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ORIGINAL PAPER

Natronoflexus pectinivorans gen. nov. sp. nov., an obligately anaerobic and alkaliphilic fermentative member of *Bacteroidetes* from soda lakes

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Abstract Anaerobic enrichment with pectin at pH 10 and moderate salinity inoculated with sediments from soda lakes of the Kulunda Steppe (Altai, Russia) resulted in the isolation of a novel member of the *Bacteroidetes,* strain AP1^T. The cells are long, flexible, Gram-negative rods forming pink carotenoids. The isolate is an obligate anaerobe, fermenting various carbohydrates to acetate and succinate. It can hydrolyze and utilize pectin, xylan, starch, laminarin and pullulan as growth substrates. Growth is possible in a pH range from 8 to 10.5, with an optimum at pH 9.5, and at a salinity range from 0.1 to 2 M Na^+. Phylogenetic analysis based on 16S rRNA sequences placed the isolate into the phylum *Bacteroidetes* as a separate lineage within the family *Marinilabilaceae*. On the basis of distinct phenotype and phylogeny, the soda lake isolate AP1^T is proposed to be assigned in a new genus and species *Natronoflexus pectinivorans* (=DSM24179^T = UNIQEM U807^T).

Keywords *Natronoflexus pectinivorans* · Pectin · Haloalkaliphilic · Soda lakes

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Nucleotide sequence accession number: the GenBank/EMBL accession number of the 16S rRNA gene sequences of strain AP1^T is GQ922844.

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Introduction

Pectin, a natural polymer of partially methylated galacturonic acid, is an important component of plant biomass acting as a glue for the cellulose fibrils. It is degraded by pectinolytic microorganisms to monomers, which are then utilized as a growth substrate. The pectinolytic organisms produce an extracellular enzymatic complex with a general term pectinases, the principal component of which is represented by pectate lyases (Jayani et al. 2005). The pectin hydrolysis is an important process, both for natural habitats and for industrial processing of food and textile (Kashyap et al. 2001; Sarethy et al. 2011). Most of the known pectinolytic microorganisms grow optimally at acidic and neutral pH, while evidences for pectinolysis at high pH and/or high salt are scarce and were never specifically investigated with respect to carbon cycling in soda lakes. However, due to a potential for application in the food and textile industry, pectinases with high alkali tolerance were specifically looked for in nonsalt-tolerant aerobes, such as various bacilli (Hoondal et al. 2002). There is only single evidence in the literature of a soda lake anaerobic fermentative bacterium, which is reported to grow with

pectin—*Alkaliflexus imshenetskii*. This alkaliphile belongs to the phylum *Bacteroidetes* and was enriched from a low-salt soda lake with cellobiose as substrate. It is a member of an anaerobic consortium degrading cellulose (Zhilina et al. 2004).

In this study, we focused on the microbial degradation of pectin and related polymers in anoxic sediments of highly alkaline and saline soda lakes. One of the organisms dominating the anaerobic enrichments with pectin at moderate salinity is described here as a novel haloalkaliphilic member of the phylum *Bacteroidetes*.

Methods

Samples

Samples of the top 5-cm sediments from the soda lakes Tanatar-5 (51.35N/79.40E; pH = 10.15; salinity = 180 g l^{-1}; total alkalinity = 1.60 M) and Cock Soda Lake (52.06N/79.09E; pH = 10.3; salinity = 120 g l^{-1}; total alkalinity = 0.83 M), both in Kulunda Steppe (Altai, Russia), were taken during a field campaign in July 2009 and used to enrich haloalkaliphilic pectinolytics. The two sediments were mixed in equal proportions and used as inoculum.

