NOTE

***Paenibacillus chinjuensis* sp. nov., a novel exopolysaccharide-producing bacterium**

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A novel exopolysaccharide-producing bacterium (WN9^T) was isolated from Chinju, Korea, and was identified as a member of the genus *Paenibacillus* on the basis of phenotypic characteristics and phylogenetic inference based on 16S rDNA sequence. This organism is a facultatively anaerobic, endospore-forming rod. The diamino acid found in the peptidoglycan is meso-diaminopimelic acid. The predominant menaquinone is MK-7. The major cellular fatty acid is anteiso-C_{15:0}. The G+C content is 53 mol%. The phylogenetic tree shows that strain WN9^T falls within the radiation of a cluster comprising the *Paenibacillus* species. The levels of 16S rDNA similarity between strain WN9^T and the type strains of validly described *Paenibacillus* species are 92.1–95.8%. Strain WN9^T is clearly distinguishable from some phylogenetically related *Paenibacillus* species on the basis of DNA–DNA relatedness data and phenotypic characters. Therefore, on the basis of these data, a novel species of the genus *Paenibacillus*, *Paenibacillus chinjuensis* sp. nov., is proposed. The type strain is strain WN9^T (= KCTC 8951P^T = JCM 10939^T).

Keywords: *Paenibacillus chinjuensis* sp. nov., 16S rDNA sequence analysis, chemotaxonomy

The members of the genus *Bacillus* are widely distributed in nature and have physiologically diverse characteristics (Claus & Berkeley, 1986). Some species are known to secrete a variety of extracellular enzymes, such as polysaccharide-hydrolysing enzymes, or to produce polysaccharides (Claus & Berkeley, 1986; Priest, 1977). For a long time, most aerobic, endospore-forming rods were assigned to the genus *Bacillus* (Claus & Berkeley, 1986). The results of genomic analyses have proved that such classification led to the single genus *Bacillus* becoming a systematically heterogeneous taxon (Priest, 1981; Ash *et al.*, 1991; Slepecky & Hemphill, 1991; Stackebrandt & Liesack, 1993; Nakamura, 1996). In particular, 16S rRNA sequence analyses have revealed the presence of several phylogenetically distinct lineages within the genus *Bacillus*. Subsequently, some phylogenetic groups have been proposed as the following new genera: *Allicyclobacillus* (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 *Virgibacillus* (Heyndrickx *et al.*, 1998). More recently, *Bacillus dipsosauri* was transferred to the new genus *Gracilibacillus*, together with a novel species, *Gracilibacillus halotolerans*, and *Bacillus salexigens* was reclassified in a new genus as *Salibacillus salexigens* (Wainø *et al.*, 1999).

There is now general agreement that the classification and identification of Gram-positive, endospore-forming rods should be performed by using a polyphasic taxonomic approach that integrates phylogenetic analysis based on 16S DNA sequence, genomic relatedness and extensive phenotypic characteristics (Shida *et al.*, 1997a, b; Yoon *et al.*, 1998; Tcherpakov *et al.*, 1999). Recently, a rod-shaped, endospore-forming bacterial strain (WN9^T) that produces a novel exopolysaccharide was isolated from soil from Korea and was tentatively identified as a member of the genus *Paenibacillus* (Seo *et al.*, 1999). In this study, strain WN9^T is characterized further, using a combination of phenotypic and genomic characteristics and phylogenetic analysis, to confirm its taxonomic status.

Strain WN9^T was isolated from a soil sample in Chinju, Korea, by using the dilution plating technique on a

A phylogenetic tree containing additional reference sequences is available as supplementary data at IJSEM Online (<http://ijs.sgmjournals.org/>).

The GenBank/EMBL/DBJ accession number for the 16S rDNA sequence of strain WN9^T (= KCTC 8951P^T = JCM 10939^T) is AF164345.

solid medium that contained (l^{-1}) 2.5 g yeast extract, 2.5 g malt extract, 30 g glucose, 2 g K_2HPO_4 , 0.5 g $MgSO_4 \cdot 7H_2O$ and 15 g agar (pH 7.0). The isolation procedure is described in a previous study (Seo *et al.*, 1999). For the investigation of morphological and physiological characteristics, strain WN9^T was, in most cases, cultivated on trypticase soy agar or broth (BBL) at 30 °C. Cell mass for the analyses of the cell wall and menaquinone was obtained from trypticase soy broth culture. Biomass for extraction of DNA from strain WN9^T and some reference strains was obtained using trypticase soy broth. All strains were cultivated at 30 °C on a horizontal shaker at 150 r.p.m. and the broth cultures were checked microscopically for purity before they were harvested by centrifugation. Strain WN9^T and the reference strains were also cultivated at 30 °C for 3 days on trypticase soy agar for fatty acid methyl ester analysis. Reference strains included *Paenibacillus alginolyticus* DSM 5050^T, *Paenibacillus chondroitinus* DSM 5051^T, *Paenibacillus larvae* subsp. *pulvificiens* DSM 3615^T and *Paenibacillus validus* DSM 3037^T.

