International Journal of Systematic and Evolutionary Microbiology (2001), **51**, 1245–1256 Printed in Great Britain

Alkaliphilus transvaalensis gen. nov., sp. nov., an extremely alkaliphilic bacterium isolated from a deep South African gold mine

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A novel extreme alkaliphile was isolated from a mine water containment dam at 3·2 km below land surface in an ultra-deep gold mine near Carletonville, South Africa. The cells of this bacterium were straight to slightly curved rods, motile by flagella and formed endospores. Growth was observed over the temperature range 20–50 °C (optimum 40 °C; 45 min doubling time) and pH range 8·5–12·5 (optimum pH 10·0). The novel isolate, one of the most alkaliphilic micro-organisms yet described, was a strictly anaerobic chemo-organotroph capable of utilizing proteinaceous substrates such as yeast extract, peptone, tryptone and casein. Elemental sulfur, thiosulfate or fumarate, when included as accessory electron acceptors, improved growth. The G+C content of genomic DNA was 36·4 mol%. Phylogenetic analysis based on the 16S rDNA sequence indicated that the isolate is a member of cluster XI within the low G+C Gram-positive bacteria, but only distantly related to previously described members. On the basis of physiological and molecular properties, the isolate represents a novel species, for which the name *Alkaliphilus transvaalensis* gen. nov., sp. nov. is proposed (type strain SAGM1^T = JCM 10712^T = ATCC 700919^T). The mechanism of generation of the highly alkaline microbial habitat and the possible source of the alkaliphile are discussed.

Keywords: *Alkaliphilus transvaalensis* gen. nov., sp. nov., alkaliphile, anaerobic, deep subsurface, gold mine

INTRODUCTION

The existence of micro-organisms in geologically diverse deep terrestrial and oceanic subsurface environments has been often noted and is of increasing scientific and practical interest. Microbial communities in the deep terrestrial subsurface environments play a significant role in geochemical processes and are the only life forms that have been encountered in the deeper regions of the Earth's crust. Subsurface micro-organisms with novel metabolic properties may be of potential value to industry for applications in bio-remediation and biotechnology (Bale *et al*., 1997; Boone *et al*., 1995; Chandler *et al*., 1998; Gold, 1992; Kieft *et al*., 1999; Krumholz *et al*., 1999, 1997; L'Haridon *et al*., 1995; Stetter *et al*., 1993; Stevens & McKinley, 1995; Takai & Horikoshi, 1999a, b; Whitman *et al*., 1998). The subsurface biosphere is spatially expansive and contains a vast diversity of potential microbial habitats, including many that approach the known limits of life. Populations of micro-organisms have been found in a variety of subsurface environments including hot formation water associated with deep oil reservoirs (Jeanthon *et al*., 1995; L'Haridon *et al*., 1995; Orphan *et al*., 2000; Ravot *et al*., 1995; Slobodkin *et al*., 1999; Stetter *et al*., 1993; Takahata *et al*., 2000), extremely oligotrophic ground waters in deep crystalline rocks (Stevens & McKinley, 1995), inside ancient subterranean salt

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Abbreviations: FAMEs, fatty acid methyl esters; Ga, Giga-annum; kmbls, km below land surface; MSD, mass selective detector; PLFA, phospholipid fatty acid.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of SAGM1^T is AB037677.

deposits (Grant *et al*., 1998) and the > 250 °C water of a deep geothermal water pool (Takai & Horikoshi, 1999b). In spite of the interest in these findings, many of these microbial habitats remain poorly characterized, mainly due to the difficulties associated with access and sampling. Explorations of extreme microbial communities, whether indigenous or altered by human activity, allow investigators to probe the potential genetic diversity of the subsurface biosphere. Also, the presence of extremophiles in the deep subsurface should prompt a re-evaluation of the spatial boundaries and biomass potentials of the global biosphere (Whitman *et al*., 1998).

The gold mines of South Africa are among the deepest excavations in the world and provide a unique opportunity for the direct exploration of the deep subsurface without the technical challenges and costs associated with deep drilling and coring (Fredrickson & Phelps, 1996; Russell, 1997). The stratigraphic sequence in the Transvaal region south of Johannesburg, where the sampled mines are located, has been well characterized. Beginning at the surface and extending to 0·5 km below land surface (kmbls) are the sandstones of the Pretoria Group. Below this, from 0·5 to 1·6 km, lie the 2·3 Ga (Giga-annum or 2300 million years) Transvaal Supergroup dolomites. These ultimately transition into the Ventersdorp Supergroup andesitic lavas (2·7 Ga), at this location extending to a depth of > 4 km. The gold-bearing 'reefs' within the quartzites of the Witwatersrand Supergroup (2·9 Ga) dip beneath the Ventersdorp lavas and are intersected in these mines by a series of hanging wall drives ranging in depths from ca. 2·8 to 3·4 km. These mines harbour a number of unique environments, both natural and anthropogenic, in which micro-organisms reside. These include high temperature/pressure ground water systems, endolithic habitats, hypersaline brines, and mine process and drainage waters ranging from acidic to strongly alkaline that provide opportunities for the isolation and characterization of extremophiles physiologically adapted to the geological and geophysical features of these mines.

In this paper, the cultivation, physiology, phylogeny and habitat of a novel alkaliphilic anaerobic bacterium from these mines are described. The novel organism grew at pH values as high as 12·5 and is thus one of the most alkaliphilic micro-organisms described to date. Phylogenetic analysis based on 16S rRNA sequences indicate that the isolate is a member of cluster XI within the low G+C Gram-positive bacteria; however, it bears only a distant relationship to the previously described members. The genus name *Alkaliphilus* is therefore proposed for this isolate.

METHODS

Sampling site. The water sample used to inoculate alkaliphile enrichments was collected from a containment dam located at a depth of 3·2 kmbls within the Ventersdorp Supergroup lavas. The dam was a concrete-lined basin, 4 × 4 m and 1 m deep, one-half filled with stagnant water located near the 46 level hanging wall drive of a gold mine owned and operated by Driefontein Consolidated (Carletonville, South Africa). Directly above the dam, a passage had been drilled in the mid-1980s connecting to 44 level, approximately 74 m above. No major influx of new water or other disturbance was noted during the 5 months prior to water sample collection on 5 February 1999. A 43 mm diameter borehole with a slow discharge of ground water was located in the mine wall directly over the edge of the containment dam that delivered saline ground water to the dam at a rate calculated from the flow rate at ca. 18 ml h^{-1} at the time of sampling.

