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TAXONOMIC DESCRIPTION

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Clostridium thailandense sp. nov., a novel CO_2-reducing acetogenic bacterium isolated from peatland soil

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Abstract

Some species of the genus *Clostridium* are efficient acetate producers and have been deemed useful for upgrading industrial biogas. An acetogenic, strictly anaerobic, Gram-stain-positive, subterminal endospore-forming bacterium designated strain PL3^T was isolated from peatland soil enrichments with H_2 and CO_2. Cells of strain PL3^T were 0.8–1.0×4.0–10.0 µm in size and rod-shaped. Growth of strain PL3^T occurred at pH 6.0–7.5 (optimum, pH 7.0), at 20–40 °C (optimum, 30 °C) and with 0–1.5% (w/v) NaCl (optimum, 0.5%). Biochemical analyses revealed that strain PL3^T metabolized lactose, maltose, raffinose, rhamnose, lactic acid, sorbitol, arabinose and glycerol. Acetic acid was the predominant metabolite under anaerobic respiration with H_2/CO_2. The major cellular fatty acids were $C_{16:0}$, $C_{16:1}$ *cis* 9 and $C_{17:0}$ cyc. The main polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, aminolipid and aminophospholipid. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain PL3^T belongs to the genus *Clostridium* with the highest sequence similarity to *Clostridium acidotolerans* DSM 17425^T (98.6%) followed by *Clostridium nitrophenolicum* (97.8%). The genomic DNA G+C content of strain PL3^T was 31.1 mol%.The genomic *in silico* DNA–DNA hybridization value between strain PL3^T and *C. acidotolerans* DSM 17425^T was 25.1%, with an average nucleotide identity of 80.2%. Based on phenotypic, chemotaxonomic and phylogenetic differences, strain PL3^T was suggested to represent a novel species of the genus *Clostridium*, for which the name *Clostridium thailandense* sp. nov. is proposed. The type strain is PL3^T (=DSM 111812^T=TISTR 2984^T).

Anaerobic acetogenic CO_2 reduction has a large potential in various scientific, social and industrial fields [1]. This microbial process is performed by a specific bacterial group called homoacetogenic bacteria. Acetogenic bacteria are anaerobes that are commonly found in anaerobic natural environments, such as peatland soils, lake sediments and rice paddies, where they account for 5–63, 1.8–2.0 and 2–40% of the total bacterial abundance, respectively [2–4]. The genus *Clostridium*, encompassing several acetogenic species, can convert H_2 and CO_2 to volatile fatty acids and ethanol by fixing CO_2 via the Wood–Ljungdahl pathway. Two moles of CO_2 are reduced to acetyl coenzyme A (acetyl-CoA), with acetic acid being the major end-product, and to butyric acid and ethanol [5]. Thermodynamically, the possible reactions and standard Gibbs free energy ($\Delta G°$) for H_2 and CO_2 conversion into acetic acid (Eq. 1), butyric acid (Eq. 2), and ethanol (Eq. 3) based on the Wood–Ljungdahl pathway in acetogens are well described [6]. Acetogens play an essential ecological role in anaerobic ecosystems by converting CO_2 into acetic acid [7]. Currently, there are more than 100 characterized species of acetogens categorized into 22 different genera [8]. *Clostridium aceticum*, 'C. autoethanogenum', *C. ljungdahlii* and 'C. ragsdalei' are efficient acetic acid producers, and have been predicted as important future industrial acetogenic production platforms [9].

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O\ (\triangle G° = -74.3 kj/mol^{-1}) \quad (1)$$

$$10H_2 + 4CO_2 \rightarrow C_3H_7COOH + 6H_2O\ (\triangle G° = -220 kj/mol^{-1}) \quad (2)$$

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Keywords: acetogenic bacteria; biogas upgrading; *Clostridium*; CO_2-reducing bacteria; peatland soil.

Abbreviations: ANI, average nucleotide identity; BA, basic anaerobic mineral; DDH, DNA–DNA hybridization; PYG, peptone–yeast extract–glucose.

The GenBank accession numbers for the 16S rRNA gene and genome sequence of strain PL3^T are MW330006 and JAEEGC000000000, respectively. The GenBank accession number for the genome sequence of strain DSM 17425^T is JAEEGB000000000.

