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TAXONOMIC DESCRIPTION

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Siculibacillus lacustris gen. nov., sp. nov., a new rosette-forming bacterium isolated from a freshwater crater lake (Lake St. Ana, Romania)

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Abstract

A new aerobic alphaproteobacterium, strain SA-279^T, was isolated from a water sample of a crater lake. The 16S rRNA gene sequence analysis revealed that strain SA-279^T formed a distinct lineage within the family *Ancalomicrobiaceae* and shared the highest pairwise similarity values with *Pinisolibacterravus* E9^T (96.4 %) and *Ancalomicrobiumadetum* NBRC 102456^T (94.2 %). Cells of strain SA-279^T were rod-shaped, motile, oxidase and catalase positive, and capable of forming rosettes. Its predominant fatty acids were $C_{18:1}\omega7c$ (69.0 %) and $C_{16:1}\omega7c$ (22.7 %), the major respiratory quinone was Q-10, and the main polar lipids were phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylcholine, phosphatidylglycerol, an unidentified aminophospholipid and an unidentified lipid. The G+C content of the genomic DNA of strain SA-279^T was 69.2 mol%. On the basis of the phenotypic, chemotaxonomic and molecular data, strain SA-279^T is considered to represent a new genus and species within the family *Ancalomicrobiaceae*, for which the name *Siculibacillus lacustris* gen. nov., sp. nov. is proposed. The type strain is SA-279^T (=DSM 29840^T=JCM 31761^T).

The order *Rhizobiales* (class *Alphaproteobacteria*) currently contains more than 15 families, such as 'Aurantimonadaceae', *Bartonellaceae*, *Beijerinckiaceae*, *Bradyrhizobiaceae*, *Brucellaceae*, *Chelatococcaceae*, *Cohaesibacteraceae*, *Hyphomicrobiaceae*, *Methylobacteriaceae*, *Methylocystaceae*, *Notoacmeibacteraceae*, *Phyllobacteriaceae*, *Rhizobiaceae*, *Rhodobiaceae* and *Xanthobacteraceae* [1–3]. Although many well-known genera from this order are pathogenic to humans and animals (e.g. *Bartonella*, *Brucella*), associated with plants (e.g. *Phyllobacterium*, *Rhizobium*) or inhabitants of soil (e.g. *Nitrobacter*) and wastewater-treating bioreactors (e.g. *Chelatococcus*) [2], yet-not-cultivated members of *Rhizobiales* could be important members of bacterioplankton in some aquatic environments (e.g. some lakes and special oceanic habitats [4–6]). In our recent study [7], we gave the description of a new *Rhizobium* species isolated from a water sample collected from a crater lake. In this paper, another new strain, SA-279^T, was characterized in detail, which was isolated from the same locality. Based on the obtained results, this strain is supposed to represent a novel genus for which the name *Siculibacillus lacustris* gen. nov., sp. nov. is proposed. The new genus is the member of the recently described new family, *Ancalomicrobiaceae*, which currently contains only two other genera, *Pinisolibacter* and *Ancalomicrobium* [8].

Strain SA-279^T was isolated from a freshwater crater lake, Lake St. Ana (46° 07′ 34.7″ N 25° 53′ 15.8″ E; located in Ciomad Mountains, Harghita County, Romania; in Romanian: Lacul Sfânta Ana) in August 2012. A detailed site description including the physical and chemical characteristics of the lake water is given by Felföldi *et al.* [9]. For isolation, plates containing only lake water solidified with 20 g l^{-1} agar were used. The standard dilution plating technique (spread plate method) was applied to obtain isolates by

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Keywords: rosette; *Alphaproteobacteria*; new genus; *Ancalomicrobiaceae*.
Abbreviations: AL, unidentified aminolipid(s); APL, unidentified aminophospholipid(s); DPG, diphosphatidylglycerol; GL, unidentified glycolipid(s); L, unidentified lipid(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unidentified phospholipid(s); PME, phosphatidylmonomethylethanolamine; PS, phosphatidylserine.
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incubation at room temperature (20–22 °C). Subsequently, strain SA-279^T was maintained on a modified Reasoner's 2A agar medium (mR2A, pH 5.5), which contained only a half amount of the carbon sources as given in the original description (DSMZ medium 830, www.dsmz.de; 0.25 g l^{-1} yeast extract, 0.25 g l^{-1} proteose peptone, 0.25 g l^{-1} casamino acids, 0.25 g l^{-1} glucose, 0.25 g l^{-1} soluble starch, 0.15 g l^{-1} sodium pyruvate, 0.3 g l^{-1} K_2HPO_4, 0.05 g l^{-1} $MgSO_4 \times 7H_2O$). Later, strain SA-279^T was grown on mR2A or R2A agar medium at room temperature (~22 °C). For side-by-side analyses, strains *Pleomorphomonas oryzae* DSM 16300^T and *Phreatobacter oligotrophus* DSM 25521^T were also maintained on R2A agar.

