Bacillus siamensis sp. nov., isolated from salted crab (*poo-khem*) in Thailand

Punnanee Sumpavapol,¹ Linna Tongyonk,¹ Somboon Tanasupawat,² Nipa Chokesajjawatee,³ Plearnpis Luxananil³ and Wonnop Visessanguan³

Correspondence

Somboon Tanasupawat
Somboon.T@chula.ac.th

¹Department of Food and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

²Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

³National Center for Genetic Engineering and Biotechnology, 113 Phaholyothin Rd, Klong 1, Klong Luang, Pathumthani 12120, Thailand

A Gram-positive, endospore-forming, rod-shaped bacterium, strain PD-A10^T, was isolated from salted crab (*poo-khem*) in Thailand and subjected to a taxonomic study. Phenotypic and chemotaxonomic characteristics, including phylogenetic analyses, showed that the novel strain was a member of the genus *Bacillus*. The novel strain grew in medium with 0–14 % (w/v) NaCl, at 4–55 °C and at pH 4.5–9. The predominant quinone was a menaquinone with seven isoprene units (MK-7). The major fatty acids were anteiso-C_{15:0} and anteiso-C_{17:0}. Polar lipid analysis revealed the presence of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, lysylphosphatidylglycerol, glycolipid and unknown lipids. The DNA G + C content was 41.4 mol%. The 16S rRNA gene sequence similarities between strain PD-A10^T and *Bacillus amyloliquefaciens* NBRC 15535^T, *Bacillus subtilis* DSM 10^T, *Bacillus vallismortis* DSM 11031^T and *Bacillus mojavensis* IFO 15718^T were 99.5, 99.4, 99.4 and 99.2 %, respectively. Strain PD-A10^T showed a low degree similarity of rep-PCR fingerprints and low DNA–DNA relatedness with the above-mentioned species. On the basis of the data gathered in this study, strain PD-A10^T should be classified as representing a novel species of the genus *Bacillus*, for which the name *Bacillus siamensis* sp. nov. is proposed. The type strain is PD-A10^T (=BCC 22614^T=KCTC 13613^T).

The genus *Bacillus* is a large and heterogeneous collection of aerobic or facultative anaerobic, rod-shaped, endospore-forming bacteria that are widely distributed in the environment. At the time of writing, this genus includes more than 228 species (Euzéby, 2009) of which several species have been utilized for various industrial applications ranging from the synthesis of pharmaceuticals and chemicals to food production. Species of the genus *Bacillus* continue to be dominant organisms in microbial fermentations, especially those on the US Food and Drug Administration's generally regarded as safe (GRAS) list. The ability of different species to ferment over acid, neutral and alkaline pH ranges, combined with the presence of thermophiles in the genus, has led to the development of a variety of new commercial enzyme products with the

desired temperature, pH activity and stability properties to address a variety of applications (Schallmeyer *et al.*, 2004).

Poo-khem is a type of salted crab product sold in Thailand. It is commonly produced by mixing fresh water crab, either *Somanniathelpusa* sp. or *Sesarma mederi*, with salt (8–17 %) and allowing the mixture to ferment in an earthen jar for 1 day (Phithakpol *et al.*, 1995). Due to its salty taste and characteristic aroma, *poo-khem* has been used as a condiment to enhance the taste of other foods. Though it is quite popular, no studies have been done to determine the spectrum of micro-organisms associated with the spontaneous fermentation of *poo-khem*. However, halophilic or halotolerant members of the genus *Bacillus* are likely to be the most prevalent species and to persist until the end of the fermentation, a situation similar to that found for other salted fishery products such as fish sauce and krill paste (Saisithi *et al.*, 1966; Crisan & Sands, 1975; Yoon *et al.*, 2001; Noguchi *et al.*, 2004). In this study, a Gram-positive, rod-shaped bacterium, strain PD-A10^T, isolated from *poo-khem*, was characterized by means of a polyphasic taxonomic approach and compared with other recognized

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PD-A10^T is GQ281299.

