Salinibacillus aidingensis gen. nov., sp. nov. and *Salinibacillus kushneri* sp. nov., moderately halophilic bacteria isolated from a neutral saline lake in Xin-Jiang, China

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Three Gram-positive, moderately halophilic, heterotrophic bacterial strains were isolated from a neutral saline lake in the Xin-Jiang area of China. The strains, designated 8-2^T, W11-1 and 25-7^T, were motile, spore-forming, aerobic rods and contained *meso*-diaminopimelic acid in their cell walls. Their DNA G + C contents were 37.4, 37.2 and 39.9 mol%, respectively. The main fatty acids in the cellular membranes of these novel strains were C₁₅ and C₁₇ methyl-branched. No species with validly published names showed 16S rRNA gene sequence similarity of more than 95 % with respect to these novel isolates; the most closely related species was a halophilic denitrifier, *Bacillus halodenitrificans* (94.6 %). Polyphasic taxonomic studies revealed that these strains belong to the *Bacillaceae* and are distantly related to other genera of the family. It is proposed that a new genus, *Salinibacillus*, should be created, with *Salinibacillus aidingensis* (type strain, 25-7^T = AS 1.3565^T = JCM 12389^T) as the type species. Another species, *Salinibacillus kushneri*, is also proposed, with 8-2^T (= AS 1.3566^T = JCM 12390^T) as the type strain.

Moderately halophilic bacteria (MHB) show optimal growth in media with salt concentrations between 3 and 15 % (w/v) (Kushner, 1985). Studies of Gram-positive halophilic bacteria have increased greatly in recent years. Gram-positive, moderately halophilic, aerobic or facultatively anaerobic, spore-forming bacteria constitute an important category within the moderate halophiles. Many new genera and species of Gram-positive MHB have been reported. To date, MHB are present in the genera *Bacillus* (Ventosa *et al.*, 1989; Fritze, 1996), *Halobacillus* (Spring *et al.*, 1996; Yoon *et al.*, 2003), *Virgibacillus* (Arahal *et al.*, 1999, 2000; Heyndrickx *et al.*, 1998; Heyrman *et al.*, 2003), *Gracilibacillus* (Wainø *et al.*, 1999), *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) and *Tenuibacillus* (Ren & Zhou, 2005). All these halophilic genera belong to the

family *Bacillaceae* having similar properties and close phylogenetic relationships.

The sampling site (Ai-Ding Lake), sample collection/treatment and the isolation of MHB were as described by Ren & Zhou (2005). The cellular morphology of the isolates was observed using an AOM Optical 1-20 light microscope and a transmission electron microscope (Hitachi, S-570) as described previously by Zhu *et al.* (2003). Gram staining was performed as described by Gerhardt *et al.* (1981), in parallel with a KOH test (Gegersen, 1978). Flagella were demonstrated using staining (Kodaka *et al.*, 1982) and transmission electron microscopy. The motility of the strains was determined using phase-contrast microscopy (AOM Optical 1-20 apparatus) and by culture in soft-agar medium.

General physiological and biochemical tests were performed as described by Smibert & Krieg (1981). The NaCl, temperature and pH ranges for growth of the novel isolates were determined as described previously (Ren & Zhou, 2005). Utilization of carbon and energy sources was investigated by using basal medium to which appropriate substrates (0.5 %, w/v) had been added (Xin *et al.*, 2001). Twenty-six kinds of saccharide, polysaccharide and sugar alcohol were each used as sole carbon sources. The hydrolysis of starch, casein, gelatin and Tweens 20, 40, 60 and 80 was determined on a modified HM plate (Ventosa *et al.*,

Abbreviation: MHB, moderately halophilic bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 25-7^T, W11-1 and 8-2^T are AY321436, AY321437 and AY321434, respectively.

The results of SDS-PAGE of whole-cell proteins of strains 8-2^T and W11-1 and a table showing the fatty acid compositions of strains 25-7^T, W11-1 and 8-2^T are available as supplementary material in IJSEM Online.

1982) by substituting saccharide in the medium with the appropriate substrate.

Cell mass used for DNA extraction (Sambrook *et al.*, 1989), cellular fatty acid profile determination (MIDI Sherlock microbial identification system; Microbial ID), peptidoglycan composition determination (Schleifer & Kandler, 1972) and SDS-PAGE of whole-cell proteins (Manchester *et al.*, 1990) was obtained by cultivating strains in modified HM medium (Ventosa *et al.*, 1982). The DNA G + C content was determined by using HPLC as described by Mesbah *et al.* (1989).

