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# *Natronosporangium hydrolyticum* gen. nov., sp. nov., a haloalkaliphilic polyhydrolytic actinobacterium from a soda solonchak soil in Central Asia

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ABSTRACT

During a cultural diversity survey on hydrolytic bacteria in saline alkaline soils, a hydrolytic actinobacterium strain ACPA39$^T$ was enriched and isolated in pure culture from a soda solonchak soil in southwestern Siberia. It forms a substrate mycelium with rod-shaped sporangia containing 1–3 exospores. The isolate is obligately alkaliphilic, growing at pH 7.5–10.3 (optimum at 8.5–9.0) and moderately halophilic, tolerating up to 3 M total $Na^+$ in the form of sodium carbonates. It is an obligately aerobic, organoheterotrophic, saccharolytic bacterium, utilizing various sugars and alpha/beta-glucans as growth substrates. According to the 16S rRNA gene-based phylogenetic analysis, strain ACPA39$^T$ forms a distinct branch within the family *Micromonosporaceae,* with the sequence identities below 94.5% with type strains of other genera. This is confirmed by phylogenomic analysis based on the 120 conserved single copy protein-based markers and genomic indexes (ANI, AAI). The cell-wall of ACPA39$^T$ contained *meso*-DAP, glycine, glutamic acid and alanine in a equimolar ratio, characteristic of the peptidoglycan type A1γ'. The whole-cell sugars include galactose and xylose. The major menaquinone is MK-10($H_4$). The identified polar lipids consist of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol. The polar lipid fatty acids were dominated by *anteiso*-$C_{17:0}$, *iso*-$C_{16:0}$, *iso*-$C_{17:0}$, 10 Me-$C_{18:0}$ and $C_{18:1}\omega9$. Based on the distinct phylogeny, the chemotaxonomy features and unique phenotypic properties, strain ACPA39$^T$ (DSM 106523$^T$ = VKM 2772$^T$) is classified into a new genus and species in the family *Micromonosporaceae* for which the name *Natronosporangium hydrolitycum* gen. nov., sp. nov. is proposed.
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## Introduction

Actinobacteria represent one of the dominant and well characterized phylum in soils. In contrast to eukaryotic fungi, many of the actinobacterial members are rather alkalitolerant, with that trait being especially common among the numerous *Streptomyces* and *Nocardiopsis* species [1–3]. Saline alkaline soils (soda solonchaks) with stable high pH above 9 [4–6] are ideal habitats for such actinobacteria due to a domination of sodium carbonates among the soluble salts but, currently, the prokaryotic communities of this extreme habitat are poorly investigated. To date, only a few publications report on the abundance of alkalitolerant actinomycetes belonging to genera *Streptomyces* and *Nocardiopsis* [7–12].

Our recent investigation of the diversity of culturable bacteria with hydrolytic potential inhabiting soda solonchak soils from different geographical locations in Central Asia yielded a large proportion of haloalkaliphilic actinobacteria, mainly belonging to the mycelium-forming genera *Streptomyces* and *Nocardiopsis*, and a less abundant single-cell isolates belonging to the genus *Isoptericola* [13]. In addition, two isolates clearly differed from the known haloalkaliphilic species forming newgenera in the families *Glycomycetaceae* and *Micromonosporaceae*. The former has recently

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been described as *Natronoglycomyces albus* [14]. The purpose of this work was to classify the novel haloalkaliphilic member of the *Micromonosparaceae* family, strain ACPA39$^T$.

## Materials and methods

### *Enrichment and cultivation conditions*

Strain ACPA39$^T$ was isolated from surface horizon (0–5 cm deep) of a soda solonchak soil in southwestern Siberia (Barabinskaya Steppe, Novosibirsk region, Russia; 55.06 N/81.1 E). The 1:5 water extract had a pH of 10.7, a total soluble salts content of 62 g $kg^{-1}$ and a total soluble carbonate alkalinity of 0.51 M. Enrichment and isolation of a pure culture was performed in a sodium carbonate medium containing 0.6 M total $Na^+$ at pH 10 with carboxymethylcellulose (1 g $l^{-1}$) and yeast extract (0.2 g $l^{-1}$) as substrates, as described previously [13,14]. Growth and cell/colony morphology were investigated in cultures grown during 2–28 days at 28 °C in liquid and solid media including: 1) soluble starch/yeast extract medium (SS-YE) (1 g $l^{-1}$ starch/ 0.2 g $l^{-1}$ yeast extract; pH 10 and 0.6 M total $Na^+$); 2) agar media mixed (1:1 v/v) with sodium carbonate base buffer (15 g $Na_2CO_3$, 20 g $NaHCO_3$, 3 g NaCl, 1 g $K_2HPO_4$ per liter, pH 9.5) and ISP 2 [15], R2A [16] or PYG medium (5 g peptone, 3 g yeast extract and 5 g glucose per liter). The growth pH profiling was performed with the following buffer systems (containing 0.6 M total $Na^+$): 50 mM potassium phosphate-HEPES/0.6 M NaCl for pH 5–8, 50 mM HEPES-0.6 M $NaHCO_3$ for pH 8–8.5 and sodium carbonate-bicarbonate for pH 9–11 (the step was 0.5 pH unit). The final pH were used as the actual values [11]. It must be argued, however, whether the pH profiling in some literature was performed convincingly [10,12], since the recommended use of strong buffering systems in liquid media compatible with biology and continuous monitoring of the pH changes during growth was not applied [11]. Thus, reported pH maxima of 12 are unrealistic, because there are no buffering systems maintaining stable pH above 11.

The influence of $Na^+$ (in the form of sodium cabonates, between 0 and 4 M, pH 10) and temperature (within the range from 10 to 60 °C with intervals of 5 °C; pH 10 and 0.6 M $Na^+$) on growth were investigated with starch/yeast extract-containing medium. For the N-source spectrum, the culture was grown in liquid medium at pH 9.5/0.6 M $Na^+$ and 30 °C with addition of ammonium, nitrate or urea at 4 mM total N or 0.2 g $l^{-1}$ of yeast extract peptons from meat or casein. For the antimicrobial activity tests, paper discs were soaked in the filter-sterilized culture supernatant of strain ACPA39$^T$ pregrown for two weeks on starch/yeast extract medium and the discs were placed on the surface of the LB agar preinoculated with either *E. coli* or *Micrococcus luteus* test cultures.

### *Cell morphology and chemotaxonomy*

Cell micromorphology was studied with light microscopy (Zeiss Axioplan Imaging 2, Göttingen, Germany) and transmission and scanning electron microscopy (JEOL, Japan). For thin section electron microscopy, the cell material was fixed with paraformaldehyde (3% w/v in 0.6 M NaCl) for 2 h at room temperature, postfixed by $OsO_4$ (1% w/v in 0.6 M NaCl) for 48 h at 4 °C, embedded into agar blocks, stained with uranyl acetate (1% w/v) for 1 h, dehydrated in alcohol series and finally in absolute acetone, embedded into Epoxy resin and thin sectioned on the ultramicrotome. The ultrathin sections were finally stained with lead citrate (1% w/v) for 1 h.

