Tenuibacillus multivorans gen. nov., sp. nov., a moderately halophilic bacterium isolated from saline soil in Xin-Jiang, China

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Two Gram-positive, rod-shaped, spore-forming and moderately halophilic bacteria (strains 28-1^T, 28-4), isolated from a soil sample from a neutral salt lake in Xin-Jiang, China, were characterized polyphasically. On the basis of FASTA (ungapped) analyses of 16S rRNA gene sequences, strains 28-1^T and 28-4 were shown to belong to the *Bacillaceae* and to be closely related to *Filobacillus milensis* DSM 13259^T (97.0%) and *Bacillus haloalkaliphilus* DSM 5271^T (95.7%). 16S rRNA gene sequence similarity with other recognized species was not more than 94.1%. Phylogenetic, chemotaxonomic, physiological and biochemical data supported the differentiation of these novel strains from *F. milensis* and *B. haloalkaliphilus*. Therefore these two previously unidentified strains are considered to represent a new genus and species, for which the name *Tenuibacillus multivorans* gen. nov., sp. nov. is proposed. The type strain is 28-1^T (= AS 1.3442^T = NBRC 100370^T).

Salt-adapted micro-organisms can be divided into halophilic and halotolerant. Growth of halophilic organisms requires salt in the medium, whereas halotolerant organisms can grow with or without salt. Halophilic/halotolerant micro-organisms occur globally from ocean to diverse terrestrial saline environments, including salt lakes. Numerous hypersaline lakes are found in the Xin-Jiang Uigur Autonomous Area of north-west China. In the present report, moderately halophilic bacteria (MHB) isolated from a hypersaline lake, Ai-Ding Lake, were studied polyphasically.

Ai-Ding Lake is located in the most arid area of China, where evaporation is significantly higher than precipitation. The lake is at the lowest point of elevation in China and the second lowest in the world (154 m below sea-level). Water is largely supplied from the nearby glacier of Tian-Shan Mountain. The water level in the lake varies continuously and, at times, the lake almost disappears. Ai-Ding Lake is of athalassohaline origin and is a typical chloride–sulphate saline lake with a neutral pH; the salt concentration fluctuates between 20 and 26% between seasons. The content of Mg²⁺ and Ca²⁺ in the brine is very low. The water is slightly alkaline, between pH 7.2 and 7.6. Plankton diversity is low and quantities change markedly throughout

the year. Soil in the vicinity is saline and vegetation cover is sparse with limited species variation (Yan, 1996). The geomorphology of the area means that this area represents a relatively isolated ecosystem. A previous study showed adaptations of the microflora to these unique and harsh conditions (Tohty & Xu, 2001).

MHB, according to the definition provided by Kushner (1985), have optimal growth in media containing 3–15% (w/v) salt but no growth in media without salt. Moderately halophilic, aerobic or facultatively anaerobic and spore-forming bacteria are widely distributed in hypersaline environments and represent an important part of the halophilic bacteria worldwide. Numerous *Bacillus* species have been reclassified (Lawson *et al.*, 1996; Wainø *et al.*, 1999; Arahall *et al.*, 1999, 2000; Heyrman *et al.*, 2003) and many new genera have been proposed. Hitherto, aerobic or facultatively anaerobic and spore-forming MHB are found within genera including *Bacillus* (Ventosa *et al.*, 1989; Fritze, 1996), *Halobacillus* (Spring *et al.*, 1996; Yoon *et al.*, 2003), *Virgibacillus* (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 *Paraliobacillus* (Ishikawa *et al.*, 2002). All of these genera belong to the family *Bacillaceae* of the low G + C Gram-positive bacteria group and are closely related.

Samples were collected aseptically into vessels from soil around the lake and from sediment near the lake bank.

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Abbreviation: MHB, moderately halophilic bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 28-1^T and 28-4 are AY319933 and AY646355.

Samples were packed in ice before transportation to the laboratory and then stored at -20°C until study. Soil samples (0.5 g) were suspended in saline water (10 ml, 10% NaCl solution) and supernatants were spread on modified Halophiles Moderate (HM, with 10% NaCl) plates (Ventosa *et al.*, 1982). Two isolates from these samples, strains 28-1^T and 28-4, were studied polyphasically.

