***Paenibacillus dendritiformis* sp. nov., proposal for a new pattern-forming species and its localization within a phylogenetic cluster**

Marianna Tcherpakov,¹ Eshel Ben-Jacob² and David L. Gutnick¹

Author for correspondence: David Gutnick. Tel: +972 3 640 9834. Fax: +972 3 642 5786.
e-mail: davidg@post.tau.ac.il

Department of Molecular Microbiology and Biotechnology, George S. Wise Faculty of Life Sciences¹, and School of Physics and Astronomy, Raymond and Beverly Sackler Faculty of Exact Sciences², Tel-Aviv University, Tel-Aviv 69978, Israel

A new strain capable of forming distinctive patterns during colony development was identified by using a combination of phenotypic characterization, fatty acid analysis and analysis of the 16S rRNA gene sequence. The strain formed either a branched, tip-splitting colony morphology (referred to as the T morphotype) or a chiral pattern exhibiting thinner branches with distinctive curling patterns (referred to as the C morphotype). Isolates of the T morphotype exhibited sequence identities greater than 97% to *Paenibacillus thiaminolyticus* JCM 7540. Phylogenetic analysis placed the T morphotype within the *Paenibacillus* cluster on a phylogenetic tree. On the basis of unique colony morphology and distinctive phenotypic characteristics, it is proposed that the pattern-forming isolates should be placed within a new species of *Paenibacillus*, *Paenibacillus dendritiformis* sp. nov., the type strain of which is T168^T (= 30A1^T).

Keywords: *Paenibacillus dendritiformis* sp. nov., pattern formation, tip-splitting, chiral morphology

INTRODUCTION

Certain species among the *Bacillaceae* have been shown to produce a variety of complex patterns during colony development (Ben-Jacob, 1997; Ben-Jacob & Garick, 1990; Ben-Jacob *et al.*, 1992, 1994, 1995, 1997, 1998a, b; Fox & Rossler, 1991; Kanzawa *et al.*, 1995; Matsushita & Fujikawa, 1990; Matsushita *et al.*, 1995; Matsuyama & Matsushita, 1993; Matsuyama *et al.*, 1993). Although microbial patterns are often remarkably similar to those observed in abiotic systems, such as snowflake formation or electrodeposition (Ben-Jacob, 1997; Ben-Jacob *et al.*, 1994), the spectrum of shapes and forms of microbial colonies is much larger and more sensitive to changes in conditions. The major difference between the microbial system and abiotic examples stems from the fact that growing organisms modify their environment as they develop co-operatively, excreting various metabolites and signalling molecules, changing the pH, extracting nutrients from the surface substrate and so on. In order to develop working hypotheses regarding the bio-

logical processes governing pattern formation, a generic modelling approach has been developed that is based on close inspection of microbial behaviour and includes features related to cell growth, generation time and production of extracellular wetting fluid that allows the cells to move across the very hard agar surface, as well as various types of cell–cell signalling and communication (Ben-Jacob *et al.*, 1994, 1998a, b; Gutnick & Ben-Jacob, 1998). The term morphotype is employed to describe the pattern-forming capacity of micro-organisms (Fujikawa & Matsushita, 1989; Ben-Jacob, 1997; Gutnick & Ben-Jacob, 1998). However, in order to gain a deeper understanding of the biological basis of pattern formation by various morphotypes, it is necessary to define the particular genes involved in pattern formation, their organization and control. This in turn depends on the identification of morphotypes. In this paper, the identification of two such pattern-forming isolates, their location on a phylogenetic tree and their phenotypic characterization are reported.

METHODS

Bacterial strains and growth conditions. The pattern-forming isolates used in this study were termed T168^T (tip-splitting

The EMBL accession number for the 16S rRNA gene sequence of isolate T168^T is Y16128.

