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Bacillus depressus sp. nov., isolated from soil of a sunflower field

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Abstract A Gram-stain positive, rod-shaped, endospore-forming and aerobic bacterium, designated BZ1^T, was isolated from a soil sample collected from a sunflower field in Wuyuan county, Inner Mongolia, China. On the basis of 16S rRNA gene sequence analysis, the isolate was found to be a member of the genus *Bacillus* and the close phylogenetic relatives to be *Bacillus gottheilii* WCC 4585^T, *Bacillus oceanisediminis* H2^T, *Bacillus mesonae* FJAT-13985^T and *Bacillus horneckiae* DSM 23495^T with 98.3, 98.1, 98.0 and 97.6 % sequence similarity, respectively. Strain BZ1^T was found to grow at 6–40 °C (optimum 30–33 °C), pH 6.0-9.0 (optimum pH 7.0) and 0–5.5 % (w/v) NaCl (optimum 0.5 %). The cell wall diamino acid of the peptidoglycan of strain BZ1^T was identified as *meso*-diaminopimelic acid and the predominant respiratory quinone as MK-7. The major cellular fatty acids were found to be iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{14:0}$, and the polar lipids to consist of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The novel strain was found to have a DNA G + C content 44.5 mol%. DNA–DNA hybridization with closely related strains was low. Based on phenotypic, phylogenetic and chemotaxonomic results, it is concluded that strain BZ1^T represents a novel species within the genus *Bacillus*, for which we propose the name *Bacillus depressus* sp. nov. The type strain is BZ1^T (= CGMCC 1.15124^T = KCTC 33643^T).

Keywords *Bacillus depressus* sp. nov · Soil · 16S rRNA · Polyphasic taxonomy

Introduction

The genus *Bacillus* was initially proposed by Cohn (1872) and since then the genus has been expanded with many further novel species. At the time of writing, the genus encompasses about several hundred species with validly published names (List of Prokaryotic Names with Standing in Nomenclature; http://www.bacterio.net/) and members of the genus have been found in various environments, such as *Bacillus panacisoli* CJ32^T isolated from soil (Choi and Cha 2014), *Bacillus andreesenii* 8-4-E13^T from a composit (Kosowski et al. 2014), *Bacillus gottheilii* WCC 4585^T from a pharmaceutical manufacturing site (Seiler et al. 2013), *Bacillus beringensis* BR035^T from the Bering Sea (Yu et al. 2011) and *Bacillus alkalisediminis* K1-25^T from a pond (Borsodi et al. 2011). Members of the genus

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Bacillus are Gram-stain positive and endospore-forming rods. The major fatty acid component of the members of the genus *Bacillus* is iso-$C_{15:0}$.

During the course of a study of bacterial diversity in a soil sample from a sunflower field in Wuyuan county of Inner Mongolia in China, a Gram-stain positive bacterium, designated BZ1^T, was isolated and studied here by a polyphasic characterisation. It is concluded that strain BZ1^T represents a novel species within the genus *Bacillus*, for which we propose the name *Bacillus depressus* sp. nov.

Materials and methods

Isolation and culture conditions

Strain BZ1^T was isolated from a saline-alkaline soil sample [pH 8.5; salt content 0.4 % (w/w)] collected from a sunflower field in Wuyuan county in Inner Mongolia in China (GPS coordinates 40°46′30″N and 107°36′10″E). For isolation, the soil sample was suspended in sterilised water, serially diluted and then incubated at 35 °C for 3 days on nutrient agar with the following composition: beef extract 3 g L^{-1}, peptone 5 g L^{-1}, NaCl 5 g L^{-1} and agar 15 g L^{-1} (pH 7.6). Then representative colonies were transferred to the same medium. Pure cultures were obtained by streaking repeatedly and the purified strains stored on trypticase soy agar (TSA; Difco) at 4 °C and in 20 % (v/v) glycerol suspensions at −20 °C.