Temperature and pH optima as well as salt tolerance were determined based on the observed growth intensity at 4, 10, 15, 20, 25, 28, 37, 45, 55 and 65 °C, at pH from 4 to 11 (with intervals of 0.5), and from 0 to 5 % (w/v) NaCl concentration (with intervals of 0.5 %), as described previously [10]. Colony morphology of strain SA-279^T was tested by direct observation of single colonies. Cell morphology was studied with Gram staining according to Claus [11], with phase contrast microscopy and with electron microscopy as described by Tóth *et al.* [12]. The presence of flagella was checked also as described by Heimbrook *et al.* [13], while motility was also inferred based on the spreading growth in semisolid agar [14] using mR2A medium containing 4 g l^{-1} agar. Oxidase activity was determined as described by Tarrand and Gröschel [15], while catalase reaction was examined according to Cowan and Steel [14]. Metabolic tests were performed with API 50 CH, API 20 NE and API ZYM (bioMérieux) systems according to instructions of the manufacturer, while chemotaxonomic analyses (determination of isoprenoid quinones using HPLC, cellular fatty acids using GC and polar lipids using two-dimensional TLC) were performed as described in detail previously [16].

The 16S rRNA gene sequence of strain SA-279^T was amplified using the protocol described by Felföldi *et al.* [17], and sequenced by the Biomi Ltd. (Gödöllő, Hungary). Closest related species represented by the type strains were identified by EzBioCloud's online Identify service [3], 16S rRNA gene sequences were retrieved from GenBank, and sequence alignment was performed with the ARB-SINA Alignment Service [18]. Phylogenetic analysis (including the search for the best-fit model parameters) was conducted with the MEGA7 software [19].

For the whole genome project of strain SA-279^T, genomic DNA was extracted with the DNeasy PowerLyzer Microbial Kit (Qiagen) including an RNase A treatment at 37 °C for 20 min. Illumina sequencing was performed by the Genomics Facility RTSF, Michigan State University (USA) with the following main steps: library preparation using the SMARTer ThruPLEX DNA-Seq kit (Takara); quality control using a combination of Qubit dsDNA HS assay (Thermo Fisher Scientific), 4200 TapeStation High Sensitivity DNA 1000 assay (Agilent) and the Illumina Library Quantification qPCR kit (Kapa Biosystems); sequencing which was performed in a 2×250 bp paired-end format using a MiSeq Standard v2 flow cell and a MiSeq 500 cycle v2 reagent cartridge (Illumina). Base calling was performed by Illumina Real Time Analysis (RTA) version 1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq version 2.19.1. Sequence read quality was checked with FastQC [20]. *De novo* assembly of sequence reads was performed using A5-miseq [21], which resulted 99 contigs (all contigs were longer than 500 nt) with N50 value of 120 665 nt and 85.9× genome coverage. The ContEst16S software [22] was used to check possible contamination.

Sequencing the 16S rRNA gene of strain SA-279^T resulted in a stretch of 1403 nucleotides. The closest type strains of bacterial species were identified as *Pinisolibacter ravus* E9^T with 96.4 %, *Ancalomicrobium adetum* NBRC 102456^T with 94.2 % (both strains are members of family *Ancalomicrobiaceae*), *Prosthecomicrobium hirschii* 16^T with 93.5 % (unclassified), *Kaistia algarum* LYH11^T with 92.8 % (family *Rhizobiaceae*), *Chthonobacter albigriseus* ED7^T with 92.8 %, *Pleomorphomonas oryzae* DSM 16300^T and *Oharaeibacter diazotrophicus* SM30^T both with 92.7 % (the former three strains, family *Methylocystaceae*), and *Phreatobacter oligotrophus* PI_21^T (=DSM 25521^T) (unclassified) with 92.6 % sequence similarity values. Other species shared <92.3 % pairwise similarity values [other related type strains belonged to genera *Ancylobacter* (family *Xanthobacteraceae*), *Ochrobactrum* (family *Brucellaceae*) and *Pannonibacter* (family *Rhodobacteraceae*)]. Although the closest related type strain showed slightly higher value than threshold value (95 %) suggested for the genus-level by Tindall *et al.* [23], in the case of a related genus, similar values could be found, since *Chthonobacter albigriseus* ED7^T shows 96.7 % 16S rRNA gene sequence similarity value to *Mongoliimonas terrestris* MIMtkB18^T and 96.4 % to *Oharaeibacter diazotrophicus* SM30^T. The phylogenetic analysis based on the 16S rRNA gene (Figs 1 and S1, available in the online version of this article) supported that the new strain is the member of family *Ancalomicrobiaceae*, since it formed a cluster with *Pinisolibacter* and *Ancalomicrobium* with high bootstrap support (99–100); on the other hand, moderate bootstrap values (78–88) supported that strain SA-279^T represents a separate genus from *Pinisolibacter*.

