Gracilibacillus orientalis sp. nov., a novel moderately halophilic bacterium isolated from a salt lake in Inner Mongolia, China

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Three Gram-positive, moderately halophilic, spore-forming rods, designated XH-63^T, XH-62 and EJ-15, were isolated from two salt lakes located near Xilin Hot and Ejnor, in Inner Mongolia, China. The strains were strictly aerobic and motile, with spherical, terminal and deforming endospores. They grew at pH 5.0–9.0 (optimal growth at pH 7.5), between 4 and 45 °C (optimal growth at 37 °C) and at salinities of 1–20 % (w/v) total salts, growing optimally at 10 % (w/v) salts. They had *meso*-diaminopimelic acid in the cell wall peptidoglycan and DNA G+C contents of 36.1–37.1 mol%. The polar lipid pattern of strain XH-63^T, selected as the representative strain, consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and a phospholipid and two amino phospholipids of unknown structure. This strain possessed anteiso-C_{15:0} and anteiso-C_{17:0} as the major fatty acids (altogether representing 72.5 % of total) and MK-7 as the major menaquinone. 16S rRNA gene analysis of the three strains showed that they were within the *Gracilibacillus* cluster, with highest sequence similarity (95.4–95.8 %) with *Gracilibacillus dipsosauri*. Based on a combination of phenotypic, chemotaxonomic and phylogenetic features, it is proposed that the three isolates represent a novel species of the genus *Gracilibacillus*, *Gracilibacillus orientalis* sp. nov. The type strain is strain XH-63^T (=CCM 7326^T=AS 1.4250^T=CECT 7097^T).

Moderately halophilic bacteria that grow optimally in media containing 3–15 % (w/v) salts are widely distributed in different saline habitats (Ventosa *et al.*, 1998). Taxonomically, they are a very heterogeneous physiological group, including both Gram-positive and Gram-negative microorganisms. Aerobic, spore-forming, moderately halophilic, Gram-positive rods are also taxonomically diverse and have been isolated from saline environments such as soils and aquatic habitats (Arahal & Ventosa, 2002). They were originally assigned to the genus *Bacillus*, but molecular and

chemical analyses have shown that they form several phylogenetically distinct lineages within the group classically defined as the genus *Bacillus* (Ash *et al.*, 1991; Stackebrandt & Liesack, 1993; Nielsen *et al.*, 1994). To date, moderately halophilic bacterial species are present in the genera *Bacillus* (Ventosa *et al.*, 1989), *Halobacillus* (Spring *et al.*, 1996; Yoon *et al.*, 2003), *Virgibacillus* (Heyndrickx *et al.*, 1998; Arahal *et al.*, 1999, 2000; Heyrman *et al.*, 2003), *Gracilibacillus* (Waino *et al.*, 1999), *Amphibacillus* (Zhilina *et al.*, 2001), *Filobacillus* (Schlesner *et al.*, 2001), *Jeotgalibacillus* (Yoon *et al.*, 2001), *Marinibacillus* (Yoon *et al.*, 2001), *Oceanobacillus* (Lu *et al.*, 2001), *Lentibacillus* (Yoon *et al.*, 2002; Jeon *et al.*, 2005a), *Tenuibacillus* (Ren & Zhou, 2005a), *Pontibacillus* (Lim *et al.*, 2005a, b), *Salinibacillus* (Ren & Zhou, 2005b), *Thalassobacillus* (García *et al.*, 2005) and *Alkalibacillus* (Jeon *et al.*, 2005b). All these genera belong to the family *Bacillaceae*,

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains XH-63^T, XH-62 and EJ-15 are AM040716, AM040717 and AM040718, respectively.

having similar properties and close phylogenetic relationships. Wainø *et al.* (1999) proposed the new genus *Gracilibacillus* to accommodate a bacterium isolated from the Great Salt Lake, Utah (USA), *Gracilibacillus halotolerans*, and they transferred *Bacillus dipsosauri* to this genus as *Gracilibacillus dipsosauri*. During the course of a broad study of moderately halophilic bacteria from several salt lakes in China, three moderately halophilic Gram-positive bacteria were isolated. Their phenotypic and chemotaxonomic characteristics, cell wall compositions, DNA G + C contents and 16S rRNA gene sequences have been determined. The data obtained strongly suggest that these three strains represent a novel species of the genus *Gracilibacillus* and therefore they have been classified as *Gracilibacillus orientalis* sp. nov.

