Paenibacillus hodogayensis sp. nov., capable of degrading the polysaccharide produced by *Sphaerotilus natans*

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Sphaerotilus natans is a sheathed bacterium often found in activated sludge that has a bulking problem. A bacterial strain that is able to degrade the extracellular polysaccharide produced by *S. natans* was isolated. The isolate was a spore-forming, aerobic, rod-shaped bacterium. The Gram reaction was variable or negative. The optimum growth temperature was 30 °C and the optimum pH was 8. The G + C content of the DNA was 55 mol%. The major cellular fatty acid and respiratory quinone were anteiso-C_{15:0} and MK-7, respectively. Phylogenetic analysis based on the 16S rRNA gene indicated that the isolate was a member of the genus *Paenibacillus*. The nearest relative, with a similarity of 94.2%, was *Paenibacillus koleovorans*, a bacterium capable of degrading the sheath of *S. natans*. The phenotypic characteristics of the isolate were apparently different from those of related species in the genus *Paenibacillus*. It is proposed that the isolate be designated *Paenibacillus hodogayensis* sp. nov. The type strain is SG^T (= JCM 12520^T = KCTC 3919^T).

Sphaerotilus natans is a typical aquatic filamentous bacterium that is potentially applicable to heavy metal removal (Pagnanelli *et al.*, 2002, 2003, 2004a, b). On the other hand, it is also known to be a troublesome bacterium that causes the problem of poor settling (filamentous bulking) of activated sludge (Eikelboom, 1975; Caravelli *et al.*, 2003, 2004; Contreras *et al.*, 2002; Gaval & Pernelle, 2003; van Veen *et al.*, 1978). *S. natans* forms a sheath (Eikelboom, 1975; Takeda *et al.*, 1998, 2003; van Veen *et al.*, 1978) and a slimy extracellular polysaccharide (EPS) (Gaudy & Wolfe, 1962). The EPS is a straight-chain acidic polysaccharide (Takeda *et al.*, 2002c) and is tightly associated with the sheath (Gaudy & Wolfe, 1962; Takeda *et al.*, 2002b). The bulking effects of *S. natans* may due to the production of the sheath and the EPS. Biological degradation of the sheath of *S. natans* was revealed to be achieved by the sheath-degrading bacterium *Paenibacillus koleovorans* (Takeda *et al.*, 2000, 2002a). However, degradation of the EPS has

not been attempted so far. In the present study, we isolated an EPS-degrading bacterium, and the taxonomic characterization of the isolate was carried out. The results demonstrated that the isolate is a close relative of *P. koleovorans* and should be classified within the genus *Paenibacillus* as *Paenibacillus hodogayensis* sp. nov.

The EPS of *S. natans* was produced and purified as previously reported (Takeda *et al.*, 2002b). The medium (EPS medium) used for screening for EPS-degrading bacteria was composed of the following (l⁻¹ distilled water): 0.1 g EPS, 2 g (NH₄)₂SO₄, 1 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.5 g NaCl, 0.5 g CaCO₃ and 0.01 g FeSO₄·7H₂O. Samples of damp humus (about 0.1 g) from 50 sites in the city of Yokohama and neighbouring areas in Japan were added to test tubes containing 2 ml screening medium and were incubated statically at 30 °C for 10 days. EPS-dependent growth of the micro-organisms was detected in four cultures. After subcultivation four times, the cultures were individually spread on solidified (1.5% agar) CaCO₃-free EPS medium (EPS agar) and incubated at 30 °C for 3 months. Large (about 5 mm), yellow, convex colonies were obtained from one (a sample from the campus of Yokohama National University, Hodogaya, Yokohama, Japan) of the four cultures. However, two morphologically different rod-shaped bacteria were observed in the yellow colony that could not be grown individually on EPS agar. When the colony was transferred to TYN agar (containing, l⁻¹ distilled water, 5 g tryptone, 2.5 g yeast extract, 2.5 g NaCl and 15 g agar), they were successfully isolated as two

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Abbreviation: EPS, extracellular polysaccharide.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SG^T and MH are AB179866 and AB179867, respectively.