The assembled genome of strain SA-279^T had a total length of 5.0 Mb. The G+C content of the genomic DNA of strain SA-279^T was 69.2 mol%. The full-length 16S rRNA gene sequence of strain SA-279^T obtained by Sanger method was compared with the extracted 16S rRNA gene sequence from the genome assembly and showed 100 % similarity. Base composition of genomic DNA was determined also by reversed-phase HPLC as described in detail previously [16], which resulted in the same value.

Cells of strain SA-279^T were rod-shaped, Gram-stain-negative, motile by a subpolar flagellum, capable to form rosettes (Figs S2 and S3), aerobic and mesophilic with a characteristic heterotrophic metabolism (Table S1). Some

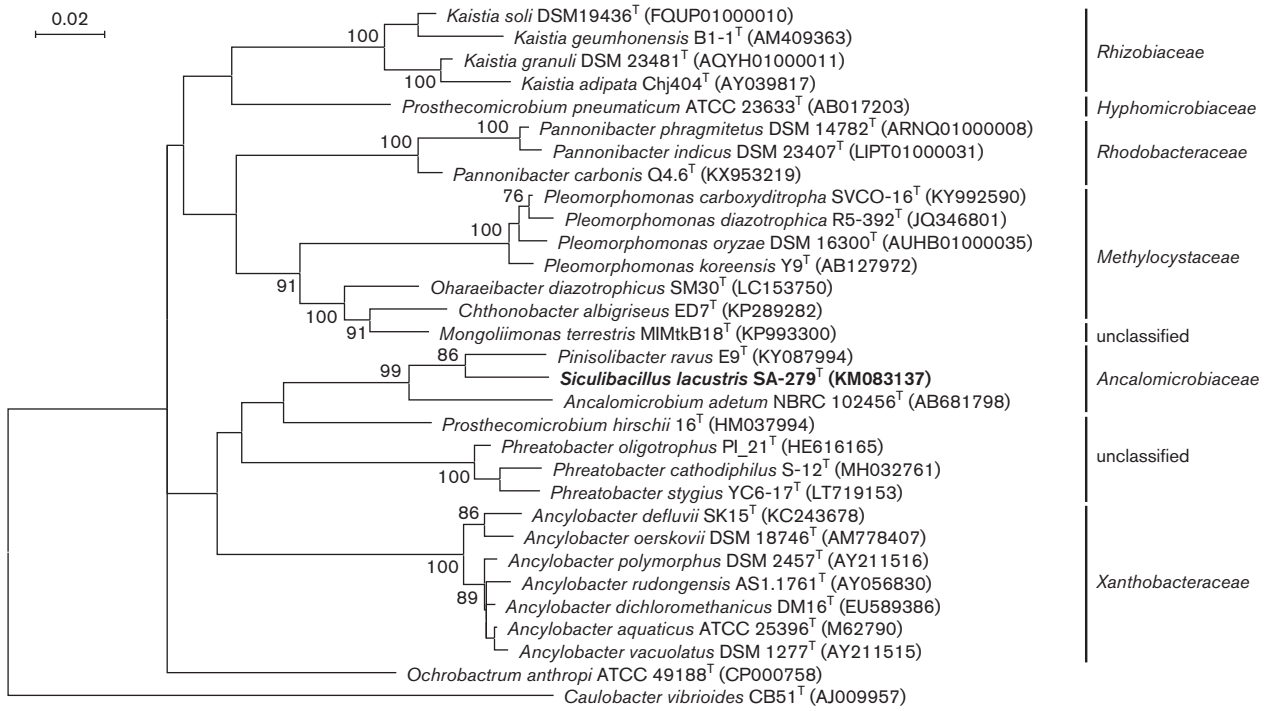

Fig. 1. Phylogenetic position of SA-279^T and related type strains based on the 16S rRNA gene. The phylogenetic tree has been reconstructed based on 1372 positions using the maximum likelihood method and the Tamura three-parameter nucleotide substitution model. Bootstrap values >70 % are shown at the nodes. GenBank accession numbers are given in parentheses. Bar, 0.02 substitutions per nucleotide.

distinguishing characters (e.g. motility, negative aesculin hydrolysis and trypsine enzyme activity and capability to use malate as a sole carbon source) which could be used for the discrimination of the new genus from related genera are given in Table 1.

