Paenibacillus stellifer sp. nov., a cyclodextrin-producing species isolated from paperboard

Irmgard Suominen,¹ Cathrin Spröer,² Peter Kämpfer,³ Frederick A. Rainey,⁴ Kari Lounatmaa⁵ and Mirja Salkinoja-Salonen¹

Correspondence

Mirja Salkinoja-Salonen
mirja.salkinoja-salonen@
helsinki.fi

¹Department of Applied Chemistry and Microbiology, PO Box 56, FIN-00014 University of Helsinki, Helsinki, Finland

²German Collection of Micro-organisms and Cell Cultures, Mascheroder Weg 1b, D-38124 Braunschweig, Germany

³Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, Heinrich-Buff-Ring 26–32, D-35392 Giessen, Germany

⁴Department of Biological Sciences, 202 Life Sciences Building, Louisiana State University, Baton Rouge, LA 70803, USA

⁵Electronics Production Technology, PO Box 3000, FIN-02015 Helsinki University of Technology, Helsinki, Finland

The microflora isolated from food-packaging board is dominated by *paenibacilli*; a number of these micro-organisms have been characterized using a polyphasic approach. The highest 16S rRNA gene similarity was found between these isolates and *Paenibacillus azotofixans* ATCC 35681^T (97.7 %). The main fatty acid of the paperboard isolates was C_{16:0} (34–45 %); straight-chain fatty acids made up 41–60 % of the total cellular fatty acids, thus distinguishing these strains from other *Paenibacillus* species. The paperboard isolates produced cyclodextrins from starch. The spore surface had a characteristic ribbed ornamentation. Spores and vegetative cells frequently had pilus-like appendages. Based on phylogenetic data and phenotypic and chemotaxonomic characteristics, it is proposed that the isolates represent a novel species, *Paenibacillus stellifer* sp. nov., with IS 1^T (=DSM 14472^T=CCUG 45566^T) as the type strain.

Gram-positive spore-formers belonging to *Bacillus* or *Paenibacillus* are known as the main contaminants of food-packaging paper and board (Pirttijärvi *et al.*, 1996; Suominen *et al.*, 1997). The strains described in this paper represent the dominant and sometimes the sole contaminant of paperboard. Data show that the isolates represent a novel species within the genus *Paenibacillus*, for which the name *Paenibacillus stellifer* sp. nov. is proposed.

The novel isolates IS 1^T, IS 2, IS 3, IS 4 and IS 5 were obtained by plating homogenized food-packaging paperboard as described by TAPPI (1990) on brain heart infusion (BHI) agar (Difco) at 28 °C. The following reference strains were used: *Paenibacillus azotofixans* DSM 5976^T, *Paenibacillus borealis* KK19^T (provided by S. Elo), *Paenibacillus* sp. KM8 (provided by S. Elo), *Paenibacillus campinasensis* KCTC

0364BP^T (provided by J.-H. Yoon), *Paenibacillus durus* DSM 1735^T, *Paenibacillus lautus* LMG 11157^T and *Paenibacillus polymyxa* strains DSM 36^T, ATCC 7047, NRRL B-369, NRRL B-375 and NRRL B-478.

Biochemical properties of strains grown on nutrient agar were determined as described by Elo *et al.* (2001) and by API 20NE and API 50CH (bioMérieux). Haemolysis was assayed on bovine blood agar plates prepared from tryptic soy agar (TSA) base (BBL) by adding 5 % (v/v) defibrinated bovine blood. Defibrination was achieved by adding 0.5 % trisodium citrate dihydrate to the blood. Plates were read after 1 and 4 days at 28 °C (Smibert & Krieg, 1994). Cyclodextrin production from starch was tested as described by Pirttijärvi *et al.* (2001) and growth on cellulose, hemicellulose or xylan as sole carbon source (10 g l⁻¹) was tested on R2A medium (Clesceri *et al.*, 1998) without glucose at 28 °C. Anaerobic growth was tested on BHI at 37 °C for 5 days (10 % H₂, 10 % CO₂, 80 % N₂).

Whole-cell fatty acid and G+C content analysis and ribotyping were done as described by Elo *et al.* (2001). For ribotyping, a molecular size marker mixture (1.1, 2.2,

The EMBL accession numbers for the 16S rDNA sequences of strains IS 1^T, IS 2, IS 3, IS 4 and IS 5 are respectively AJ316013 and AJ508713–AJ508716.

