Halomonas zhanjiangensis sp. nov., a halophilic bacterium isolated from a sea urchin

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A novel Gram-negative, slightly halophilic, catalase-positive, oxidase-negative, obligately aerobic, non-sporulating rod-shaped bacterium, designated strain JSM 078169^T, was isolated from a sea urchin (*Hemicentrotus pulcherrimus*) collected from the South China Sea. Growth occurred with 1–20 % (w/v) total salts (optimum, 3–5 %), at pH 6.0–10.5 (optimum, pH 7.5) and at 4–40 °C (optimum, 25–30 °C). The major cellular fatty acids were C_{18:1}ω7c, C_{16:0} and C_{12:0} 3-OH. The predominant respiratory quinone was Q-9 and the genomic DNA G + C content was 55.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain JSM 078169^T should be assigned to the genus *Halomonas*. The sequence similarities between the isolate and the type strains of members of the genus *Halomonas* were in the range 92.4–97.0 %. The combination of phylogenetic analysis, DNA–DNA relatedness, phenotypic characteristics and chemotaxonomic data supported the view that strain JSM 078169^T represents a novel species of the genus *Halomonas*, for which the name *Halomonas zhanjiangensis* sp. nov. is proposed, with JSM 078169^T (=CCTCC AB 208031^T=DSM 21076^T=KCTC 22279^T) as the type strain.

The genus *Halomonas*, belonging to the family *Halomonadaceae* (Franzmann *et al.*, 1988; Dobson & Franzmann, 1996; Ntougias *et al.*, 2007; Ben Ali Gam *et al.*, 2007), was first proposed by Vreeland *et al.* (1980) with the description of *Halomonas elongata*. In total, 58 species of the genus *Halomonas* have been described at the time of writing, including the recently described *Halomonas korlensis* (Li *et al.*, 2008), *Halomonas salifodinae* (Wang *et al.*, 2008) and *Halomonas daqingensis* (Wu *et al.*, 2008). The members of this genus comprise halophilic/halotolerant, chemo-organotrophic, Gram-negative rods and are widely distributed in saline habitats (Vreeland *et al.*, 1980; Dobson & Franzmann 1996; Mata *et al.*, 2002; Romanenko *et al.*, 2002; Arahall *et al.*, 2007; Xu *et al.*, 2007). *Halomonas halocynthiae* (Romanenko *et al.*, 2002) was the first described *Halomonas* species isolated from

marine invertebrates. During the investigation of the diversity of the microbial population of invertebrates inhabiting the South China Sea (Chen *et al.*, 2009a, b; Xiao *et al.*, 2009), a novel *Halomonas*-like strain, designated JSM 078169^T, was isolated from a sea urchin (*Hemicentrotus pulcherrimus*) collected from a tidal flat of Naozhou Island, near a southern Chinese city, Zhanjiang. Based on the results of a polyphasic taxonomic study, this strain is considered to represent a novel species of the genus *Halomonas*.

Strain JSM 078169^T was isolated from homogenates of a sea urchin (*Hemicentrotus pulcherrimus*) by plating 1:10 serial dilutions of the sample on marine agar 2216 (MA; Difco) cultivated at 28 °C for 1 week. After primary isolation and purification, the isolate was preserved both on MA slants at 4 °C and in marine broth 2216 (MB; Difco) supplemented with 20 % (v/v) glycerol at –80 °C. Unless otherwise indicated, morphological and physiological studies were performed with cells grown on MA at

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JSM 078169^T is FJ429198.

pH 7.5 and 28 °C. Four reference strains, *Halomonas variabilis* DSM 3051^T, *Halomonas boliviensis* DSM 15516^T, *Halomonas neptunia* DSM 15720^T and *Halomonas sulfidaeris* DSM 15722^T were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). Cell morphology was examined by using light microscopy (model BH 2; Olympus). Gram staining was carried out by using the standard Gram reaction combined with the KOH lysis test method (Gregersen, 1978). Flagella were stained according to the method of Leifson (Smibert & Krieg, 1994). Accumulation of poly- β -hydroxybutyrate was determined by using the Sudan Black staining method (Smibert & Krieg, 1994) under a light microscope. Growth was tested at various temperatures (0, 4 °C and 5–45 °C, in increments of 5 °C), pH (5.0–11.0, in increments of 0.5) and total-salt contents (0, 0.1 and 0.5 %, w/v, and 1–20 %, w/v, at increments of 1 %) on MA and in MB, prepared according to the formula of Atlas (1993). Growth was also tested with NaCl as the sole salt. Methyl red and Voges–Proskauer tests, H₂S production from L-cysteine, hydrolysis of aesculin, indole production, nitrate and nitrite reduction, phenylalanine deaminase and lysine and ornithine decarboxylases were tested as recommended by Smibert & Krieg (1994).

