Thalassobacter arenae sp. nov., isolated from sea sand in Korea

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A Gram-negative, short rod-shaped bacterium, strain GA2-M15^T, was isolated from a sea-sand sample at Homi Cape, Pohang city, Republic of Korea. 16S rRNA gene sequence analysis demonstrated that this isolate was unique, showing 95.9% sequence similarity to the type strain of *Thalassobacter stenotrophicus* and similarities of 94.0–95.2% to the type strains of species of the genera *Octadecabacter* (94.4–95.2%), *Jannaschia* (94.0–94.4%) and *Thalassobius* (94.0–94.7%). Chemotaxonomic characteristics (diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine as the major polar lipids and C_{18:1ω7c} as the predominant fatty acid) and DNA G + C content (56 mol%) were also similar to those of *Thalassobacter stenotrophicus*. 16S rRNA gene sequence similarity, physiological properties and some fatty acid components showed that strain GA2-M15^T could be differentiated from *Thalassobacter stenotrophicus*. On the basis of these results, strain GA2-M15^T is considered to represent a novel species of the genus *Thalassobacter*, for which the name *Thalassobacter arenae* sp. nov. is proposed. The type strain is GA2-M15^T (=KACC 12675^T =DSM 19593^T).

The genus *Thalassobacter* was described to accommodate a single bacterial strain isolated from Mediterranean seawater (Macián *et al.*, 2005). A subsequent polyphasic taxonomic approach revealed that the type strains of *Jannaschia cystaugens* (Adachi *et al.*, 2004) and *Thalassobacter stenotrophicus* (Macián *et al.*, 2005) were highly similar and should be considered to be members of the same species. Pujalte *et al.* (2005) thus proposed the unification of the two species as *Thalassobacter stenotrophicus* and provided an emended description of the genus *Thalassobacter*. Phylogenetically, this genus is a member of the *Alphaproteobacteria*, and is closely related to the genera *Jannaschia*, *Octadecabacter* and *Thalassobius*. *Thalassobacter* was characterized as comprising Gram-negative, strictly aerobic, chemo-organotrophic, slightly halophilic bacteria, with C_{18:1ω7c} as the major fatty acid

and phosphatidylglycerol, diphosphatidylglycerol and phosphatidylcholine as the major polar lipids.

Strain GA2-M15^T was isolated from a sea-sand sample collected from the coast of Homi Cape, Pohang city, Republic of Korea. Isolation of the strain was performed by using the standard dilution-plating method on marine agar 2216 (MA; Difco) kept at 30 °C for 10 days. The culture was maintained routinely on MA at 30 °C and was preserved as glycerol suspensions (15%, v/v) at –80 °C. Strain GA2-M15^T grew weakly on nutrient agar (NA), but did not grow on R2A, trypticase soy agar (TSA) or MacConkey agar (all from Difco). *Thalassobacter stenotrophicus* DSM 16310^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, and was used as a reference strain.

The methods described by Smibert & Krieg (1994) were used for Gram staining and to detect accumulation of polyhydroxybutyrate, the presence of catalase and oxidase, and hydrolysis of casein, DNA and starch. Hydrolysis of alginic acid (0.5%, w/v), CM-cellulose (0.1%, w/v), chitin from crab shells (1%, w/v), pectin (0.5%, w/v) and tyrosine (0.5%, w/v) was checked on MA after 14 days incubation. Cell morphology was studied by transmission

Abbreviation: Bchl *a*, bacteriochlorophyll *a*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GA2-M15^T is EU342372.

A transmission electron micrograph of a cell of strain GA2-M15^T and polar lipid profiles of strains GA2-M15^T and *Thalassobacter stenotrophicus* DSM 16310^T are available as supplementary material with the online version of this paper.