For chemotaxonomic characterization cells were grown in sodium carbonate medium at pH 9.5 and 0.6 M total $Na^+$, harvested in late exponential growth phase and freeze-dried. The cell wall fraction was obtained by hydrolysis in 0.1 M NaOH [17] followed with hydrolysis with 6 M HCl at 110 °C for 20 h. The isomer composition of diaminopimelic acid (DAP) was determined by TLC. The cell wall amino acids composition was analyzed by anion-exchange chromatography (Biotronik, Germany) equipped with the DC6a resin column (Durrum) [18]. The whole cell sugar composition was determined by anion-exchange chromatography [Biotronik, Germany; column DA-x8-11 (Durrum)] after hydrolysis of cells in 3 M trifluoroacetic acid at 100 °C for 6 h [19]. Intact membrane polar lipids (MPL) were extracted by acidic methanol and separated by two-dimensional TLC using HPTLC Silica gel 60, chloroform–methanol-water (65: 25: 4) system in the first direction and chloroform–acetic acid–methanol-water (80:15:12:4) in the second direction [20]. The identified MPL spots were compared with respective spots on chromatograms of related reference strains for which the MPL profiles were determined previously. Polar lipid fatty acids (PLFA) were extracted, saponified, methylated and purified, and the methyl esters were analyzed by a Trace GC Ultra coupled to a DSQ II single-quadrupole mass spectrometer (Thermo Scientific) and identified by using the NIST 17 mass spectral library (https://chemdata.nist.gov/ dokuwiki/doku.php?id = chemdata:nist17) [21]. Respiratory menaquinones were extracted from wet cells, purified according to Collins (1985) [22] and analyzed with a LCQ Advantage MAX mass spectrometer (Thermo Finnigan).

### *Hydrolytic activity*

The potential of ACPA33$^T$ to hydrolyze various polysaccharides was investigated at three different levels: (1) by the ability to use the polysaccharides as growth substrates; (2) by the hydrolytic activity assays either on agar plates with zone hydrolysis detection [13] or by incubation of cell suspensions in titer plates with the dye conjugated polymers as described previously for *Natronoglycomyces albus* [14]; (3) by querying the genome for the presence of glycosyl hydrolase (GH)-encoding genes using the dbCAN2 server [23].

### *Genomic sequencing, phylogenetic and genomic analyses*

Genomic DNA was extracted according to Park [24]. SMRTbell™ template library was prepared using SMRTbell® Express Template Prep Kit 2.0 PacificBiosciences (CA, USA). For preparing 10 kb libraries, 1 μg genomic DNA was end-repaired and ligated to barcoded adapters. The amplified products were pooled and sequenced in 1/16 SMRT cell on the Sequel*II* (PacificBiosciences, Menlo Park, CA, USA) resulting in 704,432 aligned subreads with mean aligned read lengths of 4.821 bp. Long read genome assembly was performed with the "Microbial Assembly" protocol included in SMRTlink version 8 using default parameters with exception of the target genome size, which was set to 5,7 Mbp. The assembly produced a single circular chromosome and two circular plasmids were obtained, afterwards rotated to the chromosomal/plasmid origin (*dnaA, parA*). Identification of replication genes has been done based on BLAST.

Primary identification of strain ACPA39$^T$ was performed using NCBI BLAST with one of two copies of the 16S rRNA gene sequence as the query.

For phylogenetic analysis based on comparison of 16S rRNA genes, the 16S rRNA gene sequence of strain ACPA39$^T$, the sequences of all species with validly published names within the *Micromonosporaceae* family as well as *Rubrobacter radiotolerans* P1 as an outgroup were aligned using MAFFT server with G-INS-i method [25]. The phylogenetic tree was constructed in MEGA7 [26] using the maximum likelihood method and the

GTR model (G + *I*, 4 categories) with 1000 bootstrap replications; all positions with less than 95% site coverage were eliminated. For extended phylogenomic analysis based on the "bac120" set of conserved single copy bacterial proteins [27], the protein sequences were identified and aligned in *in silico* proteomes of type species of each genus within *Micromonosporaceae* (*Catenuplanes* genus was excluded from analysis due to a bad quality of genome assemblies; for two multispecies genera, *Micromonospora* and *Actinoplanes*, several most distant species were selected using the GTDB-tk v.1.6.0 with reference data v.202) [28]. The alignment was treated using the trimAl v.1.4.1 with the -gt 1 parameter (full gap elimination) [29]. The phylogenomic tree was constructed in the RAxML v.8.2.12 [30] with the PROTGAMMAILG model of amino acid substitution; local support values were 1000 rapid bootstrap replications. Phylogenetic trees were visualized using iTOL v.6.3.1 [31]. The whole genome-based comparisons were performed as follows: pairwise ANI values were calculated using the *pyani* module v.0.2.8 [32] with ANIb method; AAI values – using AAI matrix calculator [33] with Diamond program [34]. The Percentage of Conserved Proteins (POCP) values were calculated using the following script: (https://github.com/hoelzer/pocp).

## Results

### *Morphological and chemotaxonomy features*

During growth on solid medium at pH 9.5 with starch and yeast extract, strain ACPA39$^T$ formed mostly substrate mycelium varying in color from grayish to brown, depending on age. In old cultures, copious number of conidia were branching off the vegetative mycelium, first in a common sporangium (as was obvious from the thin-section electron microscopy), single or in rosette-like clusters, and in a later growth stage fragmenting into two to four angular sporangiaspores with a slightly rough surface. The sporangia were also formed in liquid cultures (Fig. 1). No diffusible pigment was produced on any of the solid media tested.

The amino acids diagnostic for peptidoglycan detected in the cell wall of ACPA39$^T$ included diamino acid meso-DAP (peptidoglycan type A1γ') and also glycine, glutamate and alanine in approximately equal proportions (1 : 1 : 1 : 1 : 0.7). The cell-wall sugars included galactose and xylose. The identified MPL included diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylinositol mannoside (PIM). Those MPL species are also present in most of the *Micromonosporaceae* genera (Table 1). In addition, unidentified MPL were also present, including four phospho-, four glyco- and two aminoglycolipids (Supplementary Fig. S1). The dominant PLFA were *anteiso*-$C_{17:0}$ and *iso*-$C_{16:0}$, *iso*-$C_{17:0}$, 10 Me-$C_{18:0}$ and $C_{18:1}\omega9$ (45, 15, 7, 6.7 and 5.6% from the total, respectively). The dominant respiratory menaquinone was identified as MK-10($H_4$) (Supplementary Fig. S2), which is often present in various actinobacteria and members of the *Micromonosporaceae* in particular [35,36], while the absence of MK-9 species is not as common (Table 1).

### *Genome sequencing*

The genome of ACP39$^T$ was sequenced and assembled until completeness in the DSMZ. The final genome sequence is available in the GenBank under the numbers CP070499.1. Raw sequencing read data were deposited at NCBI SRA (698925). The genome size is 5.7 Mbp consisting of 5,177 genes encoding for 5,037 proteins. There are two *rrn* operons with identical sequences.

