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Paenibacillus campinasensis sp. nov., a cyclodextrin-producing bacterium isolated in Brazil

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An alkaliphilic, endospore-forming bacterium isolated from Brazilian soil was taxonomically studied and is proposed as a new *Paenibacillus* species. This organism (strain 324^T) was particularly distinguishable from other *Paenibacillus* species by its ability to grow optimally at pH 10 and 40 °C. The DNA G C content was 50·9 mol%. The diamino acid of the cell-wall peptidoglycan was *meso*-diaminopimelic acid. MK-7 was the predominant menaquinone and anteiso-$C_{15:0}$ was the major fatty acid. Levels of 16S rDNA similarity between strain 324^T and other *Paenibacillus* species were 90·6–95·9%. Phylogenetically, strain 324^T formed an evolutionary lineage distinct from other species within the evolutionary radiation encompassing the genus *Paenibacillus*. Based on phenotypic and chemotaxonomic properties, and phylogenetic inference, it is proposed that strain 324^T should be placed in the genus *Paenibacillus* as a new species, *Paenibacillus campinasensis*. The type strain of the new species is strain 324^T (= KCTC 0364BP^T).

Keywords: *Paenibacillus campinasensis*, alkaliphilic species, 16S rDNA analysis

INTRODUCTION

Traditionally, because of the lack of incisive differentiating criteria, all aerobic, endospore-forming organisms have been classified as *Bacillus*. This lack of differentiating criteria has also hampered recognition of new species and promoted grouping of organisms that failed to show clear-cut differences. Developments in molecular biological methods have suggested that the genus is a phylogenetically heterogeneous taxon. For example, analyses showed a wide range of G+C contents (32–69 mol%) among the DNAs of *Bacillus* species (Claus & Berkeley, 1986; Slepecky & Hemphill, 1991; Stackebrandt & Liesack, 1993). DNA reassociation studies have shown that many *Bacillus* species were composites of several genetically unrelated species (Priest, 1981; Slepecky & Hemphill, 1991; Nakamura, 1996; Shida *et al*., 1997b). Finally, 16S sequencing and resulting phylogenetic studies revealed that the genus *Bacillus* could be separated into several phylogenetically distinct genera such as *Alicyclobacillus* (Wisotzkey *et al*., 1992), *Aneurinibacillus* (Shida *et al*., 1996), *Brevibacillus* (Shida *et al*., 1996), *Halobacillus* (Spring *et al*., 1996) and *Paenibacillus* (Ash *et al*., 1993).

A bacterium was isolated in Brazil that was capable of cyclodextrin glycosyltransferase-mediated production of cyclodextrin from starch (Yim *et al*., 1997). Based on phenotypic characterization, this organism (strain 324^T) was tentatively identified as *Bacillus firmus*. Shida *et al*. (1997a) reclassified many *Bacillus* species as members of the genus *Paenibacillus*, many species of which excrete diverse assortments of polysaccharide-hydrolysing or -synthesizing enzymes. This work suggested that strain 324^T could be a *Paenibacillus* species. To rectify the tenuous classification and to establish its correct taxonomic position, strain 324^T was characterized further using 16S rDNA sequencing, chemotaxonomic and additional phenotypic methods. Based on data obtained, a new species, *Paenibacillus campinasensis*, is proposed.

METHODS

Bacterial strain and culture conditions. The isolation of strain 324^T was described in a previous study (Yim *et al*., 1997). Strain 324^T was cultivated on a medium (pH 10) that

The GenBank accession number for the sequence reported in this paper is AF021924.

contained (l^{-1}): 10 g starch; 5 g peptone; 5 g yeast extract; 1 g K_2HPO_4; 0·2 g $MgSO_4.7H_2O$; 10 g Na_2CO_3 (separately autoclaved); and 15 g agar (if needed). Strain 324^T was cultivated at 37 °C for 24 h on trypticase soy agar for fatty acid methyl ester analysis and on BUGM (Biolog) supplemented with 1% (w/v) glucose for the Biolog substrate utilization test.

