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# *Cellulosispirillum alkaliphilum,* gen. nov., sp. nov., an obligately anaerobic cellulotrophic member of the phylum *Fibrobacterota* from soda lakes

Dimitry Y. Sorokin [a,b,*], Alexander Y. Merkel [a], Tatjana V. Khizhniak [a]

[a] *Winogradsky Institute of Microbiology, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia*
[b] *Department of Biotechnology, Delft University of Technology, Delft, the Netherlands*

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ABSTRACT

Intensive microbiology studies of the past several decades of soda lakes, uncovered a rich functional diversity of haloalkaliphilic microbes driving carbon, nitrogen and sulfur cycles in these unique double-extreme habitats. One of the unexpected finding was a discovery there of aerobic extremely halophilic cellulotrophic natronoarchaea. Yet, little is still known about the identity of the soda lake bacteria able to use native cellulose as growth substrate, except for a single case of an anaerobic clostridium. In this work we present results of phenotypic and functional genomic analysis of an anaerobic bacterium, strain ANBcel5$^T$, enriched from hypersaline Siberian soda lakes with amorphous cellulose as growth substrate. Phylogenetic analysis placed the isolate into the family *Chitinispirillaceae* in the phylum *Fibrobacterota* as a new genus and species lineage with the 16S rRNA gene identity and Relative Evolutionary Divergence (RED) to its only known species *Chitinispirillum alkaliphilum* ACht6–1$^T$ of 95.2 % and 0.847, respectively. In contrast, despite obvious morphological resemblance to ACht6–1$^T$, strain ANBcel5$^T$ is a narrow cellulose-utilizing fermentative anaerobe fermenting cellulose and cellobiose to acetate, $H_2$ and succinate. It is a moderately salt-tolerant obligate alkaliphile growing optimally at 0.6 M total $Na^+$ as carbonates and pH 9.5. Functional genome analysis of the isolate revealed the presence of multiple genes encoding extracellular endocellulases from the GH families 5 and 9, three sodium-translocating membrane complexes and osmolytes *N*ε-acetyl-β-lysine and glycine betaine biosynthesis. The bacterium is proposed to be classified as *Cellulosispirillum alkaliphilum* gen. nov., sp. nov. (DSM 113825 = UQM 41584).

## Introduction

Soda lakes is a unique type of inland highly alkaline and saline habitat dominated by a specialized group of haloalkaliphilic bacteria and archaea (Grant and Jones, 2016). Intensive microbiology and molecular ecology studies of the soda lakes in the past three decades uncovered functionally rich and taxonomically diverse prokaryotic communities (Haines et al., 2023; Sorokin et al., 2014; Sorokin et al., 2015; Sorokin, 2017; Vavourakis et al., 2016, 2018, 2019; Zhao et al., 2020; Zorz et al., 2019). One of the primary steps of the carbon-cycling microbial communities is polymer degradation, and cellulose is one of the major carbohydrate polymers produced by plants and algae. So far, little was known about the identity of haloalkaliphilic prokaryotes involved in mineralization of cellulose in soda lakes. Only recently it was discovered that in hypersaline soda lakes aerobic natronoarchaea can fulfill this function, in particular the genera *Natronobiforma* and *Natronolimnobius* (Sorokin et al., 2015, 2018), whose genomes encode multiple copies of *endo*-cellulases from the GH5 and GH9 families (Elcheninov et al., 2023). However, the identity of cellulose-mineralizing bacteria in moderately saline soda lakes remained unclear. The only example of such bacteria, known so far, is a moderately salt-tolerant, anaerobic, cellulosome-forming *Clostridium alkalicellulosi* (lately reclassified as *Acetivibrio alkalicellulosi* or *Herbivorax alkalicellulosi* according to the GTDB classification) from a soda lake Hadyn in Tuva (Russia) (Zhilina et al., 2005; Zvereva et al., 2006).

Our recent efforts in obtaining a better understanding of the bacterial cellulose mineralization in moderately saline soda lakes resulted in enrichment of three haloalkaliphilic anaerobic bacterial cultures capable of utilization of various forms of insoluble cellulose as growth substrate. The primary cellulotrophic bacteria in these cultures were represented by new members of the *Clostridiales*, *Halanaerobiales* and *Fibrobacterota* (Sorokin et al., 2024). In this work we present results of phenotypic, phylogenetic and functional genomic analyses of the cellulotrophic *Fibrobacterota* strain ANBcel5$^T$, and propose to classify this

* Corresponding author at: Winogradsky Institute of Microbiology, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia.
*E-mail addresses:* soroc@inmi.ru, d.sorokin@tudelft.nl (D.Y. Sorokin).

