Marinobacter salsuginis sp. nov., isolated from the brine–seawater interface of the Shaban Deep, Red Sea

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Two moderately halophilic Gram-negative bacteria were isolated from a sample taken from the brine–seawater interface of the Shaban Deep in the Red Sea. Phylogenetic analysis of the 16S rRNA gene sequence showed that these organisms represent a novel species of the genus *Marinobacter*. Cells of the new isolates formed non-pigmented colonies and were motile by means of a single polar flagellum. Strains SD-14B^T and SD-14C grew optimally at 35–37 °C, in 5 % NaCl and at pH 7.5–8.0. The organisms were aerobic, but reduced nitrate to nitrogen under anaerobic conditions. Acid was produced from only a few carbohydrates. Ubiquinone 9 was the major respiratory quinone. The major fatty acids of strains SD-14B^T and SD-14C were C_{16:0}, C_{18:1}ω9c, summed feature 3 (C_{16:1}ω6c/C_{16:1}ω7c) and C_{12:0} 3-OH. The DNA G + C contents were 55.9 and 55.7 mol%, respectively. On the basis of the phylogenetic analyses and physiological and biochemical characteristics, it is proposed that strains SD-14B^T and SD-14C represent a novel species of the genus *Marinobacter*, with the name *Marinobacter salsuginis* sp. nov. The type strain is strain SD-14B^T (= DSM 18347^T = LMG 23697^T).

Several brine-filled deeps are present in the Red Sea. The water in the deeps is extremely saline and anoxic and well-defined brine–seawater interfaces are present, typically with steep gradients of salinity, temperature, density, O₂ and pH. The density gradient created at the brine–seawater interface also acts as an *in situ* particle trap for organic and inorganic materials from the seawater (Eder *et al.*, 2002; Ryan *et al.*, 1969; Scholten *et al.*, 2000). Such interfaces thus represent unique and highly peculiar environments, constituting a very specific biotope that might harbour extensive microbial diversity, as suggested by recent studies (Antunes *et al.*, 2003; Eder *et al.*, 1999, 2001, 2002).

New samples for microbiological studies were retrieved from the northern-most brine-filled deeps of the Red Sea during RV *Meteor* Cruise M 52/3 in 2002 (Antunes, 2003).

As a result of a subsequent microbial diversity assessment study, focusing on the brine–seawater interface of the Shaban Deep, we obtained several isolates, two of which were assigned to the genus *Marinobacter* based on 16S rRNA gene sequence analysis. This genus, belonging to the *Gammaproteobacteria*, currently comprises 15 species with validly published names: *Marinobacter algicola*, *Marinobacter aquaeolei* [later heterotypic synonym of *Marinobacter hydrocarbonoclasticus* (Márquez & Ventosa, 2005)], *Marinobacter bryozoorum*, *Marinobacter daepoensis*, *Marinobacter excellens*, *Marinobacter flavimaris*, *Marinobacter gudaonensis*, *Marinobacter hydrocarbonoclasticus*, *Marinobacter koreensis*, *Marinobacter lipolyticus*, *Marinobacter litoralis*, *Marinobacter lutaoensis*, *Marinobacter maritimus*, *Marinobacter sediminum* and *Marinobacter vinifirmus* (Gauthier *et al.*, 1992; Gorshkova *et al.*, 2003; Green *et al.*, 2006; Gu *et al.*, 2007; Kim *et al.*, 2006; Liebgott *et al.*, 2006; Martín *et al.*, 2003; Romanenko *et al.*, 2005; Shieh *et al.*, 2003; Shivaji *et al.*, 2005; Yoon *et al.*, 2003, 2004). In this study we present physiological, biochemical and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SD-14B^T is EF028328.

phylogenetic data to show that isolates SD-14B^T and SD-14C represent a novel taxon for which we propose the name *Marinobacter salsuginis* sp. nov.