Morphological and physiological tests. The morphology of cells was examined by phase-contrast microscopy. Flagellum type was examined with transmission electron microscopy using cells from 24 h culture. The cells were negatively stained with 1% (w/v) phosphotungstic acid and, after air drying, the grids were examined using a model CM-20 transmission electron microscope (Philips). Motility was observed by the hanging-drop method (Skerman, 1967). Catalase activity was determined by bubble production in a 3% (v/v) H_2O_2 solution. Oxidase activity was determined by oxidation of 1% (w/v) tetramethyl-p-phenylenediamine. Hydrolyses of gelatin, casein and starch, and production of urease were determined as described previously (Cowan & Steel, 1965). Hydrolysis of aesculin was determined according to the method of Kurup & Fink (1975). Tests for utilization of substrates as sole carbon source were performed with GP Biolog microplates containing 95 different carbon compounds. The results were checked over 48 h.

Isolation of DNA. Chromosomal DNA was isolated by a previously described method (Yoon *et al*., 1996).

Chemotaxonomic characterizations. The diamino acid of the peptidoglycan was determined by a previously described method (Komagata & Suzuki, 1987). Menaquinones were analysed as described previously (Komagata & Suzuki, 1987) by reversed-phase HPLC. Fatty acids were extracted and analysed according to the instructions of the Microbial Identification System (MIDI; Microbial ID).

Determination of G+C content. The G+C content was determined by a previously described method (Tamaoka & Komagata, 1984). DNA was enzymically hydrolysed and dephosphorylated; the resultant nucleosides were analysed by HPLC.

16S rDNA sequencing. 16S rDNA sequencing of strain 324^T was performed as described previously (Yoon *et al*., 1997). The PCR products were recovered by precipitating with 2-propanol and the strands containing phosphorylated primer from PCR products were selectively digested using λ exonuclease according to the instructions included with Strandase ssDNA Preparation kit (Novagen). The ssDNA templates produced were directly used for the sequencing reaction. The sequencing was performed as described previously (Kim *et al*., 1995) using α-^{35}S-labelled dATP and a DNA sequencing kit (US Biochemical). The sequencing primers were derived from conserved regions of previously described 16S rRNA gene sequences of eubacteria.

Phylogenetic analysis. The 16S rDNA sequence of strain 324^T was aligned with the 16S rRNA and 16S rDNA sequences of other *Paenibacillus* species and some other taxa using CLUSTAL W software (Thompson *et al*., 1994). Other reference sequences, obtained from the GenBank database, had the following accession numbers: X60632 (*Paenibacillus polymyxa*); D78318 (*Paenibacillus azotofixans*); D78476 (*Paenibacillus peoriae*); D78319 (*Paenibacillus macerans*); X77846 (*Paenibacillus durum*); D78473 (*Paenibacillus lautus* NRRL NRS-666^T); D85609 (*Paenibacillus lautus* NRRL B-377); D85394 (*P. lautus* NRRL B-379); D78470 (*Paenibacillus glucanolyticus* DSM 5162^T); D88514 (*P. glucanolyticus* DSM 5188); D85395 (*Paenibacillus chibensis*); X60625 (*Paenibacillus macquariensis*); X60630 (*Paenibacillus pabuli*); D85396 (*Paenibacillus amylolyticus*); D85397 (*Paenibacillus illinoisensis*); D78317 (*Paenibacillus alvei*); U49247 (*Paenibacillus apiarius*); D78475 (*Paenibacillus thiaminolyticus*); D78466 (*Paenibacillus curdlanolyticus*); D78471 (*Paenibacillus kobensis*); D78320 (*Paenibacillus validus*); D82064 (*Paenibacillus chondroitinus*); D78465 (*Paenibacillus aliginolyticus*); X60636 (*Paenibacillus larvae* subsp. *pulvifaciens*); X60619 (*P. larvae* subsp. *larvae*); X60646 (*Bacillus subtilis*); D16266 (*Bacillus cereus*); D78312 (*Bacillus circulans*); X60629 (*Bacillus megaterium*); D78313 (*Bacillus coagulans*); X62174 (*Halobacillus halophilus*); D82065 (*Amphibacillus xylanus*); D78455 (*Aneurinibacillus aneurinolyticus*); D78457 (*Brevibacillus brevis*); X60742 (*Alicyclobacillus acidocaldarius*); and V00348 (*Escherichia coli*). The 16S rDNA similarity values were calculated from the alignments and the evolutionary distances were calculated using the Kimura two-parameter correction with the CLUSTAL W package (Thompson *et al*., 1994). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) and the distance matrix data. A bootstrap analysis with 1000 replications for evaluating the topology of the phylogenetic tree was performed with the CLUSTAL W package (Thompson *et al*., 1994).

