NOTE

Paenibacillus koleovorans* sp. nov., able to grow on the sheath of *Sphaerotilus natans

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Two bacterial strains that are able to grow specifically on the sheath of a sheathed filamentous bacterium, *Sphaerotilus natans*, were isolated from soil samples. The sheath-degrading organisms, designated strains TB^T and TK, are facultatively anaerobic and form endospores. The Gram reaction was negative at all stages of cultivation. The optimum growth temperature and pH were 30 °C and pH 7. The DNA G+C content was 54.0–55.8 mol%. MK-7 was the predominant menaquinone and anteiso-C_{15:0} was the major fatty acid. Phylogenetic analysis based on the 16S rDNA sequences revealed that the isolates were closely related to *Paenibacillus chondroitinus*, *Paenibacillus alginolyticus*, *Paenibacillus koreensis*, *Paenibacillus validus*, *Paenibacillus larvae* subsp. *larvae* and *P. larvae* subsp. *pulvificiens*. The sequences were found to contain consensus sequences characteristic of all *Paenibacillus* species. The isolates were able to lyse and utilize the purified sheath of *S. natans* as the sole carbon and energy source. Acid was not produced from common carbon sources, allowing easy distinction from other members of *Paenibacillus*. It is concluded that the two strains represent a novel *Paenibacillus* species, for which the name *Paenibacillus koleovorans* sp. nov. is proposed. The type strain is strain TB^T (= JCM 11186^T = IAM 14926^T = KCTC 13912^T).

Keywords: *Paenibacillus koleovorans* sp. nov., degradation of bacterial sheath, *Sphaerotilus natans*

On the basis of 16S rRNA gene sequence analysis, the genus *Bacillus*, in which all rod-shaped, aerobic, endospore-forming bacteria were previously classified, has been separated into six major groups: *Alicyclobacillus* (Wisotzkey *et al.*, 1992), *Aneurinibacillus* (Shida *et al.*, 1996), *Brevibacillus* (Shida *et al.*, 1996), *Halobacillus* (Spring *et al.*, 1996), *Paenibacillus* (Ash *et al.*, 1993) and *Bacillus*. Members of the genus *Paenibacillus* are distinguishable from the other genera described above by the use of a PCR primer specific for the 16S rDNA of the genus *Paenibacillus* (Shida *et al.*, 1997). One distinctive characteristic of the genus *Paenibacillus* is the ability to degrade a variety of macromolecules including polysaccharides, DNA and

proteins (Ash *et al.*, 1993). Starch (Van der Maarel *et al.*, 2000), alginate (Nakamura, 1987), chondroitin (Nakamura, 1987), chitin (Chung *et al.*, 2000; Nielsen & Sørensen, 1997), curdlan (Kanzawa *et al.*, 1995) and other polysaccharides (Priest *et al.*, 1988) can be decomposed by *Paenibacillus* strains.

The typical filamentous, aquatic bacterium *Sphaerotilus natans* constructs a sheathed structure, a type of natural hollow fibre, which surrounds the cells. Filaments of *S. natans* are often found in activated sludge that suffers from poor sludge settlement, an operational problem commonly referred to as bulking; hence, *S. natans* has been considered to be detrimental for wastewater treatment. The sheath of *S. natans* is a complex of protein and a heteropolysaccharide that is made up of glucose and galactosamine (Takeda *et al.*, 1998a). Recently, we isolated two bacterial strains (TB^T and TK) capable of growth on the sheath, and an extracellular enzyme that can degrade the

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The GenBank/EMBL/DBJ accession numbers for the 16S rDNA sequences of strains TB^T and TK are respectively AB041720 and AB041721.

Fig. 1. Ultrastructure of strain TB^T. Cells were grown at 30 °C for 7 days on 1% (w/v) tryptone agar. Cells for thin-section electron microscopy were fixed with 5% (w/v) glutaraldehyde for 6 h and then post-fixed in 2% (w/v) osmium tetroxide at 4 °C for 3 h. The fixed cells were stained with uranyl acetate for 1 h at room temperature, dehydrated and embedded in Spurr low-viscosity resin. Thin sections of the cells were made with an ultramicrotome (Reichert ULTRACUTN) and were examined by a transmission electron microscope (Hitachi H7000). Spores with smooth (a) or spiked (b) surfaces were observed in swelled sporangia. Bars, 0.5 µm.

polysaccharide moiety of the sheath was purified and characterized (Takeda *et al.*, 2000). The isolates were tentatively identified as members of the genus *Paenibacillus*, but their precise taxonomic position was not determined. In this paper, the taxonomic characterization of the sheath-degrading bacteria is further elaborated. Based on the new data together with the previous findings, we propose that strains TB^T and TK be classified in the genus *Paenibacillus* as *Paenibacillus koleovorans* sp. nov.

