Brevibacillus fulvus sp. nov., isolated from a compost pile

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Two strains, designated K2814T and K282, were isolated from a compost pile in Japan. These strains were Gram-stain-variable, aerobic, motile and endospore-forming rods. The strains produced a characteristic brown non-diffusible pigment. The 16S rRNA gene sequences of the strains were 100 % identical and had high similarity to that of Brevibacillus levickii LMG 22481T (97.3 %). Phylogenetic analyses based on 16S rRNA gene sequences revealed that these strains belong to the genus Brevibacillus. Strains K2814T and K282 contained meso-diaminopimelic acid in their cell walls. Strains K2814T and K282 contained MK-7 (96.0 and 97.2 %, respectively) and MK-8 (4.0 and 2.8 %, respectively) as the major and minor menaquinones, respectively. Their major cellular fatty acids were anteiso-C15 : 0, anteiso-C17 : 0, iso-C15 : 0 and iso-C17 : 0. The DNA G + C contents of strains K2814T and K282 were 48.8 and 49.8 mol%, respectively. Polar lipids of strain K2814T were composed of phosphatidyl-N-methylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, an unidentified phospholipid, three unidentified polar lipids, an unidentified aminophospholipid and an unidentified aminolipid. The level of DNA–DNA relatedness between strains K2814T and K282 was 99 or 100 %, and levels between strain K2814T and the type strains of seven related species of the genus Brevibacillus, including Brevibacillus levickii LMG 22481T, were below 59 %. From the chemotaxonomic and physiological data and the levels of DNA–DNA relatedness, these two strains should be classified as representing a novel species of the genus Brevibacillus, for which the name Brevibacillus fulvus sp. nov. (type strain K2814T = JCM 18162T = ATCC BAA-2417T = DSM 25523T) is proposed.

The genus Brevibacillus was originally proposed by Shida et al. (1996) based on the description of 10 species, with Brevibacillus brevis as the type species, and was separated from the genus Bacillus. Recently, eight novel species, Brevibacillus invocatus (Logan et al., 2002), Brevibacillus limnophilus (Goto et al., 2004), Brevibacillus levickii (Allan et al., 2005), Brevibacillus ginsengisoli (Baek et al., 2006), Brevibacillus panacihumi (Kim et al., 2009), Brevibacillus fluminis (Choi et al., 2010), Brevibacillus aydinogluensis (Inan et al., 2012) and Brevibacillus nitificans (Takebe et al., 2012), were added to this genus, which thus comprises, at the time of writing, 18 recognized species. Most Brevibacillus strains have been isolated from the natural environment such as soil (Logan & De Vos, 2009). Cells of members of this genus are Gram-stain-positive or Gram-stain-variable, motile rods (Logan & De Vos, 2009). Ellipsoidal endospores are formed and swell the sporangia. Most species of this genus grow aerobically on routine media such as nutrient agar and produce flat, smooth, yellowish-grey colonies. Only Brevibacillus thermoruber produces a red pigment (Manachini et al., 1985). Chemotaxonomic characteristics of the genus include the following: the major menaquinone is MK-7; the major cellular fatty acids are anteiso-C15 : 0 and iso-C15 : 0; and the DNA G + C content ranges from 40.2 to 57.4 mol% (Logan & De Vos, 2009). Generally, determination of phenotypic characters (such as acid production from carbohydrates) of
members of the genus *Brevibacillus* is not easy (Logan & De Vos, 2009). Therefore, most species of the genus are difficult to distinguish from each other based on routine phenotypic tests.