Samples representative of the possible source waters into the dam were collected for comparative purposes. Mine service water was collected from a distribution pipe on 46 level. Water from the overlying dolomite aquifer was collected from a collection well in the neighbouring #4 shaft internal pump compartment at a depth of 1·8 kmbls. Due to the extremely slow discharge rate of the borehole nearest the containment dam, a substitute with more reliable flow characteristics was sampled several metres further along the horizontal access shaft. This borehole, drilled in 1994, extends for 120 m into the country rock, where it intersects a water-bearing andesitic dyke. The flow rate of water from this borehole was > 1500 ml h^{-1} on 2 February 1998 when the sample was collected. The borehole had been plugged and valved to exclude air for 3 months prior to sampling. A summary of select physical and chemical properties of the containment dam and possible source waters is presented in Table 1.

Sample collection and chemical analyses. Water and soft marl sediment from the base of the dam were collected for microbiological analysis in sterile 150 ml serum vials. The vials were filled to overflow, capped with butyl rubber stoppers, crimp-sealed and transported to the surface in a cooler on blue ice and held at 4 °C until processed. The *in situ* water temperature was recorded with a digital thermometer probe suspended 10 cm beneath the water surface. The *in situ* pH was measured using a hand-held Piccolo pH Meter (Hanna Instruments). Redox potential was measured on site with a platinum redox probe (Orion Research) and the value obtained was corrected according to the method of Zobell (1946). Water for oxygen determinations was collected in duplicate 250 ml BOD bottles, fixed with reagent powder pillows and titrated with $Na_2S_3O_4$ using an adaptation of the Winkler method (Wetzel & Likens, 1991).

Water for cation and anion analyses was collected in 50 ml plastic syringes and filtered with a 0·2 μm filter into 50 ml plastic bottles. Bottles for cations were pre-loaded with 2·5 ml concentrated HNO_3 as a preservative. The concentrations of select cations, Br and I were measured by inductively coupled plasma spectrometry (Activation Laboratory, Ancaster, ON, USA). Concentrations of F^-, Br^-, NO_3^-, NO_2^-, SO_4^{2-} and PO_4^{3-} were measured by ion chromatography (Dionex). Ammonia concentrations were determined according to the following protocol: 2 drops dechlorinating agent (3·5 g $Na_2S_2O_3 . 5H_2O$ l^{-1}), 1 drop EDTA solution (50 g Na . EDTA in 60 ml 4·2 M NaOH) and 2 ml Nessler reagent (100 g HgI_2 and 70 g KI added slowly to 500 ml 8 M NaOH and diluted to 1 l) were added to 22 ml sample. Samples were subsequently filtered (0·4 μm) and absorption at 425 nm was measured (Perkin Elmer model 55 spectrophotometer).

Samples for total inorganic carbon were collected in 300 ml borosilicate glass bottles, fixed with 200 μg saturated $HgCl_2$ solution and sealed with Apiezon glass stoppers. Total inorganic carbon was determined using a SOMMA system

Table 1. Summary of physical and chemical parameters of sampling site

Parameter	Containment dam	Service water	Dolomite water	Borehole water
Depth (km)	3·218	3·218	0·98	3·218
Temperature (°C)	34·2	17·2	25	37·2
pH	11·63	6·5	7·0	7·4
Eh (mV)	106	164	165	−166
Air temperature (°C)	36·3	NA	NA	NA
Air relative humidity (%)	30	NA	NA	NA
Dissolved oxygen (mg l^{-1})	3·3	8·8	1·5	ND
Total inorganic carbon (mg l^{-1})	0·86	11·3	45·45	6·79
Cl^- (mg l^{-1})	1780	217	16	22600
Br^- (mg l^{-1})	18	6·5	ND	142
NO_3^- (mg l^{-1})	17·4	128	ND	ND
SO_4^{2-} (mg l^{-1})	91	55	80	92·6
Na^+ (mg l^{-1})	391000	152000	13000	5960000
Si^+ (mg l^{-1})	ND	10100	7230	8810
Ca^{2+} (mg l^{-1})	840000	118000	69300	6789000

NA, Not analysed; ND, not detected.

and UIC model 5011 coulometer following standard methods (Johnson *et al*., 1987). Aqueous speciation and reactions were simulated using PHREEQE (Parkhurst *et al*., 1980).

Enrichment and purification. A slurry of water and sediment was used to inoculate a series of media including standard medium (SM) (described below) under a gas phase of 80% N_2 and 20% CO_2 (200 kPa) incubated at 37 °C. All tubes of SM inoculated with the sample became turbid after 2 d at 37 °C and contained motile, straight to slightly curved rods. To obtain pure cultures, the positive enrichments were streaked onto solid SM plates solidified with 1·2% (w/v) gelrite gellan gum (Sigma). After 3 d incubation at 37 °C in an anaerobic jar under a gas phase of 80% N_2 and 20% CO_2, the plates were examined in an anaerobic chamber maintained at 2% H_2, the balance in N_2 (Coy Instruments). Only one colony type was noted and well-isolated colonies were picked and inoculated into fresh liquid SM. To be assured of obtaining pure cultures, the streaking and isolation steps were repeated at least three times for each of four isolates.

Medium and culture conditions. The novel isolate was routinely cultivated in SM, consisting of mj water [10-fold-diluted MJ(-N) synthetic seawater (Takai *et al*., 2000)] containing (l^{-1}): yeast extract, 2 g; tryptone, 2 g; sodium citrate, 1 g; sodium tartrate, 1 g; $NaNO_3$, 1 g; ferric citrate, 1·2 g; $Na_2S_2O_3 . 5H_2O$, 2·48 g; $Na_2CO_3 . H_2O$, 40 g; and 10 ml trace mineral solution (Balch & Wolfe, 1976). For testing the effects of pH on growth, the pH of SM was adjusted prior to sterilization to various levels by decreasing the amount of Na_2CO_3 (below pH 10·5) or by the addition of KOH (above pH 10·5). The medium was then purged with O_2-free N_2 and autoclaved under the resulting nitrogen headspace. After autoclaving, the final pH of the medium was measured and re-adjusted with sterile KOH if necessary. The pH adjustments were performed in an anaerobic chamber under a gas phase of 2% H_2, the balance as N_2. The pH of media prepared in this manner was unaffected by bacterial growth. All anaerobic cultivations employed the techniques described by Balch *et al*. (1979). For routine cultivation, the medium was dispensed at 20% of the total volume of a given bottle or tube under a gas phase consisting of 80% N_2/20% CO_2 at 200 kPa and then tightly sealed with a butyl rubber stopper. No reducing reagents such as sodium sulfide (Na_2S) and cysteine . HCl were used.

