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Thioalkalicoccus limnaeus gen. nov., sp. nov., a new alkaliphilic purple sulfur bacterium with bacteriochlorophyll *b*

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Four strains of purple sulfur bacteria containing bacteriochlorophyll *b* were isolated from cyanobacterial mats of soda lakes in the steppe of south-east Siberia, Russia. Cells of all strains were cocci without gas vesicles. Eventually, cells with flagella were seen in the electron microscope, but motile cells were observed very rarely in cultures. Internal photosynthetic membranes were of the tubular type. Photosynthetic pigments were bacteriochlorophyll *b* and carotenoids with spectral characteristics similar to 3,4,3′,4′-tetrahydrospirilloxanthin. The bacteria were obligately phototrophic and strictly anaerobic. Hydrogen sulfide and elemental sulfur were used as photosynthetic electron donors. Thiosulfate was not used. During growth on sulfide, sulfur globules were formed as intermediate oxidation products, deposited inside the cells and centrally located. In the presence of sulfide and sodium bicarbonate, acetate, malate, propionate, pyruvate, succinate, fumarate and yeast extract were photoassimilated. Growth factors were not required. The new bacterium is an obligate alkaliphile growing at pH 8–10 with an optimum at pH 9. It showed good growth up to 6·0% sodium chloride and up to 8·5% sodium carbonates. Phenotypically, it is similar to *Thiococcus pfennigii*, but different by virtue of its alkaliphily and salt tolerance. The DNA G+C content was 63·6–64·8 mol%, compared to 69·4–69·9 mol% for *Thiococcus pfennigii*. The 16S rDNA sequence of strain A26^T was approximately 92% similar to that of *Thiococcus pfennigii* DSM 226 and therefore a new genus and species name, *Thioalkalicoccus limnaeus* gen. nov. and sp. nov., are proposed for the new bacterium.

Keywords: Phototrophic purple bacteria, *Chromatiaceae*, *Thioalkalicoccus limnaeus*, alkaliphily, bacteriochlorophyll *b*

INTRODUCTION

The first purple sulfur bacterium with bacteriochlorophyll *b* as the major photosynthetic pigment and with an *in vivo* absorption maximum at 1020 nm was described as a '*Thiococcus*' species (Eimhjellen *et al*., 1967). This bacterium differed from all other phototrophic bacteria by internal photosynthetic membranes of tubular structure. Cells were non-motile cocci depositing sulfur inside the cells and did not contain gas vesicles. Strains of *Thiococcus* were isolated from sediments of rivers, lakes and saline habitats containing sulfide. Growth occurred at pH 6·5–7·5 with an optimum at pH 7·0. This bacterium was included in the genus *Thiocapsa* Winogradsky (1888) as *Thiocapsa pfennigii* (Eimhjellen, 1970). On the basis of 16S rDNA sequences, however, it is significantly different from *Thiocapsa roseopersicina*, the type species of this genus, and it was reclassified as *Thiococcus pfennigii* (Imhoff *et al*., 1998). Until recently only few strains of *Chromatiaceae* containing bacteriochlorophyll *b* have been isolated. We have found purple sulfur bacteria containing bacteriochlorophyll *b* and tubular internal photosynthetic membranes similar to the described *Thiococcus*

The EMBL accession number for the 16S rDNA sequence of *Thioalkalicoccus limnaeus* is AJ277023.

pfennigii in phototrophic communities of soda lakes located in the Buryat Republic and Chita region of south-east Siberia, Russia. Pure cultures of these bacteria were isolated from the samples of layered microbial mats of lakes Dabasa-Nur, Gorbunka, Verkhneye Beloe and Tsaidam. This paper reports the fine structure, physiological properties and taxonomy of these new bacteria, for which the name *Thioalkalicoccus limnaeus* gen. nov., sp. nov. is proposed.

METHODS

Source of organisms. Purple sulfur bacteria, containing bacteriochlorophyll *b*, were isolated from thin (0·2–0·5 cm)-layered microbial mats that formed in the littoral of soda lakes located in the steppe of south-east Siberia, Russia. Salinity and pH of the natural samples as well as the strains isolated from these lakes are listed in Table 1.