Detailed fatty acid compositions of individual novel isolates and *P. polymyxa* strains are available as supplementary material in IJSEM Online.

3.2, 6.5, 9.6 and 48.0 kbp) was run in slots adjacent to each sample slot.

Genomic DNA extraction, PCR-mediated amplification of the rRNA gene and purification of PCR products were carried out as described previously (Rainey *et al.*, 1996). Purified PCR products were sequenced with *Taq* Dyedexy terminator cycle sequencing kits (Applied Biosystems) according to the manufacturer's protocol. An Applied Biosystems 373A DNA sequencer was used for electrophoresis of sequence reaction products. The ae2 editor (Maidak *et al.*, 1999) was used to align the 16S rRNA gene sequence determined in this study against 16S rRNA gene sequences of representatives of *Paenibacillus* species available from public databases. Pairwise evolutionary distances were computed using the correction of Jukes & Cantor (1969). The phylogenetic dendrogram was reconstructed from a distance matrix using the neighbour-joining treeing algorithm in the PHYLIP package (Felsenstein, 1993).

For negative staining, a 1-day-old culture grown on TSA (Difco) at 37 °C was allowed to attach onto the carbon grid for 1 min and stained with 1% phosphotungstic acid (pH 7.3) for 25 s. The grids were inspected on a JEOL 1200 EX TEM at an operating voltage of 60 kV. Cells grown on BHI plates for 4 days at 28 °C were examined by TEM of thin sections, cells grown on TSA plates for 4 days at 28 °C were observed by field emission SEM as described by Elo *et al.* (2001) and spores were studied by Hitachi S-4300 field emission SEM or JEOL JSM-6335F field emission SEM; voltages are given in the figure legends.

The *Paenibacillus* isolates originated from food-packaging paperboards for which the heterotrophic plate count was high in quality control analyses. They represent the main (>90%) contaminant in paperboards and stored pulps containing ground wood. Vegetative cells were rod-shaped, 0.6–0.8 × 2.5–5.0 µm. Young cells stained Gram-positive and were peritrichously flagellated (Fig. 1a). A thin section of vegetative cells showed S-layer-like material being shed from the cells (Fig. 1b). Thin sections of mature spores of the paperboard isolates showed six to seven 'spikes' on the surface (Fig. 1c), similar to spores of *P. polymyxa* (Murphy & Campbell, 1969) and *P. borealis* (Elo *et al.*, 2001). Field emission SEMs (Fig. 2a, b) reveal that the spikes were ribs ornamenting the spore surface. The ribs connected the poles of the spore (Fig. 2a). The rib pattern of the novel isolates was more similar to spores of the type strain of *P. polymyxa* (not shown in Fig. 2) than to those of *P. borealis*, in which the ornaments have a honeycomb appearance and are distributed uniformly over the spore (Elo *et al.*, 2001). The isolates produced ellipsoidal spores in swollen sporangia in the terminal region of the cell (Fig. 1c). The spores visible in Fig. 2(a, b) were covered with a slimy looking material, removable from the spores with saline-peptone diluent (0.85% NaCl, 0.1% meat peptone) (Fig. 2c). Pilus-like appendages were observed on spores as well as on vegetative

Fig. 1. (a) TEM of a 1-day-old culture of strain IS 5 after negative staining with 1% phosphotungstic acid. A high degree of peritrichous flagellation is visible. (b) Thin section of strain IS 1^T showing S-layer material being shed from vegetative cells. Sections were prepared from a colony cut from an TSA plate grown for 3 days at 28 °C. Shedding of S-layers may occur when cells are in contact with a solid surface. Operating voltage, 60 kV. (c) TEM of strain IS 5 from a 4-day-old culture showing vegetative cells and spores at different stages of maturation. Operating voltage, 60 kV. Bars, 500 nm.

cells (Fig. 2a). Appendages of similar morphology have been described for spores of *Bacillus cereus* (Kozuka & Tochikubo, 1985; Mizuki *et al.*, 1998), but not as yet for paenibacilli. Fig. 2(b) shows laminar material located on the spores; this material was shed during maturation of the spores and may represent the same material visible as the 'S-layer' in thin sections (Fig. 1b).

