Paenibacillus cisolokensis sp. nov., isolated from litter of a geyser

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A Gram-stain-positive, endospore-forming, aerobic and thermophilic bacterium, designated strain LC2-13A^T, was isolated from Cisolok geyser, West Java, Indonesia, at 50 °C. The isolate was rod-shaped and motile by means of peritrichous flagella. The major cellular fatty acids were iso-$C_{16:0}$, $C_{16:0}$ and anteiso-$C_{15:0}$ and the major quinone was menaquinone 7. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The genomic DNA G+C content was 56.6 mol% and the major diagnostic diamino acid in the cell-wall peptidoglycan was *meso*-diaminopimelic acid. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain LC2-13A^T is related most closely to *Paenibacillus kobensis* DSM 10249^T (94.86 % similarity), *Paenibacillus tarimensis* SA-7-6^T (94.77 %) and *Paenibacillus barengoltzii* SAFN-016^T (94.77 %). On the basis of phenotypic, chemotaxonomic and phylogenetic evidence, strain LC2-13A^T is affiliated to the genus *Paenibacillus*, but could be distinguished from recognized species of this genus. A novel species with the name *Paenibacillus cisolokensis* sp. nov. is thus proposed. The type strain is LC2-13A^T (=UICC B-42^T=NRRL B-65368^T=DSM 101873^T).

Ash *et al.* (1991) proposed to divide the genus *Bacillus sensu lato* into five distinct groups based on 16S rRNA gene sequence analysis and the genus *Paenibacillus* was established as group 3 (Ash *et al.*, 1991, 1993, 1994). An emended description of the genus was subsequently provided by Shida *et al.* (1997). The genus *Paenibacillus* was originally included in the family *Bacillaceae* (Ash *et al.*, 1993) and later reclassified into the family *Paenibacillaceae* (De Vos *et al.*, 2009). *Paenibacillus polymyxa* is the type species of the genus *Paenibacillus* (Stanly & Schlosser, 1947). At the time of writing, this genus comprises 174 species with validly published names (http://www.bacterio.net/paenibacillus.html).

Generally, species belonging to this genus are defined as Gram-stain-positive (although some may stain negatively) (Zhou *et al.*, 2012), aerobic or facultatively anaerobic, non-pigmented and endospore-forming bacteria, motile by means of peritrichous flagella. Menaquinone 7 (MK-7) is the major menaquinone and *meso*-diaminopimelic acid (*meso*-DAP) is the diamino acid in the cell-wall peptidoglycan. The major cellular fatty acids are anteiso-$C_{15:0}$ and iso-$C_{16:0}$ (Chou *et al.*, 2007; De Vos *et al.*, 2009; Yao *et al.*, 2014). The DNA G+C contents are in the range 39–59 mol % (Shida *et al.*, 1997; Montes *et al.*, 2004; Takeda *et al.*, 2005; Yao *et al.*, 2014). All members of the genus *Paenibacillus* for which polar lipid data are available show diphosphatidylglycerol (DPG) as the major polar lipid (Xiang *et al.*, 2014); some species also contain phosphatidylethanolamine (PE) (Kim *et al.*, 2010; Tang *et al.*, 2011) and phosphatidylglycerol (PG) (Zhou *et al.*, 2012). In this study,

Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; *meso*-DAP, *meso*-2,4-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession for the 16S rRNA gene sequence of strain LC2-13A^T is LC055784.

One supplementary figure is available with the online Supplementary Material.

the taxonomic position of strain LC2-13A^T was determined by means of a polyphasic taxonomic analysis based on the minimal standards for description of aerobic, endospore-forming bacteria (Logan *et al.*, 2009; Tindall *et al.*, 2010).

Strain LC2-13A^T was isolated from Cisolok geyser, West Java, Indonesia (6° 56′ 33″ S 106° 27′ 203″ E). Bacterial isolation was performed using ISP (International *Streptomyces* Project) 2 medium at 50 °C. ISP 2 agar medium is composed of 4 g yeast extract, 10 g malt extract, 4 g glucose and 20 g agar per litre of deionized water (pH 7.3). Litter samples were dried for a few hours by keeping them under a dried air stream, and then cut into small pieces, and each piece was placed on the agar surface, and incubated at 50 °C for 2 weeks. Single colonies were subcultivated several times to obtain pure isolates.