Enrichment and cultivation of strain AP1

Enrichment and cultivation of haloalkaliphilic strain AP1 was performed at 28°C on a mineral medium containing sodium carbonate buffer (0.5 M Na^+) with pH 10, 0.1 M NaCl, and 0.5 g l^{-1} of K_2HPO_4. The medium was supplemented with 10 mg l^{-1} of yeast extract, 4 mM NH_4Cl, 1 mM $MgSO_4$, and 1 ml l^{-1} each of acidic trace metal solution and vitamin mix (Pfennig and Lippert 1966) after sterilization. Carbohydrates were added at concentrations 1 g l^{-1} from 10% (w/v) sterile stock solutions (sugars were filter-sterilized, and polymers were autoclaved at 110°C for 30 min). Aerobic cultures were maintained in 50-ml serum bottles with a rubber stopper containing 10 ml of medium. Anaerobic cultivation was performed either in 15-ml Hungate tubes with 10-ml medium, or in 50-ml serum bottles with 40-ml medium with argon in the gas phase. The tubes and bottles were closed with butyl rubber stoppers and made anoxic by 5 cycles of evacuation-argon flushing with a final addition of 1 mM HS^- as a reductant. The solid medium was prepared by 1:1 mixing of the complete liquid medium with pectin and 4% (w/v) washed agar at 50°C. The plates were incubated in closed jars under argon with an oxygen-consuming catalyzer (Oxoid). The pH dependence was examined at a Na^+ content of 0.6 M, using the following filter-sterilized mineral medium: for pH 6–8, 0.1 M HEPES and $NaCl/NaHCO_3$; for pH 8.5–11, a mixture of sodium bicarbonate/sodium carbonate containing 0.1 M NaCl. To study the influence of salt concentration on growth, sodium carbonate media at pH 10, containing 0.1 and 2.0 M of total Na^+, were mixed in different proportions.

Analyses

Growth was measured by the increase in OD_{600}. In case of cultures grown on pectic substrates, the solids were removed before the measurements by a brief low-speed centrifugation. Fermentation products were analyzed by HPLC anionic chromatography (BioRad, HPX-87-H column at 60°C, eluent 5 mM H_2SO_4 solution at 0.6 ml min^{-1}, UV and RI detectors) after neutralization of the supernatant. Phase contrast microphotographs were obtained with a Zeiss Axioplan Imaging 2 microscope (Göttingen, Germany). For electron microscopy, the cells were negatively contrasted with 1% (w/v) neutralized phosphotungstate. Polar lipids for fatty acid composition were extracted from 1 g of wet cell pellet with acidic methanol and the fatty acid methyl esters were analyzed by GC–MS according to Zhilina et al. (1997). For the total polar lipid analysis, the cells were extracted with chloroform–methanol (1:2, v/v) on ice bath twice. After centrifugation, three phases were obtained. The polar lipid fraction was resolved by two-dimensional TLC (Kieselgel 60, 10 × 10 cm, Merck) using chloroform–methanol–water (60:25:4) in the first direction, followed by chloroform–acetic acid–methanol–water (85:15:12:4) in the second direction. The plates were sprayed with various specific reagents for detection of different phospholipids (Kates 1972). The standards of phospholipids (Sigma, USA) were used for diagram disposition of phospholipids during comparative analysis. Carotenoids were extracted from wet cell pellet by a mixture of MeOH–acetone (7:3) and the spectrum was recorded in the visible region using diode-array spectrophotometer HP8453 (Hewlett Packard, Amsterdam, The Netherlands). Catalase activity was measured iodometrically according to Sumner and Dounce (1963).

The isolation of the DNA and determination of the G+C content of DNA was performed according to Marmur (1961) and Marmur and Doty (1962), respectively. For molecular analysis, the DNA was extracted from the cells using alkaline SDS lysis at 60°C and purified with the Wizard Preps Kit (Promega, USA). The nearly complete 16S rRNA gene was obtained using the general bacterial PCR primers 11f and 1492r (Lane 1991). The sequences were aligned with sequences from GenBank using CLUSTAL W and a phylogenetic tree was reconstructed using neighbor-joining algorithm in the TREECONW program package (van de Peer and de Wachter 1994).

Results and discussion

Enrichment and isolation of a pure culture of a pectinolytic alkaliphile

When apple pectin was used as a single substrate in enrichments from two soda lake sediments at pH 10, surprisingly, active growth was obtained only under anaerobic conditions. Most probably, the sediments were too reduced to support active aerobic communities. The enrichment was dominated by a morphotype represented by long thin flexible rods that was eventually isolated in pure culture by several rounds of serial dilutions. The purity of the isolate was verified by obtaining single colony morphotypes, homogenous morphology, and by 16S rRNA sequence analysis. The isolate was designated strain AP1^T.