The three strains were isolated from water (strains XH-63^T and XH-62) and sediment (strain EJ-15) samples taken during an expedition in September 2003 of two salt lakes located near Xilin Hot and Ejnor, in Inner Mongolia, China. The lake near Xilin Hot is located at 43° 55' N 115° 37' E and the water was 24 °C, pH 8·5 and had a conductivity of 185 mS cm⁻¹. The lake near Ejnor is located at 45° 14' N 116° 31' E and the water was 28 °C, pH 7·5 and had a conductivity of 161 mS cm⁻¹. The isolation medium and the method used for isolation have been described previously (Ventosa *et al.*, 1983). These strains were cultivated in MH medium with 10% (w/v) total salts (MH-10 medium). The composition of this medium was (% w/v): NaCl, 8·1; MgCl₂, 0·7; MgSO₄, 0·96; CaCl₂, 0·036; KCl, 0·2; NaHCO₃, 0·006; NaBr, 0·0026; yeast extract (Difco), 1; proteose peptone no. 3 (Difco), 0·5; and glucose, 0·1. The

pH was adjusted to 7·2 with 1 M KOH. When necessary, solid media were prepared by adding 2·0% (w/v) Bacto-agar (Difco). *G. dipsosauri* DSM 11125^T and *G. halotolerans* DSM 11805^T were obtained from the DSMZ, Braunschweig, Germany, and cultivated according to the procedures recommended by the DSMZ.

To characterize these isolates phenotypically, standard phenotypic tests were performed, including Gram reaction, cell morphology, motility, growth under anaerobic conditions, catalase and oxidase production, as well as other tests shown in Table 1 or included in the species description. For nutritional tests, a basal medium with the following composition was used (w/v): NaCl, 10·0; KCl, 0·2; MgSO₄·7H₂O, 0·02; KNO₃, 0·1; (NH₄)₂HPO₄, 0·1; and KH₂PO₄, 0·05. To this liquid medium, a 0·1% (w/v) filter-sterilized substrate was added. Carbohydrates were used at a final concentration of 0·2% (w/v). When amino acids were used as substrate, the basal medium contained neither KNO₃ nor (NH₄)₂HPO₄. All test procedures have been previously described (Ventosa *et al.*, 1982; Quesada *et al.*, 1984; García *et al.*, 1987). Strains were Gram-positive, motile and strictly aerobic. Cells were rod-shaped with a width of 0·7–0·9 µm and length of 2·0–10·0 µm. Spherical endospores were formed at terminal positions in swollen sporangia (Fig. 1), similarly to those produced by *G. dipsosauri*, the species most closely related phylogenetically. However, the other member of the genus *Gracilibacillus*, *G. halotolerans*, produces ellipsoid endospores. The three isolates were moderately halophilic, growing in media containing 1–20% (w/v) salts and growing optimally in media

Table 1. Differential phenotypic characteristics of XH-63^T, XH-62, EJ-15, *G. dipsosauri* DSM 11125^T and *G. halotolerans* DSM 11805^T

Strains: 1, XH-63^T; 2, XH-62; 3, EJ-15; 4, *G. dipsosauri* DSM 11125^T (data from Lawson *et al.*, 1996; Wainø *et al.*, 1999; and this study); 5, *G. halotolerans* DSM 11805^T (data from Wainø *et al.*, 1999). Symbols: +, positive; –, negative; S, spherical; E, ellipsoidal; NA, not available. All strains are Gram-positive, motile rods, producing endospores at the terminal position in swollen sporangia. All of them share the following characteristics: catalase-positive; hydrolyse aesculin and starch; produce acids from glucose; and give a negative result in the methyl red and Voges–Proskauer tests.

Characteristic	1	2	3	4	5
Pigmentation	Cream	Cream	Cream	White	Creamy white
Spore shape	S	S	S	S	E
Anaerobic growth	–	–	–	+	–
Temperature range (°C)	4–45	4–45	4–45	28–50	6–50
Optimum temperature (°C)	37	37	37	45	47
NaCl range (% w/v)	3–20	3–20	1–20	0–15	0–20
Optimum growth in NaCl (% w/v)	10	10	10	3	0
Oxidase activity	–	–	–	+	+
Nitrate reduction	–	–	–	+	+
H ₂ S production	–	–	–	–	+
Hydrolysis of:					
Tween 80	–	–	–	NA	+
Urea	–	–	–	–	+

Fig. 1. Phase-contrast micrograph of strain XH-63^T showing spore-forming rods. Bar, 10 µm.

containing 10 % (w/v) salts. No growth was observed in the absence of NaCl. *G. dipsosauri* and *G. halotolerans* are able to grow in media containing 0–15 % and 0–20 % (w/v) NaCl, respectively, and thus they are considered to be extremely halotolerant, although *G. dipsosauri* was originally described as moderately halophilic by Lawson *et al.* (1996). Table 1 shows the differential characteristics between these isolates and the species *G. dipsosauri* and *G. halotolerans*.

