Halomonas daqingensis sp. nov., a moderately halophilic bacterium isolated from an oilfield soil

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A Gram-negative, moderately halophilic, short rod-shaped, aerobic bacterium with peritrichous flagellae, strain DQD2-30^T, was isolated from a soil sample contaminated with crude oil from the Daqing oilfield in Heilongjiang Province, north-eastern China. The novel strain was capable of growth at NaCl concentrations of 1–15 % (w/v) [optimum at 5–10 % (w/v)]. Phylogenetic analyses based on 16S rRNA gene sequences showed that the novel strain belonged to the genus *Halomonas* in the class *Gammaproteobacteria*; the highest 16S rRNA gene sequence similarities were with *Halomonas desiderata* DSM 9502^T (98.8 %), *Halomonas campisalis* A4^T (96.6 %) and *Halomonas gudaonensis* CGMCC 1.6133^T (95.1 %). The major cellular fatty acids of strain DQD2-30^T were C_{18:1}ω7c (43.97 %), C_{19:0} cyclo ω8c (23.37 %) and C_{16:0} (14.83 %). The predominant respiratory lipoquinone was ubiquinone with nine isoprene units (Q9). The DNA G + C content was 67.0 mol%. The DNA–DNA hybridization values of strain DQD2-30^T with the most closely related species of the genus *Halomonas* were 51.8 %, 28.4 % and 23.5 % for *H. desiderata*, *H. campisalis* and *H. gudaonensis*, respectively. Based on these analyses, strain DQD2-30^T (=CGMCC 1.6443^T=LMG 23896^T) is proposed to represent the type strain of a novel species, *Halomonas daqingensis* sp. nov.

Members of the genus *Halomonas* can grow in a wide range of salt concentrations from 0 to 25 % (w/v) or even higher (Vreeland *et al.*, 1980). In addition, *Halomonas* species possess diverse biochemical functions, such as exopolysaccharide production by *Halomonas maura* (Bouchotroch *et al.*, 2001), *Halomonas ventosae* (Martínez-Cánovas *et al.*, 2004a, c), *Halomonas anticariensis* (Martínez-Cánovas *et al.*, 2004b) and *Halomonas almeriensis* (Martínez-Checa *et al.*, 2005); polyhydroxybutyrate production by *Halomonas pantelleriensis* (Romano *et al.*, 1996); degradation of aromatic compounds by *Halomonas organivorans* (García *et al.*, 2004); denitrification by *Halomonas elongata* (Vreeland *et al.*, 1980), *Halomonas salina*, *Halomonas halodenitrificans* (Dobson & Franzmann, 1996), *Halomonas desiderata* (Berendes *et al.*, 1996), *Halomonas campisalis* (Mormile *et al.*, 1999), *Halomonas neptunia*, *Halomonas*

sulfidaeris, *Halomonas axialensis*, *Halomonas hydrothermalis* (Kaye *et al.*, 2004) and *H. maura* (Bouchotroch *et al.*, 2001), and H₂S-production by *H. salina*, *Halomonas halophila* (Dobson & Franzmann, 1996), *H. maura* (Bouchotroch *et al.*, 2001) and *H. sulfidaeris* (Kaye *et al.*, 2004). These diverse abilities have led to increasing interest in the members of the genus *Halomonas* as useful candidates for the bioremediation of polluted saline soils.

During research on effective microbial degradation of oil pollutants, we isolated a novel moderately halophilic, Gram-negative bacterium, strain DQD2-30^T, that was able to grow in crude-oil contaminated soil. A taxonomic study based on a polyphasic approach revealed that strain DQD2-30^T represents a novel species in the genus *Halomonas*.

Strain DQD2-30^T was isolated from an oil-polluted soil sample from Daqing Oilfield, Heilongjiang Province, north-eastern China. It was initially cultured with oil-production-water medium 2 (OPW2) containing 5 g peptone, 1 g yeast extract and 1 l oil/water mixture from the oil reservoir at 30 °C. The novel strain was then grown

Abbreviations: DPG, diphosphatidylglycerol; EPS, exopolysaccharides; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DQD2-30^T is EF121854.

and identified by using artificial seawater medium (ASW) containing: 5 g peptone, 1 g yeast extract, 4 g Na_2SO_4 , 0.68 g KCl, 0.1 g KBr, 0.025 g H_3BO_3 , 5.4 g $\text{MgCl}_2 \cdot \text{H}_2\text{O}$, 1.5 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.024 g $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2 g NaHCO_3 , 0.04 g Na_2HPO_4 , 0.5 g NH_4Cl , 0.002 g NaF and 1 l H_2O with 2.4 % (w/v) NaCl (pH 8.0) (Eguchi *et al.*, 1996).