31 colonies were isolated, purified and identified. Among the isolates, only one isolate was considered to represent a novel species and designated as strain BZ1^T. Routine cultivation of the strain was performed on TSA plates at 30 °C. *B. gottheilii* WCC 4585^T and *Bacillus horneckiae* DSM 23495^T were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), *Bacillus oceanisediminis* H2^T and *Bacillus mesonae* FJAT-13985^T were obtained from the China General Microbiological Culture Collection Center (CGMCC). These strains were cultured under comparable conditions and used as reference strains in the phenotypic tests.

Morphological, physiological and biochemical characteristics

Cell morphology was examined by light microscopy (CX31; Olympus). The colony morphology, size and pigmentation were observed on TSA after 48 h of incubation at 30 °C. The Gram reaction was performed according to the methods described by Gerhardt et al. (1981). Endospores were observed using the Schaeffer-Fulton staining method (Murray et al. 1994). Oxidase activity was determined using 1 % (w/v) tetramethyl-p-phenylenediamine. Catalase activity was detected by the production of oxygen bubbles with 3 % (v/v) H_2O_2. Growth at different temperatures (0–50 °C) was tested in TSB for 3–5 days. The pH range for growth was determined at various pH values (pH 3.0-12.0, in increments of 0.5 pH units) at 30 °C by culturing the strain in TSB by using the appropriate biological buffers as described by Xu et al. (2005). NaCl tolerance tests were assessed at different NaCl concentrations [0–10 % (w/v) NaCl, at intervals of 0.5 % and 11–20 % (w/v), at intervals of 1 %] for up to 14 days at 30 °C in TSB prepared according to the formula of the Difco medium except that NaCl was omitted. Sensitivity to antibiotics was examined on TSA plates using antibiotic discs (Goodfellow and Orchard 1974). Nitrate reduction, hydrolysis of casein, gelatin, starch, Tween 20, 40, 60 and 80, urease activity and H_2S production were studied as described by Smibert and Krieg (1994). Resistance to lysozyme, egg-yolk reaction, decomposition of adenine, xanthine, hypoxanthine, guanine or tyrosine were determined by the method of Logan and De Vos (2009). The tests of carbon source utilisation were determined using methods described by Gordon and Mihm (1957) and acid production from carbohydrates was determined according to Gordon et al. (1974). Nitrogen assimilation was assessed using TSB. Additional enzyme activities and other physiological properties were performed using the API 20E, API 20NE and API ZYM kits (bioMérieux) according to the manufacturer's instructions.

Chemotaxonomic characterisation

For fatty acid analyses, cells of strain BZ1^T were cultivated on TSA for 24 h at 28 °C. Fatty acid methyl esters were prepared and analysed according to the standard protocol of the Microbial Identification System (MIDI) tested by GC (Sasser 1990; Kämpfer and Kroppenstedt 1996); the database used was TSBA6. Isoprenoid quinones were isolated according to the method of Collins et al. (1987) and analysed using reversed-phase HPLC (Wu et al. 1989). For cell

wall peptidoglycan analysis, the cells were hydrolysed (4 N HCl, 100 °C, 15 h) and the peptidoglycan composition was analysed by two-dimensional ascending TLC on cellulose plates using the solvent system of Schleifer (1985). The cellular polar lipids were extracted and then examined by two-dimensional TLC and identified by using procedures described by Minnikin et al. (1984).

Molecular analysis

For 16S rRNA gene sequencing and phylogenetic analysis, extraction of genomic DNA, PCR amplification of the 16S rRNA gene and sequencing of purified PCR products were performed as described previously (Rainey et al. 1996). The 16S rRNA gene was amplified by PCR using 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′) (Weisburg et al. 1991). Sequencing reactions of the PCR products were carried out by the method of Lu et al. (2001). The resulting 16S rRNA gene sequence was compared with those sequences available from Genbank using the BLAST program and the EzTaxon server (http://eztaxon-e.ezbiocloud.net/, Kim et al. 2012). Multiple alignments with sequences of closely related species of the genus *Bacillus* was performed using the CLUSTAL_X 1.8 software (Thompson et al. 1997). Aligned sequences were analysed by using the MEGA 5.0 software (Tamura et al. 2011) to reconstruct unrooted trees with the neighbour-joining method of Saitou and Nei (1987), maximum-parsimony (Fitch 1971) and maximum-likelihood (Felsenstein 1981) methods. A distance matrix was generated using Kimura' s two-parameter model (Kimura 1980). The stability of clusters was ascertained by performing a bootstrap analysis based on 1000 replications of the sequences (Felsenstein 1985).

