Bacillus xiaoxiensis sp. nov., a slightly halophilic bacterium isolated from non-saline forest soil

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A novel Gram-stain-positive, slightly halophilic, catalase-positive, oxidase-negative, endospore-forming, motile, facultatively anaerobic, rod-shaped bacterium, designated strain JSM 081004^T, was isolated from non-saline forest soil in Xiaoxi National Natural Reserve, China. Growth occurred with 0.5–20 % (w/v) NaCl (optimum 2–4 %), at pH 6.0–10.5 (optimum pH 8.0) and at 5–40 °C (optimum 25–30 °C). *meso*-Diaminopimelic acid was present in the cell-wall peptidoglycan. The major cellular fatty acids were iso-$C_{15:0}$ and anteiso-$C_{15:0}$. Strain JSM 081004^T contained MK-7 as the predominant respiratory quinone, and diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol as the major polar lipids. The genomic DNA G+C content of strain JSM 081004^T was 40.1 mol%. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain JSM 081004^T should be assigned to the genus *Bacillus* and was most closely related to the type strains of *Bacillus lehensis* (sequence similarity 97.8 %), *Bacillus oshimensis* (97.8 %) and *Bacillus patagoniensis* (97.3 %). Phylogenetic analysis, DNA–DNA relatedness values, phenotypic characteristics and chemotaxonomic data all support the proposal of strain JSM 081004^T as a representative of a novel species of the genus *Bacillus*, for which the name *Bacillus xiaoxiensis* sp. nov. is proposed; the type strain is JSM 081004^T (=CCTCC AA 208057^T =DSM 21943^T).

Halophilic, halotolerant, alkaliphilic and/or alkalitolerant bacilli species are not only widely distributed throughout various types of saline environments (Ash *et al.*, 1991; Nielsen *et al.*, 1994; Ventosa *et al.*, 1998; Arahal & Ventosa, 2002; Romano *et al.*, 2005; Lim *et al.*, 2006a, b; Yumoto, 2007; Chen *et al.*, 2009a, b), but have also been isolated from non-saline environments (Nielsen *et al.*, 1995; Echigo *et al.*, 2005, 2007; Usami *et al.*, 2007). These bacteria have attracted increasing interest, attributable to their ability to grow under extreme conditions as well as to the potential use of their enzymes in biotechnological applications (Horikoshi, 1999; Margesin & Schinner, 2001; Nogi *et al.*, 2005; Krulwich *et al.*, 2007). During an investigation of the diversity of halophilic and halotolerant bacteria in Xiaoxi National Natural Reserve (28° 42′ 15″ to 28° 53′ 15″ N 110° 6′ 50″ to 110° 21′ 35″ E), Hunan Province, China (Chen *et al.*, 2010), a slightly halophilic, endospore-forming, Gram-stain-positive bacterium, designated strain JSM 081004^T, was isolated from a non-saline forest soil sample. Based on the results of a polyphasic taxonomic study, this strain is considered to represent a novel species of the genus *Bacillus*.

Strain JSM 081004^T was isolated from a non-saline forest soil sample by using the dilution plating technique on marine agar 2216 (MA; Difco) supplemented with 10 % (w/v) NaCl and cultivated at 28 °C for 2 weeks. After primary isolation and purification, the isolate was maintained as serial transfers on MA slants, as lyophilized

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JSM 081004^T is HM054474.

A supplementary figure is available with the online version of this paper.

cultures at 4 °C and also deep-frozen at −80 °C in 20 % (v/v) glycerol. Three type strains, *Bacillus lehensis* DSM 19099^T, *Bacillus oshimensis* DSM 18940^T and *Bacillus patagoniensis* DSM 16117^T, obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), were used as reference strains for comparison. Unless otherwise indicated, morphological, physiological, molecular and chemotaxonomic studies were performed with cells grown on MA (pH 8.0) at 30 °C.