After cells of strain DQD2-30^T had been grown on ASW plates at 30 °C for two days, cellular morphology was examined using transmission electron microscopy. The salt requirement for growth was tested with ASW medium with final NaCl concentrations of 1 %, 5 %, 10 %, 15 %, 20 %, 25 % (w/v) (pH 8.0, at 30 °C). The pH and temperature requirements for growth were determined by adjusting the final pH of the ASW medium to pH 2, 4, 6, 7, 8, 10 or 12 with HCl and NaOH (30 °C), and by incubation from 4 °C to 50 °C (at 4, 10, 15, 25, 30, 37, 40, 45, 50 and 55 °C) (5 % NaCl, pH 8.0), respectively. Strain DQD2-30^T was Gram-negative and cells were short rods with peritrichous flagellae (Fig. 1). Colonies on ASW medium were creamy, circular and opaque. Strain DQD2-30^T was strictly aerobic and grew at 1–15 % (w/v) NaCl with optimum growth at 5–10 % (w/v) NaCl. Growth temperatures ranged from 10 to 50 °C in ASW medium (pH 8.0) with optimum growth at 30 °C. The pH range for growth was pH 8.0–10.0 (optimum at pH 9.0) (Table 1).

The oxidase activity of strain DQD2-30^T was tested according to Smibert & Krieg (1994) and catalase activity was determined by using 3 % (v/v) hydrogen peroxide solution (Dong & Cai, 2001). Nitrite and nitrate reduction were tested in ASW medium by growing the strain in the

presence of NO_2^- and NO_3^- , respectively (Berendes *et al.*, 1996). Denitrification was tested by growing the strain anaerobically in the presence of NO_3^- (Zumft, 1992). Hydrolysis of starch, gelatin and Tween 80, urease activity and growth on sole carbon and nitrogen sources were examined according to the procedures of Williams *et al.* (1983) on ASW medium without organic compounds at 30 °C for 5–7 days. H_2S production was tested in ASW medium supplemented with 0.01 % L-cysteine, the indicator being a strip of paper impregnated with lead acetate placed in the neck of the tube (Clarke, 1953; Mata *et al.*, 2002). Methyl red and Voges–Proskauer tests were performed by using methyl red and Barritt's reagent (Barritt, 1936; Mata *et al.*, 2002), respectively. Strain DQD2-30^T was subjected to the phenotypic tests according to the procedures recommended by Mata *et al.* (2002), including the production of exopolysaccharides (EPS) and poly- β -hydroxybutyrate (PHB), the production of acid from a range of different carbohydrates and alcohols, ONPG, indole production, oxidation/fermentation of D-glucose, the hydrolysis of casein and DNA, activities of phenylalanine deaminase, lysine- and ornithine decarboxylases, and the ability to grow on sole nitrogen sources.

Strain DQD2-30^T was positive for activities of oxidase, catalase and urease, for the hydrolysis of Tween 80, casein and DNA, for aerobic nitrate or nitrite reduction, and for production of indole, EPS and PHB. The strain was negative in tests for H_2S production, ONPG, activities of phenylalanine deaminase, lysine decarboxylase and ornithine decarboxylase, the methyl red and Voges–Proskauer tests and for the hydrolysis of starch and gelatin. Strain DQD2-30^T could use the amino acids L-proline, L-arginine, L-alanine, L-cysteine, L-histidine, L-isoleucine, L-lysine, DL-methionine, L-serine and L-valine. Strain DQD2-30^T was able to utilize D-glucose, D-mannose, D-sorbitol, maltose, sucrose, trehalose, dextrin, D-mannitol, D-glucuronate, malonate, DL-malate, dextrin, D-galactose (weakly) and succinate as sole carbon sources in ASW medium without peptone and yeast extract, but was unable to utilize D-fructose, D-ribose, α -D-lactose, D-arabinose, cellobiose, L-sorbose, D-xylose, *myo*-inositol, salicylic acid or lactate. Strain DQD2-30^T produced acid from D-galactose, maltose, sucrose, and trehalose, but not from adonitol, α -D-lactose, D-mannitol, D-mannose, melezitose, L-rhamnose, D-sorbitol, D-fructose, L-sorbose, salicylic acid or *myo*-inositol.