The nearly full-length 16S rRNA gene sequence (1423 bp) of strain LC2-13A^{T}was analysed as described by Nurkanto *et al.* (2012). The sequence was aligned with its close relatives using the CLUSTAL W program software package (Thompson *et al.*, 1997). A neighbour-joining (Saitou & Nei, 1987) tree was reconstructed. Distances were calculated according to Kimura's two-parameter method (Kimura, 1980) and bootstrap analysis was based on 1000 resamplings (Felsenstein, 1985). The 1423 bp 16S rRNA gene sequence of strain LC2-13A^T was closely related to those of *Paenibacillus kobensis* DSM 102549^T (94.86 %), *Paenibacillus tarimensis* SA-7-6^T (94.77 %), *Paenibacillus barengoltzii* SAFN-016^T (94.77 %), *Paenibacillus xanthinilyticus* 11N27^T (94.75 %), '*Paenibacillus marinum*' THE22 (94.68 %), *Paenibacillus cellulosilyticus* PALXIL08^T (94.59 %), *Paenibacillus mendelii* C/2^T (94.54 %), *Paenibacillus oenotherae* DT7-4^T (94.48 %), *Paenibacillus timonensis* 2301032^T (94.46 %), *Paenibacillus wooponensis* WPCB018^T (94.38 %), *Paenibacillus pasadenensis* SAFN-07^T (93.95 %), *Paenibacillus daejeonensis* AP-20^T (94.37 %) and *Paenibacillus darwinianus* Br^T (94.02 %). The resulting neighbour-joining tree is shown in Fig. 1, showing that strain LC2-13A^T belongs to a cluster of *Paenibacillus* species.

Colony morphology was observed on cells grown on ISP 1 agar plates at 50 °C for 3 days. ISP 1 agar medium is composed of 5 g tryptone, 3 g yeast extract and 20 g agar per litre of deionized water (pH 7.0). The Gram reaction was determined as described by Magee *et al.* (1975). A motility test was performed using the hanging-drop method. Cell morphology and flagella were observed using a transmission electron microscope (JEM-1230; JEOL).

Sporulating cells were observed using a phase-contrast microscope (BX51M; Olympus) with cells grown at 50 °C for 5 days on ISP 2. Growth at different temperatures (30, 37, 45, 50 and 60 °C) was tested on ISP 1 for 3 days. Growth at pH 3, 4, 5, 6, 7, 8 and 9 was tested using ISP 1 medium at 50 °C for 3 days. The ISP 2 medium adjusted to different pH values was prepared with 2 M HCl or 2 M NaOH. Growth under anaerobic conditions on ISP 2 agar plates was investigated using an anaerobic chamber (Mitsubishi Gas Chemical) at 50 °C for 1 week. Growth on tripticase soy agar (TSA), R2A agar and ISP 1 was also investigated. Catalase and oxidase activities, hydrolysis of casein, gelatin, starch, aesculin, L-tyrosine and DNA, urease activity, nitrate reduction, methyl red test, Voges–Proskauer reaction, citrate utilization, production of H_2O and NH_3, and indole production were investigated by using the API 20 and API 20NE systems. Hydrolysis of cellulose, xylose and starch was tested on ISP 2. Acid production from various carbohydrates was determined by using the API 50CHB system. Enzyme activities were examined using the API ZYM system (bioMérieux) according to the manufacturer's instructions. Physiological differences between strain LC2-13A^T and the type strains of other recognized species of the genus *Paenibacillus* are given in Table 1 and in the species description below.

Strain LC2-13A^T was Gram-reaction-positive, aerobic, motile and produced endospores, which lay terminally or subterminally in swelling sporangia (Fig. 2). Cells were rod-shaped with peritrichous flagella, and cells were 0.8–0.9 μm wide and 2.0–2.4 μm long (Fig. 3). Colonies on ISP 1 medium were creamy white, smooth, circular and with approximate diameters of 3–5 mm after 3 days of growth at 50 °C. Growth was observed at temperatures and pH of 40–60 °C and pH 6.0–8.0. Optimal growth occurred at 45–50 °C and pH 6.0–7.0. Strain LC2-13A^T showed optimal growth on ISP 1 and moderate growth on ISP 2, nutrient agar (NA), R2A agar and TSA. Other characteristics of strain LC2-13A^T are listed in the species description and the differences in phenotypic characteristics between strain LC2-13A^T and the reference species are given in Table 1.