The cellular morphology of the isolates was observed by optical microscopy and transmission electron microscopy (TEM). Samples for TEM were prepared as described by Zhu *et al.* (2003). Cells were stained negatively with 1% (w/v) phosphotungstic acid; after air-drying, the grids were examined using a model H-600 transmission electron microscope (Hitachi). Gram staining was used for testing cell wall structure, in parallel with KOH testing (Gregersen, 1978). The presence of flagella was determined by staining (Kodaka *et al.*, 1982) and TEM observation. Motility was determined by phase-contrast microscopy and growth conditions were studied in soft-agar medium.

General physiological and biochemical tests were performed as described by Smibert & Krieg (1981). The range of NaCl concentration for growth was tested in aquatic HM medium with an NaCl concentration between 0% and saturated. Growth temperature of the strains in HM medium with optimal salt concentration (unless otherwise specified, cultivation was at optimal salt concentration and temperature) was determined using a TN3F temperature gradient incubator (Advantec). The pH range for growth (from pH 5.5 to 11.0 at intervals of 0.5) was determined by adding MES (5.5–6.5), PIPES (6.5–7.5), HEPES (7.0–8.0), Tricine (7.5–9.0) and CHES (9.0–11.0) into liquid modified HM medium, all at concentrations of 50 mM. Utilization of carbon and energy sources (added at 0.5%, w/v) was investigated by use of a basal medium (Xin *et al.*, 2001). Hydrolysis of starch, casein, gelatin and Tweens 20, 40, 60 and 80 was assessed on HM plates with the corresponding substrates substituting for saccharide within the medium.

Peptidoglycan composition was analysed by one-dimensional chromatography as described by Schleifer & Kandler (1972) using cellulose thin layers instead of paper. Cellular fatty acids were determined by analysing the composition of fatty acid methyl esters by GLC (determined by the DSMZ, Germany). DNA was extracted using the method of Sambrook *et al.* (1989). DNA G+C content was determined with HPLC as described by Mesbah *et al.* (1989). PCR products of the 16S rRNA gene were sequenced directly by the ABI PRISM BigDye Primer cycle sequencing kit and sequencing was performed on an ABI 3700 DNA sequencer (Applied Biosystems). Nearly complete 16S rRNA gene sequences were used to construct a phylogenetic tree with sequences of other halophilic bacilli available from GenBank. The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) and the stability of the relationship was assessed by bootstrap analysis with the TREECONW software package, as well as by

the maximum-parsimony algorithm in the Ribosomal Database Project (RDP) online analysis. The microplate DNA–DNA hybridization method was used in DNA similarity analysis as described by Ezaki *et al.* (1989) with colorimetric quantification at the optimal hybridization temperature. The microplate reader used was FLUOstar OPTIMA (BMG).

Only the 5'-end (>700 bp) of the 16S rRNA gene sequence of strain 28-4 was determined; this part contains the hypervariable region of the gene (Goto *et al.*, 2000). Using the FASTA program (ungapped), 16S rRNA gene sequence similarity between strains 28-4 and 28-1^T was 99.8%. In addition, DNA–DNA relatedness values (84 and 79% reciprocally) and the large number of shared characteristics indicated that these two strains should be classified within the same species.

Using the FASTA3 program in EBI, 16S rRNA gene sequence comparisons were made between the novel strains and other members of the *Bacillaceae*. The closest matches were with *Filobacillus milensis* (97.0% sequence similarity), the only member of the genus *Filobacillus*, and *Bacillus haloalkaliphilus* (95.7% similarity). Similarities to the species of *Gracilibacillus*, *Virgibacillus*, *Lentibacillus* and *Halobacillus* were no greater than 94.1%. In addition, DNA–DNA relatedness values of strain 28-1^T with *F. milensis* and *B. haloalkaliphilus* were 16% (23%, reciprocally) and 11% (14%, reciprocally), respectively.

Wayne *et al.* (1987) emphasized the importance of phylogeny to bacterial taxonomy, and that phylogeny should determine taxonomy. Most of the aerobic or facultatively anaerobic, spore-forming, moderately halophilic or halo-tolerant bacteria have similar phenotypic and physiological traits. Phylogenetic data predominate and are considered to be preferential in determining taxonomy among such bacteria. Several genera of moderately halophilic or halo-tolerant bacilli have previously been proposed from small numbers of strains, mainly on the basis of their phylogeny, for example *Filobacillus* (Schlesner *et al.*, 2001), *Lentibacillus* (Yoon *et al.*, 2002) and *Paraliobacillus* (Ishikawa *et al.*, 2002). On the basis of 16S rRNA gene sequence data, these organisms show the greatest degree of similarity to strains 28-1^T and 28-4.