The GenBank accession number for the 16S rDNA sequence of strain 324^T is AF021924.

RESULT AND DISCUSSION

Morphological and physiological characteristics

Cells were rod-shaped measuring 0·6–0·9 by 3·0–6·0 μm in 48 h culture grown at 37 °C. They produced ellipsoidal spores in swollen sporangia. Colonies were flat, smooth and opaque. Strain 324^T formed motile microcolonies during growth on wet agar plates. However, this characteristic was weak at 45 °C and on completely dried agar plates. Strain 324^T was facultatively anaerobic, Gram-variable and motile by means of peritrichous flagella (Fig. 1). Strain 324^T had catalase activity, but no oxidase and urease activities.

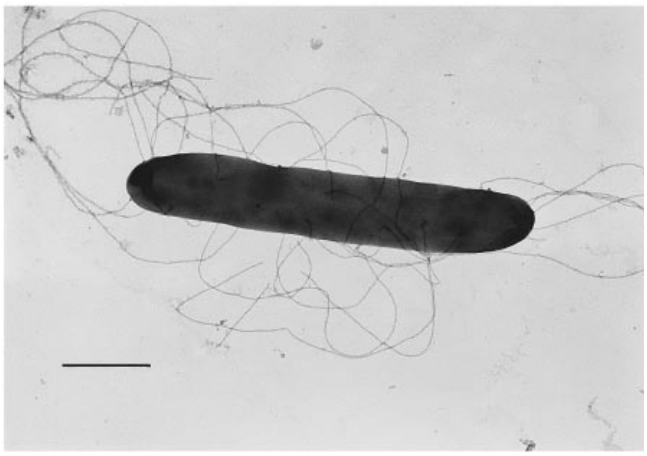

Fig. 1. Transmission electron micrograph of strain 324^T from a 24 h culture. Bar, 1 μm.

Table 1. Substrates oxidized as sole carbon source by strain 324^T

+, Oxidized; −, not oxidized; w, weak reaction.

Substrate	Reaction	Substrate	Reaction	Substrate	Reaction
Carbohydrates:		Psicose	w	Propionic acid	+
N-Acetyl-D-glucosamine	−	D-Raffinose	−	Pyruvic acid	+
N-Acetyl-D-mannosamine	−	L-Rhamnose	−	Succinamic acid	−
		D-Ribose	+	Succinic acid	−
Amygdalin	−	Salicin	−	Alcohols:	
L-Arabinose	+	Sedoheptulosan	−	2,3-Butanediol	−
Arabitol	−	D-Sorbitol	+	Glycerol	−
Arbutin	−	Stachyose	−	Amino acids:	
Cellobiose	−	Sucrose	−	D-Alanine	−
α-Cyclodextrin	−	Tagatose	+	L-Alanine	−
β-Cyclodextrin	+	D-Trehalose	−	L-Alanyl-glycine	−
Dextrin	−	Turanose	−	L-Asparagine	−
D-Fructose	+	Xylitol	−	L-Glutamic acid	−
D-Fucose	−	D-Xylose	+	Glycyl-L-glutamic acid	−
L-Fucose	−	Esters:		L-Pyroglutamic acid	−
D-Galactose	−	Methylpyruvate	−	L-Serine	−
Gentiobiose	−	Mono-methylsuccinate	−	Nucleosides:	
D-Glucose	w	D-Lactic acid methyl ester	−	Adenosine	−
Glycogen	w			2-Deoxyadenosine	−
m-Inositol	−	Detergents:		Inosine	−
Inulin	−	Tween 40	+	Thymidine	−
Lactose	−	Tween 80	−	Uridine	−
Lactulose	−	Carboxylic acids:		Nucleotides:	
Maltose	−	Acetic acid	+	Adenosine 5′-monophosphate	−
Maltotriose	−	*N*-Acetyl-L-glutamic acid	−		
Mannan	−	D-Galacturonic acid	−	Thymidine 5′-monophosphate	−
Mannitol	−	D-Gluconic acid	−		
D-Mannose	−	α-Hydroxybutyric acid	−	Uridine 5′-monophosphate	−
D-Melezitose	−	β-Hydroxybutyric acid	−		
Melibiose	+	γ-Hydroxybutyric acid	−	Phosphorylated compounds:	
Methyl α-D-galactoside	−	ρ-Hydroxyphenyl acetic acid	−	Fructose 6-phosphate	+
Methyl β-D-galactoside	−			Glucose 1-phosphate	−
3-Methylglucose	w	α-Ketoglutaric acid	−	Glucose 6-phosphate	w
Methyl α-D-glucoside	−	α-Ketovaleric acid	−	DL-α-Glycerol phosphate	−
Methyl β-D-glucoside	−	L-Lactic acid	−	Putrescine	−
Methyl α-D-mannoside	−	D-Malic acid	−	Alaninamide	−
Palatinose	−	L-Malic acid	−	Lactamide	−