A graph showing growth of strain SG^T and EPS degradation, a figure showing the effect of SG^T on *S. natans* sedimentation and a micrograph demonstrating the degradation of the sheath-associated EPS of *S. natans* are available as supplementary material in IJSEM Online.

different colonies (yellow and white). The isolate that formed the yellow colony was designated strain MH and its cell size was about $0.3 \times 0.7 \mu\text{m}$. Another isolate (designated strain SG^T) formed the white colony; its cells were larger than those of strain MH. Further experiments revealed that only strain SG^T had the ability to degrade EPS (results are available as Supplementary Fig. A in IJSEM Online). The strain was found to be able to accelerate the sedimentation of *S. natans* (Supplementary Fig. B) by removing the sheath-associated EPS without having any effect on the sheath (Supplementary Fig. C).

Strain SG^T was cultured on TYN agar at 30 °C for 3 days, and characterized taxonomically. Gram-staining was done using a Favour-GS kit (Nussui Pharmaceutical). Motility (18–24-h-old cells in TYN medium) and morphological properties were determined by phase-contrast microscopy (Axioskop; Zeiss). Anaerobic growth was tested using a BBL GasPak pouch (Becton Dickinson). The bacterium was inoculated into TYN medium and shaken at various temperatures (15–50 °C). Growth was monitored by measuring the optical density at 600 nm at intervals of 1 h; the specific growth rate was then calculated. To determine the effect of pH on growth, the bacterium was cultured in TYN medium at various pH values (pH 3.5–11.0, adjusted by adding HCl or NaOH) at 30 °C for 14 h, and the optical density was then measured. Growth in the presence of 5% NaCl was tested in TYN medium supplemented with 5% (w/v) NaCl. Strain SG^T was motile and obligately aerobic. Cells were Gram-variable but Gram-negative in old cultures. Colonies on TYN agar were white, convex and opaque. Cells were straight and rod-shaped (1.3 – $1.7 \mu\text{m}$ in width, 2.3 – $2.8 \mu\text{m}$ in length), as shown in Fig. 1(a). Flagella and elliptical endospores were shown by scanning probe microscope observation (Fig. 1b). Most sporangia were not swollen, but slightly swollen sporangia were occasionally observed with phase-contrast microscopy. Growth of strain SG^T occurred at pH 6.2–9.2, with an optimum at pH 8. The growth temperature range was 20–40 °C, with an optimum at 30 °C. No growth was observed at 50 °C or in the presence of 5% NaCl. The following characteristics were tested for as described by Takeda *et al.* (2002a): catalase and oxidase activities were positive; urease, gelatinase, arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase tests were negative; nitrate was not reduced; indole and hydrogen sulfate were not produced; citrate was not utilized; and the Voges–Proskauer reaction was negative. Acid production from various carbon sources was tested at 30 °C with API 50 CH (bioMérieux); the results are given in the species description and Table 1.

Total DNA of strain SG^T was prepared using a Genomic-Prep cells and tissue DNA isolation kit (Amersham Biosciences). The G + C content of the DNA was measured by using the HPLC method (Takeda *et al.*, 2002a). Quinone was extracted and purified according to a previously described method (Takeda *et al.*, 2004) and was identified by fast atom bombardment MS (JMS-AX500 apparatus;

Fig. 1. Scanning electron microscopy (a) and scanning probe microscopy (b) observations of strain SG^T. Strain SG^T was cultured on TYN agar for 3 days (for scanning electron microscopy observation; JSM-T100 apparatus from JEOL) or for 7 days (for scanning probe microscopy observation; SPI 3800N apparatus from Seiko Instruments). Note that flagella and an ellipsoidal spore can be seen in (b). Bars, 10 μm (a) and 1 μm (b).