Determination of the DNA G + C content of strain BZ1^T was achieved by the thermal denaturation method (Marmur and Doty 1962) using the genomic DNA of *Escherichia coli* strain K-12 as the standard for calibration. DNA–DNA hybridization was carried out based on the liquid renaturation method (De Ley et al. 1970; Huss et al. 1983; Jahnke 1992). Every hybridization experiment was performed with five replications. The highest and lowest values obtained were excluded and the means of the remaining three values were quoted as DNA–DNA relatedness values.

Results and discussion

Strain BZ1^T was observed to be Gram-stain positive rods (0.5 − 0.8 × 1.5 − 4.0 μm) and aerobic. Colonies that formed after 2 days incubation on TSA at 30 °C were observed to be circular, opaque, mud coloured and 2-5 mm in diameter. No diffusible pigment was observed. Ellipsoidal endospores were observed to be located centrally or paracentrally in swollen sporangia. Growth was found to occur at temperatures from 6 to 40 °C (optimum 30–33 °C) and pH 6.0–9.0 (optimum pH 7.0) and in the presence of up to 5.5 % (w/v) NaCl. The results for the presence of catalase, oxidase, urease and reduction of nitrate were positive. The results of hydrogen sulfide and indole production, Voges-Proskauer reaction and nitrite reduction were negative.

Strain BZ1^T was found to hydrolyse gelatin, starch, hippurate and hypoxanthine but not casein, Tween 20, 40, 60, 80, adenine, xanthine, guanine or tyrosine. Strain BZ1^T was found to be susceptible to ampicillin (10 μg), chloramphenicol (30 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), novobiocin (30 μg), penicillin (10 μg), rifampicin (5 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (30 μg), but resistant to bacitracin (0.04 μg), lyncomycin (2 μg) and nystatin (100 μ g).

The detailed physiological and biochemical characteristics of strain BZ1^T are presented in the species description and Table 1. Strain BZ1^T produces phenylalanine deaminase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase but not arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, lipase (C14), trypsin, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase or β-fucosidase; and is weak positive for valine arylamidase and cystine arylamidase. The following substrates are used as sole carbon sources: acetate, benzoate, fumarate, D-galactose, glycogen, inulin, lactic acid, oxalate, D-raffinose and succinate but not L-arabinose, cellobiose, citrate, D-fructose, D-glucose, glycerol, lactose, malate, malonate, D-mannitol, pyruvic acid, L-rhamnose, D-ribose, L-sorbose, sucrose, tartrate, D-trehalose, xylitol or D-xylose; weakly positive for erythritol. The following compounds are used

Table 1 Characteristics that distinguish strain BZ1^T from other closely related species of the genus *Bacillus*

Characteristic	1	2	3	4	5
Salt concentration for growth (%, w/v)	0–5.5	0.5–8.5	0–13.0	0–2.0	0–10.0[d]
pH for growth					
Range	6.0–9.0	7.0–9.5	6.0–10.0	5.5–9.0	7.0–10.8[d]
Optimum	7.0	8.0	7.0	7.0	7.0[d]
Temperature for growth (°C)					
Range	6–40	10–40	4–45	20–45	4–32[d]
Optimum	30–33	30	37	30	30[d]
Oxidase	+	−	+	−	−
Arginine dihydrolase	−	−	+	−	−
Hydrolysis of:					
Gelatin	+	+	+	−	+
Urea	+	−	−	−	−
Growth on:					
Citrate	−	−	−	−	+
D-Glucose	−	−	+	−	−
Maltose	−	w	w	+	+
D-Mannitol	−	−	+	−	−
Sucrose	−	−	+	+	−
Acid production from:					
Cellobiose	−	+	−	+	−
D-Fructose	w	+	−	−	−
D-Glucose	+	+	+	−	−
Inulin	+	−	+	−	−
Maltose	−	+	−	+	−
D-Mannitol	−	+	−	−	−
Raffinose	−	w	+	+	−
Sucrose	−	+	+	+	−
D-Trehalose	−	+	−	+	−
D-Xylose	+	−	−	−	−
DNA G+C content (mol%)	44.5	38.7[a]	44.8[b]	41.6[c]	35.6[d]