Cell morphology was examined by using light microscopy (model DM3000; Leica). Gram staining and KOH lysis tests were carried out according to Smibert & Krieg (1994) and Gregersen (1978), respectively. Flagella and endospores were examined according to the methods of Leifson and Schaeffer–Fulton, respectively (Smibert & Krieg, 1994). Growth was tested at various temperatures (4, 5–55 °C, in increments of 5 °C) and pH (5.0–11.0, in increments of 0.5 units) on MA as well as in nutrient broth (NB) supplemented with 2.5 % (w/v) NaCl. The buffer solutions described by Chen *et al.* (2007) were used for pH experiments. Growth in the absence of NaCl was investigated on nutrient agar (NA) and in NB prepared according to the formula of Atlas (1993) but without NaCl. Tolerance to NaCl was tested on NA as well as in NB at different NaCl concentrations [0.1 and 0.5 % (w/v), and 1–30 % (w/v) in increments of 1 %]. Methyl red and Voges–Proskauer tests, and determination of H_2S production from L-cysteine, egg yolk reaction, hydrolysis of aesculin, indole production, nitrate and nitrite reduction, and arginine dihydrolase, lysine and ornithine decarboxylase, phenylalanine deaminase and urease activities were carried out as described by Smibert & Krieg (1994). Hydrolysis of casein, cellulose, DNA, gelatin, starch, ONPG, and Tweens 20, 40, 60 and 80 was determined as described by Cowan & Steel (1965). Growth under anaerobic conditions was determined on MA supplemented with 0.5 % (w/v) glucose and with or without 0.1 % (w/v) nitrate by using the GasPak anaerobic system (BBL) according to the manufacturer's instructions. Determination of acid production from carbohydrates and utilization of carbon and nitrogen sources was performed as described by Ventosa *et al.* (1982). Motility, and catalase and oxidase activities were determined as described previously (Chen *et al.*, 2007). Other enzymic activities were assayed by using API ZYM strips (bioMérieux) according to the manufacturer's instructions with 3 % (w/v) NaCl. All physiological and biochemical tests were repeated three times.

Cells of strain JSM 081004^T were Gram-stain-positive, endospore-forming, motile, slightly halophilic, facultatively anaerobic, straight rods, with optimum growth occurring in the presence of 2–4 % (w/v) NaCl, at pH 8.0 and at 25–30 °C. Colonies were yellow-pigmented, flat, opaque with smooth, glistening surfaces and circular/slightly irregular margins, and 2–3 mm in diameter after incubation for 3–4 days at 30 °C on MA. Detailed phenotypic properties that differentiate strain JSM 081004^T from related species of the genus *Bacillus* are summarized in Table 1 and also mentioned in the species description below.

Genomic DNA was isolated according to Hopwood *et al.* (1985) and the G+C content was determined using the HPLC method (Mesbah *et al.*, 1989). The 16S rRNA gene was amplified by PCR and sequenced as described by Cui *et al.* (2001). Pairwise sequence similarities were calculated using a global alignment algorithm, implemented at the EzTaxon server (Chun *et al.*, 2007). Phylogenetic analysis was performed by using the software package MEGA version 4.1 (Tamura *et al.*, 2007) after multiple alignment of sequence data by CLUSTAL X (Thompson *et al.*, 1997). Distances were calculated using distance options according to Kimura's two-parameter model (Kimura, 1980) and clustering was performed by the neighbour-joining method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) trees were generated by using the tree-making algorithms contained in the PHYLIP package (Felsenstein, 2002). Bootstrap analysis was used to evaluate tree topology by means of 1000 resamplings (Felsenstein, 1985). After the DNA was purified to an absorbance ratio of A_{260} versus A_{280} higher than 1.8, DNA–DNA hybridization experiments were performed according to the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992) using a UV-1206 spectrophotometer (Shimadzu) equipped with a TB-85 thermo-bath. Every hybridization experiment was repeated five times and the highest and lowest values in each experiment were excluded. DNA–DNA relatedness values were expressed as the means of the remaining three values.

The DNA G+C content of strain JSM 081004^T was 40.1 mol%. The almost-complete 16S rRNA gene sequence (1467 bp) was determined. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain JSM 081004^T should be assigned to the genus *Bacillus* and was related most closely to *B. lehensis* MLB2^T (16S rRNA gene sequence similarity of 97.8 %; Ghosh *et al.*, 2007), *B. oshimensis* K11^T (97.8 %; Yumoto *et al.*, 2005) and *B. patagoniensis* PAT 05^T (97.3 %; Olivera *et al.*, 2005); sequence similarities less than 95.5 % were observed with other species of the genus *Bacillus*. The neighbour-joining phylogenetic tree further confirmed that strain JSM 081004^T was closely related phylogenetically to members of the genus *Bacillus* and formed a robust lineage with the type strains of *B. lehensis*, *B. oshimensis*, *B. patagoniensis* and *Bacillus clausii* (95.3 % similarity; Nielsen *et al.*, 1995) (Fig. 1). Topology was similar to those of the phylogenetic trees reconstructed by using maximum-likelihood and maximum-parsimony methods (Supplementary Fig. S1, available in IJSEM Online). Levels of DNA–DNA relatedness of strain JSM 081004^T with *B. lehensis* DSM 19099^T, *B. oshimensis* DSM 18940^T and *B. patagoniensis* DSM 16117^T were 18.6 % (SD of 1.8 %), 17.9 % (SD of 1.5 %) and 16.4 % (SD of 1.7 %), respectively, values that are well below the threshold value (70 %) recommended by Wayne *et al.*