All experiments described below were conducted in duplicate. In an attempt to examine whether or not potential electron donors and acceptors supported or stimulated growth, substitutions to the standard combinations of $NaNO_3$, ferric citrate and $Na_2S_2O_3$ present in SM were made. For example, nitrogen compounds (10 mM NH_4Cl or $NaNO_3$), sulfur compounds (10 mM Na_2S, cysteine . HCl, Na_2SO_4, Na_2SO_3, $Na_2S_2O_3$, $Na_2S_2O_5$ or 3% S^0), iron compounds (5 mM ferric citrate, ferric nitrilotriacetate, $FeCl_2$ and $FeSO_4$) and an organic acid (sodium fumarate) were used solely or in various combinations under differing gas phases.

For testing the effects of sea salts concentration on growth, dilutions of MJ(-N) synthetic seawater were supplemented with 0·2% (w/v) yeast extract, 0·2% tryptone, 0·1% sodium citrate, 0·1% sodium tartrate, 0·1% $NaNO_3$, 5 mM ferric citrate, 10 mM $Na_2S_2O_3 . 5H_2O$, 4% $Na_2CO_3 . H_2O$ and 10 ml trace mineral solution. Autotrophic growth was tested in mj water containing 0·1% (v/v) vitamin solution (Balch *et al*., 1979), 10 mM NH_4NO_3, 10 mM $Na_2S_2O_3 . 5H_2O$ and 4% $Na_2CO_3 . H_2O$ under a gas phase of 80% H_2/20% CO_2 (200 kPa) or 80% N_2/20% CO_2 (200 kPa) for anaerobic conditions and a gas phase of 60% H_2/10% CO_2/25% N_2/5% O_2 (200 kPa) for microaerobic conditions.

Light and electron microscopy. Cells were routinely observed by phase-contrast microscopy (Optishot 2; Nikon). Transmission electron microscopy of negatively stained cells was carried out as described by Zillig *et al*. (1990). Cells grown in SM at 37 °C to the mid-exponential phase were negatively stained with 2% (w/v) uranyl acetate and observed under a JEOL JEM-1210 electron microscope at an accelerating voltage of 80 kV.

Measurement of growth. Growth of the novel isolate was measured by direct cell count after staining with 4′,6-diamidino-2-phenylindole (Porter & Ffeig, 1980) using a Nikon Optishot microscope. Cultures were prepared in

duplicate. The cells were grown in 100 ml glass bottles (Schott Glaswerke), each containing 20 ml medium, without shaking in temperature-controlled air incubators. Growth as a function of pH was determined at 37 °C and the growth conditions for all other cultivation tests were 37 °C and pH 10·5, unless otherwise noted. H_2S and H_2 production during growth was tested using H_2S and H_2 detection tubes, respectively (GASTEC).

Nutrition. In an attempt to identify organic substrates that could support the growth of the isolate, experiments were conducted in which the yeast extract, tryptone, citrate and tartrate in SM were replaced with other organic compounds in the presence or absence of $Na_2S_2O_3$. To evaluate the potential stimulation of growth with proteinaceous substrates, various organic materials were added to medium containing yeast extract or tryptone. In these experiments, ferric citrate was removed from the media. The cells were pre-cultured in each medium prior to inoculation of the same medium. These tests were performed in duplicate at 37 °C.

Phospholipid fatty acid (PLFA) analysis. Cellular fatty acid composition was analysed using the procedures described by White & Ringelberg (1998). Cells of strain SAGM1^T were cultivated in SM at 37 °C, harvested by centrifugation, frozen at −80 °C and lyophilized. After extracting the lyophilized pellet by a modified single phase chloroform-methanol-phosphate buffer procedure (Bligh & Dyer, 1959; White *et al*., 1979), the total extractable lipid was fractionated on a silicic acid column. The polar lipid fraction was collected and transesterified into fatty acid methyl esters (FAMES) by mild alkaline methanolysis for GC analysis (Guckert *et al*., 1985; White & Ringelberg, 1998).

GC analysis of FAMES was performed with a Hewlett Packard 5890 GC equipped with a ZB-1 non-polar dimethyl-polysiloxane capillary column (50 m length, 0·25 mm ID, 0·25 μm film thickness; Phenomenex), a flame-ionization detector and a split/splitless injector using the conditions described by Ringelberg *et al*. (1997). Derivatized FAMES were dissolved in hexane, containing nonadecanoic acid methyl ester as the internal standard. The temperature programme was as follows: the initial temperature of 60 °C was held for 2 min, ramped at a rate of 10 °C min^{-1} to 150 °C, held at 150 °C for 2 min and then increased at a rate of 3 °C min^{-1} to a final temperature of 312 °C. An equal detector response was assumed for all components and identifications were made by comparison of chromatographic retention times to standards (Matreya). The identity of FAMES was verified using GC-MS.

Mass spectral verification of all lipid moieties was accomplished using a HP5971 mass selective detector (MSD) interfaced with an HP5890 series II GC equipped with a ZB-1 non-polar dimethyl-polysiloxane capillary column (60 m length, 0·25 mm ID, 0·1 μm film thickness). The temperature programme for this analysis was as follows: the initial temperature of 100 °C was immediately ramped 10 °C min^{-1} to 150 °C, held at 150 °C for 1 min and then increased at a rate of 3 °C min^{-1} to a final temperature of 282 °C, which was maintained for an additional 5 min. The MSD was run at 70 eV, using positive ion electron impact ionization.

Fatty acid nomenclature is based on the fatty acid abbreviated by the number of carbon atoms (A), a colon, number of unsaturations (B) followed by the omega symbol (ω) followed by the number of carbons (C) from the methyl end of the molecule to the position of the unsaturation (i.e. A:BωC). For monoenoic fatty acids, the A:BωC molecule is followed by the suffix '*c*' for the *cis* or '*t*' for the *trans* configuration. Branched fatty acids are described by iso (i) or anteiso (a) if the methyl branch is one or two carbons from the ω end or by the position of the methyl group from the carboxylic end of the molecule. The symbol 'br' is used if the methyl branching occurs at an undetermined position. The symbol 'cy' indicates cyclopropyl fatty acids and is followed by the total number of carbons. The location of hydroxyl groups (OH) is numbered from the carboxyl end of the fatty acid (White & Ringelberg, 1998).