This strain grew in the presence of 7% (w/v) NaCl, whereas most *Paenibacillus* species failed to grow in the presence of 5% (w/v) NaCl (Shida *et al*., 1997a). Gelatin, casein, aesculin and starch were hydrolysed. Strain 324^T grew at 10 and 45 °C, but not at 5 and 50 °C. The optimum growth temperature was 40 °C. In contrast, the optimum growth temperature for all *Paenibacillus* species, except *P. macquariensis*, was 28–30 °C (Shida *et al*., 1997a). *P. macquariensis* had an optimum growth temperature of 20–23 °C (Shida *et al*., 1997a). Strain 324^T was alkaliphilic. This strain did not grow at pH 7, but grew at pH 7·5–10·5. The optimum pH for growth was 10; members of the genus *Paenibacillus* grew optimally at pH 7·0 (Shida *et al*., 1997a). The alkaliphilic property of strain 324^T was an important phenotypic difference that distinguished strain 324^T from the known *Paenibacillus* species. The results of utilization of substrates as sole carbon sources for respiration (formation of NADH) on Biolog microplates are shown in Table 1.

Chemotaxonomic characteristics and DNA base composition

meso-Diaminopimelic acid was the diamino acid found in the cell-wall peptidoglycan of strain 324^T. This diamino acid also appeared in the members of the genus *Paenibacillus* (Shida *et al*., 1997a). Other aerobic endospore formers such as *Bacillus*, *Sporolactobacillus* and *Amphibacillus* also contained *meso*-diaminopi-

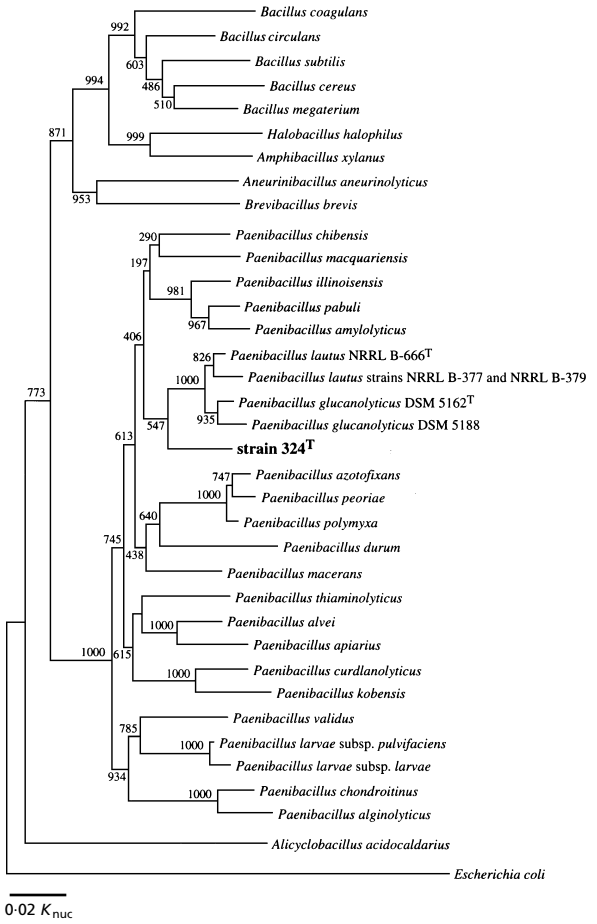

Fig. 2. Phylogenetic tree showing the position of strain 324^T in relation to *Paenibacillus* species and some rod-shaped, endospore-forming bacteria based on 16S rRNA gene sequences. Bootstrap values are indicated. Bar, 2 nucleotide substitutions per 100 nucleotides.