The isolation of strains TB^T (= JCM 11186^T = IAM 14926^T = KCTC 13912^T) and TK (= JCM 11185 = IAM 14927) was described in our earlier paper (Takeda *et al.*, 2000). Both strains were cultured at 30 °C in a medium (sheath medium) that contained (l⁻¹): 0.1 g sheath of *S. natans*, 2 g Casamino acids, 0.5 g KCl, 1 g K₂HPO₄, 0.5 g MgSO₄ · 7H₂O and 0.01 g FeSO₄ · 7H₂O. Preparation of the sheath was reported previously (Takeda *et al.*, 2000). Cell growth in liquid medium was judged by epifluorescence microscopy according to the method described previously (Takeda *et al.*, 2000). Solid cultivation was carried out using 1% (w/v) tryptone agar at 30 °C. Gram staining of

cells grown in the sheath medium was performed using a commercially available definition kit (Favour-G set-S 'Nissui'; Nissui Pharmaceutical). Motility of individual cells and morphological properties (18- to 30-h-old cells in the sheath medium) were determined by light microscope observation. Cells of strains TB^T and TK grown in the sheath medium stained Gram-negative even at an early stage of cultivation (18–24 h). Cells were rod-shaped (0.4–0.7 × 1–3.2 µm). Ellipsoidal endospores were produced in swollen sporangia (Fig. 1). The surfaces of spores were smooth (Fig. 1a) or spiked (Fig. 1b). Spores with spiked surfaces are also observed in *Paenibacillus borealis* (Elo *et al.*, 2001). Motile cells were observed occasionally during the early stages (18–24 h) of growth in the sheath medium.

Anaerobic growth was tested using a tryptone agar plate or the sheath medium. An anaerobic atmosphere was created by using a BBL GasPack pouch (Becton Dickinson). Cells grew anaerobically in the sheath medium but anaerobic growth on tryptone agar plates was not observed. The optimum growth temperature and pH and growth in the presence of 5% (w/v) NaCl were determined using the sheath medium as basal medium. Growth was inhibited when 5% (w/v) NaCl was added to the sheath medium. The optimum growth temperature and pH for both strains were 30 °C and pH 7. The strains did not grow at 10 or 45 °C or at pH 5 or 10. Catalase activity was assayed with 3% hydrogen peroxide solution. Oxidase activity was assayed by applying the cells to a moistened commercial test paper strip (Nissui Pharmaceutical). Indole production, the Voges–Proskauer reaction, nitrate reduction, citrate utilization, glucose utilization and activities of urease, arginine dehydrogenase, ornithine decarboxylase, lysine decarboxylase and gelatin hydrolysis were tested with commercial assay plates NF-18, HN-20 and EB-20 (Nissui Pharmaceutical). Prior to these tests, cells were cultured on tryptone agar plates for 5–7 days. Neither strain exhibited catalase, urease or oxidase activities. Gelatin was not hydrolysed. Arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase were negative. Nitrate was not reduced. Indole was not produced. The Voges–Proskauer reaction was negative. It was found that citrate and glucose could not be utilized.

Acid production from 49 carbon sources was tested at 30 °C with API 50 CH in combination with API 50 CHB/E medium (bioMérieux). Cells subjected to the plate assay were prepared on tryptone agar plates. Both strains exhibited a positive reaction on aesculin, a weak positive reaction on 5-ketogluconate, a variable reaction on cellobiose and negative reactions on the other carbon sources covered by the assay plate (glycerol, erythritol, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-xyloside, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, maltose,

Fig. 2. Phylogenetic positions of strains TB^T and TK among the neighbouring species of the genus *Paenibacillus* based on 16S rRNA gene sequences. Bar, 1 nucleotide substitution per 100 nucleotides. Numbers at the nodes of the tree indicate bootstrap percentages for each node of 1000 bootstrap resamplings. Database accession numbers of the reference sequences are given in parentheses.

lactose, melibiose, sucrose, trehalose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, β -gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate and 5-ketogluconate). This very limited carbon source utilization is the most distinctive feature of the two *Paenibacillus* strains, because all known *Paenibacillus* strains are able to utilize several compounds, as listed by Shida *et al.* (1997). Degradation of starch was tested on agar plates supplemented with 0.2% soluble starch and 0.2% tryptone. Degradation of starch by strains TB^T and TK were not detected by the addition of iodine solution even after incubation for 10 days at 30 °C.

