Bacillus qingdaonensis sp. nov., a moderately haloalkaliphilic bacterium isolated from a crude sea-salt sample collected near Qingdao in eastern China

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A moderately haloalkaliphilic, Gram-positive bacterium, designated as strain CM1^T, was isolated from a crude sea-salt sample collected near Qingdao in eastern China. Strain CM1^T was found to grow optimally at 37 °C and pH 9.0. It was shown to be aerobic, rod-shaped and capable of growth at salinities of 2.5–20 % (w/v) NaCl (optimum, 12 %). The genomic DNA G + C content was about 48 mol%. The major cellular fatty acids were anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} and the major isoprenoid quinones were MK-7(H₂) and MK-6(H₂). Phylogenetic analyses based on 16S rRNA gene sequences revealed that CM1^T is a member of the genus *Bacillus* and has less than 95.2 % gene sequence similarity to the most closely related strain, *Bacillus salarius* BH169^T. Its DNA–DNA reassociation value with respect to *B. salarius* BH169^T was 35.4 %. On the basis of phenotypic and molecular properties, strain CM1^T represents a novel *Bacillus* species, for which the name *Bacillus qingdaonensis* sp. nov. is proposed. The type strain is CM1^T (=CGMCC 1.6134^T=JCM 14087^T).

The genus *Bacillus* is a large taxonomic group containing species of many different physiological types. Among these, aerobic and halophilic rods have been isolated from a wide range of saline habitats, such as salt lakes, sea ice, salterns, salty foods, estuarine water and deep-sea hydrothermal vents (Agnew *et al.*, 1995; Arahall *et al.*, 1999; Lim *et al.*, 2006; Nielsen *et al.*, 1994; Ventosa *et al.*, 1989; Yoon *et al.*, 2004). Because of their potential usefulness in the production of compatible solutes or hydrolytic enzymes (Margesin & Schinner, 2001), alkaline enzymes (Horikoshi, 1971), cyclo-maltodextrin glucanotransferase (CGTase) (Yamamoto *et al.*, 1972) and cellulase (Horikoshi *et al.*, 1971), this group of bacteria has attracted a great deal of attention recently.

While screening halophilic bacteria from a crude sea-salt sample collected near Qingdao in eastern China, we isolated an aerobic, Gram-positive, moderately halophilic bacterium, designated as strain CM1^T. On the basis of its

phenotypic characteristics and genotypic data, this strain should be assigned to the genus *Bacillus* as a novel species.

CM medium (Seghal & Gibbons, 1960) at pH 7.0 and containing 12 % (w/v) NaCl was used for bacterial enrichment and growth. The sample was first inoculated into the liquid medium and cultured on a shaker (120 r.p.m.) at 37 °C in the dark until turbid, then streaked onto solid medium to obtain single colonies. The streaking was repeated several times to obtain pure single colonies. The purified strain was cultured and maintained in liquid or solid ATCC 213 medium (Garrity *et al.*, 2004) at pH 7.0, with 12 % (w/v) NaCl, in the dark.

Cell morphology was determined by using phase-contrast microscopy. Gram staining was performed according to Dussault (1955) and electron microscopy was performed with negative staining according to Bouchotroch *et al.* (2001).

Bacterial growth was determined by measuring absorbance at 660 nm at certain intervals during liquid culture. The effects of different concentrations of NaCl on the growth of strain CM1^T were tested in liquid ATCC 213 medium by measuring the absorbance at 660 nm after 7 days incubation.

The susceptibility of strain CM1^T to different antibiotics, including ampicillin, tetracycline, hygromycin, kanamycin,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CM1^T is DQ115802.

Phylogenetic trees based on maximum-parsimony and minimum-evolution analyses of the 16S rRNA gene sequences of strain CM1^T and related species are available as supplementary figures in IJSEM Online.

Fig. 1. Transmission electron micrographs of cells of strain CM1^T. Bars, 1 μm (a, c), 10 μm (b).

streptomycin, rifampicin, bacitracin, penicillin, chloramphenicol, neomycin and erythromycin (Sigma) was tested on solid ATCC 213 medium after 7 days incubation at 37 °C. Analysis of the whole-cell fatty acid pattern was performed according to previously described methods (Miller, 1982) using the Sherlock system (MIDI). Isoprenoid quinones were analysed by HPLC with a reversed-phase column according to the method of Komagata & Suzuki (1987).