Three supplementary tables and three supplementary figures are available with the online version of this article.

$$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O\ (\triangle G^\circ = -97.0 kj/mol^{-1}) \quad (3)$$

Here, we assessed peatland soil sampled from the Kuan Kreng peat swamp, Nakhon Si Thammarat Province, Thailand (8.0740145° N; 100.0331712° E), enriched in homoacetogenic consortia for simultaneous H_2 and CO_2 consumption and acetic acid production. Homoacetogens in peatland soil enrichment cultures accounted for >20% of the total bacterial abundance [10]. We isolated an acetogenic bacterial strain, designated as PL3^T, which was proposed as representing a novel species of the genus *Clostridium* based on its phenotypic and genotypic characteristics.

The peatland soil sample was collected at a depth of 20 cm and placed in sterile serum bottles for transportation to the laboratory. A basic anaerobic mineral (BA) medium containing 1.9 mM NH_4Cl, 1.7 mM NaCl, 0.5 mM $MgCl_2{\cdot}6H_2O$, 0.3 mM $CaCl_2{\cdot}2H_2O$, 1.8 mM $K_2HPO_4{\cdot}3H_2O$, 0.02 mM resazurin, 31 mM $NaHCO_3$, 1 ml l^{-1} trace elements solution and 10 µl l^{-1} vitamin mixture solution was used. The trace element and vitamin solutions were prepared as described previously [11]. The BA medium was transferred to serum bottles and flushed with nitrogen for 5 min, and the bottles were sealed with rubber plugs and autoclaved at 121 °C for 15 min. After autoclaving, the medium was reduced by adding 0.3 g l^{-1} $Na_2S{\cdot}9H_2O$ and adjusted to pH 7.0 with 1 M $NaHCO_3$. Five millilitres of the peatland soil slurry was added to serum bottles containing 45 ml BA medium and flushed with a H_2/CO_2 gas mixture at a ratio of 4:1 for 3 min [12]. The serum bottles were incubated at 30±1 °C with constant agitation at 150 r.p.m. for 7 days [8]. After 10 passages, the enriched cultures were assessed for H_2 and CO_2 consumption using a gas chromatograph equipped with a thermal conductivity detector (GC-8A, Shimadzu). The injection port, oven and detector were used at temperatures of 120, 40 and 100 °C, respectively. High-purity argon gas was used as carrier gas at a flow rate of 14 ml min^{-1}. Volatile fatty acids in the fermentation broth were analysed by a gas chromatograph equipped with a flame ionization detector (GC-17A, Shimadzu). The column, injector and detector temperatures were maintained at 85, 230 and 240 °C, respectively. High-purity helium gas was used as the carrier gas at a flow rate of 30 ml min^{-1}. Gas chromatography was conducted as previously described [13]. An enriched culture that actively consumed H_2 and CO_2 was used to isolate an acetogenic bacterium on solid BA medium using a modified Hungate roll-tube technique [14]. The enrichment culture was tenfold serially diluted in serum bottles containing 1.5% (w/v) melted BA agar medium and flushed with H_2 and CO_2 at a mixing ratio of 4:1 for 3 min. The serum bottles were rolled following the Hungate roll-tube technique and incubated at 30 °C for 7 days. Single colonies were picked using a long syringe and transferred to fresh BA medium in serum bottles with a headspace containing H_2 and CO_2 at a mixing ratio of 4:1. The roll-tube procedure was repeated several times until a pure culture was obtained. The final isolate, strain PL3^T, showed H_2 and CO_2 consumption efficiencies of 78 and 100%, respectively, when incubated for 7 days with H_2 and CO_2 in the headspace at 4:1 ratio, and produced a large amount of acetic acid (27.5 mM). For further physiological characterization and DNA extraction, strain PL3^T was subcultured in liquid BA medium containing 10 g l^{-1} peptone, 0.2 g l^{-1} yeast extract, 5 g l^{-1} glucose and 5 g l^{-1} NaCl (BA-PYG medium) with the bottle headspace containing N_2. The cells of strain PL3^T were preserved in 25% (v/v) glycerol at −80 °C for further analyses.

Cellular characteristics of strain PL3^T at the exponential growth phase were analysed using Gram staining and visualized through a light microscope (BX-90, Olympus), as well as scanning electron microscopy (SEM; JSM-7400F, JEOL). Preparation of the cells for SEM was performed following the procedure described by Erikstad *et al.* [15]. Gram staining was performed using a standard protocol [16]. Colonies on BA medium agar were 0.4–2.9 mm in diameter after incubation at 30 °C for 7 days. Colonies were creamy-white, circular, opaque and lustrous. Cells of strain PL3^T were shaped in the form of long rods, 0.8–1.0 µm wide and 4.0–10.0 µm long, stained Gram-positive and formed endospores (Fig. 1). Cells grown in the presence of organic carbon sources (BA-PYG medium) appeared shorter and wider than those grown using CO_2 as the carbon source (Fig. 1c, d). Endospores were observed in the subterminal region of the cells during the late exponential growth phase (Fig. 1b). Cells of strain PL3^T were actively motile, as judged by phase contrast microscopy of live samples, but no flagella could be revealed by SEM and negative staining.

