Halomonas daqiaonensis sp. nov., a moderately halophilic, denitrifying bacterium isolated from a littoral saltern

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Two novel Gram-negative, oxidase- and catalase-positive, rod-shaped bacterial strains, designated YCSA28^T and YCSA39, were isolated from sediment of Daqiao saltern, Jimo, Qingdao, on the east coast of China. The two strains grew optimally at 28–30 °C, at pH 7.5 and in the presence of 7–8 % (w/v) NaCl. They were assigned to the genus *Halomonas*, class *Gammaproteobacteria*, based on 16S rRNA gene sequence analysis. The major cellular fatty acids of the two strains were C_{18:1ω7c} (42.9 %), C_{16:0} (23.1 %) and C_{16:1ω7c/ω6c} (18.0 %), and Q-9 was the major ubiquinone. The G + C content of the DNA of strains YCSA28^T and YCSA39 was 63.7 and 63.9 mol%, respectively. The predominant respiratory lipoquinone, cellular fatty acid profiles and DNA G + C content of strains YCSA28^T and YCSA39 were consistent with those of recognized species of the genus *Halomonas*. Levels of DNA–DNA relatedness between strains YCSA28^T and YCSA39, between YCSA28^T and *Halomonas ventosae* Al12^T, and between YCSA39 and *H. ventosae* Al12^T were 95, 45 and 50 %, respectively. Together, these data indicated that strains YCSA28^T and YCSA39 represent a single novel species of the genus *Halomonas*, for which the name *Halomonas daqiaonensis* sp. nov. is proposed. The type strain is YCSA28^T (=CGMCC 1.9150^T =NCCB 100305^T =MCCC 1B00920^T).

The genus *Halomonas*, family *Halomonadaceae*, was established by Vreeland *et al.* (1980) based on the description of *Halomonas elongata*. The genus comprises a group of Gram-negative, rod-shaped strains (Berendes *et al.*, 1996; Yoon *et al.*, 2002; Wang *et al.*, 2007). At the time of writing, 69 species have been assigned to the genus *Halomonas* (<http://www.bacterio.cict.fr/>) and the descriptions of several new species are awaiting formal publication (<http://ijs.sgmjournals.org/>). Species assigned to the genus are mostly aerobic but some are facultatively anaerobic in the presence of nitrate (Dobson & Franzmann, 1996; Martínez-Cánovas *et al.*, 2004; González-Domenech *et al.*, 2008). The colonies of species of the genus *Halomonas* are various shades of white and yellow. Species are also characterized by the

presence of C_{16:1} plus C_{17:0} cyclo, C_{16:0}, and C_{18:1} plus C_{19:0} cyclo as major fatty acids, with ubiquinone 9 (Q-9) as the predominant isoprenoid quinone (Dobson & Franzmann, 1996; Li *et al.*, 2008; González-Domenech *et al.*, 2009). The DNA G + C content of recognized species of the genus *Halomonas* ranges from 52 mol% (*Halomonas venusta*) (Akagawa & Yamasato, 1989; Dobson & Franzmann, 1996) to 74.3 mol% (*Halomonas ventosae*) (Martínez-Cánovas *et al.*, 2004). We isolated two exopolysaccharide (EPS)-producing strains, YCSA28^T and YCSA39, from sediment of a saltern. They were considered to be members of the genus *Halomonas* based on their 16S rRNA gene sequences. The aim of this work was to determine the exact taxonomic position of strains YCSA28^T and YCSA39 based on their phenotypic, phylogenetic and genomic characteristics.

Sediment was sampled at Daqiao saltern, Jimo, Qingdao, on the east coast of China (120.82° E 36.50° N). Approximately 1 g sediment was suspended in 100 ml sterilized seawater and vortexed for 10 min. Isolates were obtained by plating serial 10-fold dilutions of the suspension onto modified marine agar medium 2216E-S

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Abbreviations: EPS, exopolysaccharide; PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains YCSA28^T and YCSA39 are FJ984862 and FJ984868, respectively.

One supplementary table and two supplementary figures are available with the online version of this paper.

(per litre seawater: 5 g tryptone, 1 g yeast extract and 15 g agar, pH 7.5) (Qu *et al.*, 2011) followed by incubation at 30 °C for 7 days. The isolates were then purified by restreaking on 2216E-S plates and incubating at 30 °C for 3 days, with the purity of the isolates examined based on cell shape under a microscope (Nikon Eclipse TS100) and colony homogeneity on the plates. The purified colonies were routinely cultured on MY medium (Moraine & Rogovin, 1966), supplemented with 7.5 % (w/w) sea salt (Rodríguez-Valera *et al.*, 1981) at 30 °C. MY medium was used for all other experiments in this study unless noted otherwise.

