Gracilibacillus halophilus sp. nov., a moderately halophilic bacterium isolated from saline soil

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A Gram-positive, moderately halophilic, moderately thermophilic, endospore-forming, catalase- and oxidase-positive, obligately aerobic bacterium, strain YIM-C55.5^T, was isolated from saline soil in the Qaidam Basin, north-west China. Cells were slender rods, motile by means of peritrichous flagella. Filamentous forms were present throughout the growth cycle. Strain YIM-C55.5^T was able to grow in the presence of 7–30% (w/v) NaCl and at pH 6.0–9.0, with optimum growth at 15% (w/v) NaCl and pH 7.0. It was able to grow at 28–60 °C, with optimum growth at 45–50 °C. *meso*-Diaminopimelic acid was present in the cell-wall peptidoglycan. The major cellular fatty acids were iso-C_{15:0}, C_{16:0}, anteiso-C_{15:0}, anteiso-C_{17:0} and C_{18:0}. Strain YIM-C55.5^T possessed menaquinone 7 (MK-7) as the predominant respiratory quinone and diphosphatidylglycerol and phosphatidylglycerol as polar lipids. The genomic DNA G + C content was 42.3 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain YIM-C55.5^T belonged to the family *Bacillaceae* and was related most closely to members of the genus *Gracilibacillus*, but with low levels of similarity to the type strains of recognized species of the genus (*Gracilibacillus orientalis* XH-63^T, 95.1%; *Gracilibacillus boracitolerans* T-16X^T, 94.0%; *Gracilibacillus dipsosauri* DD1^T, 93.7%; *Gracilibacillus halotolerans* NN^T, 93.5%). Together with phenotypic data, these results suggest that strain YIM-C55.5^T represents a novel species of the genus *Gracilibacillus*, for which the name *Gracilibacillus halophilus* sp. nov. is proposed. The type strain is YIM-C55.5^T (=DSM 17856^T =CGMCC 1.6303^T).

The genus *Gracilibacillus* was proposed by Wainø *et al.* (1999). Members of this genus are Gram-positive, motile, endospore-forming rods or filaments, with menaquinone 7 (MK-7) as the predominant respiratory quinone. At the time of writing, the genus comprises four recognized species, *Gracilibacillus halotolerans* (Wainø *et al.*, 1999), *G. dipsosauri* (Deutch, 1994; Lawson *et al.*, 1996; Wainø *et al.*, 1999), *G. orientalis* (Carrasco *et al.*, 2006) and *G. boracitolerans* (Ahmed *et al.*, 2007). In a recent study of the microbial diversity of the Qaidam Basin in Qinghai Province, north-west China, a *Gracilibacillus*-like strain, YIM-C55.5^T, was isolated from a saline soil sample collected from a beach of the Xiaochaidamu salt lake (37° 27'–37° 35' N 95° 22'–95° 33' E). Based on the results of a polyphasic taxonomic study, this strain is considered to represent a novel species of the genus *Gracilibacillus*.

Strain YIM-C55.5^T was isolated from a saline soil sample by plating 1:10 serial dilutions of the sample on a basic high-salt medium (HSM, pH 7.5) agar supplemented with 20% NaCl (w/v) (HSMA20, pH 7.5) at 55 °C for 1 week. The basic HSM medium, constructed based on marine agar 2216 (Atlas, 1993), consisted of (per litre distilled water): 5.0 g casein peptone, 2.0 g yeast extract (Oxoid), 1.0 g lactalbumin hydrolysate (Oxoid), 8.0 g Na₂SO₄, 6.4 g MgCl₂·6H₂O, 4.0 g KCl, 2.0 g MgSO₄·7H₂O, 2.0 g CaCl₂, 0.1 g iron(III) citrate, 0.16 g Na₂CO₃, 0.08 g KBr, 34.0 mg SrCl₂, 22.0 mg H₃BO₃, 4.0 mg sodium silicate, 2.4 mg NaF, 1.6 mg (NH₄)NO₃ and 8.0 mg Na₂HPO₄. The strain was maintained on slants of HSM agar supplemented with 15% (w/v) NaCl (HSMA15) at 4 °C and was also preserved in Difco marine broth 2216 (MB) supplemented with 20% (v/v) glycerol at –80 °C.

Cell morphology was examined by light microscopy (model BH 2; Olympus). Gram staining was carried out

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM-C55.5^T is EU135704.

by using the standard Gram reaction combined with the KOH lysis test method (Gregersen, 1978). Flagella and endospores were stained according to the methods of Leifson and Schaeffer-Fulton, respectively (Smibert & Krieg, 1981). Motility was observed on MB supplemented with 7% (w/v) NaCl, solidified with 0.3% agar under high moisture conditions and in a hanging-drop preparation under a $\times 100$ oil-immersion objective lens.