Strains SD-14B^T and SD-14C were isolated from samples from the Shaban Deep, Red Sea, during *Meteor* Cruise M 52/3, in 2002. Enrichment cultures were established from sample SD-14 (15.2 % NaCl, pH 6.0, 23.8 °C *in situ* temperature), collected at a depth of 1327 m from the brine–seawater interface of the eastern basin (26° 12.7' N 35° 21.5' E). Tubes (capacity 28 ml) were filled with 10 ml Marine broth 2216 (Difco), inoculated with 0.2 ml of the original sample and incubated at 30 °C. When turbidity was observed (after approximately 1 week), the cultures were streaked on Marine agar. The cultures were purified by subculture using the same medium and the isolates were stored at –70 °C in growth medium with 15 % (w/v) glycerol. *Thermus* medium with 5 % NaCl (Williams & da Costa, 1992) was used for the majority of the tests because of the high turbidity of Marine broth and the consequent difficulty in recording results. The type strains of *M. algicola* (DSM 16394^T), *M. bryozorum* (DSM 15401^T), *M. daepoensis* (DSM 16072^T), *M. excellens* (CIP 107686^T), *M. flavimaris* (DSM 16070^T), *M. hydrocarbonoclasticus* (DSM 8798^T), *M. litoralis* (CIP 108099^T), *M. lipolyticus* (DSM 15157^T), *M. lutaoensis* (CIP 108251^T), *M. maritimus* (CIP 108870^T) and *M. sediminum* (DSM 15400^T) were used for comparative purposes.

Unless stated otherwise, all morphological examinations and biochemical and tolerance tests were performed as described previously (Santos *et al.*, 1989; Nunes *et al.*, 1992) using *Thermus* liquid medium and *Thermus* agar containing 5 % (w/v) NaCl at pH 7.5, with incubation at 37 °C for up to 5 days. The NaCl range for growth of the organisms was determined in liquid medium containing 0–20 % (w/v) NaCl, in a reciprocal shaker. The temperature range for growth was determined using the same medium at 10–50 °C. The pH range for growth was determined at 37 °C using the same medium with 50 mM MES, HEPES, TAPS, CAPSO and CAPS at pH 6–10.

Single carbon source assimilation tests were performed using a minimal medium composed of *Thermus* basal salts containing 5 % (w/v) NaCl to which filter-sterilized ammonium chloride (0.05 %, w/v) and the carbon source (0.1 %, w/v) were added. Growth of the strains was examined by measuring the turbidity of cultures incubated at 37 °C in 20 ml screw-capped tubes containing 10 ml medium for up to 5 days. The same procedure was used to test degradation of aliphatic hydrocarbons, but the cultures were incubated for up to 3 weeks. Acid production and enzymic tests were performed using the API 50 CHB/E system and API ZYM test strips (bioMérieux), respectively, as recommended by the manufacturer, but with the salinity adjusted to 5 % (w/v) NaCl and incubation at 37 °C for up to 5 days. The ability of the strains to grow with several electron acceptors was examined using *Thermus* basal salts containing 5 % (w/v) NaCl at 37 °C and pH 7.5. Pyruvate and glucose (30 mM)

were used as electron donors to examine growth under an N₂ atmosphere, coupled to the reduction of nitrate, nitrite, sulfate, thiosulfate, sulfur and Fe(III) (each at 10 mM, except nitrite which was at 1.0 mM). Control cultures without an electron acceptor were also tested for growth.

Lipoquinones were extracted from freeze-dried cells and purified by TLC as described by Tindall (1989). Cultures for fatty acid analysis were grown on Marine agar, containing 5.0 % (w/v) NaCl, in sealed plastic bags submerged in a waterbath at 37 °C for 48 h. Extraction, identification and quantification of the fatty acid methyl esters, as well as numerical analysis of the fatty acid profiles, were performed by using the standard MIS library Generation software (Microbial ID).

