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Paenibacillus glycanilyticus sp. nov., a novel species that degrades heteropolysaccharide produced by the cyanobacterium *Nostoc commune*

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A novel bacterial strain, DS-1^T, was isolated that degrades heteropolysaccharide produced by the cyanobacterium *Nostoc commune*. The isolate was identified by a combination of phenotypic characterization, cellular fatty acid analysis, DNA base composition, DNA–DNA hybridization and 16S rRNA gene sequence analysis. Phylogenetic analysis placed strain DS-1^T within the *Paenibacillus* cluster on a phylogenetic tree and the phenotypic characteristics of this strain appear to be similar to those of *Paenibacillus curdlanolyticus* IFO 15724^T and *Paenibacillus kobensis* IFO 15729^T. The strain was distinguished from *P. curdlanolyticus* IFO 15724^T and *P. kobensis* IFO 15729^T by its ability to degrade the polysaccharide of *Nostoc commune*, by assimilation of rhamnose, inositol and L-fucose and by its wide range of optimal growth temperature (28–37 °C). Like other *Paenibacillus* species, this strain contains anteiso-$C_{15:0}$ as a major cellular fatty acid, and it has a DNA G+C content of 50·5 mol%. Based on these results, it is concluded that this isolate should be placed within a novel species of *Paenibacillus*, *Paenibacillus glycanilyticus* sp. nov., with the type strain DS-1^T (= IFO 16618^T = JCM 11221^T = NRRL B-23455^T).

Keywords: *Paenibacillus glycanilyticus* sp. nov., Congo red staining assay, polysaccharide-degrading microbe, *Nostoc commune*

INTRODUCTION

Nostoc commune, a nitrogen-fixing filamentous cyanobacterium, produces copious amounts of slimy extracellular polysaccharide around its cells. Although this polysaccharide is thought to play an important role in the desiccation tolerance (Hill *et al*., 1994) and UV tolerance (Scherer *et al*., 1988) of this cyanobacterium, little is known about its chemical structure, primarily because of the structural complexity. Polysaccharide-degrading enzymes are one of the most useful tools for the structural study of complex polysaccharides. Helm *et al*. (2000) have tried to degrade the viscous polysaccharides of *Nostoc commune* DRH-1 by using conventional enzymes, but their attempt was unsuccessful.

In the course of our structural study of the polysaccharides of *Nostoc commune*, we first tried to obtain a microbe that could degrade this polysaccharide. In this paper, we report a soil bacterium, DS-1^T, with the ability to degrade β-linked *Nostoc commune* polysaccharide. Based on the sequence of the 16S rRNA genes, phenotypic characterization, DNA base composition, DNA–DNA hybridization and cellular fatty acid composition analysis, we have concluded that this isolate represents a novel species of *Paenibacillus*, *Paenibacillus glycanilyticus* sp. nov.

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A phylogenetic tree based on 16S rRNA gene sequences including a wider sample of the relatives of *Paenibacillus glycanilyticus* sp. nov. is available in IJSEM Online (http://ijs.sgmjournals.org/).

The DDBJ accession number for the 16S rRNA gene sequence of isolate DS-1^T is AB042938.

METHODS

Preparation of cyanobacterial polysaccharide. Field-grown *Nostoc commune* samples were washed with tap water and soaked in methanol at room temperature to remove lipophilic substances. The cells were then dried at room temperature for 3 days and ground in a mortar. The resulting powder was extracted with boiling water for 3 h. The mucilaginous suspension was filtered to remove insoluble material and the polysaccharide fraction was recovered by ethanol precipitation (final ethanol concentration 75%, v/v). After centrifugation (10000 ***g***, 4 °C for 15 min), the precipitate was dissolved in distilled water, dialysed and lyophilized to obtain the crude polysaccharide.

