Terribacillus saccharophilus gen. nov., sp. nov. and *Terribacillus halophilus* sp. nov., spore-forming bacteria isolated from field soil in Japan

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Three strains, 002-048^T, RB589 and 002-051^T, isolated from field soil in Japan, were characterized using a polyphasic approach. The isolates were Gram-positive, strictly aerobic, non-motile rods that formed ellipsoidal, subterminal endospores. The chemotaxonomic characteristics of these isolates included the presence of *meso*-diaminopimelic acid as the cell-wall peptidoglycan, anteiso-C_{15:0} and anteiso-C_{17:0} as the major cellular fatty acids and MK-7 as the predominant menaquinone. The DNA G + C content was 44–46 mol%. Phylogenetic analyses based on 16S rRNA gene sequences revealed that the isolates represented an independent lineage that is distinct from related taxa and exhibited less than 94.3% sequence similarity with respect to those taxa. Moreover, a DNA–DNA hybridization analysis showed that the three isolates represented two species. On the basis of their phenotypic and phylogenetic distinctiveness, the isolates represent two species within a novel genus, for which the names *Terribacillus saccharophilus* gen. nov., sp. nov. and *Terribacillus halophilus* sp. nov. are proposed. The type strain of *T. saccharophilus* is 002-048^T (=IAM 15309^T = KCTC 13936^T) and the type strain of *T. halophilus* is 002-051^T (=IAM 15310^T = KCTC 13937^T).

The aerobic, spore-forming, Gram-positive, rod-shaped, moderately halophilic bacteria are a taxonomically diverse group whose members have been isolated from various environments. Phylogenetic and chemotaxonomic analyses have shown that halotolerant and moderately halophilic bacteria constitute several phylogenetically distinct lineages scattered throughout the genus *Bacillus* (Ash *et al.*, 1991), described as the genera *Halobacillus* (Spring *et al.*, 1996), *Virgibacillus* (Heyndrickx *et al.*, 1998), *Gracilibacillus* (Wainø *et al.*, 1999), *Oceanobacillus* (Lu *et al.*, 2001), *Lentibacillus* (Yoon *et al.*, 2002), *Thalassobacillus* (García *et al.*, 2005) and *Pontibacillus* (Lim *et al.*, 2005b). Three isolates with similar characteristics, 002-048^T, RB589 and 002-051^T, were isolated from field soil in Japan. On the basis of partial 16S rRNA gene sequence analyses, the strains were found to be closely related to members of the genera *Oceanobacillus*, *Halobacillus*, *Thalassobacillus*, *Virgibacillus*

and *Gracilibacillus*. The aim of the present study was to elucidate the taxonomic position of the isolates, using polyphasic taxonomy (physiological and chemotaxonomic analyses, determination of the fatty acid composition, the quinone system and the G + C content, and 16S rRNA gene sequence analysis). On the basis of these data, it is proposed that the three isolates represent two species within a novel genus, *Terribacillus saccharophilus* gen. nov., sp. nov. and *Terribacillus halophilus* sp. nov.

Strains 002-048^T, RB589 and 002-051^T were obtained using the dilution plating technique on plate-count agar (Merck). Cell morphology and motility were examined by using phase-contrast microscopy (BX60 microscope; Olympus). Growth under anaerobic conditions was determined after 1 week incubation in an AnaeroPack (Mitsubishi Gas Chemical). Catalase activity was determined by means of bubble production from a 3% H₂O₂ solution. Oxidase activity was determined by using cytochrome oxidase paper (Nissui Pharmaceutical). The API 20E and API 50CH microtest galleries (bioMérieux) were used to determine the physiological and biochemical characteristics. All suspension media were supplemented with 0.5 × Herbst's artificial

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 002-048^T, RB589 and 002-051^T are AB243845, AB243847 and AB243849, respectively.

The major fatty acids of strains 002-048^T, RB589 and 002-051^T are listed in a supplementary table available in IJSEM Online.