Morphology and identification

The cells of AP1^T are long thin rods, 0.25–0.3 × 3–10 μm, bending and gliding when solid surfaces (e.g., agar, pectin particles) are present. The cell wall is of the Gram-negative type and the cells are covered by a slime-like layer (Fig. 1). In young cultures, the cells were mostly single and suspended, while in the old cultures they started to aggregate and rapidly lysed. No cyst-like round bodies appeared during cell lysis in old cultures. Colonies were mucoid, convex, up to 2 mm, and pink. The concentrated cell biomass was red, due to the presence of carotenoids (Supplementary Fig. 1).

Phylogenetic analysis based on 16S rRNA sequences placed AP1 into the phylum *Bacteroidetes* as a novel, well separated lineage within the family *Marinilabiliaceae* (Ludwig et al. 2008) with 90–91% sequence similarity to the representatives of the genera *Marinilabilia*, *Anaerophaga, Alkaliflexus,* and "Geofilum" (Fig. 2). One of the non-described xylanolytic isolates from soda Soap Lake (State, Washington), annotated as "Alkalitalea" in GenBank (HQ191474), is a closest relative of strain AP1^T (ca. 98% sequence similarity) and probably belongs to the same genus.

A comparison of the polar lipid fatty acids composition of strain AP1^T with its relatives from the family "*Marinilabiliacea*" demonstrated a general trait of domination of 2–3 isomers of C_{15} (Table 1). However, there are two complications that prevent more detailed comparison between the members of this family: firstly, the AP1 profile could be directly compared only with *Alkaliflexus*, since both were grown at high pH; secondly, the new profiles for *Alkaliflexus* and *Marinilabilia* appeared in a recent description of a novel member of the family (*Geofilum*) (Miyazaki et al. 2011), which differed substantially from the original profiles. It is difficult to judge which of these data are more trustworthy. We can only comment that in the *Geofilum* paper, neither the growth conditions nor the source of the reference strains are indicated.

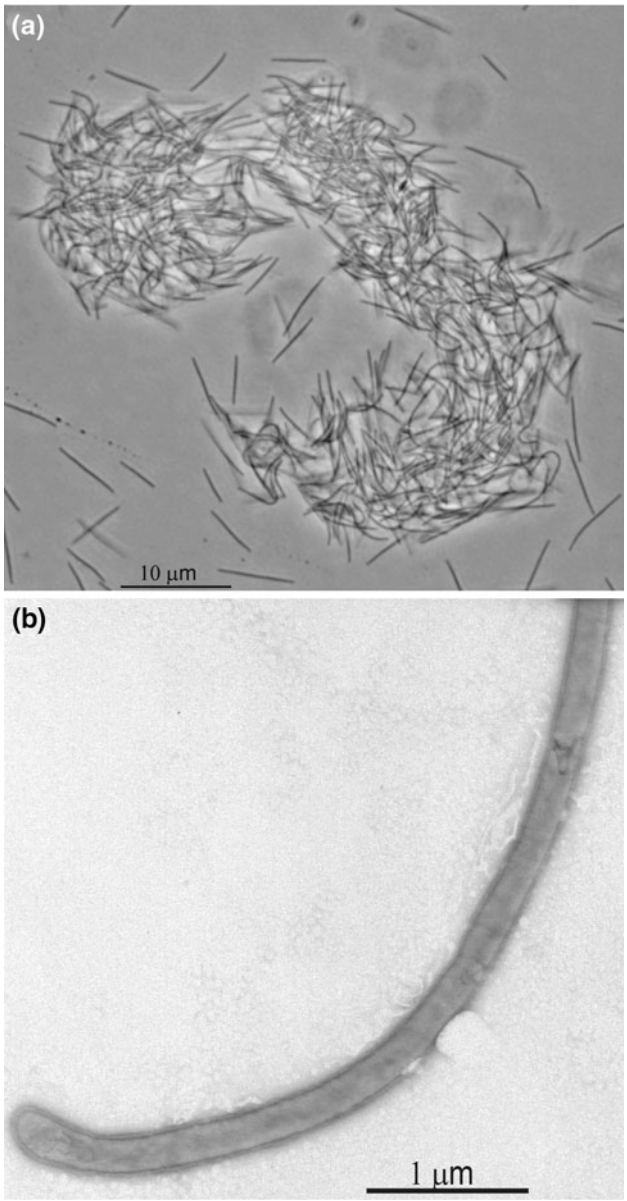

Fig. 1 Morphology of strain AP 1 grown with galacturonic acid at pH 10. **a** Phase contrast, **b** electron microphotograph of positively stained cells

The polar lipid profile of strain AP1 contained phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine (PS), two unidentified aminophospholipids, an aminolipid, and 4 different unidentified phospholipids (Supplementary Fig. 2).