Isolation and cultivation of the organism. Isolation of the polysaccharide-degrading microbe was guided by a Congo red staining assay (Teather & Wood, 1982). The soil samples were suspended in distilled water and inoculated on agar plates (1·2% agar) containing 0·2% polysaccharide in BG-11 medium (Rippka *et al*., 1979) with the following composition (l^{-1} distilled water): 1·5 g $NaNO_3$, 0·04 g $K_2HPO_4 . 3H_2O$, 0·075 g $MgSO_4 . 7H_2O$, 0·036 g $CaCl_2 . 2H_2O$, 0·02 g Na_2CO_3 and a trace metal mixture including cobalt [2·86 g H_3BO_3, 1·81 g $MnCl_2 . 4H_2O$, 0·222 g $ZnSO_4 . 7H_2O$, 0·39 g $Na_2MoO_4 . 2H_2O$, 0·079 g $CuSO_4 . 5H_2O$, 0·0494 g $Co(NO_3)_2 . 6H_2O$]. After incubation for 2 days at 30 °C, the plates were flooded with an aqueous solution of Congo red (0·1%, w/v) for 15 min. The Congo red solution was then poured off and the plates were further flooded with 1 M NaCl for 15 min. A microbe strain, DS-1^T, that produced a clear zone around the colony was isolated from a soil sample from Osaka University Campus.

Three type strains, *Paenibacillus curdlanolyticus* IFO 15724^T, *Paenibacillus kobensis* IFO 15729^T and *Paenibacillus polymyxa* IAM 13419^T, were used as reference strains. For taxonomic study, all strains including isolate DS-1^T were grown on IFO medium 702 containing 1% polypeptone, 0·2% yeast extract and 0·1% $MgSO_4 . 7H_2O$ at 30 °C for 3 days.

TLC of degradation products. The isolated microbe DS-1^T was inoculated into BG-11 medium (Rippka *et al*., 1979) containing 0·2% cyanobacterial polysaccharides and cultured at 30 °C for 3 days using a rotary shaker. After centrifugation (10000 ***g***, 4 °C for 30 min), the supernatant was concentrated under vacuum. The degradation products were analysed by silica gel TLC (Merck no. 5554 aluminium plate) with a solvent system of 1-butanol/acetic acid/water (2:1:1, by vol.) together with oligosaccharide standards. Spots were visualized by heating at 110 °C for 5 min after spraying with orcinol reagent.

16S rRNA analysis. Genomic DNA of isolate DS-1^T was prepared according to an established protocol (Sambrook *et al*., 1989). Gene fragments specific to 16S rRNA-encoding regions were amplified by PCR, as described previously (Kawasaki *et al*., 1993), using primers 20F (5′-GAGTTTGATCCTGGCTCAG-3′; positions 9–27) and 1500R (5′-GTTACCTTGTTACGACTT-3′; positions 1509–1492). The numbers of positions in the rRNA gene fragments were based on the *Escherichia coli* numbering system (accession number V00348; Brosius *et al*., 1981).

Amplified 16S rRNA genes were sequenced directly with an ABI PRISM Bigdye Terminator cycle sequencing ready reaction kit on an ABI PRISM model 310 Genetic Analyzer. The following six primers were used for sequencing: 20F, 1500R, 520F (5′-CAGCAGCCGCGGTAATAC-3′; positions 519–536), 520R (5′-GTATTACCGCGGCTGCTG-3′; positions 536–519), 920F (5′-AAACTCAAATGAATTGACGG-3′; positions 907–926) and 920R (5′-CCGTCAATTCATTTGAGTTT-3′; positions 926–907). The sequence data were applied to a BLAST homology search (Altschul *et al*., 1997) to obtain highly similar sequences. Multiple alignments of the sequences were carried out with the program CLUSTAL W version 1.7 (Thompson *et al*., 1994). Distance matrices for the aligned sequences were calculated using the two-parameter method of Kimura (1980). The neighbour-joining method was used to construct a phylogenetic tree (Saitou & Nei, 1987). The robustness of individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985).

Phenotypic characterization. Phenotypic characterization was carried out as described by Takagi *et al*. (1993). The carbon-source assimilation of strain DS-1^T and reference strains was checked by using the API 50 CH carbon-source assimilation test kit (bioMérieux) according to the manufacturer's instructions.

Determination of DNA base compositions and DNA–DNA hybridization. DNA was obtained by the method of Saito & Miura (1963). The G+C contents of the DNAs were determined by the method of Mesbah *et al*. (1989). DNA similarities were determined by fluorometric DNA–DNA hybridization in microdilution wells, as described by Ezaki *et al*. (1989). DNA–DNA hybridization was carried out at 45 °C for 2 h.