### *Phylogenetic analysis*

According to 16S rRNA gene sequence BLAST search, strain ACPA39$^T$ is a member of the *Actinobacteria,* family *Micromonosporaceae* [36,37] with the highest 16S rRNA gene sequence identity arond 94% with the type strain CNH643$^T$ of marine species *Salinispora arenicola* and *Actinoplanes subglobosus*. 16S rRNA gene sequence-based phylogenetic analysis showed that ACPA39$^T$ forms a separate branch within the family *Micromonosporaceae* but several key nodes are not significantly supported. A search for more closely related 16S rRNA gene sequences among the uncultured database identified a few clones with sequence identities 97–98% to ACPA39$^T$, indicating that they might belong to the same new genus as ACPA39$^T$. In particular, two clones from the saline alkaline lakes Texcoco and Alchichica in Mexico (TX1A 103 and Alchichica AQ22 1B 75) were the closest to ACPA39$^T$ (Supplementary Fig. S3).

A more advanced phylogenomic approach based on 120 conserved protein markers (bac120 set) yielded a tree with a more stable branching order and revealed that ACPA39$^T$ forms a novel genus-level lineage clustering in an independent group with the genus *Pilimelia* as the closest relative. This branch is positioned between two large monophyletic clusters, one with the genus *Catellatospora*, together with its closely related genera and the other with the multispecies genus *Actinoplanes* (Fig. 2). A search of the metagenomic databases did not identify any apparent uncultured members belonging to the ACPA39$^T$ lineage, most probably because saline soda soils are not yet represented in such databases (Fig. 2).

The calculated AAI values between ACPA39$^T$ and members of the related genera from the *Micromonosporaceae* with available genome sequences ranged from 55.8% to 62.8% (Table S1a), which is below 65%, the threshold proposed for different genera [38]. The POCP values were 40.9–51.5% (Table S1b); thus maximum PCOP between the strain ACPA39$^T$ and relatives was slightly higher than the genus-level boundary – 50% [39]. But there are many works revealing that strict boundary of 50% was not appropriate for genera delineation within some taxa [40–45]. The ANI values (72.4–75.3%) were close to the median and mean intergeneric values (75.5% and 75.7%, respectively) calculated for representatives of this family (Table S1c).

Moreover, numerous probable misclassifications in the family that would need to be addressed in the future were uncovered using the phylogenomic approach. These include the genera *Couchioplanes*, *Krasilnikovia*, *Mangrovihabitans* and *Pseudosporangium,* which most probably belong to the genus *Actinoplanes*; the genera *Allorhizocola* and *Rhizocola* – as species of the genus *Catelliglobosispora;* the genera *Plantactinospora*, *Polymorphospora* and *Salinispora* - as species of the type genus *Micromonospora.*

### *Growth physiology and hydrolytic potential*

Growth at different pH values and Na-carbonate concentrations was assessed on starch/yeast extract medium at 0.6 M total $Na^+$ (for variable pH) and at pH 10 (for different salinities). The strain grew optimally at low salt (0.1–0.3 M $Na^+$) but with the high maximum tolerance of up to 3 M $Na^+$ (Fig. 3a). ACPA39$^T$ started to grow actively within a pH range from 7.5 to 10.3 (these are the final pH values) and had a moderately alkaline optimum at pH 8.2–9.0 (Fig. 3b), which qualifies this organism as an obligate alkaliphile. The genome search identified a gene locus coding for a single (multisubunit) sodium/proton antiporter MrpA-B/CDEFG as prerequisite for maintaining a near-neutral pH inside the cells of alkaliphiles [46]. *Salinispora arenicola* is the only member of the family *Micromonosporaceae* reported as able to grow at extremely high pH values up to 12, despite being isolated from a pH-neutral habitat [12]. However, as mentioned in the introduction,