Strain: 1, Strain BZ1^T; 2, *B. gottheilii* WCC 4585^T; 3, *B. oceanisediminis* H2^T; 4, *B. mesonae* FJAT-13985^T; 5, *B. horneckiae* DSM 23495^T. Data were obtained from this study except where marked. +, positive; −, negative; w, weakly positive

[a] Data from Seiler et al. (2013)

[b] Data from Zhang et al. (2010)

[c] Data from Liu et al. (2014)

[d] Data from Vaishampayan et al. (2010)

as sole nitrogen sources: L-cysteine and L-tyrosine but not L-alanine, L-arginine, L-aspartate, glycine, L-hydroxyproline, L-leucine, L-lysine, L-ornithine, L-phenylalanine, L-proline, L-tryptophan or L-valine. Acid is produced from aesculin, arbutin, D-fructose, D-glucose, inulin, D-ribose, L-sorbose and D-xylose but not from L-arabinose, D-cellobiose, erythritol, D-galactose, glycerol, inositol, lactose, maltose, D-mannitol, D-raffinose, L-rhamnose, sorbitol, starch, sucrose, D-trehalose or xylitol.

The main cellular fatty acids of the novel strain were identified as iso-$C_{15:0}$ (32.1 %), anteiso-$C_{15:0}$ (28.8 %) and iso-$C_{14:0}$ (17.0 %); these iso- and anteiso-branched fatty acids of the 14–17-carbon series are typical of those observed in profiles of the type strains of members of the genus *Bacillus*

Table 2 Cellular fatty acid composition (%) of strain BZ1^T and closely related type strains of the genus *Bacillus*

Fatty acid (%)	1	2	3	4	5
iso-$C_{14:0}$	17.0	3.6	9.7	3.1	4.0
$C_{14:0}$	2.3	3.8	0.6	0.6	1.1
iso-$C_{15:0}$	32.1	28.2	35.7	40.8	54.3
anteiso-$C_{15:0}$	28.8	35.0	9.6	23.3	19.9
$C_{16:1}$ $\omega 7c$ alcohol	4.1	2.2	10.0	1.0	6.8
iso-$C_{16:0}$	4.0	4.4	9.2	3.8	2.5
$C_{16:1}$ $\omega 11c$	2.0	4.7	2.4	4.6	2.0
$C_{16:0}$	1.8	5.9	1.8	4.8	0.5
iso-$C_{17:1}$ $\omega 10c$	0.4	–	2.8	2.3	2.3
iso-$C_{17:0}$	0.6	1.4	2.7	6.2	1.3
anteiso-$C_{17:0}$	2.0	9.5	2.8	3.7	2.3
Summed Feature 4*	1.2	1.3	6.7	0.9	–

Strain: 1, Strain BZ1^T; 2, *B. gottheilii* WCC 4585^T; 3, *B. oceanisediminis* H2^T; 4, *B. mesonae* FJAT-13985^T; 5, *B. horneckiae* DSM 23495^T. All the data were obtained under the same conditions. –, Less than limit of detection

* Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System (MIDI). Summed feature 4 consisted of iso-$C_{17:1}$I and/or anteiso-$C_{17:1}$B

(Kämpfer 1994; Albert et al. 2005). However, strain BZ1^T could be differentiated significantly from the closely related species *B. gottheilii* WCC 4585^T in the amount of iso-$C_{14:0}$ (17.0 % vs. 3.6 %) and anteiso-$C_{17:0}$ (2.0 % vs. 9.5 %), as shown in Table 2. In particular, the amount of iso-$C_{14:0}$ in strain BZ1^T was higher than that of the closely related species. There were notable differences observed in the percentages of other fatty acids between strain BZ1^T and *B. gottheilii* WCC 4585^T, confirming that strain BZ1^T could be distinguished from *B. gottheilii* WCC 4585^T.