electron microscopy (model 912AB; LEO) and phase-contrast microscopy (AXIO; Zeiss), with cells grown for 3 days at 30 °C on MA. The temperature range for growth was tested on MA from 5 to 45 °C at increments of 5 °C. The pH range for growth was determined in the range pH 5–10 at increments of 1 pH unit by using marine broth buffered with citrate/phosphate buffer or Tris/HCl buffer (Breznak & Costilow, 1994). The salinity range for growth was tested on MA. The minimal amount of salts required for growth was determined on diluted MA containing 0.34, 0.68, 0.85, 1.02, 1.36, 1.7, 2.04, 2.55 and 3.06 % (w/v) total salts (dilution factors of 0.1, 0.2, 0.25, 0.3, 0.4, 0.5, 0.6, 0.75 and 0.9, respectively). The loss of nutrients and agar by dilution was compensated for by adding the appropriate amounts of peptone, yeast extract and agar. The highest salinity that supported growth was determined on MA plus NaCl up to 6, 7, 8, 9 and 10 % (w/v) total salinity [2.6, 3.6, 4.6, 5.6 and 6.6 % (w/v) NaCl, respectively]. To determine ionic requirements for growth, salt-tolerance agar [STA: 1 % (w/v) tryptone, 0.3 % (w/v) yeast extract and 1.5 % (w/v) agar] was supplemented with the following combinations of salts: (i) 2 % (w/v) NaCl; (ii) 2 % (w/v) NaCl, 0.9 % (w/v) MgCl₂·6H₂O, 0.2 % (w/v) CaCl₂·2H₂O and 0.06 % (w/v) KCl (all concentrations equal to those found in MB for these four salts); and (iii) 2 % (w/v) NaCl, 0.9 % (w/v) MgSO₄·7H₂O, 0.2 % (w/v) CaCl₂·2H₂O and 0.06 % (w/v) KCl. Anaerobic growth was tested on MA incubated in an anaerobic jar (BBL). Carbon utilization was tested on basal medium agar [BMA: 50 mM, Tris/HCl, pH 7.5; 190 mM, NH₄Cl; 0.33 mM K₂HPO₄·3H₂O; 0.1 mM FeSO₄·H₂O; and 1.5 % Bacto agar (Difco) in half-strength artificial seawater; Baumann & Baumann, 1981] containing 0.2 % carbon source. BMA was supplemented with 0.01 % yeast extract. Tests in the API 20NE and API ZYM (bioMérieux) commercial systems were performed according to the manufacturer's instructions.

Cells of strain GA2-M15^T were Gram-negative, short rods with polar flagella (see Supplementary Fig. S1 in IJSEM Online). In the minimal salinity test for growth, restriction of growth was observed from 0.85 % NaCl (w/v), and no growth was observed in the presence of 0.34 % NaCl (w/v). In the maximum salinity test for growth, restriction of growth was observed from 8 % (w/v) total salinity, and no growth was observed above 10 % (w/v) total salinity. In tests of ionic requirements for growth, strain GA2-M15^T showed good growth on STA with four added salts [media (ii) and (iii)] whereas it was not able to grow on STA or on STA plus 2 % NaCl [medium (i)]. Assimilation of substrates within the API 20NE system was not observed, indicating that the minimal medium as used here was not appropriate for tests of growth. A comparison of the phenotypic properties of strain GA2-M15^T and *Thalassobacter stenotrophicus* DSM 16310^T is given in Table 1. Strain GA2-M15^T was more versatile as regards substrate utilization and enzyme activities than *Thalassobacter stenotrophicus*.

For the measurement of bacteriochlorophyll *a* (Bchl *a*), cells were grown in the dark on MA and MB medium.

Table 1. Differential phenotypic characteristics between strain GA2-M15^T and *Thalassobacter stenotrophicus* DSM 16310^T

The two strains are strictly aerobic, motile and positive for polyhydroxybutyrate accumulation, catalase and oxidase. They assimilate D-glucose, L-glutamate, β-hydroxybutyrate, D-mannitol, pyruvate and L-serine, but not L-alanine, L-arabinose, L-aspartate, cellobiose, glycine, L-histidine, lactose, L-leucine, maltose, L-rhamnose, D-ribose, sucrose, L-threonine or trehalose. The two strains are positive for aesculin hydrolysis, but negative for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase and gelatin hydrolysis (API 20NE test strips). +, Positive; –, negative or not detected (or comprising <1 % of the total fatty acids).

Characteristic	GA2-M15 ^T	<i>Thalassobacter stenotrophicus</i> DSM 16310 ^T
DNA G + C content (mol%)	56	59*
β-Galactosidase	+	–
Assimilation of:		
Citrate	+	–
D-Fructose	+	–†
D-Galactose	+	–
Glycerol	+	–†
D-Mannose	+	–
Melibiose	+	–
Propionate	+	–
Enzyme activities (API ZYM)		
Alkaline phosphatase	–	+
Esterase lipase (C8)	+	–
α-Glucosidase	+	–
β-Glucosidase	+	–
Fatty acids (%)		
C _{10:0} 3-OH	3.7	5.4
C _{16:0}	10.4	1.1
C _{18:0}	1.2	4.5
C _{18:1ω7c}	74.3	70.6
C _{18:1ω7c} 11-methyl	5.9	6.7
C _{18:1ω9c}	–	2.7
Summed feature 7‡	–	2.5
Unknown 11.799	3.0	4.5

*Data from Macián *et al.* (2005).

†Reported as positive by Pujalte *et al.* (2005).

‡Summed feature 7 comprises unknown 18.846 and/or C_{19:1ω6c}.

Extraction of Bchl *a* was attempted with acetone (Takaichi *et al.*, 1991), acetone/methanol (1:1, v/v) (Lafay *et al.*, 1995) and acetone/methanol (7:2, v/v) (Hiraishi *et al.*, 1998). However, cells were not disrupted in any of these solvents, and extraction of Bchl *a* was not possible.