The isolates grew aerobically at 15–40 °C and anaerobically on BHI at 37 °C. The whole-cell fatty acid compositions of the paperboard isolates (grown at 28 °C) and phylogenetically related *Paenibacillus* species are summarized in Table 1; detailed fatty acid compositions of individual novel isolates and *P. polymyxa* strains are available as supplementary material in IJSEM Online. The main

Fig. 2. Field emission SEMs of strain IS 1^T. (a) Appendages attached to spores as well as to vegetative cells. (b) Layered material on the spores. (c) Mature spore with a regular pattern of ribs connecting the poles of the spore. Operating voltages were 15 kV for (a) and (b) and 3 kV for (c). Bars, 500 nm.

Table 1. Whole-cell fatty acid compositions of the novel isolates and selected *Paenibacillus* strains

Values are percentages of total cellular fatty acids, with means (ranges) given for the novel isolates and *P. polymyxa*. Strains/taxa: 1, novel isolates ($n=5$); 2, *P. azotofixans* DSM 5976^T; 3, *P. borealis* KK19^T; 4, *Paenibacillus* sp. KM8; 5, *P. campinasensis* KCTC 0364BP^T; 6, *P. durus* DSM 1735^T; 7, *P. lautus* LMG 11157^T; 8, *P. polymyxa* ($n=5$). Detailed fatty acid compositions of individual novel isolates and *P. polymyxa* strains are available as supplementary material in IJSEM Online.

Fatty acid	1	2	3	4	5	6	7	8
Saturated straight-chain								
C _{14:0}	9.0 (6.0–12.5)	7.4	18.6	7.3	3.4	4.6	2.8	1.3 (1.0–1.8)
C _{15:0}	1.5 (0.5–3.0)	3.4	<0.2	<0.2	0.8	7.7	0.7	0.5 (0.3–0.7)
C _{16:0}	40.6 (34.0–44.6)	20.5	10.0	12.4	24.9	17.4	34.0	5.9 (4.1–7.5)
Saturated iso-branched								
C _{13:0}	<0.2 (<0.2–0.4)	<0.2	<0.2	0.5	<0.2	<0.2	<0.2	<0.2
C _{14:0}	3.1 (2.8–3.5)	6.9	4.9	8.6	0.9	5.4	1.6	1.5 (0.9–2.0)
C _{15:0}	10.4 (8.2–13.0)	6.4	11.5	15.0	14.1	2.9	7.6	7.1 (5.2–9.0)
C _{16:0}	5.6 (5.1–5.9)	8.0	10.5	5.7	3.3	8.8	4.6	9.8 (5.6–14.2)
C _{17:0}	2.4 (1.7–3.4)	0.8	4.9	1.8	6.8	0.9	4.8	4.1 (2.5–5.0)
Saturated anteiso-branched								
C _{13:0}	<0.2 (<0.2–0.2)	1.0	<0.2	<0.2	<0.2	0.8	<0.2	<0.2
C _{15:0}	25.2 (20.2–31.7)	42.4	35.2	46.5	37.1	47.6	37.0	58.2 (55.2–61.0)
C _{17:0}	1.5 (1.2–1.9)	2.0	2.1	1.3	6.8	2.7	4.7	9.6 (8.5–12.6)
Unsaturated								
C _{16:1} ω11c	<0.2	<0.2	1.3	0.4	<0.2	<0.2	1.7	1.1 (0.5–1.4)
C _{18:1} ω9c	<0.2	<0.2	0.4	<0.2	<0.2	<0.2	<0.2	<0.2
Other*	<0.5 (<0.2–0.8)	1.2	0.6	0.5	0.3	1.2	0.5	<0.8 (<0.2–2.0)

*Includes C_{12:0}, C_{16:1}ω7c alcohol, C_{17:0}, C_{17:1} iso ω10c, C_{17:1} iso I/anteiso B, C_{17:1} anteiso B/iso I and C_{18:0}.

fatty acid (34–45 %) of the paperboard isolates was C_{16:0}, thus differentiating the paperboard isolates from all *Paenibacillus* species except *P. lautus*. Straight-chain fatty acids in the paperboard isolates made up 41–60 % total fatty acids, whereas, for the other *paenibacilli*, this sum was <40 %. The iso:anteiso ratio was lower (2.3–2.5) for the paperboard isolates and their phylogenetic relatives *P. azotofixans*, *P. borealis* and *P. durus* than for other *Paenibacillus* species (including *P. lautus*), where it was 3.1–16.4.