The morphology of the cells was examined by phase-contrast microscopy and transmission electron microscopy. The flagellum type was examined by transmission electron microscopy using cells from exponentially growing culture, as described previously (Yoon *et al.*, 1999). Motility was determined with an optical microscope, using the hanging-drop technique (Skerman, 1967). Catalase activity was determined by bubble production in a 3% (v/v) hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1% *p*-aminodimethylaniline oxalate. Hydrolysis of casein, gelatin, starch, hypoxanthine, tyrosine, Tween 80 and xanthine and production of urease were determined as described previously (Cowan & Steel, 1965). Hydrolysis of aesculin and nitrate reduction were tested as described by Lanyi (1987). Hydrolysis of arbutin was tested with the method of Kurup & Fink (1975). Hydrolysis of elastin was determined according to the method of Williams *et al.* (1983). Tolerance to sodium chloride was measured on trypticase soy broth, at concentrations of 1–8% (w/v). Acid production from some carbohydrates was detected by using the method of Hugh & Leifson (1953).

Chromosomal DNA was isolated and purified according to the method of Yoon *et al.* (1996), with the exception that ribonuclease T1 was used together with ribonuclease A. The isomer type of diamino acid of the peptidoglycan was analysed by the method of Komagata & Suzuki (1987). Menaquinones were analysed as described previously (Komagata & Suzuki, 1987), using reverse-phase HPLC. For quantitative analysis of cellular fatty acid compositions, a loop of cell mass was harvested and fatty acid methyl esters were prepared and identified according to the instructions of the Microbial Identification System (MIDI). The G+C content of the DNA was determined by using the method of Tamaoka & Komagata (1984). DNA was hydrolysed and the resultant nucleotides were

Fig. 1. Transmission electron micrograph of strain WN9^T from exponentially growing culture. Bar, 1 µm.

analysed by reverse-phase HPLC. A DNA–DNA hybridization study was performed fluorometrically by using the method of Ezaki *et al.* (1989), with photobiotin-labelled DNA probes and microdilution wells.

Sequencing of 16S rDNA was performed as described previously (Yoon *et al.*, 2000). Alignment of sequences was carried out with the CLUSTAL W software (Thompson *et al.*, 1994). Gaps at the 5' and 3' ends of the alignment were omitted from further analyses. Evolutionary distance matrices were calculated by using the algorithm of Jukes & Cantor (1969) with the DNADIST program within the PHYLIP package (Felsenstein, 1993). A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) as implemented within the NEIGHBOR program of the same package. The stability of relationships was assessed by a bootstrap analysis of 1000 datasets by using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package. GenBank and EMBL accession numbers for reference 16S rDNA sequences used in this analysis are given in Fig. 2.

Strain WN9^T was Gram-positive but Gram-variable in old cultures. It formed peritrichously flagellated rods, 0.8–1.1 µm wide and 3.0–5.0 µm long, in 24 h culture on trypticase soy agar at 30 °C (Fig. 1). Ellipsoidal spores were observed in swollen sporangia. Colonies were light pink, smooth, glossy, circular and convex after 3–4 days of growth on trypticase soy agar. Colonies became flat with a slightly irregular form as the culture aged. Growth of strain WN9^T occurred at 20 and 45 °C, but not at 15 or 50 °C. The optimal growth temperature was in the range 30–37 °C. Strain WN9^T grew well at pH 6.5–7.3 but growth was

Table 1. Distinctive phenotypic properties of some *Paenibacillus* species and strain WN9^T

Taxa are listed as: 1, *P. alginolyticus*; 2, *P. chondroitinus*; 3, *P. larvae* subsp. *larvae*; 4, *P. larvae* subsp. *pulvifaciens*; 5, *P. validus*; 6, strain WN9^T. Data are from this study and from Nakamura (1987), Heyndrickx *et al.* (1995, 1996), Shida *et al.* (1997a) and Pettersson *et al.* (1999). Strain WN9^T and all other *Paenibacillus* species shown have oval-shaped spores, swollen sporangia and are positive for acid production from D-glucose, D-ribose and trehalose. +, Positive reaction; –, negative reaction; v, variable reaction; NT, not tested.

