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ORIGINAL PAPER

Gracilibacillus eburneus sp.nov., a moderately halophilic bacterium isolated from Xinjiang province, China

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Abstract

A novel Gram-staining positive, moderately halophilic, endospore-forming, motile, rod-shaped and strictly aerobic strain, designated YIM 93565^T, was isolated from a salt lake in Xinjiang province of China and subjected to a polyphasic taxonomic study. Strain YIM 93565^T grew in the range of pH 6.0–9.0 (optimum pH 7.0), 10–45 °C (optimum 35–40 °C) and at salinities of 2–24% (w/v) NaCl (optimum 7–10%). Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain YIM 93565^T clustered with members of the genera *Gracilibacillus* and form a clade with *Gracilibacillus bigeumensis* KCTC 13130^T (95.6% similarity) and *Gracilibacillus halophilus* DSM 17856^T (94.9%), which was well separated from others. The DNA G+C content of this novel strain was 36.8 mol%. The major fatty acids were anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{16:0}$ and anteiso-$C_{17:0}$ and its polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, one unidentified glycolipid and two unidentified phospholipids. The predominant menaquinone was MK-7. The cell-wall peptidoglycan was based on *meso*-diaminopimelic acid. Based on the results of phylogenetic, physiological and chemotaxonomic comparative analyses, the isolate is assigned to a novel species of the genus *Gracilibacillus*, for which the name *Gracilibacillus eburneus* sp. nov. is proposed, with the type strain YIM 93565^T (=DSM 23710^T=CCTCC AB 2013249^T).

Keywords *Gracilibacillus eburneus* sp. nov. · Polyphasic taxonomy · 16S rRNA gene

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Hui-Lin Guan and Yun-Jiao Zhang contributed equally to this study.

The GenBank accession number for the 16S rRNA gene sequence of strain YIM 93565^T is KF548095. The 'digital protologue' database (DPD) TaxonNumber of strain YIM 93565^T is TA00291 (http://imedea.uib-csic.es/dprotologue/).

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Introduction

The genus *Gracilibacillus*, belonging to the family *Bacillaceae*, was proposed by Wainø et al. (1999). Till the time of writing this manuscript, the genus *Gracilibacillus* comprised 12 species with validly published names (http://www.bacterio.net/gracilibacillus.html; Parte 2014). They have similar phenotypic and chemotaxonomic features and close phylogenetic relationship. The genus *Gracilibacillus* was previously characterized as embracing Gram-positive,

motile, endospore-forming rods. The peptidoglycan type is A1γ, with *meso*-diaminopimelic acid as the diagnostic diamino acid. MK-7 is the predominant menaquinone. The polar lipids consist of diphosphatidylglycerol and phosphatidylglycerol. The major cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{16:0}$ and anteiso-$C_{17:0}$ (Kim et al. 2012b).

Moderately halophilic bacteria are a group of organisms that grow optimally in media containing 3.0–15.0% (w/v) salts and are widely distributed in hypersaline habitats such as salterns, salt lakes, soda lakes and seawater (Kushner 1978). During a project to isolate halophilic bacterial strains from a soil sample of Lop Nur salt lake in Xinjiang province (China), one moderately halophilic strain, designated YIM 93565^T, was isolated. The present study reports results of a polyphasic taxonomic study of strain YIM 93565^T.

Materials and methods

Isolation and maintenance of organism

Strain YIM 93565^T was isolated from a soil sample of Lop Nur salt lake in Xinjiang province of China (39° 36′N 89° 45′E). For the isolation, serial dilutions of the sample were spread on Glucose-Tryptone-Yeast (GTY) medium (Tang et al. 2010) and incubated at 37 °C for 2 weeks. Colonies were picked and repeatedly re-streaked onto modified GTY medium containing 7.0% (w/v) NaCl, until purity was confirmed. Strain YIM 93565^T was routinely cultured on modified GTY medium at 37 °C and maintained on modified GTY slants at 4 °C, and as glycerol suspension (20%, v/v) at − 80 °C for long-term preservation. The reference type strains *Gracilibacillus bigeumensis* KCTC 13130^T and *Gracilibacillus halophilus* DSM 17856^T were obtained from the Korean Collection for Type Cultures (KCTC) and the German Collection of Microorganisms and Cell Cultures (DSMZ), and used in all physiological, biochemical and chemotaxonomic analysis.