Fig. 1. Representative patterns of (a) isolate T168^T (tip-splitting) and (b) isolate C168 (chiral). Pattern formation was carried out as described in the text.

morphotype) and C168 (chiral morphotype) (Fig. 1). Both morphotypes were isolated several times in the laboratory of E. Ben-Jacob. When cultures of *Bacillus subtilis* 168 were spotted on thin agar plates containing 12 ml agar (1.0–1.75%) and peptone broth (0.2%), the strains routinely grew as compact colonies. Occasionally, at frequencies of about 0.1–0.01%, bursts of growth exhibiting a new colony morphology emerged. Cells were picked from this new region of growth and restreaked on LB plates several times in order to purify isolates. The T morphotype was isolated

on 1.5% agar and the C morphotype was isolated on 1% agar. Bacteria were grown at 37 °C on LB broth, containing 10 g bactotryptone, 5 g yeast extract and 10 g NaCl l⁻¹ distilled water. Working stocks were cultured on LB agar (LB broth solidified with 2.0% agar) plates (containing about 25 ml medium) for 24 h at 37 °C. Strains were stored at 4 °C.

Phenotypic characterization. Standard phenotypic characterization (Steel, 1961; Edwards & Ewing, 1972; Gordon *et al.*, 1973) of isolates T168^T and C168 presented in Table 2 was carried out in part at the ATCC (Manassas, VA, USA). Cellular fatty acid analysis was carried out with the assistance of Analytical Services (Williston, VT, USA) by using high-resolution GC to compare the fatty acid profile of the isolates with a known standard in the database with the MIDI/Hewlett Packard Microbial Identification System.

Pattern formation assay. Pattern formation assays were carried out on thin agar plates containing varying concentrations of agar and peptone broth (Difco) as previously described (Ben-Jacob *et al.*, 1992). Tip-splitting patterns (formed by morphotype T168^T) were typically observed on plates containing 1.5% agar and 0.2% peptone. Chiral patterns (formed by morphotype C168) were generally observed on plates containing 0.8–1.2% agar and 0.2% peptone. Typically, 12 ml agar-containing medium was added to each plate and dried at room temperature until the initial weight had decreased by 4.0–5.0%. Overnight cultures of the various strains were grown for 24 h at 37 °C in LB broth and 5 µl aliquots were spotted onto the centre of the plate. Plates were incubated at 37 °C. Visualization of the colony morphology was enhanced with Coomassie blue staining as previously described (Ben-Jacob *et al.*, 1992).

Sequencing of 16S rRNA genes. Genomic DNA was prepared from 50 ml overnight cultures grown at 37 °C according to an established protocol (Sambrook *et al.*, 1989). PCR amplification of the 16S rRNA was done as described previously by Di Cello *et al.* (1997) by using conserved eubacterial sequences from the 5' and 3' ends of the 16S rRNA gene. The forward primer was 5' AGAGTTTGA-TCCTGGCTCAG 3' and the reverse primer was 5' TAC-GGCTACCTTGTTACGACTT 3'. PCR was performed with an Eppendorf Mastercycler 5330 instrument (Eppendorf-Netheler). Amplified 16S rRNA gene fragments were purified with a QIAquick Spin PCR Purification kit (Qiagen) and used as sequencing templates. Sequencing was carried out with an automatic DNA sequencer (ABI Prism 377; Perkin-Elmer).

Comparison of 16S rRNA gene sequences. Sequences determined in this study were compared with 16S rRNA gene sequences obtained from the EMBL, GenBank, DDBJ and PDB databases. Multiple alignment of sequences, calculation of nucleotide substitution rates and construction of a neighbour-joining phylogenetic tree were carried out with the CLUSTAL W program version 1.6. Alignment gaps and unidentified base positions were not taken into account in the calculations. Sequence identities shown in Table 1 were determined with the BESTFIT program of the GCG package.

RESULTS

Pattern formation by T and C morphotypes

The distinguishing characteristic of the isolates T168^T and C168 is their capacity to form unique, complex

Table 1. 16S rRNA sequence identities to morphotype T168^T

The 16S rRNA sequence was determined for a PCR-amplified fragment from genomic DNA of strain T168^T as described in the text. The 16S rRNA sequence was compared with each of the other sequences by using the BESTFIT program from the GCG package.