Genomic DNA of strain PL3^T was extracted using a GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). The 16S rRNA gene was amplified using the universal bacterial primers (27F and 1492R) as previously described [17]. The PCR product was purified using the ExoSAP-IT Cleanup kit (Sigma-Aldrich) and then sequenced using the ABI Prism Big-Dye Terminator kit (Perkin Elmer) and an ABI PRISM capillary sequencer at the University of Bergen Sequencing Facility (Bergen, Norway). The obtained sequence was analysed using BLASTN to identify the closest relatives. The sequences of the most similar strains were retrieved from the GenBank database and the EzBioCloud web service (www.ezbiocloud.net) [18]. The Molecular Evolutionary Genetics Analysis (MEGA) software package version 7.0 [19] was used for reconstructing a phylogenetic tree using the neighbour-joining algorithm [20]. Bootstrap values were calculated based on 1000 random replicates to evaluate the neighbour-joining tree branches, as described previously [21]. *Caloramator fevidus* ATCC43204^T was selected as an outgroup [22]. The evolutionary distances were computed using the Tamura–Nei method [23]. Phylogenetic analysis based on the 16S rRNA gene sequence showed that strain PL3^T was a member of the genus *Clostridium* (Fig. 2). It was most closely related to *C. aciditolerans* DSM 17425^T (98.6% similarity) followed by *C. nitrophenolicum* 1D^T (97.8% similarity) and *C. magnum* DSM 2767^T (96.7% similarity). Strain PL3^T fell outside two clusters of known acetogens, including *C. scatologenes* ATCC 25775^T, *C. drakei* DSM 12750^T and *C. carboxidivorans* DSM 15243^T, and a cluster of the acetogens 'C. ragsdalei' DSM 15248^T, 'C. coskatii' PTA-10522^T, *C. ljungdahlii* DSM 13528^T and 'C.

