Paenibacillus favisporus sp. nov., a xylanolytic bacterium isolated from cow faeces

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During a search for xylan-degrading micro-organisms, a sporulated bacterium was recovered from recent and old cow dung and rectal samples. The isolates were identified as members of a novel species of the genus *Paenibacillus*, based on 16S rRNA gene sequences. According to the results of phylogenetic analysis, the most closely related species was *Paenibacillus azoreducens*. Phenotypic and chemotaxonomic analyses and DNA–DNA hybridization experiments also showed that the isolates belonged to a novel species of the genus *Paenibacillus*. The novel species is a facultatively anaerobic, motile, Gram-variable, sporulated rod. The spores of this rod-shaped micro-organism occur in slightly swollen sporangia and are honeycomb-shaped. The main fatty acid is anteiso-branched C_{15:0}. Growth was observed with many carbohydrates, including xylan, as the only carbon source and gas production was not observed from glucose. The novel species produces a wide variety of hydrolytic enzymes, such as xylanases, cellulases, amylases, gelatinase, urease and β -galactosidase. On the contrary, it does not produce caseinase, phenylalanine deaminase or lysine decarboxylase. According to the data obtained in this work, the strains belong to a novel species, for which the name *Paenibacillus favisporus* sp. nov. is proposed (type strain, GMP01^T = LMG 20987^T = CECT 5760^T).

Xylan is a heterogeneous polymer that is composed of (1,4)-linked β -D-xylosyl residues. It is very abundant in nature, being the major component of hemicelluloses in monocotyledon cell walls. Xylanases (1,4- β -D-xylanohydrolases) are a group of xylanolytic enzymes that hydrolyse xylan to xylan-oligosaccharides and xylose; such degradation of xylan is industrially important in diverse fields, such as enzyme-aided bleaching of paper (Viikari *et al.*, 1994), production of fuels or chemicals such as ethanol or lactate from plant biomass (Lamed *et al.*, 1988), animal feed additives (Annison, 1992), bread-making (Maat *et al.*, 1992) and, in combination with pectinase and cellulase, in degumming of plant fibre sources (Puchart *et al.*, 1999). Several micro-organisms have been described as xylanase producers; many of these belong to the Gram-positive bacteria within the actinomycetes (Ruiz-Arribas *et al.*, 1995; Busch & Stutzenberger, 1997; Beg *et al.*, 2000; Chamberlain

& Crawford, 2000; Rivas *et al.*, 2003a) and bacilli (Tabernero *et al.*, 1995; Nishimoto *et al.*, 2002; Sá-Pereira *et al.*, 2002; Tseng *et al.*, 2002).

The bacilli are a wide group of micro-organisms that are characterized by endospore formation. Currently, this group includes several families and genera, many of which belonged to the former genus *Bacillus*, which has been separated into several novel genera that belong to several families. This is the case for the genus *Paenibacillus*, which was proposed by Ash *et al.* (1994) and belongs to the family 'Paenibacillaceae'. Members of the genus *Paenibacillus* are facultatively anaerobic organisms that produce spores in swollen sporangia and have DNA G + C contents that range from 45 to 54 mol%. Some of these bacteria excrete diverse assortments of extracellular polysaccharide-hydrolysing enzymes, including xylanases (Zamost *et al.*, 1991; Morales *et al.*, 1995; Hespell, 1996; Aÿ *et al.*, 1998; Nielsen & Sorensen, 1997; Lee *et al.*, 2000).

Taking into account the fact that xylan is a polysaccharide that is present in plant cell walls, we searched for xylan-hydrolysing bacteria in the faeces of ruminant animals. In

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GMP01^T is AY208751.

Micrographs and an expanded phylogenetic tree are available as supplementary material in IJSEM Online.

the present work, we describe the isolation and identification of a novel xylan-degrading species of the genus *Paenibacillus*.

Isolation of strains was always done in YNBX medium that contained xylan as the carbon source (6.7 g yeast nitrogen base l⁻¹, 7 g xylan l⁻¹ and 20 g agar l⁻¹), as specified by Esteban *et al.* (1983). The three strains from this study were isolated from old (GMP01^T=USC13^T) and recent (GMP02=USC14) cow dung in Salamanca, Spain, and from rectal samples (GMP03=USC15) of a cow in León, Spain. Routine growth of all strains was done in tryptic soy broth and, unless otherwise stated, the temperature for both growth and biochemical tests was 37 °C. Cultures in liquid medium were carried out routinely at 37 °C in a rotary shaker at 250 r.p.m. The three colonies were of an aqueous aspect, translucent, rounded and convex. As they aged, a brown spot could be observed at the centre of the colony. The three strains were isolated on YNBX plates and showed a high xylanolytic activity.