Analysis of the quinones showed that the novel strain contains MK-7 as the predominant respiratory quinone. The diagnostic diamino acid in the cell wall peptidoglycan was identified as *meso*-diaminopimelic acid, which is typical of the large majority of members of the genus *Bacillus* (Priest et al. 1988). The major polar lipids present in strain BZ1^T were identified as diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine (Supplementary Fig. S1). The DNA G + C content of the genomic DNA of strain BZ1^T was determined to be 44.5 mol%. This value is in the range of the genomic G + C contents of the members of the genus *Bacillus* (Slepecky and Hemphill 2006).

The 16S rRNA gene sequence of strain BZ1^T was determined (1459 bp; GenBank/EMBL/DDBJ accession number KP259553). Phylogenetic analysis based on the neighbour-joining method revealed that strain BZ1^T was included in the clusters of species of the genus *Bacillus* (Fig. 1). The relative position of the novel isolate was also confirmed in the trees constructed using maximum-parsimony and maximum-likelihood methods (data not shown). The close relatives in terms of pairwise 16S rRNA gene sequence similarity were *B. gottheilii* WCC 4585^T (98.3 %), *B. oceanisediminis* H2^T (98.1 %), *B. mesonae* FJAT-13985^T (98.0 %), *B. horneckiae* DSM 23495^T (97.6 %), *Bacillus drentensis* LMG 21831^T (97.5 %), *Bacillus firmus* NCIMB 9366^T (97.5 %), and *Bacillus purgationiresistens* DS22^T (97.4 %), respectively. Low DNA–DNA relatedness was observed between strain BZ1^T and its close phylogenetic neighbours: 33.1 ± 1.2 % with *B. gottheilii* WCC 4585^T and 28.6 ± 0.7 % with *B. oceanisediminis* H2^T. These values are below the threshold value of 70 % recommended by Wayne et al. (1987) for assignment of strains to the same species.

Therefore, on the basis of the above-mentioned phenotypic, phylogenetic and genetic data, strain BZ1^T should be placed in the genus *Bacillus*. However, strain BZ1^T could tolerate lower NaCl than all the reference strains except for *B. mesonae* FJAT-13985^T. Strain BZ1^T could be separated from its close relative *B. gottheilii* WCC 4585^T in several phenotypic properties, such as colony colour, cell size, positive for activity of oxidase and urease, hydrolysis of complex substrates, as well as assimilation of carbon substrates. The strain differed from *B. oceanisediminis* H2^T in its inability to produce arginine dihydrolase and, unlike *B. mesonae* FJAT-13985^T, it is able to hydrolyse gelatin. Strain BZ1^T is able to produce acid from D-glucose, inulin and D-xylose, in contrast to *B. mesonae* FJAT-13985^T and *B. horneckiae* DSM 23495^T. Characteristics useful for differentiating between strain BZ1^T and the reference strains are shown in Tables 1 and 2. Thus, strain BZ1^T should be classified as the type strain of a novel species of the genus *Bacillus*, for which the name *Bacillus depressus* sp. nov. is proposed.

Description of *Bacillus depressus* sp. nov

Bacillus depressus (de.pres′sus. L. masc. adj. *depressus*, depressed in shape, referring to the colonies).