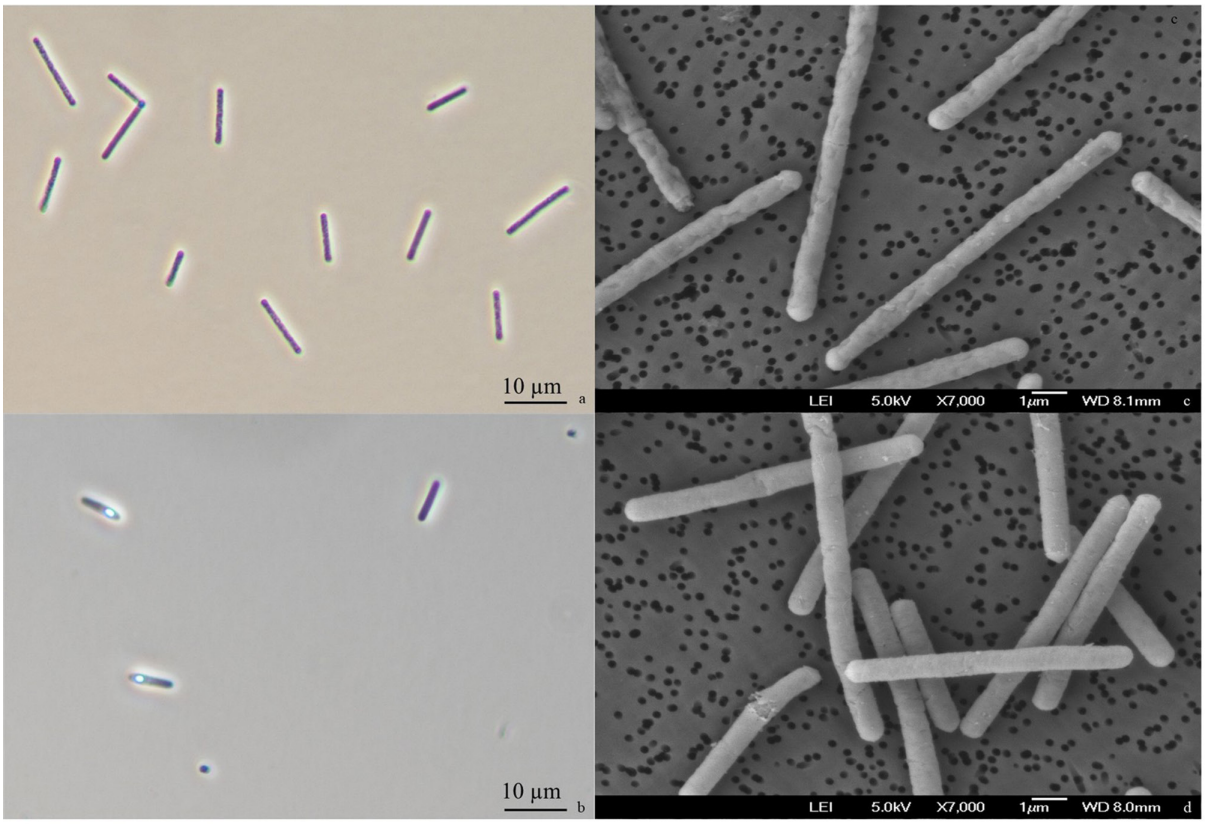

Fig. 1. Morphology strain PL3^T cells. (a, b) Phase contrast microscopy of the strain grown in basal broth (basic anaerobic mineral medium) with H_2 and CO_2 as substrates reveals a typical rod-shaped cell (a) containing subterminal endospores (b). (c, d) Scanning electron microscope image of the strain grown in basal broth with H_2 and CO_2 as substrate (c) or peptone–yeast extract–glucose medium (d).

autoethanogenum' DSM 10061^T [1]. The 16S rRNA gene sequence similarities between strain PL3^T and other *Clostridium* species were below the recommended 98.7% threshold value for species differentiation [24], thus suggesting that strain PL3^T represents a novel *Clostridium* species. This was also supported by the high bootstrap values, including the lineage separation from two of its closest relatives (98%). *C. aciditolerans* DSM 17425^T was selected as a reference strain for further taxonomic identification.

Further, we determined the optimal growth conditions, including the temperature, pH and NaCl tolerance ranges, via triplicate experiments using BA-PYG medium. Growth was measured in the temperature range of 15–45 °C at intervals of 5 °C. The pH dependency was measured in the pH range of pH 3.0–8.0 at intervals of 0.5 pH units using appropriate 20.0 mM buffers: Na_2HPO_4–citric acid for pH 3.0–5.5, 2-(*N*-morpholino) ethanesulfonic acid for pH 6.0–6.5, piperazine–*N*,*N*′-bis(2-ethanesulfonic acid) for pH 7.0–7.5, and Tris–HCl solution for pH 8.0. HCl (1 M) or NaOH (1 M) was used to adjust the pH as described previously [25]. The dependency of bacterial cell growth on NaCl concentration was measured using concentrations ranging from 0 to 2.0% (w/v) at intervals of 0.5%. The inoculation amount was 10% (v/v). Cell growth was monitored using a spectrophotometer (DU730, Beckman-Coulter) with absorbance measured at 600 nm. Substrate utilization and enzyme activities were analysed using the API 20A and API ZYM kits (bioMérieux), respectively, according to the manufacturer's protocols.

Active growth of strain PL3^T occurred at pH 6.0–7.5 with an apparent optimal growth at pH 7.0 (Fig. S1a, available in the online version of this article). No growth was detected at pH ≤5.5 and ≥8.0. Strain PL3^T grew at 20–40 °C, with an apparent optimal growth at 30 °C (Fig. S1b). Bacterial growth was not detected below 20 or above 40 °C. Optimal growth was noted at 0.5% NaCl (w/v), with active bacterial growth in the presence of 0–1.5% NaCl (Fig. S1c). Compared with *C. aciditolerans*, strain PL3^T differed slightly with respect to the optimal temperature (35 vs. 30 °C), salinity (0 vs. 0.5% NaCl) and pH (7.5 vs. 7.0), as well as the parametric ranges (Table 1). In addition, cells of *C. aciditolerans* stained Gram-negative. The cellular characteristics of *C. nitrophenolicum* differ from those of *C. aciditolerans* and strain PL3^T, but are similar with respect to growth conditions (temperature, pH and salinity) (Table 1). The other two close relatives, *C. magnum* and *C. scatologenes*, tolerate significantly lower temperatures, ≥15 and ≥18 °C, respectively, while their pH and salinity requirements are similar to those of strain PL3^T and *C. aciditolerans*. The physiological characteristics of strain PL3^T differed significantly from *C. magnum* and *C. scatologenes*. The major fermentation metabolites from glucose in BA medium with 0.5% NaCl of strain PL3^T were acetic acid and butyric acid (Table 1). Apart from H_2 and CO_2,