Table 1. Differential characteristics of the novel strains and members of phylogenetically related genera

Taxa: 1, strains 002-048^T, RB589 and 002-051^T; 2, *Oceanobacillus*; 3, *Virgibacillus*; 4, *Lentibacillus*; 5, *Halobacillus*; 6, *Thalassobacillus*; 7, *Pontibacillus*; 8, *Gracilibacillus*. Data are from Lu *et al.* (2001), Lee *et al.* (2006), Heyndrickx *et al.* (1998), Yoon *et al.* (2002), Spring *et al.* (1996), García *et al.* (2005), Lim *et al.* (2005a, b) and Wainø *et al.* (1999). Symbols: +, positive; –, negative; V, varied according to strain; ND, not detected.

Characteristic	1	2	3	4	5	6	7	8
Spore shape*	E, S	E	E, S	S, O	E, S	E	S	E, S
Spore position†	ST, T	ST, T	ST, T	T	C, L, ST	C	T	T
Motility	–	+	+	+	+	+	+	+
Anaerobic growth	–	V	+	–	–	–	–	V
Maximum growth temperature (°C)	45	42	50	>40	>40	45	45	50
Requirement for NaCl	–	–	V	+	V	+	+	V
Maximum NaCl concentration for growth (%)	18	22	>10	>20	>20	20	15	>15
Oxidase activity	–	+	V	+	+	–	V	+
Reduction of nitrate	–	–	V	+	–	+	V	+
Voges–Proskauer test	+	–	–	ND	–	–	ND	–
Diamino acid in murein‡	<i>m</i> -DAP	ND	<i>m</i> -DAP	<i>m</i> -DAP	Orn	<i>m</i> -DAP	<i>m</i> -DAP	<i>m</i> -DAP
DNA G + C content (mol%)	44–46	36–40	37–38	44	40–45	42	41–42	38–39
Major fatty acid	anteiso-C _{15:0} , anteiso-C _{17:0}	anteiso-C _{15:0}	iso-C _{15:0} , anteiso-C _{15:0}	anteiso-C _{15:0} , iso-C _{16:0}	anteiso-C _{15:0} , iso-C _{16:0}	anteiso-C _{15:0} , iso-C _{16:0} , iso-C _{15:0}	iso-C _{15:0} , anteiso-C _{15:0}	anteiso-C _{15:0}

*E, Ellipsoidal; S, spherical; O, oval.

†C, Central; L, lateral; T, terminal; ST, subterminal.

‡*m*-DAP, *meso*-diaminopimelic acid; Orn, ornithine.

seawater. Herbst's artificial seawater contains the following (per l distilled water): NaCl, 30 g; KCl, 0.7 g; MgSO₄·7H₂O, 5.3 g; CaSO₄·2H₂O, 1.3 g; and MgCl₂·6H₂O, 10.8 g. The API tests were read after 48 h incubation at 30 °C. The results of the morphological, biochemical and physiological analyses are shown in Tables 1 and 2.

The 16S rRNA gene sequences were determined using the 16S rRNA Gene Kit (Applied Biosystems) according to the protocols of the manufacturer. The 16S rRNA gene sequences of strains 002-048^T, RB589 and 002-051^T were used for a BLAST search via the National Center for Biotechnology Information. The sequences obtained were aligned using the CLUSTAL W software package (Thompson *et al.*, 1994), and evolutionary distances and *K*_{nuc} values (Kimura, 1980) were generated. Alignment gaps and ambiguous bases were not taken into consideration when the 1387 bases of the 16S rRNA gene sequence were compared. The phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985), with 1000 replicates. The similarity values were calculated using MEGA3 (Kumar *et al.*, 2004). Almost-complete 16S rRNA gene sequences of strains 002-048^T, RB589 and 002-051^T were subjected to comparative analysis. The 16S rRNA gene sequence similarities for strain 002-048^T with respect to

RB589 and 002-051^T were 100 and 99.2 %, respectively. Strains 002-048^T and 002-051^T exhibited 16S rRNA gene sequence similarity values of 94.3 and 93.6 %, respectively,

Table 2. Differential characteristics of strain 002-048^T and strain 002-051^T

Strains: 1, 002-048^T; 2, 002-051^T. +, Positive; –, negative.