Physiological and biochemical tests were performed as described by Buchanan & Gibbons (1974) and Dong & Cai (2001). Anaerobic growth of strain CM1^T with nitrate as the electron acceptor was tested according to the method of Mancinelli & Hochstein (1986). Growth with DMSO and by the fermentation of L-arginine as alternative electron acceptors was tested as described by Oren *et al.* (1997) and Oren & Trüper (1990) using closed tubes fully filled with the growth medium and then kept in the dark for 3 weeks before comparison of the growth with that found on medium lacking the test compounds. The DNA G + C content was determined by the thermal denaturation method (Sly *et al.*, 1986) using a Beckman UV-800 spectrophotometer equipped with a TB-85 thermostat. We used the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992) to perform DNA–DNA hybridization experiments.

PCR amplification of the 16S rRNA gene was performed according to Reddy *et al.* (2003). The PCR product was ligated to the T-vector and transformed into *Escherichia coli* JM109 for purification and sequencing.

The sequences used for comparison with the 16S rRNA gene sequence from strain CM1^T were obtained from GenBank by using the BLAST program. The sequence from strain CM1^T was aligned with closely related 16S rRNA gene sequences using CLUSTAL_X software (version 1.83; Thompson *et al.*,

Table 1. Fatty acid content (% of total fatty acids) of strain CM1^T

Fatty acid	Content (%)
C _{12:0}	0.05
C _{14:0}	1.29
C _{14:0} 2-OH	0.23
C _{15:0}	1.22
C _{15:1ω6c}	0.10
C _{16:0}	7.72
C _{16:1ω7c}	0.82
C _{17:0}	0.72
C _{18:0}	2.12
C _{18:1ω9c}	0.94
C _{18:1ω7c}	0.87
C _{16:1ω7c} alcohol	0.17
anteiso-C _{13:0}	0.17
iso-C _{14:0}	1.63
iso-C _{14:0} 3-OH	0.21
iso-C _{15:0}	5.88
anteiso-C _{15:0}	34.78
iso-C _{15:1} I	0.50
anteiso-C _{15:1} A	0.31
iso-C _{16:0}	13.99
anteiso-C _{17:0}	21.43
iso-C _{17:0}	4.38
iso-C _{18:0}	0.38
iso-C _{19:0}	0.10

Table 2. Differential characteristics of strain CM1^T and closely related strains

Strains: 1, CM1^T; 2, *B. salarius* DSM 16461^T (data from Lim *et al.*, 2006); 3, *Bacillus saliphilus* DSM 15402^T (Romano *et al.*, 2005); 4, *Bacillus clarkii* DSM 8720^T (Nielsen *et al.*, 1995). +, Positive; –, negative; NR, not reported; w, weakly positive. All strains are positive for the Gram reaction and negative for the hydrolysis of starch and anaerobic growth.

Characteristic	1	2	3	4
Cell morphology	Rods	Rods	Cocci	Rods
Cell size (µm)	0.3–0.4 × 1.8–3.2	0.5–0.7 × 1.3–1.9	0.8–0.9	0.6–0.7 × 2.0–5.0
Colony colour	Cream–white to yellow	Cream	Yellow	Cream–white to dark yellow
Spore formation	–	+	–	+
Oxidase	–	+	+	–
Catalase	+	+	w	+
Growth at pH 10	+	–	+	+
NaCl concentrations for growth (% w/v)				
Minimum	2.5	3	>0	0.5
Optimum	12	10–12	16	NR
Maximum	20	20	25	16
Optimum temperature for growth (°C)	37	30	37	30
Hydrolysis of:				
Gelatin	–	NR	+	+
Urea	+	–	NR	NR
Tween 20	–	NR	NR	–
Tween 80	–	–	NR	+
Nitrate reduction	+	–	+	+
Major cellular fatty acids (% of total)	anteiso-C _{15:0} (34.78), anteiso-C _{17:0} (21.43), iso-C _{16:0} (13.99), C _{16:0} (7.72)	anteiso-C _{15:0} (53.13), anteiso-C _{17:0} (18.67), iso-C _{15:0} (8.95), iso-C _{16:0} (6.6)	anteiso-C _{15:0} (89), iso-C _{15:0} , C _{17:0}	NR
Quinone composition (% of total)	MK-7(H ₂) (95.15), MK-6(H ₂) (4.85)	MK-7	MK-7(H ₄) (15), MK-7(H ₂) (70), DMK-7(H ₂) (15)	NR
DNA G + C content (mol%)	48	43	48.4	43.0