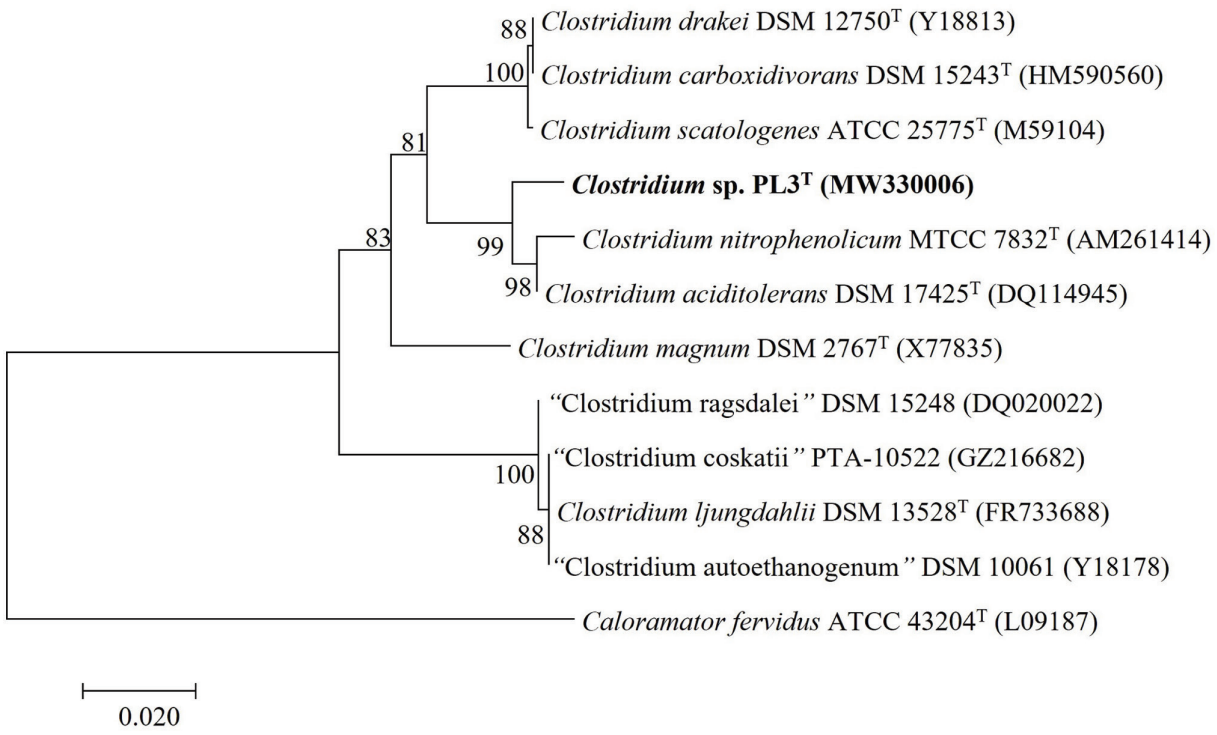

Fig. 2. Neighbour-joining phylogenetic tree reconstructed using 16S rRNA gene sequences showing the relationships between strain PL3^T and the related type strains of the genus *Clostridium*. Bootstrap values (>50%) based on 1000 replications are shown at the branch nodes. *Caloramator fervidus* ATCC43204^T represents the outgroup. GenBank accession numbers of the 16S rRNA sequences are presented in parentheses. Scale bar, 2% sequence divergence.

strain PL3^T could utilize lactose, maltose, raffinose, rhamnose, lactic acid, sorbitol, arabinose, glycerol, xylose, ribose, fructose and glucose, but not trehalose or sucrose, as assessed using the API 20A kit (Table 1). In addition to not being able to utilize H_2 and CO_2, *C. aciditolerans* DSM 17425^T differed from strain PL3^T by not being able to utilize rhamnose, lactic acid, sorbitol or arabinose, as well as in catalase and indole production (Table 1). *C. nitrophenolicum* differed from strain PL3^T in nine substrate utilization tests (Table 1). Therefore, the physiological characteristics of strain PL3^T differed significantly from two of its closest relatives. All of the strains could utilize fructose and glucose. Except for *C. scatologenes* ATCC 25775^T, strain PL3^T and the reference strains were positive for gelatin hydrolysis. Furthermore, strain PL3^T was negative for catalase activity, urea hydrolysis and indole production, but its closest type strain, *C. aciditolerans* DSM 17425^T was positive for catalase and indole production. Strain PL3^T and *C. aciditolerans* DSM 17425^T were positive for alkaline phosphatase, esterase and acid phosphatase activities (Table 1). Strain PL3^T was positive for esterase and lipase activities, whereas *C. aciditolerans* DSM 17425^T was negative. Moreover, strain PL3^T was negative for naphthol-AS-BI-phosphohydrolase activity, whereas *C. aciditolerans* DSM 17425^T was positive. In conclusion, strain PL3^T differed from its closest relatives in numerous physiological and biochemical characteristics, thereby bolstering the notion of strain PL3^T being a novel distinct *Clostridium* species.