Cells of strain YIM-C55.5^T were Gram-positive thin rods, approximately 4–8 μm long by 0.3–0.5 μm wide, motile by means of peritrichous flagella, and occurred predominantly in flexible chains (5–10 cells). Filamentous forms were present throughout the growth cycle, especially in young cultures. Terminal ellipsoidal spores were observed in swollen sporangia.

DNA was isolated according to the method of Hopwood *et al.* (1985) and the G+C content was determined by the thermal denaturation method (Mandel & Marmur, 1968) with a Shimadzu UV-visible spectrophotometer (UV1601). Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of PCR products were performed as described by Cui *et al.* (2001). The resulting 16S rRNA gene sequence was compared with sequences obtained from public databases (GenBank/EMBL/DBJ) to find the most closely related species. Phylogenetic analyses were performed by using the software package MEGA version 3.1 (Kumar *et al.*, 2004) after multiple alignment of the sequence data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (corrected based on Kimura's two-parameter model; Kimura, 1980) were calculated and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) trees (not shown) were generated by using the treeing algorithms contained in the PHYLIP package (Felsenstein, 1993). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by means of 1000 resamplings (Felsenstein, 1985).

The DNA G+C content of strain YIM-C55.5^T was 42.3 mol%. The almost-complete 16S rRNA gene sequence (1514 bp) of strain YIM-C55.5^T was determined. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain YIM-C55.5^T was closely related to the type strains of the four recognized species of the genus *Gracilibacillus* (Wainø *et al.* 1999), and these five strains formed a distinct clade in the phylogenetic tree, in which strain YIM-C55.5^T occupied a distinct branch (Fig. 1). Strain YIM-C55.5^T showed relatively low levels of 16S rRNA gene sequence similarity to the type strains of the four recognized species of this genus *Gracilibacillus* (*G. orientalis* XH-63^T, 95.1%; *G. boracitolerans* T-16X^T, 94.0%; *G. dipsosauri* DD1^T, 93.7%; *G. halotolerans* NN^T, 93.5%). It has been suggested that bacterial strains showing less than 97% 16S rRNA gene sequence similarity can be considered to be members of different species (Stackebrandt & Goebel, 1994). It is therefore evident from

the phylogenetic data that strain YIM-C55.5^T represents a previously unknown species of the genus *Gracilibacillus*.

Growth was tested at different temperatures (4, 10, 20, 28, 37, 40, 45, 50, 55, 60 and 65 °C) on HSMA15 and at different pH values (5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) in MB supplemented with 15% (w/v) NaCl. For pH tolerance experiments, the buffer solutions described by Chen *et al.* (2007) were used. Tolerance of/requirement for salts was determined on HSMA supplemented with 0–30% (w/v) NaCl, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ or KCl, and on other media as controls, i.e. nutrient agar, trypticase soy agar (BBL) and ISP medium 2 agar (Shirling & Gottlieb, 1966). Hydrolysis of polymers, urease activity, nitrate reduction and Voges-Proskauer and methyl red tests were determined as described by Gerhardt *et al.* (1981), Ventosa *et al.* (1982) and Atlas (1993). Growth under anaerobic conditions, resistance to antibiotics and catalase and oxidase activities were tested as described by Chen *et al.* (2007). Substrate utilization as sole carbon and energy source, activities of constitutive enzymes and other physiological characteristics were examined by using API 20E, API 50CH (with API 50 CH B/E medium) and API ZYM strips (bioMérieux) and GP2 MicroPlates (Biolog) according to the manufacturers' instructions. All suspension media were supplemented with 15% (w/v) NaCl and incubated at 45 °C.

Strain YIM-C55.5^T was catalase- and oxidase-positive and obligately aerobic. It was moderately halophilic; the optimum NaCl concentration for growth was 15%, with growth observed at NaCl concentrations of 7–30% (Kushner, 1993). No growth was observed when NaCl was replaced with MgCl_2 or KCl in the medium. Strain YIM-C55.5^T was moderately thermophilic; optimum growth was observed at 45–50 °C on HSMA15, with a temperature range for growth of 28–60 °C. No growth was observed at 25 °C. The results of other phenotypic tests are given in the species description and in Table 1.

Amino acids of whole-cell hydrolysates were analysed as described by Hasegawa *et al.* (1983). Polar lipids were extracted by the method of Minnikin *et al.* (1979) and were identified by two-dimensional TLC and spraying with specific reagents (Collins & Jones, 1980). Isoprenoid quinones were analysed by HPLC as described by Groth *et al.* (1996). The fatty acid composition was determined as described by Sasser (1990) by using the Microbial Identification System (MIDI; Microbial ID) with cells grown in 10% MH (moderate halophile) medium (Garabito *et al.*, 1997) in flasks on a rotary shaker at 200 r.p.m. and 45 °C for 2 days.