Genomic DNA was prepared according to the method of Hiraishi (1992). The 16S rRNA gene was amplified with universal primers 27F and 1492R as described by Lane (1991). The PCR product was purified by using the Wizard SV Gel and PCR Clean-Up System (Promega), and was sequenced directly in both directions with universal primers 27F and 1492R on a 3730XL automated DNA Analyser (Applied Biosystems). Sequence similarity searching of the 16S rRNA gene was carried out online against the GenBank DNA database with the BLASTN program (Altschul *et al.*, 1990). Similar sequences were retrieved from GenBank (Benson *et al.*, 2009). After multiple alignment with BioEdit version 7.0.9 (T. Hall, Ibis Biosciences, Carlsbad, CA, USA), phylogenetic trees were reconstructed with the neighbour-joining (Saitou & Nei, 1987) and minimum-evolution (Rzhetsky & Nei, 1992) methods by using MEGA version 4.0 (Tamura *et al.*, 2007), and distances were evaluated according to the Kimura two-parameter model (Kimura, 1983).

The complete 16S rRNA gene sequences of strains YCSA28^T (1426 bp) and YCSA39 (1419 bp) were almost identical to each other. The closest relative was *H. ventosae* Al12^T, similarity between the 16S rRNA gene sequence of *H. ventosae* Al12^T and the consensus sequence of strains YCSA28^T and YCSA39 reaching 96.9 %. The two novel strains were also closely related to members of the genera *Chromohalobacter*, *Modicisalibacter*, *Salinicola*, *Aidingimonas* and *Kushneria* in the family *Halomonadaceae* (93–95 % 16S rRNA gene sequence similarity). According to the cut-off range of 98–99 % set by Stackebrandt & Ebers (2006), strains YCSA28^T and YCSA39 are thus considered to represent a novel species. In the neighbour-joining phylogenetic tree (Fig. 1), strains YCSA28^T and YCSA39 formed a monophyletic clade with 99 % bootstrap support within the genus *Halomonas*. The tree reconstructed with the minimum-evolution method (see Supplementary Fig. S1 in IJSEM Online) was similar to the neighbour-joining tree. In addition, the consensus 16S rRNA gene sequence of the two novel strains contained 15 signature nucleotides specific for the family *Halomonadaceae* and four for the genus *Halomonas* (Dobson & Franzmann, 1996). Therefore, strains YCSA28^T and YCSA39 were recognized as representatives of a novel species in the genus *Halomonas*.

DNA–DNA hybridization to determine genomic similarity among strains YCSA28^T, YCSA39 and *H. ventosae* Al12^T

was performed as described by Liu & Shao (2005). Levels of DNA–DNA relatedness between strains YCSA28^T and YCSA39, strain YCSA28^T and *H. ventosae* Al12^T, and strain YCSA39 and *H. ventosae* Al12^T were 95, 45 and 50 %, respectively. The high level of relatedness between strains YCSA28^T and YCSA39 indicated that they belonged to the same species. According to the cut-off value of 70 % recognized by Wayne *et al.* (1987) for discriminating bacterial species, strains YCSA28^T and YCSA39 represent a novel species.

Procedures for phenotypic characterization followed Mata *et al.* (2002) and Arahall *et al.* (2007). Phenotypic characteristics tested included acid production, carbon source utilization, H₂S production from L-cysteine, indole production, methyl red and Voges–Proskauer reaction, urease activity, growth on MacConkey agar or cetrimide agar, nitrate and nitrite reduction, ONPG, respiration in the presence of fumarate, nitrate and nitrite, DNase activity, haemolysis, and hydrolysis of gelatin, starch, casein, Tween 20, Tween 80, tyrosine and aesculin. The Gram reaction was checked by standard Gram staining (Dong & Cai, 2001) and KOH tests (Buck, 1982). Catalase activity was determined by adding 10 volumes of H₂O₂ to each strain culture (48 h) on solid MH medium (Dong & Cai, 2001). Oxidase activity was determined by oxidation of 1 % *p*-aminodimethylaniline oxalate (Dong & Cai, 2001). Cellular morphology and the presence of flagella were observed by transmission electron microscopy (H-7000; Hitachi). Poly-β-hydroxybutyrate (PHB) granules were observed with epifluorescence microscopy (CKX41; Olympus) after staining with Nile blue A (Dong & Cai, 2001; Ostle & Holt, 1982). EPS production was observed by using the phenol/sulfuric acid method with glucose as a standard (Zhou *et al.*, 2007). The temperature range for growth was determined by incubating at 5, 10, 15, 20, 25, 30, 35, 38, 40, 42 and 45 °C. Salinity tolerance was determined in MH medium with 0, 0.5, 1, 3, 5, 7.5, 10, 15, 20, 25 and 30 % (w/v) NaCl. pH tolerance was determined in MH medium at pH 3–10 (intervals of 1 pH unit). The media were adjusted to different pH with HCl and NaOH (Mata *et al.*, 2002). Anaerobic growth was tested on solid MH medium by using an MGC AnaeroPack Series (MGC). Antibiotic susceptibility was tested with the disc diffusion method as described by Lai & Shao (2008). Enzyme activity was tested by using API ZYM strips (bioMérieux) according to the manufacturer's instructions, except that NaCl concentration was adjusted to 7.5 %. For comparison, *H. ventosae* Al12^T was analysed in parallel with the two novel strains.