Table 1. Characteristics used to distinguish strain JSM 081004^T from the type strains of phylogenetically related species of the genus *Bacillus*

Strains: 1, *B. xiaoxiensis* sp. nov. JSM 081004^T; 2, *B. lehensis* DSM 19099^T; 3, *B. oshimensis* DSM 18940^T; 4, *B. patagoniensis* DSM 16117^T. All strains are endospore-forming, Gram-stain-positive rods that can grow in the presence of 1–15 % (w/v) NaCl, at pH 7.0–10.5 and at 10–40 °C. All strains are positive for catalase activity and hydrolysis of casein, gelatin, starch and Tween 20. All strains are negative for: egg yolk reaction; indole and H_2S production; hydrolysis of cellulose and ONPG; nitrite reduction; methyl red and Voges–Proskauer tests; and arginine dihydrolase, phenylalanine deaminase, and lysine and ornithine decarboxylase activities. All data were obtained from this study unless indicated otherwise. +, Positive; −, negative.

Characteristic	1	2	3	4
Colony pigmentation	Yellow	Creamy yellow	Creamy	Creamy white
Spore shape	Ellipsoidal	Oval	Ellipsoidal	Oval
Spore position	Central to subterminal	Subterminal	Terminal	Subterminal
Sporangium	Slightly swollen	Unswollen	Unswollen	Swollen
Motility	+	+	−	+
Facultatively anaerobic	+	−	−	−
Nitrate reduction	+	−	−	−
Oxidase	−	+	+	−
Urease	−	+	+	+
Citrate utilization	−	−	−	+
Growth conditions				
NaCl range (%, w/v)	0.5–20	0.5–15	0–18	1–25
NaCl optimum (%, w/v)	2–4	5–8	2–4	6–10
pH range	6.0–10.5	6.5–10.5	7.0–11.0	6.5–10.5
pH optimum	8.0	8.0	8.5	7.5–8.0
Temperature range (°C)	5–40	10–40	10–40	5–40
Temperature optimum (°C)	25–30	30	25–30	25–30
Hydrolysis of:				
DNA	−	−	+	−
Aesculin	+	+	−	−
Tween 40	−	+	+	+
Tween 60	−	+	+	+
Tween 80	−	−	+	−
Polar lipids*	DPG, PG, PE, PL	DPG, PG, PE, 2PL	DPG, PG, PE, PI, PIM, PL	DPG, PG, PE, PI, PIM, PL
DNA G+C content (mol%)†	40.1	41.4	40.8	39.7

*DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; PL, unidentified phospholipid.

†Data for the type strains of *B. lehensis*, *B. oshimensis* and *B. patagoniensis* were obtained from Ghosh *et al.* (2007), Yumoto *et al.* (2005) and Olivera *et al.* (2005), respectively.

(1987) for the definition of members of a species. Therefore, it would appear that, on the basis of phylogenetic and DNA–DNA hybridization data, strain JSM 081004^T represents a novel species of the genus *Bacillus* according to accepted criteria (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

Amino acids of whole-cell hydrolysates were analysed by TLC as described by Hasegawa *et al.* (1983). Isoprenoid quinones were analysed by HPLC as described by Groth *et al.* (1996). Polar lipids were extracted according to the method of Minnikin *et al.* (1979) and identified by two-dimensional TLC; total lipid material and specific functional groups were detected using Dittmer–Lester reagent (phosphate-containing lipids), ninhydrin (free amino groups), Dragendorff reagent (quaternary nitrogen) and anisaldehyde/sulfuric acid (glycolipids) (Dittmer & Lester, 1964; Vaskovsky *et al.*, 1975; Ryu & MacCoss, 1979; Collins & Jones, 1980). Fatty acids were determined according to Sasser (1990) using the Microbial Identification System (Microbial ID) with cells grown in marine broth 2216 (Difco) in flasks on a rotary shaker (with shaking at 200 r.p.m.) at 30 °C for 2 days.

