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Characterization of cellulase producing *Bacillus* and *Paenibacillus* strains from Thai soils

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ABSTRACT

Seventeen strains of cellulase producing bacteria were isolated from soil samples collected in Nan province, Thailand. They exhibited cellulase activity as a clear zone surrounded their colonies grown on carboxymethyl cellulose (CMC) agar medium ranged from 0.63 to 2.95 cm in diameter. Their hydrolysis capacity values were 1.65 - 7.55. The bacteria isolated were divided into 2 groups and belonged to genus *Bacillus* and *Paenibacillus* based on their phenotypic characteristics and chemotaxonomic characteristics such as *meso*-diaminopimelic in cell wall peptidoglycan and menaquinones of MK-7. *Bacillus* strains, P3-1 and P4-6 in Group I, produced maximum cellulase at 0.015 U ml^{-1}. Their optimal pH and temperature for enzyme production and enzyme activity were 7.0 and 50 ^{o}C, respectively. The strains P3-1 and P4-6 were closely related to *Bacillus velesensis* LMG 22478^{T} (100% similarity) whereas representative strain, S10-4 in Group II, was closely related to *P. cellulositrophicus* KCTC 13135^{T} (98.7% similarity) based on 16S rRNA gene sequence**.** On the basis of their phenotypic, chemotaxonomic characteristics and phylogenetic analysis of 16S rRNA gene sequence, the strains P3-1 and P4-6 were identified as *B. velesensis* and the strain S10-4 was a novel species in the genus *Paenibacillus*.

INTRODUCTION

Cellulose is a linear chain of D-glucose units linked together by β-1,4-glycosidic bond (Salmon, 1997). The β-1,4-glycosidic linkages in the cellulose can be hydrolysed by cellulolytic enzyme, cellulase. The cellulase is a group of enzymes which composes of at least three different enzymes. Endoglucanase (1,4-β-D-glucan -4-glucanohydrolase, E.C. 3.2.1.4) attacks randomly internal linkages within the cellulose chain, creating free chain ends, exoglucanase (1,4-β-D-glucan cellobiohydrolase, E.C. 3.2.1.91) hydrolyses cellulose from the free chain ends creating mainly cellobiose as an end product, and β-glucosidase (E.C. 3.2.1.21) hydrolyses the cellobiose to glucose. Harmonization of exoglucanase and β-glucosidase activities is important for glucose liberation from cellulose because accumulation of cellobiose strongly inhibits exoglucanase activity (Beguin and Aubert, 1994; Harjunpaa, 1998). During the past two decades, usage of enzyme in industrial process has significantly increased. The cellulase has been used in various industries for example; paper production, juice clarification, lignocellulosic ethanol production, extraction of valuable components from plant cells, and nutritional improvement of animal feed, etc (Bhat, 2000; Csiszar *et al*., 2001; Haki and Rakshit 2003; Lynd *et al*., 2002; Vielle and Ziekus, 2001). However, difference of applications requires cellulase (s) which is different in particular properties. Several microorganisms; bacteria, yeast and fungi; have been reported as cellulase producers. Bacterial cellulase is more thermostable than fungal cellulase. Optimal pH for fungal cellulase activity is between 4 and 6 (Zhu *et al.*, 1982; Yazdi *et al.*, 1990). While, alkaline pH has been reported as optimal pH for bacterial cellulase activity (Ruttersmith and Daniel, 1993). Extracellular cellulases of several bacteria have been studied and characterized *e.g*. *Clostridium*, *Caldocellum*, and *Acidothermus* (Bergquist *et al.*, 1999), *Acetovibrio* (Ding *et al.*, 1999), *Ruminococcus* (Aurilia *et al.*, 2000), *Sinorhizobium* (Chen *et al.*, 2004), *Cellulomonas*, *Micrococcus* and *Bacillus* (Immanuel *et al.*, 2006).

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In this investigation, cellulase producing bacteria were isolated from soils in Nan province, Thailand to screen for bacterial cellulase with particular property. The bacteria isolated were also identified based on their phenotypic and chemotaxonomic characteristics including 16S rRNA gene sequence analysis.