A TLC plate showing the polar lipids for strain PD-A10^T is available as a supplementary figure with the online version of this paper.

species. Strain PD-A10^T exhibited characteristics that were sufficiently different from those of other recognized species of endospore-forming rods to distinguish it as a novel species of the genus *Bacillus*.

Strain PD-A10^T was isolated by means of a standard dilution plating technique from salted crab (*poo-kem*) collected from a local market in Nakornpathom Province, Thailand. The sample was plated on agar plates of modified Luria–Bertani (MLB) medium consisting of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 2.5% (w/v) glucose, 5.0% (w/v) NaCl and 1.5% (w/v) agar (pH 7.2) (Kim *et al.*, 1997) and was incubated at 37 °C for 24 h. A pure culture was obtained by repeated transfers of separate colonies on agar plates of the same medium. The reference strains used were *Bacillus amyloliquefaciens* KCTC 1660^T, *B. subtilis* subsp. *subtilis* KCTC 3135^T, *B. vallismortis* KCTC 3707^T, *B. licheniformis* KCTC 1918^T, *B. mojavensis* KCTC 3706^T and *B. velezensis* KCTC 13012^T. Unless otherwise stated, strains were grown in MLB medium and cultivated at 37 °C for 24 h.

Morphological, cultural, physiological and biochemical characteristics were determined as described by Claus & Berkeley (1986) and Barrow & Feltham (1993) unless otherwise indicated. Colonies grown on trypticase soy agar (TSA) were examined for their morphological and cultural characteristics including cell shape, colonial appearance, endospore formation and pigmentation after incubation at 37 °C for 2 days. The Gram reaction was determined by the method of Hucker & Conn (1923). Flagella were examined by using the staining method described by Rhodes (1958). Growth was tested on MacConkey agar and bile salt medium. Anaerobic growth was tested by incubating cultures on TSA plates with and without nitrate in an anaerobic jar with Anaerocult (Merck). Tests for catalase, oxidase, the hydrolysis of casein, aesculin, gelatin, starch, Tween 80 and tyrosine, DNase and urease activities, the methyl red/Voges–Proskauer (MR–VP) reaction, reduction of nitrate, phenylalanine deaminase activity and utilization of citrate were performed. H₂S production, indole production and cell motility were examined using sulphide–indole–motility (SIM) medium (Barrow & Feltham, 1993). Growth in various salt concentrations (0–20% NaCl), at different pH values (4–11) and at various temperatures (4–60 °C) were tested in nutrient broth. All tests were carried out by incubating the cultures at 37 °C, except for investigations into the effect of temperature upon growth. Acid production profiles from carbohydrates were obtained with the API 50 CH system (bioMérieux) after growth in 50 CHB medium as described by Logan & Berkeley (1984). Tubes from the API 50 CH gallery were also examined for gas bubbles. API 20 ZYM tests (bioMérieux) were performed according to the manufacturer's instructions.

Cell biomass for analyses of isoprenoid quinone composition and isomers of diaminopimelic acid in the cell wall was obtained from cultivation in TSB at 37 °C and

determined according to the methods of Komagata & Suzuki (1987). Polar lipids were extracted from freeze-dried cells (300 mg), separated by two-dimensional silica-gel TLC and detected with appropriate detection reagents (Tindall, 1990a, b). Cellular fatty acid content was determined by fatty acid methyl ester analysis according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (MIS; Sasser, 1990) at the Identification Service of the DSMZ (Braunschweig, Germany). Fatty acid content was compared to the fatty acid database in the Microbial Identification System, Sherlock version 4.5 (MIDI).