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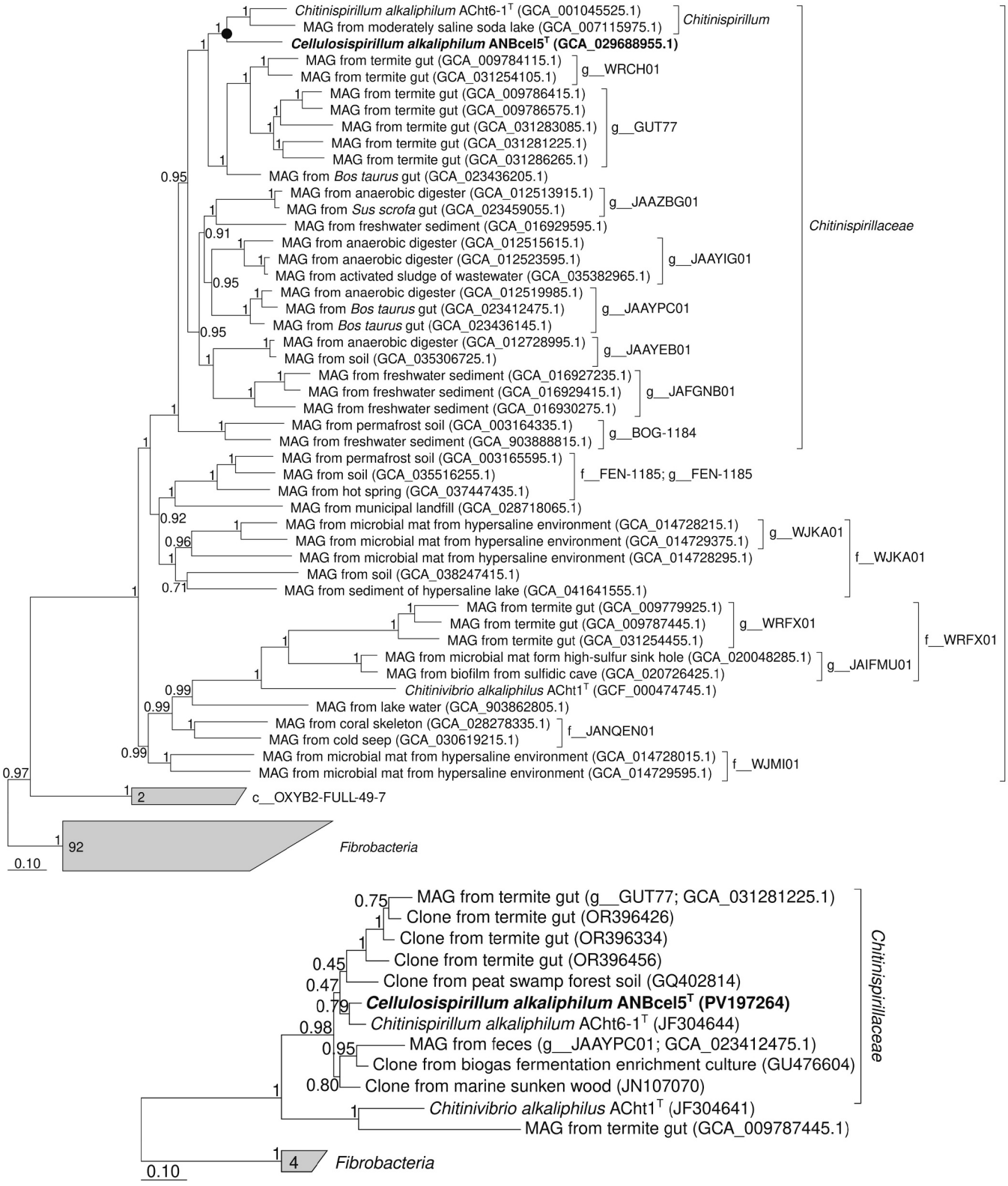


**Fig. 1.** Phylogenetic position of strain ANBcel5 within the phylum *Fibrobacterota* based on (A) concatenated amino acid sequences of 120 bacterial single copy conserved marker proteins (the length of the alignment is 29,541 aa) or (B) on 16S rRNA gene sequences. The tree was constructed using the maximum likelihood method (Minh et al., 2020). Branch support values from ultrafast bootstrap approximation are shown at the nodes. Numbers inside the trapezoids denote the number of genomes in a given cluster. Representatives of all genera of the family *Chitinispirillaceae* from the GTDB 10-RS226 database are included in the tree. All taxon names are given according to the GTDB 10-RS226 database. Bar, 0.1 change per position. ● – RED value 0.843. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