Analysis of cellular fatty acids of strain LC2-13A^{T}and reference strains was performed according to the instructions of the Microbial Identification System (Sherlock TSBA Library version 3.80; Microbial ID) (Sasser, 1990). The strains were cultivated for 72 h in tripticase soy broth (TSB) at 50 °C until the bacteria reached mid-exponential phase of growth. Cell walls were prepared using the methods described by Schleifer & Kandler (1972). Briefly, dry cells were mechanically disrupted with an ultrasonic oscillator, and precipitates obtained by centrifuging at 18 000 r.p.m for 30 min were heated at 100 °C for 40 min in 3 % SDS solution, washed with warm water three times and freeze-dried. The amino acid composition of complete cell-wall hydrolysates was determined by developing preparations on cellulose TLC plates (Tokyo Kasei), using two-dimensional descending chromatography as described by Harper & Davis (1979). Menaquinones were extracted as described by Collins *et al.* (1977) from lyophilized cells grown on ISP 2 at 50 °C for 3 days and analysed by HPLC. Polar lipids of strain LC2-13A^T were determined by two-dimensional TLC as described by Minnikin *et al.* (1979, 1984). The DNA G +C content of strain LC2-13A^T was determined by HPLC according to the method of Tamaoka & Komagata (1984).

The major fatty acids (>5 %) of strain LC2-13A^T comprised iso-$C_{16:0}$ (23.4 %), $C_{16:0}$ (23.2 %), anteiso-$C_{15:0}$ (21.5 %) and iso-$C_{15:0}$ (11.4 %) (Table 2). The cell-wall hydrolysate contained *meso*-DAP as the major diamino acid. The major quinone of strain LC2-13A^T was MK-7. The predominant