Several bacteria were isolated and characterized from a newly developed hyperthermal composting machine in Japan (Iida et al., 2005; Hatayama et al., 2005a, b, 2006). In this process, organic wastes are subjected to rapid composting at high temperature (over 100 °C) using a hyperthermal composting machine followed by a further piling step in air. In this latter step, the temperature of the compost piles was kept at approximately 60 °C for several weeks by the heat generated metabolically by the microorganisms growing in the compost piles. After easily metabolized organic compounds in the piles had been thoroughly utilized by the micro-organisms, the temperature of the piles decreased to ambient temperature, indicating maturation of the compost piles. In a previous study, we succeeded in isolating a novel thermophilic bacterium from this composting process by cultivating on a microbial corpses (MC) agar plate (Hatayama et al., 2006). We further isolated micro-organisms from this composting process, using mature composts as an isolation source, with MC agar plates, and strains K2814T and K282 were obtained by cultivating aerobically at 28 °C. From similarity searches based on their 16S rRNA gene sequences, strains K2814T and K282 showed a close relationship to members of the genus *Brevibacillus*. Therefore, the taxonomic positions of strains K2814T and K282 were investigated using polyphasic approaches, including phylogenetic analyses based on 16S rRNA gene sequences, and chemotaxonomic, physiological and DNA–DNA hybridization analyses. This study was performed based on the minimal standards for describing new taxa of aerobic, endospore-forming bacteria (Logan et al., 2009).

Although strains K2814T and K282 were isolated with MC agar plates, this medium was not suitable for maintenance of these strains and for further physiological experiments because it contained other microbial corpses. Therefore, growth of these strains was tested with several media. On solid media, both strains grew well on nutrient agar (Difco), R2A (Reasoner & Geldreich, 1985), GYE (Manachini et al., 1985), TRE/1 (Manachini et al., 1985) and MTY [containing 1.0 g malt extract (Difco), 5.0 g Bacto tryptone (Difco), 1.0 g yeast extract (Difco), 10 ml Hutner’s vitamin-free mineral base (Stanier et al., 1966), 990 ml distilled water and 15 g agar, pH 6.0] agars, but no growth was observed on Luria–Bertani or CYC agar (Lacey & Cross, 1989). In liquid culture, both strains grew well in GYE broth, weakly in MTY and TRE/1 broths, but not in nutrient broth. Therefore, we used GYE and MTY media for further cultivation and maintenance of the strains.

The temperature range for growth was tested on GYE and MTY agar plates at 20, 25, 30, 37, 40, 45, 50, 55 and 60 °C. The pH range for growth was determined in GYE broth that was adjusted to pH 3.0–11.0 (in increments of 1 pH unit) using 10 mM citrate buffer (pH 3.0–4.0), 10 mM MES (pH 5.0–6.0), 10 mM HEPES (pH 7.0–8.0), 10 mM CHES (pH 9.0–10.0) and 10 mM CAPS (pH 11.0).

Growth of the strains was observed at 20–55 °C (with an optimum at 40–45 °C) on GYE and MTY agar plates. These strains grew over a pH range of 5.0–9.0 (with an optimum pH range of 5.0–6.0) in GYE broth. Strains K2814T and K282 produced a brown non-diffusible pigment. Strain K2814T formed colonies that were circular, glossy, flat, had a brown centre and light yellow ochre edge, and were 1–4 mm in diameter on MTY agar plates after 3 days at 45 °C, and strain K282 formed colonies that were circular, glossy, flat, had a light brown centre and beige edge, and were 1–4 mm in diameter. Both strains formed colonies that were circular, glossy, flat, cream yellow and 1–4 mm in diameter on GYE agar plates after 3 days at 45 °C.

Cell morphology and motility were observed using a DMLB phase-contrast microscope (Leica) and an S-4200 scanning electron microscope (Hitachi), using cells grown in GYE and MTY broths. Flagella staining was conducted by the method of Toda (1928). Gram reaction of the cells was tested by the non-staining (KOH) method (Buck, 1982) and by using a Live Baclight Bacterial Gram Stain kit (Invitrogen). Cells of strains K2814T and K282 were Gram-stain-variable rods [Gram-stain-negative by the non-staining (KOH) method], 0.5–0.8 μm wide and 2.0–5.0 μm long (Fig. S1A available in IJSEM Online) and motile, and occurred singly or in pairs. The two strains formed ellipsoidal endospores (1.0–1.2 μm wide and 2.0–2.2 μm long) located terminally within a swollen sporangium after 2 days of cultivation at 37 °C (Fig. S1B). The rate of endospore formation was higher in cells grown on MTY agar plates (pH 6.0) than on GYE agar plates (pH 6.0) in both strains. Strain K2814T formed peritrichous flagella after 1 day of cultivation at 37 °C in GYE medium (Fig. S1C).