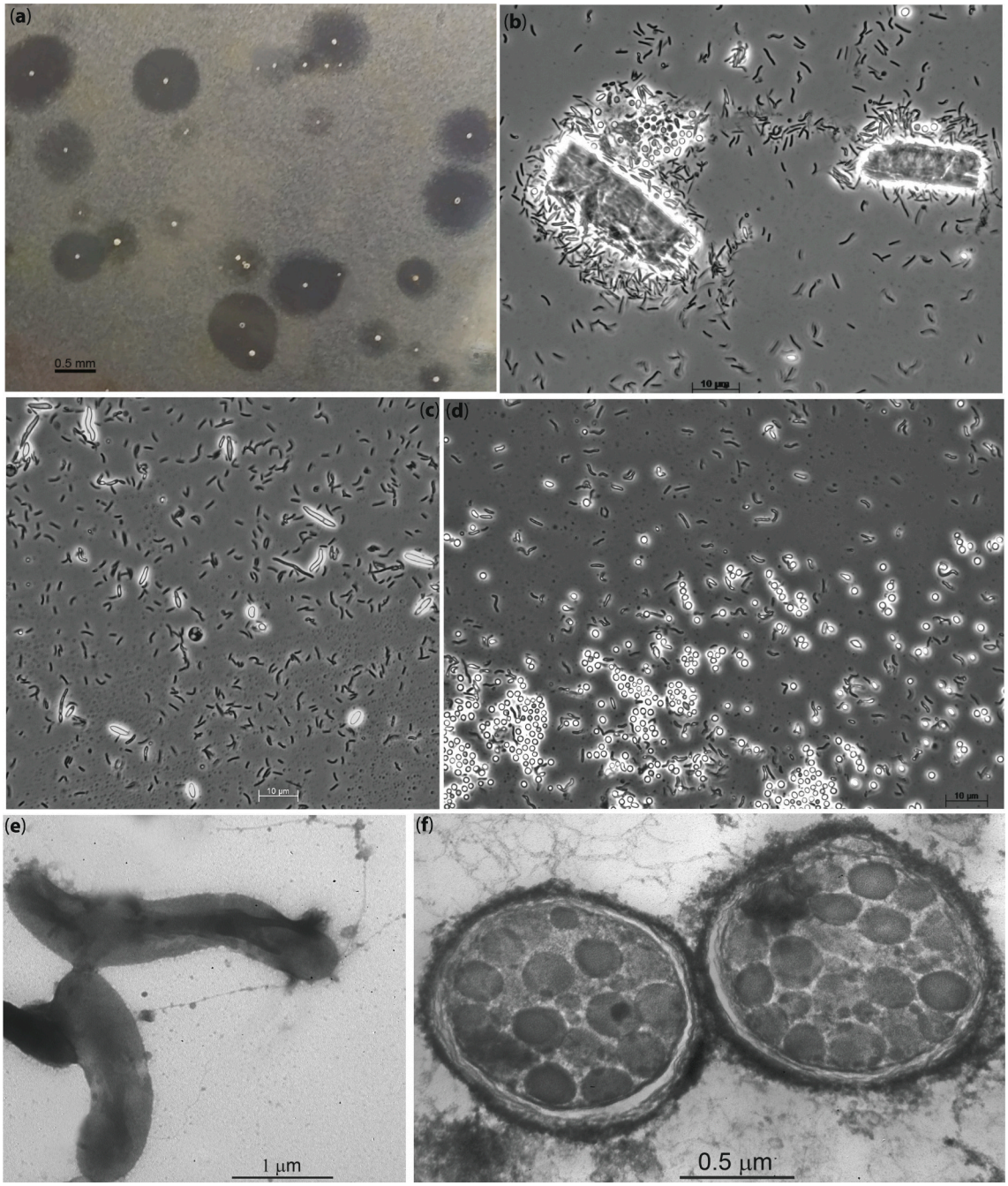


**Fig. 2.** Colonies inside soft agar dissolving amorphous cellulose (**a**), and cell morphology in phase contrast (**b-d**) and electron microscopy (**e-f**) of strain ANBcel5 grown in liquid culture with amorphous cellulose at 1 M total $Na^+$, pH 9.5 and 30 °C. (**b**) colonization of cellulose fragment; (**c-d**) free cells with various stages of cyst formation; (**e**) transmission electron microscopy showing flagellation; (**f**) thin section electron microscopy showing ultrastructure of cysts-like refractive cocci.

bacterium in a a new genus and species in the family *Chitinispirillaceae*.

## Material and methods

### *Strain origin*

Strain ANBcel5$^T$ has been obtained from an enrichment culture with amorphous cellulose (ac) inoculated by a combined sample of anaerobic sediments from several soda lakes collected in July 2020 and 2021 in the Kulunda Steppe (Altai region, Russia). The brines pH varied from 9.7 to 10.5, total salt – from 40 to 200 g $l^{-1}$ and carbonate alkalinity – from 0.4 to 2.0 M (Sorokin et al., 2024).

### *Phenotypic characterization*

The routine culturing was performed in sodium bicarbonate-carbonate base medium containing 0.6 M total $Na^+$, 1 g $l^{-1}$ $K_2HPO_4$ and 20 mg $l^{-1}$ yeast extract (final pH of 9.5 after autoclaving at 120 °C). After sterilization, the base medium was supplemented with 1 mM $MgSO_4$, 4 mM $NH_4Cl$ and 1 ml each of acidic trace metal (DSMZ S5704) and vitamin mix (DSMZ S56780 (Pfennig and Lippert, 1966; Pfennig and Trüper, 1992). Anaerobic cultivation was conducted as described previously (Sorokin et al., 2024). For pH profiling at optimal salinity, a combination of K-phosphate and HEPES buffers (50 mM each) was used for the pH range from 6 to 8 and a bicarbonate-carbonate buffer for the pH from 8 to 11. The final pH values at the end of experiment were taken

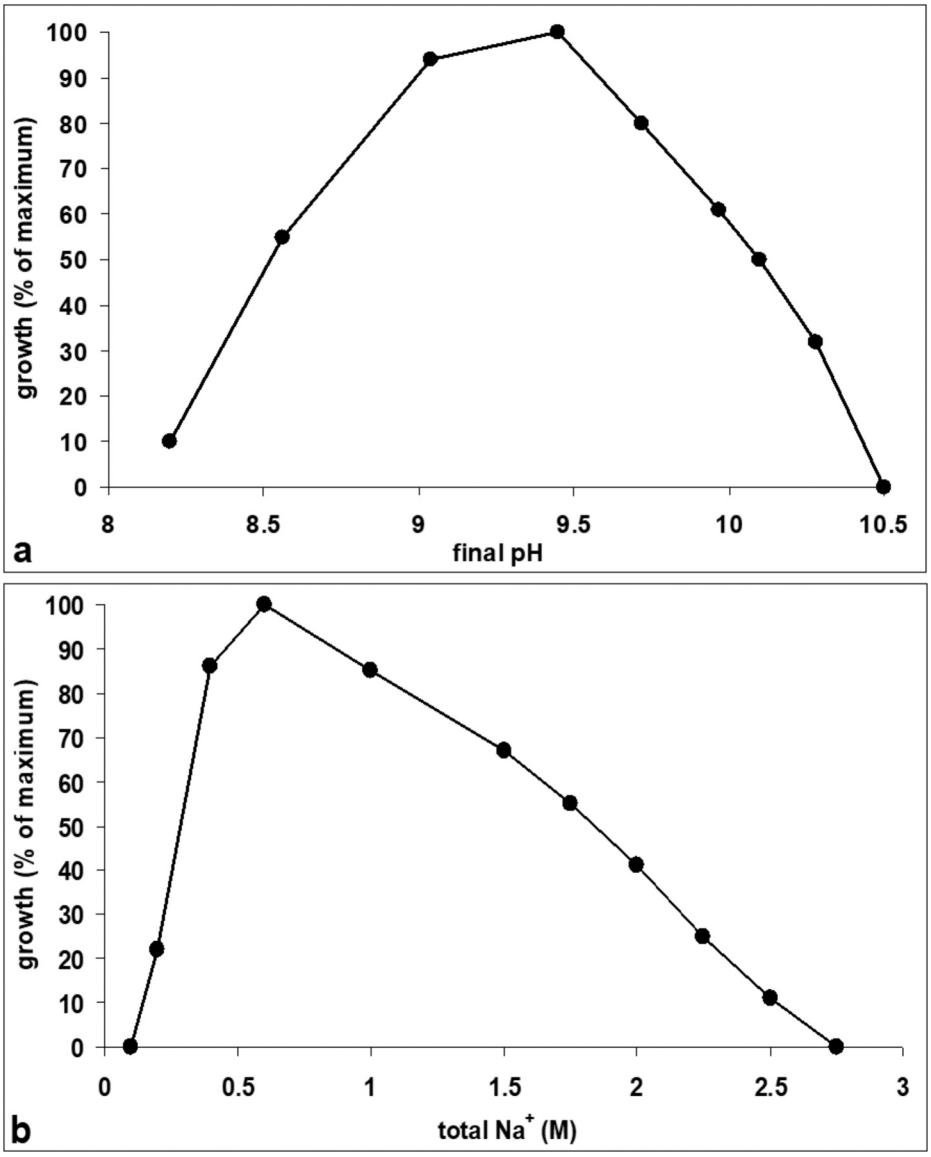


**Fig. 3.** pH (at 1 M $Na^+$) and salinity as sodium carbonates (at pH 9.5) profiles for growth of strain ANBcel5 on cellobiose at 30 °C (average values from a duplicate experiment).