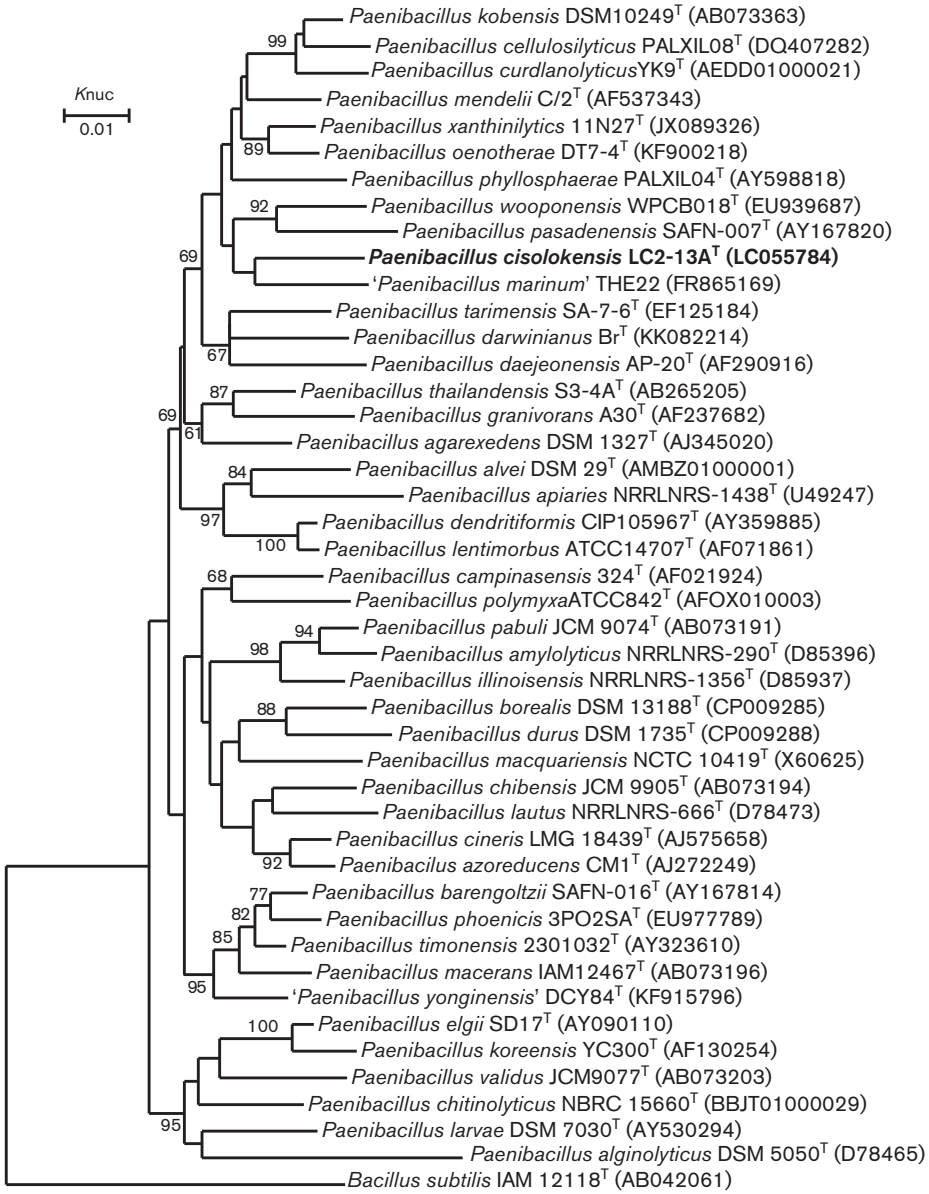

Fig. 1. Neighbour-joining tree based on partial 16S rRNA gene sequences. Bootstrap values above 60 % (based on 1000 replications) are shown at branch points. The sequence of *Bacillus subtilis* IAM 12118^{T}was included as the outgroup. Bar, 0.02 substitutions per nucleotide position.

menaquinone and cell-wall diamino acid was the same as that of the type species and other members of the genus *Paenibacillus* (De Vos *et al.*, 2009; Yao *et al.*, 2014). The polar lipids of strain LC2-13A^T consisted of DPG, PG, PE, three unknown phospholipids, three unknown aminophospholipids and three unknown lipids (Fig. S1, available in the online Supplementary Material). The DNA G+C content of strain LC2-13A^T was 56.6 mol%, which was similar to those of related species of the genus *Paenibacillus*.

Although strain LC2-13A^T shared many features with existing species of the genus *Paenibacillus*, phenotypic and chemotaxonomic results provide evidence to demonstrate that it represents a novel species of the genus *Paenibacillus*.

During 16S rRNA gene sequence analysis, the pairwise comparison indicated that strain LC2-13A^T shared a similarity of less than 94. 86 % with all recognized species of the genus *Paenibacillus*. These similarity values suggested that strain LC2-13A^T could be considered as a representative of a novel species as sequence divergence values of more than 3 % are recommended for species delineation (Stackebrandt & Goebel, 1994).

Taken together, the genotypic and phenotypic data presented in this study dsemonstrate that the novel

Table 1. Differential characteristics between strain LC2-13A^T and related *Paenibacillus* species

Strains: 1, LC2-13A^T; 2, *P. tarimensis* DSM 19409^T (Wang *et al.*, 2008); 3, *P. daejeonensis* DSM 15491^T (Lee *et al.*, 2002); 4, *P. darwinianus* Br^T (Dsouza *et al.*, 2014); 5, *P. wooponensis* WPCB018^T (Baik *et al.*, 2011); 6, '*P. marinum*' THE22 (Bouraou *et al.*, 2013); 7, *P. pasadenensis* SAFN-007^T (Osman *et al.*, 2006). V, Variable; W, weakly positive; ND, not determined; m, monotrichous; p, peritrichous.

	1	2	3	4	5	6	7
Gram stain	+	V	V	V	+	+	+
Flagella arrangement	p	p	p	m	p	Non-motile	p
Oxidase	+	−	+	−	+	−	+
Optimum growth temperature (°C)	50	37	30	18	30–35	55	ND
Growth at pH 5.7	+	−	−	−	+	+	ND
DNA G+C content (mol%)	56.6	53.7	53	55.6	56.0	56.0	ND
Hydrolysis of starch	+	−	+	+	−	+	ND
Acid production from:							
D-Arabinose	+	W	−	−	−	ND	ND
L-Arabinose	+	W	−	−	−	+	−
D-Fructose	+	−	−	+	+	ND	ND
D-Mannose	−	+	−	−	+	ND	ND
L-Rhamnose	+	−	−	−	−	ND	−
Inositol	+	−	−	−	−	ND	ND
Melezitose	−	W	−	−	+	ND	ND
Glycogen	−	+	+	−	−	ND	ND
Turanose	+	W	−	−	−	ND	ND
D-Tagatose	−	+	−	−	−	ND	ND
Melibiose	−	+	ND	ND	−	ND	−
Raffinose	−	+	+	−	−	ND	ND
Starch	−	+	+	−	−	ND	ND
Assimilation of:							
D-Glucose	−	ND	ND	ND	−	+	+
L-Arabinose	+	ND	ND	ND	+	−	+
D-Mannose	−	ND	ND	ND	+	+	+
D-Mannitol	−	ND	ND	ND	+	+	+
N-Acetyl-glucosamine	−	ND	ND	ND	+	+	+
Potassium gluconate	−	ND	ND	ND	−	+	−
n-Capric acid	−	ND	ND	ND	+	−	−
Adipate	−	ND	ND	ND	+	−	−
DL-Malate	−	ND	ND	ND	+	−	+
Sodium citrate	−	ND	ND	ND	+	−	−
Phenyl acetic acid	−	ND	ND	ND	+	−	−
D-Sorbitol	+	ND	ND	ND	−	−	−
Sucrose	−	ND	ND	ND	−	+	−
Melibiose	+	ND	ND	ND	−	+	+
D-Amygdalin	+	ND	ND	ND	−	+	−