Metabolic characteristics

Strain AP1 is an obligately anaerobic bacterium that grows by fermentation of a wide range of carbohydrates. The

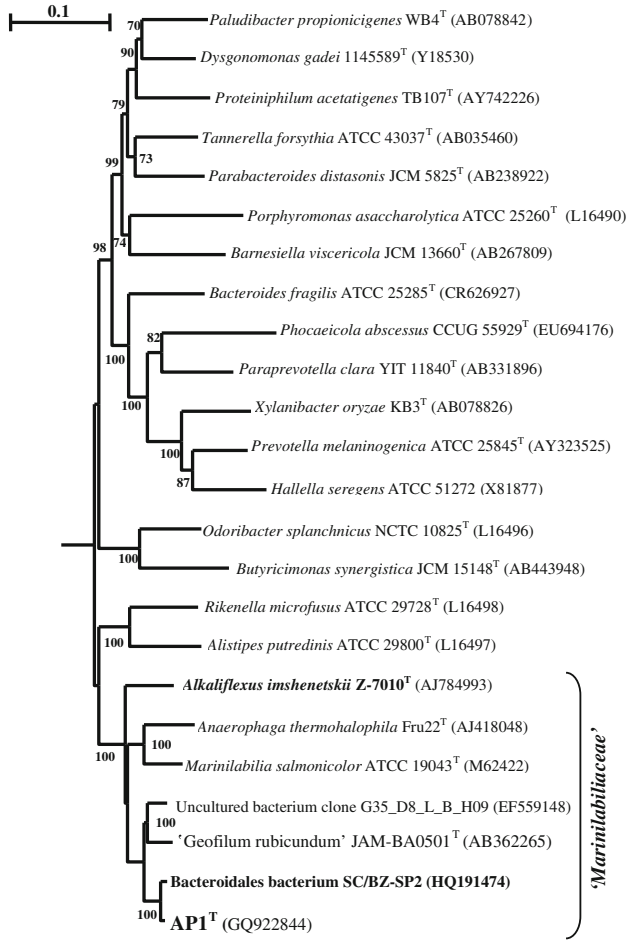

Fig. 2 Phylogenetic position of strain AP1 within the order *Bacteroidales* based on 16S rRNA gene sequence analysis. Tree topography and evolutionary distances are obtained by the neighbor-joining method with Jukes and Cantor distances. The *scale bar* represents 5 nucleotide changes per 100 nucleotides. The *numbers on the nodes* indicate bootstrap values above 70%

fastest growth was observed with the pectin monomer galacturonic acid and with xylose (μ_{max} at pH 10 and 30°C = 0.30 and 0.28 h^{-1}, respectively). The final products of galacturonic acid fermentations were acetate and succinate. Apart from the monomeric sugars, the bacterium was able to grow with polysaccharides, such as pectin, xylan, pullulan, laminarin, starch and glycogen, thus being a typical representative of the hydrolytic *Bacteroidetes* (Thomas et al. 2011). Of the variety of pectic substrates, it grew with polygalacturonate, polypectate and pectate. It did not grow in the presence of oxygen in the gas phase and growth was stimulated by the addition of a reductant. The maximal growth temperature with galacturonic acid as substrate was 41°C. Catalase activity was present, but at a much lower level than in the aerotolerant fermentative alkaliphile *Natronobacillus* (Sorokin et al. 2008).