To determine the phylogenetic positions of strains K2814T and K282, their 16S rRNA gene sequences were analysed. DNA extraction, PCR amplification of the 16S rRNA gene and sequencing were performed as previously described (Hatayama et al., 2005a). Similarity-based searches were performed using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). The 16S rRNA gene sequences (approx. 1400 bp) of strains K2814T and K282 were aligned with those of the type strains of species of the genus *Brevibacillus* using the CLUSTAL X software package (Thompson et al., 1997). Evolutionary distances were calculated using the Kimura two-parameter method (Kimura, 1980), and were then used by the MEGA5 program (Tamura et al., 2011) to reconstruct a phylogenetic tree through the neighbour-joining method (Saitou & Nei, 1987) with bootstrap values (Felsenstein, 1985) based on 1000 replications. Another phylogenetic tree was reconstructed with the maximum-parsimony method (Nei & Kumar, 2000) in the MEGA5 program, with bootstrap values based on 1000 replications.

The 16S rRNA gene sequences of strains K2814T and K282 were 100 % identical, and showed highest similarity to that
of *Brevibacillus levickii* LMG 22481<sup>T</sup> (97.3 %) and high similarities to those of *Brevibacillus centrosorus* DSM 8445<sup>T</sup> (96.7 %), *Brevibacillus panacihumi* DCY35<sup>T</sup> (96.6 %), *Brevibacillus borstelensis* NRRL NRS-818<sup>T</sup> (96.6 %), *Brevibacillus ginsengisoli* Gsoil 3088<sup>T</sup> (96.6 %) and *Brevibacillus thermoruber* DSM 7064<sup>T</sup> (96.6 %). Phylogenetic analyses based on 16S rRNA gene sequences, performed by the neighbour-joining and maximum-parsimony methods, indicated that these strains belong to the genus *Brevibacillus*, and their closest phylogenetic neighbours were *Brevibacillus levickii*, *Brevibacillus thermoruber* and *Brevibacillus borstelensis* (Fig. 1).

Physiological and chemotaxonomic characteristics of strains K2814<sup>T</sup> and K282 were determined and compared with those of *Brevibacillus levickii* LMG 22481<sup>T</sup>, *Brevibacillus thermoruber* DSM 7064<sup>T</sup>, *Brevibacillus borstelensis* DSM 6347<sup>T</sup>, *Brevibacillus ginsengisoli* DSM 21346<sup>T</sup>, *Brevibacillus panacihumi* DSM 22869<sup>T</sup>, and the type strain of the type species of this genus, *Brevibacillus brevis* DSM 30<sup>T</sup>, as reference strains. Anaerobic growth, the Voges–Proskauer reaction and citrate utilization were tested according to traditional methods (Claus & Berkeley, 1986). Hydrolysis of aesculin, casein, DNA, starch, L-tyrosine and Tween 80, and H<sub>2</sub>S production were assayed at 40 °C according to Barrow & Feltham (1993), using MTY agar medium containing (per litre) 1 g aesculin and 0.5 g ferric citrate, 1 % (w/v) casein, 0.2 g DNA from salmon sperm (Wako Pure Chemicals), 1 % (w/v) soluble starch, 5 g L-tyrosine, 0.5 % (v/v) Tween 80 (MP Biomedicals), and 0.3 g Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 5H<sub>2</sub>O and 0.2 g FeCl<sub>2</sub>·4H<sub>2</sub>O, respectively. Some physiological characteristics were determined with the API 20 NE system (bioMérieux). Acid production from carbon sources was tested with the API 50 CH system (bioMérieux) and with medium for acid production from carbohydrates [containing (per litre) 1 g (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.2 g KCl, 0.2 g MgSO<sub>4</sub>, 0.2 g yeast extract, 15 g agar, 15 ml 0.04 % (w/v) bromocresol purple solution and 0.5 % (w/v) carbohydrate, pH 7.0] (Claus & Berkeley, 1986). The results of API 20 NE and API 50 CH tests were evaluated after 48 h incubation at 40 °C. Carbon source utilization was evaluated with Biolog GP2 MicroPlate assays (Biolog) after 24 h of incubation at 40 °C. NaCl tolerance was assayed on GYE agar plates (pH 6.0) containing 1–10 % (w/v) NaCl. Catalase activity was tested with hydrogen peroxide solution at a concentration of 3 % (w/w) in H<sub>2</sub>O (Sigma). Oxidase activity was tested with oxidase reagent (bioMérieux). Strains K2814<sup>T</sup> and K282 did not produce acid from any

