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Alcalilimnicola halodurans gen. nov., sp. nov., an alkaliphilic, moderately halophilic and extremely halotolerant bacterium, isolated from sediments of soda-depositing Lake Natron, East Africa Rift Valley

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An alkaliphilic, halotolerant, Gram-negative, heterotrophic, aerobic and rod-shaped organism was isolated from drying soda and at a water-covered site of Lake Natron, Tanzania, by means of the most-probable-number technique developed for anoxygenic, phototrophic sulfur bacteria. It had an absolute requirement for alkalinity, but not for salinity; growth occurred at salt concentrations of 0–28% (w/v), with optimal growth at 3–8% (w/v) NaCl. The bacterium preferentially metabolized volatile fatty acids and required vitamins for growth. The name *Alcalilimnicola halodurans* gen. nov., sp. nov. is proposed for the novel isolate, placed in the *γ-Proteobacteria* within the family *Ectothiorhodospiraceae* on the basis of analysis of the 16S rDNA sequence, polar lipids, fatty acids and DNA base composition. Although *Alcalilimnicola halodurans* is closely related to the extreme anoxygenic, phototrophic sulfur bacteria of the genus *Halorhodospira*, it is not phototrophic.

Keywords: *Alcalilimnicola halodurans*, alkaliphilic and halotolerant bacterium, sediments, soda-depositing Lake Natron, East African Rift Valley

INTRODUCTION

Alkaline soda lakes and soda deserts are found in many parts of the world but, because of their poor accessibility in most cases, they have not been characterized extensively to date. These environments are characterized by high concentrations of sodium carbonate complexes. In the course of their formation, other salts, particularly NaCl, also concentrate, generally giving rise to environments that are both alkaline and saline to varying degrees. The Kenyan–Tanzanian Rift Valley, an area of active volcanism, contains a number of soda lakes that represent the most alkaline naturally occurring environments on Earth. Their salinities range from around 5% (w/v) total salts, in the case of the more northerly lakes Bogoria, Nakuru, Elmenteita and Sonachi, to saturation in the southern lakes of Magadi and Natron, with roughly equal proportions of Na_2CO_3 and NaCl as the major salts. These soda lakes are supposed to be the habitats of relict microbial communities and are regarded as possible centres of the origin of microbial diversity (Zhilina & Zavarzin, 1994). The concentrated brines of the hypersaline Lake Magadi have been shown to support a dense and diverse population of aerobic, organotrophic, halophilic and alkaliphilic representatives of major bacterial and archaeal phyla (reviewed recently by Duckworth *et al*., 1996, 2000; Jones *et al*., 1998; Grant *et al*., 1999). Anaerobic decomposition, which dominates in these lakes, is performed by the anaerobic, alkaliphilic microbial community that has not yet been studied in detail, in spite of its possible

Abbreviations: APG, acyl phosphatidylglycerol; CID, collision-induced dissociation; CL, cardiolipin; FAB, fast atom bombardment; GPA, glycerophosphatic acid; PC, phosphatidylcholine; PE, phosphatidylethylamine; PG, phosphatidylglycerol; PLFA, phospholipid ester-linked fatty acid.

The EMBL accession numbers for the 16S rRNA gene sequences of *Alcalilimnicola halodurans* 34Alc^T and isolates Ph-C1 and Ph-C2 are AJ404972, AJ278688 and AJ278687, respectively.

biotechnological applications (Zhilina & Zavarzin, 1994).

During the identification of anoxygenic, phototrophic sulfur bacteria that were numerically dominant within naturally occurring microbial communities of Lake Natron trona/mud and sediment samples, we isolated an accompanying organotrophic γ-proteobacterium as a persistent contaminant of phototrophic enrichments. We describe here the phenotypic characteristics of the isolates, as well as their phylogenetic assignment, based upon their 16S rRNA sequences, polar lipids, fatty acids and DNA base compositions. The isolates constitute a novel species within a new genus and, considering their origin and exceptional halotolerance, the name *Alcalilimnicola halodurans* is proposed. Strain 34Alc^T is designated as the type strain.

METHODS

Isolation. Sediment samples were collected from the Tanzanian section of the soda-depositing Lake Natron in a dry period, both under trona (site 1: 2° 32′ S and 36° 04′ E) and at a water-covered site in the lake (site 2: 2° 35′ S and 36° 02′ E) (Fig. 1). The pH and temperature at the two collecting sites were recorded as 11·2 and 57 °C and 10·5 and 42 °C, respectively. Sterile, 200 ml wide-mouthed glass cores were gently screwed entirely into the sediments, left for 30 min and then sealed with Teflon-coated rubber stoppers held in place by screw caps. The cores were transported immediately to Germany for further study. The cores were opened from the bottom inside a glove box (Coy) in a 10 % H_2/90 % N_2 atmosphere (Zhilina & Zavarzin, 1994) and subcores were taken by insertion of sterile glass tubes into the core centres up to the upper stopper. The tubes were then sectioned and 5 ml sediment, corresponding to a depth of approximately 5–6 cm from the surface, was removed aseptically from each tube and homogenized with 5 ml enrichment medium. Replicate 10-fold dilutions of the sediment slurries were made anaerobically in aluminium-sealed Hungate tubes (Bellco Glass). The tubes were incubated at 37 °C and 1000–2000 lux until colour changes due to growth of phototrophs ceased (approx. 1 month). Tubes were scored positive or negative on the basis of this colour change. Positive tubes representing the highest dilution were subsequently plated on agar and cultivated under both anoxic and aerobic conditions at 2000 lux. Both phototrophic isolates and the small colourless colonies were taken for further investigation.

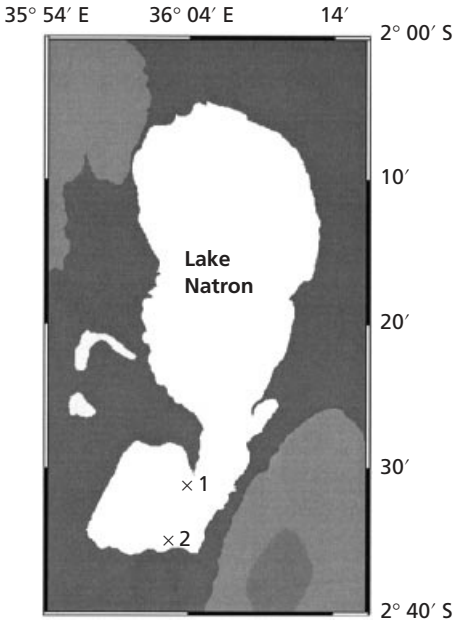

Fig. 1. Location of sampling sites 1 and 2, respectively corresponding to the trona- and water-covered areas.