thermophilic bacterium LC2-13A^T represents a novel species of the genus *Paenibacillus,* for which the name *Paenibacillus cisolokensis* sp. nov. is proposed.

Description of *Paenibacillus cisolokensis* sp. nov.

Paenibacillus cisolokensis (ci.so.lok.en'sis. N.L. masc. adj. *cisolokensis* pertaining to Cisolok geyser, West Java, Indonesia, where the type strain was isolated).

Cells are Gram-reaction-positive, aerobic, endospore-forming rods with peritrichous flagella. The rods are 0.8–0.9 μm wide and 2.0–2.4 μm long. Colonies on ISP 1 medium are white, circular, flat, smooth and 2–3 in diameter after incubation at 50 °C for 3 days. Grows on ISP 1, ISP 2 and TSA. Growth occurs at 37–55 °C (optimal at 45–50 °C), at pH 6.0–9.0 (optimal at 6.0–7.0) and in TSB with 0–2.0 % (w/v) NaCl. Positive for oxidase and catalase activities, hydrolysis of starch, cellulose, xylan, aesculin, gelatin and chitin, nitrate reduction, acid

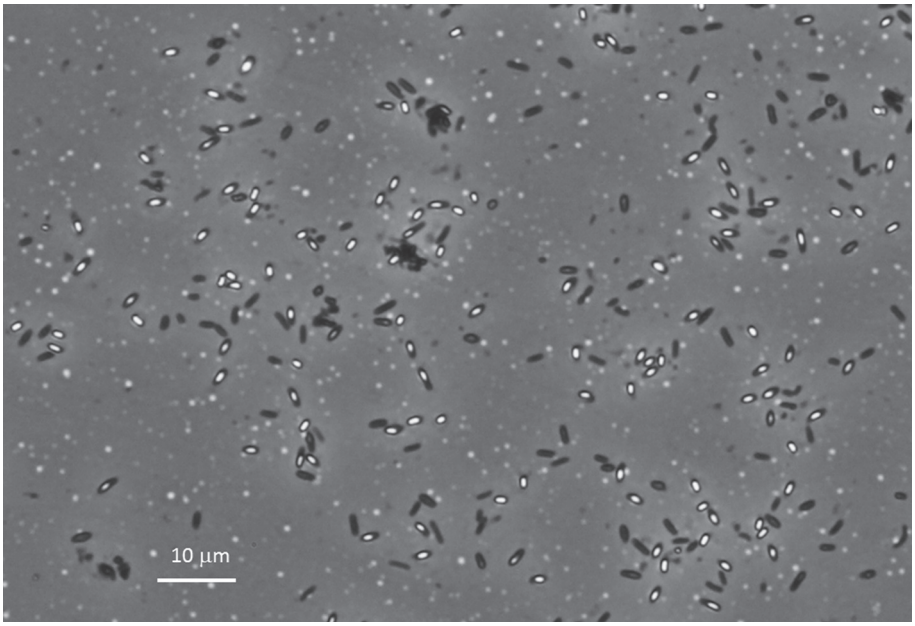

Fig. 2. Phase-contrast light micrograph of cells of strain LC2-13A^T grown on ISP 1 agar at 50 °C for 3 days. Bar, 10 μm.