Morphological, physiological and biochemical characterization

Gram staining was carried out by the standard Gram reaction combined with the KOH lysis test (Cerny 1978). Cell motility was detected by the presence of turbidity throughout the semisolid medium (Leifson 1960) and the hanging-drop method with an optical microscope (Skerman 1967). Cell morphology was determined on cultures grown for 24, 48 and 72 h and examined by transmission electron microscopy (Hitachi H-7650). The presence of endospores was determined by phase-contrast microscopy. The growth temperature was tested at 5–65 °C in increments of 5 °C. For NaCl tolerance test, GTY medium was used as the basal medium and the salt concentrations ranging from 0 to 30.0% (w/v) at an interval of 1.0% were tested. The pH growth range was investigated between 4.0 and 10.0 at an interval of 1 pH unit using the buffer systems of 0.1 M citric acid/0.1 M sodium citrate (pH 4.0–5.0), 0.1 M KH_2PO_4/0.1 M NaOH (pH 6.0–8.0), 0.1 M $NaHCO_3$/0.1 M Na_2CO_3 (pH 9.0–10.0). Anaerobic growth was tested on modified GTY medium using the GasPak Anaerobic System (BBL) according to the manufacturer's instructions at 37 °C for 6 days. Catalase activity was determined by bubbles production after the addition of a drop of 3% H_2O_2 to the tested colony. Oxidase activity was observed by oxidation of tetramethyl-*p*-phenylenediamine on filter paper. Other enzymatic activities were determined using API ZYM biological kits (bioMérieux) according to the manufacturer's instructions, and the cell suspension of the tests was prepared in distilled water supplemented with 7% (w/v) NaCl. Nitrate reduction, hydrolysis of cellulose, gelatin, starch, urea and Tweens 20, 40, 60 and 80 were performed following the methods of Cowan and Steel (1965). H_2S and indole production were assayed according to the protocols as described by Smibert and Krieg (1994). Methyl red and Voges-Proskauer tests were performed as described by Smibert and Krieg (1981). Carbon and nitrogen source utilization was tested using Biolog GEN III MicroPlates according to the manufacturer's instructions. Acid production from various carbohydrates was determined using API 50 CH biological kit (bioMérieux) according to the manufacturer's instructions, and the API 50 CHB/E medium (bioMérieux) of the test was supplemented with 7.0% (w/v) NaCl. Antibiotics susceptibility was determined by the disc diffusion plate method (Bauer et al. 1966). Similar physiological and biochemical studies were done for the reference strain, unless otherwise mentioned in the data.

Chemotaxonomy

For chemotaxonomic analysis, strains YIM 93565^T and *Gracilibacillus bigeumensis* KCTC 13130^T were grown on modified GTY containing 7.0% (w/v) NaCl at 37 °C and *Gracilibacillus halophilus* DSM 17856^T was grown on modified GTY containing 15.0% (w/v) NaCl at 45 °C until they reached the late exponential phase. Cellular fatty acids were extracted, methylated and analyzed using the Microbial Identification System (MIDI) according to the manufacturer's instructions (Sasser 1990). The fatty acid methyl esters were analyzed by the Microbial Identification software package of TSBA6 (Sherlock Version 6.1). Cellular menaquinones were extracted and purified as described by Collins et al. (1977) and were analyzed by HPLC (Kroppenstedt 1982). Polar lipids were extracted according to the method of Minnikin et al. (1984) and identified by two-dimensional thin-layer chromatography (TLC) and spraying with appropriate

Table 1 Differential characteristics of strain YIM 93565^T and type strains of related *Gracilibacillus* species

Characteristics	*G. eburneus* YIM 93565T	*G. halophilus* DSM 17856T	*G. bigeumensis* KCTC 13130T
NaCl (%, w/v)			
Range	2–24	7–30	1–22
Optimum	7–10	15	7
Temperature (ºC)			
Range	10–45	30–60	10–50
Optimum	35–40	45–50	35–40
pH			
Range	6.0–9.0	6.0–9.0	6.0–9.0
Optimum	7.0	7.0	8.0
Nitrate reduction	+	+	−
Urease activity	+	−	−
Hydrolysis of			
Gelatin	−	+	+
Starch	+	+	−
Tween 80	−	+	−
Growth on carbon and nitrogen source (Biolog GEN III MicroPlates)			
Acetic acid	−	+	−
L-Alanine	+	+	−
L-Aspartic Acid	−	+	−
D-Cellobiose	+	−	−
D-Galactose	+	w	+
Glycerol	w	+	+
L-Rhamnose	+	+	−
D-Trehalose	+	+	−
Acid production from (API 50CH)			
D-Cellobiose	+	−	−
D-Galactose	−	−	+
Glycogen	−	+	−
Lactose	+	−	+
L-Rhamnose	w	+	−
Salicin	−	−	+
D-Sorbitol	+	+	−
D-Trehalose	+	+	−
D-Turanose	+	−	−
D-Xylose	−	+	−
Enzyme activity (API ZYM)			
Cystine arylamidase	+	+	−
Leucine arylamidase	−	+	−
α-Fucosidase	−	−	+
α-Glucosidase	+	+	−
Lipase (C14)	−	−	w
α-Mannosidase	−	+	+