Nutritional and growth characteristics. Basal media described by Imhoff & Trüper (1977) were used throughout the studies. A high-salt medium with 150 g total salt l^{-1} was used for samples collected at site 1, while low-salt medium (35 g total salt l^{-1}) was chosen for the isolation experiments from site 2 samples. Aliquots of 1 ml vitamin solution 'VA' and 1 ml trace element solution 'SLA' (Imhoff & Trüper, 1977) were added to 1 l batches of medium after sterilization. We subsequently found that a more optimal medium for growth of *Alcalilimnicola halodurans* was modified DSMZ medium 430 (430M) (http://www.dsmz.de/media/med430.htm), in which the salinity was decreased to 70 g NaCl l^{-1} and 1 g sodium acetate l^{-1} was replaced by 10 g glucose l^{-1}. The temperature range for growth was determined in 430M medium at temperatures between 4 and 60 °C; growth was scored as optical density at 600 nm. Growth under microaerophilic and anaerobic conditions was tested in uniformly inoculated agar tubes. pH and salinity optima were determined and growth was compared with that of cultivation at optimal conditions (pH 9·5; 5 % salinity). Five replicate test cultures of each strain were analysed after three serial transfers in identical media. General phenotypic characterization of isolates was carried out as described previously (Yakimov *et al.*, 1998; Golyshina *et al.*, 2000). Unless stated otherwise, all tests were carried out in 430M medium at pH 9·5 and 37 °C. Antibiotic susceptibility was tested by spreading bacterial suspensions on 430M plates containing 30 g NaCl l^{-1} and applying the following antibiotic discs (Difco): ampicillin (25 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), streptomycin (10 μg), sulfomethoxazole (25 μg), trimethoprim (15 μg), tetracycline (30 μg) and vancomycin (30 μg).

Electron microscopy. Exponential-phase cells grown in 430M liquid medium supplemented with 30 g NaCl l^{-1} were collected by centrifugation and fixed in 5 % glutaraldehyde buffered with 50 mM PBS, pH 7·1. Negative staining, shadow casting, embedding and ultrathin sectioning were carried out according to methods described previously (Yakimov *et al.*, 1998; Golyshina *et al.*, 2000).

Polar lipid fatty acid and pigment analysis. Lipids were extracted by a modified Bligh–Dyer procedure and fatty acid methyl esters were generated and analysed by gas chromatography, as described previously (Vancanneyt *et al.*, 1996). Absorption spectra of whole cells and of chloroform/methanol cellular extracts were analysed with a Biochrom 4060.

Fast atom bombardment mass spectrometry (FAB-MS). FAB-MS in the negative mode was performed on the first of two

mass spectrometers of a tandem high-resolution instrument of $E_1B_1E_2B_2$ configuration (JMS-HX/HX110A; JEOL) at 10 kV accelerating voltage. Resolution was set to 1:1500. The JEOL FAB gun was operated at 6 kV with xenon as the FAB gas. A mixture of triethanolamine and tetramethylurea (Japanese matrix) was used as matrix.

Tandem mass spectrometry (MS-MS). Negative daughter ion spectra were recorded using all four sectors of the tandem mass spectrometer. High-energy collision-induced dissociation (CID) took place in the third field-free region. Helium served as the collision gas at a pressure sufficient to reduce the precursor ion signal to 30% of the original value. The collision cell was operated at ground potential. Resolution of MS-MS was set to 1:1000. FAB-CID spectra (linked scans of MS-MS at constant B/E ratio) were recorded at 300 Hz filtering with a JEOL DA 7000 data system.

G+C content. The G+C content of DNA isolated from strain 34Alc^T was determined directly by HPLC with a Nucleosil 100-5 C_{18} column (Macherey-Nagel) according to methods described previously (Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984). Purified non-methylated lambda phage DNA (Sigma) was used as a control.

16S rRNA gene sequence determination and analysis of phylogenetic relationships. DNA was isolated from 5 ml late exponential-phase cells grown in 430M medium, using the CTAB miniprep protocol for bacterial genomic DNA preparations, as described previously (Yakimov *et al.*, 1998). PCR amplification of the 16S rRNA genes was performed with an ABI 9600 thermocycler (PE Applied Biosystems) using the forward primer 16F27 (5′-AGAGTTTGATC-MTGGCTCAG-3′) and the reverse primer 16R1492 (5′-TACGGYTACCTTGTTACGACTT-3′). The amplified product was purified with QIAquick PCR purification columns (Qiagen). Direct sequence determination of the purified rDNA was carried out using a Prism Ready Reaction DyeDeoxy Terminator sequencing kit according to the protocols of the manufacturer (Perkin-Elmer Applied Biosystems) and an automated DNA sequencer model 377 (Applied Biosystems). Both template strands were sequenced at least twice. Analysis of the sequences obtained was performed using SIMILARITY_MATRIX version 1.1, SEQUENCE_MATCH version 2.7 and SEQUENCE_ALIGN version 1.7 from the Ribosomal Database Project (RDP) and BLAST (Altschul *et al.*, 1997). These analyses were used to estimate the degree of similarity to other 16S rRNA gene sequences (Table 4). Nucleotide sequences were further aligned manually to 16S rRNA sequence data from the RDP according to primary and secondary structure considerations, using Se-Al sequence alignment editor version 1.0 alpha 1 (Rambaut, 1996). Further phylogenetic analyses were restricted to nucleotide positions that were unambiguously alignable in all sequences. Alignments were exported as PHYLIP 3.5 interleaved file types to make multiple bootstrapped datasets from the data obtained by means of the SEQBOOT program. Phylogenetic analyses were performed using parsimony (DNAPARS) and neighbour-joining (NEIGHBOR) methods. Random order input of sequences, single jumbling and the global rearrangement option were used to avoid potential bias introduced by the order of sequence addition. Least-squares distances were obtained using the NEIGHBOR program with similarity values obtained after using DNADIST with maximum-likelihood analysis and with multiple datasets options. The resulting tree files were analysed by using the CONSENSE program to provide confidence estimates for phylogenetic tree topologies and to make a majority rule consensus tree. All phylogenetic programs used were from PHYLIP version 3.573c (Felsenstein, 1993). The RNA helix secondary structure was drawn using the loopDloop software (Gilbert, 1992).