MATERIALS AND METHODS

Isolation and screening methods

A total of 9 soil samples were collected from Pua and Santisuk districts, Nan province, Thailand (Table 1). Cellulase producing bacteria were isolated from the soil samples not later than 24 hours after collection by an enrichment culture method. The soil sample (0.1 g) was put into a 10 ml of cellulose powder medium (CP, cellulose power 1 g, peptone 5 g, yeast extract 1 g, K_2HPO_4 4 g, $MgSO_4.7H_2O$ 1 g, KCl 0.2 g, $FeSO_4.7H_2O$ 0.02 g in 1000 ml distilled water, pH 7.0) and incubated on a rotary shaker at 200 rpm, 40°C for 2 days. One milliliter of the culture was transferred to fresh CP medium and incubated at the same above condition for 2 more times.

The enriched cultures or their dilutions (0.1 ml) were dropped and spreaded on the CP agar medium and incubated at 40°C for 2 days. Cellulase producing capability of the cultures was screened qualitatively by method described by Teather and Wood (1982). Their colonies grown on carboxymethyl cellulose (CMC)-basal (CMC 5 g, $(NH_4)_2 SO_4$ 1 g, yeast extract 1 g in 1000 ml distilled water) agar medium at 40°C for 2 days were flooded with 0.1% (w/v) Congo red solution for 1 min and then washed with 0.1 M NaCl. Colonies surrounded by clear zone were selected as cellulase producing isolates and then they were purified by streak plate method. Hydrolysis capacity (HC) value was calculated from clear zone diameter divided by colony diameter.

Cellulase production capability was also quantitatively determined by inoculating single colony into 10 ml of carboxymethyl cellulose medium (CMC medium, the CP medium which cellulose power was replaced by CMC) and incubated at 40°C, 200 rpm for 2 days. Three milliliters of the cultures were transferred into 30 ml of CMC medium and incubated at the same aboved condition for 2 days. Supernatants obtained after centrifugation of the cultures at 13,300 x g , 4° C for 15 min were used as crude enzyme for cellulase activity assay.

Cellulase activity assay

Cellulase activity assay was done by the method described by Ghose (1987). Reaction mixture composed of 0.5 ml of 2% (w/v) carboxymethyl cellulose in 100 mM sodium phosphate buffer pH 7.0 , and 0.5 ml of crude enzyme were incubated at 40°C for 30 min. The amount of reducing sugar released was quantified by Somogyi-Nelson method (Nelson, 1944; Somogyi, 1952) using glucose as authentic sugar. The reaction stopped immediately after addition of enzyme solution was used as a reaction blank. One unit of cellulase was defined as the amount of enzyme yielding 1 micromole of glucose within 1 min under the assay condition.

Identification methods

Cell morphology, colonial appearance, spore formation, and pigmentation of the cellulase producing bacteria isolated grown on CMC agar medium at 37°C for 1 day were examined. Catalase, oxidase, hydrolysis of L-arginine, aesculin, casein, gelatin, starch, tyrosine and deoxyribonuclease (DNase) activity; MR-VP, indole test, nitrate reduction, Simmon citrate test, Triple Sugar Iron agar (TSI), dihydroxyacetone from glycerol, urease activity and acid from carbohydrates were determined as described by Barrow and Feltham (1993).

Growth at different pH (5, 6, 8 and 9), in 3 and 5% NaCl and at different temperatures (10 °C, 15 °C, 20 °C, 37 °C, 45 °C, 50 °C, 55 °C and 60 °C) were investigated. All tests were carried out by using C medium (polypeptone 5 g, yeast extract 1 g, K_2HPO_4 4 g, $MgSO_4.7H_2O$ 1 g, KCl 0.2 g, $FeSO_4.7H_2O$ 0.02 g in 1000 ml distilled water) as a basal medium and incubated at 37°C, except for the investigation of the effect of temperatures. Diaminopimelic acid in the cell wall and menaquinone were determined as described by Komagata and Suzuki (1987).

DNAs were isolated from cells grown on C agar plate for 18 to 48 h, and purified by the method of Saito and Miura (1963). DNA base composition was determined by the method of Tamaoka and Komagata (1984).