Species and strain	Accession no.	Identity (%)	Reference
<i>P. thiaminolyticus</i> JCM 7540	D88513	97.3	Shida <i>et al.</i> (1997a)
<i>P. thiaminolyticus</i> JCM 8360 ^T	D78745	96.6	Shida <i>et al.</i> (1997a)
<i>P. alvei</i> ATCC 6344	X57304	96.1	Fox & Rossler (1991)
<i>P. apiarius</i> NRRL NRS-1438	U49247	94.3	Nakamura (1996)
<i>P. illinoisensis</i> NRRL NRS-1356 ^T	D85397	93.5	Shida <i>et al.</i> (1997b)
<i>P. curdlanolyticus</i> IFO 15724 ^T	D78466	93.5	Shida <i>et al.</i> (1997a)
<i>P. kobensis</i> IFO 15729 ^T	D78471	93.0	Shida <i>et al.</i> (1997a)
<i>P. larvae</i> ATCC 9545	X60619	91.8	Ash <i>et al.</i> (1991)
<i>Brevibacillus thermoruber</i> DSM7064	Z26921	90.1	Rainey <i>et al.</i> (1994)
<i>Bacillus subtilis</i> NCDO 1769	X60646	89.2	Ash <i>et al.</i> (1991)
<i>Bacillus</i> sp. DSM 8718	X76442	89.1	Nielsen <i>et al.</i> (1994)
<i>Bacillus alcalophilus</i> DSM 1485 ^T	X76436	88.8	Nielsen <i>et al.</i> (1994)
<i>Bacillus cereus</i> IAM 12605	D16266	88.4	Suzuki & Yamasato (1994)
<i>Bacillus coagulans</i> IAM 12463	D16267	87.6	Suzuki & Yamasato (1994)
<i>Bacillus stearothermophilus</i> T10	X57309	87.5	Fox & Rossler (1991)
<i>Bacillus megaterium</i> IAM 13418	D16273	87.3	Suzuki & Yamasato (1994)

patterns during colony development under specific conditions, determined primarily by the nutrient concentration and hardness of the agar surface. The patterns vary somewhat as these parameters are modified (Ben-Jacob, 1997; Ben-Jacob *et al.*, 1995). The term morphotype has been used to identify the capacity of a strain to form a unique characteristic pattern under particular conditions (Ben-Jacob, 1997; Ben-Jacob *et al.*, 1998a, b; Gutnick & Ben-Jacob, 1998). When spotted onto the centre of plates containing only a small volume of medium (about 12 ml), but at agar concentrations greater than about 1.5%, cells of strain T168^T swarmed out from the centre to form characteristic branch patterns that exhibit a tip-splitting colony morphology (Fig. 1a). The isolate is thus referred to as T morphotype. After about 48 h with agar concentrations of about 1.2% or lower, a burst of new branches from the growing tips is frequently observed. These new growing branches, which are characteristically much thinner, all curl with the same handedness, to yield a chiral morphology, characteristic of C morphotypes. This chiral morphology is characteristic of isolate C168, which was isolated from the chiral region of the branched pattern (Ben-Jacob *et al.*, 1995). When respoated onto plates containing the lower agar concentration, the resulting colonies developed with chiral morphology without going through the 48 h adaptation period (Fig. 1b). Previous results have demonstrated that C168 developed as a result of a morphotype transition from T to C occurring under specific conditions of lower agar concentration (Gutnick & Ben-Jacob, 1998).

Identification of the morphotypes

The complete nucleotide sequence of the 16S rRNA gene from isolate T168^T was determined. The highest sequence identities to morphotype T168^T are shown in Table 1. Highest identities were found for *Paenibacillus thiaminolyticus* JCM 7540 (97.3%) and *P. thiaminolyticus* JCM 8360^T (96.6%). Other representative strains exhibiting high sequence identities, between 96 and 91.8%, included in order of identity *Paenibacillus alvei* ATCC 6344, *Paenibacillus apiarius* NRRL NRS-1438, *Paenibacillus illinoisensis* NRRL NRS-1356^T, *Paenibacillus curdlanolyticus* IFO 15724^T, *Paenibacillus kobensis* IFO 15729^T and *Paenibacillus larvae* ATCC 9545. In addition, identities ranging from 90.1 to 88.8% were found for *Brevibacillus thermoruber* DSM 7064, *Bacillus* sp. DSM 8718 and *Bacillus alcalophilus* 1485^T. In a phylogenetic tree, the T morphotype was clearly located within the distinct *Paenibacillus* cluster (Fig. 2) that was reported previously (Shida *et al.*, 1997a).