JEOL). Cellular fatty acids were identified by using previously described methods (Takeda *et al.*, 2004). The DNA G + C content of strain SG^T was 55.0 mol%. Most *Paenibacillus* strains exhibit a G + C content of 39–54 mol% (Shida *et al.*, 1997), although the strains of *Paenibacillus dendritiformis* (Tcherpakov *et al.*, 1999) and *P. koleovorans* (Takeda *et al.*, 2002a) have a rather higher G + C content, of 54–55 mol%. The major respiratory quinone was MK-7(H₂). The major cellular fatty acids were anteiso-C_{15:0} (39.7%), C_{15:0} (19.2%), iso-C_{16:0} (9.6%) and C_{16:0} (7.6%). *Paenibacillus* strains are reported to have MK-7 as the major respiratory quinone and anteiso-C_{15:0} as the major fatty acid (Shida *et al.*, 1997).

The 16S rRNA gene sequences of strains SG^T (1488 bp) and MH (1345 bp) were amplified by PCRs with a universal

Table 1. Phenotypic characteristics of strain SG^T and closely related *Paenibacillus* species

Taxa: 1, *P. validus*; 2, *P. larvae* subsp. *larvae*; 3, *P. larvae* subsp. *pulvificiens*; 4, *P. chinjuensis*; 5, *P. chondroitinus*; 6, *P. koleovorans*; 7, strain SG^T. Reference data are from Nakamura (1987), Heyndrickx *et al.* (1995, 1996), Yoon *et al.* (2002) and Takeda *et al.* (2002a). +, Positive; –, negative; V, variation between strains; +/–, variation within strains; NT, not tested.

Characteristic	1	2	3	4	5	6	7
Anaerobic growth	–	+	+	+	–	+	–
Swollen sporangia	+	+/–	+/–	+	+	+	+/–
Catalase	+	–	–	+	+	–	+
Oxidase	–	NT	NT	+	–	–	+
Urease	–	–	–	–	+	–	–
Arginine dihydrolase	–	–	+	NT	–	–	–
Gelatin hydrolysis	–	+	+	+	NT	–	–
Acid production from:							
D-Xylose	+	–	–	–	+	–	–
D-Fructose	+	V	V	–	+	–	–
Mannitol	+	–	+	–	+	–	+
Cellobiose	–	–	–	+	+	–	+
Lactose	–	–	–	–	+	–	+
Glycerol	+	+	+	–	NT	–	+
Galactose	+	V	V	–	+	–	–
D-Glucose	+	+	+	+	+	–	+
L-Arabinose	–	–	–	–	+	–	–
D-Mannose	V	+	+	+	+	–	–
Rhamnose	–	–	–	–	+	–	–
Salicin	–	+	–	–	+	–	+
Sucrose	+	–	–	NT	+	–	+
Growth at 50 °C	+	–	–	–	–	–	–
Optimum growth temp. (°C)	42–50	35–37	28–30	30–37	28–30	30	30
DNA G+C content (mol%)	51–52	42.3	42–43	53	47–48	54.0–55.8	55
Major fatty acids (>10%)	anteiso-C _{15:0} , iso-C _{15:0}	anteiso-C _{15:0} , iso-C _{15:0} , anteiso-C _{17:0}	anteiso-C _{15:0} , iso-C _{15:0} , anteiso-C _{17:0}	anteiso-C _{15:0}	anteiso-C _{15:0} , iso-C _{15:0} , iso-C _{16:0}	anteiso-C _{15:0} , C _{15:0} , C _{16:0}	anteiso-C _{15:0} , C _{15:0}

primer (8F: 5'-AGAGTTTGATCATGGCTCAG-3') (Hallbeck *et al.*, 1995) in combination with universal primer 1392R (5'-ACGGGCGGTGTGTAC-3') (Hallbeck *et al.*, 1995) or 1522R (5'-AAGGAGGTGATCCAGCCGCA-3') (Ash *et al.*, 1993). The PCR products were cloned into pCR-TOPO (Invitrogen) and the nucleotide sequences were subsequently determined using vector-specific primers (Invitrogen) and a series of primers designed by Hallbeck *et al.* (1995). Phylogenetic analysis (neighbour-joining method) was done according to previously described methods (Takeda *et al.*, 2000a). Sequences specific for the genus *Paenibacillus*, the PAEN515F primer-binding site (Shida *et al.*, 1997) and the 22-base sequence (Ash *et al.*, 1993), were found in the sequence determined for strain SG^T. The closest relative of strain MH was *Chryseobacterium indoltheticum* (AY468448), a species that has been inadequately characterized (Vandamme *et al.*, 1994), which showed a high level of similarity (97.5%). A phylogenetic analysis revealed that strain SG^T could be placed in the cluster that included *P. koleovorans*, *Paenibacillus chondroitinus*, *Paenibacillus alginolyticus*, *Paenibacillus larvae* subsp.