**Fig. 1.** Phylogenetic tree based on the 16S rRNA gene sequences of strains K2814<sup>T</sup> and K282 and type strains of related species. The tree was drawn using the neighbour-joining method. Bootstrap values are calculated from 1000 repeats and those greater than 50 % are shown at branch points. Numbers in parentheses are accession numbers of the GenBank/EMBL/DDBJ database. Filled circles indicate that the corresponding nodes were also recovered in the tree drawn using the maximum-parsimony method (not shown). Bar, 0.01 substitutions per nucleotide position.

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of the carbon sources in the API 50 CH system. By contrast, strains K2814ᵀ and K282 produced acid from several carbon sources after aerobic cultivation on the medium for acid production from carbohydrates for 2 weeks at 40 °C (Table 1). Strains K2814ᵀ and K282 were positive for utilization of l-malic acid in the Biolog GP2 MicroPlate assay when 0.4 % NaCl solution was used for their suspensions. However, they did not react to any of the substrates in the Biolog GP2 MicroPlate assay when GN/GP inoculation fluid (Biolog) was used for suspensions. As the cell suspensions became clear within 2 h, the cells seemed to be lysed in the GN/GP inoculation fluid. Positive oxidase reactions were observed in strains K2814ᵀ and K282 and all reference strains when cells were grown on MTY agar, but negative reactions were observed in strains K2814ᵀ and K282 and some of the reference strains when cells were grown on GYE agar. The physiological characteristics of strains K2814ᵀ and K282 are summarized in Table 1 and in the species description.