into consideration. The salinity range was investigated at optimal pH using carbonate buffer system with the total $Na^+$ range from 0.1 to 3 M. Cellobiose was used as a substrate (1 g $l^{-1}$) for both types of profiling. Fermentation products in culture supernatant and in the gas phase were analyzed by HPLC and GC, respectively, as described by Sorokin et al. (2024).

Transmission electron microscopy was applied to examine flagellation and ultrastructural organization of the cells grown with ac. The cells were centrifuged (after gravity separation of residual ac particles), resuspended in 0.6 M NaCl and fixed with para-formaldehyde (final concentration 3 %, *v*/v) at room temperature for 2 h, centrifuged and resuspended in the same NaCl solution and positively contrasted with 1 % (*w/v*) uranyl acetate for 30 s. For ultrastructural examination, a second fixation (after formaldehyde removal) was done with 1 % (w/v) $OsO_4$ for overnight at 4 °C, then the agar-immobilized cells were dehydrated in ethanol series, epoxidated and subjected to ultramicrotome thin sectioning. The obtained preparations were poststained in lead citrate and uranyl acetate (1 % each). The cells were examined in Jeol JEM-1400 electron microscope (Japan).

Phospholipid fatty acid (PLFA) analysis was carried out by DSMZ Service (DSMZ, Braunschweig, Germany). The freeze-dried biomass was extracted, saponified and methylated according to standard protocols of the Microbial Identification System (MIDI Inc.; version 6.1) according to Sasser (1990). Compounds were separated an HP-5 ms UI 30 m × 250 µm × 0.25 µm column (Agilent, Santa Clara, CA, USA) and analyzed by the an GC–MS 7000D (Agilent Technologies, USA).

### *Genome sequencing and annotation*

Genomic DNA was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, United States). Shotgun WGS libraries were prepared with the KAPA HyperPlus Library Preparation Kit (KAPA Biosystems, UK). The genome sequencing was performed using NovaSeq 6000 system (Illumina, San Diego, CA, USA). The genome assemblage was performed with Unicycler v.0.5.0 (Wick et al., 2017) and automatically annotated by the PGAP (Tatusova et al., 2016) in GenBank. The 16S rRNA gene sequence was separately sequenced by Sanger sequencing and completely matched the 16S rRNA gene sequence from the genome. Genome was deposited in GenBank (GCA_029688955.1) under BioProject number PRJNA283172.

**Table 1**
Comparative properties of strain ANBcel5$^T$ and its closest related *Chitinispirillum alkaliphilum*[a].

| Property | **ANBcel5$^T$** | *Chitininspirillum alkaliphilum* ACht6–1$^T$ |
|---|---|---|
| **Morphological features** | | |
| Cell shape | motile vibrio-spirilla | motile vibrio-spirilla |
| Vegetative cell size (μm) | 0.5 × 2–12 | 0.4–0.5 × 2–10 |
| Glycogen-accumulating cysts | + | + |
| Relation to oxygen | obligate (fermentative) anaerobe | obligate (fermentative) anaerobe |
| **Growth substrates** | | |
| polysaccharides | cellulose<br>lichenan (w)<br>barley beta-glucan (w) | chitin<br>-<br>- |
| sugars | cellobiose | - |
| Salinity range (opt.) M $Na^+$ as carbonates | 0.2–2.5 (0.6) | 0.4–1.75 (0.5–1.0) |
| Final pH range (opt.)* | 8.2–10.3 (9.0–9.5) | 8.0–10.5 (9.5–10.0) |
| Maximum growth temperature (°C) | 45 | 43 |
| Predominant polar lipid fatty acids | *ai*C15:0; C14:0; *i*C16:0; C16:0 | *ai*C15:0; C16:0; C14:0; *i*C16:0 |
| *Key genomic properties* | | |
| Genome size (Mbp) | 4.65 | 4.43 |
| G + C (%, whole genome) | 41.5 | 42.9 |
| **Sodium-translocating** | | |
| membrane pumps: | + | + |
| Rnf complex | − | + |
| Oxaloacetate decarboxylase | + | + |
| V-type ATPase | + | + |
| Pyrophosphatase | + | − |
| Multisubunit sodium:proton antiporter Mnh | | |
| *Nε*-acetyl-β-lysine synthesis (osmolyte) | + | +* |
| 2-step *N*-methylation of glycine to glycine betaine (osmolyte) | + (fusion of 2 *N*-methyl- transferases) | − |
| 2-step oxidation of choline to osmolyte glycine betaine (BetAB) | − | + |
| Nitrogenase operon | + | − |
| Glycogen synthesis/degradation genes | 7 | 10** |
| Source of isolation | anoxic sediments from soda lakes in southeastern Siberia | anoxic sediments from hypersaline alkaline lakes in Wadi an Natrun (Egypt) |

[a] Sorokin et al., 2016; Sorokin et al., 2020; *measured at the end of experiment; **found/corrected in this work; (w), weak growth.

### *Phylogenetic analyses*

120 single copy conserved bacterial marker proteins (120bac) were used for phylogenomic reconstruction (Rinke et al., 2021), aligned with the GTDB-Tk v2.4.0 (Chaumeil et al., 2022) and trimmed by trimAl 2.0 (29541) aa length) using "-automated1" and "-gt 0.85" modes (Capella-Gutiérrez et al., 2009). The 16S rRNA-based and phylogenetic trees were constructed with the IQ-TREE2 program v3.0 (Minh et al., 2020) with fast model selection via ModelFinder (Kalyaanamoorthy et al., 2017) and ultrafast bootstrap approximation (Minh et al., 2013). The AAI were calculated according to Kim et al. (2021). Relative evolutionary divergence (RED) values were calculated using GTDB-Tk v2.4.1 (Chaumeil et al., 2022) and PhyloRank (https://github.com/dparks1134/PhyloRank).