DNA was extracted and purified from whole cells by the phenol method of Saito & Miura (1963). The DNA G+C content was determined by the method of Tamaoka & Komagata (1984), with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. 16S rRNA gene sequencing was carried out using the methods reported by Shida *et al.* (1996) and Takagi *et al.* (1993). The 16S rRNA gene was amplified by PCR with the universal bacterial primers, 16S-27 F (5'-AGAGTTTGATCMTGGCTCAG-3') annealed at positions 8–27 and 16S-1539 R (5'-CCAGTGAGCAGAGTGACG-3') annealed at positions 1522–1539 (*Escherichia coli* numbering according to Brosius *et al.*, 1978). The amplification reaction was performed with a volume of 25 µl, using the Takara *Ex Taq* DNA polymerase and buffer system (Takara Mirus Bio Corporation). The final PCR mixture comprised 1 × *Ex Taq* buffer (with 1.5 mM MgCl₂), 200 µM of each deoxynucleoside triphosphate, 0.2 µM of each primer, 1 unit *Ex Taq* DNA polymerase and 50 ng template DNA. Amplification was carried out in a thermocycler (GeneAmp PCR System 2400, PE Biosystems) with the following cycling program: initial denaturation at 94 °C for 5 min followed by 25 cycles of 94 °C for 10 s, annealing at 58 °C for 30 s, and extension at 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The PCR product was purified using a QIAquick-PCR purification kit (Qiagen). The double-stranded DNA was sequenced with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions, by the use of the following four primers; 16S-357 R (5'-CTGCTGCCT-CCCGTAG-3') annealed at positions 342–357, 16S-802 R (5'-TACCAGGGTATCTAATCC-3') annealed at positions 785–802, 16S-1115 R (5'-AGGGTTGCGCTCGTTG-3') annealed at positions 1000–1115 and 16S-1539 R (5'-CCAGTGAGCAGAGTGACG-3') annealed at positions 1522–1539. The PCR products were sequenced with an ABI PRISM 377 Genetic Analyzer (Applied Biosystems). The 16S rRNA gene sequence obtained (1525 bases) was deposited in the GenBank/EMBL/DDBJ databases with accession no. GQ281299. The sequence was aligned along with selected sequences from the GenBank/EMBL/DDBJ databases by using the CLUSTAL_X program (version 1.81) (Thompson *et al.*, 1997). Gaps and ambiguous bases were eliminated from the calculations. The distance matrices for

the aligned sequences were calculated by the two-parameter method of Kimura (1980). A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) with the MEGA program (version 2.1) (Kumar *et al.*, 2001). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein (1985) based on 1000 samplings. Repetitive sequence based PCR (rep-PCR) fingerprinting, as described by Versalovic *et al.* (1994), was carried out with (GTG)₅ primer (5'-GTGGTGGTGGTGGT-3') (Gevers *et al.*, 2001) and the PCR and electrophoresis conditions were modified from Chokesajjawatee *et al.* (2008). The amplification reaction was carried out in a volume of 50 µl and the final PCR mixture comprised 1 × *Ex Taq* buffer (with 1.5 mM MgCl₂), 200 µM of each deoxynucleoside triphosphate, 800 nM primer, 1.25 units *Ex Taq* DNA polymerase and 50 ng template DNA. Amplification was carried out in a Peltier PTC-200 thermal cycler (MJ Research Inc.) with the following cycling program: initial denaturation at 95 °C for 5 min followed by 35 cycles of 94 °C for 45 s, annealing at 40 °C for 1 min, and extension at 65 °C for 10 min, and a final extension step at 65 °C for 20 min. A 5 µl aliquot of the PCR product was electrophoresed for 2.4 h at 4 V cm⁻¹ on a 1 % (w/v) agarose gel in 0.5 × TBE buffer (0.045 M Tris base, 0.045 M boric acid, 0.001 M EDTA, pH 8.0). After staining with ethidium bromide (0.5 µg ml⁻¹), the fingerprint patterns were captured using

a laser scanner Typhoon 9410 (Amersham Pharmacia Biotech). The resulting fingerprints were analysed using the GelComparII v.4.5 computer package (Applied Maths). The similarities in the fingerprint patterns were calculated using the Pearson correlation coefficient and the cluster analysis was performed using the unweighted-pair group method using average linkages (UPGMA). The different dendrograms were interpreted visually to set the delineation level separately for each species. DNA–DNA hybridization was carried out by the photobiotin-labelling method with microdilution wells at 43 °C for 5 h (Ezaki *et al.*, 1989).