Character	1	2	3	4	5	6
Anaerobic growth	–	–	+	+	–	+
Catalase	+	+	–	–	+	+
Oxidase	–	–	NT	–	–	+
Nitrate reduction	–	–	V	V	V	–
Hydrolysis of:						
Casein	–	–	+	+	NT	+
Gelatin	NT	NT	+	+	–	+
Starch	+	+	–	–	NT	+
Urea	+	+	–	–	–	–
Tween 80	+	+	NT	NT	–	+
Decomposition of tyrosine	–	–	–	–	NT	–
Acid production from:						
L-Arabinose	+	+	–	–	–	–
D-Cellobiose	+	+	–	–	V	+
D-Fructose	+	+	V	V	+	–
D-Galactose	+	+	V	V	+	–
Glycerol	NT	NT	+	+	+	–
Inulin	NT	NT	–	–	V	–
Lactose	+	+	–	–	V	–
Maltose	+	+	–	–	+	+
D-Mannitol	+	+	–	+	+	–
D-Mannose	+	+	+	+	V	+
D-Raffinose	NT	NT	–	–	V	–
L-Rhamnose	+	+	–	–	–	–
Salicin	+	+	+	–	–	–
D-Sorbitol	–	–	–	–	V	–
Starch	NT	NT	–	–	V	–
D-Xylose	+	+	–	–	+	–
Growth at/in:						
2% NaCl	NT	NT	+	+	NT	–
50 °C	–	–	–	–	+	–
pH 5.6	+	+	–	–	NT	+
Optimum growth temperature (°C)	28–30	28–30	35–37	28–30	28–35	30–37
G + C content (mol %)	47–49	47–48	42–43	42–43	50–52	53

inhibited at pH 4.5 and 9. The strain failed to grow on trypticase soy broth containing more than 2% (w/v) NaCl. Growth occurred under anaerobic conditions. Strain WN9^T had catalase, oxidase and DNase activities but no urease activity. Arbutin, casein, aesculin, gelatin, starch and Tween 80 were hydrolysed, but no hydrolysis of elastin, hypoxanthine, tyrosine and xanthine was observed. Acid was produced from D-cellobiose, D-glucose, maltose, D-mannose, D-ribose and trehalose but not from L-arabinose, D-fructose, D-galactose, glycerol, inulin, lactose, D-mannitol, D-raffinose, L-rhamnose, salicin, D-sorbitol, starch or D-xylose. The phenotypic properties of strain WN9^T are summarized in Table 1, together with those of some *Paenibacillus* species.

Strain WN9^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The predominant isoprenoid quinone was unsaturated menaquinone with seven isoprene units (MK-7). The cellular fatty acid profile of strain WN9^T is shown in Table 2, together with those of *P. alginolyticus* DSM 5050^T, *P. chondroitinus* DSM 5051^T, *P. larvae* subsp. *pulvifaciens* DSM 3615^T and *P. validus* DSM 3037^T. Strain WN9^T had a cellular fatty acid profile containing large amounts of saturated and branched fatty acids and anteiso-C_{15:0} (66.1%) as the major fatty acid (Table 2). This fatty acid profile is similar to those of the type strains of *Paenibacillus* species used in this study, but there are small differences between them in terms of the proportions of

Table 2. Cellular fatty acid profiles of some *Paenibacillus* species and strain WN9^T

Taxa are listed as: 1, *P. alginolyticus* DSM 5050^T; 2, *P. chondroitinus* DSM 5051^T; 3, *P. larvae* subsp. *pulvifaciens* DSM 3615^T; 4, *P. validus* DSM 3037^T; 5, strain WN9^T.