Chemotaxonomic data for strain YIM-C55.5^T were consistent with its assignment to the genus *Gracilibacillus* (Wainø *et al.* 1999). The peptidoglycan of strain YIM-C55.5^T contained *meso*-diaminopimelic acid. The fatty acid profiles of strain YIM-C55.5^T and the type strains of related species are given in Table 2. The major fatty acids of strain YIM-C55.5^T were iso-C_{15:0} (22.9%), C_{16:0} (19.9%), anteiso-C_{15:0} (14.5%), anteiso-C_{17:0} (10.6%) and C_{18:0}

Fig. 1. Phylogenetic dendrogram, based on 16S rRNA gene sequence analysis, constructed according to the neighbour-joining method, showing the phylogenetic position of strain YIM-C55.5^T within the genus *Gracilibacillus*. 'm' and 'p' indicate branches that were also found with the maximum-likelihood (Felsenstein, 1981) or parsimony (Kluge & Farris, 1969) algorithms, respectively; asterisks indicate branches that were recovered with all three methods. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50 % are given. Bar, 0.01 substitutions per nucleotide position.

(10.3 %). MK-7 (94.0 %) was the predominant menaquinone, with MK-6 (4.5 %) and MK-8 (1.5 %) present in minor amounts. Diphosphatidylglycerol and phosphatidylglycerol were found as polar lipids.

The phenotypic data supported the placement of strain YIM-C55.5^T in the genus *Gracilibacillus* (Wainø *et al.*, 1999). However, the novel strain differed markedly from the four recognized *Gracilibacillus* species by having a comparatively high NaCl concentration for optimum growth (15 %, w/v) and a high maximum temperature for growth (60 °C; Table 1). Strain YIM-C55.5^T also differed with respect to its fatty acid profile, in which there were significant amounts of unbranched saturated components (making up 34.5 % of the total), whereas the amount of anteiso-C_{15:0} (14.5 %) was noticeably less than that of the four type strains of recognized *Gracilibacillus* species (Table 2).

On the basis of the phylogenetic analysis and phenotypic distinctiveness presented here, we suggest that strain YIM-C55.5^T represents a novel species of the genus *Gracilibacillus*, for which the name *Gracilibacillus halophilus* sp. nov. is proposed.

Description of *Gracilibacillus halophilus* sp. nov.

Gracilibacillus halophilus (ha.lo'phi.us. Gr. n. *hals* salt; N.L. masc. adj. *philus* from Gr. adj. *philos* loving; N.L. masc. adj. *halophilus* salt-loving).

Cells are Gram-positive, catalase- and oxidase-positive, obligately aerobic, thin rods (4–8 × 0.3–0.5 µm) that occur predominantly in flexible chains (5–10 cells). Cells are motile by means of peritrichous flagella. Filamentous forms

are present throughout the growth cycle. They bear ellipsoidal endospores that lie in terminal swollen sporangia. After 3 days on HSMA15 (pH 7.0) at 45 °C, colonies are circular, creamy white and translucent with slightly irregular margins and 1–1.5 mm in diameter. No diffusible pigments are produced. Growth occurs at pH 6.0–9.0 (optimum pH 7.0). Moderately halophilic, with optimum growth at 15 % (w/v) NaCl and a growth range of 7–30 % (w/v) NaCl. Moderately thermophilic, with optimum growth at 45–50 °C and a growth temperature range of 28–60 °C. Positive for hydrolysis of chitin, aesculin, gelatin, starch and Tween 80, but negative for indole and H₂S production, Voges–Proskauer and methyl red tests and hydrolysis of casein and urea. Nitrate is reduced to nitrite. Resistant to gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (20 µg), polymyxin B (30 µg) and streptomycin (10 µg), but sensitive to ampicillin (30 µg), chloramphenicol (30 µg), lincomycin (2 µg), novobiocin (30 µg), rifampicin (5 µg) and tetracycline (30 µg). Positive for oxidation of arabinose, inositol, sorbitol, amygdalin, rhamnose, sucrose and melibiose and fermentation of glucose, but negative for citrate utilization (API 20E). Acids are produced from D-arabinose, D-ribose, D-tagatose, potassium 2-ketogluconate and potassium 5-ketogluconate and acids are produced weakly from D-glucose, glycogen, D-fructose, L-sorbose, L-rhamnose, inositol, D-mannitol, D-sorbitol, amygdalin, melibiose and sucrose (API 50CH). Oxidizes acetic acid, amygdalin, L-arabinose, D-fructose, D-glucose, glycerol, glycogen, myo-inositol, maltose, D-mannitol, D-mannose, melibiose, raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose, trehalose, D-xylose, L-alanine and L-asparagine (Biolog GP2). Constitutive enzymes expressed are alkaline phosphatase,