The 16S rDNA gene sequence was amplified, purified and analysed as described previously (Tanasupawat *et al.*, 2004). The sequences determined (1489-1545 bp) were aligned with the selected sequences obtained from the GenBank/ EMBL/DDBJ database employing CLUSTAL_X version 1.81 (Thompson *et al.*, 1997). The alignment was manually edited to remove gaps and ambiguous nucleotides prior to construction of phylogenetic tree. The phylogenetic tree was constructed by neighbor-joining method (Saitou and Nei, 1987) using MEGA programme version 2.1 (Kumar *et al.*, 2001). Confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

RESULTS AND DISCUSSION

Isolation and screening

Seventeen isolates of cellulase producing bacteria which were isolated from soil samples collected in Pua and Santisuk districts, Nan province, Thailand by enrichment culture method at 40 °C exhibited cellulase activity as a clear zone surrounded their colonies grown on carboxymethyl cellulose (CMC) agar medium ranged from 0.63 to 2.95 cm in diameter and hydrolysis capacity (HC) values of 1.65 - 7.55. Isolate S10-4 gave maximum hydrolysis capacity value at 7.55 (Table 1).

Table. 1: Sample location, isolate no. and cellulase activity on CMC-basal agar of bacteria isolated.

District/ Province	Isolate no.	Clear zone diameter (cm)	HC* value	Cellulase activity (Uml^{-1})	Identification
Pua/ Nan	P1-2	2.60	2.32	0.006	*Bacillus* sp.
	P1-3	1.85	2.89	0.008	*Paenibacillus* sp.
	P1-9	1.65	5.16	0.001	*Paenibacillus* sp.
	P3-1	2.95	2.81	0.015	*Bacillus* sp.
	P4-6	2.00	5.71	0.015	*Bacillus* sp.
	P5-5	0.63	2.63	0.004	*Bacillus* sp.
	P5-7	1.63	4.29	0.002	*Bacillus* sp.
	P5-8	1.18	5.36	0.001	*Bacillus* sp.
	P6-3	1.90	5.43	0.004	*Bacillus* sp.
	P6-5	0.57	1.84	0.003	*Bacillus* sp.
	P6-6	2.82	3.92	0.007	*Bacillus* sp.
	P7-5	2.70	2.41	0.007	*Bacillus* sp.
Santisuk/ Nan	S8-1	0.75	1.74	0.004	*Paenibacillus* sp.
	S8-4	2.11	4.14	0.003	*Paenibacillus* sp.
	S9-2	0.70	2.33	0.001	*Bacillus* sp.
	S10-2	0.51	1.65	0.01	*Bacillus* sp.
	S10-4	2.34	7.55	0.006	*Paenibacillus* sp.

*HC (hydrolysis capacity) was calculated from clear zone diameter divided by colony diameter.

Identification of isolates

All bacteria isolated were spore forming, Gram positive rods. They were divided into 2 groups based on their phenotypic and chemotaxonomic characteristics. All isolates were positive for catalase, oxidase, hydrolysis of L-arginine and grew at pH 5-8. Most of the isolates grew at 15, 20, 45 and 50 ^{o} C. All were negative for indole production, methyl red (MR), and dihydroxyacetone formation. They showed variable reaction on growth in the presence of 3-5% NaCl; DNAase, VP, citrate utilization, nitrate reduction, TSI, hydrolysis of gelatin, esculin, casein, L-tyrosine, starch, Tween 80; and on acid production from D-amygdalin, L-arabinose, D-cellobiose, D-galactose, inulin, lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, salicin, sucrose, D-trehalose, and D-xylose. All did not produce acids from gluconate, glycerol, inositol, ∞-methyl-D-glucoside, D-melezitose, raffinose, L-rhamnose, D-sorbitol, and L-sorbose (Table 2).

Table. 2: Differential characteristics of *Bacillus* (Group I) and *Paenibacillus* (Group II) isolates.