RESULTS

Habitat and isolation

Isolates of the purple phototrophic bacteria Ph-C1 and Ph-C2, which deposit elemental sulfur outside the cells, were obtained from the last positive dilutions of sediment slurries derived respectively from the trona mud (site 1) and water sediment (site 2) cores taken from the southern part of Lake Natron (Fig. 1). These environments were anoxic, strongly alkaline (pH 10–11) and varied in salinity from 20% (w/v) (site 1) to 2% (w/v) (site 2). After plating Ph-C1 and Ph-C2 on high- and low-salt solid media, non-pigmented, flat, irregular-shaped colonies, 2–3 mm in diameter, appeared among the typical purple colonies after 72 h of cultivation. The colonies were identical on both low- and high-salt media and two isolates, 3S and 34Alc^T, were selected for further studies.

Cell morphology and ultrastructural analysis

Microscopic examination showed that isolates 3S and 34Alc^T were non-spore-forming, motile rods, about 1·5 μm wide and 2–6 μm long. Mid-exponentially growing cells exhibited a single, polar flagellum (monopolar-monotrichous) with a length of about 7 μm (Fig. 2a). Under the growth conditions used (430M medium supplemented with 2 g sodium acetate l^{-1}), the bacteria produced an extracellular matrix of non-fibrillar appearance that surrounded the cell completely when observed from air-dried, Pt/C shadow-cast preparations (data not shown). Cross-sectioned cells showed a cytoplasmic matrix of general ribosomal density and the bacterial chromosome, which characteristically appeared condensed and electron-transparent (Fig. 2b, open arrowheads), was often intercalated by electron-dense matter (Fig. 2b, edm). This electron-dense matter often terminated as dark granules, similar to polyphosphate granules. Though the cells were grown under conditions that favoured phototrophy (anoxic cultivation at 2000 lux on 430M supplemented with 1 g sodium acetate l^{-1}), neither intracellular vesicles nor membrane stacks, characteristic of phototrophic micro-organisms, were detected. Analysis of absorption spectra of whole cells and cellular extracts failed to reveal any maxima indicative of the presence of photosynthetic pigments (data not shown). Large amounts of electron-translucent intracellular storage material, similar to poly-hydroxybutyrate, appeared as granules (Fig. 2b), 50–300 nm in diameter, representing 80% or more of the cell contents. The granules did not show a strong tendency to coalesce and were generally separated by narrow, dark-stained clefts (Fig. 2b, filled arrow-

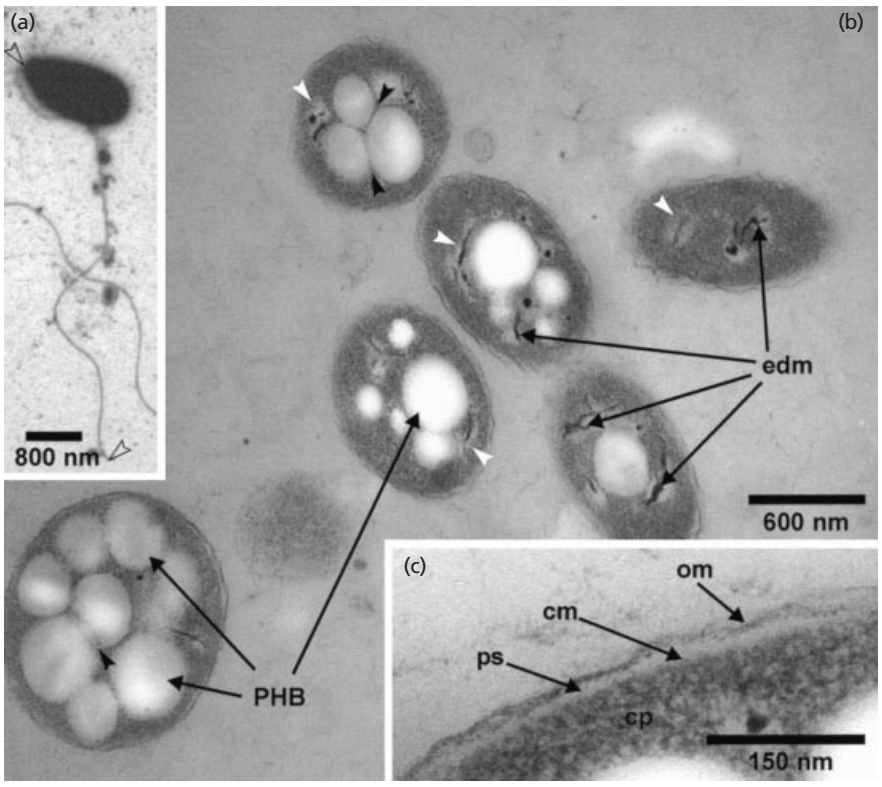

Fig. 2. Ultrastructural analysis of *Alcalilimnicola halodurans* 34Alc^T. (a) Negatively stained cell shows a single flagellum (open arrowheads), which is polarly inserted. (b) General view of cross- and longitudinally sectioned cells, which contain mostly electron-translucent storage inclusions of polyhydroxybutyrate (PHB). Filled arrowheads point to the interspace of the PHB granules. The condensed chromosome (open arrowheads) is often interspersed with electron-dense matter (edm), occasionally showing a polyphosphate-like granularity. (c) Detailed view of the Gram-negative cell wall. The outer membrane (om), the periplasmic space (ps), the cytoplasmic membrane (cm) and the cytoplasm (cp) are indicated.

heads). The bacterial cell wall is Gram-negative, as indicated by the presence of an outer membrane and an electron-translucent periplasmic space (Fig. 2c, om, ps). The murein sacculus could not be seen as a fine electron-dense line within the periplasm.

Cultural and physiological characterization

In general, the two isolates exhibited identical phenotypic characteristics; organism 34Alc^T was designated as the type strain. The isolates were aerobic, catalase- and oxidase-positive, capable of growth at salt concentrations between 0 and 28 % (w/v), with salt optimum at concentrations between 3 and 8 % (w/v) (Fig. 3a). They were obligate alkaliphiles, with pH optima for growth higher than 9·5 (Fig. 3b) and a temperature range between 20 and 50 °C. The strains could also grow anaerobically with or without nitrate and were able to utilize a limited number of simple organic compounds, such as short chain fatty acids, alcohols and sugars (Table 1). Growth was stimulated by addition of yeast extract and vitamins. Strains were insensitive to ampicillin, gentamicin, kanamycin, nalidixic acid, streptomycin, trimethoprim, tetracycline and vancomycin, but sensitive to chloramphenicol and sulfomethoxazole, at the concentrations tested.

Analysis of polar lipids

Five different types of polar lipids were identified, namely phosphatidylglycerol (PG), phosphatidylethylamine (PE), phosphatidylcholine (PC), acyl phosphatidylglycerol (APG) and cardiolipin (CL) (Fig. 4). Their structures were elucidated with the aid of CID-MS.