Phenotypic characterization

Further evidence supporting the placement of the T morphotype within the genus *Paenibacillus* emerged from a study of several phenotypic characters (Table 2) as well as analysis of cellular fatty acids (Table 3). Cells of strain T168^T are motile, Gram-negative rods found either singly or in short chains, with one endospore formed in the subterminal or terminal region of the cell. The colonies on LB agar plates are translucent

Fig. 2. Phylogenetic relationships of *Paenibacillus* species and other closely related bacteria based on 16S rDNA gene sequences. The branching pattern was generated by the neighbour-joining method. Bar, 0.01 nucleotide substitutions per site.

and irregular with low convex elevation, a glistening, smooth surface and an entire margin (not shown). Most of the biochemical and nutritional features observed, as well as the G+C content, have been reported as characteristic for various species and subspecies of *Paenibacillus* (Shida *et al.*, 1997a). These characteristics were compared with those reported for *P. thiaminolyticus*, the species showing the highest sequence identity to the T morphotype (Table 2). A number of differences were observed (in bold in Table 2). For example, T168^T was unable to reduce nitrate, decompose tyrosine, use citrate as a carbon source or ferment either mannitol or L-arabinose. In contrast, *P. thiaminolyticus* was reported to exhibit positive reactions for all of these traits with the exception of mannitol and L-arabinose fermentation, which were variable (Shida *et al.*, 1997a). Moreover, the optimum growth temperature for the pattern-forming strain was 37 °C, while that of *P. thiaminolyticus* was 28 °C. Other differences between the closely related *Paenibacillus* species were found for urea hydrolysis, litmus milk reaction and growth in the presence of 5% NaCl (Table 2). The T morphotype showed clear positive

reactions for these features, while *P. thiaminolyticus* was reported to be either negative (urea hydrolysis and litmus milk) or variable (5% NaCl). Ignoring the variable behaviour for some of the traits, the morphotype showed the same characteristics as *P. thiaminolyticus* in 74% (23 of 31) of the phenotypic tests.

Cellular fatty acid analysis

The cellular fatty acid composition of the T morphotype is shown in Table 3 and compared with the distribution of fatty acids previously determined for a variety of *Bacillus* and *Paenibacillus* strains (Shida *et al.*, 1997a). The major fatty acid of the T morphotype was found to be the anteiso-branched C_{15:0}, which constituted 43% of the total. As illustrated in Table 3, this value is within the range previously determined for members of the genus *Paenibacillus* (Shida *et al.*, 1997a).

DISCUSSION

In recent years, a number of rod-shaped, endospore-forming strains previously associated with the genus *Bacillus* have been transferred to the genus *Paenibacillus*, which was shown by analysis of the 16S rRNA sequences to form a specific monophyletic cluster (Ash *et al.*, 1993; Heyndrickx *et al.*, 1995, 1996a, b; Nakamura, 1996; Shida *et al.*, 1997b). In this regard, phylogenetic analysis of the 16S rRNA sequence from the pattern-forming T168^T morphotype placed it within the *Paenibacillus* cluster. Intracluster identities were greater than 90% for the pattern-forming strain, which is within the range (between 89.9 and 97.1%) previously reported for this cluster (Shida *et al.*, 1997a, b). It should be noted that no reported 16S rRNA sequence from any of the *Paenibacillus* species showed identities greater than 97.2% when compared with the 16S rRNA sequences from T168^T (see Table 1). In this regard, a putative classification of the T (and C) morphotypes was recently reported (Rudner *et al.*, 1998). In our opinion, these morphotypes were mistakenly classified as members of the genus *Bacillus*. Moreover, no evidence was presented in that work to justify the placement of the two morphotypes into different species.

Phenotypic characterization of morphotype T168^T and cellular fatty acid analysis suggested that the strain is likely to be a member of the genus *Paenibacillus* and most closely resembles *P. thiaminolyticus*. However, as shown in Table 2, about 26% of the phenotypic characters were different for the T morphotype when compared with the profile for *P. thiaminolyticus*.