Cells of strain PD-A10^T were Gram-positive, facultatively anaerobic, endospore-forming rods that were 0.3–0.6 µm wide and 1.5–3.5 µm long after 48 h culture on TSA. Colonies formed on agar plates were creamy white, mucoid, translucent, raised, had entire margins and were 3–4 mm in diameter after 2 days incubation at 37 °C. In TSB, a thin film formed at the surface whilst the rest of the medium was uniformly cloudy. Strain PD-A10^T could be differentiated from other phylogenetically related species of the genus *Bacillus* by means of some phenotypic characteristics (Table 1). For example, strain PD-A10^T was halo-acidotolerant and was able to grow at acidic pH values and in the presence of NaCl up to 14 % (w/v). In addition, strain PD-A10^T was able to grow over a wider range of temperatures, especially at 55 °C, when compared with related species.

Table 1. Differential characteristics of strain PD-A10^T and related species of the genus *Bacillus*

Strains: 1, PD-A10^T; 2, *B. amyloliquefaciens* KCTC 1660^T; 3, *B. subtilis* subsp. *subtilis* KCTC 3135^T; 4, *B. vallismortis* KCTC 3707^T; 5, *B. mojavensis* KCTC 3706^T; 6, *B. licheniformis* KCTC 1918^T. Data were obtained in this study unless otherwise indicated. +, Positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5	6
Oxidase activity	–	+	+	+	+	+
Citrate utilization	–	+	+	+	+	+
Temperature for growth (°C):						
Maximum	55	50	50	50	50	55
Minimum	4	10	10	10	10	15
Growth in the presence of NaCl (%):						
Maximum	14	10	12	10	12	10
Growth at pH:						
Minimum	4.5	6	6	6	5	6
Acid from:						
D-Arabinose	–	+	+	+	ND	+
Gentiobiose	+	–	–	–	–	–
Glycogen	+	–	–	–	–	–
Lactose	+	+	–	–	–	–
Melibiose	–	+	+	–	–	+
Methyl α-D-glucoside	+	+	+	–	–	+
Trehalose	–	+	+	+	+	+
Turanose	–	–	+	–	–	+
D-Xylose	+	–	–	–	+	–
DNA G + C content (mol%)	41.4	45.4 ^a *	42.9 ^b	43.7 ^c	43.4 ^d	45.3 ^c

*Data from: a, Priest *et al.* (1988); b, Claus & Berkeley (1986); c, Roberts *et al.* (1996); d, Roberts *et al.* (1994).

Strain PD-A10^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan and had menaquinone with seven isoprene units (MK-7) as the predominant isoprenoid quinone. The cellular fatty acid profiles showed a large amount of branched fatty acids; the major components were anteiso-C_{15:0} (42.35 %) and anteiso-C_{17:0} (22.65 %) (Table 2). Cellular fatty acid profiles, particularly major fatty acid(s), distinguished strain PD-A10^T from the closely related species in the genus *Bacillus*. The genus *Bacillus* comprises variable species having anteiso-C_{15:0} and iso-C_{15:0} as the major fatty acids (Shida *et al.*, 1997 and Yoon *et al.*, 2001). Accordingly, strain PD-A10^T was distinct from some groups of species within the genus *Bacillus*. The DNA G+C content of strain PD-A10^T was 41.4 mol%. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, lysylphosphatidylglycerol, glycolipid, an unknown aminophospholipid, four unknown aminolipids and two unknown lipids were found (see Supplementary Fig. S1 in IJSEM Online).