Table 1 Comparison of the PLFA profiles of strain AP1 and its relatives

FA	**AP1^T**	*Alkaliflexus imshenetskii*		*Marinilabilia salmonicolor*		"*Geofilum rubicundum*"[c]
		1[a]	2[c]	1[b]	2[c]	
i13:0		1.4	0.5		19.3	
ai13:0		1.1			3.8	
i14:0	1.2	1.1	0.9		6.1	5.6
14:0					0.8	1.3
i15:1ω6	0.9					
i15:0	**19.4**	**29.4**	**37.2**	**11.3**	**39.0**	**23.7**
ai15:0	**16.9**	**11.6**	**33.2**	**31.7**	**17.6**	**33.4**
15:1		4.1				
15:0	**16.2**	**39.0**	1.3	**19.5**		
3OH-i15	1.7		1.3		6.8	4.8
3OH-ai15	0.5				0.6	1.8
15cyc	5.6					
i16:0	2.8	1.0	0.9	2.1		2.1
16:1ω5	0.7					
16:1ω7			0.7			4.0
16:1ω9			1.2			
16:0	0.6	1.0	6.6	**12.2**		2.8
i16:1	2.1					
OH-i16	3.6				0.9	7.0
OH-16:0	1.1		1.0			2.9
17:0				3.4		
17:1			1.9			
i17:0	0.6					
ai17:0			0.8	2.0		
ai17:1 ω7	3.2	3.5				
cyc17:0	3.8	4.0				
3OH-17:0	2.0					
3OH-i17:0	**11.0**		3.1	3.3	0.8	3.5
3OH-ai17:0	4.4		1.8			3.1
18:0			3.4	4.6		
18:1				5.4	5.4	
18:2				4.6		1.2
18:1ω7						2.3

The dominant FA are in bold. Only the values above 0.5% are presented. Reference strains: [a] (Zhilina et al. 2004), [b] (Suzuki et al. 1999), [c] (Miyazaki et al. 2011)

Influence of pH and sodium on growth and activity

With galacturonic acid as substrate, AP1 was able to grow at a pH between 8 and 10.5 with an optimum at 9.5 (Fig. 3a). The growth was chloride independent. In sodium carbonate buffer at pH 10, growth was possible between 0.1 and 1.5 M total Na^+ with an optimum at 0.4–0.6 M (Fig. 3b). According to these characteristics, the organism belongs to the moderately salt-tolerant obligate alkaliphiles.

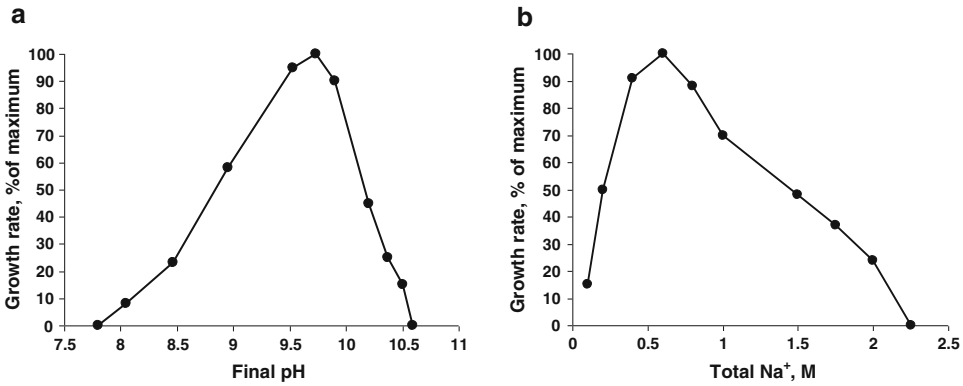

Fig. 3 Influence of pH at 0.6 M Na^+ (**a**) and of sodium carbonate at pH 10 (**b**) on anaerobic growth of strains AP1 with galacturonic acid

Table 2 Phenotypic comparison of strain AP1 with the closest described relatives

Characteristic	$AP1^T$	*Alkaliflexus imshenetskii*[a]	"*Geofilum rubicundum*"[b]	*Marinilabilia salmonicolor*[c]	*Anaerophaga thermohalophila*[d]
Cell size (μm)	0.25–0.3 × 3–10	0.25–0.4 × 4–10	0.2–0.4 × 4.0–22.0	0.3–0.5 × 2–6	0.3 × 4–8
Motility	Gliding	Gliding	Gliding	Gliding	−
Pigmentation	Pink	Pink	Salmon pink	Yellow to orange	Orange–red
Fermentation products	Acetate, succinate	Acetate, succinate, propionate, formate	n.d.	Acetate, succinate, propionate, lactate, H_2	Acetate, succinate, propionate
Aerobic growth	−	−	+	+	−
Catalase activity	Weak	+	+	+	−
Substrates					
Galacturonic acid	+	+	n.d.	n.d.	n.d.
Xylose	+	+	+	+	+
Cellobiose	+	+	+	+	+
Fructose	+	−	+	+	+
Lactose	−	−	−	+	+
Glucosamine	+	+	n.d.	n.d.	n.d.
N-acetyl glucosamine	+	+	n.d.	n.d.	n.d.
Pectin, xylan, pullulan	+	+	xylan−	n.d.	n.d.
Maximal growth temperature	41	45	36	n.d.	55
pH range (optimum)	8.0–10.5 (9.5)	7.2–10.2 (8.5)	6.9–9.3 (7.8)	Neutrophile	Neutrophile
Salt range (M Na^+)	0.2–2.0	0–0.88	0–1.0	0.17–0.5	0.33–2.0
G+C content (mol %)	40.6	44.3	42.9	37	41.8
Habitat	Soda lakes	Soda lake	Marine	Marine	Saline oil field