It is recommended, therefore, that morphotype T168^T be placed in a new species within the *Paenibacillus* cluster. Since the most distinguishing characteristic of the pattern-forming T morphotype is its branched colony morphology, it is suggested that the new species should be called *Paenibacillus dendritiformis* sp. nov.

Table 2. Distinctive phenotypic characters of T morphotype and *P. thiaminolyticus*

Only T168^T is shown, as no differences were found between T168^T and C168 for any characters examined. Data for *P. thiaminolyticus* are from Nakamura (1990) and Shida *et al.* (1997a). Characters shown in bold differ between T168^T and *P. thiaminolyticus*. NT, Not tested; v, variable.

Character	T168 ^T	<i>P. thiaminolyticus</i>
Spore shape	Round, cylindrical or oval	Oval
Swollen sporangia	+	+
Anaerobic growth	+	+
Catalase	+	+
Oxidase	+	+
Nitrate reduction	—	+
Production of:		
Acetylmethylcarbinol	—	—
Indole	+	+
Dihydroxyacetone	—	—
pH in Voges-Proskauer broth	<6.0	4.9–5.5
Decomposition of:		
Tyrosine	—	+
Thiamine	NT	+
Hydrolysis of:		
Casein	+	+
Starch	+	+
Urea	+	—
Tween 80	—	—
Egg yolk lecithin	Weak	—
Utilization of:		
Citrate	—	+
Succinate	+	+
Acetate	+	+
Fumarate	+	+
L-Malate	+	+
Litmus milk	+	No change
Optimum growth temperature (°C)	37	28
Growth at pH 5.6	+	—
Growth in the presence of:		
0.001 % lysozyme	NT	—
5% NaCl	+	v
Fermentation of:		
D-Glucose	+	+
L-Arabinose	—	v
Mannitol	—	v
D-Xylose	—	—
G + C content (mol %)	55	52–54

One feature of several members of the genus *Paenibacillus* is that they produce extracellular polysaccharide-degrading enzymes (Shida *et al.*, 1997a; Nakamura, 1987; Alexander & Priest, 1989; Claus & Berkeley, 1986; Priest *et al.*, 1988). Some of these strains, such as *P. kobensis* IFO 15729, *Paenibacillus glucanolyticus* DSMZ 5162^T, *Paenibacillus lautus* NRRL NRS-666^T and *P. curdlanolyticus* IFO 15724^T, are located quite close to morphotype T168^T on the phylogenetic tree. Experiments are currently in progress in this laboratory to determine whether any of the

other members of the *Paenibacillus* cluster exhibit morphotype pattern-forming characteristics and, if so, whether this behaviour can be studied using the generic models developed previously (Ben-Jacob, 1997; Ben-Jacob *et al.*, 1998a, b; Gutnick & Ben-Jacob, 1998).

Preliminary results in this regard have demonstrated that at least nine different species of *Bacillus* and two different species of *Paenibacillus* fail to form patterns under the conditions described here (M. Tcherpakov, unpublished results). The two *Paenibacillus* species, *P.*

Table 3. Cellular fatty acid composition of T168^T and several species of the genera *Paenibacillus* and *Bacillus*

The percentage of the total cellular fatty acid content is shown. Data for fatty acid composition of strains of *Bacillus* and *Paenibacillus* were taken from Shida *et al.* (1997a). Data for fatty acid composition of the T morphotype was obtained as described in Methods. ND, Not detected.