Isolation and base composition of DNA. DNA was prepared as described by Marmur & Doty (1962). The G+C content of DNA was determined by direct analysis of deoxyribonucleotides by HPLC (Tamaoka & Komagata, 1984). Non-methylated DNA from bacteriophage lambda (49·8 mol% G+C; TaKaRa) (Sanger *et al*., 1982) was used as a reference.

Amplification of 16S rRNA gene and sequence determination. The 16S rRNA gene (rDNA) was amplified by the PCR method using Bac 27F and 1492R primers (DeLong, 1992; Lane, 1991). The 1·5 kb PCR product was directly sequenced by the dideoxynucleotide chain termination method using a DNA sequencer (model 377; Perkin Elmer/Applied Biosystems). The rDNA sequence was analysed using the gapped-BLAST search algorithm (Altschul *et al*., 1997; Benson *et al*., 2000) to estimate the degree of similarity to other bacterial 16S rDNA sequences.

Data analysis. The nearly complete sequence (1393 bp) of the 16S rDNA of SAGM1^T was manually aligned to 16S rDNA data from the RDP based on primary and secondary structure considerations using the Genetic Data Environment multiple sequence editor. Phylogenetic analyses were restricted to nucleotide positions that could be unambiguously aligned (Takai & Horikoshi, 1999a, b; Takai & Sako, 1999). Least-squares distance matrix analysis (Olsen *et al*., 1986), based on evolutionary distances, was performed using the correction of Kimura (1980). Neighbour-joining and maximum-likelihood analyses were performed using the PHYLIP package (version 3.5; obtained from J. Felsenstein, University of Washington, Seattle, WA, USA). Bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies.

RESULTS

Enrichment and purification

Bacterial enrichments from the mixed alkaline water/sediment sample contained only motile, straight to slightly curved rods after incubation for 2 d. Using the anaerobic plating technique, four rod-shaped pure strains were successfully obtained. The purity of this culture was routinely confirmed by microscopic examination and repeated partial sequencing of the 16S rRNA gene using several PCR primers. Isolate SAGM1^T(= JCM 10712^T = ATCC 700919^T) was the first of several pure cultures obtained and was used exclusively in this study.

Morphology

Cells were straight to slightly curved rods with a mean length of 3–6 μm and a width of approximately 0·4–0·7 μm (Fig. 1a). As observed by light microscopy,

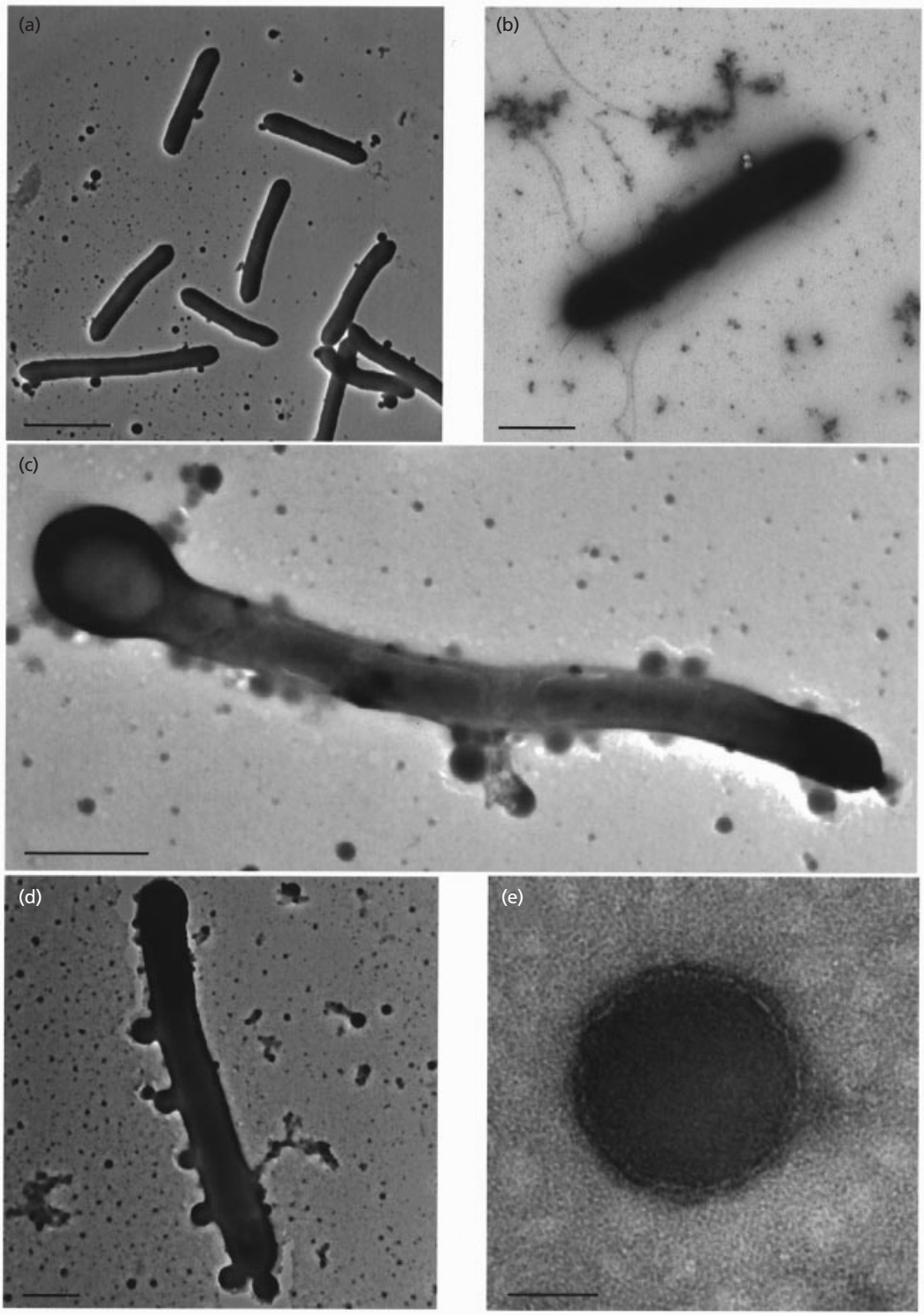

Fig. 1. Electron micrographs of negatively stained cells of *Alkaliphilus transvaalensis* in the mid-exponential phase of growth (a; bar, 2 μm), cells with flagella (b; bar, 1 μm), a swollen cell forming a spore in the stationary phase of growth (c; bar, 1 μm), a cell with several budding bodies (d; bar, 0·5 μm) and a budding body 'vesicle' observed in the medium (e; bar, 50 nm).

the cells were motile and transmission electron microscopy of negatively stained cells indicated that the cells possessed multiple flagella (Fig. 1b). In the exponential phase of growth, single and paired rods predominated. In the late exponential and stationary phases of growth, the rods tended to form chains of

Table 2. Effects of the gas phase and inorganic substances on autotrophic or heterotrophic growth of SAGM1^T

The temperature for cultivation was 37 °C and the pH of all media was 10·5 at room temperature. NG, No growth.