Bacterial 16S rRNA gene universal primers were used (forward 27: 5'-AGA GTT TGA TCC TGG CTC AGG-3'; reverse 1492: 5'-ACG GCA ACC TTG TTA CGA GTT-3'). PCR products of 16S rRNA genes were sequenced directly using the ABI PRISM BigDye Primer cycle sequencing kit (Applied Biosystems) with an ABI 3700 DNA sequencer (Applied Biosystems). Almost-complete 16S rRNA gene sequences were used to construct a phylogenetic tree using GenBank sequences of related halophilic genera. The tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) and the stability of relationships was assessed by bootstrap analysis with the TREECONW software package, and by using the maximum-parsimony algorithm in the Ribosomal Database Project online analysis (<http://rdp.cme.msu.edu/index.jsp>). The Microplate DNA-DNA hybridization method was used for DNA similarity analysis, as described by Ezaki *et al.* (1989) except that colorimetric quantification was used. The microplate reader used was a FLUOstar OPTIMA (BMG).

Morphological characteristics, utilization of carbon sources and physiological/biochemical traits are given in the genus and species descriptions and in Table 1. The diamino acid in the cell walls of strains 8-2^T, 25-7^T and W11-1 was *meso*-diaminopimelic acid. Details of the fatty acid compositions of the three strains are available in a supplementary table in IJSEM Online.

The 16S rRNA gene sequences obtained were more than 1450 nt in length. According to a FASTA search (European Bioinformatics Institute, <http://www.ebi.ac.uk/fasta33/>), no species with validly published names showed relatedness above 95% with respect to the three novel isolates. Of the species with validly published names, *Bacillus halodenitrificans* was the one most closely related to the novel isolates (approximately 94.6% similarity). Strains 8-2^T and W11-1 were closely related (99.9% similarity) and showed 98.1 and 98.2% relatedness, respectively, with respect to the 16S sequence of strain 25-7^T.

A phylogenetic tree was constructed using the neighbour-joining method by aligning 16S rRNA gene sequences of the novel isolates and other moderately halophilic and spore-forming bacilli, with *Bacillus subtilis* as the outgroup (Fig. 1). The three novel isolates were located on an isolated branch away from other MHB species. The bootstrap values at each branch point as well as maximum-parsimony algorithm online analysis (data not shown) are sufficient to support such alignment. The most closely related species with a validly published name, *B. halodenitrificans*, grouped with species of the genera *Virgibacillus*, *Lentibacillus* and *Oceanobacillus* in another cluster. We doubted

Table 1. Differential characteristics of strains 8-2^T, 25-7^T, W11-1 and *B. halodenitrificans*

Species/strains: 1, *B. halodenitrificans* (data from Denariáz *et al.*, 1989); 2, 8-2^T; 3, W11-1; 4, 25-7^T. Symbols: +, positive; −, negative; NA, not available; v, variable; w, weak. All strains are motile rods and share the following characteristics: no growth in medium without salt; show catalase activity; lack urease and DNase activity; give a negative result in the Voges-Proskauer test; and hydrolyse gelatin and casein but not Tween 80.

Characteristic	1	2	3	4
Gram reaction	v	+	+	+
Spore shape/position*	No spores	S, T	S, T	S, T
Type of flagellation	Polar or lateral	Single, polar	Peritrichous	Peritrichous
Optimal NaCl concn (% w/v)	3–8	12–15	12	10
Anaerobic growth	+	−	−	−
Optimal pH	7.4	7.0–8.0	7.0–8.0	6.5–7.5
pH range	5.8–9.6	6.0–8.0	6.0–10.0	6.0–10.0
Optimal temp. (°C)	38	40–47	40–44	39–43
Growth below 20 °C	+	−	−	−
Reduction of nitrate to nitrite	+	−	−	−
Production of H ₂ S	ND	−	+	−
Production of NH ₃	ND	+	−	−
Oxidase activity	+	−	+	+
Acid production from D-glucose	w	−	+	−
Hydrolysis of starch	−	+	−	+

*Abbreviations: S, spherical; T, terminal.

Fig. 1. Neighbour-joining tree constructed on the basis of 16S rRNA gene sequences of the three novel isolates and some Gram-positive, spore-forming, halophilic species. Bootstrap values (expressed as percentages of 1000 replications) greater than 70 % are shown at the branch points. GenBank/EMBL/DDBJ accession numbers of 16S rRNA gene sequences are shown in parentheses. Bar, 0.02 substitutions per nucleotide position.

the classification of *B. halodenitrificans* because of the phylogenetic relationship of this species with the genus *Virgibacillus* and the genus *Bacillus*. This confusion was resolved by Yoon *et al.* (2004), who reclassified *B. halodenitrificans* with a novel isolate into the genus *Virgibacillus*.