Characteristics	Group I (12)	P3-1, P4-6	Group II (5)	S10-4
Anaerobic growth	- (+3)	+	-(+1)	-
Growth at 50^{o} C	+	+	+(-2)	-
Growth at 55^{o} C	-	-	-(+1)	-
at pH 5-8	+	+	+	+
at pH 9	+ (-2)	+	+	+
Growth in 5% NaCl	+ (-2)	+	+	+
Hydrolysis:				
Casein	+ (-3)	+	-(+2)	-
Esculin	+ (-2)	+	+	+
Gelatin	+	+	+(-2)	-
Starch	+ (-4)	+	-(+2)	-
DNA	+ (-5)	+	-	-
Tyrosine	- (+2)	-	-	-
Tween 80	- (+1)	-	-	-
Urea	+ (-2)	+	+	+
Citrate utilization	- (+4)	+ (-1)	-(+1)	-
Nitrate reduction	+ (-5)	+	+(-1)	+
Voges-Proskauer	+ (-4)	+	+(-1)	-
Acid from:				
D-Amygdalin	+ (-5)	+	+(-1)	+
L-Arabinose	- (+4)	-	+	+
D-Cellobiose	+ (-2)	+	+	+
D-Galactose	- (+3)	-	+(-1)	+
Inulin	- (+5)	+	+(-1)	+
Lactose	+ (-6)	-	+(-2)	+
D-Maltose	+ (-2)	-	+(-2)	+
D-Mannitol	- (+2)	+	-(+1)	-
D-Mannose	+	+	+(-1)	+
D-Melibiose	- (+2)	-	+(-1)	+
Salicin	- (+5)	-	+(-2)	+
Sucrose	+ (-1)	+	+(-2)	+
D-Trehalose	+ (-4)	-	+	+
D-Xylose	+ (-5)	-	+(-1)	+

+, positive; -, negative. Numbers in parentheses indicate the number of isolates showing the reaction.

Group I contained 12 isolates. All were catalase and oxidase positive. They showed irregular, raised, entire undulate margin, smooth, dull, white colour colonies. Some strains showed circular, raised, entire margin, smooth, dull, white cream colour colonies. Isolate P4-6 produced endospore when grown on carboxymethyl cellulose agar medium at 37 ^{o} C for 2 days (Figure 1).

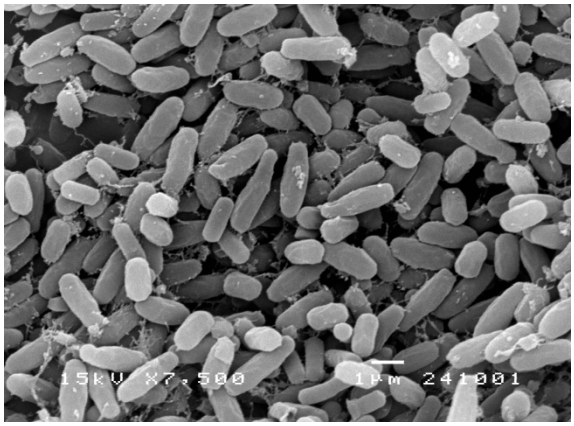

Fig. 1: Scanning electron micrograph of isolate P4-6 grown on carboxymethyl cellulose agar medium at 37 ^{o} C for 2 days.

Representative isolates, P3-1 (1490 bp) and P4-6 (1489 bp), were closely related to *Bacillus velesensis* LMG 22478^{T} (100% similarity), *Bacillus polyfermenticus* CJ6^{T} (99.7% similarity) and *Bacillus subtilis* DSM 10^{T} (99.7% similarity) based on 16S rRNA gene sequence (Figure 2). They contained *meso*-diaminopimelic acid in the cell wall peptidoglycan. Therefore, they were identified as *Bacillus velezensis* (Ruiz-García *et al.*, 2005).

Group II contained 5 isolates. Colonies were circular, raised, entire margin, smooth, dull, white cream colour. Bacteria in this group showed similar characteristics, however they could be differentiated from each other. Representative isolate, S10-4, contained *meso*-diaminopimelic acid in cell wall peptidoglycan and had 53.5 mol% of DNA G+C content. This strain (1545 bp) was closely related to *P. cellulositrophicus* KCTC 13135^{T} (98.7% similarity), *P. favisporus* LMG 20987^{T} (98.6% similarity) and *P. cineris* LMG 18439^{T} (98.6% similarity) based on 16S rRNA gene sequence (Figure 2) (Logan *et al.*, 2004). It will be proposed as a novel species after DNA-DNA hybridization experiment has been carried out.