Medium	Electron acceptor/nitrogen source*	Gas phase	Max. cell yield (cells ml^{-1})
SM	$Na_2S_2O_3$ + $NaNO_3$ or NH_4Cl	80 % N_2/20 % CO_2 (2 atm)	$3{\cdot}0 \times 10^9$ (black precipitate)
SM (without $Na_2S_2O_3$)	$NaNO_3$ or NH_4Cl	80 % N_2/20 % CO_2 (2 atm)	$5{\cdot}0 \times 10^8$
SM	$Na_2S_2O_3$ + $NaNO_3$ or NH_4Cl	Air	NG
SM	$Na_2S_2O_3$ + $NaNO_3$ or NH_4Cl	95 % N_2/5 % O_2 (2 atm)	NG
SM (without organic nutrients)†	$Na_2S_2O_3$ + $NaNO_3$	80 % N_2/20 % CO_2 (2 atm)	NG
SM (without organic nutrients)†	$Na_2S_2O_3$ + $NaNO_3$	80 % H_2/20 % CO_2 (2 atm)	NG
SM	$Na_2S_2O_3$ + $NaNO_3$	80 % H_2/20 % CO_2 (2 atm)	$2{\cdot}0 \times 10^9$ (black precipitate)
SM (without $Na_2S_2O_3$)	$NaNO_3$	80 % H_2/20 % CO_2 (2 atm)	$8{\cdot}0 \times 10^7$
SM (without $Na_2S_2O_3$)	S^0 + $NaNO_3$	80 % N_2/20 % CO_2 (2 atm)	$1{\cdot}0 \times 10^9$ (black precipitate)
SM (without $Na_2S_2O_3$)	S^0 + $NaNO_3$	80 % H_2/20 % CO_2 (2 atm)	$1{\cdot}0 \times 10^9$ (black precipitate)
SM (without $Na_2S_2O_3$)	Fumarate + $NaNO_3$	80 % N_2/20 % CO_2 (2 atm)	$1{\cdot}0 \times 10^9$
SM (without $Na_2S_2O_3$)	Fumarate + $NaNO_3$	80 % H_2/20 % CO_2 (2 atm)	$1{\cdot}0 \times 10^9$

* Each substance was added to the medium at a concentration of 10 mM for $Na_2S_2O_3$, $NaNO_3$ or NH_4Cl, 3 % (w/v) for S^0 and 20 mM for fumarate.

† Yeast extract, tryptone, sodium citrate, sodium tartrate and ferric citrate were removed from SM.

four to six cells and swelled to form spherical terminal spores (Fig. 1c). Spore formation was enhanced when the isolate was grown in medium containing carbohydrates such as starch and glucose in addition to the yeast extract. The cells stained Gram-positive; electron microscopy revealed that cell division occurred inside the outer envelope (Fig. 1c). Spherical budding resulted in vesicle-like structures with a mean diameter 100–200 nm. These structures were observed attached to cells or free in the culture medium at all phases of growth (Fig. 1d). The 'vesicles' were surrounded by a cell membrane-like structure (Fig. 1e).

Growth parameters

The novel isolate grew only under strictly anaerobic culture conditions (Table 2) and had a strictly heterotrophic metabolism; it did not grow under any of the autotrophic culture conditions tested. Heterotrophic growth of the isolate was unaffected by changes in the H_2 concentration in the gas phase when either $Na_2S_2O_3$ or S^0 was provided. However, the presence of high concentrations of H_2 in the headspace resulted in strong inhibition of growth in the absence of $Na_2S_2O_3$ and S^0 (Table 2). Hydrogen gas was produced during growth in the absence of $Na_2S_2O_3$ and S^0, whereas hydrogen sulfide was produced as a major gas product in the presence of either sulfur compound.

The isolate grew at 20–50 °C, with optimum growth at 40 °C (Fig. 2). The generation time at 40 °C was about 45 min at pH 10·5. No growth was observed at 55 °C or higher. Growth of the new isolate at 37 °C occurred between pH 8·5 and 12·5, with optimum growth at approximately pH 10·0 (Fig. 2). No growth was detected below pH 8·5 or above pH 12·5. The novel

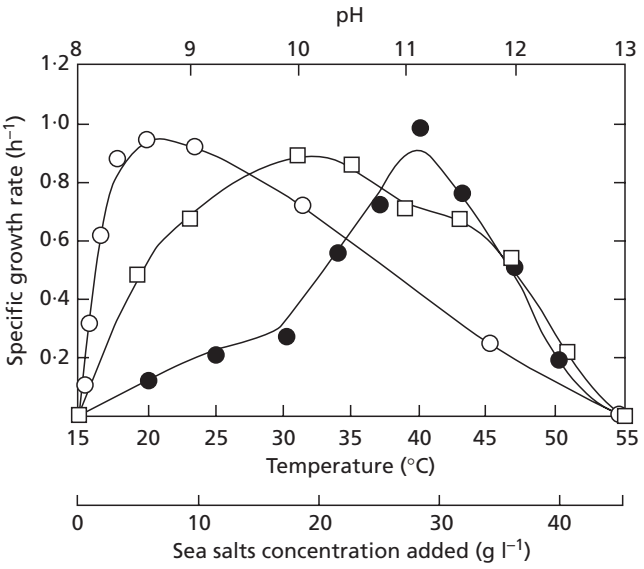

Fig. 2. Influence of temperature (●), pH (□) and sea salts concentration (○) on growth of *Alkaliphilus transvaalensis*. Growth was determined in SM at pH 10·5. No growth occurred below 20 °C or above 50 °C. Growth was determined in SM at 37 °C over a range of pH values. Growth was determined in varying dilutions of MJ(-N) synthetic seawater supplemented with 0·2 % (w/v) yeast extract, 0·2 % tryptone, 0·1 % sodium citrate, 0·1 % sodium tartrate, 0·1 % $NaNO_3$, 5 mM ferric citrate, 10 mM $Na_2S_2O_3 . 5H_2O$, 4 % $Na_2CO_3 . H_2O$ and 10 ml trace mineral solution at 37 °C and pH 10·5. No growth was observed at a sea salts concentration above 33 g l^{-1}.

isolate did not require sea salts for growth, but grew at sea salts concentrations up to 33 g l^{-1}, with optimum growth at $\sim$ 5 g l^{-1} at 37 °C and pH 10·5, corresponding to a concentration of about 10^{-1} of the ionic strength of seawater (Fig. 2).