melic acid in their cell-walls (Shida *et al*., 1997a). The major isoprenoid quinone was MK-7, which is the major menaquinone generally found in aerobic, endospore-forming rods. Anteiso-$C_{15:0}$ (53%), the predominant fatty acid found in the genus *Paenibacillus*, was also the major component of strain 324^T fatty acids. Although other aerobic, endospore-forming genera also contained large amounts of anteiso-$C_{15:0}$ fatty acid, they simultaneously contained significant amounts of other fatty acids (Shida *et al*., 1997a).

The G+C content of strain 324^T was 50·9 mol%, a level included within the range found in the members of the genus *Paenibacillus* (Shida *et al*., 1997a).

Phylogenetic analysis

Although the preceding chemotaxonomic, G+C content and biochemical analyses did not establish a definitive taxonomic position for strain 324^T, they suggested *Paenibacillus* as a possible classification. 16S rDNA was sequenced to verify the suggestion. An almost complete 16S rDNA sequence (1508 bp), which corresponds to a region between positions 28 and 1524 by comparison with the *E. coli* 16S rRNA gene, was directly sequenced. The phylogenetic tree (Fig. 2) constructed from the sequence data shows that strain 324^T appeared within the evolutionary radiation encompassing the genus *Paenibacillus* and occupied a distinct phylogenetic position within the genus. Levels of 16S rDNA similarity between strain 324^T and the *Paenibacillus* species were 90·6–95·9%. The highest 16S rDNA sequence similarities of 95·2–95·9% were observed between strain 324^T and *P. lautus*, and between strain 324^T and *P. glucanolyticus*. The phylogenetic definition states that 'strains with approximately 70% or greater DNA–DNA relatedness' are members of the same species (Wayne *et al*., 1987). According to the available compilation of data by Stackebrandt & Goebel (1994), organisms that have less than 97·0% 16S rDNA or 16S rRNA similarities will not reassociate to more than 60%, no matter which hybridization method is used. The phylogenetic study clearly established that strain 324^T was a *Paenibacillus* species.

The phenotypic, chemotaxonomic and phylogenetic data showed that strain 324^T belonged to the genus *Paenibacillus*. Moreover, phenotypic uniqueness imparted by the alkaliphilic and moderately thermophilic nature of the strain and genetic distinctiveness inferred from the phylogenetic study warranted the proposal of strain 324^T as a new species, *Paenibacillus campinasensis* sp. nov.

Description of *Paenibacillus campinasensis* sp. nov.

Paenibacillus campinasensis (cam.pi.na.sen′sis. M.L. adj. *campinasensis* referring to Campinas, the city where the College of Food Engineering, State University of Campinas, Brazil, is located).

Cells are rods measuring 0·6–0·9 by 3·0–6·0 μm and motile by means of peritrichous flagella. Ellipsoidal spores are formed in swollen sporangia. Colonies are flat, smooth and opaque. Forms motile microcolonies on wet agar plates. Facultatively anaerobic and Gram-variable. Catalase-positive and oxidase- and urease-negative. Growth occurs in the presence of 7% NaCl. Gelatin, casein, aesculin and starch are hydrolysed. Utilizes L-arabinose, *β*-cyclodextrin, D-fructose, D-glucose, melibiose, 3-methylglucose, psicose, D-ribose, D-sorbitol, tagatose, D-xylose, Tween 40, acetic acid, propionic acid, pyruvic acid, fructose 6-phosphate and glucose 6-phosphate as sole carbon sources for respiration. Substrates which are not utilized are shown in Table 1. Grows at 10 and 45 °C, but not at 5 and 50 °C; optimum temperature is 40 °C. Alkaliphilic. Does not grow at pH 7. Grows at pH 7·5–10·5; optimum pH is 10. Cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The major isoprenoid quinone is a menaquinone, MK-7. The major fatty acid is anteiso-$C_{15:0}$. The G+C content is 50·9 mol% (de-

termined by HPLC). The type strain is 324^T, which was isolated in Brazil. The type strain has been deposited in the Korean Collection for Type Cultures as KCTC 0364BP^T.

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