The novel isolates differed from *P. azotofixans*, *P. durus* and *P. borealis* in gluconate assimilation and hydrolysis of pNP β -D-galactopyranoside and L-proline pNA (Table 2). The novel isolates had similar reactions to *P. azotofixans*, *P. durus* and *P. borealis* for 48 of the 65 tested substrates. Additional tests were performed for the novel isolates, and results are given in Table 2. The isolates were catalase-positive, oxidase-negative and did not reduce nitrate to nitrite or nitrogen. None of the isolates showed haemolytic activity. Carboxymethylcellulose, hemicellulose and xylan were not used as sole carbon sources by the paperboard isolates. The five isolates degraded starch and produced cyclodextrins (Fig. 3), an important pathway for spoiling starch in the paper industry (Pirttijärvi *et al.*, 2001). The isolates represent a novel taxon with starch-degrading properties in the paper machine environment. van der Maarel *et al.* (2000) described a starch-degrading *Paenibacillus* species, *Paenibacillus granivorans*, but it is not known whether it produces cyclodextrins.

The ribofragment patterns (Fig. 4) obtained for the isolates by *EcoRI* and *PvuII* digestion differed clearly from those of phylogenetically closely related species (*P. borealis*, *Paenibacillus* sp. KM8, *P. campinasensis*, *P. azotofixans*, *P. lautus*, *P. polymyxa*, *P. durus*). The patterns generated for isolates IS 1^T, IS 2 and IS 4 with *PvuII* were identical, and these strains shared seven bands with IS 3 and eight bands with IS 5. With *EcoRI*, all strains shared nine of 11 bands. Isolates IS 2 and IS 4 had identical *EcoRI* and *PvuII* ribotypes and shared identical whole-cell fatty acid compositions, indicating that they may be clones. Isolates IS 1^T, IS 3 and IS 5 each had a different combination of *EcoRI* and *PvuII* ribotypes, demonstrating they were isolates representing independent strains.

An almost-complete 16S rRNA gene sequence of 1519 nt (positions 27–1532, *Escherichia coli* numbering; Brosius *et al.*, 1978) was determined for strain IS 1^T. Phylogenetic analyses based on a dataset consisting of 1255 unambiguous nucleotides between positions 45 and 1431 showed that strain IS 1^T represented a novel lineage within the radiation of the genus *Paenibacillus* (Fig. 5). Pairwise similarity values ranged between 90.0 % (*Paenibacillus kobensis* IFO 15729^T, *Paenibacillus dendritiformis* T168^T) and 96.8 % (*P. borealis* DSM 18188^T). This novel lineage clusters with *P. azotofixans* (Seldin *et al.*, 1984), *P. borealis*, *Paenibacillus odorifer* and *Paenibacillus graminis*, having 97.7, 97.2, 97.4 and

Table 2. Biochemical characteristics of the novel isolates and type strains of selected *Paenibacillus* species

Strains: 1, paperboard isolates ($n=5$); 2, *P. azotofixans* DSM 5976^T; 3, *P. durus* DSM 1735^T; 4, *P. borealis* KK19^T. None of the strains utilized L-rhamnose, D-ribose, adonitol, i-inositol, putrescine, propionate, *cis*-aconitate, citrate, fumarate, 2-oxoglutarate, adipate, 4-aminobutyrate, azelate, glutarate, DL-3-hydroxybutyrate, itaconate, DL-lactate, mesaconate, suberate, L-alanine, β -alanine, L-aspartate, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-tryptophan, 3-hydroxybenzoate or phenylacetate. Strains IS 1^T, IS 2, IS 3, IS 4 and IS 5 did not utilize glycerol, erythritol, D-arabinose, methyl β -D-xyloside, L-sorbose, dulcitol, methyl α -D-mannoside, methyl α -D-glucoside, inulin, melizitose, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate or 5-ketogluconate. All strains utilized D-fructose, D-galactose, D-glucose, D-mannose, D-maltose, α -D-melibiose, D-sucrose, salicin and D-trehalose. Strains IS 1^T, IS 2, IS 3, IS 4 and IS 5 utilized L-xylose, amygdalin, lactose, D-raffinose, starch, glycogen, β -gentiobiose and D-turanose. Strains IS 1^T, IS 2, IS 3, IS 4 and IS 5 did not assimilate glucose, arabinose, mannose, mannitol, N-acetylglucosamine, maltose, gluconate, caprate, adipate, malate, citrate or phenylacetate. All strains hydrolysed aesculin and pNP β -D-glucopyranoside. None of the strains hydrolysed pNP β -D-glucuronide, pNP phenylphosphonate, pNP phosphorylcholine, 2-deoxythymidine-5'-pNP phosphate, L-alanine pNA or L-glutamate- γ -carboxy pNA. pNP, *p*-nitrophenyl; pNA, *p*-nitroanilide.