Table 1. Differential characteristics between strain YIM-C55.5^T and its closest phylogenetic relatives in the genus *Gracilibacillus*

Strains: 1, YIM-C55.5^T (data from the present study); 2, *G. orientalis* XH-63^T (Carrasco *et al.*, 2006); 3, *G. boracitolerans* T-16X^T (Ahmed *et al.*, 2007); 4, *G. dipsosauri* DSM 11125^T (Deutch, 1994; Lawson *et al.*, 1996; Wainø *et al.*, 1999; Carrasco *et al.*, 2006); 5, *G. halotolerans* DSM 11805^T (Wainø *et al.*, 1999). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5
Colony pigmentation	Creamy white	Cream	Light pink to red	White	Creamy white
Morphology	Thin rods, filaments	Thin rods	Thin rods, filaments	Thin rods, filaments	Thin rods, filaments
Spore shape*	E	S	S/E	S	E
NaCl range (optimum) (%)	7–30 (15)	1–20 (10)	0–11 (0.5–3)	0–15 (3)	0–20 (0)
Temperature range (optimum) (°C)	28–60 (45–50)	4–45 (37)	16–37 (25–28)	28–50 (45)	6–50 (47)
pH range (optimum)	6.0–9.0 (7.0)	5.0–9.0 (7.5)	6.0–9.7 (7.5–8.5)	ND (7.5)	5.0–10.0 (7.5)
Oxidase activity	+	–	+	+	+
H ₂ S production	–	–	–	–	+
Nitrate reduction	+	–	–	+	+
Hydrolysis of:					
Gelatin	+	+	–	+	+
Tween 80	+	–	ND	ND	+
Urea	–	–	–	–	+
DNA G + C content (mol%)	42.3	37.1	35.8	39.4	38

*E, Ellipsoidal; S, spherical.

Table 2. Whole-cell fatty acid compositions of strain YIM-C55.5^T and type strains of related *Gracilibacillus* species

Strains: 1, YIM-C55.5^T (data from the present study); 2, *G. orientalis* XH-63^T (Carrasco *et al.*, 2006); 3, *G. boracitolerans* T-16X^T (Ahmed *et al.*, 2007); 4, *G. dipsosauri* DSM 11125^T (Wainø *et al.*, 1999); 5, *G. halotolerans* DSM 11805^T (Wainø *et al.*, 1999). Fatty acid methyl esters were determined with cells grown in 10 % MH medium (Garabito *et al.*, 1997) except for *G. boracitolerans* DSM 17256^T, for which MB plus 1.5 % NaCl was used. Data are expressed as percentages of total fatty acids; those components representing less than 1.0 % are not shown. –, Not detected; tr, trace amount; ND, no data available.

Fatty acid	1	2	3	4	5
Unbranched saturated					
C _{14:0}	3.1	ND	tr	tr	tr
C _{15:0}	tr	ND	2.0	tr	tr
C _{16:0}	19.9	4.9	5.6	7.2	9.1
C _{17:0}	1.2	3.2	tr	ND	ND
C _{18:0}	10.3	ND	tr	ND	ND
Branched saturated					
iso-C _{14:0}	tr	1.0	tr	tr	9.0
iso-C _{15:0}	22.9	8.5	14.5	17.6	4.2
anteiso-C _{15:0}	14.5	51.9	50.8	39.1	56.2
iso-C _{16:0}	2.2	3.3	1.8	4.7	7.1
iso-C _{17:0}	6.7	2.4	2.0	7.3	–
anteiso-C _{17:0}	10.6	20.6	18.4	24.2	14.9
Unsaturated					
C _{16:1} ω7c alcohol	1.1	ND	–	ND	ND
C _{16:1} ω11c	–	1.9	tr	ND	ND
C _{18:1} ω7c	1.1	ND	ND	ND	ND
C _{18:1} ω9c	2.1	ND	tr	ND	ND

esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, α -chymotrypsin, acid phosphatase, β -galactosidase, naphthol-AS-BI-phosphohydrolase, α -glucosidase, β -glucosidase and α -mannosidase (API ZYM). Menaquinone 7 (MK-7) is the predominant respiratory quinone. The polar lipids consist of diphosphatidylglycerol and phosphatidylglycerol. The major cellular fatty acids (making up 77.9% of the total in the type strain) are iso-C_{15:0}, C_{16:0}, anteiso-C_{15:0}, anteiso-C_{17:0} and C_{18:0}. Contains meso-diaminopimelic acid in the cell-wall peptidoglycan. The genomic DNA G+C content of the type strain is 42.3 mol%.

The type strain, YIM-C55.5^T (=DSM 17856^T =CGMCC 1.6303^T), was isolated from a saline soil sample collected from a beach of the Xiaochaidamu salt lake in the Qaidam Basin, Qinghai Province, north-west China.

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