Characteristic	1	2
Cell size (µm)	0.7–1.0 × 2.3–4.8	0.6–0.8 × 2.8–4.8
Maximum NaCl concn tolerated (%)	16	19
pH range	6.0–10.0	5.0–10.0
Acid produced from:		
Glucose	+	–
Sucrose	+	–
Assimilation of:		
L-Arabinose	–	+
Galactose	+	–
Melibiose	+	–
Trehalose	+	–
Raffinose	+	–
DNA G + C content (mol%)	44.0	45.8

with respect to *Oceanobacillus picturae*, their closest phylogenetic relative, and the similarity values with respect to other taxa were less than 93.7 %. 16S rRNA gene sequence similarities of less than 95 % between two bacteria indicate that they belong to different genera (Ludwig *et al.*, 1998). Moreover, the phylogenetic analysis (Fig. 1) indicated that the three isolates formed a strong cluster, with 100 % bootstrap support, representing a distinct lineage. The topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms were similar to those of the tree constructed by neighbour-joining analysis (data not shown).

DNA–DNA hybridization was performed using the photo-biotin-labelling method of Ezaki *et al.* (1989), with a multi-well plate reader (CytoFluor; PerSeptive Biosystems). The values for DNA–DNA hybridization between strain 002-048^T and the other isolates were 99.9 % (RB589) and 35.4 % (002-051^T). Therefore, strains 002-048^T and RB589 should be incorporated into a single species, and strain 002-051^T should be considered as representing a separate species relative to strain 002-048^T (Stackebrandt *et al.*, 2002).

Genomic DNA was prepared according to the method of Marmur (1961), and the DNA G + C content was measured by HPLC according to the method described by Mesbah *et al.* (1989). The cellular fatty acids were identified with the Sherlock Microbial Identification System (MIDI). Bacterial strains were grown on marine agar 2216 (Difco) medium for 3 days at 25 °C. The analysis of the cell-wall peptidoglycan was carried out using the methods of Schleifer & Kandler (1972). Respiratory quinone analyses were performed

according to the method indicated by Collins & Jones (1981). The chemotaxonomic characteristics determined were also congruent with the phylogenetic data. The DNA G + C contents of 002-048^T, RB589 and 002-051^T ranged from 44 to 46 mol%. Similar values were found in other low-G + C, Gram-positive bacteria. The major fatty acids of the novel strains were anteiso-C_{15:0} (47.6–63.5 %) and anteiso-C_{17:0} (17.9–34.8 %) (see Supplementary Table S1 in IJSEM Online). The two type strains, 002-048^T and 002-051^T, can be differentiated from each other by the amounts of anteiso-C_{15:0} and anteiso-C_{17:0} present (Supplementary Table S1 in IJSEM Online). The cell-wall peptidoglycan of strains 002-048^T and 002-051^T was based on *meso*-diaminopimelic acid, which corresponds with that of the phylogenetically closest neighbours (with the exception of members of the genus *Halobacillus*, which contain D-ornithine). The major isoprenoid quinone was MK-7, as for the other phylogenetically related genera.

The three isolates showed very low levels of 16S rRNA gene sequence similarity with respect to other taxa and formed a distinct lineage. Moreover, the isolates could be differentiated from their phylogenetically related neighbours on the basis of phenotypic characteristics (Table 1), such as motility, acetoin production, oxidase activity and nitrate reduction. The DNA G + C contents of the isolates were in the range 44–46 mol%, which is 4 % lower than the values for members of the genera *Virgibacillus* (36–40 mol%), *Lentibacillus* (37–38 mol%) and *Gracilibacillus* (38–39 mol%). The diamino acid in the murein of the isolates is *meso*-diaminopimelic acid (as opposed to ornithine for members of the genus *Halobacillus*). The cells of the isolates

Fig. 1. Phylogenetic positions based on neighbour-joining of the 16S rRNA gene sequences of three isolates and other related taxa. Numbers at the nodes indicate the percentages of occurrence in 1000 bootstrapped trees; only those values greater than 70 % are shown. Bar, 0.01 substitution per 100 nt.

are non-motile, whereas most of the phylogenetically related genera (except *Thalassobacillus*) comprise members that are motile. The isolates reduce nitrates, but the members of the genus *Thalassobacillus* do not.