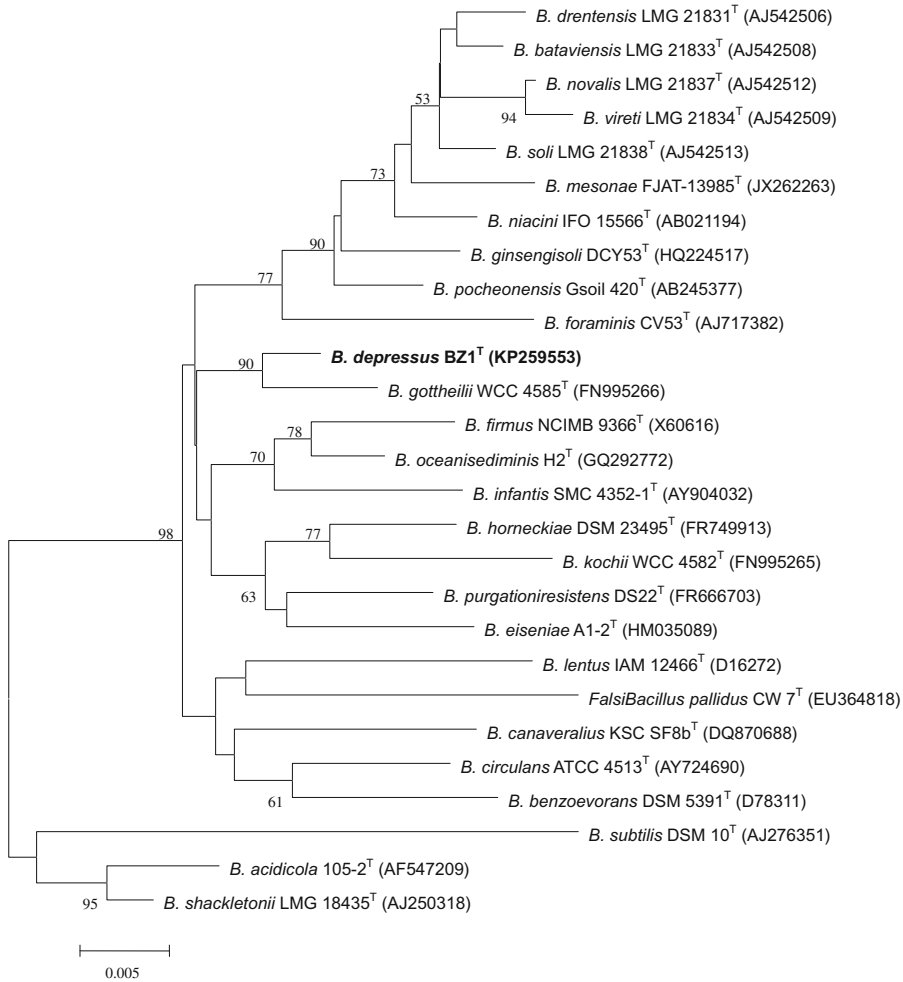

Fig. 1 Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing the relationships among strain BZ1^T and other related type strains in the genus *Bacillus*. Bootstrap values (>50 %) based on 1000 replicates are shown at branch nodes. *Bar*, 0.005 substitutions per nucleotide position

Cells are Gram-stain positive, strictly aerobic rods, 0.5-0.8 μm in width and 1.5-4.0 μm long. Colonies are circular, opaque, mud coloured and 2-5 mm in diameter after 2 days incubation on TSA at 30 °C. Ellipsoidal endospores are observed centrally or paracentrally in swollen sporangia. Growth occurs at pH 6.0-9.0 and the optimal value for growth is pH 7.0; the temperature range for growth is 6–40 °C, optimum growth occurs at 30–33 °C; tolerant of up to 5.5 % (w/v) NaCl and optimum NaCl for growth is 0.5 % (w/v). Oxidase positive and catalase positive. Under aerobic conditions, nitrate is reduced, but nitrite not. Hydrogen sulfide and indole production, Voges-Proskauer and citrate utilisation are negative. Hydrolyses aesculin, starch, gelatin, urea, hippurate and hypoxanthine but not casein, Tween 20, 40, 60, 80, adenine, xanthine, guanine or tyrosine. The cell wall peptidoglycan contains *meso*-diaminopimalic acid and the predominant respiratory quinone is MK-7. The principal fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{14:0.}$ The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The DNA G + C content of the type strain is 44.5 mol%.

The type strain, BZ1^T(= CGMCC 1.15124^T - = KCTC 33643^T), was isolated from a soil sample in Wuyuan county, Inner Mongolia, China. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BZ1^T is KP259553.

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