All strains share the following characteristics: positive for acid phosphatase, catalase, β-galactosidase, β-glucosidase and oxidase but negative for methyl red and Voges-Proskauer test. H_2S and indole are not produced. Cellulose, tween 20, 40 and 60 are not hydrolyzed. Acid is produced from aesculin, arabinose, D-glucose, glycerol, maltose, D-mannose, D-raffinose and sucrose. All data were from this study. +, positive; −, negative; W, weak

detection reagents (Collins and Jones 1980). Amino acids of whole-cell hydrolysates were analyzed as described by Hasegawa et al. (1983).

Phylogenetic analysis and G + C content determination

The genomic DNA was extracted and purified according to the method as described by Marmur (1961). PCR amplification of the 16S rRNA gene was carried out using the universal primers 27f and 1492r (Lane 1991). The PCR product was purified using a PCR purification kit according to the manufacturer instructions (Sangon, Shanghai), and the sequencing work was carried out by Invitrigen (Shanghai). Calculations of levels of 16S rRNA gene sequence similarity between strain YIM 93565^T and related taxa were carried out using the EzTaxon-e database (Kim et al. 2012a). Sequences retrieved from GenBank database were aligned using the CLUSTAL_X (Thompson et al. 1997) software and the alignment was corrected manually. Phylogenetic analyses were performed using three tree-making algorithms: neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Fitch 1971) methods. The NJ, MP and ML phylogenetic trees were reconstructed using MEGA version 5.0 (Tamura et al. 2011). Kimura's two-parameter model was used to calculate evolutionary distance matrices of the phylogenetic trees (Kimura 1980). The topology of the phylogenetic trees was assessed by the bootstrap analysis based on 1000 replications (Felsenstein 1985). The genomic G + C content (mol %) of strain YIM 93565^T was determined by high-performance liquid chromatography (HPLC) following the method of Mesbah et al. (1989).

Results and discussion

Morphological, physiological and biochemical characterization

The observations in this study indicated that strain YIM 93565^T showed the similar morphological characteristics to the closely related strains. The physiological and biochemical characteristics of strain YIM 93565^T that differed from two reference strains are shown in Table 1. Other detailed phenotypic characteristics of strain YIM 93565^T are given in the species description. These features also suggest that strain YIM 93565^T should represent a novel species of the genus *Gracilibacillus*.

Chemotaxonomic characteristics

The predominant menaquinone of strain YIM 93565^T was MK-7, the same as for other *Gracilibacillus* species. The cellular fatty acid profiles of strain YIM 93565^T and two reference strains showed similar compositions under the growth conditions of this study. The major fatty acids of the three strains were anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{16:0}$ and anteiso-$C_{17:0}$, and moderate amounts of $C_{18:0}$, iso-$C_{16:0}$ and iso-$C_{17:0}$ were also observed. Cellular fatty acid composition of strain YIM 93565^T indicates that the isolate matches the genus *Gracilibacillus*. Detailed fatty acid comparison of the three strains is displayed in Table 2. The polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, one unidentified glycolipid and two unidentified phospholipids (Fig. S4). This polar lipid profile is typical for the genus *Gracilibacillus*. *meso*-Diaminopimelic acid was present in the cell-wall peptidoglycan. The chemotaxonomic results of strain YIM 93565^T determined in this study are basically consistent with the previous described characteristics of the genus *Gracilibacillus*.