Hydrolysis of casein, cellulose, DNA, gelatin, starch, Tweens 20, 40, 60 and 80 and urea were determined as described by Cowan & Steel (1965). Motility, growth under anaerobic conditions, antibiotic-susceptibility tests, and catalase and oxidase activities were determined as described previously (Chen *et al.*, 2007). Other enzymic activities were tested by using the API ZYM system (bioMérieux) according to the manufacturer's instructions except that the suspension medium was supplemented with 4 % (w/v) NaCl. Acid production from carbohydrates and utilization of substrates as sole carbon or nitrogen sources and other phenotypic characteristics were determined as recommended by Ventosa *et al.* (1982) and Mata *et al.* (2002).

Strain JSM 078169^T was able to grow with 1–20 % (w/v) total salts (optimum, 3–5 %) and at pH 6.0–10.5 (optimum, pH 7.5) and 4–40 °C (optimum, 25–30 °C). Colonies were yellow-pigmented, flat and non-translucent with glistening surfaces and circular/slightly irregular margins, 2–3 mm in diameter after incubation for 3–5 days at 28 °C on MA. Cells were Gram-negative, non-sporulating, catalase-positive, oxidase-negative, motile, aerobic rods. The results of other phenotypic tests are summarized in the species description and in Table 1.

Table 1. Characteristics that distinguish strain JSM 078169^T from phylogenetically related *Halomonas* species

Strains: 1, JSM 078169^T (*H. zhanjiangensis* sp. nov.; data from this study); 2, *H. variabilis* DSM 3051^T (Fendrich, 1988; Mata *et al.*, 2002); 3, *H. boliviensis* LC1^T (Quillaguamán *et al.*, 2004); 4, *H. neptunia* Eplumel^T (Kaye *et al.*, 2004); 5, *H. sulfidaeris* Esulfide1^T (Kaye *et al.*, 2004). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5
Colony pigmentation	Yellow	Cream	Cream	Cream	Cream
Oxidase	–	+	ND	+	+
Temperature range (optimum) (°C)	4–40 (25–30)	15–37 (32)	0–45 (25–30)	–1 to 35 (30)	–1 to 35 (20–35)
pH range (optimum)	6–10.5 (7.5)	6–9 (ND)	6–11 (7.5)	5–12 (7–8)	5–10 (7–8)
Salt range (optimum) (% w/v)	1–20 (3–5)	1–25 (10)	0–25 (5)	0.5–27 (2–3)	0.5–24 (2–3)
Nitrate reduction	+	–	+	+	+
Respiration on nitrate	–	–	ND	+	+
Facultative anaerobe	–	–	–	+	+
H ₂ S production	–	+	–	–	–
Acid production from:					
D-Fructose	+	–	ND	–	–
D-Galactose	–	–	ND	+	+
D-Glucose	+	–	ND	+	+
Maltose	+	–	ND	–	+
Sucrose	+	–	ND	–	–
Utilization of:					
Citrate	+	+	–	+	–
D-Glucose	+	+	+	+	–
Maltose	+	+	+	+	–
D-Mannose	–	+	+	+	–
L-Serine	+	+	ND	–	–
DNA G + C content (mol%)	55.8	61	51.4	57.3	56.0

Genomic DNA was isolated according to Hopwood *et al.* (1985) and the DNA G+C content was determined using the HPLC method (Mesbah *et al.*, 1989). The 16S rRNA gene was amplified and sequenced as described by Cui *et al.* (2001). The resulting 16S rRNA gene sequence was compared with sequences obtained from public databases (GenBank/EMBL/DDBJ) to find the most closely related species. Pairwise sequence similarities were calculated using a global alignment algorithm, implemented at the EzTaxon server (Chun *et al.*, 2007). Phylogenetic analysis was performed by using the software package MEGA version 4.1 (Tamura *et al.*, 2007) after multiple alignment of sequence data by using CLUSTAL_X (Thompson *et al.*, 1997). Distances were calculated using distance options according to Kimura's two-parameter model (Kimura, 1980) and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Maximum-parsimony (Kluge & Farris, 1969) and minimum-evolution (Felsenstein, 1997) trees (not shown) were generated by means of methods contained within the software package

MEGA version 4.1 (Tamura *et al.*, 2007). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by means of 1000 resamplings (Felsenstein, 1985). DNA-DNA hybridization was carried out by using photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989).