16S rRNA gene-based analysis of the distribution of ANBcel5-related bacteria in GenBank database (Benson et al., 2013) was performed by nucleotide BLAST (Altschul et al., 1997) using core_nt database and by analyzing the first 5000 results. Analysis in SILVA SSU 138.2 database (Quast et al., 2013) was performed by BLAST using 16S rRNA gene of ANBcel5$^T$ as a query and all 21,404 sequences belonging to *Fibrobacterota* phylum as a subject. A search among high-throughput sequencing data for 16S rRNA gene regions was performed by IMNGS (Lagkouvardos et al., 2016) using 97 % similarity threshold and 200 bp as a minimum size threshold. Presence of ANBcel5-related MAGs in GenBank was analyzed by AAI (Kim et al., 2021) using genome blast against 684 genome sequences of *Fibrobacterota* phylum available in GenBank. Presence of ANBcel5-related MAGs in GTDB was analyzed by GTDB-Tk 2.4.1 de_novo_wf (Chaumeil et al., 2022) and entire GTDB 10-RS226 database (Parks et al., 2022). Publicly available metagenomic data from the SRA database (https://www.ncbi.nlm.nih.gov/sra) were analyzed for the presence of the ANBcel5-related bacteria using the YACHT (Koslicki et al., 2024).

### *Functional genomic analysis*

Identification of genes encoding polysaccharide-active enzymes according to classification of the CAZy database (Drula et al., 2022) was first performed using the dbCAN3 portal (Zheng et al., 2023) and then manually verified by Blast search in UniProt along with SignalP 6.0 server (Teufel et al., 2022) to predict the presence of signal peptides. For other key functional complexes, their presence was first analyzed manually in the translated protein assembly and then the identity was verified in UniProt using model proteins with proven function as references.

## Results and discussion

### *Phylogenetic analysis*

The isolate was first identified by its 16S rRNA gene sequence indicating that its closest cultured relative was *Chitinispirillum alkaliphilum* ACht6–1$^T$ (class *Chitinivibrionia*, *Fibrobacterota*), a chitin-utilizing haloalkaliphilic bacterium from Egyptian haloalkaline lakes (Sorokin et al., 2016), with the sequence identity of 95.2 % (Supplementary **Fig. S1**). Further phylogenomic analysis based on 120 bacterial conserved protein markers (Parks et al., 2018) confirmed the preliminary placement of ANBcel5$^T$ into the family *Chitinispirillaceae* as a new genus-level lineage (Fig. 1) with a RED value of 0.843, which is much lower than the median RED value for genus (0.921 according to GTDB RS226). With the 16S rRNA gene and AAI identity scores (see below) at near-threshold levels for genus delineation, we rely on the phylogenomic analysis and the RED value to delineate a new genus.

To search for MAGs belonging to this new-genus lineage, we analyzed not only GTDB RS226, but also performed AAI analysis with all 684 genome assemblies of the *Fibrobacterota* phylum in GenBank (Taxonomy ID: 65842, Feb. 2025). But AAI analysis did not reveal any additional ANBcel5-related genomes. ACht6–1$^T$ and its related MAG GCA_007115975 from sediments of a moderately saline Cock Soda Lake in the Kulunda Steppe (Vavourakis et al., 2019) showed the highest AAI values (64.4 and 64.0 %, respectively, proteome coverage around 60 %) (Fig. 1). The new lineage represented by strain ANBcel5$^T$ proved to be rarely detected in molecular ecological studies. The 16S rRNA gene-based analysis of the distribution of this bacterium in Silva and GenBank databases only found one sequence (KU651808) that was more than 97 % identical to the 16S rRNA gene of the strain ANBcel5$^T$ detected in anaerobic full-scale reactor (Calusinska et al., 2018). A search of the high-throughput sequencing database (SRA) revealed the presence of ANBcel5-related phylotypes (>97 % identity) in several samples of hydrocarbon contaminated marine microbial mats (Vigneron et al., 2024). However, their abundance did not exceed 0.05 %. Thus, in order to detect any related bacteria, publicly available metagenomic sequencing database was searched next. Using *k*-mers-based coverage analysis approach (Koslicki et al., 2024) we analyzed the water and sediment metagenomes of Cock Soda lake – moderately saline soda lake

**Table 2**
Extracellular (mostly Sec/SPI signal peptides) polysaccharide hydrolases encoded in the genome of strain ANBcel5$^T$.