Colony-forming capability was tested at 30 °C on nutrient agar (Difco) and on agar plates supplemented with 1% (w/v) beef extract, tryptone, yeast extract, peptone or Casamino acids. Utilization of these compounds was also examined in liquid medium (10 g organic compound, 0.2 g MgSO₄ · 7H₂O, 0.5 g CaCO₃ and 0.1 g K₂HPO₄ l⁻¹). Utilization of several complex organic compounds was examined. Strains TB^T and TK grew weakly in liquid media containing tryptone, yeast extract or peptone. Colonies were formed on agar plates of these compounds. Both strains were isolated on 0.02% beef extract agar plates as small and thin colonies (Takeda *et al.*, 2000), but neither colony formation nor cell proliferation occurred on 1% beef extract. Casamino acids did not support cell growth either in liquid or solid media and cell growth did not occur when the sheath was omitted from the sheath medium, suggesting that Casamino acids are utilized only as a source of nitrogen. Growth was not observed in nutrient broth or on nutrient agar. Two different colony morphotypes were observed on 1% tryptone agar plates. One was flat and translucent and the other was convex and opaque. The convex and opaque colonies were dominant on 1% tryptone, whereas most colonies formed on 0.02% tryptone were flat and translucent. Nearly equal numbers of spores was observed microscopically in the two colony types, indicating that the difference was not related to the extent of sporulation.

For chemotaxonomic characterization, strains TB^T and TK were cultured on 1% tryptone agar plates. Total DNA was extracted according to the procedure of Saito & Miura (1963) and was digested with P1 nuclease (Yamasa Shoyu). The G+C content of the digested DNA was measured by HPLC (Kamagata &

Mikami, 1991). The reference standard was an equimolar mixture of deoxyribonucleotides (Yamasa Shoyu). Quinones were extracted from lyophilized cells (grown on tryptone agar plates) with chloroform/methanol (2:1). The extract was passed through a Sep-Pak Plus column (Waters) and analysed by reverse-phase HPLC for identification and quantification (Tamaoka *et al.*, 1983). Cellular fatty acids were converted to methyl esters by treatment with anhydrous methanolic HCl. The methyl esters were analysed using a Hitachi M7200A GC/3DQMS system equipped with a DB-5ms capillary column (30 m by 0.25 mm; J&W Scientific) coated with (5%-phenyl)-methylpolysiloxane (thickness 250 nm). Helium was used as the carrier gas at a flow rate of 1.5 ml min⁻¹. The column temperature was held at 100 °C for 1 min and then increased to 280 °C at a rate of 18 °C min⁻¹ and held at 280 °C for 12 min. The predominant fatty acid methyl esters in whole-cell methanolysates of strain TB^T were anteiso-C_{15:0} (37.4%), C_{16:0} (22.8%) and C_{15:0} (13.3%). The major isoprenoid quinone of strain TB^T was MK-7. These results are in accordance with the previous report (Shida *et al.*, 1997) that *Paenibacillus* strains have anteiso-C_{15:0} as the major cellular fatty acid and MK-7 as the respiratory quinone. The G+C contents of the DNA of strains TB^T and TK were 54.0 and 55.8 mol%. Most *Paenibacillus* strains exhibit a G+C content of 39–54 mol% (Shida *et al.*, 1997), although strains of *Paenibacillus dendritiformis* have a rather higher G+C content (55 mol%; Tcherpakov *et al.*, 1999).

Nearly the full length of the 16S rDNA sequence was determined previously (Takeda *et al.*, 2000). It was aligned with reference sequences by CLUSTAL W (Thompson *et al.*, 1994). Alignment gaps and ambiguous bases were not taken into consideration and 1336 bases were compared. A phylogenetic tree was constructed from the evolutionary distance matrix calculated by the neighbour-joining method (Saitou & Nei, 1987). The neighbour-joining analysis was performed with the TREECON program (Van de Peer & De Wachter, 1994, 1997). Bootstrap resampling analysis (Felsenstein, 1985) for 1000 replicates was performed to estimate the topology confidence. Using DNA prepared from strain TB^T or TK as a template, PCR amplification of part of the 16S DNA was done with a combination of the *Paenibacillus*-specific forward primer PAEN513F (Shida *et al.*, 1997) and a universal reverse primer, 1392R (Hallbeck *et al.*, 1993; Takeda

Table 1. Distinctive phenotypic characteristics of strains TB^T and TK and phylogenetically related members of the genus *Paenibacillus*

Taxa are indicated as: 1, strains TB^T and TK; 2, *P. alginolyticus* (data from Nakamura, 1987); 3, *P. chondroitinus* (Nakamura, 1987); 4, *P. koreensis* (Chung *et al.*, 2000); 5, *P. larvae* subsp. *larvae* (Heyndrickx *et al.*, 1996); 6, *P. larvae* subsp. *pulvifaciens* (Heyndrickx *et al.*, 1996); 7, *P. validus* (Heyndrickx *et al.*, 1995). NT, Not tested; v, variable.