The isoprenoid quinones of strain SA-279^T were Q-10 and Q-9 in the ratio 94 : 4. The fatty acid pattern of strain SA-279^T was predominated by $C_{18:1}\omega7c$ (69.0 %) and $C_{16:1}\omega7c$ (22.7 %), while $C_{16:0}$ (6.4 %) was also present in a notable amount (Table S2). The dominance of fatty acid $C_{18:1}\omega7c$ and ubiquinone Q-10 is a characteristic chemotaxonomic trait in the case of other related members of *Rhizobiales* (Table 1, Table S2). The polar lipid profile of strain SA-279^T was dominated by phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME), phosphatidylcholine (PC), phosphatidylglycerol (PG) and an unidentified aminophospholipid (AL), while an unidentified lipid (L) was also detected (Fig. S4). The lack of diphosphatidylglycerol (DPG) distinguishes the new strain from the members of closest related genera, *Pinisolibacter* and *Ancalomicrobium* (Table 1).

In conclusion, based on the data discussed above, strain SA-279^T is considered to represent a novel genus and a novel species within family *Ancalomicrobiaceae*, for which the name *Siculibacillus lacustris* gen. nov., sp. nov. is proposed.

DESCRIPTION OF *SICULIBACILLUS* GEN. NOV.

Siculibacillus [Si.cu.li.ba.cil′lus, M.L. masc. pl. n. *Siculi* Székely, referring to people living in Terra Siculorum (i.e. Transylvania, Romania) from where the type strain was isolated, L. masc. n. *bacillus* a rod and also a bacterial generic name); N.L. masc. n. *Siculibacillus*, Székely bacillus)].

Cells are Gram-negative, motile rods and capable to form rosettes. Aerobic and mesophilic. Oxidase- and catalase-positive. The major respiratory quinone is Q-10. Major cellular fatty acids are $C_{18:1}\omega7c$ and $C_{16:1}\omega7c$. Polar lipids are dominated by PE, PME, PC, PG, APL and L.

The type species is *Siculibacillus lacustris*.

DESCRIPTION OF *SICULIBACILLUS LACUSTRIS* SP. NOV.

Siculibacillus lacustris (la.cus′tris. N.L. masc. adj. *lacustris* of a lake)

Cells are rod-shaped (0.6–0.8×1.3–2.5 µm) and motile. Colonies on mR2A medium are greyish-white in colour, circular and raised with a diameter of 1–2 mm. Growth occurs at 15–37 °C (with an optimum between 20–28 °C) and pH 5.0–7.5 (optimum, pH 5.0–6.0). Positive for acid phosphatase (weak), alkaline phosphatase, esterase (C4), esterase lipase (C8), naphthol-AS-BI-phosphohydrolase and urease

Table 1. Differential characteristics of *Siculibacillus* and related genera

Genera: 1, *Siculibacillus* (this study); 2, *Pinisolibacter* [8]; 3, *Ancalomicrobium* [8, 24]; 4, Putative new genus represented by *Prosthecomicrobium hirschii* 16^T (as suggested in Yee *et al.* [25]) [8, 25–27]; 5, *Chthonobacter* [28]; 6, *Pleomorphomonas* [29–32]; 7, *Oharaeibacter* [33]; 8, *Phreatobacter* (this study, [34–36]); 9, *Pannonibacter* [37–39]. Polar lipids: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PME, phosphatidylmonomethylethanolamine; PS, phosphatidylserine; APL, unidentified aminophospholipid(s); AL, unidentified aminolipid(s); GL, unidentified glycolipid(s); PL, unidentified phospholipid(s); L, unidentified lipid. Fatty acids in parentheses are present but not reaching 10 % in all type strains (or contradictory data are available in the literature). Test results and polar lipids shown in parentheses were detected only in one type strain. In all cases, the major isoprenoid quinone is Q-10. +, Present; −, absent; W, weak reaction; ND, no data. Most data are not available for *Pleomorphomonas carboxyditropha* SVCO-16^T.