| Locus tag (MDG581+) | Family | Protein name | Possible substrate | Length (aa) |
|---|---|---|---|---|
| *Glycosyl hydrolases (GH families)* | | | | |
| 3904/6148 | GH5_2 + CBM4 | | | 654/560 |
| 5485/5520 | GH5_2 + CBM6 | | | 350/577 |
| 5484 | GH5_2 + 2 x CBM6 | | | 766 |
| 4302/6925 | GH5_2 | *endo*-1,4-beta-glucanase | | 587/605 |
| 6390 | GH5_5 | | | 432 |
| 5195 | GH9 | | **cellulose** | 837 |
| 5657/6058 | GH9 + CBM4 | | | 817/870 |
| 6341 | GH9 | cellulase | | 1882 |
| 6348 | GH9 + CBM4 | | | 787 |
| 6752 | GH9 + CBM32 | | | 848 |
| 7026 | GH9 | | | 598 |
| 4020 | GH5_55 + CBM4 | endo-1,4-beta-glucanase/mannanase | **cellulose,** mannan | 654 |
| 3944 | GH51_3 | endo-1,4-beta-glucanase/xylanase | **cellulose**, xylan | 444 |
| 4081 | GH51_3 + 2 x CBM11 + CBM30 | endo-1,4-beta-glucanase/xylanase | **cellulose**, xylan | 1042 |
| 6526 | GH51_3 | endo-1,4-beta-glucanase/xylanase | **cellulose**, xylan | 541 |
| 3642 | GH10 + 2 x CBM22 | endo-1,4-beta xylanase | xylan | 834 |
| 6733 | GH11 + CBM4 | endo-1,4-beta xylanase | xylan | 596 |
| 3643 | GH43_16 + CBM4 + CBM6 | endo-1,4-beta xylanase, beta-xylosidase | xylan, xylobiose | 730 |
| 6421 | GH30 + CBM4 | exo-1,4-beta-xylanase/xylobiohydrolase | xylan, xylobiose | 683 |
| 3524 | GH45_1 | xyloglucan-specific endo-1,4-beta-glucanase | xyloglucan | 487 |
| 3650 | GH45_1 | | | 500 |
| 6351 | GH74 | | | 871 |
| 3509 | GH8 + CBM4 | endo-1,3/1,4-beta-glucanase | lichenan/laminarin | 705 |
| 3652 | GH16_25 | endo-1,3/1,4-beta-glucanase (lichenase) | lichenan | 440 |
| 6977 | GH148 | endo-1,3/1,4-beta-glucanase (lichenase) | lichenan | 856 |
| 6494 | GH16_3 | endo-1,3- glucanase/laminarinase | laminarin | 506 |
| 6615 | GH16_3 | endo-1,3- glucanase/laminarinase | laminarin | 576 |
| 4024 | GH5_4 | endo-1,4-beta-glucanase | glucomannan | 677 |
| 5915 | GH26 | | | 360 |
| 6751 | GH5_8 + CBM4 | endo-1,4-beta-mannanase | beta-mannan | 687 |
| 7020 | GH5_10 | | | 679 |
| 6618 | GH18 | endo-chitinase | chitin | 477 |
| *Polysaccharide lyases (PL families)* | | | | |
| 4712 | PL21_1 | heparin sulfate lyase | heparin | 909 |
| 6667 | PL11_1 | rhamogalacturonan lyase | rhamogalacturonan | 873 |
| 6739 | PL1_5+ PL1 + CBM13 | pectate lyase | pectin | 1118 |
| 6897 | PL9_1 | pectin lyase | pectin | 804 |
| 5844/6113 | 3 x CBM4 | | | 755/811 |
| 6529 | CBM4 | Carbohydrate-binding modules | **cellulose** | 750 |
| 6996/6997 | CBM4 | | | 809/586 |
| 4241 | CBM9 | | **cellulose**, xylan | 526 |
| 6986 | CBM11 | | 1,3/1,4-beta-glucans | 765 |
| 6668 | CBM88 | | galactomannan | 748 |

in the same area whereby ANBcel5$^T$ originated (Vavourakis et al., 2019). The results indicated the absence of ANBcel5-related MAGs in this ecotope (at the time of sampling at least, which was in 2013).

We run a genome search of MAGs closest to the isolate for the presence of key functional marker genes encoding beta-glucan-active glucanases, including the MAG GCA_007115975 clustered with ACht6–1$^T$ and a somewhat more remotely related group represented in the GTDB by the putative genus abbreviated as WRCH01, which includes two MAGs from termite guts, one from French Guiana (GCA_009784115) and another – from China (GCA_031254105) (Fig. 1). All three completely lacked the chitininase gene markers, while, obviously, belonging to cellulotrophic bacteria. The GCA_007115975 encodes 4 x GH5 (5_2 and 5_25 subfamilies) and 4 x GH9 families cellulases. The GCA_009784115 has 5 x GH5_2, 2 x GH5_4, 2 x GH8 and 2 x GH9 cellulase families; a GH74 and 3 x GH45_1 xyloglucanases and 3 endoxylanases of the GH10/GH11 families. The GCA_031254105 features 6 x GH5_2, 2 x GH5_4, 2 x GH8 and 5 x GH9 cellulase families; 2 x GH74 and 3 x GH45_1 xyloglucanases; a xylan-utilization locus including endoxylanases of the GH10 and GH11 and *exo*-xylanases of the GH43_10, GH43_16 and GH43_29 subfamilies. All of the proteins have a Sec/SPI-SPII signal peptide indicating their extracellular localization. This suggests that the genus *Chitinispirillum* is more likely an exception with its chitin specialization among the members of this phylogenetic cluster of *Fibrobacterota*.

### *Cell morphology and chemotaxonomy*

ANBcel5$^T$ formed tiny white colonies inside soft agar with ac surrounded by visible cellulose degradation zone (Fig. 2 **a**). In liquid culture with various forms of insoluble cellulose, growth started on the bottom cellulose layer with massive cell colonization of the cellulose fragments (Fig. 2 **b**). After visible fracturing of the cellulose layer, cells started to move to the upper cellulose-free medium layer. Young cells were mostly motile vibrio-spirilla (Fig. 2 **c, e**), while later on some of the cells started to acquire refractivity, swelling to ellipsoids and, finally, becoming highly refractive cocci (Fig. 2 **c-d**). All such transformations were very similar to what was observed in the chitinotrophic ACht6–1$^T$. Thin sectioning showed that in both these bacteria the highly refractive coccoid cells looked like cysts, with much thicker cell wall than the young cells and completely packed with storage granules (Fig. 2 **e**). The genomes of both organisms feature large repertoire of genes coding for glycogen biosynthesis and its degradation, including glycogen/starch synthase/phosphorylase (GlgA/GlgP) and glycogen branching-debranching alpha-1,4/1,6-glucanases (GlgB/GlgX). On the other hand, the genes for PHA synthesis are absent. This prompts to assume that the storage polymer is glycogen.