F. milensis is a moderately halophilic bacillus, with an unusual murein type, L-ornithine, for cross-linking (Schlesner *et al.*, 2001). By contrast, the diamino acid in the murein of strains 28-1^T and 28-4 was *meso*-diaminopimelic acid, which is common in members of *Bacillus* and many related genera, for example *Virgibacillus*, *Lentibacillus* and *Paraliobacillus*. In the neighbour-joining tree, *Filobacillus milensis* and strain 28-1^T group in the same cluster (Fig. 1), with a bootstrap value for this cluster of 84%. This phylogenetic topology was also supported by maximum-parsimony analysis, producing a similar bootstrap value (data not shown). The difference of murein type between *F. milensis* and strains 28-1^T and 28-4 suggested they should be

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences of several spore-forming, halophilic or halotolerant species. Bootstrap values (expressed as percentages of 1000 replications) greater than 80 % are shown at the branch points. GenBank accession numbers are given in parentheses. Bar, 0.02 substitutions per nucleotide position.

separated into a different taxon. The major fatty acids of *F. milensis* cultivated on modified HM agar were anteiso- $C_{15:0}$ (35.2 %), iso- $C_{15:0}$ (27.0 %) and anteiso- $C_{17:0}$ (12.3 %), whereas the major fatty acids of isolate 28-1^T were iso- $C_{15:0}$ (64.7 %), anteiso- $C_{15:0}$ (12.7 %) and iso- $C_{17:0}$ (8.3 %). In addition to the phylogenetic and biochemical data mentioned above, differences in position

of flagella, Gram-staining, growth in media without NaCl and oxidase activity supported their separation (Table 1).

In the neighbour-joining tree (Fig. 1), strain 28-1^T and the alkaliphilic, extremely halotolerant species *B. haloalkaliphilus* (Fritze, 1996) were on a lower branch with 100 % bootstrap value at the node. This topology was also supported by a maximum-parsimony algorithm, and the clustering fidelity was supported by bootstrap analysis at a confidence level of 100 % (data not shown). Although there are many similar phenotypic characteristics between the novel strains and *B. haloalkaliphilus*, some important traits that distinguish them remain. For example, iso- $C_{16:0}$ is one of the major fatty acids (4 % of total composition) of strain 28-1^T, but is not found in *B. haloalkaliphilus*; Gram reaction of *B. haloalkaliphilus* is variable but strain 28-1^T is positive; and a notable difference is that *B. haloalkaliphilus* is an obligately alkaliphilic species without growth at pH 7.0 and with optimal growth at pH 9.7, whereas strain 28-1^T is neutrophilic with no growth above pH 9.0.

Other aerobic or facultatively anaerobic, spore-forming and moderately halophilic bacilli, such as *Gracilibacillus*, *Virgibacillus*, *Lentibacillus* and *Halobacillus* species, were distantly related to the novel isolates (16S rRNA gene sequence similarity of less than 94.1 %). This can be seen

Table 1. Distinguishing characteristics of *Tenuibacillus multivorans* gen. nov., sp. nov. and related taxa

Taxa: 1, *Filobacillus* (data from Schlesner *et al.*, 2001); 2, *Bacillus haloalkaliphilus* (Fritze, 1996); 3, *Virgibacillus* (Heyndrickx *et al.*, 1998; Proom & Knight, 1950); 4, *Lentibacillus* (Yoon *et al.*, 2002); 5, *Gracilibacillus* (Heyrman *et al.*, 2003); 6, *Tenuibacillus* (this study). +, Positive; –, negative; (+) weakly positive; V, variable; NA, not available; *m*-Dpm, *meso*-diaminopimelic acid; Orn, ornithine. All taxa are motile, have swollen sporangia and are catalase-positive.