Analyses of whole-cell fatty acid patterns followed the methods given for the MIDI system (Microbial ID) (Kroppenstedt, 1985; Sasser, 1990) after cells were grown on MA medium for 4 days at 30 °C. Polar lipids were analysed according to Minnikin *et al.* (1984). The DNA G + C content was determined by using HPLC (Mesbah

et al., 1989). The predominant cellular fatty acid of strain GA2-M15^T was C_{18:1}ω7c (74.3 %), followed by C_{16:0} (10.4 %) and lesser amounts of C_{18:1}ω7c 11-methyl (5.9 %), C_{10:0} 3-OH (3.7 %) and unknown 11.799 (3.0 %). Strain GA2-M15^T contained a greater proportion of C_{16:0} than *Thalassobacter stenotrophicus* DSM 16310^T (10.4 and 1.1 %, respectively). The polar lipid profiles of the two strains (Supplementary Fig. S2) were similar, containing diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine. The presence of phosphatidylethanolamine in *Thalassobacter stenotrophicus* DSM 16310^T in the present study was in contrast to the results of Pujalte *et al.* (2005). The DNA G+C content of strain GA2-M15^T was 56 mol%, compared with 59 mol% for *Thalassobacter stenotrophicus* DSM 16310^T.

Genomic DNA was extracted by using a DNeasy Tissue kit (Qiagen) following the manufacturer's instructions. Sequencing of the 16S rRNA gene was performed as described by Kwon *et al.* (2003). The 16S rRNA gene sequence of strain GA2-M15^T determined in this study was a continuous stretch of 1396 bp. Multiple alignments were performed by using the program CLUSTAL W (Thompson *et al.*, 1994). Evolutionary distances were calculated by using Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were constructed according to the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods by using the program MEGA3 (Kumar *et al.*, 2004), with bootstrap values based on 1000 replications (Felsenstein, 1985). Based on 16S rRNA gene sequences, strain GA2-M15^T was related most closely to the type strain of *Thalassobacter stenotrophicus* (95.9 % similarity). Members of the genera *Octadecabacter* (94.4–95.2 % 16S rRNA gene sequence similarity), *Jannaschia*

(94.0–94.4 %) and *Thalassobius* (94.0–94.7 %) were also revealed as close phylogenetic neighbours of strain GA2-M15^T. According to the neighbour-joining tree (Fig. 1), strain GA2-M15^T clustered with *Thalassobacter stenotrophicus* CECT 5294^T, albeit with a rather low bootstrap value (30 %).

On the basis of the results presented here, strain GA2-M15^T is considered to represent a novel species of the genus *Thalassobacter*, for which the name *Thalassobacter arenae* sp. nov. is proposed.

Description of *Thalassobacter arenae* sp. nov.

Thalassobacter arenae (a.re'nae. L. gen. n. *arenae* of sand).

Cells are strictly aerobic, Gram-negative, short rods (0.7–1.2 µm wide and 1.2–2.4 µm long). Motile by means of polar flagella. Accumulates polyhydroxybutyrate. Catalase- and oxidase-positive. Does not reduce nitrate or ferment glucose. Colonies are deep-brown, circular and with clear margins. Grows at 5–35 °C (optimum 30 °C) and at pH 6–9 (optimum pH 7). Grows in the presence of 0.85–8 % NaCl (w/v), but not at or below 0.34 % or at or above 10 % NaCl (w/v). Hydrolyses aesculin and hydrolyses tyrosine weakly, but does not hydrolyse alginate, casein, chitin, CM-cellulose, DNA, gelatin, pectin, starch or urea. Assimilates citrate, D-fructose, D-galactose, D-glucose, L-glutamate, glycerol, β-hydroxybutyrate, D-mannitol, D-mannose, melibiose, propionate, pyruvate, L-serine, L-tyrosine and D-xylose, but not L-alanine, L-arabinose, L-aspartate, cellobiose, glycine, L-histidine, lactose, L-leucine, maltose, L-rhamnose, D-ribose, sucrose, L-threonine or trehalose. Positive for β-galactosidase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase,

Fig. 1. Neighbour-joining phylogenetic tree constructed based on a comparative analysis of 16S rRNA gene sequences showing the relationships between strain GA2-M15^T and related species. Bootstrap values (expressed as percentages of 1000 replications) of >50 % are shown at branch points. Circles indicate branches replicated in the maximum-parsimony tree. Bar, 0.01 substitutions per site.

but negative for indole production, arginine dihydrolase, alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, α -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase (API 20NE and API ZYM test strips). The major fatty acids are C_{18:1} ω 7c and C_{16:0}. The predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine. The G+C content of the DNA of the type strain is 56.0 mol%.

The type strain, GA2-M15^T (=KACC 12675^T =DSM 19593^T), was isolated from a sea-sand sample from Homi Cape, Pohang city, Republic of Korea.

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