1997). The phylogenetic tree was constructed by using neighbour-joining (Saitou & Nei, 1987), maximum-parsimony and minimum-evolution methods in MEGA, version 3.1 (Kumar *et al.*, 2001, 2004). The confidence values for the branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Cells of strain CM1^T were Gram-positive rods, 0.3–0.4 µm wide and 1.8–3.2 µm long (Fig. 1). The colonies were cream–white to yellow in colour, wet and smooth-surfaced with clear edges on ATCC 213 medium containing 12 % (w/v) NaCl. Strain CM1^T grew as single cells or clusters (Fig. 1). The novel strain did not produce spores, even in the presence of 0.1 g MnCl₂ ml^{–1} and under other conditions used for stimulating spore formation (Hageman *et al.*, 1984).

Strain CM1^T grew at salt concentrations in the range 2.5–20 % (w/v) NaCl, 12 % being the optimum concentration. It grew at pH 6.5–10.5 in 12 % (w/v) NaCl, pH 9.0 being the optimum. The permissive temperature for growth was between 25 and 45 °C, the optimum value being 37 °C.

Growth of strain CM1^T could be inhibited by the antibiotics rifampicin, penicillin, hygromycin, ampicillin and bacitracin. No inhibition was observed when the strain was grown in the presence of kanamycin, erythromycin, streptomycin, tetracycline, chloramphenicol or neomycin.

The major fatty acids of strain CM5^T were anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}; the fatty acid profile is shown in Table 1. The major isoprenoid quinones were MK-7(H₂) (95.15 %) and MK-6(H₂) (4.85 %).

A comparison of the phenotypic and physiological properties of strain CM1^T with those of the type strains of three closely related *Bacillus* species is shown in Table 2. Strain CM1^T had a DNA G + C content of 48 mol% and had a DNA–DNA reassociation value of 35.4 % with *Bacillus salarius* BH169^T.

The full sequence (1572 bases) of the 16S rRNA gene of strain CM1^T was determined. The 16S rRNA gene phylogenetic analysis showed that strain CM1^T was most similar to *B. salarius* BH169^T (95.2 %). The sequence similarities

Fig. 2. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of strain CM1^T and the type strains of related taxa. *Paenibacillus polymyxa* was used as an outgroup. Bootstrap percentages (based on 1000 replicates) greater than 50% are shown at branch points. Bar, 0.01 changes per nucleotide position.

with respect to other *Bacillus* species with validly published names were below 91.6%. Evaluation of the tree topology showed that strain CM1^T forms a branch with *B. salarius* BH169^T (Fig. 2 and Supplementary Figs S1 and S2 available in IJSEM Online).

In summary, our molecular phylogenetic analysis and phenotypic characterization of strain CM1^T clearly show that this strain is distinguishable from closely related species within the genus *Bacillus* (Table 1). In conclusion, strain CM1^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus qingdaonensis* sp. nov. is proposed.

Description of *Bacillus qingdaonensis* sp. nov.

Bacillus qingdaonensis (qing.da.o.nen'sis. N.L. masc. adj. *qingdaonensis* pertaining to Qingdao, the name of the place from which the type strain was isolated).

Cells are rod-shaped, 0.3–0.4 × 1.8–3.2 µm and Gram-positive. Colonies are cream-white to yellow, smooth and circular. Aerobic, non-spore-forming and non-motile (non-flagellated). Grows at 25–45 °C and at 2.5–20% (w/v) NaCl, with optima around 37 °C and 12% (w/v) NaCl. The pH range for growth is 6.5–10.5 in 12% (w/v) NaCl (optimum,

pH 9.0). Oxidase-negative and catalase-positive. Negative for hydrolysis of soluble starch, gelatin and Tweens 20 and 80 and for production of H₂S and indole. Positive for hydrolysis of urea and arginine and for nitrate reduction. Utilizes, and produces acid, from sucrose, D-glucose, D-fructose, D-galactose, cellobiose, D-mannose, D-mannitol, D-galactose, lactose, D-xylose and dextran as sole carbon sources. Voges-Proskauer and methyl red reactions are negative. The major fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The major isoprenoid quinones are MK-7(H₂) and MK-6(H₂). The DNA G + C content of the type strain is 48 mol%.

The type strain, CM1^T (= CGMCC 1.6134^T = JCM 14087^T), was isolated from a crude sea-salt sample collected near Qingdao in eastern China.

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