On the basis of the phylogenetic, physiological and chemotaxonomic data, therefore, strains 002-048^T, RB589 and 002-051^T belong to two species within a novel genus, for which the names *Terribacillus saccharophilus* gen. nov., sp. nov. (strains 002-048^T and RB589) and *Terribacillus halophilus* sp. nov. (strain 002-051^T) are proposed.

Description of *Terribacillus* gen. nov.

Terribacillus [Ter.ri.ba.cil'lus. L. n. *terra* earth; L. masc. n. *bacillus* a small staff; N.L. masc. n. *Terribacillus* earth (soil) bacillus (rod)].

Cells are non-motile, Gram-positive, aerobic rods. Ellipsoidal endospores are formed subterminally within swollen sporangia. Colonies are circular and convex. Catalase-positive. H₂S and indole are not produced. Nitrate is not reduced to nitrite. Gelatin is liquefied. Acetoin is produced. Urease and β -galactosidase are absent. The DNA G+C content is 44–46 mol%. The major cellular fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0}. MK-7 is the major component of the quinone system. The type species of the genus is *Terribacillus saccharophilus*.

Description of *Terribacillus saccharophilus* sp. nov.

Terribacillus saccharophilus (sac.cha.ro.phi'lus. Gr. n. *sak-khar* -aros sugar; Gr. adj. *philos* loving; N.L. masc. adj. *saccharophilus* sugar-loving).

The characteristics are the same as those given in the description of the genus, with the following additions. Cells are 0.7–1.0 × 2.3–4.8 µm in size. Colonies grown on trypticase soy agar containing 0.5 × Herbst's artificial seawater are circular, convex and pale yellow. Temperature range for growth is 5–45 °C; pH range for growth is 6.0–10.0. Growth occurs without NaCl and in the presence of 16 % (w/v) NaCl. Optimum NaCl concentration for growth is 1–5 % (w/v). Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are absent. Citrate utilization is negative. Utilizes glycerol, galactose, glucose, fructose, mannose, rhamnose, mannitol, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, lactose, sucrose, gentiobiose and D-tagatose but does not utilize erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β -D-xyloside, sorbose, dulcitol, inositol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, maltose, inulin, melezitose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Acid is produced from glucose, mannitol and sucrose, but not from inositol, sorbitol, rhamnose, melibiose, amygdalin or arabinose. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid.

The predominant menaquinone system is MK-7. The major cellular fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0}. The DNA G+C content of the type strain is 44.0 mol%.

The type strain, 002-048^T (=IAM 15309^T = KCTC 13936^T), was isolated from field soil from Tama City (Tokyo, Japan). The reference strain, RB589, was isolated from field soil in Saitama City (Japan).

Description of *Terribacillus halophilus* sp. nov.

Terribacillus halophilus (ha.lo.phi'lus. Gr. n. *hals* salt; Gr. adj. *philos* loving; N.L. masc. adj. *halophilus* salt-loving).

The characteristics are the same as those given in the description of the genus, with the following additions. Cells are 0.6–0.8 × 2.8–4.8 µm in size. Colonies grown on trypticase soy agar containing 0.5 × Herbst's artificial seawater are circular, convex and pale yellow. Temperature range for growth is 5–45 °C; pH range for growth is 5.0–10.0. Growth occurs without NaCl and in the presence of 19 % (w/v) NaCl. Optimum NaCl concentration for growth is 1–5 % (w/v). Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and citrate utilization. Utilizes glycerol, L-arabinose, glucose, fructose, mannose, rhamnose, mannitol, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, lactose, sucrose, gentiobiose and D-tagatose but does not utilize erythritol, D-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β -D-xyloside, galactose, sorbose, dulcitol, inositol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, maltose, melibiose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Acid is produced from mannitol, but not from glucose, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin or arabinose. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The major quinone system is MK-7. The major cellular fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0}. The DNA G+C content of the type strain is 45.8 mol%.

The type strain, 002-051^T (=IAM 15310^T = KCTC 13937^T), was isolated from field soil in Tama City (Tokyo, Japan).

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