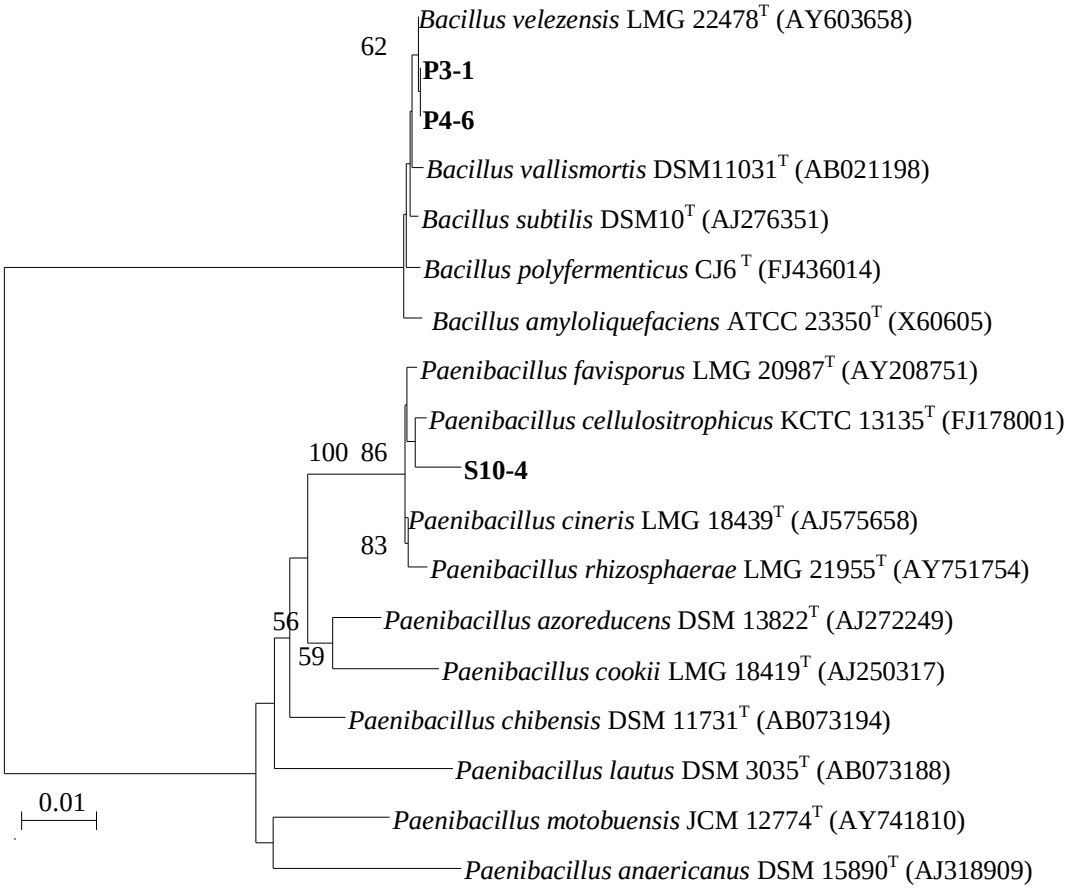

Fig. 2: Phylogenetic tree based on 16S rRNA gene sequence, showing relationship between isolates P3-1, P4-6, S10-4 and related *Bacillus* and *Paenibacillus* species.