Isolation and cultivation. The basal medium used for isolation of the phototrophic sulfur bacteria contained (per litre distilled water): 0·5 g KH_2PO_4; 5 g NaCl; 0·5 g NH_4Cl; 0·2 g $MgCl_2.6H_2O$; 0·05 g $CaCl_2.2H_2O$; 5 g $NaHCO_3$; 5 g Na_2CO_3; 0·5 g sodium acetate; 0·5 g sodium malate; 0·1 g yeast extract; 0·7 g $Na_2S.9H_2O$; 20 μg vitamin B_{12}; 1 ml trace element solution SL4 (Pfennig & Lippert, 1966). The pH was adjusted to 9·0–9·5.

Pure cultures were obtained by repeated deep agar (0·8 %) dilution series. Purity of cultures was checked microscopically and by inoculation in agar medium under aerobic conditions in the dark. Pure cultures were grown phototrophically in screw-capped bottles at 20–25 °C and a light intensity of 2000 lx. Repeated addition of sulfide feeding solution was used to obtain high cell yields. Carbon sources were added at concentrations of 0·3 or 0·5 g l^{-1}. Growth was either followed by quantifying the pigment content in extracts of acetone/methanol (7:2, v/v) at 470 nm or as optical density at 650 nm. For the determination of the pH optimum, growth was measured as optical density at 650 nm, as elemental sulfur was completely consumed after the second feeding of the cultures with sodium sulfide. Because of the interrelated requirements for alkalinity (sodium carbonates) and salinity (sodium chloride) and the apparent requirement for the sodium ion, growth dependence on sodium chloride was tested in the presence of small amounts of sodium carbonate (0·5 %) and that on sodium carbonates in the presence of small amounts of sodium chloride (0·05 %). Growth under identical conditions was followed over at least four consecutive transfers in all growth experiments.

Microscopy. Cell morphology was studied by light and electron microscopy. Intact cells were stained with 1 % phosphotungstic acid. Ultrathin sections were prepared as described by Ryter & Kellenberger (1958). Cells were viewed with a JEOL electron microscope JEM 100C.

Pigment and sulfur analyses. Cells were suspended in 50 % glycerol for measuring the absorption spectra of living cells which were recorded with an SPh-56 spectrophotometer (Lomo). In addition, pigments were extracted with acetone/methanol (7:2) and absorption spectra of these extracts were also recorded. Sulfide was measured colorimetrically (Trüper & Schlegel, 1964) and sulfate was determined densitometrically (Dodgson, 1961).

DNA analysis. DNA was isolated by the method of Marmur (1961). The DNA base composition was determined by thermal denaturation according to Owen *et al*. (1969). Cell material for 16S rDNA sequencing was taken from 1–2 ml of well grown liquid cultures. DNA was extracted and purified by using the Qiagen genomic DNA buffer set. PCR amplification and 16S rDNA sequencing was done as described previously (Imhoff *et al*., 1998). Recombinant *Taq* polymerase was used for PCR, which was started with the primers 5′-GTTTGATCCTGGCTCAG-3′ and 5′-TACCTTGTTACGACTTCA-3′ (positions 11–27 and 1489–1506, respectively, according to the *Escherichia coli* 16S rRNA numbering of the International Union of Biochemistry). Sequences were obtained by cycle sequencing with the SequiTherm sequencing kit (Biozym) and the chain-termination reaction (Sanger *et al*., 1977) using an automated laser fluorescence sequencer (Pharmacia). Sequences were aligned using the CLUSTAL W program (Thompson *et al*., 1994). The alignment length was from position 29 to 1381 (*E. coli* numbering). The distance matrix was calculated on the basis of the algorithm according to Jukes & Cantor (1969) with the DNADIST program within the PHYLIP package (Felsenstein, 1989). The FITCH program in the PHYLIP package fitted a tree to the evolutionary distances.