Table 3. Substrates stimulating growth of *Alkaliphilus transvaalensis*

Cells were grown at 37 °C under a gas phase of 80 % N_2/20 % CO_2 (200 kPa) and the pH of all media was adjusted to 10·5 at room temperature. Medium I was equivalent to SM except that yeast extract, sodium citrate, sodium tartrate and ferric citrate were removed; other components were added at the concentrations given in the table. +++, $> 2{\cdot}0 \times 10^9$ cells ml^{-1}; ++, $> 8{\cdot}0 \times 10^8$ and $< 2{\cdot}0 \times 10^9$ cells ml^{-1}; +, $< 8{\cdot}0 \times 10^8$ cells ml^{-1}. Results for medium I alone or with any of the substrates added were '+'.

Substrate added	Medium I + 10 mM $Na_2S_2O_3$
None	++
Starch (0·2 %, w/v)	++
Sugars (20 mM)*	++
Citrate (20 mM)	++
Tartrate (20 mM)	+++
Succinate (20 mM)	+++
Malate (20 mM)	+++
Oxalate (20 mM)	++
Lactate (20 mM)	++
Acetate (20 mM)	++
Formate (20 mM)	++
Dimethyl formamide (0·1 %, v/v)	+
DMSO (0·1 %, v/v)	++
Glycerol (0·1 %, v/v)	++
n-Butanol (0·1 %, v/v)	+++
n-Propanol (0·1 %, v/v)	++
Ethanol (0·1 %, v/v)	++
Methanol (0·1 %, v/v)	++

* Sugars were tested with cellobiose, maltose, lactose, trehalose, sucrose, glucose, galactose, fructose, xylose and ribose at a concentration of 20 mM.

Nutrition

Organic substrates that supported heterotrophic growth were determined in the presence or absence of $Na_2S_2O_3$. In either case, the isolate grew in medium containing proteinaceous complex substrates such as yeast extract, peptone, tryptone and casein as a sole energy source. The presence of $Na_2S_2O_3$ improved growth with the proteinaceous substrates and induced black precipitates (presumably iron sulfide). S^0 and fumarate also improved growth with the proteinaceous substrates (Table 2, Table 3). Growth on the proteinaceous substrates increased with increasing concentration. No growth occurred with starch, Casamino acids, sugars (cellobiose, maltose, lactose, trehalose, sucrose, glucose, galactose, fructose, xylose or ribose), any of 20 amino acids, any of the organic acids tested (tartrate, succinate, malate, lactate, pyruvate, fumarate, formate or acetate), any of alcohols tested (n-butanol, n-propanol, ethanol or methanol), glycerol, DMSO or dimethyl formamide as sole energy sources.

To examine potential substrates improving fermentative growth with proteinaceous substrates, the effect of organic substances on growth was examined in the presence or absence of thiosulfate. Ravot *et al.* (1996) and Takai & Horikoshi (2000) have reported that the growth of extremely thermophilic, fermentative bacteria (*Thermosipho* members) utilizing only complex proteinaceous substrates as a sole energy source was greatly improved by the addition of carbohydrates. Unlike the growth of *Thermosipho* members, carbohydrates had no effect on cell yield during the growth with yeast extract or tryptone (Table 3). However, the addition of tartrate, succinate, malate or n-butanol enhanced the maximum cell yield during growth with the proteinaceous substrates in the presence of $Na_2S_2O_3$ (Table 3).

PLFA analysis and DNA base composition

GC analysis of the cellular fatty acids of the isolate revealed the following composition: 2·3 % iC13:0, 1·7 % C14:0, 9·2 % iC15:1ω7*c*, 51·6 % iC15:0, 2·8 % aC15:0, 1·9 % iC16:1ω7*c*, 3·9 % C16:0, 7·2 % iC17:1ω7*c*, 12·2 % iC17:0, 1·1 % iC18:1ω9*c* and 2·0 % C18:0. In general, the major branched-chain acids in *Clostridium* species contain 15, 16 or 17 carbon atoms and include minor proportions of monoenoic fatty acids. For example, *Clostridium symbiosum* has high proportions (44 %) of straight-chain, odd-chain fatty acids (O'Leary & Wilkinson, 1988). Branched-chain fatty acids are the predominant fatty acids (55–75 %) in thermophilic species such as *Clostridium thermocellum* (mainly i16:0), '*Clostridium tartarivorum*' and *Clostridium thermosaccharolyticum* (both mainly i15:0) (Chan *et al.*, 1971). The fatty acid profiles of SAGM1^T were similar to those observed for the thermophilic clostridia. The G+C content of the genomic DNA of strain SAGM1^T was 36·4 ± 0·5 mol %.

Phylogenetic analyses

The rDNA sequence of the strain SAGM1^T was most closely related to those of members of cluster XI within the low G+C Gram-positive bacteria (Cato *et al.*, 1986; Collins *et al.*, 1994; Rainey & Stackebrandt, 1993) that include *Clostridium felsineum* (91·8 %) (Cato *et al.*, 1986; Mahony *et al.*, 1977), *Clostridium formicoaceticum* (Cato *et al.*, 1986; Ravot *et al.*, 1999) (90·1 %), *Clostridium halophilum* (87·8 %) (Andreesen *et al.*, 1970), *Acidaminobacter hydrogenoformans* (88·3 %) (Fendrich *et al.*, 1990), *Fusibacter paucivorans* (89·1 %) (Meijer *et al.*, 1999) and *Tindallia magadiensis* (90·4 %) (Kevbrin *et al.*, 1998). This result indicates that the novel isolate belongs to cluster XI within the low G+C Gram-positive group, even though it is phylogenetically distant from any of the genera within cluster XI. To determine the phylogenetic relationship of the isolate to other cluster XI members, phylogenetic trees were constructed based on the neighbour-joining and maximum-likelihood methods.