Strain GMP01^T was grown in YNBX medium for 48 h to check for motility by phase-contrast microscopy. Cells were also stained according to the classical Gram procedure described by Doetsch (1981). For electron microscopy, cells were grown in liquid YED (yeast extract/glucose) medium for 3 days at 180 r.p.m. and 28 °C. Samples were fixed overnight in 2% (v/v) glutaraldehyde. Sections were mounted according to Reynolds (1963) and examined with a Philips EM 300 electron microscope. For scanning microscopy, cells and spores were treated with 2% uranyl acetate and observed under a Philips PSEM 500 electron microscope. Cells of strain GMP01^T are straight, rod-shaped and generally observed singly, but can also occur as a group of 3–4 cells in a chain-like structure. Actively growing cells had a mean length of 2–3 µm and a mean width of 0.5–0.7 µm. They are Gram-variable and exhibit motility by polar and subpolar flagella, frequently at both poles. The organism is sporulated and its spores are resistant to dry heat (the number of viable cells does not vary when treated at 150 °C for 10 min). Spores are easily stained with Gram-stain. They are central or central-terminal, 1 µm long and 0.7 µm wide and slightly distend the sporangium wall. When observed by scanning electron microscopy, the spore surface resembles a honeycomb and according to the thin sections visualized by transmission electron microscopy, they exhibit a star-shape morphology (see Figs A and B, available as supplementary material in IJSEM Online).

For 16S rDNA sequencing, DNA extraction was carried out as described previously (Rivas *et al.*, 2001). Amplification and sequencing of 16S rDNA were performed according to a method described previously (Rivas *et al.*, 2003a). The sequence obtained was compared with those from GenBank by using the FASTA program (Pearson & Lipman, 1988). Sequences were aligned by using CLUSTAL W software (Thompson *et al.*, 1997). Distances were calculated according to the method of Kimura (1980). Phylogenetic trees were

inferred by using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was based on 1000 resamplings. The MEGA 2.1.0 package (Kumar *et al.*, 2001) was used for all analyses. Trees were rooted by using *Lactobacillus delbrueckii* subsp. *lactis* DSM 20072^T as the outgroup.

The complete 16S rDNA sequence of isolate GMP01^T (GenBank accession no. AY208751) was obtained. The complete sequences of strains GMP02 (AF346495) and GMP03 (AY308758) had 100% similarity to that of GMP01^T. Comparison against 16S rRNA gene sequences held in GenBank indicates that the organism is related phylogenetically to members of the family 'Paenibacillaceae'. Fig. 1 shows the phylogenetic tree obtained by the neighbour-joining method (an expanded tree is available as supplementary material in IJSEM Online). The most closely related species is *Paenibacillus azoreducens*, with 97.7% sequence similarity. Therefore, DNA–DNA hybridization was performed with the type strain of this species.

Phenotypic characterization was performed according to the standard methods described by Logan & Berkeley (1984) and Claus & Berkeley (1986), using *P. azoreducens* DSM 13822^T as the reference. Cellulases were detected after 7 days incubation on plates that contained 0.5% CM-cellulose as the carbon source, 0.3% yeast extract and 1.5% agar. Plates were stained with 1% Congo red solution in water. Other physiological and biochemical tests were done by using the API 20E system (bioMérieux), following the manufacturer's instructions. For testing natural antibiotic resistance, the following antibiotics were used: erythromycin (15 µg), penicillin (10 µg), cephalixin (30 µg), oxacillin (5 µg), bacitracin (10 µg), piperacillin (100 µg), tobramycin (10 µg), gentamicin (10 µg), cefoxitin (30 µg) and dibekacin (10 µg). Discs that contained each antibiotic were placed onto plates with antibiotic medium 1 agar (Difco) that had previously been inoculated with the appropriate strain. According to the data obtained, the three strains showed the same phenotypic characteristics. Table 1 shows the phenotypic properties of the novel species, as well as those of the most closely related species in the genus *Paenibacillus*. Strains of the novel species are different from phylogenetically related species in anaerobic growth, oxidase, nitrate reduction, growth at 50 °C and fermentation of glucose and L-arabinose (Table 1).

Analysis of cellular hydroxy fatty acids was performed from a culture of strain GMP01^T grown for 24 h in TSA medium (Merck) at 28 °C, as described previously (Rivas *et al.*, 2003b). Results of the chemotaxonomic analyses are shown in Table 2. According to published data, the cellular fatty acid pattern of strain GMP01^T is similar to those of phylogenetically closely related species of the genus *Paenibacillus*. The main fatty acid is anteiso-branched C_{15:0}, which comprises 48.8% of the total. This value is lower than those reported for other species of *Paenibacillus*, but higher than that reported for *P. azoreducens* (Meehan *et al.*, 2001).