The low level of 16S rRNA gene sequence similarity (<95 %) between species with validly published names and the novel isolates, as well as the differences in morphology, physiology and biochemistry, indicated the separation of the novel isolates from other MHB bacilli. Consequently, DNA–DNA reassociation studies were not necessary (Stackebrandt & Goebel, 1994). DNA–DNA hybridization assays were performed to evaluate the relatedness among the three novel isolates. Each assay was performed at least three times and reciprocal tests were performed for reference. The DNA similarities between these novel isolates were as follows: 8-2^T with 25-7^T, 29.5 %; 8-2^T with W11-1, 72.1 %; and 25-7^T with W11-1, 36.5 %. The almost-identical 16S rRNA gene sequences, phylogeny (Fig. 1) and DNA–DNA hybridization values suggested that strains 8-2^T and W11-1 should be classified in the same species, but some physiological traits (e.g. flagellation, oxidase activity and the carbohydrate-utilization profile) did not support this view. SDS-PAGE of whole-cell protein was then performed to clarify the relationship between these isolates. The results for strains 8-2^T and W11-1 (see the supplementary figure available in IJSEM Online) indicated that they should be classified as strains of the same species.

The genus *Bacillus* is a heterogeneous group composed of members with diverse properties (i.e. acidophiles, thermophiles, alkaliphiles and halophiles and so on). Many attempts have been made to obtain a coherent taxonomy of this genus (Ash *et al.*, 1991; Kämpfer, 1994; Goto *et al.*, 2000). Polyphasic classification methods combined with phenotypic and phylogenetic analyses of *Bacillus* and related species have begun to untangle the jumbled taxonomy of this genus, and many new genera of bacilli (including halophilic groups) have emerged from within the genus *Bacillus* in recent years.

We isolated many bacilli from the hypersaline Ai-Ding Lake. In the present research, three moderately halophilic, aerobic, spore-forming, Gram-positive bacteria were classified by using polyphasic taxonomy. According to the phylogenetic analysis, no known related species has 16S rRNA gene sequence similarity of more than 95 % with respect to these novel isolates. The phylogenetic tree (Fig. 1) shows that the isolates were distantly related to other spore-forming halophilic bacteria with validly published names. According to the FASTA search, the halophilic organism *B. halodenitrificans* was the species most closely related (around 94.6 % similarity) to the novel isolates, but it was also distant from the novel strains in the phylogenetic tree and differed in many respects (e.g. Gram reaction, sporangia, growth under anaerobic conditions; see Table 1) from the novel isolates. The levels of 16S rRNA gene sequence similarity between the three novel strains and species of the genus *Virgibacillus* (including *Virgibacillus marismortui*, *Virgibacillus pantothenticus*, *Virgibacillus carmonensis* and *Virgibacillus picturae*) were close to that between the novel strains and *B. halodenitrificans*, being around 94.4 %. All of these species clustered with *B. halodenitrificans*, *Lentibacillus* and *Oceanobacillus* on another branch (Fig. 1). Other MHB genera were more distantly related to the novel strains. Despite the different cultural conditions used to study the novel isolates and other genera of halophilic/halotolerant bacilli, the fatty acid profiles obtained differed considerably (for details, see the supplementary table available in IJSEM Online), suggesting taxonomic distance. In addition, features such as different diagnostic amino acids in the cell walls (e.g. L-lysine for *Filobacillus*, Orn-D-Asp for *Halobacillus* and L-lysine for *Jeotgalibacillus* and *Marinibacillus*) also support the results of the phylogenetic analysis.

On the basis of the data above, especially with regard to the phylogenetic analysis, the novel strains should be classified within a new genus, for which we propose the name *Salinibacillus*. The new genus comprises two species: *Salinibacillus kushneri*, containing strains 8-2^T and W11-1, and

Salinibacillus aidingensis, with 25-7^T as the type strain. The type species of this new genus is *Salinibacillus aidingensis*.

Description of *Salinibacillus* gen. nov.

Salinibacillus [Sa.li.ni.ba.cil'lus. N.L. adj. *salinus* salted; L. n. *bacillus* rod; N.L. masc. n. *Salinibacillus* salted rod, salt (-loving) bacterium].

Cells are Gram-positive, obligately aerobic rods and motile by means of polar flagellum or peritrichous flagella. Moderately halophilic; no growth without NaCl in medium. Catalase-positive and oxidase-variable. Acid production from glucose and hydrolysis of starch are variable. Production of NH₃ and H₂S are variable within this genus. Major fatty acids are iso-C_{15:0}, anteiso-C_{15:0} and anteiso-C_{17:0}. Directly cross-linked amino acid in peptidoglycan is *meso*-diaminopimelic acid.