DNA for the determination of the G + C content was isolated as described by Nielsen *et al.* (1995). The G + C content of the DNA was determined by using HPLC, as described by Mesbah *et al.* (1989). Extraction of genomic DNA for 16S rRNA gene sequence determination, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described previously (Rainey *et al.*, 1996). Purified products were electrophoresed using a model 310 Genetic Analyzer (Applied Biosystems). The 16S rRNA gene sequences were aligned with representative reference sequences of members of the genus *Marinobacter* and related taxa using MEGA version 3.1 (Kumar *et al.*, 2004). The method of Jukes & Cantor (1969) was used to calculate evolutionary distances. Phylogenetic dendrograms and bootstrap analyses were generated using various algorithms contained in the PHYLIP package (Felsenstein, 1993).

16S rRNA gene sequence analysis revealed that strains SD-14B^T and SD-14C were members of the *Gammaproteobacteria*, being most closely related to species of the genus *Marinobacter* (93.3–98.0 % pairwise 16S rRNA gene sequence similarity). The 16S rRNA gene sequences of strains SD-14B^T and SD-14C were identical. These 16S rRNA gene sequences showed highest similarity to those of *M. algicola* (97.9 %), *M. flavimaris* (97.8 %), *M. lipolyticus* (97.6 %) and *M. sediminum* (97.6 %). Fig. 1 shows the equidistant branching of these species, which was supported by low bootstrap values (< 50 %).

The results of the physiological and chemotaxonomic characterizations are given in Table 1 and in the species description. Several physiological and biochemical characteristics distinguished strains SD-14B^T and SD-14C from the type strains of other species of the genus *Marinobacter* (Table 1). For example, the new isolates were able to grow on glucose and glycerol but were not able to use the other carbohydrates and polyols examined. On the other hand, the organism used the majority of the organic acids tested but only a few amino acids. Some of the aliphatic hydrocarbons examined, namely n-hexadecane, dodecane, n-decane, heptane, hexane and petroleum ether, were assimilated by strains SD-14B^T and SD-14C. Interestingly, petroleum-impregnated sediments have been retrieved

Fig. 1. Phylogenetic dendrogram based on 16S rRNA gene sequence comparisons. The dendrogram was reconstructed from evolutionary distances using the neighbour-joining method. Bar, 1 inferred nucleotide substitution per 100 nucleotides. *Microbulbifer hydrolyticus* DSM 11525^T was used as an outgroup.

Table 1. Characteristics that distinguish strain SD-14B^T (*Marinobacter salsuginis* sp. nov.) from phylogenetically related species

Data were obtained from Green *et al.* (2006), Kim *et al.* (2006), Yoon *et al.* (2004) and this study. All taxa degraded Tween 80, were unable to use sucrose or D-mannose and contained ubiquinone 9. +, Positive; –, negative; W, weak reaction; ND, not determined.

Characteristic	Strain SD-14B ^T	<i>M. algicola</i>	<i>M. flavimaris</i>
Temperature range (optimum) for growth (°C)	10–45 (35–37)	5–40 (25–30)	4–45 (37)
NaCl range (optimum) for growth (%)	1–20 (5)	1–9 (3–6)	1–20 (2–6)
Presence of:			
Valine arylamidase	W	ND	–
Cystine arylamidase	W	ND	–
Degradation of:			
Casein	+	–	–
Gelatin	+	–	–
Starch	–	+	–
Fermentation of D-glucose	+	ND	–
Utilization of:			
D-Glucose	+	+	–
D-Fructose	–	+	+
Glycerol	+	+	–
Citric acid	–	+	–
L-Alanine	+	+	–
L-Glutamate	+	+	–
n-Hexadecane	+	+	–
Nitrate reduction	+	–	+
Nitrite reduction	+	–	–
DNA G + C content (mol%)	55.9	57.0	58.0

previously from the Shaban Deep (Michaelis *et al.*, 1990), which could serve as carbon sources for these organisms. The utilization of aliphatic hydrocarbons has also been reported for other species of this genus, with some minor differences being observed in the assimilation profiles.