larvae, *P. larvae* subsp. *pulvificiens*, *Paenibacillus chinjuensis*, *Paenibacillus koreensis*, *Paenibacillus validus* and *Paenibacillus naphthalenovorans* (Fig. 2). The sequence similarity values for the type strains of the genus *Paenibacillus* ranged from 94.2% (*P. koleovorans*) to 89.9% (*P. dendritiformis*). The closest relative of strain SG^T, *P. koleovorans*, is capable of degrading the sheath of *S. natans* (Takeda *et al.*, 2000, 2002a). It is interesting that two bacteria involved in the degradation of extracellular macromolecules of *S. natans* are closely related. Table 1 summarizes the properties of strain SG^T that can be used to differentiate it from phylogenetically related species of the genus *Paenibacillus*. Strain SG^T produced acids from several carbohydrates but *P. koleovorans* does not. Most strains of the genus *Paenibacillus* form swollen sporangia, but strain SG^T formed non-swollen or only slightly swollen sporangia. A few strains of *P. larvae* subsp. *larvae* and *P. larvae* subsp. *pulvificiens* are known to form non-swollen sporangia (Heyndrickx *et al.*, 1996). The DNA G+C content of these subspecies of *P. larvae* (42–43 mol%) is much lower than that of strain SG^T (55%), allowing easy discrimination. The genetic and

Fig. 2. Phylogenetic position of strain SG^T among related *Paenibacillus* species. Bootstrap values (percentages of 1000 replications) are shown at branch points. Accession numbers are indicated in parentheses. Bar, 0.02 substitutions per nucleotide.

phenotypic features of strain SG^T suggest that it represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus hodogayensis* sp. nov. is proposed.

Description of *Paenibacillus hodogayensis* sp. nov.

Paenibacillus hodogayensis (ho.do.ga.yen'sis. N.L. masc. adj. *hodogayensis* relating to Hodogaya, the name of a district in Yokohama, Japan, the geographical origin of isolation of the type strain).

Cells are rod-shaped (1.3–1.7 × 2.3–2.8 µm) and motile. Gram-variable or Gram-negative. Ellipsoidal spores are formed in non-swollen or slightly swollen sporangia. Growth occurs only under aerobic conditions. Colonies on TYN agar are white, convex and opaque. The optimum growth temperature is 30 °C. The optimum pH for growth is 8. No growth occurs at 50 °C or in the presence of 5 % (w/v) NaCl. The Voges–Proskauer reaction is negative. Catalase- and oxidase-positive. Nitrate is not reduced. Indole is not produced. Citrate is not utilized. Acids are produced from glycerol, methyl β-xyloside, glucose, mannitol, aesculin, cellobiose, maltose, melibiose, sucrose, trehalose and turanose. Small amounts of acid are produced from ribose, methyl α-mannoside, methyl α-glucoside, N-acetylglucosamine, amygdalin, salicin, lactose and 5-ketogluconate. Acid is not produced from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, galactose, fructose, mannose, sorbose, rhamnose, dulcitol, inositol, sorbitol, arbutin, inulin, melezitose, raffinose, starch, glycogen, xylitol, β-gentiobiose, lyxose, tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate or 2-ketogluconate. Acidic exopolysaccharide from *S. natans* is degraded and utilized. The predominant cellular fatty acid is anteiso-C_{15:0}. The major quinone is MK-7(H₂). The DNA G+C content is 55 mol%. The PAEN515F binding site exists in the 16S rRNA gene.

The type strain is SG^T (= JCM 12520^T = KCTC 3919^T).

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