Characteristic	1	2	3	4	5	6
Spore shape, position*	S, T	S, T	E/S, T/ST	S/E, T	E/S, T	S, T
Gram reaction	–	–	+	V	+	+
Flagella	Single lateral	NA	NA	Single	Peritrichous	Single polar
Anaerobic growth	–	–	V	–	V	–
Optimal growth temperature (°C)	33–38	NA	28–37	30	45–47	36–41
Optimal NaCl concentration (%)	8–14	NA	4–10	4–8	0 or 15	5–8
NaCl growth range (%)	2–23	0–25	0–25	2–23	0–20	1–20
Growth in medium with no NaCl	–/(+)	–/(+)	V	–	+	–
Growth at pH 7	+	–	+	+	+	+
Alkaliphilic	–	+	–	–	–	–
Oxidase	–	+	V	+	+	+
Acid produced from glucose	–	–	V	–	+	–
Hydrolysis of:						
Aesculin	–	NA	+	–	+	+
Starch	–	(+)	V	–	+	–
Casein	–	–/+	+	+	–	+
Diamino acid in murein	L-Orn	<i>m</i> -Dpm	<i>m</i> -Dpm	<i>m</i> -Dpm	<i>m</i> -Dpm	<i>m</i> -Dpm
DNA G + C content (mol%)	35	37–38	36–43	44	38–39	36.5–37
Major fatty acid(s)	anteiso- $C_{15:0}$, iso- $C_{15:0}$	iso- $C_{15:0}$, anteiso- $C_{15:0}$	anteiso- $C_{15:0}$	anteiso- $C_{15:0}$, iso- $C_{16:0}$	anteiso- $C_{15:0}$, anteiso- $C_{16:0}$	iso- $C_{15:0}$, anteiso- $C_{15:0}$

*E, Ellipsoidal; S, spherical; ST, subterminal; T, terminal.

from the phylogenetic relationship in the neighbour-joining tree (Fig. 1). Further differences can be observed in the morphological, chemotaxonomic and physiological characteristics (Table 1).

Two strains are not ideal for description of the diversity of a new genus, especially as its nearest genus, *Filobacillus*, was based on only one strain. A more complete understanding must await isolation of further strains. Based on the data, a new genus seems to be the best solution for classifying these strains. We propose the name *Tenuibacillus* gen. nov. with type species *Tenuibacillus multivorans* sp. nov.

Description of *Tenuibacillus* gen. nov.

Tenuibacillus (Te.nu.i.ba.cil'lus. L. adj. *tenuis* slender, thin, slim; L. n. *bacillus* small rod; N.L. masc. n. *Tenuibacillus* a slender rod).

Gram-positive, aerobic, organotrophic, rod-shaped cells (about $0.3\text{--}0.5 \times 2.0\text{--}6.0\text{ }\mu\text{m}$). Cells are motile with a single polar flagellum. Spores are spherical, terminally located, and sporangium swollen. No growth in media without NaCl. Nitrate is not reduced to nitrite. Catalase- and oxidase-positive; phosphoesterase- and cellulase-negative. Production of H_2S but not NH_3 . Methyl red and Voges-Proskauer tests are negative. The major fatty acids are iso- $\text{C}_{15:0}$ (65%), anteiso- $\text{C}_{15:0}$ (13%), iso- $\text{C}_{17:0}$ (8%) and iso- $\text{C}_{16:0}$ (4%). DNA G + C content is 36.5–37 mol%. The diamino acid in peptidoglycan is *meso*-diaminopimelic acid. The type species is *Tenuibacillus multivorans*.

Description of *Tenuibacillus multivorans* sp. nov.

Tenuibacillus multivorans (mul.ti.vor'ans. L. part. adj. *multus* many, numerous; L. v. *voro* to devour, swallow; N.L. part. adj. *multivorans* devouring numerous kinds of substrates).

In addition to characteristics given above for the genus, the followings are characteristic of *T. multivorans*. Gram staining of fresh culture is positive but variable in old culture. KOH test is negative. Cells are motile by one polar flagellum. After 2 days growth, colonies are circular, translucent, convex and 1–2 mm in diameter; older colonies become brown from the centre outwards on HM medium. Optimal NaCl concentration for growth is 5% for strain 28-1^T (8% for strain 28-4); NaCl growth range is 1–20% and no growth is observed at either 37 or 20 °C in rich media without NaCl. Temperature range for growth is 21–42 °C (optimum 36–41 °C). pH range for growth is 6.5–9.0 (optimum 7.0–8.0). Able to utilize but not produce acid from all saccharides, polysaccharides and sugar alcohols tested, including arabinose, xylose, D-fructose, glucose, D-mannose, rhamnose, DL-sorbose, cellobiose, D-galactose, lactose, maltose, melibiose, sucrose, trehalose, melezitose, D-raffinose, inulin, dulcitol, erythritol, glycerol, inositol, mannitol and salicin. Hydrolyses gelatin, casein, aesculin and Tweens 40 and 60, but not starch or Tweens 20 or 80.

The type strain is strain 28-1^T (=AS 1.3442^T=NBRC 100370^T), isolated from a hypersaline lake, Ai-Ding Lake, in the Xin-Jiang Uigur Autonomous Area of north-west China.

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