Cellular fatty acid composition. Cells of strain DS-1^T, *P. curdlanolyticus* IFO 15724^T, *P. kobensis* IFO 15729^T and *P. polymyxa* IAM 13419^T were cultivated in IFO medium 702 at 30 °C for 4 days. Preparation and identification of cellular fatty acids were carried out as described by Komagata & Suzuki (1987). Standards of fatty acid methyl esters were obtained from Nippon Fine Chemical Co. Samples were analysed by using a QP-5000 GC/MS spectrometer (Shimadzu).

RESULTS AND DISCUSSION

Congo red dye forms an insoluble, deep-pink complex with β-linked polysaccharides (Cantwell & McConnell, 1983). In this study, bacterial strains that could degrade *Nostoc commune* polysaccharides were screened for by the clear zone assay using this dye, as this polysaccharide is considered to have a β-1,4-linked xylogalactoglucan backbone (Helm *et al*., 2000). A bacterial strain, DS-1^T, was isolated by this method, and an obvious clear zone was observed around colonies after washing out unbound dye. Fig. 1 shows the TLC profile of the degradation products (oligosaccharides) of this isolate. The cyanobacterial polysaccharide was degraded completely to oligosaccharides, ranging from tetra- to hexasaccharides.

Strain DS-1^T was found to be Gram-positive, with its cells being motile rods more than 0·8 μm in diameter. Ellipsoidal spores are formed in swollen sporangia (Fig. 2).

Phylogenetic analysis based on 16S rRNA sequences was performed to determine the phylogenetic position

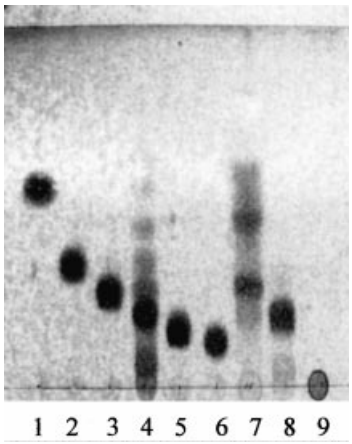

Fig. 1. TLC profiles of oligosaccharides. Lanes: 1, glucose; 2, isomaltotriose; 3, isomaltotetraose; 4, isomaltopentaose; 5, isomaltohexaose; 6, isomaltoheptaose; 7, acid hydrolysate of cyanobacterial polysaccharide; 8, products of degradation of cyanobacterial polysaccharide by strain DS-1^T; 9, cyanobacterial polysaccharide.

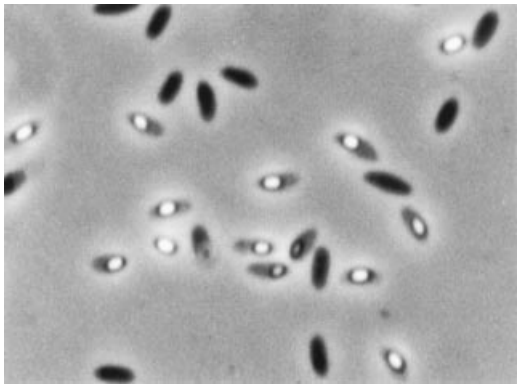

Fig. 2. Phase-contrast micrograph of sporulating cells of strain DS-1^T. Cells were cultured on IFO medium 802 containing 1% polypeptone, 0·2% yeast extract, 1·5% agar and 0·1% $MgSO_4 . 7H_2O$ at 30 °C for 3 days.

of strain DS-1^T. Fig. 3 shows that strain DS-1^T belongs to the genus *Paenibacillus* and is closely related to *P. curdlanolyticus* IFO 15724^T and *P. kobensis* IFO 15729^T with a high bootstrap value (95·0%). The same results were obtained when the phylogenetic tree was constructed based on a wider sample of species (available as supplementary material in IJSEM Online at http://ijs.sgmjournals.org/).

The phenotypic characters of strain DS-1^T were compared with those of *P. curdlanolyticus* IFO 15724^T, *P. kobensis* IFO 15729^T and *P. polymyxa* IAM 13419^T. Obvious differences were observed in colony colour and optimum growth temperature. Colonies of strain DS-1^T were pinkish yellow when grown on agar plates, whereas colonies of the other strains were white. Strain DS-1^T was found to grow well at a comparatively wide range of temperatures (28–37 °C), whereas the other type strains had an optimum growth temperature of 30 °C (Table 1).