Chemotaxonomic data for strain JSM 081004^T were consistent with assignment of the strain to the genus *Bacillus*. The strain possessed a cell-wall type based on *meso*-diaminopimelic acid as the diagnostic diamino acid.

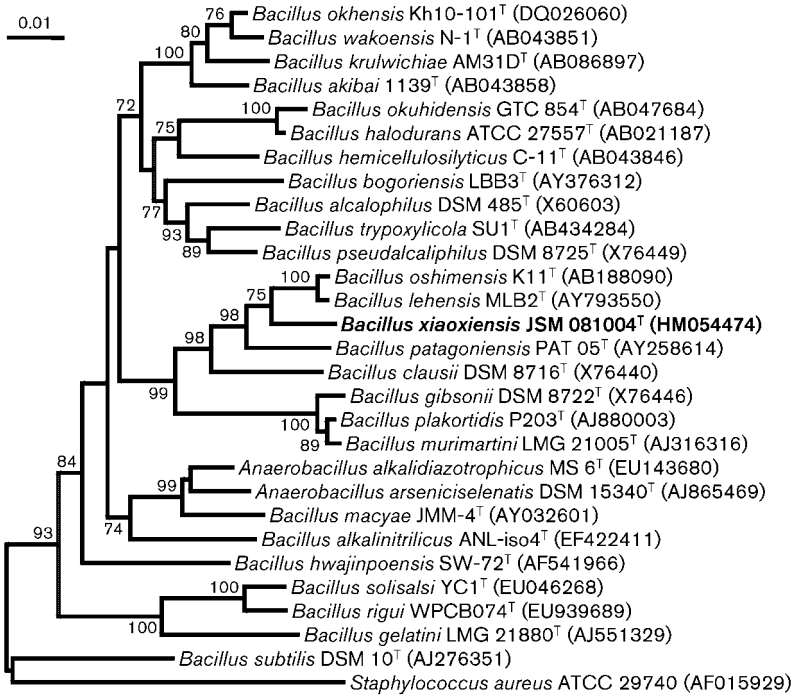

Fig. 1. Phylogenetic tree showing the position of strain JSM 081004^T and related taxa based on 16S rRNA gene sequence analysis reconstructed by using the neighbour-joining method. Numbers at nodes are bootstrap percentages (>70 %) based on a neighbour-joining analysis of 1000 resampled datasets. Bar, 1 substitution per 100 nt.

Strain JSM 081004^T contained MK-7 (96.4 %) as the predominant menaquinone, with MK-6 (1.1 %) and MK-8 (2.5 %) present in minor amounts. The polar lipids of this strain consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and one unknown phospholipid (Table 1). The fatty acid profile of strain JSM 081004^T was similar to those of the type strains of the three phylogenetically related species of the genus *Bacillus*, although there were differences in the proportions of some components (Table 2). The fatty acid profile of strain JSM 081004^T contained the major compounds iso-$C_{15:0}$ (75.4 %) and anteiso-$C_{15:0}$ (11.5 %), which are characteristic of numerous members of the genus *Bacillus* (Kämpfer, 1994).

The results of the phylogenetic analysis and of morphological and chemotaxonomic investigations supported the affiliation of strain JSM 081004^T to the genus *Bacillus*. However, the yellow pigmentation of strain JSM 081004^T, as well as the ability to grow under anaerobic conditions and reduce nitrate to nitrite, together with several other phenotypic characteristics and chemotaxonomic data, differentiated the isolate clearly from its phylogenetic relatives (Tables 1 and 2). In conclusion, phylogenetic analysis based on 16S rRNA gene sequences, DNA–DNA relatedness results, and phenotypic and chemotaxonomic data presented here support the proposal that strain JSM 081004^T represents a novel species of the genus *Bacillus*, *Bacillus xiaoxiensis* sp. nov.

Description of *Bacillus xiaoxiensis* sp. nov.

Bacillus xiaoxiensis (xi.a.o.xi.en′sis. N.L. masc. adj. *xiaoxiensis* pertaining to Xiaoxi National Natural Reserve,

Table 2. Fatty acid compositions of strain JSM 081004^T and related species of the genus *Bacillus*

Strains: 1, *B. xiaoxiensis* sp. nov. JSM 081004^T; 2, *B. lehensis* DSM 19099^T; 3, *B. oshimensis* DSM 18940^T; 4, *B. patagoniensis* DSM 16117^T. Data are percentages of the total fatty acid content. −, Not detected. All data were from this study.