production from glucose and urease, β-D-galactosidase, arginine dihydrolylase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase, but negative for indole production, citrate utilization, H_2S production and Voges–Proskauer test. In API 20NE and API 20E systems, the following substrates are utilized as sole carbon and/or nitrogen sources: L-arabinose, maltose, D-sorbitol, melibiose and D-amygdalin; the following substrates are not utilized: D-mannose, D-mannitol, *N*-acetyl-glucosamine, *n*-capric acid, adipic acid, DL-malic acid, sodium succinic acid and phenyl acetic acid. In the API ZYM system, the following enzyme activities are positive: alkaline phosphatase, esterase (C4), esterase (C8), leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase and α-glucosidase; the following enzyme activities are negative: lipase (C14), cystine arylamidase, β-glucosidase, *N*-acetyl-β-galactosaminidase, α-mannosidase and α-fucosidase. In the API 50CHB system, acid is produced from D-arabinose, L-arabinose, D-xylose, D-adonitol, methyl β-D-xylopyranoside, D-glucose, D-fructose, L-rhamnose, inositol, D-mannitol, D-sorbitol, methyl β-D-mannopyranoside, methyl β-D-glucopyranoside, amygdalin, aesculin ferric citrate, salicin, cellobiose, maltose, D-lactose, sucrose, trehalose, gentiobiose, turanose and L-fucose, but not from glycerol, erythritol, ribose, L-xylose, D-galactose, D-mannose, L-sorbose, galactitol, *N*-acetyl-glucosamine, arbutin, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. The major quinone is MK-7. The diagnostic diamino acid of the cell-wall peptidoglycan is *meso*-DAP. The major polar lipids are DPG, PG and PE. The major cellular fatty acids are iso-$C_{16:0}$, $C_{16:0}$ and anteiso-$C_{15:0}$.

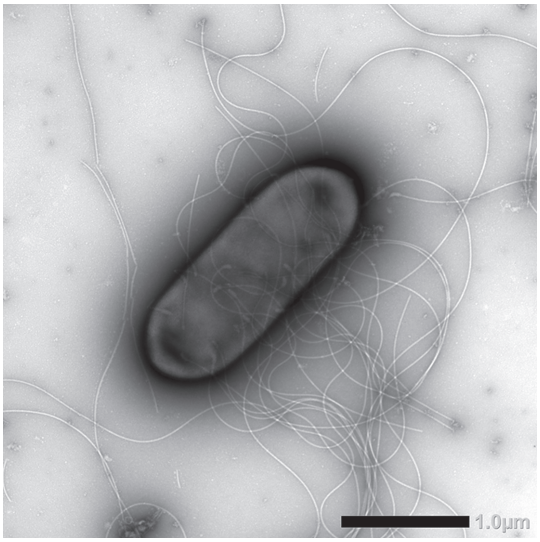

Fig. 3. Transmission electron micrograph of a negativey stained cell of strain LC2-13A^T grown on TSA at 50 °C for 3 days. Bar, 1 μm.

The type strain is LC2-13A^T (=UICC B-42^T=NRRL B-65368^T=DSM 101873^T), isolated from fallen leaf of Cisolok geyser, West Java, Indonesia. The DNA G+C content of the type strain is 56.6 mol%.

Table 2. Cellular fatty acid composition of strain LC2-13A^T and related *Paenibacillus* species

Strains: 1, LC2-13A^T; 2, *P. tarimensis* DSM 19409^T (Wang *et al.*, 2008); 3, *P. daejeonensis* DSM 15491^T (Lee *et al.*, 2002); 4, *P. darwinianus* Br^T (Dsouza *et al.*, 2014); 5, *P. wooponensis* WPCB018^T (Baik *et al.*, 2011); 6, '*P. marinum*' THE22 (Bouraou *et al.*, 2013). TR, Trace amount.

	1	2	3	4	5	6
$C_{12:0}$	1.3	–	–	–	0.6	–
$C_{14:0}$	3.5	–	–	1.0	2.6	TR
$C_{15:0}$	2.5	–	TR	0.6	4.6	TR
$C_{16:0}$	23.2	8.4	11.7	5.7	20.9	19.6
$C_{17:0}$	2.7	2.1	16.1	–	1.4	–
$C_{18:0}$	1.0	46.6	16.8	1.7	18.1	–
iso-$C_{14:0}$	4.5	–	–	1.2	0.8	TR
iso-$C_{15:0}$	11.4	TR	–	1.4	4.6	1.2
anteiso-$C_{15:0}$	21.5	TR	TR	63.4	32.2	19.2
iso-$C_{16:0}$	23.4	–	3.6	–	5.2	34.5
iso-$C_{17:0}$	4.2	3.3	8.1	TR	0.8	2.43
anteiso-$C_{17:0}$	–	30.5	32.3	4.5	3	18.1
$C_{16:1}\omega 11c$	–	1.6	2.3	8.1	–	TR

Acknowledgements

This work was funded by Hibah Pusat Riset Unggulan Universitas Indonesia 2015 Research Grant to W. S. Direktorat Riset dan Pengabdian Masyarakat Universitas Indonesia.

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