The carbon-source assimilation pattern of strain DS-1^T was similar to those of other *Paenibacillus* species, except for rhamnose, inositol and L-fucose assimilation (Table 2).

The major cellular fatty acid of strain DS-1^T, *P. curdlanolyticus* IFO 15724^T, *P. kobensis* IFO 15729^T and *P. polymyxa* IAM 13419^T was found to be anteiso-branched $C_{15:0}$ (Table 3), which is present as a dominant cellular fatty acid in all members of the genus *Paenibacillus* (Shida *et al.*, 1997).

Analyses of DNA relatedness between strain DS-1^T and *P. curdlanolyticus* IFO 15724^T, *P. kobensis* IFO 15729^T and *P. polymyxa* IAM 13419^T gave low DNA-similarity values between 2·0 and 10%.

We also examined the polysaccharide-degrading activity of these strains. All strains except for isolate DS-1^T could not degrade *Nostoc commune* polysaccharides on agar plate medium.

A novel species, *Paenibacillus granivorans* A30^T (van der Maarel *et al.*, 2000), which degrades native potato starch granules, has recently been proposed. In the present study, the phylogenetic lineage of *P. granivorans* A30^T was combined with the lineage constructed for DS-1^T, *P. curdlanolyticus* IFO 15724^T and *P. kobensis* IFO 15729^T. The similarity between the 16S rRNA sequences of strain DS-1^T and *P. granivorans* A30^T was 94·8% in a comparison of 1448 bases, except for a gap, indicating that strain DS-1^T represents a species distinct from *P. granivorans* A30^T. Some phenotypic characters were also found to be different for strain DS-1^T when compared with the profile for *P. granivorans* A30^T. Unlike *P. granivorans* A30^T, strain DS-1^T can grow at pH 5·7 and assimilate mannitol. Optimal growth was obtained at 37 °C for *P. granivorans* A30^T and at 28–37 °C for strain DS-1^T. The DNA G+C contents were significantly different for strain DS-1^T (50·5 mol%) and *P. granivorans* A30^T (47·8 mol%).

On the basis of the results described above, we propose that strain DS-1^T be assigned to a novel species within the *Paenibacillus* cluster, *Paenibacillus glycanilyticus* sp. nov.

Description of *Paenibacillus glycanilyticus* sp. nov.

Paenibacillus glycanilyticus (gly.can.i.ly′ti.cus. N.L. n. *glycanum* glycan, a heteropolysaccharide; Gr. adj. *lyticus* dissolving; N.L. adj. *glycanilyticus* degrading heteropolysaccharide).

Cells are rod-shaped, 0·5–0·8 μm by 3·0–5·0 μm. Gram-positive. Cells are motile by means of peritrichous flagella. Ellipsoidal spores are formed in swollen

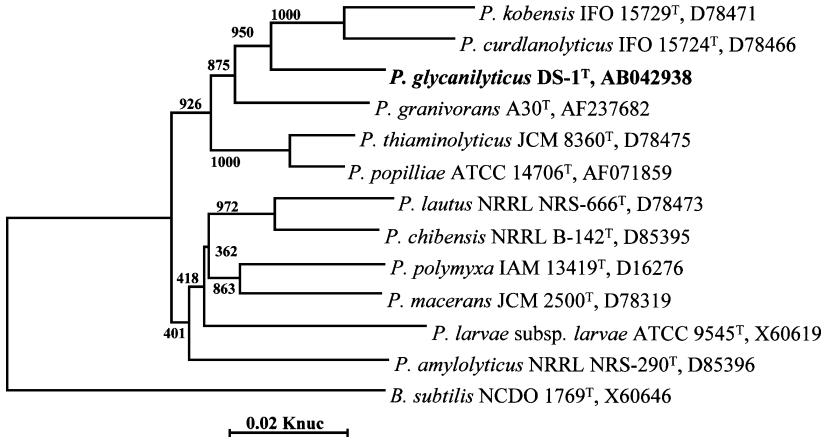

Fig. 3. Phylogenetic relationship of *Paenibacillus glycanilyticus* sp. nov. to members of the genus *Paenibacillus* and *Bacillus subtilis*. The phylogenetic tree is based on a comparison of 16S rRNA gene sequences (1237 bases) and was constructed using the neighbour-joining method. The sequences were retrieved from the DDBJ, the Center for Information Biology and the National Institute of Genetics. A tree including a wider sample of the relatives of the novel species is available as supplementary material in IJSEM Online (http://ijs.sgmjournals.org/).