Diagnostically important fragments arise by cleavage of the phosphatidyl–polar head group bond. In phospholipids such as PG or PC, cleavages that are characteristic of the polar head are known to occur in the negative and positive FAB modes (Heller *et al.*, 1988). The resulting ions from such phospholipids have the structures of glycerophosphatic acids (GPA) and can be observed in the normal FAB as well as in the CID spectra (e.g. m/z 673, 675 and 699). The fact that ions of this type with varying m/z can be formed from one parent ion, as in the case of m/z 1307, indicates the presence of isobaric species due to the presence of different fatty acids. Additional CID experiments of the GPA anions allow the identification

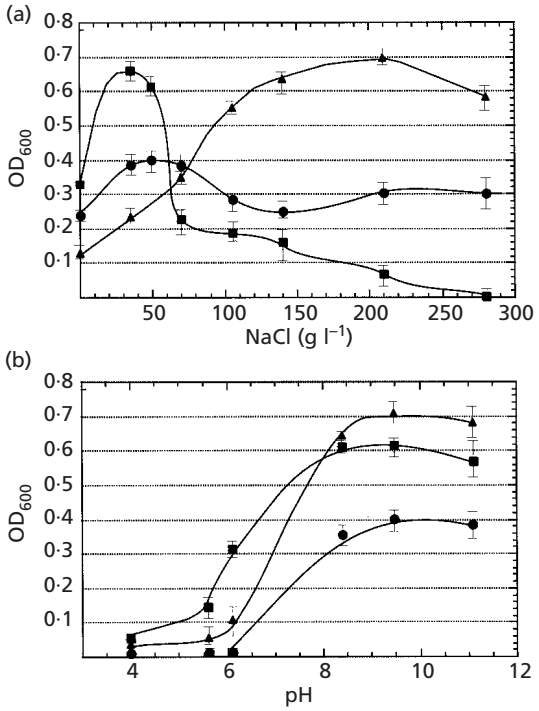

Fig. 3. Growth of *Alcalilimnicola halodurans* 34Alc^T (●) and two isolated phototrophs, Ph-C1 (▲) and Ph-C2 (■), on modified 430M medium. (a) Influence of salinity on the growth of isolates at 35 °C and pH 9·5. (b) Influence of pH on growth at 35 °C and 5% salinity (isolates 34Alc^T and Ph-C2) or 20% salinity (Ph-C1).

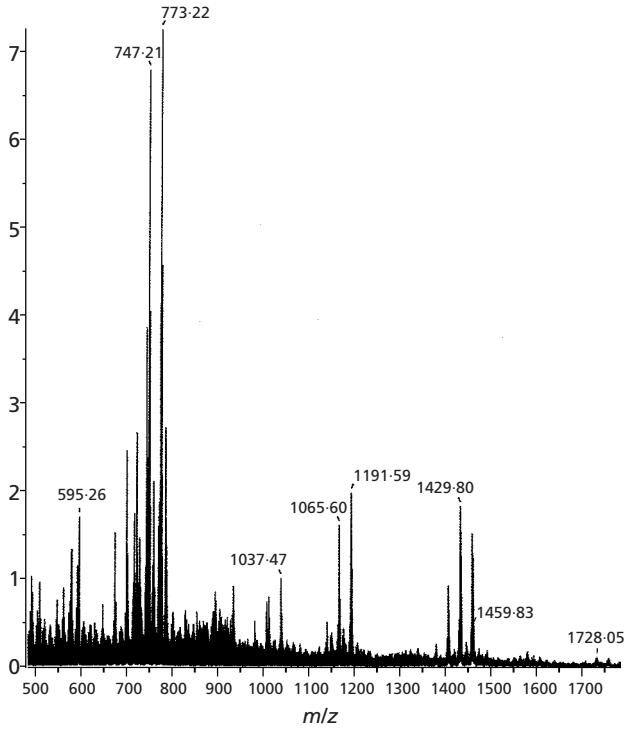

Fig. 4. (−)-FAB mass spectrum of the total lipid fraction of *Alcalilimnicola halodurans* 34Alc^T. The following features can be observed: acyl ions in the range *m/z* 250–300 (not shown); PG around *m/z* 750; APG at *m/z* 1038; lyso-CLs at *m/z* 1166 and 1192; CLs around *m/z* 1420. For more details see text.

Table 1. Utilization of organic carbon compounds as sole sources of carbon and energy by *Alcalilimnicola halodurans* 34Alc^T and *Arhodomonas aquaeolei* HA-1^T

Data for *Arhodomonas aquaeolei* were taken from Adkins *et al.* (1993). +, Positive growth; −, no growth. The following compounds were utilized by both organisms: acetate, butyrate, DL-*β*-hydroxybutyrate, *γ*-amino-*n*-butyrate, crotonate, ethanol, fumarate, D-gluconate, glycerol, lactate, propionate, pyruvate, succinate, valerate and D-xylose. Methanol was not used by either organism. A complete list of the organic carbon sources used is given in the species description.

Compound	*Alcalilimnicola halodurans* 34Alc^T	*Arhodomonas aquaeolei* HA-1^T
γ-Hydroxybutyrate	+	−
Citrate	+	−
D-Fructose	+	−
D-Galactose	+	−
D-Glucose	+	−
L-Glutamic acid	−	+
L-Glutamine	−	+
D-Malate	+	−
D-Mannitol	+	−
Sucrose	+	−

of these fatty acids (Murphy & Harrison, 1994). CID of the $(M–H)^-$ ion from these GPA yielded abundant carboxylate anions from both the *sn*-1 and *sn*-2 positions, thus allowing the identification of the fatty acids attached to the different GPAs. In addition, there were neutral losses of the *sn*-2 and *sn*-1 substituents as free carboxylic acids as well as loss of each fatty acyl group as a substituted ketene. Furthermore, the positions of the fatty acids on the glycerol backbone can be determined. For the fatty acid positioned at *sn*-2, neutral loss as free fatty acid, as well as substituted ketene, is more frequent than for that at *sn*-1 (Murphy & Harrison, 1994). With this method, the structure of the GPA anions and hence the structure of the phospholipids was identified (Niepel *et al.*, 1998). Table 2 summarizes the analysis of 17 different phospholipids identified in strain 34Alc^T. Six different PGs and, in each case, only one PE, PC and APG could be found. CLs were the most diverse class of phospholipids detected in strain 34Alc^T; eight different compounds of this type were observed and their structure elucidated. While cardiolipin CL14 is also found in *Arthrobacter atrocyaneaus* LMG 3814^T (Niepel *et al.*, 1997), three of these cardiolipins, CL13, CL15 and CL16, are compounds that have not been described previously.