Genomic DNAs were prepared using the method described by Marmur (1961). PCR amplifications of the 16S rRNA gene were carried out using methods that have been previously described in detail (Mellado *et al.*, 1995). Sequencing was performed using an automated DNA sequencer model 3100 (Applied Biosystems). The 16S rRNA gene sequence analyses were performed with the aid of the ARB software package (Ludwig & Strunk, 1996). 16S rRNA gene sequences were aligned and the alignment was confirmed and checked

against both the primary and secondary structure of the 16S rRNA molecule using the alignment tool of the ARB software package. Phylogenetic trees were constructed using three different methods, maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987), algorithms integrated in the ARB software for phylogenetic inference.

Almost-complete 16S rRNA gene sequences of strains XH-63^T, XH-62 and EJ-15 were obtained and used for initial BLAST searches in GenBank and phylogenetic analysis. Phylogenetic analysis was conducted with sequences of representative strains of the family *Bacillaceae* and related taxa. A tree constructed by maximum-parsimony analysis clearly showed that the three isolates were part of a cluster that included the genera *Gracilibacillus* and *Paraliobacillus*, as shown in Fig. 2. Topologies of phylogenetic trees built using the maximum-likelihood and neighbour-joining algorithms were similar to those of the tree constructed by maximum-parsimony analysis (data not shown). The 16S rRNA gene sequences of the three novel isolates shared 99.9–100 % similarity to each other and are on the same phylogenetic branch. The nearest known relatives of the strains XH-63^T, XH-62 and EJ-15 were *G. dipsosauri* (95.7, 95.6 and 95.8 % 16S rRNA gene sequence similarity, respectively) and *G. halotolerans* (95.4, 95.3 and 95.4 % 16S rRNA gene sequence similarity, respectively). The other closely related relative was *Paraliobacillus ryukyuensis* (94.8, 94.7 and 94.9 % similarity, respectively).

For determination of the DNA base composition of the three isolates, DNAs were extracted and purified by the method of Marmur (1961) and the G+C contents of the DNAs were determined from the midpoint value of the thermal denaturation profile (Marmur & Doty, 1962) using the equation of Owen & Hill (1979). The genomic DNA G+C

Fig. 2. Maximum-parsimony tree, based on 16S rRNA gene sequence comparison, showing the relationship of strains XH-63^T, XH-62 and EJ-15 to related species. Accession numbers of the sequences used in this study are shown in parentheses. *Bacillus subtilis* was used as an outgroup. Bar, 0.02 substitution per nucleotide position.

contents of strains XH-63^T, XH-62 and EJ-15 were 37.1, 36.1 and 37.0 mol%, respectively. These values are within the range of G+C described for the species of the genus *Gracilibacillus* and are similar to those described for *G. dipsosauri* (39.4 mol%; Lawson *et al.*, 1996) and *G. halotolerans* (38.0 mol%; Wainø *et al.*, 1999). On the other hand, these values are similar to that described for *P. ryukyuensis* (35.6 mol%; Ishikawa *et al.*, 2002). DNA–DNA hybridization was carried out to evaluate the genomic DNA relatedness of the three isolates following the competition procedure of Johnson (1994), described in detail elsewhere (Arahal *et al.*, 2001). Hybridization experiments were carried out under optimal conditions, at a temperature of 44.3 °C, which is within the limits of validity for the filter method (De Ley & Tijtgaat, 1970). DNA–DNA hybridization values of strain XH-63^T with XH-62 and EJ-15 were 98 and 94 %, respectively. These data clearly support the idea that the three strains are members of a single species (Stackebrandt *et al.*, 2002).

Preparation of cell walls from the three strains and analysis of peptidoglycan structures were carried out using methods described by Schleifer (1985), with the modification that TLC on cellulose sheets was performed instead of paper chromatography. Our isolates possessed a cell wall type based on *meso*-diaminopimelic acid, which was in common with those of *G. dipsosauri*, *G. halotolerans* and the great majority of endospore-forming, Gram-positive bacilli.

The cellular fatty acids of strain XH-63^T, selected as a representative strain of the isolates, were analysed with the MIDI system (Microbial ID). Cells were cultured in MH-10 medium at 37 °C for 24 h. The predominant fatty acids of strain XH-63^T were anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{15:0}, C_{16:0}, iso-C_{16:0} and C_{17:0}. This fatty acid profile is quite similar to those of the species of *Gracilibacillus* and is in accordance to those previously reported for these two species (Wainø *et al.*, 1999), although a number of differences could be seen in the distribution of the minor fatty acids (Table 2).

Respiratory lipoquinone analysis and analysis of polar lipids of strain XH-63^T were carried out by the Identification Service of the DSMZ. The predominant isoprenoid quinone of this strain was menaquinone-7 (MK-7), which is typical of the group classically defined as the genus *Bacillus*. With respect to the polar lipid pattern, strain XH-63^T contained diphosphatidylglycerol and phosphatidylglycerol as the major polar lipids. Phosphatidylethanolamine, a phospholipid and two amino phospholipids of unknown structure were also detected as minor components. In the case of species of the genus *Gracilibacillus*, the major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and two phospholipids of unknown structure; however, phosphatidylethanolamine has not been reported for either *G. dipsosauri* or *G. halotolerans* (Wainø *et al.*, 1999).