Strains YCSA28^T and YCSA39 were similar in terms of morphological, cultural, physiological and biochemical characteristics. The two strains were Gram-negative, oxidase- and catalase-positive, and rod-shaped with several peritrichous flagella (see Supplementary Fig. S2). They did not produce acids from sugars and they were positive for motility, nitrate and nitrite reduction, phenylalanine deaminase and PHB accumulation. These characteristics

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strains YCSA28^T and YCSA39 among neighbouring species of the family *Halomonadaceae*. Bootstrap values (percentages of 1000 replications) are shown at nodes; only values >50% are shown. Bar, 0.005 nt substitution rate (K_{nuc}) units.

were similar to those for *H. ventosae* Al12^T. However, the novel strains differed from *H. ventosae* Al12^T in colony pigmentation, pH range and temperature range for growth, and H₂S generation from L-cysteine. Differential phenotypic characteristics between the novel strains and *H. ventosae* Al12^T are given in Table 1, and detailed phenotypic characterization of the novel strains is given in the species description below.

Isoprenoid quinones were obtained from whole cells and were determined with HPLC according to Komagata & Suzuki (1987). The predominant respiratory lipoquinone of strains YCSA28^T and YCSA39 was ubiquinone with nine isoprene units (Q-9), as in recognized species of the genus *Halomonas*. The G + C content of the DNA was determined with the methods described by Mesbah & Whitman (1989) by using reversed-phase HPLC; values for strains YCSA28^T and YCSA39 were 63.7 and 63.9 mol%, respectively, which are within the range known for the genus (54–74.3 mol%) (Romanenko *et al.*, 2002; Martínez-Cánovas *et al.*, 2004; González-Domenech *et al.*, 2009). Fatty acid methyl esters were prepared and analysed by using the standard Microbial Identification System (MIDI) for automated gas chromatographic analysis (Sasser, 1990). The fatty acids of *H. ventosae* Al12^T were profiled in parallel with those of strain YCSA28^T. The predominant cellular fatty acids of strain YCSA28^T were C_{18:1}ω7c (42.9%), C_{16:0} (23.1%) and C_{16:1}ω7c/ω6c (18.0%); these are also the predominant components in *H. ventosae* Al12^T, and are characteristic of recognized species of the genus *Halomonas*. However, the two strains were significantly different from each other in the content of C_{18:1}ω7c (Supplementary Table S1). As the fatty acid profiles of strain YCSA28^T and *H. ventosae* Al12^T were determined under the same conditions, this difference

could also be used to distinguish strain YCSA28^T from *H. ventosae* Al12^T.

The combined phylogenetic, DNA–DNA relatedness, fatty acid and phenotypic data presented thus suggest that strains YCSA28^T and YCSA39 represent a novel species of the genus *Halomonas*, for which the name *Halomonas daqiaonensis* sp. nov. is proposed.

Description of *Halomonas daqiaonensis* sp. nov.

Halomonas daqiaonensis (da.qia.o.nen'sis. N.L. fem. adj. *daqiaonensis* of the Daqiao saltern, where the type strain was isolated).

Cells are Gram-negative, oxidase- and catalase-positive rods (0.3–0.6 × 1.2–2.2 μm) with several peritrichous flagella, appearing either singly or in pairs. Colonies are cream-yellow, smooth and circular with entire edges on MY solid medium. Produces EPS but does not form endospores or capsule. Cells are moderately halophilic, capable of growing at 2–15% (w/v) NaCl, with optimum growth occurring at 7–8% (w/v) NaCl; no growth occurs in the absence of salt. Growth occurs at 5–40 °C and pH 6–9 (optimum growth at 28–30 °C and pH 7.5). Indole, methyl red, Voges–Proskauer, O/F and ONPG tests are negative. Positive for aerobic reduction of selenite, nitrate and nitrite. Metabolism is respiratory, with oxygen, nitrate or nitrite as terminal electron acceptor. No respiration with fumarate. Accumulates PHB granules. Hydrolyses aesculin, but not casein, gelatin, starch, Tween 20, Tween 80 or urea. Does not produce H₂S from L-cysteine. Blood is not haemolysed. No growth on MacConkey agar or cetrimide agar. Cells do not produce acids from sugars (adonitol, L-arabinose, D-fructose, D-galactose, D-glucose, *myo*-inositol,

Table 1. Differential characteristics between strains YCSA28^T and YCSA39 and their closest phylogenetic neighbourStrains: 1, YCSA28^T/YCSA39; 2, *H. ventosae* Al12^T.