Chemotaxonomic characteristics (cellular fatty acids and polar lipids) were determined at the Leibniz Instiut DSMZ (Braunschweig, Germany). Strain PL3^T and reference strain *C. aciditolerans* DSM 17425^T were cultured in BA-PYG medium under anaerobic conditions at 30 °C. Biomass for whole cellular fatty acid and polar lipid measurements was collected by centrifugation at 6500 r.p.m. at 4 °C for 20 min. Cellular fatty acids were saponified, methylated and extracted as previously described [26, 27], and identified using the Sherlock MIS software version 6.1 (MIDI). Polar lipids were extracted and analysed following the method described by Tindall *et al.* [28]. The total polar lipid profiles revealed phosphatidylethanolamine, aminophospholipid, diphosphatidylglycerol and aminolipid as the major polar lipids in strain PL3^T, whereas those in *C. aciditolerans* DSM 17425^T were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol (Fig. S2). Therefore, strain PL3^T differed from *C. aciditolerans* DSM 17425^T by containing the additional unidentified aminolipid. The major cellular fatty acids in strain PL3^T were $C_{16:0}$, $C_{16:1}$ *cis* 9 and $C_{17:0}$ cyc, which accounted for 74.5% of the total fatty acids. Strain PL3^T and reference strains *C. aciditolerans* DSM 17425^T, *C. nitrophenolicum* DSM 21057^T, *C. scatologenes* DSM 757^T and *C. carboxidivorans* DSM 15243^T all shared $C_{16:0}$ as the major fatty acid (Table 2). A significant amount of $C_{17:0}$ cyc was detected in strain PL3^T, whereas it was absent in *C. aciditolerans* DSM 17425^T. Furthermore, the content of $C_{16:1}$*cis* 9 was significantly higher in strain PL3^T than in any of the

Table 1. Morphological, physiological, and biochemical characteristics of strain PL3^T and its most closely related species

Strain: 1, PL3^T (this study); 2, *Clostridium aciditolerans* DSM 17425^T [34]; 3, *Clostridium nitrophenolicum* DSM 21057^T [35]; 4, *Clostridium magnum* DSM 2767^T [36, 37]; 5, *Clostridium scatologenes* ATCC 25775T [38] . –, Negative; +, positive; NR, not reported.

Characteristic	1	2	3	4	5
Source	Peatland sediment	Constructed wetland sediment	Subsurface soil	Olive mill wastewater	Coal mine pond sediment
Gram stain	+	–	+	–	+
Cell size (μm)	0.8–1.0×4.0–10.0	0.5–1.0×3.0–9.0	3.5–5.0×0.6–0.9	1.0–4.0×4–16	1.0–3.0×3.0–21.0
Spore location	Subterminal	Subterminal	Central	Elliptical	Terminal
Growth temperature (optimum; °C)	20–40 (34)	20–45 (29)	20–45 (34)	15–45 (27)	18–42 (31)
Growth pH (optimum)	6.0–7.5 (7.0)	3.8–8.9 (7.5)	6.5–8.0 (7.2)	6.0–7.5 (7.0)	4.6–8.0 (6.0)
NaCl for growth (optimum; %)	0–1.5 (0.5)	0–1.5 (0)	0–1.0 (0)	0–3 (1.5)	0–5 (2.0)
Gelatin hydrolysis	+	+	+	+	–
Catalase activity	–	+	–	NR	–
Urea hydrolysis	–	–	–	+	NR
Indole production	–	+	+	–	–
Substrate utilization:					
H_2/CO_2	+	–	NR	+	+
Lactose	+	+	–	NR	+
Maltose	+	+	–	–	+
Raffinose	+	+	–	NR	+
Trehalose	–	–	+	NR	–
Rhamnose	+	–	+	NR	+
Sucrose	–	+	–	+	+
Lactic acid	+	–	–	–	+
Sorbitol	+	–	+	NR	+
Arabinose	+	–	–	–	+
Glycerol	+	+	+	–	+
Xylose	+	+	–	+	+
Ribose	+	+	–	–	+
Enzyme activity:					
Alkaline phosphatase	+	+	NR	NR	NR
Esterase	+	+	NR	NR	NR
Esterase lipase	+	–	NR	NR	NR
Acid phosphatase	+	+	NR	NR	NR
Naphthol-AS-BI-phosphohydrolase	–	+	NR	NR	NR
Major fermentation metabolites from glucose	Acetic acid and butyric acid	Acetic acid, butyric acid, ethanol	Formate, acetate pyruvate	Acetic acid	Acetic acid, butyric acid
DNA G+C content (mol%)	31.1	31.3	35.5	29.1	31