Phylogenetic analysis

The 16S rRNA gene sequence of strain YIM 93565^T obtained in this study was 1539 bp and the GenBank accession number is KF548095. Phylogenetic analysis based on the NJ phylogenetic tree (Fig. 1) displayed that strain YIM 93565^T clustered with members of the genera

Table 2 Cellular fatty acids composition of strain YIM 93565^T and type strains of related *Gracilibacillus* species

Fatty acid	*G. eburneus* YIM 93565T	*G. halophilus* DSM 17856T	*G. bigeumensis* KCTC 13130T
Unbranched saturated			
$C_{16:0}$	9.21	13.48	10.73
$C_{17:0}$	1.42	1.49	1.95
$C_{18:0}$	2.35	3.65	1.44
Branched saturated			
iso-$C_{14:0}$	1.85	0.43	1.22
iso-$C_{15:0}$	30.47	33.65	27.85
Anteiso-$C_{15:0}$	27.03	13.87	22.92
iso-$C_{16:0}$	3.98	2.68	4.99
iso-$C_{17:0}$	7.28	13.23	8.58
Anteiso-$C_{17:0}$	13.77	14.83	15.43
Unsaturated			
$C_{16:1}$ $\omega 11c$	0.70	ND	1.02

Data are percentages of total fatty acids; fatty acids that represent less than 1.0% in all strains are omitted. Fatty acids were determined in this study; the three strains were incubated to the late exponential phase