**Table 3**
Proteins potentially involved in haloalkaliphilic adaptation encoded in the genome of strain ANBcel5$^T$.

| Locus tag (MDG581+) | Protein | Putative function |
|---|---|---|
| *Osmoprotection* | | |
| 4456 | lysine 2,3-aminomutase | Biosynthesis of osmolyte *N*ε-acetyl-β-lysine |
| 4457 | β-lysine *N*-acetyltransferase | |
| 4473 | *N*-methyltransferase of glycine to betaine (fusion of two genes) | Biosynthesis of osmolyte betaine from glycine |
| 6190 | BetT | High affinity glycine betaine exporter |
| 6729; 6736–6738 | OpuAC; OpuAD-OpuAB-OpuAA | Glycine-betaine ABC transporter |
| *pH-ion homeostasis* | | |
| 3769–3775 | MnhAB1B2CDEF | Multisubunite sodium:proton antiporter |
| 5907/6976 | CPA2 | $Na^+$: $H^+$ antiporter |
| 6140 | NhaC | $Na^+$: $H^+$ antiporter |
| 6187 | NhaP/CPA1 | $K^+$: $H^+$ antiporter |
| 4450–4451 | TrkAH | Potassium uptake ($K^+$: $H^+$ symporter) |
| 6004/6828 | Kch | Potassium channel |
| 79,736; 79,928 | CaCA | $Ca^{2+}$: $Na^+$ antiporter |
| *Sodium-translocating membrane pumps* | | |
| 4622–4628 | NtpABCDEIK | V-type ATPase ($Na^+$out) |
| 5604 | HppA | Pyrophosphatase ($Na^+$out) |
| 7005–7010 | RnfABCDEG | NADH-Fd oxidoreductase ($Na^+$ out) |
| *Hydrogenases* | | |
| 5781–5785/ 5789 | HfsABCD/E | Cytoplasmic [FeFe] ferredoxin-dependent hydrogenase |
| 5299–5306 | HydABCD/EFG | Cytoplasmic NAD(H)-dependent hydrogenase |
| *Other functional complexes* | | |
| 6386 6387 | formate *C*-acetyltransferase pyruvate-formate lyase activation protein | Pyruvate-formate lyase |
| 3811, 4237, 4408, 4494, 6753 4578, 5448 | Glycogen/starch synthase/ phosphorylase<br>Glycogen debranching proteins | Glycogen synthesis/degradation |
| 6391–6397 | NifHDK/P-II N-regulators/ NifEN chaperons | Nitrogenase complex |

The dominant membrane phospholipid fatty acids in ANBcel5$^T$ were quite similar to those detected in ACht6–1, albeit at different proportions, including (in order of abundance) saturated *anteiso*C15:0, C14:0, *iso*C16:0 and C16:0 (Supplementary **Table S1**).

### *Phenotypic characteristics*

Growth tests indicated that ANBcel5$^T$ is an obligate anaerobic, saccharolytic, fermentative bacterium utilizing very limited number of carbohydrates as carbon and energy source. In fact, vigorous growth was observed only with two substrates: (a) cellulose, especially ac, but also less rapid with more crystalline forms, including filter paper, Avicel and Sigma celluloses; (b) – cellobiose, a soluble dimer of cellulose. The major fermentation products on both substrates were acetate, succinate and $H_2$. Furthermore, weak growth was also possible with two other glucose polymers – soluble barley 1,4/1,6-beta-glucan and insoluble lichenan (beta-1,3/1,4 -glucan). Tested but not utilized glucans included: chitin, xyloglucan, laminarin, beech and birch xylans, gluco- and galactomannans, beta-mannan, galactan, arabinogalactan, arabinan, arabinoxylan, alginate, polygalacturonate, rhamnogalacturonan, hyaluronate, heparin, chondroitin, levan, inulin, starch and glycogen.

The results of salt (as sodium carbonates at pH 9.5) and pH (at 0.6 M total $Na^+$) profiling indicated that the isolate is a moderately salt-tolerant obligate alkaliphile with the salinity range for growth on

**Table 4**
Protologue of *Cellulosispirillum* gen. nov., and *Cellulosispirillum alkaliphilum* sp. nov.

| Parameter | genus: ***Cellulosispirillum*** | Species: ***alkaliphilum*** |
|---|---|---|
| Family name | *Chitinispirillaceae* | |
| Genus name | *Cellulosispirillum* | |
| Species name | | *Cellulosispirillum alkaliphilum* |
| Status | gen. nov. | sp. nov. |
| Type species of the genus | *Cellulosispirillum alkaliphilum* | yes |
| Etymology | [Cel.lu.lo.si.spi.ril'lum N.L. n. *cellulosum*, cellulose; Gr. fem. n. *spira* spiral; N.L. dim. neut. n. *spirillum* a small spiral. N.L. neut. n. *Cellulosispirillum*, a spirillum utilizing cellulose] | [al.ka.li'phi.lum M.L. n. *alkali* soda ash; N.L. masc. adj. *philus* (from Gr. masc. adj. *philos* loving); N.L. neut. adj. *alkaliphilum* loving alkaline conditions] |
| Description of a new taxon | The genus includes moderately salt-tolerant and alkaliphilic anaerobic heterotrophic bacteria specialized on utilization of cellulose as the growth substrate. Currently it includes a single species. A member of the family *Chitinispirillaceae*, phylum *Fibrobacterota*. The type species is *Cellulosispirillum alkaliphilum*. Found in soda lakes. | Cells are spirilla of variable length, 0.5 × 2–12 μm with the Gram-negative cell-wall structure and motile with 1–2 subpolar flagella. Form ellipsoid/spherical cysts apparently filled with glycogen granules. Formed tiny white colonies inside soft agar with amorphous cellulose clearance zones. Strictly anaerobic fermentative saccharolytic bacterium growing only with two substrates – cellulose and cellobiose and producing acetate, $H_2$ and succinate as major fermentation products. Weak growth was also observed with lichenan and Barley beta-glucan. Obligately alkaliphilic, with a pH range for growth between 8.2 and 10.3 (opt. at pH 9.0–9.5) and moderately salt-tolerant, with a total $Na^+$ range for growth from 0.2 to 2.5 M (opt. at 0.4–0.6 M). Mesophilic, with a maximum growth temperature at 45 °C. The membrane phospholipid fatty acids are dominated by *ai*-C15:0 and C14:0 (70 % of the total), followed by less abundant C16:0 and *i*C16:0. The species description is based on the properties of a single strain ANBcel5$^T$ isolated from soda lakes in southeastern Siberia (Altai region, Russia). |
| Type strain | | ANBcel5$^T$ |
| Culture collection numbers | | DSMZ 113825; UQM 41584 |
| Genome status | | Draft |
| GenBank genome assembly | | GCA_029688955 |
| Genome size (Mbp) | | 4.7 |
| 16S-rRNA gene accession number in the GenBank | | PV197264 |
| Country of origine | | Russian Federation |
| Region | | Altai |
| Habitat | | Anoxic sediments from soda lakes |
| Latitude | | N52°06'/N51°37'/N51°40' |