Fatty acid	1	2	3	4
Saturated				
$C_{14:0}$	0.5	1.2	0.8	0.4
$C_{16:0}$	1.5	3.4	1.9	0.8
$C_{18:0}$	0.4	1.3	0.3	−
Unsaturated				
$C_{16:1}\omega7c$ alcohol	0.8	0.6	0.3	3.7
$C_{16:1}\omega11c$	0.2	−	0.2	0.6
Branched				
iso-$C_{13:0}$	0.4	0.3	0.3	0.2
iso-$C_{14:0}$	3.7	6.7	6.6	7.9
iso-$C_{15:0}$	74.5	63.9	63.9	50.7
anteiso-$C_{15:0}$	11.5	13.3	16.8	24.6
iso-$C_{16:0}$	1.7	3.4	2.8	3.5
iso-$C_{17:0}$	2.5	3.9	3.8	2.8
anteiso-$C_{17:0}$	0.9	1.4	1.4	2.4
iso-$C_{17:1}\omega10c$	−	−	−	0.7
iso-$C_{18:0}$	0.2	−	−	−
$C_{18:1}\omega9c$	1.0	0.5	0.4	0.1
Summed feature 4*	−	−	−	1.0

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 4 comprises iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.

China, the source of the sample from which the type strain was isolated).

Cells are Gram-stain-positive, catalase-positive, oxidase-negative, slightly halophilic, facultatively anaerobic, straight rods, approximately 0.6–1.2 μm wide and 3.0–5.0 μm long, occurring singly, as pairs or as short chains, producing ellipsoidal endospores that lie in central to subterminal, slightly swollen sporangia. Motile by means of peritrichous flagella. Colonies are yellow-pigmented, flat and opaque, have smooth, glistening surfaces and circular/slightly irregular margins, and are 2–3 mm in diameter on MA. No diffusible pigments are produced. Growth occurs with 0.5–20 % (w/v) NaCl (optimum 2–4 %), at pH 6.0–10.5 (optimum pH 8.0) and at 5–40 °C (optimum 25–30 °C). Nitrate is reduced to nitrite, but nitrite is not further reduced. Negative for egg yolk reaction, methyl red, Voges–Proskauer, H_2S and indole production tests. Aesculin, casein, gelatin, starch and Tween 20 are hydrolysed, but cellulose, DNA, ONPG, and Tweens 40, 60 and 80 are not. Acids are produced from amygdalin, D-glucose, glycerol, glycogen, maltose, D-mannitol, melibiose, raffinose, starch and sucrose, but not from *N*-acetylglucosamine, adonitol, L-arabinose, cellobiose, dulcitol, D-fructose, D-galactose, *myo*-inositol, lactose, D-mannose, melezitose, L-rhamnose, D-ribose, D-salicin, D-sorbitol, trehalose or D-xylose. The following compounds are utilized as sole sources of carbon and energy or sole sources of carbon, nitrogen and energy: D-glucose, glycogen, D-mannose, D-xylose, amygdalin, glycerol, D-salicin, acetate and L-asparagine. The following are not utilized: L-arabinose, cellobiose, dextrin, D-fructose, D-galactose, lactose, maltose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, sucrose, trehalose, adonitol, D-arabitol, *myo*-inositol, D-mannitol, D-sorbitol, butyrate, citrate, gluconate, propionate, succinate, *N*-acetylglucosamine, L-alanine, L-arginine, L-glutamic acid, glycine, L-histidine, hydroxy L-proline, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-serine and L-valine. Alkaline phosphatase, α-chymotrypsin, esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase are expressed constitutively; acid phosphatase, arginine dihydrolase, cystine arylamidase, α-fucosidase, α- and β-galactosidase, α- and β-glucosidase, *N*-acetyl-β-glucosaminidase, β-glucuronidase, lipase (C14), lysine decarboxylase, α-mannosidase, ornithine decarboxylase, phenylalanine deaminase, trypsin, urease and valine arylamidase activities are not observed. *meso*-Diaminopimelic acid is present in the cell-wall peptidoglycan as the diagnostic diamino acid. Possesses MK-7 as the predominant menaquinone, and diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol as the major polar lipids. Major fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$.

The type strain is JSM 081004^T (=CCTCC AA 208057^T =DSM 21943^T), isolated from non-saline forest soil in Xiaoxi National Natural Reserve, China. The DNA G+C content of the type strain is 40.1 mol% (HPLC method).

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