Fig. 1. Comparative sequence analysis of 16S rDNA from *Paenibacillus favisporus* GMP01^T and representative strains of the genus *Paenibacillus* from GenBank, using the neighbour-joining method. Significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 2 nt substitutions per 100 nt.

DNA was isolated by following the method of Marmur (1961). G+C content was determined by hydrolysing the DNA enzymically and quantifying the nucleosides by HPLC, as reported previously (Cerdà-Cuéllar *et al.*, 1997). Pooled standard deviation (SD) of the experiment was 0.5. The DNA G+C content of strain GMP01^T was 53 mol%. This value is similar to that obtained for *P. azoreducens* (Meehan *et al.*, 2001).

DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with the modifications described by Huss *et al.* (1983) and Escara & Hutton (1980). The results of DNA–DNA hybridization showed 100% relatedness between strains GMP01^T and GMP02 and 17.1% between strain GMP01^T and *P. azoreducens* DSM 13822^T. These results indicate that strain GMP01^T does not belong to *P. azoreducens*, when the recommended threshold value of 70% DNA–DNA similarity for definition of species is considered (Wayne *et al.*, 1987).

Therefore, on the basis of phylogenetic, chemotaxonomic and phenotypic data, we propose that isolate GMP01^T (= LMG 20987^T = CECT 5760^T) should be classified as the type strain of a novel species, *Paenibacillus favisporus* sp. nov.

Description of *Paenibacillus favisporus* sp. nov.

Paenibacillus favisporus (fa.vi.spo'rus. L. n. *favus* a honeycomb; Gr. n. *spora* a seed, spore; N.L. masc. adj. *favisporus* referring to the honeycomb form of spores).

Gram-variable, motile, aerobic or facultatively anaerobic, chemo-organotrophic, xylanolytic bacteria. Colonies on YED medium are circular, convex, white with a central brown spot, translucent and usually 1–3 mm in diameter within 48 h at 37 °C. Optimal growth temperature is 37 °C; optimal growth pH is 7. Oxidase- and catalase-positive. Spore-forming rods, 2–3 µm long and 0.5–0.7 µm wide. Spores slightly swell the sporangia and they are in sub-terminal position in cells. Spores have a ornamentation similar to that of honeycomb. This species is phylogenetically most closely related to *P. azoreducens*. DNA G+C content of the type strain is 53 mol%. The main fatty acid is anteiso-branched C_{15:0}. Gas is not produced from D-glucose. Acid is produced from D-glucose, sucrose, D-mannose, lactose, raffinose, rhamnose, melibiose, maltose, xylose and mannitol. Xylan, CM-cellulose and starch are utilized as carbon sources. By contrast, no growth occurs in L-arabinose, citrate, inositol, sorbitol, glucitol or xylitol as carbon sources. Xylanases, cellulases, gelatinase, urease, amylase and β-galactosidase are produced actively, but

Table 1. Distinctive phenotypic characteristics of species phylogenetically related to *Paenibacillus favisporus* sp. nov.

Species: 1, *Paenibacillus polymyxa* ATCC 842^T; 2, *Paenibacillus azotofixans* ATCC 35681^T; 3, *Paenibacillus peoriae* LMG 14832^T; 4, *Paenibacillus macerans* ATCC 8244^T; 5, *Paenibacillus lautus* NRRL NRS-666^T; 6, *Paenibacillus amylolyticus* NRRL NRS-290^T; 7, *Paenibacillus macquariensis* ATCC 23464^T; 8, *Paenibacillus pabuli* NRRL NRS-924^T; 9, *Paenibacillus campinasensis* KCTC 0364BP^T; 10, *Paenibacillus dendritiformis* T168^T; 11, *Paenibacillus illinoisensis* NRRL NRS-1356^T; 12, *Paenibacillus chibensis* NRRL B-142^T; 13, *Paenibacillus glucanolyticus* DSM 5162^T; 14, *P. azoreducens* DSM 13822^T; 15, *Paenibacillus turicensis* MOL722^T; 16, *Paenibacillus graminis* RSA19^T; 17, *Paenibacillus odorifer* TOD45^T; 18, *Paenibacillus borealis* KK19^T; 19, *Paenibacillus jamilae* B.3^T; 20, *Paenibacillus terrae* AM141^T; 21, *Paenibacillus kribbensis* AM49^T; 22, *Paenibacillus brasiliensis* PB172^T; 23, *Paenibacillus favisporus* GMP01^T. Data are from Meehan *et al.* (2001) except where indicated. +, Positive; −, negative; V, variable; W, weak; NR, not recorded.