Phylogenetic analysis based on the 16S rRNA sequence, cell wall type and respiratory lipoquinone analysis clearly suggest that our isolates belong to the genus *Gracilibacillus*.

Table 2. Cellular fatty acid content of strain XH-63^T, *G. dipsosauri* and *G. halotolerans*

Strains: 1, XH-63^T; 2, *G. dipsosauri* DSM 11125^T; 3, *G. halotolerans* DSM 11805^T. Values are percentages of total fatty acids; values less than 1 % are not shown. ND, Not detected; TR, trace amount.

Fatty acid	1	2*	3*
Straight chain			
C _{16:0}	4.9	7.2	9.0
C _{17:0}	3.2	ND	ND
Branched			
iso-C _{14:0}	1.0	TR	9.0
iso-C _{15:0}	8.5	17.6	4.2
anteiso-C _{15:0}	51.9	39.1	56.1
iso-C _{16:0}	3.3	4.7	7.0
iso-C _{17:0}	2.4	7.2	ND
anteiso-C _{17:0}	20.6	24.2	14.6
Unsaturated			
C _{16:1} 11c	1.89	ND	ND

*Data from Wainø *et al.* (1999).

Furthermore, phenotypic features (temperature and salt ranges for growth, optimal salt concentration for growth, and absence of oxidase and nitrate reductase activities), DNA base composition, fatty acid profile and polar lipid patterns clearly differentiate the three isolates from *G. dipsosauri* and *G. halotolerans*. In conclusion, it is proposed that the novel strains represent a novel species of the genus *Gracilibacillus*, for which the name *Gracilibacillus orientalis* sp. nov. is proposed.

Description of *Gracilibacillus orientalis* sp. nov.

Gracilibacillus orientalis (o.ri.en.ta'lis. L. adj. *orientalis* eastern, bacterium inhabiting the East).

Gram-positive rods, 0.7–0.9 × 2.0–10.0 µm. Motile, spherical endospores are produced at a terminal position in swollen sporangia. Strictly aerobic. Colonies are 0.3–0.6 mm in diameter, cream, circular, opaque and entire on MH-10 medium after 2 days of cultivation. Moderately halophilic, growing in a wide range (1–20 %, w/v) of salt concentrations, with optimal growth at 10 % (w/v) salts. No growth in the absence of NaCl. Grows at 4–45 °C (optimal at 37 °C) and pH 5.0–9.0 (optimal at pH 7.5). Strictly aerobic. Catalase-positive and oxidase-negative. Nitrate is not reduced to nitrite. Acid is produced from arabinose, galactose, glycerol, D-glucose, D-fructose, D-lactose, D-mannitol, D-xylose, maltose, D-trehalose and sucrose. Aesculin, gelatin and starch are hydrolysed; casein, Tween 80 and urea are not hydrolysed. H₂S is not produced. Indole, methyl red, phenylalanine deaminase, Simmons citrate, arginine dihydrolase, and lysine and ornithine decarboxylase tests are negative. Phosphatase test is positive. The following

compounds are utilized as sole carbon and energy sources: acetate, citrate, formate, fumarate, D-fucose, lactose, propanol, D-sorbitol and valerate. The following compounds are not utilized as sole carbon and energy sources: D-arabinose, D-cellobiose, D-galactose, maltose, D-mannose, D-melibiose, D-melzitose, L-raffinose, D-trehalose, D-xylose, butanol, ethanol, methanol, benzoate, propionate and succinate. The following compounds are not used as sole carbon, nitrogen and energy sources: L-alanine, L-arginine, aspartic acid, L-cysteine, phenylalanine, glutamic acid, DL-lysine, L-methionine, L-ornithine, L-threonine, tryptophan and L-serine. Susceptible to bacitracin (10 U), chloramphenicol (30 µg), erythromycin (15 µg) and rifampicin (30 µg). Resistant to ampicillin (10 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), neomycin (10 µg), novobiocin (30 µg) and penicillin (10 U). The cell wall contains peptidoglycan of the *meso*-diaminopimelic acid type. Major isoprenoid quinone is MK-7. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and a phospholipid and two amino phospholipids of unknown structure. Additional characteristics of the strains are listed in Table 1. Cellular fatty acid composition is given in Table 2.

The type strain is XH-63^T (=CCM 7326^T = AS 1.4250^T = CECT 7097^T), isolated from a salt lake near Xilin Hot in Inner Mongolia, China.

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