Table 2. Major cellular fatty acids in strain PL3^T and its most closely related species

Strain: 1, PL3^T (this study); 2, *Clostridium aciditolerans* DSM 17425^T (this study); 3, *Clostridium nitrophenolicum* DSM 21057^T [35]; 4, *Clostridium scatologenes* DSM 757^T; 5, *Clostridium carboxidivorans* DSM 15243^T [39]. The fatty acids having content higher than 10% of the total fatty acid content are highlighted in bold. Summed features represent groups of two fatty acids that could not be resolved by Sherlock MIS software (MIDI). For instance, summed feature 2 comprises iso-$C_{16:1}$/$C_{14:0}$ 3-OH, summed feature 3 comprises $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH, and summed feature 4 comprises iso-$C_{17:1}$ I/anteiso B. –, Not detected; DMA, dimethylacetal.

Cellular fatty acids (%)	1	2	3	4	5
$C_{11:0}$	–	–	–	4.1	**6.2**
$C_{13:0}$	–	0.9	–	–	–
$C_{14:0}$ DMA	0.9	0.4	–	1.2	2.3
$C_{14:0}$	4.2	**16.1**	**10.0**	4.8	1.7
$C_{15:0}$	–	**13.4**	–	1.7	4.0
$C_{16:0}$ DMA	7.3	4.5	–	9.3	6.2
$C_{16:0}$	**33.8**	**26.1**	**28.0**	**46.2**	**44.9**
$C_{16:1}$*cis* 9 DMA	4.1	3.5	–	3.4	0.9
$C_{16:1}$*cis* 9	**20.5**	**10.1**	–	6.6	4.5
$C_{16:1}$*cis* 11	2.3	0.9	–	–	–
$C_{17:0}$ cyc	**20.2**	–	–	8.4	7.5
$C_{17:0}$ cyc DMA	2.6	–	–	8.7	5.5
$C_{18:0}$	0.4	0.9	8.0	–	1.2
Summed feature 2	–	–	4.8	–	–
Summed feature 3	–	–	**10.8**	–	–
Summed feature 4	–	–	**23.1**	–	–

reference strains. Thus, the significant qualitative and quantitative differences in cellular fatty acid and polar lipid compositions between strain PL3^T and its closest relatives support the proposal of strain PL3^T as a distinct *Clostridium* species.

The genomes of strain PL3^T and *C. aciditolerans* DSM 17425^T were sequenced using the Illumina MiSeq sequencing platform at Eurofins Genomics (https://eurofinsgenomics.eu/). Raw reads were assembled using CLC Genomics Workbench 20.1 (CLC Bio, Qiagen). The draft genomes of strain PL3^T and *C. aciditolerans* DSM 17425^T contained 6485355 bp and 5262995 bp, with G+C content of 31.1 and 31.3mol%, respectively. They contained more than 4500 genes. The general genome features of strain PL3^T and *C. aciditolerans* DSM 17425^T are presented in Table S1. The average nucleotide identity (ANI) of strain PL3^T was compared with those of *C. aciditolerans* DSM 17425^T and other *Clostridium* strains using the EzBioCloud web service [29]. The genome sequence of *C. nitrophenolicum* was not available from the public domain and thus was not included in the comparison of these species. However, the biochemical characteristics and cellular fatty acid profiles indicated that *C. nitrophenolicum* DSM 21057^T and strain PL3^T were clearly distinct. The ANI value of strain PL3^T as compared to *C. aciditolerans* DSM 17425^T was 80.2% (Table S2). Accordingly, the values obtained for *in silico* DNA–DNA hybridization (DDH) between strain PL3^T and other close relatives varied from 26.0 to 21.8% (Table S3) using Genome-to-Genome Distance Calculator (GGDC) version 2.1 (Leibniz Instiut DSMZ). Strain PL3^T could be considered a new species because its ANI and DDH values compared with other species were significantly lower than the recommended ANI and DDH threshold values of 96% [30] and 70% [31], respectively. A phylogenomic tree reconstructed using the TYGS server (https://tygs.dsmz.de/) also supports strain PL3^T as a species distinct from other *Clostridium* species based on whole genome sequence similarity (Fig. S3). As predicted, the key genes for the reductive synthesis of acetyl-CoA from CO_2 through the Wood–Ljungdahl pathway [32] were found in the genome of strain PL3^T, including CO dehydrogenase (*acsA* and *cooC*), formate dehydrogenase (*fdh*), acetyl-CoA synthase (*acsB*), phosphotransacetylase (*pta*) and acetate kinase (*ack*) genes. Furthermore, the key genes involved in the arginine deiminase pathway, including *arcA*, *arcB*, *arcC* and *arcD* [32], were found in the strain PL3^T genome. The arginine deiminase pathway is involved in energy conservation in acetogens, such as *C. scatologenes* ATCC 25775^T, 'C. autoethanogenum' DSM 10061, 'C. coskatii' PTA-10552 and *C. ljungdahlii* DSM 13528^T [33]. The draft genome sequence contained a complete set of flagellar genes.