Characteristic	1	2	3	4
Assimilation of:				
N-Acetyl-D-glucosamine	—	—	—	+
L-Arabinose	+	—	—	+
<i>p</i> -Arbutin	—	—	—	+
D-Cellobiose	+	—	+	+
Gluconate	—	+	+	+
D-Xylose	+	—	—	+
Maltitol	—	+	+	+
D-Mannitol	—	—	+	—
D-Sorbitol	—	+	—	+
Acetate	—	+	—	—
L-Malate	—	—	—	+
Pyruvate	—	+	+	—
4-Hydroxybenzoate	—	—	+	—
Hydrolysis of:				
pNP β -D-galactopyranoside	+	—	—	—
pNP α -glucopyranoside	—	+	—	+
bis-pNP phosphate	—	—	+	+
L-Proline pNA	+	—	—	—

95.2 % 16S rRNA gene sequence similarity, respectively, to these species. In partial sequence analysis (497 nt; positions 24–510, *E. coli* numbering), strain IS 5 showed 99.6 % similarity to strains IS 1^T, IS 2, IS 3 and IS 4; the latter four strains were 100 % similar to each other. This indicates that all five strains belong to one

Fig. 3. Production of cyclodextrins by strain IS 1^T on a starch plate. Cyclodextrins precipitated as a zone inside the clearing zone where starch was hydrolysed.

species. The DNA G+C content of strain IS 1^T was 55.6 mol%.

The novel isolates can be distinguished from their closest phylogenetic relatives by whole-cell fatty acid composition, ribotyping with *Eco*RI or *Pvu*II, assimilation of gluconate and hydrolysis of pNP β-D-galactopyranoside and L-proline pNA.

Description of *Paenibacillus stellifer* sp. nov.

Paenibacillus stellifer (stel'li.fer. L. adj. *stellifer* star-carrying, referring to the presence of star-shaped spores).

Gram-positive, facultatively anaerobic rods (0.6–0.8 × 2.5–5.0 μm), motile by means of peritrichous flagella. Ellipsoidal endospores form in swollen sporangia in the terminal region of the cell. Mature spores have a rib pattern connecting the poles of the spore. Spores as well as vegetative cells have pilus-like appendages. Biochemical features are specified in Table 2. The temperature range for growth is 15–40 °C. Non-haemolytic. Catalase-positive. Oxidase-negative. Nitrate is not reduced to nitrite or nitrogen. The DNA G+C content of the type strain is 55.6 mol%. The major fatty acid is C_{16:0} (34–45 % total fatty acids) and the ratio of C_{15:0} iso: anteiso (28 °C) is 2.3–2.5. Produces cyclodextrins from potato starch.

The type strain, IS 1^T (=DSM 14472^T=CCUG 45566^T), was isolated from food-packaging paperboard.

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Fig. 4. Ribotyping of the five novel isolates and 11 *Paenibacillus* reference strains cut with *Eco*RI (a) and *Pvu*II (b). Fragments are shown that hybridized with a phosphorescently labelled probe containing the *E. coli* operon of 5S, 16S and 23S rRNA genes and the intergenic regions.

Fig. 5. Neighbour-joining tree of 16S rDNAs showing the phylogenetic position of strain IS 1^T compared with species of the genus *Paenibacillus*. Sequences were selected from a comparison with 16S rDNA sequences of all described *Paenibacillus* species. Sequences of members of the *Bacillus*/*Staphylococcus* group were used to root the dendrogram. Bootstrap analyses were made with 100 cycles. Bar, 2 substitutions per 100 nt.

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