Species and strain	Saturated acids								Unsaturated acids	
	Straight-chain			Iso-branched			Anteiso-branched	C _{16:1} ω11	Iso-C _{17:1} ω10	
	C _{14:0}	C _{15:0}	C _{16:0}	C _{15:0}	C _{16:0}	C _{17:0}	C _{15:0}			C _{17:0}
Morphotype T168 ^T	0.7	3.3	6.3	6.0	4.6	7.5	43.2	20.6	2.7	1.4
<i>Paenibacillus thiaminolyticus</i>										
JCM 8360 ^T	0.9	0.6	10.5	10.5	5.8	6.5	42.4	11.6	5.2	1.7
JCM 7540	0.5	0.5	8.5	10.7	6.3	7.1	45.3	16.4	1.3	1.0
<i>Paenibacillus alvei</i>										
IFO 3343 ^T	2.3	1.6	15.3	12.3	2.3	3.3	53.9	3.3	1.3	0.5
NRRL NRS-811	1.8	2.0	12.2	9.2	2.5	2.0	60.3	4.0	0.8	0.2
<i>Paenibacillus kobensis</i>										
IFO 15729 ^T	1.0	2.8	12.2	2.0	8.8	0.5	65.7	3.2	ND	ND
IFO 15730	0.7	2.7	8.3	1.7	16.2	0.7	61.1	4.1	ND	ND
<i>Paenibacillus apiarius</i>										
NRRL NRS-1438 ^T	0.6	2.2	4.8	8.3	4.2	5.5	60.5	16.3	0.8	0.2
NRRL NRS-1578	0.5	1.4	4.6	6.1	5.3	6.0	59.3	16.3	0.2	0.3
<i>Paenibacillus larvae</i> subsp. <i>pulvifaciens</i>										
IFO 15408 ^T	0.6	1.3	6.4	9.5	5.3	4.5	48.8	20.7	0.8	ND
NRRL B-14152	0.2	1.6	4.7	8.0	1.3	7.2	40.8	30.2	1.0	ND
<i>Bacillus subtilis</i> JCM 1465 ^T	0.3	2.3	1.3	28.4	2.0	6.1	44.5	6.8	ND	0.5
<i>Bacillus cereus</i> JCM 2152 ^T	3.1	4.9	2.4	48.7	2.7	6.2	3.8	0.7	4.4	2.8
<i>Bacillus stearothermophilus</i> JCM 2501 ^T	ND	ND	3.4	27.5	5.6	17.2	7.8	38.6	ND	ND
<i>Bacillus sphaericus</i> JCM 2502 ^T	1.4	6.8	1.3	53.4	6.9	2.1	8.8	0.9	ND	ND

alvei IFO 3343^T and *P. apiarius* NRRL NRS-1578, that showed no pattern-forming characteristics are quite closely linked to the T morphotype on the phylogenetic tree.

Description of *Paenibacillus dendritiformis* sp. nov.

Paenibacillus dendritiformis (den.dri.ti.for'mis. G. n. *dendron* tree; M.L. adj. *formis* shaped).

Rod-shaped 0.5–1.0 µm by 2.0–3.0 µm (T morphotype) or 1.0–1.5 µm by 4.0–6.0 µm (C morphotype). Gram-negative. Motile. Round, cylindrical or oval spores formed in swelling sporangia. On 2.0% agar plates containing 0.5% yeast extract, 1.0% bacto-tryptone and 1.0% NaCl, colonies are non-pigmented, translucent, thin, smooth, regular, entire and 1.0–2.0 mm in diameter. Catalase and oxidase are produced. Facultatively anaerobic. Acetylmethylcarbinol is not produced. Indole is produced. The pH in Voges–Proskauer broth (test for acetylmethylcarbinol production) is less than 6.0. Nitrate is not reduced to nitrite. Starch and casein are hydrolysed. Urea is decomposed. Egg yolk lecithin is weakly decomposed. Tween 80 is not decomposed. There is no degradation of tyrosine. Acetate, fumarate, malate and succinate are utilized. Citrate is not utilized. Growth occurs at

pH 5.6 or 5.7. There is change in litmus milk in 7 d. Grows in the presence of 5% NaCl. Optimal growth temperature is 37 °C; weak growth at 20 °C; maximum growth temperature is 45 °C. Acid but no gas is produced from cellobiose, glucose, mannose, sucrose and trehalose. No acid is produced from arabinose, fructose, lactose, mannitol or xylose. The G+C content is 55 mol %. Isolated occasionally from cultures of *Bacillus subtilis* 168. The ability to form complex symmetrical tip-splitting (T) or chiral (C) patterns on thin agar plates containing between 0.7 and 1.75% agar is a characteristic of this species. Morphotype transition from T to C is generally observed on thin agar plates containing up to 1.2% agar, while reverse transitions from C to T are observed on agar plates containing at least 1.5% agar. Such transitions generally require a period of about 48 h at 30 °C.

Isolate T168^T was deposited as the type strain in the Bacillus Genetic Stock Center, Columbus, OH, USA, as strain 30A1^T.

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