(*continued on next page*)

**Table 4** (*continued*)

| Parameter | genus: ***Cellulosispirillum*** | Species: ***alkaliphilum*** |
|---|---|---|
| Longitude | | E79°09'/E79°50'/ E79°54' |
| Sampling dates | | July 2020 and July 2021 |
| pH of the samples | | 9.7–10.5 |
| Salinity of the samples | | 40–200 |
| Number of strains in study | | 1 |

cellobiose from 0.2 to 2.5 M total $Na^+$ (optimum at 0.4–0.6 M) and the (actual measured) pH from 8.2 to 10.3 (optimum at 9.0–9.5) (Fig. 3). Maximum temperature for growth with cellobiose at optimal salt-pH conditions was 45 °C (optimum at 30 °C). The properties of ANBcel5$^T$ in comparison to ACht6–1$^T$ are shown in Table 1. The major difference is their utilized polysaccharide speciation, one for cellulose and another for chitin. There are also a few important differences in the presence of complexes potentially involved in haloalkaliphilic adaptation, which are discussed below.

*Functional genome analysis*

According to the results of genome scan in the dbCAN portal, ANBcel5$^T$ can secrete a large array of extracellular glycoside hydrolases (GH) and a few polysaccharide lyases (PL) listed in Table 2. Among them, a special significance belonged to two GH families – GH5 and GH9, which mostly include cellulases. The genome encodes eight *endo*-cellulases with or without carbohydrate-binding domains (CBM) from the subfamilies GH5_2 and GH5_5 and five from the GH9. Furthermore, there are several GH51_3 subfamily, whose substrate might also be cellulose. Such multitude of cellulases is in a good agreement with the cellulose being the major growth substrate for ANBcel5$^T$. A presence of several endo-beta-1,3/1,4 glucanases from the GH families 8, 16_4 and 148 is also consistent with the ability of the isolate to grow (albeit weakly), on lichenan. On the other hand, the genome also encodes several GH families whose potential substrates are xylan (GH10, 11 and 30), xyloglucan (GH45_1 and 74) and laminarin (GH16_3) with no obvious role as the bacterium failed to grow with any of those polysaccharides. Same is true for the presence of PL-encoding genes and a single endo-chitinase GH18. One of the possibilities, also considered for the exclusively chitin-specialized ACht6–1$^T$ (Sorokin et al., 2016), is that these enzymes would still be necessary for a narrowly-specialized polysacharidolytics to get an access to their growth substrate at native conditions.

The genes coding for biosynthesis of osmolytes most commonly produced by salt-tolerant prokaryotes (Roberts, 2005) identified a potential pathway for production of *Nε*-acetyl-β-lysine present in genomes of both ANBcel5$^T$ and ACht6–1$^T$ (**Tables 1** and **3**). In addition, both organisms have potential for biosynthesis of a second common osmolyte – glycine betaine, but by different pathways. In ANBcel5$^T$, the pathway includes a 3-step *N*-methylation of glycine (Nyyssölä et al., 2001). However, in contrast to classical two SAM-radical families methylases (glycine>sarcosine and sarcosine to betaine), it has a fusion of the two enzymes. In ACht6–1$^T$, the pathway consists of a 2-step oxidation of choline to glycine betaine via betaine aldehyde. Apart of the *de-novo* synthesis, external glycine betaine can also be imported by a high-affinity BCC transporter BetT and a glycine betaine ABC transporter in both bacteria.

With respect to the alkaliphilic adaptation-ion homeostasis, only ANBcel5$^T$ encodes a multisubunit $Na^+$: $H^+$ antiporters MnhABCDEF (lacking the G subunit) usually present in most haloalkaliphiles, while both ANBcel5$^T$ and ACht6–1$^T$ have several single-subunit $Na^+/K^+$: $H^+$ antiporters and a $K^+$: $H^+$ symporter TrkAH. Furthermore, both genomes encode three membrane sodium-translocating pumps commonly maintaining cytoplasmic sodium homeostasis in anaerobic fermentative alkaliphiles: a RnfABCDEG complex acting as the ferredoxin-NADH oxidoreductase, a V-type ATPase NtpABCDEIK and a pyrophosphatase HppA. In addition, ACht6–1$^T$ also has a membrane oxalacetate decarboxylase OadABG acting as a primary sodium pump (Buckel, 2001) (**Tables 1** and **3**).

Finally, a remarkable difference between the new isolate and ACht6–1$^T$ was the presence in the former of a nitrogenase operon encoding three catalytic proteins NifDHK and four auxiliary genes (regulators and chaperons). Therefore, acetylene test was performed for nitrogenase activity with the cells pregrown at N-limited conditions (5 mM cellobiose +0.5 mM ammonium, instead of 4 mM in the routinely used medium). Acetylene was added at 2 % (*v*/v) into the gas phase when the culture stopped growing and ethylene formation was followed by gas chromatography. Only traces of ethylene were detectable after 2 days incubation (<0.1 %) indicative of negative results.

On the basis of distant phylogenomic and unique phenotypic properties, the novel soda lake cellulotrophic bacterium strain ANBcel5$^T$ is suggested to form a new genus and species *Cellulosispirillum alkaliphilum* within the family *Chitinispirillaceae*. The new genus and species protologues are presented in Table 4.

## CRediT authorship contribution statement

**Dimitry Y. Sorokin:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Alexander Y. Merkel:** Writing – original draft, Methodology, Formal analysis, Data curation. **Tatjana V. Khizhniak:** Writing – original draft, Investigation.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2025.126623.

## Data availability

Data will be made available on request.

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