Fatty acid	1	2	3	4	5
Saturated fatty acids:					
C _{13:0} 2OH					0.6
C _{14:0}	1.3	1.3	0.7	1.7	0.8
C _{15:0}	1.4	2.7	4.9	1.5	4.5
C _{16:0}	3.7	4.1	4.7	7.2	5.3
C _{17:0}			0.9	0.2	0.3
C _{18:0}	0.2	0.5	0.2	0.3	
Unsaturated fatty acids:					
C _{16:1} ω7c alcohol	0.4			0.9	
C _{16:1} ω11c				2.6	
C _{18:1} ω9c	0.2	0.2		0.3	
Branched fatty acids:					
iso-C _{13:0}		0.2	0.1	0.1	
iso-C _{13:0} 3OH					0.3
anteiso-C _{13:0}				0.1	0.2
iso-C _{14:0}	1.5	5.4	0.6	2.3	2.2
iso-C _{15:0}	13.1	12.9	16.6	15.1	5.0
anteiso-C _{15:0}	62.6	54.3	38.2	52.7	66.1
iso-C _{16:0}	7.6	13.0	7.4	6.3	7.0
anteiso-C _{16:0}			0.4		
iso-C _{17:0}	3.4	2.3	7.5	3.3	1.6
anteiso-C _{17:0}	4.7	3.0	16.3	4.6	6.1
iso-C _{17:1} ω10c				0.5	
Summed features:*					
3			1.4		
4				0.3	

* Summed features represent groups of two or three fatty acids that could not be separated by GLC with the Microbial Identification System (MIDI). Summed feature 3 contained one or more of following fatty acids: iso-C_{15:0} 2OH and/or C_{16:1} ω7c. Summed feature 4 contained one or more of following fatty acids: iso-C_{17:1} I and/or anteiso-C_{17:1} B.

anteiso-C_{15:0} and iso-C_{15:0} (Table 2). The G + C content of strain WN9^T is 53 mol %, which is in the range for known *Paenibacillus* species (Shida *et al.*, 1997a).

The almost complete 16S rDNA sequence of strain WN9^T was determined in this study. This sequence comprised 1488 nucleotides, corresponding to approximately 96 % of the *Escherichia coli* 16S rRNA sequence. The 16S rDNA sequence of strain WN9^T was compared with those of all validly described *Paenibacillus* species, representatives of some related taxa and *E. coli* (as the outgroup). The phylogenetic tree shows that strain WN9^T falls within the evolutionary radiation enclosed by the genus *Paenibacillus* and forms a coherent cluster with the type strains of *P. validus*, *P. larvae*, *P. chondroitinus* and *P. alginolyticus*, particularly with *P. validus* DSM 3037^T (Fig. 2). The relationship between this cluster and the cluster comprising other *Paenibacillus* species is supported by a bootstrap resampling value of 100 % (Fig. 2). In particular, the relationship between strain WN9^T and

P. validus DSM 3037^T is supported by a relatively high bootstrap value (87.4 %). A tree containing additional reference sequences is available as supplementary material at IJSEM Online (<http://ijs.sgmjournals.org/>). Strain WN9^T also exhibited the highest degree of relatedness to *P. validus* DSM 3037^T, sharing 95.8 % 16S rDNA similarity. The 16S rDNA similarity values between strain WN9^T and the type strains of other validly described *Paenibacillus* species are in the range 92.1–95.2 %. DNA–DNA hybridization was performed between strain WN9^T and the type strains of some *Paenibacillus* species that formed a coherent cluster with high bootstrap values in the phylogenetic analysis. Strain WN9^T exhibited levels of DNA–DNA relatedness of 3.7, 3.0, 4.0 and 7.2 % with *P. alginolyticus* DSM 5050^T, *P. chondroitinus* DSM 5051^T, *P. larvae* subsp. *pulvifaciens* DSM 3615^T and *P. validus* DSM 3037^T, respectively.

The combination of phenotypic, chemotaxonomic and phylogenetic data shows that strain WN9^T belongs to

Fig. 2. Phylogenetic tree based on 16S rDNA sequences, showing the positions of strain WN9^T and other *Paenibacillus* species. Bar, 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points. A tree containing additional reference sequences is available as supplemental material at IJSEM Online (<http://ijs.sgmjournals.org/>).