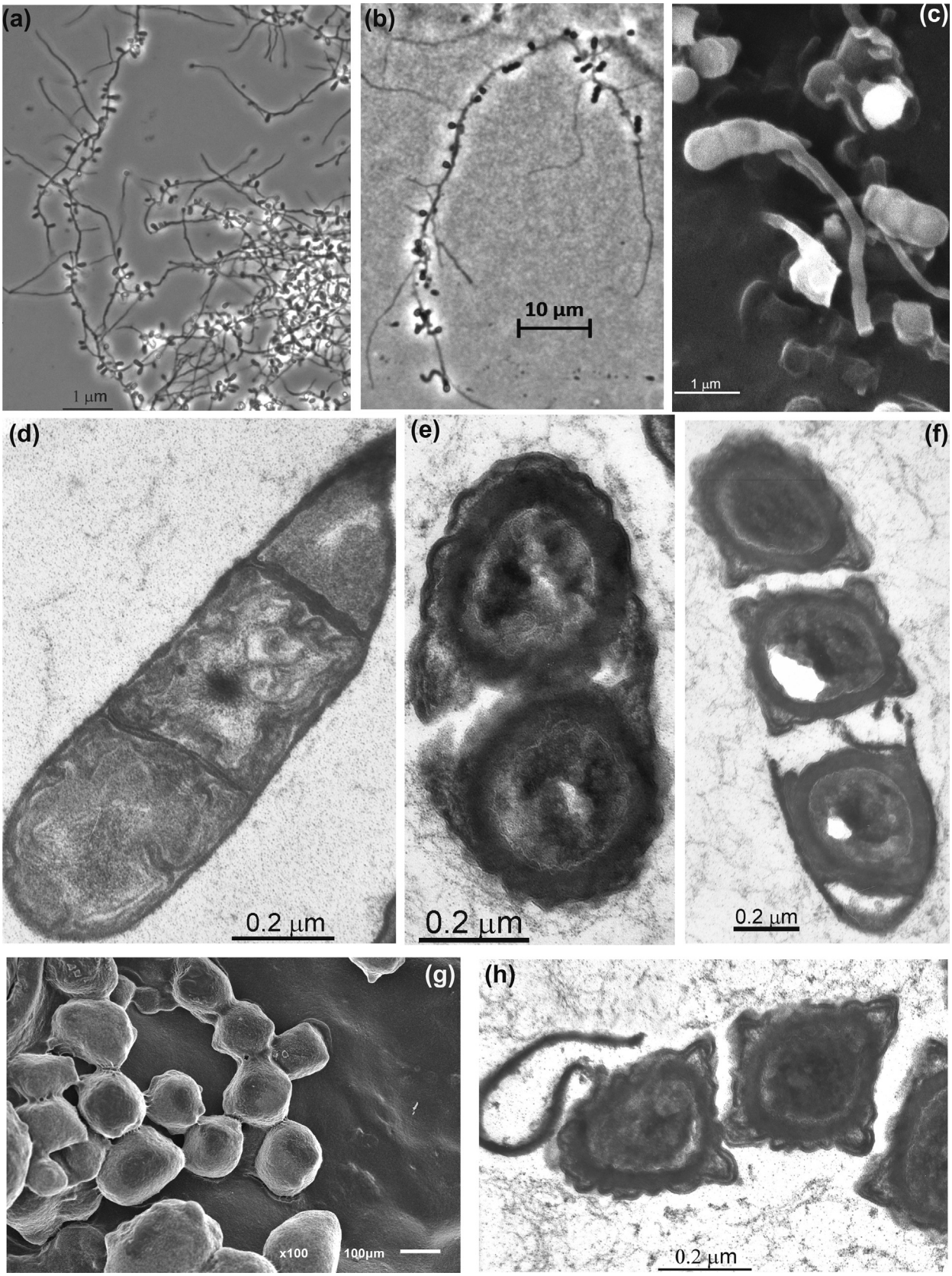



**Fig. 1.** Cell morphology of strain ACPA39$^T$ growing on starch/yeast extract medium at pH 10 and 0.6 M total $Na^+$. (a–b), phase contrast microscopy showing formation of sporangia in young cultures (a) and defragmentation of sporangia into separate spores in old culture (b); (c, g), scanning electron microphotographs showing sporangia and separate spores; (d–f, h), thin section electron microscopy showing a sporangium with spores (d), release of spores from sporangium (e) and separated mature exospores (h).

**Table 1**
Phenotypic comparison of haloalkaliphilic strain ACPA39$^T$ from soda soils with the phylogenetically closely related genera in the family *Micromonosporaceae* (data from [12,36,47–53].

| Property | ***"Natronosporangium"*** | *Pilimelia* | *Catellatospora* | *Catelliglobosispora* | *Hamadaea* | *Actinoplanes* | *Salinispora* |
|---|---|---|---|---|---|---|---|
| Number of species with validly published names | 1 | 3 | 8 | 1 | 2 | 50 | 9 |
| Aerial mycelium | Gray to brown, poor | Yellow, poor | No | No | No | No or rudimentary | No |
| Substrate mycelium | Abundant, branching, brown | Abundant, yellow-orange | Abundant, yellow | Abundant, yellow | Abundant, yellow | Abundant, color variable with orange as dominant | Abundant, branching, from orange to black |
| Exospores | 2–4 in sporangia, angular, 0.2–0.6 μm, slightly rough | Sporangia with multiple spores, motile | Chains without sporangia | Chains without sporangia | Chains without sporangia | Multiple globular in sporangia, motile; 1.1–1.6 μm diameter | In grape-like bunches, round, 0.8–3.8 μm, smooth |
| Peptidoglycan diagnostic amino acids | *meso*-DAP: glutamate : glycine: alanine (1 : 1 : 1 : 0.7) | *meso*-DAP; glycine | *meso*-DAP; 3-OH-meso-DAP; glycine | *meso*-DAP; glycine | *meso*-DAP; 3-OH-meso-DAP; glycine | *meso*-DAP, glycine; 3-OH-meso-DAP (V) | *meso*-DAP; glycine |
| Cell wall sugars | Galactose, xylose | Arabinose, xylose | galactose, xylose, arabinose, ribose, glucose, mannose; rhamnose (V) | galactose, xylose, arabinose, glucose, mannose, rhamnose | galactose, xylose, arabinose, ribose, glucose, mannose; rhamnose | Xylose; galactose, arabinose (V) | galactose, xylose; arabinose, ribose, glucose (V) |
| Identified polar lipids | PG, DPG, PE, PI | PE, PI | DPG, PE, PI; PIM (V) | | DPG, PE, PI, PIM; PME(V) | PE; DPG, PG, PI, PIM (V) | DPG, PE, PI; PME; PG and PIM (V) |
| Dominant menaquinons | 10($H_4$) | 9($H_{2,}H_4$) | 10($H_6$;$H_8$); 9($H_4$; $H_6$) | 10($H_4$) | 9($H_6$) | 9($H_4$); 9($H_6$) (V) | 9($H_4$) |
| Dominant PLFA (in order of abundance) | *anteiso*-$C_{17:0}$, *iso*-$C_{16:0}$ | $C_{15:0}$; *iso*-$C_{15:0}$; $C_{17:0}$ | $C_{17:0}$, $C_{17:1}\omega 8c$, *iso*-$C_{15:0}$, *iso*-$C_{16:0}$ | $C_{17:0}$, *iso*-$C_{15:0}$, *iso*-$C_{16:0}$ | *iso*-$C_{15:0}$, *anteiso*-$C_{17:0}$; *iso*-$C_{16:0}$; *anteiso*-$C_{15:0}$ | *iso*-$C_{15:0}$, *iso*-$C_{16:0}$, *anteiso*-$C_{17:0}$ | *iso*-$C_{16:0}$, *iso*-$C_{15:0}$, |
| Polysaccharide as growth substrates | starch, dextrin, cyclodextrin, glycogen, pullulan, inulin, amorphous cellulose, laminarin, lichenan, barley glucan, xylane, pachyman, beta-mannan, glucomannan, xyloglycan, arabinoxylan | - | Starch (V) | Starch | Starch | Starch/dextrin, chitin, pectin (multiple); cellulose (few) | Starch, chitin |
| Sugar utilized for growth | Glucose, galactose, rhamnose, dextrose, arabinose, maltose, sucrose, cellobiose, trehalose, melizitose, raffinose, lactose | - | Glucose, galactose, arabinose; maltose, melibiose, ribose, rhamnose, lactose, mannose, sucrose, trehalose, xylose, mannitol (all V) | Glucose, galactose, arabinose; maltose, melibiose, ribose, rhamnose, lactose, mannose, sucrose, trehalose, xylose, mannitol (V) | Glucose, arabinose melibiose, xylose, rhamnose, mannose, sucrose, trehalose; galactose, ribose, maltose, raffinose, fructose, cellobiose (V) | Glucose, galactose, fructose, arabinose, mannose, maltose, melibiose, xylose, sucrose, trehalose; lactose, melizitose, raffinose, rhamnose, ribose, cellobiose sorbitol, inositol, mannitol (V) | Cellobiose, melezitose, lactose |
| Proteolytic activity | Casein, gelatin | Keratine, casein, gelatin (V) | Gelatin (V) | Casein, gelatine | Gelatin (V) | Casein, gelatine | Casein, elastin, gelatin: (all V) |
| Lipase activity | - (olive oil) | NR | NR | NR | +Tween80 | +Tween80 (NR in most species) | V (Tween80) |
| Oxidase/catalase | w/+ | NR/NR | NR/+ | +/+ | +/+ | V/V | V/+ |
| pH range (optimum) | 7.5–10.3 (8.5) | 5.0–7.8 (6.5–7.5) | 6–9 | 6–9 | 6–9 | 6–10* | 6–12* (7.5–9.5*) |

(*continued on next page*)

