiduii AS1T, A. stellipolaris ANT 69T and A. hispanica F-32T were taken from Baumann et al. (1984), Yoon et al. (2003), Yoon et al. (2004), Ivanova et al. (2013), Chiu et al. (2007), Van Trappen et al. (2004) and Martinez-Checa et al. (2005), respectively.

Table 1. Differential phenotypic characteristics between strain 9a2T and related species of the genus Alteromonas

Strains: 1, 9a2T; 2, A. macleodii JCM 20772T; 3, A. marina JCM 11804T; 4, A. litorea CIP 109921T; 5, A. australica JCM 13896T; 6, A. stellipolaris LMG 21861T; 7, A. hispanica LMG 22958T. All data were taken from this study except where indicated. All strains were positive for production of oxidase, catalase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase and acid phosphatase, acid production from glucose, cellobiose, maltose and lactose, and hydrolysis of aesculin. All strains were negative for Gram staining, production of indole, urease, arginine dihydrolase, β-glucosidase, β-glucuronidase, cystine arylamidase, α-mannosidase and α-fucosidase, acid production from rhamnose, and assimilation of formic acid, itaconic acid and propionic acid. +, Positive; –, negative; W, weakly positive.
identification of isoprenoid quinones, the quinones of the isolates were extracted from freeze-dried cells according to the method of Nishijima et al. (1997) and analysed by HPLC (Waters 600 series). Ubiquinone-8 (Q-8) was identified as the only quinone of strain 9a2T. For fatty acid analysis, strain 9a2T and the reference strains were grown in MB at 27 °C for 24 h. Whole-cell fatty acids were extracted from 100 mg of freeze-dried cells esterified by acid methanolysis and analysed by GC (model GC 353; GL Sciences) equipped with a 0.25 mm (i.d.) × 100 m, 0.2 μm film SP-2560 column (Spelco). Fatty acids were identified by comparing them with fatty acid methyl esters purchased from Spelco and GL Sciences, and using GC/MS (model INCOS 50; Thermo Scientific) connected to a model 3400 GLC machine (Varian). The fatty acid composition of strain 9a2T is detailed in Table 2. These data were obtained using the same growth conditions for all strains. The cellular fatty acid profiles of strain 9a2T and the type strains of A. macleodii, A. marina, A. litorea, A. australica, A. simiduii, A. stellipolaris and A. hispanica consisted mainly of C16:0 and C18:1ω9c. The fatty acid profile of strain 9a2T clearly resembles those determined for other marine genera of the Gammaproteobacteria, for example Pseudoalteromonas and Glaciecola (Ivanova et al., 2000).

Bacterial DNA was prepared according to the method of Marmur (1961). The DNA obtained was digested with nuclease P1 (Yama Shoyu) and resulting nucleotides were separated by HPLC (Tamaoka & Komagata, 1984). The DNA G+C content of strain 9a2T was 46.6 mol%, which is consistent with values reported for the genus Alteromonas (44–48 mol%; Bowman & McMeekin, 2005).

The level of DNA–DNA relatedness was determined fluorometrically according to the method of Ezaki et al. (1989) using photobiotin-labelled DNA probes and black microplates. DNA–DNA hybridizations between strain 9a2T and A. macleodii JCM 20772T, A. marina JCM 11804T, A. litorea JCM 12188T, A. australica CIP 109921T, A. simiduii JCM 11804T and A. macroleonis JCM 20772T, A. marina JCM 11804T, A. litorea JCM 12188T, A. australica CIP 109921T, A. simiduii JCM 13896T, A. stellipolaris LMG 21861T, A. hispanica LMG 22958T, ND, Not detected. Data are based on the results of this study.
JCM 13896T, A. stellipolaris LMG 21861T and A. hispanica LMG 22958T were performed. DNA–DNA hybridization data indicated that the new isolate was distinct from A. macroloidii JCM 20772T (49% relatedness), A. marina JCM 11804T (67%), A. litorea JCM 12188T (36%), A. australica CIP 109921T (36%), A. simidii JCM 13896T (23%), A. stellipolaris LMG 21861T (27%) and A. hispanica LMG 22958T (26%). Furthermore, A. macroloidii JCM 20772T and A. marina JCM 11804T were used to generate probes, and the DNA–DNA relatedness values with strain 9A2T were 21 and 68%, respectively. These are below the value of 70% that is considered to be the threshold for the delineation of species (Wayne et al., 1987).

On the basis of this polyphasic taxonomic analysis, strain 9A2T is considered to represent a novel species of the genus Alteromonas, for which the name Alteromonas gracilis sp. nov. is proposed.

**Description of Alteromonas gracilis sp. nov.**

*Alteromonas gracilis* (gra’ci. L. fem. adj. gracilis long and narrow).

Cells are rod-shaped (mean size, 2.5 × 0.46 μm), Gram-stain-negative and motile. Colonies are circular, smooth, convex and mucoid with entire margins. Positive for oxidase and catalase. Does not produce pigment or endospores. Growth occurs in media with 0.5–9% (w/v) NaCl, with the optimum at 1.5–2%. Temperature for growth ranges from 5 to 43 °C, with the optimum at about 37 °C. The pH for growth ranges from 6.0 to 9.0, with the optimum at pH 7.0–8.0. Produces EPS and H2S.

The major fatty acids are mainly of C16:1ω7c, C18:1ω9c and C16:0. The major isoprenoid quinone is ubiquinone-8 (Q-8).

The type strain is 9A2T (=JCM 30236T=NCIMB 14947T), which was isolated from sediment of the Pacific Ocean at a water depth of 6310 m. The DNA G + C content of the type strain is 46.6 mol% (HPLC).

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**References**