An almost-complete 16S rRNA gene sequence of strain PD-A10^T comprising 1525 nt was determined directly after PCR amplification. In a phylogenetic tree based on 16S rRNA gene sequences, strain PD-A10^T formed a cluster with *Bacillus amyloliquefaciens* NBRC 15535^T within the genus *Bacillus* (Fig. 1). 16S rRNA gene sequence similarities between strain PD-A10^T and *B. amyloliquefaciens* NBRC 15535^T, *B. subtilis* subsp. *subtilis* DSM 10^T, *B. vallismortis* DSM 11031^T and *B. mojavensis* IFO 15718^T were 99.5, 99.4,

99.4 and 99.2 %, respectively. The relationship between strain PD-A10^T and closely related species of the genus *Bacillus* was also determined by the rep-PCR fingerprinting method, a useful technique for determining inter- and intraspecies relatedness (Versalovic *et al.*, 1994 and Gevers *et al.*, 2001). The fingerprints obtained with primer (GTG)₅ are shown in Fig. 2. The (GTG)₅-PCR patterns resulted in the delineation of three (GTG)₅-PCR clusters with Pearson's correlation coefficient values of 50.6 %, comprising four, two and one isolates. Within the clusters, the (GTG)₅ patterns showed delineation of the strains examined at Pearson's correlation coefficient values of 70, 72.8 and 74 % indicating that they were different genotypically and belonged to separate distinct species. Nick *et al.* (1999) and Rademaker *et al.* (2000) have compared rep-PCR genomic fingerprint analysis with DNA–DNA relatedness; they suggested that the two techniques yield results that are in close agreement. Heyrman *et al.* (2003) reported that rep-PCR fingerprinting can be used as a genomic screening method to differentiate strains at the species level and to select representatives for DNA–DNA reassociation experiments. A DNA–DNA hybridization study revealed that strain PD-A10^T showed only low levels of DNA–DNA relatedness to *B. amyloliquefaciens* KCTC 1660^T (39.46 %), *B. vallismortis* KCTC 3707^T (27.43 %) and *B. subtilis* subsp. *subtilis* KCTC 3135^T (25.34 %) as shown in Table 3. Thus, strain PD-A10^T is not a member of these recognized species of the genus *Bacillus*.

In conclusion, on the basis of the above physiological and biochemical data, molecular phylogenetic results, cellular fatty acid compositions and DNA G+C content, strain PD-A10^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus siamensis* sp. nov. is proposed.

Description of *Bacillus siamensis* sp. nov.

Bacillus siamensis (si.am.en'sis. N.L. masc. adj. *siamensis* pertaining to Siam, the old name of Thailand, from where the type strain was isolated).

Cells are Gram-positive, facultatively anaerobic and rod-shaped, measuring 0.3–0.6 × 1.5–3.5 µm. Cells occur singly, in pairs and occasionally in short chains. They are motile with peritrichous flagella. Ellipsoidal endospores are produced at central or subterminal positions in swollen sporangia. Colonies are creamy white, mucoid, translucent, raised, have an entire margin and are 3–4 mm in diameter after 2 days incubation at 37 °C on TSA. In liquid medium, a thin film is formed at the surface whilst the rest of the medium is uniformly cloudy. Anaerobic growth occurs. Grows on MacConkey agar but not on bile salt medium. Produces catalase but not oxidase. Positive result in tests for the hydrolysis of casein, aesculin, gelatin, DNA and starch, for nitrate reduction and with the Voges–Proskauer test. Negative results in tests for hydrolysis of tyrosine and Tween 80, production of H₂S and indole, urease activity

Table 2. Cellular fatty acid profiles (%) of strain PD-A10^T and related species of the genus *Bacillus*

Strains: 1, PD-A10^T; 2, *B. amyloliquefaciens* KCTC 1660^T; 3, *B. subtilis* subsp. *subtilis* KCTC 3135^T; 4, *B. vallismortis* KCTC 3707^T; 5, *B. mojavensis* KCTC 3706^T; 6, *B. licheniformis* KCTC 1918^T. Data were obtained in this study by the Identification Service of the DSMZ (Braunschweig, Germany). The relative amount of each fatty acid is expressed as a percentage of the total fatty acid. –, Value below limit of detection.