**Table 1** (*continued*)

| Property | ***"Natronosporangium"*** | *Pilimelia* | *Catellatospora* | *Catelliglobosispora* | *Hamadaea* | *Actinoplanes* | *Salinispora* |
|---|---|---|---|---|---|---|---|
| Salt range (optimum), M of total $Na^+$ | 0.1–3.0 (0.1–0.3) | nonhalophilic | nonhalophilic | nonhalophilic | nonhalophilic | Nonhalophilic; max. toleance – 0.5 M NaCl | 0–1.3 (0.1–0.3) |
| Temperature range (optimum) | 20–40 (25) | 10–38 (20–30) | 15–35 (30) | 20–37 | 4–40 (30–37) | 10–35 (23–28) | 10–30 (20–28) |
| G + C % | 70.2 (genome) | 70.1 (Tm) | 70.7–71.4 (HPLC) | 70.0 (Tm) | 69.4–70.6 (HPLC) | 69–73 (Tm) | 69.1–70 (genome) |
| Habitat | Soda solonchak soil | Moderately acidic and neutral soils | | | | | Marine |

*final pH was not determined, the results were obtained on agar unbuffered media; **the range is not given; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PME, phatidylmethylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositolmannosyl; (V) - variable property; NR - not reported; w, weak positive.

it is highly improbable: the pH profiling was done on a solid medium, which in no way can provide stable pH conditions; the final pH was not measured; the medium was not properly buffered and, anyhow, there are no buffering systems to maintain such pH values. Furthermore, the highest reported pH maximum for bacteria measured in a pH-controlled chemostate culture is 11.3.

Since strain ACPA39$^T$ was obtained from bioprospecting polyhydrolytic haloalkaliphiles [13], its hydrolytic potential was extensively characterized by three different approaches. The cultivation-based and activity measurement-based approaches showed coherent, but slightly variable results, summarized in Table 2 and Supplementary Fig. S4. The growth experiments demonstrated that ACPA39$^T$ could utilize as growth substrate various alpha-glucans, including starch, dextrin, glycogen, cyclodextrin, pullulan and even broader range of beta-glucans, including amorphous cellulose, barley glucan, laminarin, lichenan, xylan, xyloglucan, arabinoxylan, pachyman, beta-mannan, gluco- and galactomannans, inulin. Most of them are consistent with the activity tests on plates and against dye-conjugated polysaccharides. The genome search identified a large gene inventory of glycosylhydrolases, polysaccharide lyases and carboxylesterase family enzymes (GH, PL and CE) involved in various polysaccharide hydrolysis (Table 2; Supplementary Table S2). However, in some cases, as for chitin and pectin, for example, the genome content was not supported by the negative results in growth and plate activity tests. Overall, the results demonstrated a strong polydrolytic potential of the novel haloalkaliphilic actinobacterium. Similar genome analysis for a presence of genes encoding the polysaccharide-specific enzymes have recently been performed in multiple available genomes of the genus *Salinispora* and yielded comparable results indicating that most of the species of these marine actinomycetes should be polyhydrolytic [47]. However, whether these actinobacteria are actually able to grow with those polysaccharides, apart from the confirmed cellulose and chitin, remains to be tested.

Among the soluble sugars, growth was observed with glucose, galactose, rhamnose, dextrose, arabinose, maltose, sucrose, trehalose, melezitose, raffinose, lactose and cellobiose. The sugars tested, but not utilized included mannose, sorbose, glucuronic and galacturonic acids, xylose, ribose and sugar alcohols mannitol and inositol.

ACPA39$^T$ showed a strong positive reaction for catalase (test with 3% $H_2O_2$) and a weak positive oxidase activity (colony assay with 0.1% tetramethyl-p-phenylendiamine HCl). Genome search identified a presence of two oxidases in ACPA39$^T$: a *bd* quinol oxidase, often present in low oxygen-tolerant organisms, and a 4-subunit heme-copper superfamily cytochrome *c* oxidase $aa_3$. But the latter was not encoded (as is usually the case) in a single genomic locus.

Ammonium and amino acids (but not nitrate or urea) can be used as the N-source. Investigation of antimicrobial activity of the isolate using disk-plate technique showed negative results with haloalkaliphilic *Bacillus horikoshii* and *Halomonas* sp. (isolated from soda lakes) and with freshwater bacteria (*E. coli*, *Pseudomonas* sp., *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus*).

### *Osmoprotection mechanism based on the genomic analysis*

Since the nature of organic osmolyte was not investigated by a direct chemical analysis, an attempt was made to find the known pathways in the genome. The analysis clearly indicated that ACPA39$^T$ is using glycine betaine (GB) and related tertiary amines for osmoprotection, both importing them from environment and also by synthesizing GB *de novo*. Moreover, the genome contains a set of genes encoding a pathway of GB mineralization allowing to reutilize it as the energy and C source. The encoded import