The type species is *Salinibacillus aidingensis*.

Description of *Salinibacillus aidingensis* sp. nov.

Salinibacillus aidingensis (ai.ding.en'sis. N.L. masc. adj. *aidingensis* relating to Ai-Ding Lake, Xin-Jiang, China, where the organism was isolated).

Colonies are white, slightly convex, smooth and 1–2 mm in diameter with regular margins (after 2 days cultivation). Cells are approximately 0.3–0.5 × 1–2 µm in size. Salt and temperature ranges for growth are 5–20% (w/v) and 28–49 °C, respectively. The pH range for optimal growth is 6.5–7.5. Methyl red and Voges–Proskauer tests are negative. Negative for DNase and urease activity, but positive for phosphoesterase activity. The following carbohydrates are utilized: cellobiose, D-mannose, L-sorbose, inulin, (–)-D-fructose, (+)-D-raffinose, glucose, D-galactose, salicin, lactose, sucrose, aesculin, maltose, mannitol, melibiose, D-sorbose, trehalose, dulcitol, glycerol, inositol, erythritol, melezitose, starch, arabinose, rhamnose and xylose. Acid is produced from fermentation of cellobiose, (–)-D-fructose, D-galactose, glycerol, maltose, rhamnose and sucrose. Gelatin, casein, aesculin and Tween 40 are hydrolysed, but Tweens 20, 60 and 80 are not. The DNA G + C content is 39.9 mol%. anteiso-C_{15:0} is the major fatty acid (39.0%), followed by anteiso-C_{17:0} (23.7%), iso-C_{15:0} (18.4%) and iso-C_{16:0} (9.8%). The detailed fatty acid composition is given in the supplementary table available in IJSEM Online. Additional characteristics are listed in Table 1.

The type strain is 25-7^T (=AS 1.3565^T=JCM 12389^T).

Description of *Salinibacillus kushneri* sp. nov.

Salinibacillus kushneri (kush.ner.i. N.L. gen. n. of Kushner, in honour of Professor Donn J. Kushner, for his contribution to halophile microbiology).

Colonies of both strains are white, slightly convex, smooth, 1–2 mm in diameter, with indented margins (after 2 days cultivation). Cells of 8-2^T are 0.4–0.6 × 2.5–3.0 µm in size

and those of W11-1 are 0.35 × 1.35–3.5 µm in size. Salt ranges for growth of strains 8-2^T and W11-1 are 1–30% (w/v) and 5–23% (w/v), respectively; temperature ranges for growth are 20–50 °C and 20–52 °C, respectively. The pH range for optimal growth is 7.0–8.0. Methyl red and Voges–Proskauer tests are negative. Negative for DNase and urease activity, but positive for phosphoesterase activity. Strain W11-1 utilizes all carbohydrates tested [cellobiose, D-mannose, L-sorbose, inulin, (–)-D-fructose, (+)-D-raffinose, glucose, D-galactose, salicin, lactose, sucrose, aesculin, maltose, mannitol, melibiose, D-sorbose, trehalose, dulcitol, glycerol, inositol, erythritol, melezitose, starch, arabinose, rhamnose and xylose], but 8-2^T cannot utilize D-arabinose, rhamnose or xylose. Strain W11-1 produces acid from fermentation of cellobiose, maltose, mannitol, (–)-D-fructose, trehalose and glycerol, while strain 8-2^T produces acid from fermentation of cellobiose, (–)-D-fructose, D-mannose, L-sorbose and trehalose. Gelatin, casein, aesculin and Tween 40 are hydrolysed, but Tweens 20, 60 and 80 are not. Branched C_{15:0} and C_{17:0} are the major fatty acids (8-2^T: iso-C_{15:0}, 28.0%; anteiso-C_{15:0}, 23.9%; anteiso-C_{17:0}, 18.4%; W11-1: anteiso-C_{15:0}, 27.2%; iso-C_{15:0}, 24.9%; anteiso-C_{17:0}, 17.8%). The detailed fatty acid composition is given in the supplementary table available in IJSEM Online. The DNA G + C contents of 8-2^T and W11-1 are 37.4 and 37.2 mol%, respectively. Additional characteristics of the strains are listed in Table 1.

The type strain is 8-2^T (=AS 1.3566^T=JCM 12390^T); a reference strain is also available (W11-1=AS 1.3567=JCM 12391).

Acknowledgements

We wish to thank Dr Shuang-Jiang Liu (Institute of Microbiology, Chinese Academy of Sciences) for his valuable advice. We thank the DSMZ for the fatty acid analyses. This work was supported by grant KSCS2-3-01 from the Chinese Academy of Sciences and National 973 grant no. 2004CB719600.

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