Characteristic	1	2	3	4	5	6	7
Anaerobic growth	+	—	—	—	+	+	—
Catalase	—	+	+	+	—	—	+
Oxidase	—	—	—	+	NT	NT	—
Urease	—	+	+	—	—	—	—
Nitrate reduction	—	—	—	+	v	v	v
Indole production	—	—	—	—	+	+	—
Citrate utilization	—	+	—	—	—	—	—
Starch hydrolysis	—	+	+	+	—	—	NT
Acid production from:							
D-Glucose	—	+	+	+	+	+	+
L-Arabinose	—	+	+	+	—	—	+
Mannitol	—	+	+	+	—	+	+
D-Xylose	—	+	+	—	—	—	+
Glycerol	—	NT	NT	NT	+	+	+
Raffinose	—	NT	NT	+	—	—	v
Optimum growth temp. (°C)	30	28–30	28–30	38–40	35–37	28–30	28–35
Growth at 50 °C	—	—	—	+	—	—	+
DNA G + C content (mol %)	54.0–55.8	47–49	47–48	54	42–43	42–43	50–52

et al., 1998b), designed to bind to positions 1406–1392 (*Escherichia coli* numbering; Brosius *et al.*, 1981). The purity and size of the amplified product was judged by agarose electrophoresis. Phylogenetic analysis based on the 16S rDNA sequences revealed that strains TB^T and TK could be placed into the cluster of *Paenibacillus* species (Fig. 2). The 16S rDNA sequence similarity between the two strains was 99.5%, indicating that they should be classified into the same species (Goebel & Stackebrandt, 1994). Both strains were located in a monophyletic clade that also included *Paenibacillus alginolyticus*, *Paenibacillus chondroitinus*, *Paenibacillus koreensis*, *Paenibacillus larvae* subsp. *larvae*, *P. larvae* subsp. *pulvifaciens* and *Paenibacillus validus*. A 0.9 kb fragment was amplified from DNA of strains TB^T and TK by PCR using primers PAEN515F and 1392R. In addition, 16S rDNAs of strains TB^T and TK were found to contain the *Paenibacillus*-specific 22-base sequence 5'-TCGATACCCTTGGTGCCGAAGT-3' (Ash *et al.*, 1993), in the variable region V5. The closest relatives of strain TB^T other than strain TK were *P. larvae* subsp. *larvae* (93.4% similarity) and *P. larvae* subsp. *pulvifaciens* (93.7% similarity). However, these two relatives produce indole and exhibit rather low G + C contents of 42–43 mol % (Heyndrickx *et al.*, 1996), which differ from those of strains TB^T and TK (Table 1). Strains TB^T and TK are also distinguishable from the other species and subspecies of the genus *Paenibacillus* on the basis of the phenotypic characteristics summarized in Table 1. The genetic and phenotypic features suggest

that strains TB^T and TK represent a novel species in the genus *Paenibacillus*. The name *Paenibacillus koleovorans* sp. nov. is therefore proposed for these strains.

Description of *Paenibacillus koleovorans* sp. nov.

Paenibacillus koleovorans (ko.le.o.vo'rans. Gr. n. *koleon* sheath; L. v. *voro* to devour, N.L. part. adj. *koleovorans* sheath-devouring).

Cells are rod-shaped (0.4–0.7 × 1–3.2 µm). Motile cells are observed in the early stages of cultivation grown on the sheath of *Sphaerotilus natans*. Ellipsoidal spores are formed in swollen sporangia. Facultatively anaerobic and Gram-negative. Two types of creamy-grey colonies are formed when grown on tryptone: one is flat and translucent and the other is convex and opaque. Optimum growth temperature is 30 °C. Optimum pH is 7. Growth does not occur in the presence of 5% (w/v) NaCl. No growth in nutrient broth. Catalase, urease and oxidase activities are negative. Gelatin is not hydrolysed. Citrate and glucose are not utilized. Arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase are negative. Nitrate is not reduced. Indole is not produced. The Voges–Proskauer reaction is negative. The sheath of *Sphaerotilus natans* is degraded and utilized. Most carbohydrates, organic acids and amino acids are not oxidized, as described above. Predominant fatty acids are anteiso-C_{15:0}, C_{16:0} and C_{15:0}. The major isoprenoid quinone is MK-7. The G + C content is

54.0–55.8 mol %. The PAEN515F binding site exists in the 16S rRNA gene.

The type strain is strain TB^T (= JCM 11186^T = IAM 14926^T = KCTC 13912^T).

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