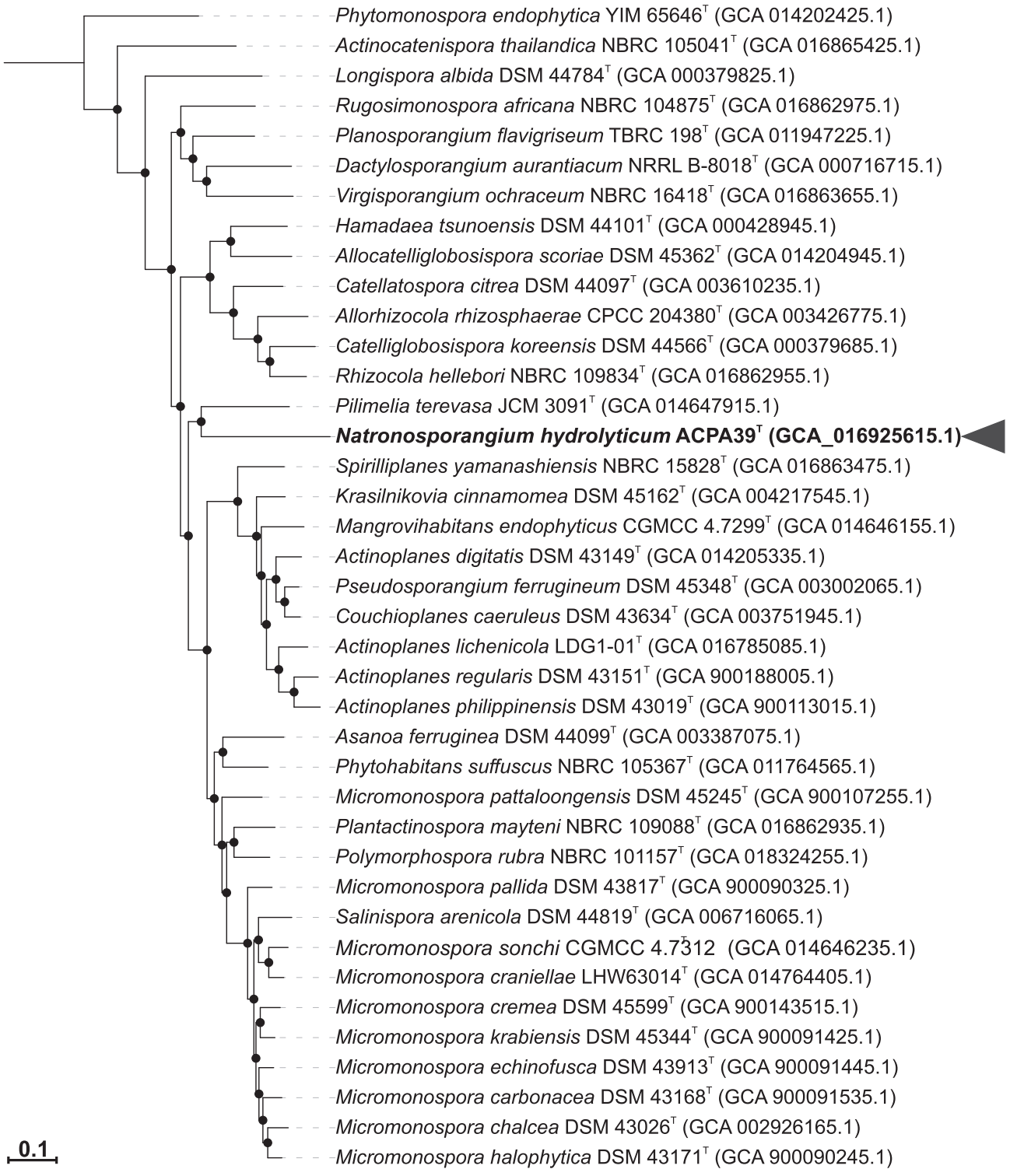



**Fig. 2.** Maximum likelihood phylogenetic tree based on concatenated alignment of 120 conserved single copy bacterial proteins and showing position of strain ACPA39$^T$ (in bold) within the family *Micromonosporaceae*. The branch lengths correspond to the number of substitutions per site with corrections associated with the models. The black circles at nodes indicate that the percentage of corresponding support values was above 50. *Rubrobacter radiotolerans* P1$^T$ was used as an outgroup (not shown).

transporters include two ABC Opu transporters, one specific for GB (Opu AA/AB/AC) and another one with a broader tertiary amines specificity for GB/choline/carnithine import (OpuCA/CB/CD). In addition, two single-subunit GB transporters are present - OpuD and a sodium/GB simporter BetL. The imported tertiary amines can, apparently be mineralized to glycine with the released methyl groups being oxidized to $CO_2$ with the following encoded proteins identified: (1) Oxidative conversion of choline (trimethylethanolamine) into GB via choline dehydrogenase BetA and betaine aldehyde dehydrogenase BetB (2 copies); (2) 3-step demethylation of GB via dimethylglycine and sarcosine to glycine by a GB-homocystein methyltransferase, forming dimethylglycine and methionine, followed by dimethylglycine and sarcosine demethylation with the respective homocystein-dependent methyltransferases. Sarcosine can also be oxidized to glycine by sarcosine oxidase SoxAB. The released methyl groups are oxidized to $CO_2$

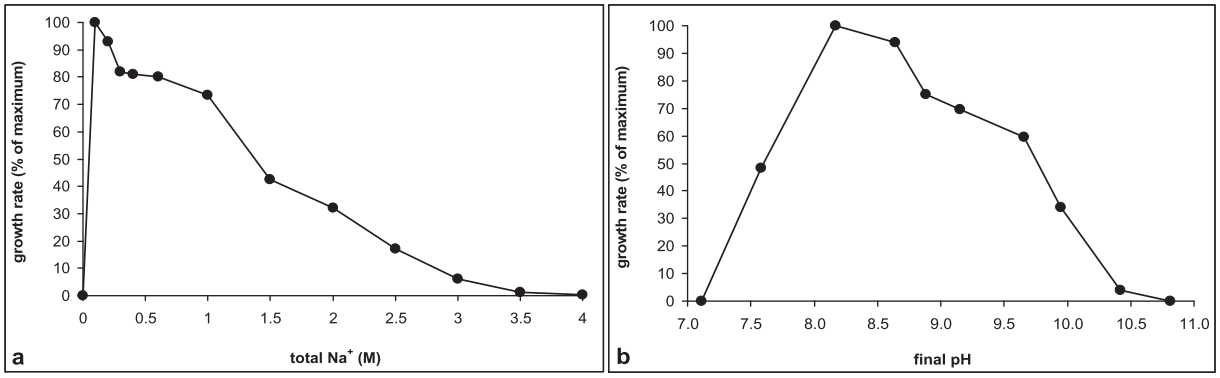



**Fig. 3.** Influence of total $Na^+$ (as carbonates) at pH 10 (a) and pH (final values are shown) at 0.6 M total $Na^+$ (b) on growth of ACPA22$^T$ and ACPA33$^T$ on starch/yeast extract medium. The data are mean results from duplicate cultures.

via the $C_1$ pathway, including: 5,10-CH3-THF dehydrogenase MetH > methenyltetrahydrofolate dehydrogenase/cyclohydrolase FolD > 5-formyltetrahydrofolate cyclo-ligase Mhs > formate dehydrogenase FdoGHI. The catalytic subunits of the latter, FdoGH, are located in the cytoplasme, while FdoI is a diheme b membrane subunit, which is unusual for the membrane-bound formate dehydrogenases. A potential pathway of the de novo GB biosynthesis from glycine includes three sequential methylation steps with homocystein-dependent methyl transferases: glycine-sarcosine methyltransefarase BmsA (2 copies) and glycine/sarcosine/dimethylglycine N-methyltransferase, a fusion version of the two methyltransferase homologues of *Halorhodospira halophila*, which can form GB directly from glycine. A gene encoding the ectoine (a common organic osmolyte) synthase EctC is also present in the genome, but the other two subunits (EctAB) necessary for the ectoine biosynthesis are not encoded. Apart from organic osmo-

**Table 2**
Polymer-degrading potential of strain ACPA39$^T$ in growth experiments, activity tests with colony plate assay and incubations with dye-conjugated polymers and the genome-based prediction GH, glycosyl hydrolases; CE – carboxylesterases; PL – polysaccharide lyases; nd, not determined.