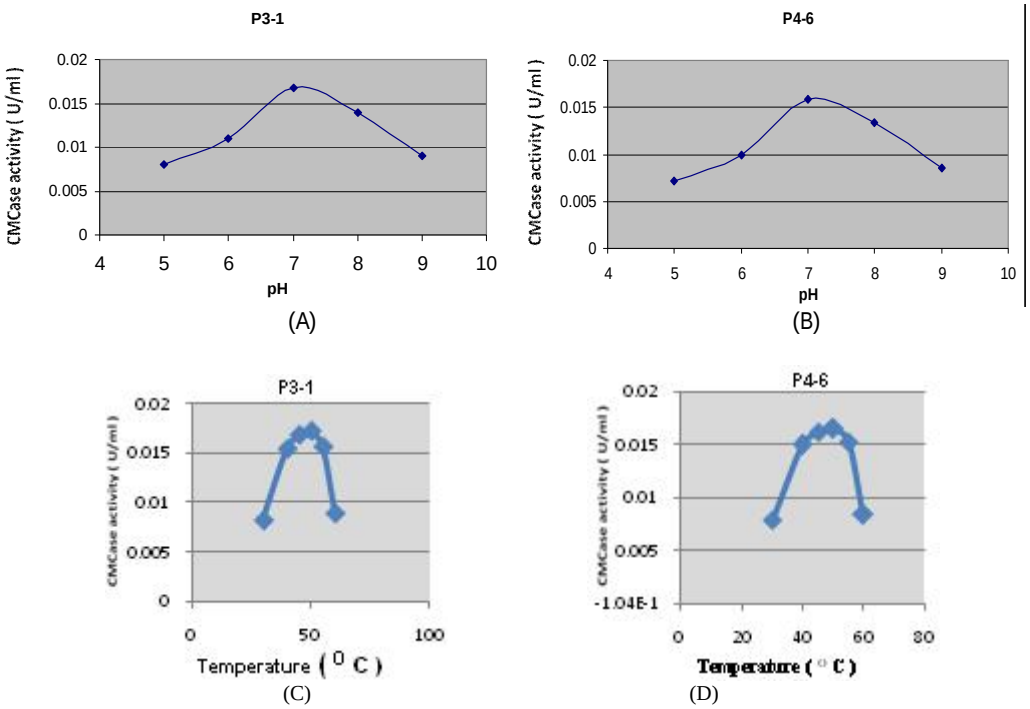

Fig. 3: Effect of pH and temperature on cellulase production of the isolates, P3-1 (A, C) and P4-6 (B, D).

Cellulase activity

The isolates P3-1 and P4-6 gave the highest cellulase activity (0.015 U ml^{-1}) when grown in carboxymethyl cellulose (CMC) broth. Their optimal pH and temperature for cellulase production were at 7 and 50 ^{o}C, respectively.

Several strains of *Bacillus* including *B. brevis, B. firmus, B. polymyxa, B. pumilus, B. subtilis, B. circulans* were reported as cellulase producing bacteria (Priest, 1977; Hakamada *et al.*, 2002; Sa-Pereira *et al*., 2002). Studies of *Bacillus* cellulase were lagged far behind that of fungal cellulase due to the fact that most *Bacillus* cellulase hydrolyses synthetic carboxymethyl cellulose (CMC) but barely hydrolyses crystalline form of cellulose. Ito (1989) isolated alkaline cellulase producing *Bacillus* and showed that this bacterial cellulase was an effective additive of laundry detergents. Other bacterial alkaline cellulase from *Bacillus* sp. (Eudo *et al.*, 2001), *B. circulans* (Hakamada *et al.*, 2002) and *Paenibacillus* sp. (Ogawa *et al.*, 2007) were reported. In addition, Kawai *et al.* (1988) reported that neutrophilic *Bacillus* produced alkaline cellulase. By this study, *Bacillus* and *Paenibacillus* strains isolated were found to be different from previous report based on their phenotypic characteristics (Table 2). Ten *Bacillus* and 2 *Paenibacillus* strains were isolated from Pua district, while 2 *Bacillus* strains and 3 *Paenibacillus* strains were isolated from Santisuk district. *Paenibacillus* sp. S10-4 isolated from Santisuk district showed high cellulase activity on CMC agar plate but low cellulase activity in CMC broth compared to *Bacillus* strains P3-1 and P4-6 that isolated from Pua district.

CONCLUSION

Seventeen cellulase producing bacteria were isolated from soil samples collected in Nan provice, Thailand by enrichment culture method at 40^{o}C. They were all spore forming, Gram-positive rods. Based on their morphological, cultural, physiological and biochemical characterictics including chemotaxonomic characterictics, they were divided into 2 groups. Representative strains of each group were selected and characterized by 16S rRNA gene sequence analysis. Phylogenetic tree constructed of the representative strains revealed that they were *Bacillus* strains and a novel species *Paenibacillus*. Highest cellulase producing isolates (0.015 U ml^{-1}) were belonged to genus *Bacillus*. Their cellulase had an optimal pH and temperature at 7 and 50^{o}C, respectively. Isolate with the highest hydrolysis capacity (7.55) belonged to genus *Paenibacillus*.

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