Genomic DNA was extracted according to the previously described method of Marmur (1961) from cells grown in ASW medium for 2 days at 30 °C that had been washed and resuspended in buffer. Purity of the DNA was assessed by the A_{280}/A_{260} and A_{230}/A_{260} ratios (Johnson, 1994). Phylogenetic analyses based on 16S rRNA gene sequences were performed as described by Bouchotroch *et al.* (2001). The 16S rRNA gene was amplified with universal bacterial primers corresponding to *Escherichia coli* position 8F (5'-AGAGTTTGATCCTGGCTCAG) and 1492R (5'-GGTTACCTTGTTACGACTT) (Embley, 1991). The 16S

Fig. 1. Transmission electron micrograph of a negatively stained cell of strain DQD2-30^T. Arrows show the flagellae. Bar, 0.5 μm .

Table 1. Physiological and biochemical characteristics that differentiate strain DQD2-30^T from closely related species of the genus *Halomonas*

Taxa: 1, DQD2-30^T; 2, *H. gudaonensis*; 3, *H. desiderata*; 4, *H. campisalis*. Data for carbon source utilization are from the present study and from Berendes *et al.* (1996), Mormile *et al.* (1999) and Wang *et al.* (2007). All strains are Gram-negative rods, that are positive in tests for catalase and oxidase and for the utilization of sucrose, trehalose, DL-malate, D-gluconate, D-sorbitol, succinate, L-proline, L-arginine and L-alanine. All strains are negative for hydrolysis of gelatin and starch and for the utilization of D-ribose, D-arabinose, D-fructose, cellobiose, L-sorbose, D-xylose and lactate. –, Negative; +, positive; +/-, weakly positive; ND, not determined.

Characteristic	1	2	3	4
Cell size (µm)	0.7–0.8 × 1.0–1.2	0.3–4.0 × 0.7–1.6	0.4–0.6 × 1.0–2.6	1.0 × 3–5
Flagellation	Peritrichous	Single lateral	Peritrichous	Absent
EPS	+	+	ND	ND
PHB	+	ND	+	ND
Optimum temperature (°C)	30	30	37	30
Temperature range (°C)	10–50	10–42	10–45	4–50
Optimum pH	9.0	8.0	9.7	9.5
pH range	8.0–10.0	8.0–9.0	7.0–11.0	6.0–11.0
Optimum NaCl (%)	5.0–10.0	10.0–15.0	9.0	8.0
NaCl range (%)	1–15.0	1.0–20.0	1–18.0	1.0–20.0
Oxidation/fermentation of D-glucose	Oxidation	ND	ND	ND
Hydrolysis of casein and DNA	+	ND	ND	ND
ONPG	–	ND	ND	ND
Indole production	+	–	–	–
Phenylalanine deaminase	–	–	+	–
Lysine decarboxylases	–	ND	ND	ND
Ornithine decarboxylases	–	ND	ND	ND
Growth on:				
D-Glucose	+	+	+	+/-
D-Galactose	+/-	+	+/-	–
Malonate	+	+/-	+	+/-
<i>myo</i> -Inositol	–	+/-	+	+
D-Mannitol	+	+	+	+/-
Dextrin	+	+/-	+	+/-
α-D-Lactose	–	+	+/-	–
D-Mannose	+	+	+	–
Maltose	+	+	+	–
Salicylic acid	–	ND	ND	ND
Hydrolysis of:				
Urea	+	+	–	+
Tween 80	+	–	–	–
Aerobic NO ₃ [–] reduction	+	+	+/-	+
Aerobic NO ₂ [–] reduction	+	+/-	–	+/-
Anaerobic NO ₃ [–] reduction	–	+	+	–
H ₂ S production	–	+	–	–
Growth on:				
L-Cysteine	+	+	ND	ND
L-Histidine	+/-	ND	ND	ND
L-Isoleucine	+	+	ND	ND
L-Lysine	+	–	ND	ND
DL-Methionine	+	ND	ND	ND
L-Serine	+	ND	ND	ND
L-Valine	+	ND	ND	ND
Production of acid from				
Adonitol	–	ND	ND	ND
D-Galactose	+	–	ND	–
Lactose	–	–	ND	–
Maltose	+	–	ND	ND
Mannitol	–	+	ND	ND
D-(+)-Mannose	–	–	ND	ND

Table 1. cont.