Fatty acid	1	2	3	4	5	6
Straight-chain saturated						
C _{14:0}	0.56	0.70	–	–	–	0.48
C _{16:0}	6.41	5.40	3.65	2.71	2.05	3.67
Branch-chain saturated						
iso-C _{14:0}	1.42	1.48	0.87	1.07	0.98	0.87
iso-C _{15:0}	9.80	28.63	24.08	24.60	22.33	32.36
iso-C _{16:0}	6.47	3.12	2.47	4.06	2.56	3.74
iso-C _{17:0}	9.81	13.18	11.53	14.43	8.92	8.42
anteiso-C _{15:0}	42.35	33.10	38.48	37.50	42.51	34.08
anteiso-C _{17:0}	22.65	8.65	12.52	12.07	12.53	11.64
Monounsaturated						
C _{16:1} ω11c	–	2.73	1.75	–	–	1.43
iso-C _{17:1} ω10c	–	2.05	2.41	–	–	1.47

Fig. 1. Phylogenetic relationships of strain PD-A10^T, some species of the genus *Bacillus* and related taxa based on 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbour-joining method. Bootstrap values (expressed as percentages of 1000 replications) >60 % are shown at the branch points. Bar, 0.01 substitutions per nucleotide position.

and the methyl-red reaction. Growth occurs at 4 °C and 55 °C; optimal growth temperature is 37 °C. Growth occurs between pH 4.5 and 9.0 and the optimal pH is 6–7. Growth occurs in the presence of 0–14 % (w/v) NaCl and optimally with no NaCl. Phenylalanine and tryptophan deaminase are not produced. When assayed with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase are present, but lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are absent. Acid is produced from glycerol, L-arabinose, D-ribose,

D-xylose, D-glucose, D-fructose, inositol, D-mannitol, D-sorbitol, methyl α -D-glucopyranoside, amygdalin, arbutin, aesculin ferric citrate, salicin, cellobiose, maltose, lactose, sucrose, raffinose, starch, glycogen and gentiobiose. Acid is not produced from erythritol, D-arabinose, L-xylose, D-adonitol, methyl β -D-xylopyranoside, D-galactose, D-mannose, L-sorbose, L-rhamnose, dulcitol, methyl α -D-mannopyranoside, *N*-acetylglucosamine, trehalose, inulin, melezitose, xylitol, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-7. The major fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{17:0} and

Fig. 2. (GTG)₅-PCR banding patterns of strain PD-A10^T and related species of the genus *Bacillus*. The dendrogram was generated after cluster analysis of the digitized fingerprints and was derived from UPGMA linkage of Pearson's correlation similarity coefficients.

Table 3. DNA–DNA relatedness of strain PD-A10^T and related species of the genus *Bacillus*

ND, Not determined.

Strain	% DNA–DNA reassociation with*		
	1	2	3
1. PD-A10 ^T	100	46.54	25.17
2. <i>B. amyloliquefaciens</i> KCTC 1660 ^T	39.46	100	ND
3. <i>B. subtilis</i> subsp. <i>subtilis</i> KCTC 3135 ^T	25.34	ND	100
<i>B. vallismortis</i> KCTC 3707 ^T	27.43	ND	ND

*Means of the values from three determinations.

iso-C_{15:0}. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, lysylphosphatidylglycerol, glycolipid, an unknown aminophospholipid, four unknown aminolipids and two unknown lipids.

The type strain, PD-A10^T (=BCC 22614^T=KCTC 13613^T), was isolated from salted crab (*poo-khem*). The DNA G + C content of the type strain is 41.4 mol%.

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