ND not detected

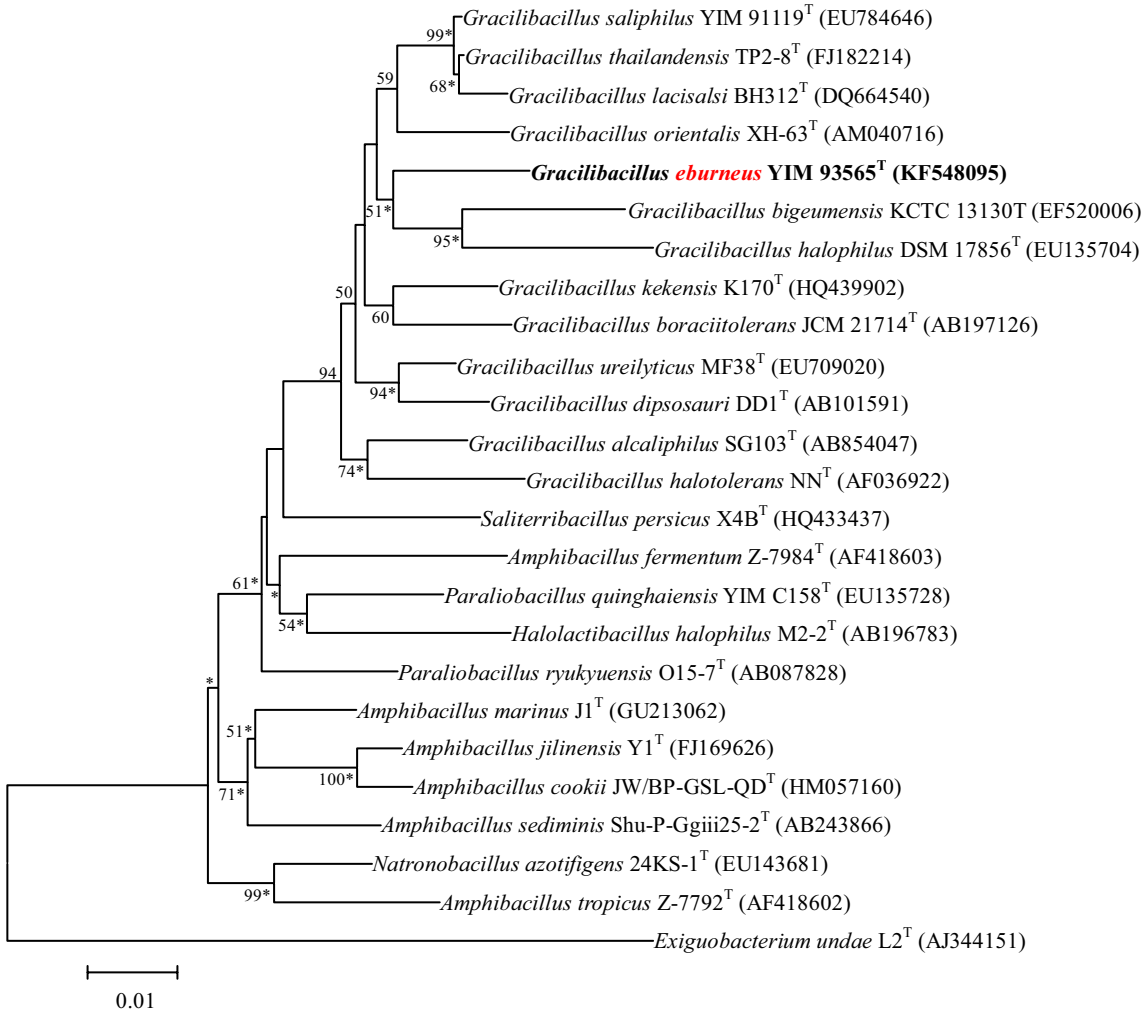

Fig. 1 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence analysis shows the position of strain YIM 93565^T. Numbers on branch nodes are bootstrap values (1000 resamplings, only values above 50% are shown). Asterisks denote nodes that were also recovered using the maximum parsimony and maximum likelihood methods. The sequence of *Exiguobacterium undae* L2^T was used as outgroup. Bar 0.01 substitutions per nucleotide position

Gracilibacillus and form a clade with *Gracilibacillus bigeumensis* KCTC 13130^T and *Gracilibacillus halophilus* DSM 17856^T, which was well separated from others, but with low levels of similarity to the two type strains (*Gracilibacillus bigeumensis* KCTC 13130^T, 95.6% and *Gracilibacillus halophilus* DSM 17856^T, 94.9%). The stability was further supported by ML and MP trees (Fig. S1, S2). The DNA G + C content of this novel was 36.8 mol% which was within the range of the DNA base content of the genus *Gracilibacillus* (35.0–43.0 mol%). Phylogenetic analysis results described above indicated that the new isolate YIM 93565^T should be a candidate of novel species in the genus *Gracilibacillus*.

Description of Gracilibacillus eburneus sp. nov

Gracilibacillus eburneus (e.bur.ne'us. L. masc. adj. eburneus white as ivory).

Cells are Gram-positive, strictly aerobic, endospore-forming, rods (0.5–0.8 μm × 1.6–2.2 μm, Fig. S3). Cells are motile by means of peritrichous flagella. Spherical endospores are produced at the terminal position in swollen sporangia (Fig. S5). Colonies are slightly convex, translucent and white as ivory on modified GTY medium at 37 °C for 3 days. Growth occurs at 10–45 °C and pH 6.0–9.0, with optimal growth at 35–40 °C and pH 7.0. Moderately halophilic, growth is not observed without NaCl or at NaCl concentrations lower than 2.0% (w/v), and growth is observed in the presence of 2.0–24.0% (w/v) NaCl (optimum 7.0–10.0%, w/v). It is positive for catalase, oxidase, urease

activity, nitrate reduction, but negative for methyl red and Voges-Proskauer test. Starch is hydrolyzed, but cellulose, gelatin, tween 20, 40, 60 and 80 are not. H_2S and indole are not produced. The following compounds are utilized as sole carbon or nitrogen sources: L-alanine, D-cellobiose, D-fructose, D-galactose, D-gluconic, D-glucose, glycerol, D-lactose, L-malic acid, D-maltose, D-mannitol, D-mannose, D-raffinose, L-rhamnose, D-serine, sorbitol, sucrose, D-trehalose and D-turanose (Biolog GEN III MicroPlates). Cells are sensitive to ampicillin, bacitracin, chloramphenicol, erythromycin, penicillin G, rifampicin, tetracycline, vancomycin, but resistant to gentamicin and neomycin. In the API ZYM system, it is positive for acid phosphatase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, esterase lipase (C8), β-galactosidase, α-glucosidase, β-glucosidase and naphthol-AS-BI-phosphohydrolase. Acid is produced from aesculin, amygdalin, arabinose, D-cellobiose, D-fructose, D-glucose, glycerol, lactose, maltose, D-mannitol, D-mannose, melibiose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose and D-turanose (API 50CH). The predominant menaquinone of strain YIM 93565^T is MK-7. The major fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{16:0}$ and anteiso-$C_{17:0}$. The polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol, one unidentified glycolipid and two unidentified phospholipids. *meso*-Diaminopimelic acid is present in the cell-wall peptidoglycan. The DNA G + C content of this novel is 36.8 mol%.

The type strain YIM 93565^T (= DSM 23710^T = CCTCC AB 2013249^T) was isolated from a salt lake in Xinjiang province of China. The GenBank accession number for the 16S rRNA gene sequence of strain YIM 93565^T is KF548095.

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