Test	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15*	16†	17†	18‡	19§	20	21	22¶	23
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
Optimum growth temp.	30	30–37	30	30	28–30	28–30	20	28–30	40	37	37	37	NR	37	37–42	10–35	5–35	28	30	30	30–37	30–32	37
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	NR	+	−	+	+	+	+	+	+	+	+
Oxidase	−	−	−	NR	−	−	NR	−	−	+	−	−	+	−	−	−	−	−	−	−	−	NR	+
Nitrate reduction	+	−	+	+	+	+	−	−	NR	−	−	+	V	−	−	+	+	−	+	+	+	+	+
Production of:																							
Acetylmethylcarbinol	+	+	+	−	−	−	−	−	NR	−	−	−	NR	−	+	NR	NR	−	+	NR	NR	+	−
Indole	−	−	−	−	−	−	−	−	NR	+	NR	−	NR	−	−	NR	NR	−	NR	NR	NR	NR	−
Dihydroxyacetone	+	−	−	−	NR	NR	−	NR	NR	−	NR	−	NR	−	NR	NR	NR	−	NR	NR	NR	−	−
pH in V–P broth#	4.5–6.8	4.5–5.1	5.5–6.6	4.5–5.0	<5.5	<5.5	<6	<5.5	NR	<6	5.0–5.2	4.6–4.7	NR	4.6–4.7	NR	NR	NR	NR	NR	NR	NR	NR	5.0
Tyrosinase	−	−	−	−	−	−	−	−	NR	−	−	−	NR	−	NR	NR	NR	NR	NR	−	−	NR	−
Caseinase	+	−	+	−	−	−	−	V	+	+	+	−	V	−	NR	−	NR	+	+	+	+	+	−
Amylases	+	−	+	+	+	+	+	+	+	+	+	+	NR	+	NR	+	+	−	+	+	+	+	+
Citrate	−	−	+	V	−	−	−	−	NR	−	−	−	V	−	−	−	NR	−	−	W	W	+	−
Growth at/in:																							
pH 5.6	+	+	+	+	−	+	−	+	−	+	+	+	+	+	+	NR	NR	+	+	+	+	+	+
50 °C	−	−	−	+	−	−	−	−	−	−	+	+	NR	+	−	−	−	NR	−	−	−	−	−
5% NaCl	−	−	−	−	+	−	−	V	+	+	−	−	+	−	+	NR	NR	−	−	−	−	−	W
Utilization of:																							
L-Arabinose	+	−	+	+	+	+	−	+	+	−	+	+	V	−	+	+	+	+	+	+	−	−	−
Mannitol	+	+	+	+	+	+	+	+	−	−	+	+	NR	+	−	+	−	V	+	+	+	+	+
D-Xylose	+	−	+	+	+	+	+	+	+	−	+	+	NR	+	+	+	+	+	+	+	−	−	+

*Bosshard *et al.* (2002).†Berge *et al.* (2002).‡Elo *et al.* (2001).§Aguilera *et al.* (2001).||Yoon *et al.* (2003).¶von der Weid *et al.* (2002).

#V–P broth, Voges-Proskauer broth.

Table 2. Cellular fatty acid composition of strain GMP01^T and phylogenetically related species of the genus *Paenibacillus*

Species: 1, *Paenibacillus favisporus* GMP01^T; 2, *P. azoreducens*; 3, *P. chibensis*; 4, *P. illinoisensis*; 5, *P. macquariensis*. Data from Meehan *et al.* (2001).

Fatty acid	1	2	3	4	5
Straight-chain:					
C _{10:0}	1.2	3.5	0.3	2.0	0.7
C _{15:0}	3.6	0.1	0.6	1.0	1.2
C _{16:0}	8.7	22.1	5.3	24.7	2.9
Iso-branched:					
C _{14:0}	0.9	0.8	0.8	1.8	0.8
C _{15:0}	6.5	5.9	4.0	1.5	5.2
C _{16:0}	11.1	8.6	12.2	6.3	2.6
Anteiso-branched:					
C _{17:0}	3.3	5.6	3.2	1.4	0.4
C _{15:0}	48.8	33.9	57.8	56.6	81.0
C _{17:0}	15.0	19.8	14.3	4.9	0.7

caseinase, phenylalanine deaminase, lysine decarboxylase, tyrosinase, indole, dihydroxyacetone, hydrogen sulfide, acetylmethylcarbinol and acetoin (Voges–Proskauer medium) are not produced. Nitrate is reduced to nitrite. Susceptible to erythromycin, penicillin, oxacillin, bacitracin, piperacillin, gentamicin and cefoxitin; resistant to tobramycin, cephalixin and dibekacin.

The type strain, GMP01^T (= LMG 20987^T = CECT 5760^T), was isolated from old cow dung in Salamanca, Spain.

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