The phospholipid ester-linked fatty acid (PLFA) patterns contained features indicative of members of

Table 2. Polar lipids identified in the cell extract of strain *Alcalilimnicola halodurans* 34Alc^T

PG, Phosphatidylglycerol; PE, phosphatidylethylamine; PC, phosphatidylcholine; APG, acyl phosphatidylglycerol; CL, cardiolipin.

No.	Mass	Type	R1-COOH (*sn*-1)	R1-COOH (*sn*-2)	R1-COOH (*sn*-1′)	R1-COOH (*sn*-2′)
1	722	PG	16:0	16:0		
2	743	PE	18:1	18:1		
3	748	PG	18:1	16:0		
4	748	PG	18:0	16:1		
5	750	PG	18:0	16:0		
6	774	PG	18:1	18:1		
7	776	PG	18:0	18:1		
8	785	PC	18:1	18:1		
9	1038	APG	18:1	18:1	18:1	
10	1166	lyso-CL	18:1	16:0	18:1	
11	1166	lyso-CL	18:1	18:1	16:0	
12	1192	lyso-CL	18:1	18:1	18:1	
13	1404	CL	16:0	18:1	16:0	18:1
14	1404	CL	18:1	18:1	16:0	16:0
15	1430	CL	18:1	16:0	18:1	18:1
16	1430	CL	18:0	16:1	18:1	18:1
17	1456	CL	18:1	18:1	18:1	18:1

Table 3. Comparison of PLFA composition among strains of slightly, moderately/extremely and extremely halophilic phototrophs and *Alcalilimnicola halodurans* 34Alc^T

Taxa are identified as: 1, *Alcalilimnicola halodurans* 34Alc^T; 2, *Halorhodospira halophila* BN9626; 3, *Halorhodospira halophila* DSM 244^T; 4, *Ectothiorhodospira shaposhnikovi* DSM 243^T; 5, *Ectothiorhodospira mobilis* DSM 237^T. Values are percentages of the total amount of fatty acids isolated from cellular phospholipids. Data were taken from this study (34Alc^T), Thiemann & Imhoff (1991) (BN9626) and Thiemann & Imhoff (1996) (DSM 244^T, DSM 243^T and DSM 237^T). tr, Trace (< 0·05%).

Fatty acid	1	2	3	4	5
C14:0	4·1	4·6	0·3	2·5	1·2
C14:0-3OH	0·3	—	—	—	—
C16:1ω9	0·7	tr	0·6	4·6	2·7
C16:0	23·4	18·0	10·8	21·6	35·7
C17:0	1·1	—	—	—	—
Summed feature 1*	54·0	54·9	62·8	59·4	16·5
C18:0	12·1	13·9	11·7	5·6	7·6
C19:0d8,9	0·6	2·9	5·8	tr	33·8
C20:1ω9	0·4	—	1·8	0·5	—
C22:0	0·7	3·9	—	—	—
Total PLFA detected (%)	97·4	98·2	93·8	94·2	97·5

* C18:1ω7/C18:1ω9.

the halophilic γ-proteobacteria (Table 3). The predominant PLFAs were 16:0, summed feature C18:1ω7/C18:1ω9 and C18:0. These fatty acids accounted for more than 89·5% of the total PLFAs detected. Such a profile is typical for moderately and extremely halophilic members of genus *Halorhodospira* and no clear distinction was observed between *Alcalilimnicola halodurans* 34Alc^T and *Halorhodospira halophila* BN9626 (Table 3).

DNA base composition

The G+C content of the *Alcalilimnicola halodurans* 34Alc^T DNA was shown to be 65·6 mol%.

Molecular phylogenetic analysis

Sequence similarity values for *Alcalilimnicola halodurans* 34Alc^T (1478 bases) and selected reference organisms (S_ab $\geqslant$ 0·900) belonging to the γ-*Proteobacteria* are shown in Table 4. The results of the 16S rDNA sequence analysis indicated that the genus *Alcalilimnicola* is a separate genus and, together with the genera *Arhodomonas* and *Nitrococcus*, represents a deeply branching non-phototrophic lineage within the family *Ectothiorhodospiraceae* (Fig. 5). *Alcalilimnicola halodurans* 34Alc^T is specifically related to '*Alkalispirillum mobilis*' (98% identity), an undescribed non-phototrophic member of the family *Ectothiorhodospiraceae*, the partial 16S rRNA sequence of which is available as accession no. AF114783. The 16S rRNA of *Alcalilimnicola halodurans* has structural similarities to that of the purple sulfur bacteria. The loop starting at position 420 is UGCG, and an additional C is present in the loop covering position 1361 (*Escherichia coli* numbering), features not found in other members of the γ-

Table 4. Similarity values observed by comparison of the 16S rRNA sequence of *Alcalilimnicola halodurans* 34Alc^T with those of most similar RDP database sequences

Only sequences giving S_ab $\geqslant$ 0·900 are shown.

Organism	Accession no.	Length (bp)	Similarity score (S_ab)
'*Alkalispirillum mobilis*'	AF114783	1464	0·987
Halorhodospira halophila SL 1 (= DSM 244^T)	M26630	1494	0·941
Arhodomonas aquaeolei HA-1^T (= ATCC 49307^T)	M26631	1487	0·939
Halorhodospira halophila BN 9624	X93474	1470	0·935
Nitrococcus mobilis ATCC 25380^T	L35510	1483	0·931
Halorhodospira halophila BN 9630	X93484	1500	0·929
Halorhodospira halochloris BN 9851 (= 51/12)	X93483	1480	0·922
Thiorhodovibrio sp.	Y12368	1289	0·921
Ectothiorhodospira shaposhnikovii DSM 243^T	M59151	1483	0·915
Thiorhodovibrio winogradskyi MBIC 2776^T (= DSM 6702^T)	AB016986	1456	0·915
Thiorhodovibrio winogradskyi SSP1^T (= DSM 6702^T)	AJ006214	1382	0·914
'*Thiorhodovibrio sibirica*'	AJ010297	1365	0·911
Ectothiorhodospira vacuolata BN 9512^T (= DSM 2111^T)	X93478	1473	0·909
Ectothiorhodospira shaposhnikovii BN 9912	X93480	1464	0·908
Symbiont of *Solemya reidi* gill	L25709	1413	0·907
Ectothiorhodospira haloalkaliphila BN 9903^T	X93479	1507	0·906
Ectothiorhodospira 'Bogoria Red'	AF084511	1492	0·906
Rhabdochromatium marinum 8315^T (= DSM 5261^T)	X84316	1452	0·905
Ectothiorhodospira haloalkaliphila BN 9902	X93475	1465	0·902
Ectothiorhodospira marismortui BN 9410^T (= DSM 4180^T)	X93482	1467	0·902
Lamprocystis roseopersicina DSM 229^T	AJ006063	1393	0·901
Thiocystis violacea 2711^T (= DSM 207^T)	Y11315	1450	0·900