Table 1. Phenotypic characteristics of strain DS-1^T and related strains

All of the strains were positive for catalase and growth at pH 5·7. All of the strains were negative for utilization of citrate, succinate, acetate, fumarate and L-malate, for oxidase and for growth at 50 °C and in the presence of 5% NaCl.

Characteristic	Strain DS-1^T	*P. curdlanolyticus* IFO 15724^T	*P. kobensis* IFO 15729^T	*P. polymyxa* IAM 13419^T
Colony colour	Pinkish yellow	White	White	White
Anaerobic growth	−	−	−	+
Litmus milk	−	−	−	+
Optimum growth temperature (°C)	28–37	30	30	30
DNA G+C content (mol%)	50·5	51·7	51·1	44·2

Table 2. Carbon-source assimilation profiles of strain DS-1^T and related strains

The following carbon sources were utilized by all four strains: L-arabinose, D-xylose, methyl β-D-xyloside, galactose, D-glucose, methyl α-D-glucoside, amygdalin, aesculin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, D-raffinose, starch, glycogen and β-gentibiose. The following carbon sources were not utilized by any of the four strains: erythritol, D-arabinose, L-xylose, adonitol, L-sorbose, dulcitol, sorbitol, inulin, xylitol, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate.

Carbon source	Strain DS-1^T	*P. curdlanolyticus* IFO 15724^T	*P. kobensis* IFO 15729^T	*P. polymyxa* IAM 13419^T
Glycerol	+	+	−	+
Ribose	+	−	−	+
D-Fructose	+	+	−	+
D-Mannose	+	−	−	+
Rhamnose	+	−	−	−
Inositol	+	−	−	−
Mannitol	+	−	−	+
Methyl α-D-mannoside	+	+	−	−
N-Acetyl glucosamine	+	+	+	−
Arbutin	+	+	−	+
Salicin	+	+	−	+
Melezitose	+	+	+	−
D-Turanose	+	+	−	+
L-Fucose	+	−	−	−

Table 3. Cellular fatty acid compositions of strain DS-1^T and related strains

Fatty acid	Strain DS-1^T	*P. curdlanolyticus* IFO 15724^T	*P. kobensis* IFO 15729^T	*P. polymyxa* IAM 15729^T
Straight-chain saturated				
$C_{14:0}$	1·0	0·8	1·2	2·9
$C_{15:0}$	3·2	0·4	0·9	0·8
$C_{16:0}$	13·0	0·6	16·1	15·6
$C_{17:0}$	1·3	9·9	0·2	0·3
Iso-branched saturated				
$C_{14:0}$	2·5	1·8	2·3	2·4
$C_{15:0}$	5·0	5·4	6·1	9·7
$C_{16:0}$	21·8	15·4	14·5	8·6
$C_{17:0}$	4·8	16·4	5·2	8·5
Anteiso-branched saturated				
$C_{15:0}$	38·0	44·6	38·5	41·3
$C_{17:0}$	9·4	4·7	14·0	8·4
Unsaturated				
$C_{16:1}$	ND	ND	1·0	1·5

ND, Not detected.

sporangia. Colonies are flat, smooth, circular, entire and pinkish yellow. Catalase is produced. Oxidase is not produced. Facultatively aerobic. Citrate, succinate, acetate, fumarate and malate are not utilized. Growth occurs at pH 5·7. There is no change in litmus milk. Does not grow in the presence of 5% NaCl. Optimal growth is obtained at 28–37 °C. No growth at 50 °C. The DNA G+C content is 50·5 mol%. The major cellular fatty acid component is anteiso-$C_{15:0}$. The following carbon sources are assimilated: glycerol, L-arabinose, ribose, D-xylose, methyl β-D-xyloside, galactose, D-glucose, D-fructose, D-mannose, rhamnose, inositol, mannitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetyl glucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, D-raffinose, starch, glycogen, β-gentibiose, D-turanose and L-fucose. The following carbon sources are not assimilated: erythritol, D-arabinose, L-xylose, adonitol, L-sorbose, dulcitol, sorbitol, inulin, xylitol, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate.

The type strain is strain DS-1^T (= IFO 16618^T = JCM 11221^T = NRRL B-23455^T), isolated from soil.

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