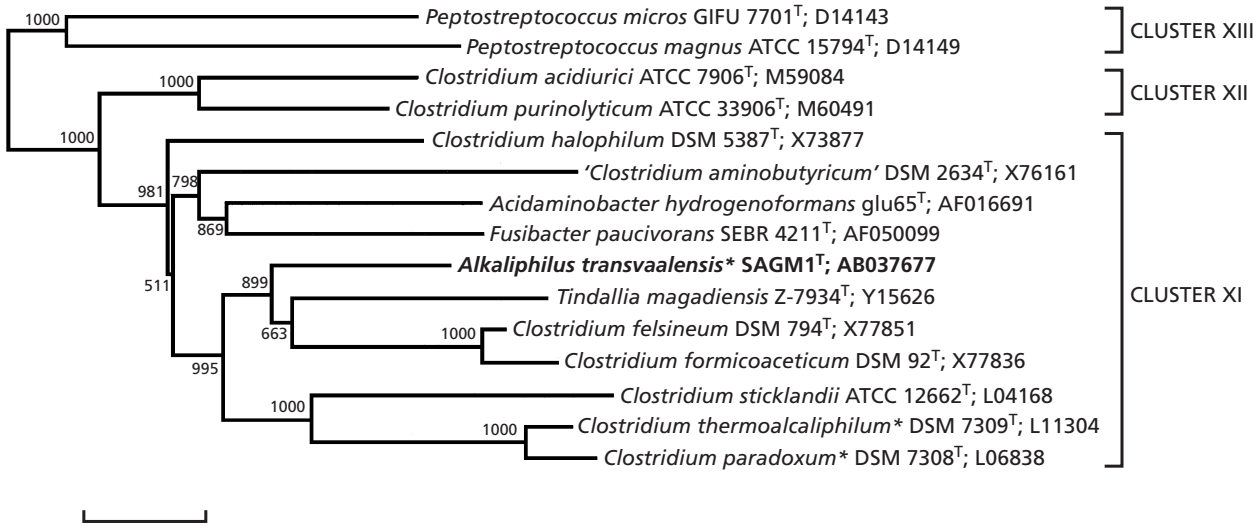

Fig. 3. Phylogenetic tree of representative members of cluster XI within the low G+C Gram-positive group inferred from 16S rDNA sequences by the neighbour-joining method using 1280 homologous sequence positions for each organism. Asterisks indicate true alkaliphiles not growing in neutral pH range. Each number represents the bootstrap value for branching (1000 replicates). Bar, 2 substitutions per 100 nt.

Phylogenetic analyses by the neighbour-joining and maximum-likelihood methods revealed identical topology, placing this organism prior to the divergence between *Tindallia* and *Clostridium* [according to Collins *et al*. (1994), *C. felsineum* and *C. formicoaceticum* should be classified as members of a new genus in a new family within clostridia and their close relatives] and distantly from any other genera (Fig. 3). On the basis of its phylogenetic placement, the novel isolate is likely of an ancestral lineage to *T. magadiensis* and *C. felsineum*, *C. formicoaceticum* representing a new genus within cluster XI. The bootstrap confidence estimate also revealed high significance in the placement of SAGM1^T (Fig. 3).

DISCUSSION

Strain SAGM1^T was isolated from alkaline waters within a containment dam located in a deep gold mine in South Africa. The organism was an extreme alkaliphile, growing only at pH 8·5–12·5. To the best of our knowledge, this bacterium is one of the most alkaliphilic micro-organisms yet described (Horikoshi, 1999; Krulwich & Guffanti, 1989). The isolate was a strictly anaerobic heterotroph, capable of growth using proteinaceous substrates such as yeast extract, peptone, tryptone and casein. Elemental sulfur, thiosulfate and fumarate were reduced during growth and significantly improved the growth yield and rate. Although fermentative metabolism is common among members of cluster XI and typical within the members of clostridia and close relatives, SAGM1^T displayed a distinctive fermentative growth, utilizing only complex proteinaceous substrates as sole energy sources.

Comparisons of physiological and chemotaxonomic properties indicated that SAGM1^T was distinct from other members of cluster XI within the low G+C Gram-positive group. Among the members of cluster XI, several true alkaliphilic species have been previously described (Collins *et al*., 1994; Kevbrin *et al*., 1998; Li *et al*., 1994, 1993). *Clostridium paradoxum* and *Clostridium thermoalcaliphilum* are thermophilic alkaliphiles, isolated from sewage plants, that grow at pH 8·0–10·5 (Li *et al*., 1994, 1993). While this group conforms to a physiotypic unit on the basis of pH range for growth, the known members differ markedly from SAGM1^T in temperature range for growth, substrate utilization patterns and chemical taxonomic properties (Table 4). *T. magadiensis* is an alkaliphilic anaerobic ammonifier that was isolated from soda deposits in hypersaline Lake Magadi, Kenya (Kevbrin *et al*., 1998). This organism is also a true alkaliphile, like SAGM1^T, but has a lower pH range for growth (Table 4) and can only ferment a few amino acids (Kevbrin *et al*., 1998). Although the novel isolate is most closely related to *C. felsineum* based on16S rDNA sequence similarity, SAGM1^T differs from this species in several important ways. Unlike SAGM1^T, *C. felsineum* is neutrophilic and able to ferment various sugars, grows over a narrower temperature range (25–45 °C) and has a lower genomic DNA G+C content (Table 4) (Cato *et al*., 1986; Mahony *et al*., 1977). Phylogenetic analysis revealed that SAGM1^T occupies a distant relationship from previously described members of cluster XI, bifurcating at the genus level. On the basis of the distinct physiological and phylogenetic features of the isolate, a novel genus, *Alkaliphilus*, is therefore proposed. The type species is *Alkaliphilus transvaalensis* and the type strain is *Alkaliphilus transvaalensis* SAGM1^T(= JCM 10712^T = ATCC 700919^T).

Table 4. Comparison of properties among closely related strains of the genera of *Alkaliphilus*, *Tindallia* and *Clostridium*

Character	*Alkaliphilus transvaalensis* SAGM^T	*Tindallia magadiensis* Z-7934^T	*Clostridium felsineum* DSM 794^T
Cell size (μm)	3–6 × 0·4–0·7	1·2–2·5 × 0·5–0·6	3·1–25·7 × 0·5–1·3
Motility	+	−	+
Spore formation	+	−	+
Cell wall structure	Gram-positive	Gram-positive	Gram-positive
Temperature optimum (°C)	40	37	37
pH optimum	10·0	8·5	Neutral pH
pH upper limit	12·5	10·5	ND
External electron acceptor	Elemental sulfur, thiosulfate, fumarate	DMSO, ferric iron	ND
Substrate			
Tryptone or peptone	+	+	+
Casein	+	−	−
Fructose	−	−	+
Galactose	−	−	+
Mannose	−	−	+
Sucrose	−	−	+
Xylose	−	−	+
Arginine	−	+	ND
Ornithine	−	+	ND
Pyruvate	−	+	+
Citrate	−	+	ND
G + C content (mol %)	36·4	37·6	26·0
Reference	This study	Kevbrin *et al.* (1998)	Mahony *et al.* (1977); Cato *et al.* (1986)

ND, Not determined.