Profiles of menaquinones and cellular fatty acids were determined by TechnoSuruga Laboratory (Shizuoka, Japan). Menaquinones were extracted from cells grown aerobically in GYE broth (pH 6.0) for 1 day at 40 °C according to Nishijima et al. (1997), and analysed by HPLC. Cellular fatty acids were analysed from cells grown aerobically on GYE agar plates (pH 6.0) for 1 day at 40 °C, corresponding to the mid- to late-exponential growth phase. Analysis of cellular fatty acids was performed using an HP 6890 series GC system (Agilent Technologies) equipped with an HP Ultra 2 column (25.0 m x 0.2 mm x 0.33 μm) (Agilent Technologies) and Sherlock Microbial Identification System (Version 4.5) (Minnikin et al., 1984). Polar lipids were extracted from cells grown aerobically in GYE broth (pH 6.0) for 1 day at 40 °C, and examined by two-dimensional TLC (Minnikin et al., 1984). Polar lipids were detected by staining with molybdatophosphoric acid (Merck Millipore) for total lipids, molybdenum blue (Sigma) for phospholipids, α-naphthol sulphuric acid for glycolipids or ninhydrin for aminolipids. The composition of amino acids in the cell wall and the DNA G+C content were determined as previously described (Hatayama et al., 2005b). Strains K2814ᵀ and K282 contained MK-7 (96.0 and 97.2 %, respectively) as the major menaquinone and MK-8 (4.0 and 2.8 %, respectively) as the minor menaquinone. Phylogenetically related Brevibacillus levickii DSM 22481ᵀ and Brevibacillus thermoruber DSM 7064ᵀ also contained MK-7 (98.8 and 99.5 %, respectively) as the major menaquinone and MK-8 (1.2 and 0.5 %, respectively) as the minor menaquinone. The major cellular fatty acids of strains K2814ᵀ and K282 were anteiso-C₁₅:₀ (43.7 and 37.5 % of the total, respectively), anteiso-C₁₇:₀ (19.8 and 20.6 %), iso-C₁₅:₀ (16.0 and 17.1 %) and iso-C₁₇:₀ (11.7 and 17.3 %) (percentages of other cellular fatty acids are shown in Table S1). Polar lipids of strain K2814ᵀ were phosphatidyl-N-methylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidyethanolamine, an unidentified phospholipid, three unidentified polar lipids, an unidentified aminophospholipid and an unidentified aminolipid (Fig. S2). Strains K2814ᵀ and K282 and the seven reference strains contained meso-diaminopimelic acid in their cell walls. The DNA G+C contents of strains K2814ᵀ and K282 were 48.8 and 49.8 mol%, respectively. These values were close to that of Brevibacillus levickii (48.3–50.3 mol%; Allan et al., 2005) and within the range of values reported for the genus Brevibacillus (Logan & De Vos, 2009).

DNA–DNA hybridization between strains K2814ᵀ and K282 and the seven reference strains was conducted according to the method of Ezaki et al. (1989). The level of DNA–DNA relatedness between strains K2814ᵀ and K282 was 99 or 100 %, indicating that these two strains belong to one species. By contrast, levels of relatedness between strain K2814ᵀ and the other reference strains were less than 59 % (<32 % for Brevibacillus levickii LMG 22481ᵀ; <41 % for Brevibacillus thermoruber DSM 7064ᵀ; <39 % for Brevibacillus borstelensis DSM 6347ᵀ; <41 % for Brevibacillus centrosorus DSM 8445ᵀ; <59 % for Brevibacillus ginsengisoli DSM 21346ᵀ; <42 % for Brevibacillus panachium DSM 22869ᵀ; <43 % for Brevibacillus brevis DSM 30ᵀ) (Table S2). These values were below the 70 % cut-off point recommended for the assignment of bacterial strains to the same genomic species (Wayne et al., 1987).

Several chemotaxonomic characteristics, such as major menaquinone, major cellular fatty acids, cell-wall composition and DNA G+C content, of strains K2814ᵀ and K282 agreed with those of Brevibacillus brevis and other members of the genus Brevibacillus. The phylogenetic positions of strains K2814ᵀ and K282 indicated that these strains are members of the genus Brevibacillus. Several physiological characteristics, the profile of cellular fatty acids, levels of 16S rRNA gene sequence similarity and the result of DNA–DNA hybridization indicated that strains K2814ᵀ and K282 should be classified in a single species separate from recognized species. In particular, strains K2814ᵀ and K282 were easily distinguished from other species of the genus Brevibacillus based on production of a brown non-diffusible pigment. Therefore, we propose that strains K2814ᵀ and K282 should be assigned to a novel species of the genus Brevibacillus, for which the name Brevibacillus fulvus sp. nov. is proposed.