Ubiquinone 9 was the major respiratory quinone. The fatty acids of strains SD-14B^T and SD-14C and their relative proportions were consistent with the inclusion of the strains within the genus *Marinobacter*. A standardized analysis of the fatty acid methyl ester profiles of recognized species of the genus *Marinobacter* was performed to assess small differences among the organisms. Interestingly, a good correlation was observed between data from the phylogenetic and fatty acid methyl ester analyses (data not shown).

The major fatty acids of strains SD-14B^T and SD-14C were C_{16:0}, C_{18:1ω9c}, summed feature 3 (C_{16:1ω6c}/C_{16:1ω7c}) and C_{12:0} 3-OH. These fatty acids are also predominant components of other species of the genus *Marinobacter* and

are present in similar relative proportions in most species, except *M. bryozorum* (Table 2). However, some differences exist that allow differentiation of many of the species from each other. The fatty acid profile of strains SD-14B^T and SD-14C was very similar to those of the type strains of *M. sediminum* and *M. flavimaris*, but these organisms could be easily distinguished from each other by physiological and biochemical characteristics.

Our results show that strains SD-14B^T and SD-14C can be distinguished from other recognized species of the genus *Marinobacter*. We therefore propose that the two strains represent a novel species, with the name *Marinobacter salsuginis* sp. nov.

Description of *Marinobacter salsuginis* sp. nov.

Marinobacter salsuginis (sal.su'gi.nis. L. gen. n. *salsuginis* from salt water, brine, pertaining to the environment from which the strain was isolated).

Table 2. Fatty acid compositions (%) of *Marinobacter* strains

Strains: 1, SD-14B^T/SD-14C (*Marinobacter salsuginis* sp. nov.); 2, *M. algicola* DG893^T; 3, *M. flavimaris* SW-145^T; 4, *M. sediminum* R65^T; 5, *M. lipolyticus* SM-19^T; 6, *M. maritimus* CK 47^T; 7, *M. gudaonensis* SL014B61A^T (grown at 28 °C for 72 h; data from Gu *et al.*, 2007); 8, *M. bryozorum* 50-11^T; 9, *M. koreensis* DD-M3^T (grown at 28 °C for 48 h; data from Kim *et al.*, 2006); 10, *M. lutaoensis* T5054^T; 11, *M. hydrocarbonoclasticus* SP.17^T; 12, *M. daepoensis* SW-156^T; 13, *M. excellens* KMM 3809^T; 14, *M. litoralis* SW-45^T. Values for fatty acids present at levels of less than 0.5 % are not shown.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14
C _{10:0}		0.7		2.1	1.6			1.1	1.2	0.6	1.4	0.8		3.2
C _{12:0}	7.3	7.6	7.7	5.3	7.7	7.9	2.8	5.0	8.6	6.8	5.0	6.8	5.7	5.0
C _{11:0} 3-OH	0.5	0.8	0.7	1.0	0.7							0.5	0.9	
C _{13:0}												0.5	1.5	
C _{12:0} 3-OH	9.3	10.6	10.2	9.8	12.6	10.3	3.4	10.8	8.3	9.5	9.8	9.8	10.1	10.5
C _{14:0}	1.1	0.5	0.9	0.7	0.6		1.3	0.8	2.1	3.0	2.2	1.7	3.1	1.8
C _{13:0} 3-OH													2.0	0.5
C _{15:0} iso													0.8	
C _{16:0} N alcohol							7.1							
C _{16:1ω7c} alcohol			0.5										0.6	
C _{16:1ω9c}	10.5	5.4	9.1	7.5	11.1	4.7	6.4	2.2	11.3	14.9	7.5	12.4	9.7	13.0
Summed feature 3*	13.1	17.6	12.5	14.9	5.2	16.7	7.3	0.7	20.5	3.5	5.1	5.9	4.4	5.7
C _{16:1ω5c}										0.5			0.5	
C _{16:0}	22.9	25.5	22.4	20.6	23.3	21.9	21.2	14.8	23.5	27.7	22.6	22.0	15.9	2.2
C _{16:0} 10-methyl	2.6	4.6	0.9	2.8	4.5	2.2	2.5		1.6	0.9	1.4	1.4	1.3	
C _{17:0}	0.5	0.7	0.8	1.3									0.9	0.7
C _{17:1 ω8c}	3.8	2.7	4.2	4.9	2.8	1.4	1.1	1.7	2.7	1.9	3.2	4.1	6.7	2.2
C _{17:0}	3.4	2.3	3.2	3.9	3.4	1.1	0.9	4.0	1.0	2.1	3.1	5.3	5.5	4.6
C _{17:0} 10-methyl				0.9			4.5							
C _{18:3ω6c} (6,9,12)							8.5							
C _{18:1ω9c}	17.2	8.8	17.3	13.6	15.6	24.1	20.3	43.4	11.1	19.8	31.5	23.4	26.1	25.6
Summed feature 8*	3.0	6.5	1.3	3.9	4.3	2.3	5.4	0.9	2.6	0.7	1.6	1.3		0.9
C _{18:0}	2.9	3.8	3.0	5.0	3.9	5.3	5.6	5.5	1.2	2.9	2.7	3.1	1.7	4.7
Summed feature 7*							7.8			2.1				
C _{19:0} cyclo ω8c			2.0											