The environment (see Table 1 for geochemical properties) from which SAGM1^T was derived is best characterized by a very high pH (11·6), high Ca^{2+}, low dissolved inorganic carbon (pCO_2 of 10–11 atm) and supersaturation with respect to calcite. This suggests that the pH of the water is being controlled by the dissociation of $Ca(OH)_2$ which is being acquired by the dissolution of portlandite in the cement of the containment dam. This hypothesis was verified by equilibrating service water with portlandite and with calcite at a saturation indices of 1 or 10 at 34 °C in a closed system (i.e. pCO_2 was not fixed). The service water was equilibrated with mine air before it was added to the portlandite. The partial pressure of CO_2 in the mine was 10–2·3 atm. (S. Kotelnikova, personal communication). This elevated the pH of the service water and reduced the total dissolved inorganic carbon slightly, but had little effect on the outcome of the simulation. The log of the equilibrium constant at 25 °C and enthalpy used for the portlandite dissociation reaction were 23 and 30·7, respectively (HSC Chemistry, version 2.0). The simulation yielded a pH of 12·1, a pCO_2 of 10–11·5 atm and a calcium concentration equal to that measured (Table 1). Closed system equilibrium, however, produced only 5×10^{-4} mol calcite l^{-1}; this value is insufficient to account for the mass of carbonate crust and marl at the base of the containment dam. In contrast, in an open system disequilibrium, where a constant source of inorganic carbon is allowed, the continuous precipitation of carbonate and dissolution of portlandite would account for the observed calcite accumulation. Geochemical analyses indicated that the *in situ* pH of ground water in the area of the containment dam is within the growth range (Fig. 2) of SAGM1^T. Although the original source of SAGM1^T is difficult to pinpoint, it is possible that this and similar anaerobic alkaliphiles are endemic to deep alkaline ground waters in the region. Alkaliphilic micro-organisms may be widely distributed in deep ground waters as alkaline pH values are common for deep ground waters that have been in contact with rock for extensive periods. It is proposed that SAGM1^T functions as a consumer of dissolved and particulate organic matter in deep alkaline ground waters, the source of which is likely to be primary production by autotrophic micro-organisms that utilize geological sources of energy such as H_2 (Pedersen, 1998; Stevens & McKinley, 1995). However, further investigations will be required to determine the extent to which *Alkaliphilus transvaalensis* and other alkaliphilic micro-organisms inhabit deep, alkaline ground waters and the specific niche(s) they occupy.

Description of *Alkaliphilus* gen. nov.

Alkaliphilus (Al.ka.li′phil.us. N.L. n. *alkali* from Arabic *al-qaliy* the ashes of saltwort; Gr. adj. *philos* friendly; N.L. adj. *Alkaliphilus* liking alkaline environments).

Straight to slightly curved rod, motile with flagella. Gram-positive and spore-forming. Anaerobic and alkaliphilic heterotroph, able to utilize proteinaceous substrates such as yeast extract, peptone, tryptone and casein. Elemental sulfur, thiosulfate and fumarate stimulate growth as electron acceptors. G+C content of genomic DNA is about 36%. The major phospholipid fatty acids are iC15:1ω7*c*, iC15:0, iC17:1ω7*c*, iC17:0, common PLFA in Gram-positive bacteria. On the basis of 16S rRNA gene analysis, the genus *Alkaliphilus* is most closely related to the genera *Tindallia* and *Clostridium* and is a member of the cluster XI within the low G+C Gram-positive group. Members of the genus *Alkaliphilus* occur in deep subsurface alkaline environments. The type species is *Alkaliphilus transvaalensis*.

Description of *Alkaliphilus transvaalensis* sp. nov.

Alkaliphilus transvaalensis (trans.vaa.len′sis. N.L. gen. n. *transvaalensis* of Transvaal, a region of South Africa).

Straight to slightly curved rod, with a mean length of 3–6 μm and a width of about 0·4–0·7 μm. Cells occur singly or in pairs. Exhibits motility with flagella. Gram-positive and spore-forming. Strictly anaerobic. The temperature range for growth is 20–50 °C, with the optimum being 40 °C. The pH range for growth is 8·5–12·5, with optimum growth occurring at pH 10·0. Sea salts enhance, but are not absolutely required, for growth; optimum growth occurs at 5 g l^{-1}. Growth occurs with yeast extract, peptone, tryptone or casein. Elemental sulfur, thiosulfate and fumarate stimulate growth as electron acceptors. The major phospholipid fatty acids are 2·3% iC13:0, 1·7% C14:0, 9·2% iC15:1ω7*c*, 51·6% iC15:0, 2·8% aC15:0, 1·9% iC16:1ω7*c*, 3·9% C16:0, 7·2% iC17:1ω7*c*, 12·2% iC17:0, 1·1% iC18:1ω9*c* and 2·0% C18:0. G+C content of genomic DNA is about 36·4 mol% (HPLC method). 16S rDNA sequence exhibits 89–91% similarity to those of members of the genera *Clostridium*, *Tindallia*, *Acidaminobacter* and *Fusibacter*. The organism was isolated from a highly alkaline water pool at a depth of 3·2 kmbls in the East Driefontein gold mine 5 shaft located southwest of Johannesburg near Carletonville, South Africa. The type strain is SAGM1^T(= JCM 10712^T = ATCC 700919^T).

ACKNOWLEDGEMENTS

Thanks to Driefontein Consolidated for access to their mines. To Dawie Nel and the other members of the East Driefontein Geology Department for help in interpreting the mine stratigraphy. We wish to thank Dr Hans Trüper for his advice on the naming of *Alkaliphilus transvaalensis*. Thanks also to Jennifer Alexander, Margaret Grant and others in the Microbiology Department at the University of Witwatersrand for scientific support. We also wish to acknowledge the help of Jennifer Bowas at the University of Tennessee in support of the PLFA analyses. This research was supported by grant EAR-9714214 from the National Science Foundation LExEn program to Princeton University (T.C.O.) and by the National Geographic Society grant 6339-98 to T.C.O. for travel support.

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