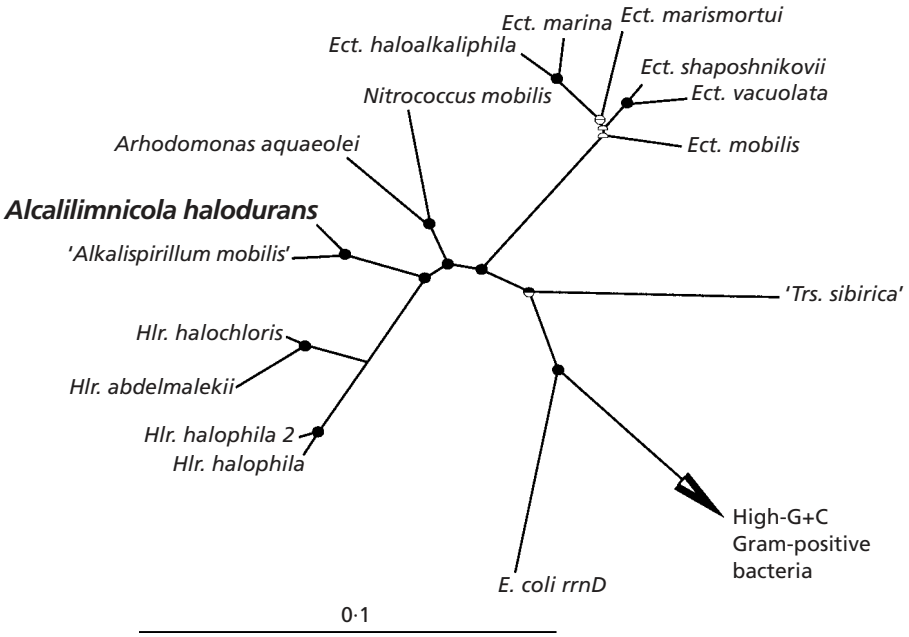

Fig. 5. Estimated phylogenetic position of *Alcalilimnicola halodurans* 34Alc^T among the members of family *Ectothiorhodospiraceae*, derived from 16S rRNA gene sequence comparisons. The neighbour-joining tree is based on 1450 nucleotide positions. The tree is rooted with *Cellulomonas cellulans* and *Escherichia coli* was used as the outgroup. Abbreviations: *E.*, *Escherichia*; *Ect.*, *Ectothiorhodospira*; *Hlr.*, *Halorhodospira*; *Trs.*, *Thiorhodospira*. Bootstrap values of 100 resamplings obtained from the CONSENSE program are shown as the upper (maximum-likelihood) and lower (parsimony) halves of circles. Branching points supported by bootstrap values > 70 % are indicated by filled hemispheres; open hemispheres indicate branching points that are supported by bootstrap values of 50–70 %. Branching points without hemispheres were not resolved (bootstrap values < 50 %). Bar, 10 % sequence divergence.

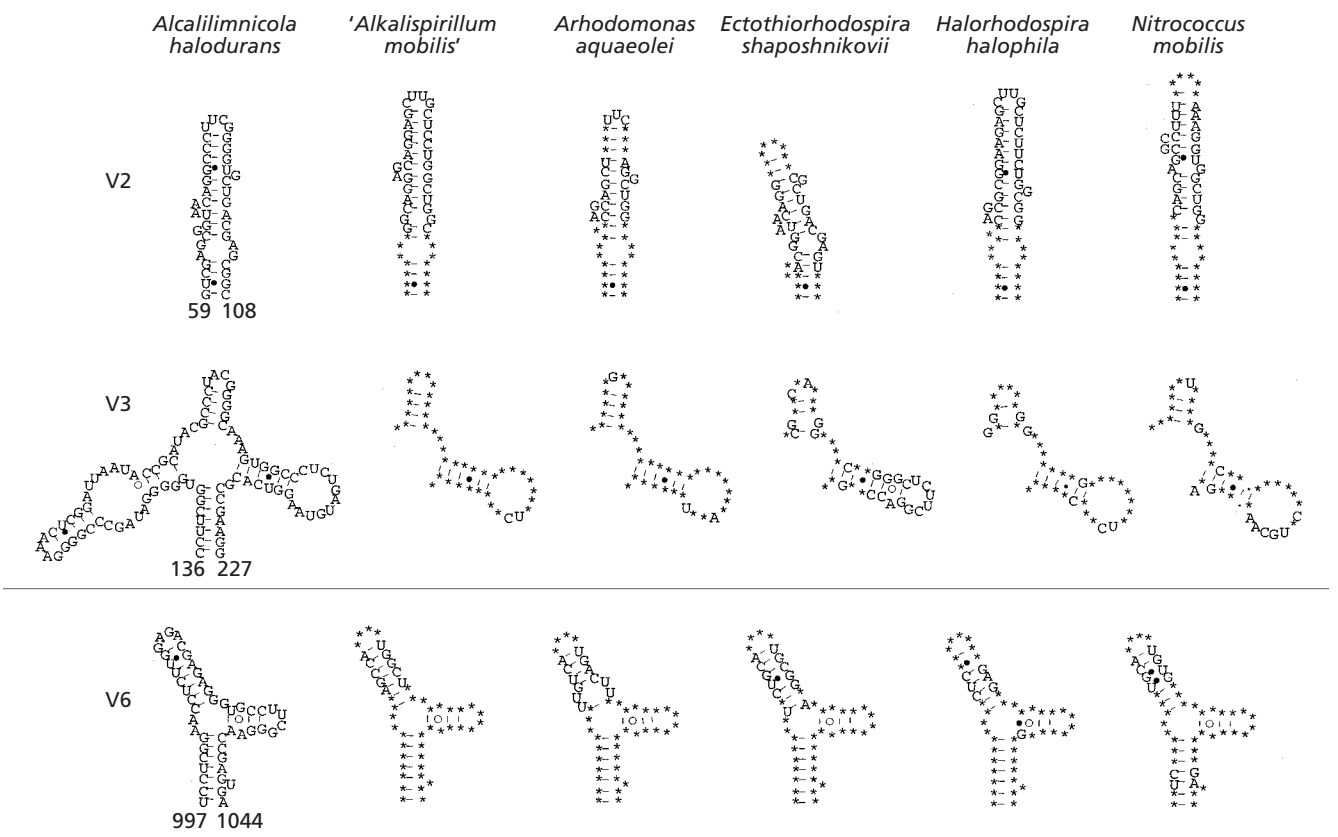

Fig. 6. Secondary structures of three 16S rRNA variable helices, V2, V3 and V6 (Woese, 1987), illustrating the differences between *Alcalilimnicola* and other representative genera within the *Ectothiorhodospiraceae*. Nucleotides shown indicate the signature sequences that deviate from that of *Alcalilimnicola*. Identical positions are shown by asterisks. Position numbering shown below the *Alcalilimnicola* helices corresponds to *Escherichia coli* numbering.