Characteristic	1	2	3	4
Melezitose	–	–	ND	ND
L-Rhamnose	–	–	ND	–
Sucrose	+	–	ND	ND
D-Sorbitol	–	+	ND	ND
Trehalose	+	–	ND	ND

rRNA gene sequence of strain DQD2-30^T was compared with those available in GenBank using the BLAST program (NCBI) to determine the approximate phylogenetic affiliation. The 16S rRNA gene sequence of strain DQD2-30^T was aligned with related species of the genus *Halomonas* and phylogenetic trees were constructed using the neighbour-joining, maximum-parsimony and maximum-likelihood methods in the MEGA software (Kumar *et al.*, 2004). The genomic DNA G+C content of strain DQD2-30^T was estimated from the midpoint value (T_m) of the thermal denaturation profile, as described by Martínez-Cánovas *et al.* (2004b). DNA–DNA hybridization of strain DQD2-30^T was performed in triplicate following the methods of De Ley *et al.* (1970) and Huß *et al.* (1983). It was performed with strain DQD2-30^T and the most closely related strains, *H. desiderata* DSM 9502^T, *H. campisalis* A4^T and *H. gudaonensis* LMG 23610^T.

The DNA G+C content of strain DQD2-30^T was 67.0 mol%, within the range of 52–68 mol% that has already been reported for the genus *Halomonas* (Franzmann & Tindall, 1990). The nearly complete 16S rRNA gene sequence (1422 bp) of strain DQD2-30^T was determined and analysed. The results revealed that strain DQD2-30^T was a member of the genus *Halomonas* and had close phylogenetic relationships with *H. desiderata* DSM 9502^T (Berendes *et al.*, 1996), *H. campisalis* A4^T (Mormile *et al.*, 1999) and *H. gudaonensis* LMG 23610^T (Wang *et al.*, 2007) with 16S rRNA gene sequence similarity values of 98.8 %, 96.6 % and 95.1 %, respectively. A phylogenetic tree was constructed and it clearly showed the position of strain DQD2-30^T within the genus *Halomonas*, with the novel strain forming a cluster with *H. campisalis*, *H. desiderata* and *H. gudaonensis* (Fig. 2). Further DNA–DNA hybridization experiments between strain DQD2-30^T and *H. desiderata*, *H. campisalis* and *H. gudaonensis* revealed that the DNA–DNA relatedness values were 51.8 ± 0.05 %, 28.41 ± 0.06 % and 23.47 ± 0.06 %, respectively.

The chemotaxonomic study of strain DQD2-30^T included analysis of the cellular fatty acids, polar lipids and quinones. Cellular fatty acid analysis was carried out as described by Komagata & Suzuki (1987) and tested using GC/MS following the instructions of the Microbial Identification System (MIDI). Polar lipid analysis was performed following the polar lipid extraction procedure and tested by single and two-dimensional TLC on Merck silica gel 60 F254 aluminium-backed TLC plates according to the methods of Kates (1986) and Collins *et al.* (1980).

Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987) and using an HPLC fitted with a reversed-phase column (Shim-pack, VP-ODS, Shimadzu). Ubiquinone 9 was the only quinone found and the predominant polar lipids found in cells of strain DQD2-30^T were phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and phosphatidylethanolamine (PE). The main cellular fatty acids were C_{18:1}ω7c (43.97 %), C_{19:0} cyclo ω8c (23.37 %) and C_{16:0} (14.83 %). The contents of C_{12:0} 3-OH, C_{14:0}, summed feature 3 (C_{16:1}ω7c and/or iso C_{15:0} 2-OH), C_{10:0} and C_{17:0} cyclo were less than 10 % (Table 2).

Tests of antimicrobial susceptibility were performed by using the disc diffusion (Kirby–Bauer) method with the antimicrobial compounds suggested by Mata *et al.* (2002). Results showed that strain DQD2-30^T was sensitive to (amounts per disc) cefoxitin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), kanamycin (30 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg), polymyxin B (300 U), rifampicin (5 µg), streptomycin (300 µg) and tobramycin (10 µg), but was resistant to amoxicillin (10 µg), ampicillin (10 µg), carbenicillin (100 µg), cefotaxime (30 µg), streptomycin (10 µg), sulfamethoxazole (300 µg) and trimethoprim-sulfamethoxazole (1.25 µg/23.75 µg).

The above phenotypic, phylogenetic and genotypic results indicate that the new isolate DQD2-30^T represents a novel species of the genus *Halomonas*, for which the name *Halomonas daqingensis* sp. nov. is proposed.

Description of *Halomonas daqingensis* sp. nov.

Halomonas daqingensis (da.qing.en'sis. N.L. fem. adj. *daqingensis* pertaining to Daqing, north-eastern China, where the strain was isolated).