n.d. not determined

[a] (Zhilina et al. 2004), [b] (Miyazaki et al. 2011), [c] (Suzuki et al. 1999), [d] (Denger et al. 2003)

In the overall characteristics, the pectinolytic strain $AP1^T$ isolated from the sediments of southeastern Siberian soda lakes resembles *Alkaliflexis imshenetskii*—a low salt-tolerant alkaliphilic saccharolytic bacterium isolated from a Transbaikal soda lake (Zhilina et al. 2004) (Table 2). However, the large phylogenetic distance and several phenotypic differences allow the novel isolate to be assigned to a separate genus and species for which the name *Natronoflexus pectinivorans* is suggested.

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Appendix 1: Description of *Natronoflexus* gen. nov

(Na.tro.no.flex'us; N.L. n. *natron* (arbitrarily derived from the Arabic n. *natrun* or *natron*) soda, sodium carbonate; N.L. pref. *natrono-*, pertaining to soda; L. masc. n. *flexus* a bending, N. L. masc. n. *Natronoflexus* bending/flexible cells living in soda.).

Gram-negative long flexible rods. Obligately anaerobic with fermentative metabolism. Utilize various monomeric and polymeric carbohydrates. Obligately alkaliphilic and moderately salt-tolerant. Belong to the family '*Marinilabiliaceae*' in the phylum *Bacteroidetes*. Habitats—soda lakes. The type species is *Natronoflexus pectinivorans*.

Appendix 2: Description of *Natronoflexus pectinivorans* sp. nov

(N.L. n. *pectinum*, pectin; L. part. adj. *vorans*, devouring; N.L. part. adj. *pectinivorans*, pectin-devouring).

Cells are long flexible rods, 0.25–0.3 × 3–10 μm, single or in bundles, capable of gliding movement on solid surfaces. Gram-negative, contain carotenoids with absorption peaks in methanol-acetone at 468 (shoulder), 492 (main), and 523 nm. Strictly anaerobic fermentative saccharolytic bacterium, utilizing the following carbohydrates: D-galacturonic acid, D-glucuronic acid, dextrose, fructose, glucose, α,α-trehalose, α-CH_3-glycoside, 2-deoxyglucose, D-mannose, sucrose, D-maltose, D-cellobiose, D-glucosamine, *N*-acetyl glucosamine, galactose, xylose, glycogen, starch, pectin, laminarin, pullulan. Substrates not utilized: lactose, melibiose, melisitose, arabinose, arabinite, glycerol, L-sorbose, *m*-erithritol, *m*-inositol, D-raffinose, dextrin, D-rhamnose, D-ribose, alginate, CMC, cellulose, agar. The final products of galacturonic acid fermentation are acetate and succinate. Obligately alkaliphilic with a pH range for growth between 8.0 and 10.5, and an optimum at pH 9.5. Moderately salt tolerant with a range from 0.1 to 2.0 M Na^+ (optimum at 0.4–0.6 M). Mesophilic, with a maximum temperature for growth at 41°C and an optimum at 30°C. The polar lipids consist of unidentified phospho-, amino- and aminophospholipids, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. The predominant fatty acids in the polar membrane lipids are isomers of $C_{15:0}$. The G+C content of the genomic DNA is 40.6 mol% (T_m). The type strain is $AP1^T$ ($DSM24179^T$ = $UNIQEM\ U807^T$). Isolated from sediments of soda lakes in southwestern Siberia. The GenBank 16S rRNA gene sequence accession number is GQ922844.

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