| Polymer | Growth | Activity | CAZymes genes found in genome | |
|---|---|---|---|---|
| | | | CAZymes family | Possible function |
| **α-linked glucans** | | | | |
| trehalose | + | + | GH15 | trehalase |
| | | | 2xGH65 | trehalose phosphorylase |
| amylose, dextrin, cyclodextrin, pullulan glycogen | + | + | 6xGH13; GH133 | glycosidases with activity against alpha-linked glucans |
| dextran (*Leuconostoc*) | - | - | - | dextranase |
| **β-linked polysaccharides** | | | | |
| carboxymethyl cellulose | - | + | 2x GH5; GH6; GH9 | endo-beta-1,4-glucanase |
| amorphous cellulose | + (slow) | + | 2xGH6; GH48 | exoglucanase |
| | | | GH94 | cellobiose phosphorylase |
| | | | 3xGH1; 4xGH3 | beta-glucosidase |
| | | | 3xAA10 | lytic cellulose monooxygenase |
| barley beta-glucan | + | + | 2xGH16 | beta-1,3–1,4 glucanase |
| laminarin | + | + | 3xGH1; 4xGH3 | beta-glucosidase |
| lichenan | + | nd | | |
| pachyman | + | + | | |
| xylan (beech/birch) | + | + | 7xGH10; 2xGH11; 2xGH141 | endo-beta-1,4-xylanase |
| | | | 2xGH39; GH43 | beta-xylosidase |
| | | | 3xAA7 | xylooligosaccharide oxidase |
| | | | 3xCE1 | acetylxylan esterase |
| amorphous chitin | - | nd | 2xGH18, GH23 | endochitinase |
| | | | GH3 | beta-hexosaminidase |
| | | | AA10 | lytic chitin monooxygenase |
| | | | CE14 | diacetylchitobiose deacetylase |
| **Other polysaccharides including heteropolysaccharides** | | | | |
| pectin (apple, citrus) | - | - | 3xPL1 | pectate lyase |
| rhamnogalacturonan | nd | + | PL26 | rhamnogalacturonan exolyase |
| alginate | - | nd | - | |
| beta-mannan | + | + | GH5; GH26 | endo-beta-1,4-mannosidase |
| galactomannan | + | + | 2x GH5; GH6; GH9 | endo-beta-1,4-glucanase |
| glucomannan | + | nd | GH130 | beta-1,4-mannooligosaccharide phosphorylase |
| | | | GH2 | beta-mannosidase |
| | | | GH27; 2xGH36 | alpha-galactosidase |
| galactan | - | - | GH2; 2xGH42 | beta-galactosidase |
| xyloglucan | + | nd | 2x GH5; GH6; GH9 | endo-beta-1,4-glucanase |
| | | | 3xGH1; 4xGH3 | beta-glucosidase |
| arabinan | - | - | GH43; GH51; GH62 | alpha-L-arabinosidase |
| arabinoxylan | + | + | 7xGH10; 2xGH11; 2xGH141 | endo-beta-1,4-xylanase |
| | | | GH43; GH51; GH62 | alpha-L-arabinosidase |
| arabinogalactan | - | nd | - | |
| inulin | + | nd | GH32 | inulinase |

lytes, there is also a set of genes coding for potassium uptake transporters usually active in salt-tolerant microorganisms, including potassium uptake proteins TrkHA, and potassium/proton antiporters KefB and NhaP.

Overall, on the basis of the results of phylogenomic analysis and distinct phenotypic features, we propose to classify the actinobacterium isolate ACPA39$^T$ from a soda solonchak soil into a new genus and species *Natronosporangium hydrolyticum* gen. nov. sp. nov. Its comparative properties with the closely related genera of the *Micromonosporaceae* family is presented in Table 1. The common features shared with the related genera include absence of aerial mycelium; meso-DAP and lysine in the peptidoglycan; xylose as the whole cell sugar; diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol as the dominant membrane phospholipids; *iso*-C16:0 among the dominant PLFA and utilization of starch as growth substrate. The major difference is that ACPA39$^T$ is the only obligate alkaliphilic member of this family described so far originating from a permanently haloalkaline habitat and only a second genus (in addition to the marine genus *Salinispora*) with a moderate to high salt tolerance. Furthermore, lack of the MK-9 respiratory lipoquinones, a major domination of the *anteiso*-17:0 in the PLFA profile and the polar lipids phatidylglycerol and diphosphatidylglycerol differentiate ACPA39$^T$ from mostly related genus *Pilimelia*. Finally, ACPA39$^T$ has a remarkably broad range of polysaccharide-hydrolyzing enzymes encoded in the genome whose activity (at least partially) is confirmed in growth and activity experiments. Although this is not uncommon for actinobacteria in general, this organism can clearly be characterized as a polyhydrolytic.

The overall phylogenomic and phenotypic characteristics obtained for strain ACPA39 suggest that it can be classified in a new genus and species within the current margins of actinobacterial family *Micromonosporacea* for which we suggest the name *Natronosporangium hydrolyticum* ge. nov., sp. nov. The results of phylogenomic analysis also indicated the necessity for a more deep phylogenetic reevaluation of the *Micromonosporaceae* family structure using the advances of the whole genome sequencing.

The new genus and species protologues are presented in Table 3.

**Table 3**
*Natronosporangium hydrolyticum* gen. nov., sp. nov.: protologue.

| Parameter | Genus: *Natronosporangium* gen. nov. | Species: *Natronosporangium hydrolyticum* sp. nov. |
|---|---|---|
| Date created | 2021-09-09 | 2021-09-09 |
| Species name | | *hydrolyticum* |
| Genus name | *Natronosporangium* | |
| Taxon status | gen. nov. | sp. nov. |
| Etymology | Na.tro.no.spo.ran'gi.um. Gr. n. *natron*, arbitrarily derived from the Arabic n. *natrun* or *natron*, soda; Gr. fam. n. *spora* a seed, a spore; Gr. neut. n. *angeion* (Latin transliteration *angium*), vessel; N.L. neut. n. *Natronosporangium* a soda-loving organism with finger-shaped, spore-containing vessels (sporangia) | hy.dro.ly'ti.cum. Gr.neut. n. *hydor,* water; Gr. masc. adj. *lytikos,* dissolving, splitting; N.L. neut. adj. *hydrolyticum,* splitting with [by] water, referring to the hydrolytic activity of the bacterium |
| Description of the new taxon | Aerobic, saccharolytic, Gram-positive and filamentous actinomycetes forming mostly a well developed branched substrate mycelium with sporangia containing 2–4 spores. Peptidoglycan contains meso-DAP as the diamino acid. The membrane phospholipids include The only respiratory quinon is MK-10($H_4$) and the major PLFA is a17:0. Main phenotypic features include high pH-salt tolerance and broad spectrum of hydrolyzed polysaccharides. A member of the family *Micromonosporaceae*. The type species is *Natronosporangium hydrolyticum*. The closest phylogenetic relatives are the genera *Pilimelia* and *Catellatospora*. | The aerial micelium is not developed. The substrate mycelium is highly branched, brownish in old cultures due to a formation of 2–4 spores encased in common sporangia, arranged either single or in rosettes aggregates. The spores are 0.2–0.6 μm, angular and slightly rough. Soluble pigments are not produced. The cells wall aminoacids include meso-DAP, glutamate, glycine and alanine in approximately equal proportion. The cell wall sugars are represented by arabinose, galactose and xylose. The identified membrane phospholipids include diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylglycerol. The unidentified lipids include 4 phospho-, 4 glyco- and 2 aminophospho -lipids. The dominant PLFA include (in order of abundance) *anteiso*-$C_{17:0}$, *iso*-$C_{16:0}$, *iso*-$C_{17:0}$, 10-Me-$C_{18:0}$ and *anteiso*-$C_{17:1}\omega8$. The respiratory menaquinons include MK-10($H_4$) (80%) and MK-11($H_4$) (20%). The following sugars are utilized as substrate : glucose, galactose, rhamnose, dextrose, arabinose, maltose, sucrose, trehalose, melezitose, raffinose and lactose. The following polysaccharides are hydrolyzed: amylose, dextrin, pullulan, glycogen, pachyman, laminarin, lichenan, amorphous cellulose, carboxymethyl - and carboxyethyl-cellolose, Barley beta-glucan, xylane, pachyman, beta-mannan, glycomannan, xyloglycan, arabinoxylan. Also hydrolyze casein and gelatin, but not olive oil. Ammonium is utilized as N-source. Oxidase is weak positive, catalase is positive. Antibiotic activity is absent. Salt-tolerant, with a range of total $Na^+$ for growth from 0.1 to 3.0 M (optimum at 0.1–0.3 M) and obligately alkaliphilic, with a pH range for growth from 7.5 to 10.5 (optimum at 8.5). At pH 10, the growth temperature range is 20–40 °C (optimum at 25). The G + C content of the DNA is 70.2% (genome). The type strain (ACPA39$^T$ = DSM 106523 $^T$ = VKM 2772 $^T$) was isolated from a soda solonchak soil in the southwestern Siberia (Russia). The 16S rRNA gene sequence and the genome sequence accession numbers in the GenBank are KY775645 and CP070499.1, respectively. |