Proteobacteria (Adkins *et al.*, 1993). Additionally, analysis of some 16S rRNA hypervariable signature regions used previously to distinguish the genera *Ectothiorhodospira* and *Halorhodospira* (Imhoff & Süling, 1996) showed clearly the distinct position of *Alcalilimnicola* among all of the genera belonging to the *Ectothiorhodospiraceae* (Fig. 6).

DISCUSSION

The Kenyan–Tanzanian Rift Valley contains a number of soda lakes that represent the most alkaline naturally occurring environments on Earth. In the dry period, the majority of these lakes are partially covered by a white precipitate of trona (usually as $Na_2CO_3 . 10H_2O$ or $NaHCO_3 . Na_2CO_3 . 2H_2O$), about 2–5 cm thick, below which a highly alkaline brine (pH 10–12) and black precipitate of trona mixed with mud occur. Surface trona temperatures rise to 65 °C as a result of solar activity. Despite the extreme nature of such environments, they are characterized by a high primary productivity, presumably because of the unlimited supply of CO_2 and high light intensities, which are optimal for blooms of cyanobacteria and anaerobic phototrophic organisms of the family *Ectothiorhodospiraceae*. The latter group is very important for the ecology of such lakes because of its additional participation in the sulfur cycle, i.e. oxidation of the H_2S produced by organo- and lithotrophic, haloalkaliphilic, sulfate-reducing bacteria, a poorly studied functional group of the trophic network (Zhilina & Zavarzin, 1994). Only one bacterium representing this group of lithotrophic organisms, *Desulfonatronovibrio hydrogenovorans*, has to date been isolated in pure culture from Lake Magadi sediments (Zhilina *et al.*, 1997). Haloalkaliphilic isolates *Ectothiorhodospira* spp. and *Halorhodospira* spp., which constitute a distinct lineage of the phototrophic sulfur bacteria, have, however, been studied extensively (Thiemann & Imhoff, 1996).

In comparison with other East African Rift Valley soda lakes, the microbial diversity of Lake Natron, especially its southern part, has so far received little attention. The active volcano Ol Doinyo Lengai (2878 m), located close to this Tanzanian section of the lake, contains basalts rich in sodium and potassium and is so alkaline that its lava resembles washing soda. In order to isolate and characterize novel alkaliphilic microbes from this section of the lake, sediment samples were collected in the dry period from both

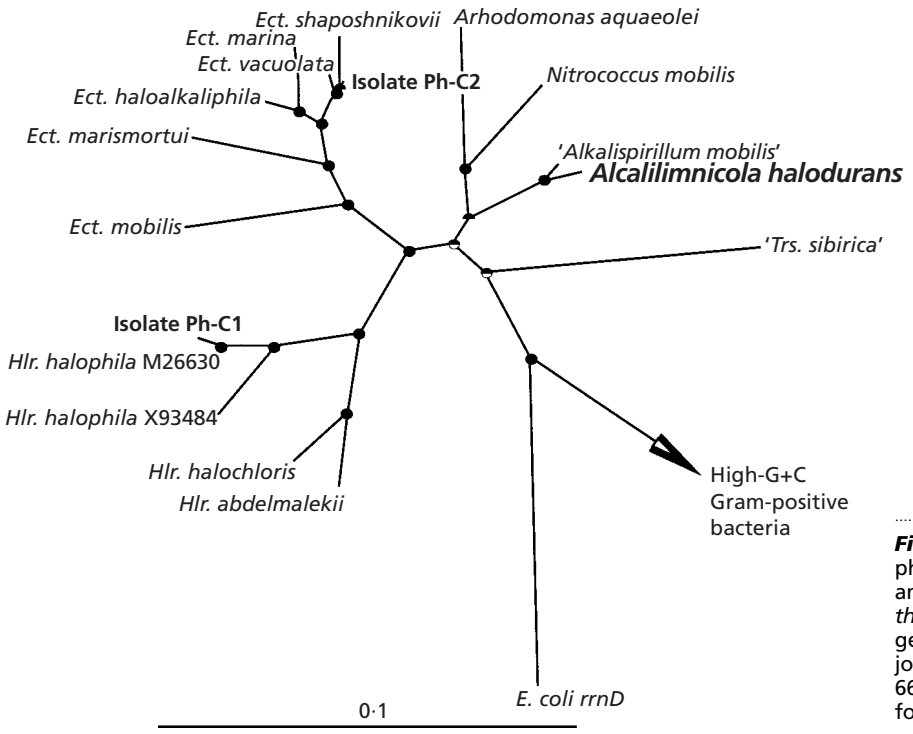

Fig. 7. Estimated phylogenetic position of phototrophic isolates Ph-C1 and Ph-C2 among the members of family *Ectothiorhodospiraceae*, derived from 16S rRNA gene sequence comparisons. The neighbour-joining tree is based on the analysis of 665 nucleotide positions. See Fig. 5 legend for further details.

underneath the drying soda salts and from water-covered sites of the lake. These environments are strongly alkaline (pH > 10) and vary in salinity from 20% (w/v) (site 1) to 2% (w/v) (site 2). Numerically abundant anaerobic sulfur phototrophic bacteria were isolated by extinction dilution culture. Two different purple phototrophic organisms that deposited elemental sulfur outside the cells were obtained from the last positive dilutions of sediment slurries obtained from trona mud (10^{-6}) and water sediments (10^{-5}). Both isolates were obligate alkaliphiles, but differed in their salt requirements: Ph-C1 represents the group of most haloalkaliphilic, anaerobic, phototrophic bacteria known so far, whereas the growth of Ph-C2 was strongly inhibited at salinity higher than 70 g NaCl l^{-1}. Partial sequencing of their 16S rRNA genes revealed that Ph-C1 and Ph-C2 were closely affiliated to the extremely halophilic *Halorhodospira halophila* and to the moderately halophilic *Ectothiorhodospira vacuolata*, respectively (Fig. 7).