In conclusion, strain PL3^T could be distinguished from its relatives in terms of its optimal growth temperature and NaCl concentration, substrate utilization, enzyme activity, and chemotaxonomic characteristics (Table 1). Strain PL3^T grew optimally at

30 °C with 0.5% (w/v) NaCl and could utilize H_2 and CO_2 in acetogenic metabolism, and other substrates, including rhamnose, lactic acid, sorbitol, and arabinose. It did not produce indole and was positive for naphthol-AS-BI-phosphohydrolase activity. The polar lipid profile of strain PL3^T differed significantly from that of *C. aciditolerans* DSM 17425^T, with a larger diversity of components (Fig. S2). The main cellular fatty acids were $C_{16:0}$, $C_{16:1}$*cis* 9 and $C_{17:0}$ cyc, with $C_{17:0}$ cyc being absent in the closest relatives, *C. aciditolerans* DSM 17425^T and *C. nitrophenolicum* DSM 21057^T. In addition, the genome sequence of strain PL3^T, in terms of ANI and *in silico* DDH values, was significantly different from that of *C. aciditolerans* DSM 17425^T. Therefore, based on the phenotypic and genotypic characteristics, we suggest that strain PL3^T should be classified as a novel acetogenic species of the genus *Clostridium*, with the proposed name *Clostridium thailandense* sp. nov. Based on this finding, strain PL3^T will be applied for reducing CO_2 in biogas using H_2 as an electron donor to produce acetic acid and upgrading methane content in biogas to above 95% that could be used as a vehicle fuel.

DESCRIPTION OF *CLOSTRIDIUM THAILANDENSE* SP. NOV.

Clostridium thailandense (thai.lan.den'se. N.L. neut. adj. thailandense, pertaining to Thailand).

Cells are Gram-stain-positive and rod-shaped, with a size of 0.8–1.0×4.0–10.0 µm and with subterminal endospores. Cells grow under the following conditions: pH 6.0–7.5 (optimal, pH 7.0); 20–40 °C (optimal, 30 °C); and 0–1.5% (w/v) NaCl (optimal, 0.5%). Colonies on BA medium agar were 0.4–2.9 mm in diameter after growth at temperature 30 °C and incubation for 7 days. Colonies were creamy-white, circular, opaque and lustrous. The strain is strictly anaerobic with the ability to utilize H_2 and CO_2 as energy and carbon sources. Acetic acid is the predominant metabolite of CO_2 reduction with H_2. It is negative for catalase, urea and indole tests but tests positive for gelatin hydrolysis, alkaline phosphatase, esterase (C4), esterase lipase and acid phosphatase activities, confirmed by the presence of the respective enzyme-coding genes. Cells metabolize cellobiose, lactose, maltose, fructose, raffinose, rhamnose, lactic acid, sorbitol, arabinose, galactose, glucose, mannose and glycerol. The major cellular fatty acids are $C_{16:0}$, $C_{16:1}$ cis 9 and $C_{17:0}$ cyc. The main polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, aminolipid and aminophospholipid. The genomic DNA G+C content of the type strain is 31.1 mol%. The type strain is PL3^T (=DSM 111812^T=TISTR 2984^T), which was isolated from peatland soil collected from Kuan Kreng peat swamp, Nakhon Si Thammarat Province, Thailand.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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