All strains were positive for motility, oxidase, nitrate/nitrite reduction, phenylalanine deaminase and PHB accumulation, but negative for indole, methyl red, Voges–Proskauer, O/F and blood haemolysis tests. None showed growth on MacConkey agar or cetrimide agar. All strains were negative for hydrolysis of gelatin, starch, Tween 80 and urea. In API ZYM tests, all the strains were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine aminopeptidase, valine aminopeptidase and cystine aminopeptidase; negative for other enzyme tests except for those noted in the table below. w, Weakly positive. Data were taken from Martínez-Cánovas *et al.* (2004) and this study.

Characteristic	1	2
Colony pigmentation	Cream–yellow	Cream
pH range	6–9	6–10
Temperature range (°C)	5–40	10–45
H ₂ S generation	–	+
ONPG	–	+
Growth on:*		
Aesculin	+	–
D-Xylose	–	+
myo-Inositol	–	+
Sorbitol	–	+
L-Arabinose	+	–
Adonitol	–	+
D-Mannitol	–	+
Malonate	–	+
L-Serine	–	+
Hydrolysis of Tween 20	–	+
Enzyme activities (API ZYM)		
Acid phosphatase	–	+
α-Glucosidase	–	+
Valine aminopeptidase	w	+
Susceptibility to antimicrobial agents		
Oxacillin (1 µg)	+	–
Vancomycin (30 µg)	+	–
Ampicillin (10 µg)	+	–
Lincomycin (2 µg)	–	+
DNA G + C content (mol%)	63.7–63.9	74.3†

*When supplied as the sole source of carbon and energy or sole source of carbon, nitrogen and energy.

†Data from Martínez-Cánovas *et al.* (2004).

lactose, maltose, D-mannitol, D-mannose, melezitose, L-rhamnose, sucrose, D-salicin, D-sorbitol, sorbose or trehalose). Positive for phenylalanine deaminase and ornithine decarboxylase, but not lysine decarboxylase. Susceptible to ciprofloxacin (5 µg), nalidixic acid (30 µg), doxycycline (30 µg), erythromycin (15 µg), cefalexin (30 mg), carbenicillin (100 µg), streptomycin (10 µg), tetracycline (30 µg), novobiocin (30 µg), rifampicin (5 µg), penicillin G (10 IU), chloramphenicol (30 µg), ampicillin (10 µg), neomycin (30 µg), kanamycin (30 µg), vancomycin (30 µg), oxacillin

(1 µg) and polymyxin B (300 IU), but resistant to lincomycin (2 µg) and clindamycin (2 µg). In the API ZYM system, positive for alkaline phosphatase and leucine aminopeptidase, weakly positive for esterase (C4), lipase (C14), esterase lipase (C8), valine aminopeptidase and cystine aminopeptidase, and negative for acid phosphatase, α-mannosidase, naphthol-AS-BI-phosphoamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucosidase, trypsin, N-acetyl-β-glucosaminidase, β-glucuronidase, α-glucosidase and α-fucosidase. Utilizes L-arabinose, ribose, citrate, D-fructose, lactose, D-galactose, gluconate, D-glucose, maltose, succinate, acetate, fumarate, glycerol and propionate as sole carbon and energy source, but not D-mannose, cellobiose, adonitol, sorbitol, starch, aesculin, ethanol, formate, D-mannitol, myo-inositol, malonate, D-salicin or trehalose. Utilizes L-alanine and L-valine as sole sources of carbon, nitrogen and energy, but not DL-isoleucine, L-cysteine, L-lysine, L-histidine or L-methionine. The predominant cellular fatty acids are C_{18:1ω7c}, C_{16:0} and C_{16:1ω7c/ω6c}. The predominant isoprenoid quinone is Q-9. The DNA G + C content of the type strain is 63.7 mol%.

The type strain, YCSA28^T (=CGMCC 1.9150^T =NCCB 100305^T =MCCC 1B00920^T), was isolated from sediment of Daqiao saltern, Jimo, Qingdao, on the east coast of China. YCSA39, isolated from the same source, is a second strain of the species.

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