*Summed features: 3, C_{16:1ω6c}/C_{16:1ω7c}; 7, C_{19:0} cyclo ω10c/C_{19:1ω6c}; 8, C_{18:1ω6c}/C_{18:1ω7c}.

Gram-negative, non-spore-forming, rod-shaped cells (1 µm in width and 2–4 µm in length). Motile by means of a single polar flagellum. Colonies on Marine agar are round and whitish (2–3 mm). NaCl is required for growth; the NaCl range for growth is 1–20% (w/v), with optimum growth occurring at about 5% (w/v) NaCl. Growth occurs at 10–45 °C (optimum, 35–37 °C). pH range for growth is about 6.5–9.5 (optimum, pH 7.5–8.0). Heterotrophic and facultatively anaerobic. Grows without yeast extract or growth factors. Produces acid from glycerol, D-glucose, D-fructose and 5-ketogluconate; acid is not produced from D-melibiose, D-lactose, maltose or mannitol or any of the other substrates tested. Denitrifies in medium supplemented with pyruvate or D-glucose. Oxidase- and catalase-positive. Gelatin, casein and Tweens 20, 40, 60 and 80 are hydrolysed. Arbutin, DNA, aesculin, hippurate, starch and xylan are not hydrolysed. Acetate, glucose, lactate, malate, fumarate, glycerol, L-alanine, L-glutamate, L-glutamine, L-phenylalanine, proline, pyruvate, succinic acid and 2-oxoglutarate are assimilated. Fructose, galactose, mannose, L-sorbose, L-rhamnose, ribose, xylose, L-arabinose, maltose, lactose, cellobiose, melezitose, raffinose, sucrose, ribitol, sorbitol, arabitol, mannitol, erythritol, xylitol, glucuronate, citrate, formic acid, glycine, aspartate, arginine, asparagine, histidine, lysine, methionine, serine, tryptophan, ethanol, benzoate and methanol are not used. Aliphatic hydrocarbons used include n-decane, n-hexadecane, heptane, hexane and petroleum ether, but L-chlorobutane, dodecane and toluene are not used. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase are detected. Weak reactions are also detected for valine arylamidase, cystine arylamidase and acid phosphatase. Major cellular fatty acids are C_{16:0}, C_{18:1ω9c}, summed feature 3 (C_{16:1ω6c}/C_{16:1ω7c}) and C_{12:0} 3-OH. Ubiquinone 9 is the major isoprenoid quinone. The DNA G + C content of the type strain is 55.9 mol%.

Strains SD-14B^T and SD-14C were isolated from the brine-seawater interface of the Shaban Deep, Red Sea. The type strain is SD-14B^T (= DSM 18347^T = LMG 23697^T).

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