(*continued on next page*)

**Table 3** (*continued*)

| Parameter | Genus: *Natronosporangium* gen. nov. | Species: *Natronosporangium hydrolyticum* sp. nov. |
|---|---|---|
| Authors (AUT) | Dimitry Y. Sorokin, Alexander I. Elcheninov, Tatiana V. Khijniak, Alicia P. Zaharycheva, Richard L. Hahnke, Olga V. Boueva, Elena V. Ariskina, Boyke Bunk, Lyudmila I. Evtushenko, Ilya V. Kublanov | |
| Title (TITL) | *Natronosporangium hydrolyticum* gen. nov., sp. nov., haloalkaliphilic actinobacterium from a soda solonchak soil in Central Asia | |
| Journal (JOUR) | Systematic and Applied Microbiology | |
| Corresponding author (COAU) | Dimitry Y. Sorokin | |
| E-mail of corresponding author (EMAU) | d.sorokin@tudelft; soroc@inmi.ru | |
| Designation of the type strain (TYPE) | | **ACPA39** |
| Strain collection numbers (COLN) | | DSM 106523; VKM 2772 |
| 16S rRNA gene accession number (16 SR) | | KY775646 |
| Alternative house-keeping genes: gene [accession numbers] (HKGN) | | 120 bacterial single-copy conserved protein markers |
| Genome status (GSTA) | | Complete, circular chromosome (accession CP070499.1) |
| GC % (GGCM) | | 70.2 (genome) |
| Country of origin (COUN) | Russian Federation | Russian Federation |
| Region of origin (REGI) | | Novosibirsk region |
| Date of isolation (DATI) | | December 2004 |
| Source of isolation (SOUR) | Saline alkaline soils | Soda solonchak soil |
| Sampling dates (DATS) | | August 2003 |
| Geographic location (GEOL) | Southwestern Siberia | Southwestern Siberia, Barabinskaya Steppe |
| Latitude (LATI) | | 54.61 N |
| Longtitude (LONG) | | 76.89 E |
| Depth (DEPT) | | 0–0.1 m |
| Temperature of the sample (TEMS) | | 22 °C |
| pH of the sample (PHSA) | | 10.7 |
| Salinity of the sample (SALS) | | 62 g/kg |
| Number of strains in study (NSTR) | | 1 |
| Source of isolation of non-type strains (SAMP) | | – |
| Growth medium, incubation conditions (CULT) | Alkaline media based on sodium carbonates, pH from 9 to 10, salinity 0.3–3 M $Na^+$ | Alkaline media based on sodium carbonates containing 0.3–0.6 M total $Na^+$ with pH 9.5–10; incubation – 37 °C; starch as C, energy and yeast extract as N-source |
| Conditions of preservation (PRES) | Deep freezing in 15% glycerol (v/v) | Deep freezing in 15% glycerol (v/v) |
| Gram stain (GRAM) | Positive | Positive |
| Cell shape (CSHA) | Mycelial, exospores | Moderately branched mycelium; on solid media - substrate mycelium, darkening with ageing; forms sporangia with up to 3 linearly aligned nonmotile angular exospores with smooth surface |
| Cell size (CSZI) | | 0.4–6 μm in diameter |
| Motility (MOTY) | | nonmotile |
| Motility type (MOTK) | | - |
| Type of flagellation (TFLA) | | - |
| Sporulation (SPOR) | | Exospores in sporangia |
| Colony morphology (COLM) | | Light to dark grey, up to 2 mm; substrate mycelium only |
| Temperature range for growth (TEMR) | | 20–40 °C |
| Lowest temperature for growth (TEML) | | 20 °C |
| Highest temperature for growth(TEMH) | mesophilic | 40 |
| Optimal temperature for growth (TEMO) | >20 | 25 |
| Lowest pH for growth (PHLO) | >7.0 | 7.5 |
| Highest pH for growth (PHHI) | >9 | 10.3 |
| Optimum pH for growth (PHOP) | | 8.5 |
| pH category (PHCA) | Alkaliphilic | Obligate alkaliphile |
| Lowest salt concentration for growth (SALL) | | 0.1 M total $Na^+$ |
| Highest salt concentration for growth (SALH) | | 3.0 total $Na^+$ |
| Optimum salt concentration for growth (SALO) | | 0.1–0.3 total $Na^+$ |
| Other salts important for growth | Sodium carbonates | Sodium carbonates |
| Salinity category (SALC) | Halophilic | High salt-tolerant |
| Relation to oxygene (OREL) | Aerobic | Obligate aerobe |
| $O_2$ conditions for strain testing (OCON) | | Air |
| Carbon source used (class) (CSUC) | Carbohydrates | Carbohydrates, proteins |
| Specific compounds (CSUC) | Polysaccharides (alpha/beta-glucans) | Sugars: cellobiose. Polysaccharides: cyclodextrin, glycogen, pullulan, inulin, amorphous cellulose, laminarin, lichenan, barley glucan, xylane, pachyman, beta-mannan, glucomannan, xyloglycan, arabinoxylan. |
| Nitrogen source (NSOU) | | Ammonium, yeast extract |
| Terminal electron acceptor (ELAC) | $O_2$ | $O_2$ |
| Energy metabolism (EMET) | chemoorganoheterophic | chemoorganoheterophic |
| Phospholipids (PHOS) | | PG, DPG, PE, PI, PIM |
| Glycolipids (GLYC) | Glyco- and aminoglycolipids | 5 unidentified glycolipids |
| Respiratory quinons | MK-10 | MK-10(H4) |
| Habitat (HABT) | Saline alkaline soils | Soda solonchak soil, s-w Siberia |
| Extraordinary feautres (EXTR) | Polyhydrolytics | Utilize large number of alpha/beta-glucans for growth at haloalkaline conditions |

The 16S rRNA gene and whole genome sequences of strains ACPA39$^T$ have been deposited in the NCBI GenBank under the numbers KY775646 and CP070499.1, respectively. Raw read data were deposited at NCBI SRA (698925).

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2022.126307.

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