Characteristic	1	2	3	4	5	6	7	8	9
Family	*Ancalo-microbiaceae*	*Ancalo-microbiaceae*	*Ancalo-microbiaceae*	Unclassified	*Methylo-cystaceae*	*Methylocystaceae*	*Methylocystaceae*	Unclassified	*Rhodobacteraceae*
Number of species*	1	1	1	1	1	4	1	3	3
Colony colour	Greyish-white	Straw-coloured	ND	Light pink	Greyish-white	Pale-white, white	White	Whitish, pale yellow	Whitish cream
Motility	+	−	−	+	−	−	+	+	+
Aesculin hydrolysis	−	+	+	−	+	+	−	+/−	−/+
Alkaline phosphatase activity	+	+	W	W	−	+	ND	+	(+)
Citrate utilization	+	+	+	−	ND	−	−	−	+
Malate utilization	+	−	−	+	+	+/-	+	−	ND
Trypsine activity	−	+	+	−	+	+	ND	+/−	(+)
Urease activity	+	+	+	−	+	+/-	−	+/−	+
Major fatty acids (at least 10 %)†	$C_{18:1}\omega7c$, $C_{16:1}\omega7c$	SF8, SF3, $C_{16:0}$	SF8, $C_{14:0}$ 2-OH, $C_{16:0}$	$C_{18:1}\omega7c$, $C_{16:1}\omega7c$, $C_{16:0}$	SF8, SF2	$C_{18:1}\omega7c$/SF8, (cyclo$C_{19:0}\omega8c$, $C_{16:0}$, $C_{18:0}$)	SF8, cyclo$C_{19:0}\omega8c$	$C_{18:1}\omega7c$, 11-methyl-$C_{18:1}\omega7c$, (SF3)	$C_{18:1}\,\omega7c$
Detected polar lipids‡	PE, PME, PC, APL, PG, L	PE, PME, PC, DPG, PG, L	PE, DPG, PG, PC, L	PG, PME, PC	PC, PG, PE, APL, PL	PC, PE, PME, PG, DPG	ND	PC, PE, DPG, L (PL, GL, PG)	PG, PC, DPG, PE, PL, AL (PME, PS, L)
DNA G+C content (mol%)	69.2	68.4	70.4	68.9	71.8	63.1–66.4	74.6	64.4–69.3	63.3–64.6
Isolation source of type strains	Lake water	Soil	Freshwater	Pond	Grass-field soil	Paddy soil, root tissue, contaminated culture, anaerobic sludge	Rhizosphere	Ultrapure water, pieces of wood, microbial fuel cell	Reed rhizome, hot spring, coal mine water

*Based on the search performed with Prokaryotic Nomenclature Up-To-Date [40] on 17 February 2019.

†SF2, summed feature 2 (consisted of $C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I); SF3, summed feature 3 (consisted of $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$); SF8, summed feature 8 (consisted of $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$).

‡Not available for *Ohareibacter diazotrophicus* SM30^T and *Pleomorphomonas koreensis* Y9^T.

enzyme activities; assimilation of D-arabinose, L-arabinose, citrate, D-fructose, L-fucose, gluconate (weak), D-glucose, D-lyxose, D-mannitol (weak), D-mannose, malate, maltose (weak), L-rhamnose, D-ribose (weak) and D-xylose. Negative for α-chymotrypsine, cystine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase, gelatinase, α-glucosidase, β-glucosidase, β-glucuronidase, leucine arylamidase, lipase (C14), α-mannosidase, *N*-acetyl-β-glucosaminidase and trypsine enzyme activities; assimilation of adipate, D-adonitol, aesculin, amygdalin, D-arabitol, L-arabitol, L-arginine, arbutin, capric acid, cellobiose, dulcitol, erythritol, D-fucose, D-galactose, gentiobiose, glycerol, glycogen, inositol, inulin, 2-ketogluconate, 5-ketogluconate, lactose, melezitose, melibiose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, N-acetylglucosamine, phenylacetic acid, raffinose, salicin, D-sorbitol, L-sorbose, starch, sucrose, D-tagatose, trehalose, turanose, xylitol and L-xylose.

The G+C content of the genomic DNA is 69.2 mol%.

The type strain is SA-279^T (=DSM 29840^T=JCM 31761^T), which was isolated from lake water.

The GenBank accession numbers for the 16S rRNA gene and the genome sequence of strain SA-279^T are KM083137 and SJFN00000000, respectively.

EMENDED DESCRIPTION OF THE FAMILY *ANCALOMICROBIACEAE* DAHAL *ET AL.* 2018

The description of family the *Ancalomicrobiaceae* is as given by Dahal *et al.* [8], with the following amendments. Cells are motile or non-motile. The major polar lipids are PE, PC, PME, PG and DPG.

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

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