The DNA G+C content of strain JSM 078169^T was 55.8 mol%. An almost-complete 16S rRNA gene sequence (1446 bp) was determined. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain JSM 078169^T belonged to the genus *Halomonas*, being related most closely to the type strains of *H. variabilis* (sequence similarity 97.0%), *H. boliviensis* (96.9%), *H. neptunia* (96.7%) and *H. sulfidaeris* (96.5%). These five strains constituted an independent subcluster in the phylogenetic tree, in which strain JSM 078169^T occupied a distinct lineage branching at the periphery of the subcluster (Fig. 1). The sequence similarities between the isolate and the type strains of the other recognized *Halomonas* species were in

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence analysis and constructed using the neighbour-joining method showing the phylogenetic positions of strain JSM 078169^T and other related taxa. 'm' or 'p' indicate branches that were also found with the minimum-evolution (Felsenstein, 1997) or maximum-parsimony (Kluge & Farris, 1969) algorithms, respectively; asterisks indicate branches that were recovered with all three methods. Numbers at nodes are bootstrap percentages (>40%) based on a neighbour-joining analysis of 1000 resampled datasets. Bar, 2 substitutions per 100 nucleotides.

the range 92.4–97.0 %. To establish the precise taxonomic position of strain JSM 078169^T, DNA–DNA hybridizations were performed between the novel isolate and the type strains of *H. variabilis*, *H. boliviensis*, *H. neptunia* and *H. sulfidaeris*, and the levels of DNA–DNA relatedness between them were 19.5, 15.4, 13.6 and 18.6 %, respectively. These values were far below the threshold value of 70 % recommended by Wayne *et al.* (1987) for assignment of strains to the same species. Based on the results of the phylogenetic analysis and DNA–DNA hybridization, it was evident that strain JSM 078169^T represents a previously unknown species of the genus *Halomonas* (Wayne *et al.*, 1987; Stackebrandt & Goebel 1994).

Isoprenoid quinones were analysed by HPLC as described by Groth *et al.* (1996). Fatty acids were determined for the novel isolate, as well as for the four reference strains, *H. variabilis* DSM 3051^T, *Halomonas b.* DSM 15516^T, *H. neptunia* DSM 15720^T and *H. sulfidaeris* DSM 15722^T, as described by Sasser (1990) using the Microbial Identification System (MIDI; Microbial ID) with cells grown in MB in flasks on a rotary shaker (200 r.p.m.) at 28 °C for 3 days. Chemotaxonomic data for strain JSM 078169^T were consistent with its assignment to the genus *Halomonas*. The strain contained Q-9 (96.6 %) as the predominant respiratory quinone, with Q-7 (1.2 %), Q-8 (1.4 %) and Q-10 (0.7 %) present in minor amounts. The fatty acid profile of strain JSM 078169^T was similar to those of the type strains of the four phylogenetically related *Halomonas* species (Table 2). The major fatty acids of strain JSM 078169^T were C_{18:1}ω7c (48.9 %), C_{16:0} (17.0 %) and C_{12:0} 3-OH (10.7 %).

Table 2. Fatty acid compositions of strain JSM 078169^T and related *Halomonas* species

Strains: 1, JSM 078169^T (*H. zhanjiangensis* sp. nov.); 2, *H. variabilis* DSM 3051^T; 3, *H. boliviensis* DSM 15516^T; 4, *H. neptunia* DSM 15720^T; 5, *H. sulfidaeris* DSM 15722^T. Data are percentages of the total fatty acids. Fatty acids that represented <0.5 % for all strains are omitted. –, Not detected.

Fatty acid	1	2	3	4	5
C _{10:0}	0.5	2.4	3.4	2.2	2.0
C _{10:0} 3-OH	–	2.1	0.1	0.1	0.3
C _{12:0}	0.9	1.6	1.2	1.9	1.3
C _{12:0} 3-OH	10.7	12.6	8.5	11.1	15.0
C _{14:0}	9.2	3.6	1.1	0.5	0.5
iso-C _{16:0}	0.9	1.0	–	–	–
C _{16:1} ω6c/ω7c	8.2	3.9	13.9	3.9	9.5
C _{16:0}	17.0	13.1	20.7	20.1	8.3
anteiso-C _{17:0}	0.5	0.8	0.1	–	–
C _{17:1} ω8c	0.5	–	–	–	–
C _{17:0}	0.7	1.1	0.3	0.3	0.2
C _{17:0} cyclo	–	7.5	10.7	13.0	0.3
C _{18:1} ω7c	48.9	36.1	26.7	29.8	59.2
C _{19:0} cyclo ω8c	–	12.5	11.1	15.3	0.9