When the last positive dilutions were plated on solid media with corresponding salinities and cultivated under anoxic conditions in the light, two types of colonies appeared. The first type was typical of purple phototrophic bacteria, while the second type, identical on both low- and high-salt media, was characterized by small, flat, non-pigmented, irregularly shaped colonies. The phenotypes of these isolates, designated 3S and 34Alc^T (such as isolation from an alkaline environment, restricted profile of nutrients, moderate halophily, extreme halotolerance and high G+C content) are similar in many respects to those of members of the phototrophic genus *Halorhodospira*. Analysis of their polar lipids revealed the presence of 17 different phospholipids, including eight different cardiolipins, the most diverse class of detected phospholipids. Three of these cardiolipins, CL13, CL15 and CL16, have not been described previously. Such cardiolipin diversity has only been described previously for halophilic strains of *Halorhodospira halophila* (Imhoff & Thiemann, 1991). The PLFA profile of *Alcalilimnicola halodurans* 34Alc^T is also similar to those of moderately/extremely halophilic members of the genus *Halorhodospira*. However, *Alcalilimnicola halodurans* is not a phototrophic organism and does not have any intracellular structures or photosynthetic pigments typical of phototrophic bacteria.

The results of the 16S rRNA sequence analysis showed that *Alcalilimnicola halodurans* represents a deeply branching lineage within the family *Ectothiorhodospiraceae* in the *γ-Proteobacteria* of the eubacterial kingdom and is a separate genus, related specifically to the undescribed '*Alkalispirillum mobilis*' and to *Arhodomonas aquaeolei* (97·74 and 92·33% identity in a 1472 bp overlap, respectively). *Arhodomonas aquaeolei* is an obligate halophile with the pH range of growth from 6·0 to 8·0 and can be additionally differentiated from *Alcalilimnicola halodurans* by nutritional requirements and PFLA profile.

Since there are significant phenotypic and phylogenetic differences between our isolate (strain 34Alc^T) and the previously described members of the family *Ectothiorhodospiraceae*, we propose a novel genus and species, *Alcalilimnicola halodurans* gen. nov., sp. nov., for this organism.

Description of *Alcalilimnicola* gen. nov.

Alcalilimnicola (Al.ca.li.lim.ni′co.la. N.L. n. *alcali* from Arabic n. *al qaliy* soda ash; Gr. n. *limnos* lake; L. suffix *cola* a dweller of; N.L. masc. n. *Alcalilimnicola* a dweller of alkaline lakes).

Gram-negative, short, straight, oval-shaped cells occurring singly or in pairs. Motile by means of a single polarly inserted flagellum. Non-spore-forming and non-encapsulated cells, usually producing an extracellular matrix of non-fibrillar appearance. Respiratory metabolism. Catalase- and oxidase-positive. Nitrate but not nitrite is reduced. Non-phototrophic, without any observable internal photosynthetic membranes. No photosynthetic pigments are produced under conditions that favour the phototrophic mode of metabolism. Optimal growth is observed under alkaline conditions (pH > 8·5). Members of this genus are moderately halophilic and extremely halotolerant organisms. Storage products are detected as large amounts of electron-translucent intracellular storage material, similar to polyhydroxybutyrate. Phylogenetically, the genus is placed in the *γ-Proteobacteria* within the family *Ectothiorhodospiraceae*. The G+C content of the DNA of the type strain of the type species is 65·6 mol%. The type species is found in extremely alkaline sediments of Lake Natron with moderate to extremely high salinities. The type and only species is *Alcalilimnicola halodurans*.

Description of *Alcalilimnicola halodurans* sp. nov.

Alcalilimnicola halodurans (ha.lo.du′rans. Gr. n. *hals* salt; L. pres. part. *durans* enduring; N.L. part. adj. *halodurans* salt-enduring).

Gram-negative, non-spore-forming, oval-shaped rods that are about 1·5 μm wide and 2–6 μm long and occur singly or in pairs. Motile by means of a single polar flagellum about 7 μm long. Colonies on DSM 430M medium are transparent and flat; they are 2–3 mm in diameter after 72 h of incubation. The optimal pH for growth is above 8·5; the organism grows in the presence of 0–28% (w/v) NaCl and at temperatures between 20 and 55 °C; optimal growth occurs at 35 °C and 3–8% (w/v) NaCl. Aerobic. Respiratory metabolism with oxygen as the terminal electron acceptor. Nitrate is reduced to nitrite. Catalase- and oxidase-positive. Strains exhibit tweenase activity, while agarase, amylase, arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, gelatinase and aesculinase activities are not detected. Growth is poor on complex bacteriological media and media containing peptides and amino acids. Cells can utilize a limited number of simple organic compounds, such as short-chain fatty acids, alcohols and some sugars, namely acetic acid, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-glucuronic acid, *β*-hydroxybutyric acid, *γ*-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, DL-lactic acid, succinic acid, succinamic acid, urocanic acid, inosine, 2-aminoethanol, 2,3-butanediol, glucose 6-phosphate, cyclodextrin, dextrin, Tween 80, *N*-acetyl D-galactosamine, *N*-acetyl D-glucosamine, L-arabinose, D-arabitol, cellobiose, *i*-erythritol, fructose, L-fucose, D-galactose, gentibiose, α-D-glucose, α-lactose, α-D-lactose lactulose, L-maltose, D-mannitol, D-mannose, D-melibiose, methyl *β*-D-glucoside, D-psicose, L-rhamnose, turanose and methylpyruvate. The following compounds are not utilized as sole carbon and energy sources: adonitol, D-sorbitol, L-sorbite, sucrose, xylitol, D-xylose, D-trehalose, monomethyl succinate, *m*-inositol, raffinose, formic acid, D-gluconic acid, D-glucosaminic acid, α-hydroxybutyric acid, α-ketoglutaric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, alaninamide, glucuronamide, D-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, aminobutyric acid, uridine, thymidine, phenylethylamine, putrescine, glycerol, DL-α-glycerol phosphate, glucose 1-phosphate, glycogen and Tween 40. Cells are susceptible to chloramphenicol and sulfomethoxazole and insensitive to ampicillin, gentamicin, kanamycin, nalidixic acid, streptomycin, trimethoprim, tetracycline and vancomycin. The major components of the PLFA are $C_{16:0}$ (23·4%), $C_{18:1}$ (54·0%) and $C_{18:0}$ (12·1%). Main phospholipids are phosphatidylglycerols and cardiolipins while some phosphatidylethylamines and phosphatidylcholines are also present. The G+C content of the DNA is 65·6 mol%.

The only and type strain is strain 34Alc^T (= DSM 13718^T = LMG 20111^T), which was isolated from the sediments of the soda-depositing Lake Natron, Tanzania.

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