the genus *Paenibacillus*. Strain WN9^T exhibited the closest phylogenetic affiliation to *Paenibacillus* species in comparative 16S rDNA sequence analysis. Phylogenetic inference places strain WN9^T within the radiation of the cluster containing the *Paenibacillus* species (Fig. 2). Phylogenetic analysis based on 16S rDNA sequences is now recognized as the most important method for inferring the relationships of Gram-positive, endospore-forming rods (Ash *et al.*, 1991, 1993; Wisotzkey *et al.*, 1992; Shida *et al.*, 1996; Spring *et al.*, 1996). Nevertheless, chemotaxonomic characterization may be necessary to support the result of the monothetic classification by 16S rDNA analysis. Chemotaxonomic properties and G+C content do not necessarily allow clear differentiation between aerobic or facultatively anaerobic, rod-shaped, endospore-forming genera. However, the genus *Paenibacillus* is differentiated from other related genera primarily by the composition of major fatty acids (Shida *et al.*, 1997a). The genus *Paenibacillus* has anteiso-C_{15:0} as the major fatty acid, whereas other rod-shaped, endospore-forming genera have been known to contain significant amounts of additional fatty acids or to have another fatty acid as the major fatty acid (Shida *et al.*, 1997a; Wainø *et al.*, 1999). The cellular fatty acid profile obtained for strain WN9^T, together with the types of diaminopimelic acid and the predominant menaquinone, are consistent with the result of phylogenetic classification.

Strain WN9^T exhibited levels of 16S rDNA similarity of 92.1–95.8% with the type strains of all validly described *Paenibacillus* species. According to the data compilation of Stackebrandt & Goebel (1994), strains with a difference in 16S rDNA sequence of more than 3% can be considered as different species. Accordingly, the levels of nucleotide similarity obtained in this study are low enough to categorize strain WN9^T as a distinct species within the genus *Paenibacillus*. Phenotypic characteristics of strain WN9^T were compared with those of some *Paenibacillus* species that form a coherent phylogenetic cluster. As shown in Table 1, the physiological characteristics of strain WN9^T are

different from those of these *Paenibacillus* species. The levels of DNA–DNA relatedness between strain WN9^T and the type strains of some phylogenetically related *Paenibacillus* species were such that strain WN9^T could be distinguished genetically from those *Paenibacillus* species (Wayne *et al.*, 1987). Although not all *Paenibacillus* species were used in DNA–DNA relatedness tests, those used in this study were considered to be sufficient because they and strain WN9^T form a coherent phylogenetic cluster, the relationship of which to other *Paenibacillus* species is supported by bootstrap analysis at a confidence level of 100% (Fig. 2). Differences in some phenotypic characteristics and genetic distinctiveness, together with phylogenetic data from 16S rDNA sequences, indicate that strain WN9^T is separate from previously described *Paenibacillus* species.

On the basis of the data presented above, strain WN9^T should be placed in the genus *Paenibacillus* as a novel species, for which we propose the name *Paenibacillus chinjuensis* sp. nov.

Description of *Paenibacillus chinjuensis* sp. nov.

Paenibacillus chinjuensis (chin.ju.en'sis. N.L. adj. *chinjuensis* of Chinju, the city in Korea where the type strain was isolated).

Cells are facultatively anaerobic rods, 0.8–1.1 µm wide and 3.0–5.0 µm long, in 24 h culture on trypticase soy agar. Ellipsoidal spores are formed in swollen sporangia. Cells are Gram-positive, but Gram-variable in old cultures. Motile by means of peritrichous flagella. Colonies are light pink, smooth, glossy, circular and convex after 3–4 days on trypticase soy agar. Colonies are flat and slightly irregular in old cultures. Catalase-, oxidase- and DNase-positive. Urease-negative. Arbutin, casein, aesculin, gelatin, starch and Tween 80 are hydrolysed. Elastin, hypoxanthine, tyrosine and xanthine are not hydrolysed. Acid is produced from D-cellobiose, D-glucose, maltose, D-mannose, D-ribose and trehalose. No acid is produced from L-arabinose,

D-fructose, D-galactose, glycerol, inulin, lactose, D-mannitol, D-raffinose, L-rhamnose, salicin, D-sorbitol, starch or D-xylose. No growth occurs in the presence of more than 2% (w/v) NaCl. Growth occurs at 20 and 45 °C but not at 15 or 50 °C. The optimal temperature for growth is 30–37 °C. The optimal pH for growth is 6.5–7.3. Growth is very slow or inhibited below pH 4.5 and above pH 9. Cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-7. The major fatty acid is anteiso-C_{15:0}. The G + C content is 53 mol% (determined by HPLC). Isolated from soil in Chinju, Korea. The type strain is strain WN9^T, which has been deposited in the Korean Collection for Type Cultures as KCTC 8951P^T and the Japan Collection of Microorganisms as JCM 10939^T.

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