The results of the phylogenetic analysis and chemotaxonomic studies supported the view that strain JSM 078169^T should be assigned to the genus *Halomonas*. However, the yellow pigmentation and the absence of oxidase activity, as well as the ability to produce acids from D-fructose and sucrose, differentiated the novel isolate markedly from its phylogenetically related *Halomonas* species (Table 1). Strain JSM 078169^T could also be distinguished from its closest phylogenetic neighbour *H. variabilis* by their discriminative fatty acid profiles, in which C_{17:0} cyclo and C_{19:0} cyclo ω8c were not detected in the fatty acid pool of strain JSM 078169^T, whereas significant amounts of these two fatty acids were present in the type strain of *H. variabilis* (Table 2). Moreover, strain JSM 078169^T could be clearly distinguished from its closest phylogenetic neighbour by some other discriminative taxonomic markers, such as its capacity of nitrate reduction and acid production from D-glucose, maltose and sucrose and the comparatively higher margin of the DNA G+C content (~5 mol%) between them (Table 1). Together with the low levels of DNA–DNA relatedness between strain JSM 078169^T and the type strains of the phylogenetically related *Halomonas* species, the results of the polyphasic taxonomic study presented here allowed us to assign the isolate as representing a novel species, for which we propose the name *Halomonas zhanjiangensis* sp. nov.

Description of *Halomonas zhanjiangensis* sp. nov.

Halomonas zhanjiangensis (zhan.ji.ang.en'sis. N.L. fem. adj. *zhanjiangensis* pertaining to Zhanjiang, a city in China near where the sample was collected).

Cells are slightly halophilic, strictly aerobic, non-sporulating, catalase-positive, oxidase-negative, Gram-negative rods, approximately 0.7–1.4 µm wide and 1.5–2.5 µm long, occurring singly or as doublets and motile with peritrichous flagella. Accumulate poly-β-hydroxybutyrate, but do not produce exopolysaccharide. Colonies are yellow-pigmented, flat and non-translucent with glistening surfaces and circular/slightly irregular margins, 2–3 mm in diameter after incubation on MA at 28 °C for 3–5 days. No diffusible pigments are produced. Growth occurs in 1–20 % (w/v) total salts (optimum, 3–5 %), at pH 6.0–10.5 (optimum, pH 7.5) and 4–40 °C (optimum, 25–30 °C). No growth occurs in the absence of NaCl or with NaCl as the sole salt. Positive for nitrate reduction and oxidation of glucose, but negative for nitrite reduction, fermentation of glucose, phenylalanine deaminase and lysine and ornithine decarboxylases. Respiration on nitrate, nitrite and fumarate under anaerobic conditions is not observed. H₂S is not produced from L-cysteine. Indole production and methyl red and Voges–Proskauer tests are negative. Tween 60 is hydrolysed, but aesculin, casein, cellulose, DNA, gelatin, starch, Tweens 20, 40 and 80 and urea are not. Acids are produced from D-fructose, D-glucose, maltose, sucrose and trehalose, but not from L-arabinose, cellobiose, dulcitol, D-galactose, D-lactose, D-mannose, melezitose, melibiose,

raffinose, L-rhamnose, D-ribose, D-salicin, starch, D-xylose, adonitol, glycerol, *myo*-inositol, D-mannitol or D-sorbitol. The following compounds are utilized as sole source of carbon and energy or sole source of carbon, nitrogen and energy: L-arabinose, D-fructose, D-glucose, maltose, acetate, butyrate, citrate, pyruvate, L-alanine, L-asparagine, L-glutamic acid, L-leucine, L-proline and L-serine; the following substances are not utilized: aesculin, cellobiose, dextrin, D-galactose, D-lactose, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-salicin, starch, sucrose, trehalose, D-xylose, adonitol, D-arabitol, ethanol, glycerol, *myo*-inositol, D-mannitol, D-sorbitol, benzoate, caprylate, formate, fumarate, gluconate, hippurate, malate, malonate, propionate, succinate, L-arginine, L-cysteine, glycine, L-histidine, hydroxy-L-proline, L-isoleucine, L-methionine, L-threonine, L-tryptophan and L-valine. Constitutive enzymes expressed are acid phosphatase, alkaline phosphatase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase; *N*-acetyl- β -glucosaminidase, α -chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), α -fucosidase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, β -glucuronidase, lipase (C14), α -mannosidase and trypsin are not observed (API ZYM). Susceptible to (μ g per disc): ampicillin (30), carbenicillin (30), gentamicin (10), nalidixic acid (20), polymixin B (30), rifampicin (5) and streptomycin (10), but not to chloramphenicol (30), kanamycin (30), lincomycin (2), nitrofurantoin (30), novobiocin (30), tetracycline (30) or tobramycin (10). Major fatty acids are C_{18:1} ω 7c, C_{16:0} and C_{12:0} 3-OH. Predominant respiratory quinone is Q-9. The DNA G+C content of the type strain is 55.8 mol% (HPLC).

The type strain, JSM 078169^T (=CCTCC AB 208031^T=DSM 21076^T=KCTC 22279^T), was isolated from homogenates of a sea urchin (*Hemicentrotus pulcherrimus*) collected from a tidal flat of Naozhou Island in the South China Sea, near a southern city, Zhanjiang.

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