Cells are Gram-negative, strictly aerobic, short rods (0.7–0.8 × 1.0–1.2 µm) with peritrichous flagellae. Growth occurs at temperatures between 10 and 50 °C (optimum growth at 30 °C), at pH 8.0–10.0 (optimum at pH 9.0) and at NaCl concentrations from 1 to 15 % (w/v) (optimum at 5–10 %). Gives a positive reaction in tests for oxidase, catalase and urease, for the hydrolysis of Tween 80, casein and DNA, for aerobic nitrate or nitrite reduction and for the production of indole, EPS and PHB, but is negative with respect to H₂S production, ONPG, activities of phenylalanine deaminase, lysine decarboxylase and ornithine decarboxylase, the methyl red and Voges–

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the members of the genus *Halomonas* and related genera within the family *Halomonadaceae*. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 50 % bootstrap support. Other algorithms including maximum-parsimony and maximum-likelihood were also used to compare and determine the stability of the phylogenetic trees and gave almost the same topology as shown in this figure. Bar, 0.02 expected changes per site.

Table 2. Chemotaxonomic characteristics of strain DQD2-30^T and the related species *H. gudaonensis*, *H. desiderata* and *H. campisalis*

Taxa: 1, DQD2-30^T; 2, *H. gudaonensis*; 3, *H. desiderata*; 4, *H. campisalis*. Cells of all tested strains were grown in the same ASW medium at 30 °C for two days before harvest and further analyses. Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained C_{16:1ω7c} and/or iso C_{15:0} 2-OH. All taxa had Q9 as the respiratory quinone.

Fatty acid	1	2	3	4
C _{10:0}	2.63	0.98	1.83	2.76
Unknown 11.799	—	2.98	—	—
C _{12:0}	0.85	1.25	—	0.86
C _{12:0} 3-OH	5.59	8.87	6.27	5.87
C _{14:0}	3.42	—	3.03	3.35
Summed feature 3	2.82	4.44	1.77	8.07
C _{16:0}	14.83	19.66	18.03	16.44
C _{17:0} cyclo	1.08	2.70	1.12	2.96
C _{17:0}	0.35	0.56	0.42	0.28
C _{18:0}	0.56	0.65	0.84	—
C _{18:1ω7c}	43.97	28.61	35.58	49.12
11 methyl C _{18:1ω7c}	0.54	0.82	0.78	—
C _{19:0} cyclo ω8c	23.37	27.97	29.68	10.28
C _{20:2ω6,9c}	—	0.51	0.65	—
Polar lipids	PG, PE, DPG	PG, PE, DPG, glycolipid	PG, PE, DPG	PG, PE, DPG

Proskauer tests and for the hydrolysis of starch and gelatin. Grows on amino acids L-proline, L-arginine, L-alanine, L-cysteine, L-histidine, L-isoleucine, L-lysine, DL-methionine, L-serine and L-valine. Utilizes D-glucose, D-mannose, D-sorbitol, maltose, sucrose, trehalose, dextrin, D-mannitol, D-gluconate, malonate, DL-malate, dextrin, D-galactose (weakly) and succinate as sole carbon sources in ASW medium without peptone and yeast extract, but it does not utilize D-fructose, D-ribose, α -D-lactose, D-arabinose, cellobiose, L-sorbose, D-xylose, *myo*-inositol, salicylic acid or lactate. Produces acid from D-galactose, maltose, sucrose and trehalose, but not from adonitol, α -D-lactose, D-mannitol, D-mannose, melezitose, L-rhamnose, D-sorbitol, D-fructose, L-sorbose, salicylic acid or *myo*-inositol. Sensitive to cefoxitin (30 μ g), chloramphenicol (30 μ g), erythromycin (15 μ g), kanamycin (30 μ g), nalidixic acid (30 μ g), nitrofurantoin (300 μ g), polymyxin B (300 U), rifampicin (5 μ g), streptomycin (300 μ g), tobramycin (10 μ g), resistant to amoxicillin (10 μ g), ampicillin (10 μ g), carbenicillin (100 μ g), cefotaxime (30 μ g), streptomycin (10 μ g), sulfamethoxazole (300 μ g) and trimethoprim-sulfamethoxazole (1.25 μ g/23.75 μ g). Q9 is the only quinone and PG, DPG and PE are the predominant polar lipids. The main cellular fatty acids are C_{18:1} ω 7c, C_{19:0} cyclo ω 8c and C_{16:0}.

The type strain, DQD2-30^T (=CGMCC 1.6443^T=LMG 23896^T), was isolated from a soil sample contaminated with crude oil from the Daqing oilfield, Heilongjiang Province, China. The DNA G + C content of the type strain is 67.0 mol%.

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