**Description of Brevibacillus fulvus sp. nov.**

*Brevibacillus fulvus* (ful′vus. L. masc. adj, fulvus tawny, and by extension brown, reflecting the colour of colonies). Cells are aerobic, Gram-stain-variable rods (0.5–0.8 x 2.0–5.0 μm), occurring singly or in pairs, and are motile in GYE and MTY broths by means of peritrichous flagella. They bear ellipsoidal endospores (1.0–1.2 x 2.0–2.2 μm) located terminally within a swollen sporangium. Colonies are circular, glossy and flat, have a brown (or light brown) centre and light yellow ochre (or beige) edge, and are 1–4 mm in diameter on MTY agar plates, and are circular, glossy, flat, cream yellow and 1–4 mm in diameter on GYE agar plates after 3 days at 45 °C. Growth occurs at pH 5.0–9.0 (with an optimum pH range of 5.0–6.0), at 20–55 °C (with an optimum at 40–45 °C) and in the presence...
Table 1. Phenotypic characteristics that differentiate strains K2814\textsuperscript{T} and K282 from seven type strains of phylogenetically related species of the genus Brevibacillus

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<td>Pigmentation</td>
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<td>NaCl tolerance (%)</td>
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<td>0–2</td>
<td>0–2</td>
<td>0–1†</td>
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<td>Catalase activity</td>
<td>+</td>
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<td>–§</td>
<td>–</td>
<td>+</td>
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<td>Nitrate reduction</td>
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<td>Aesculin</td>
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<td>Casein</td>
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<td>H\textsubscript{2}S production</td>
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<td>Acid production from:§</td>
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*No growth in the presence of 2 % NaCl was reported by Shida et al. (1995).
†Growth in the presence of 2 % NaCl was reported by Baek et al. (2006).
§No growth in the presence of more than 2 % NaCl and positive for acid production from mannitol and L-rhamnose were described by Kim et al. (2009).
§Weakly positive for catalase, hydrolysis of starch, and variable for nitrate reduction were reported by Allan et al. (2005).
||Brown pigment was produced.
¶Data were determined using the medium for acid production from carbohydrates.
#Weakly positive for acid production from D-fructose and mannitol were described by Logan & De Vos (2009).
**Weakly positive for acid production from D-mannitol was described by Logan & De Vos (2009).

of 0–1 % NaCl. Produces a brown non-diffusible pigment. Positive for hydrolysis of aesculine, DNA and casein, but negative for hydrolysis of starch, Tween 80 and L-tyrosine in MTY medium. Produces H\textsubscript{2}S in MTY medium. Positive for catalase and oxidase (grown on MTY agar plates). Positive for citrate utilization. Negative for anaerobic growth and the Voges–Proskauer reaction. Hydrolysis of gelatin is positive, but urease, arginine dihydrolase, β-galactosidase, β-glucosidase, nitrate reduction, indole production and fermentation are negative in tests using API 20NE. In medium for acid production from carbohydrates, acid production is positive from L-rhamnose, variable from (the type strain is negative) D-fructose, D-galactose, D-glucose, D-mannitol, D-ribose, sucrose and D-xylose, and negative from D-arabinose, L-arabinose, glycerol, maltose, D-mannose, melibiose, raffinose, D-sorbitol, L-sorbosé and trehalase. Negative for all reactions in the acid production test of API 50 CH. Positive for utilization of L-malic acid in the Biolog GP2 MicroPlate assay (0.4 % NaCl instead of GN/GP inoculation fluid as the solution for cell suspension). The major menaquinone is MK-7 and the minor menaquinone is MK-8. The major cellular fatty acids are anteiso-C\textsubscript{15}:0, anteiso-C\textsubscript{17}:0, iso-C\textsubscript{15}:0 and iso-C\textsubscript{17}:0. The cell wall contains meso-diaminopimelic acid. Polar lipids consist of phosphatidyl-N-methylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, an unidentified phospholipid, three unidentified polar lipids, an unidentified aminophospholipid and an unidentified aminolipid. The DNA G+C content is 48.8–49.8 mol%. The type strain, K2814\textsuperscript{T} (=JCM 18162\textsuperscript{T} =ATCC BAA-2417\textsuperscript{T} =DSM 25523\textsuperscript{T}), was isolated from a compost pile undergoing a hyperthermal composting process in Okinawa Prefecture, Japan. The DNA G+C content of the type strain is 48.8 mol%. K282 is an additional strain of the species.

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References