RESULTS

Natural habitat and isolation

Samples were collected from cyanobacterial mats and microbial biofilms in the littoral of several soda lakes in south-east Siberia, Russia (Dabasa-Nur, Gorbunka, Verkhneye Beloe and Tsaidam) with salinity ranging from approximately 6 to 16 g l^{-1} and the pH from 9·5 to 10·1 (Table 1). The microbial films were dominated by filamentous cyanobacteria. Among anoxygenic phototrophic bacteria, representatives of *Ectothiorhodospiraceae* were dominant and, in addition, bacteria resembling species of *Rhodobacter*, *Allochromatium*, *Thiocystis* and *Thiocapsa*, as well as green filamentous bacteria like *Oscillochloris*, were quite abundant. In lake Verkhneye Beloe, *Thiorhodospira*

Table 1. Total salt content and pH of four Siberian soda lakes, strains of the new bacterium isolated from the lakes and their DNA G+C content

Lake	Dabasa-Nur	Gorbunka	Verkhneye Beloe	Tsaidam
Total salinity (g l^{-1})	10·0	6·1	7·5	15·8
pH	9·5	10·0	10·1	10·1
Isolate	A14	A18	A26^T	A31
G+C content (mol %)	64·1–64·4	64·2–64·6	64·0–64·5	63·6–64·8

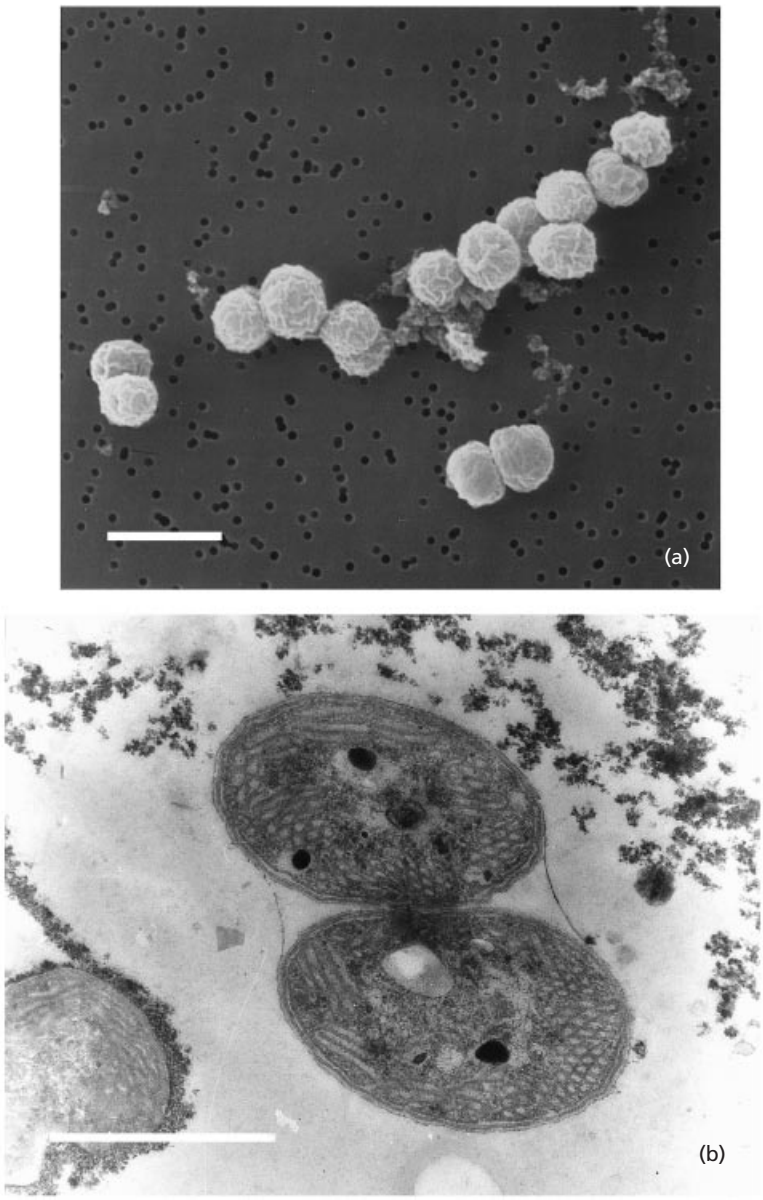

Fig. 1. (a) Scanning electron photomicrograph of cells of strain A26^T showing spherical shape and diplococcus-like division stages. Bar, 2 μm. (b) Electron microphotograph of an ultra-thin section of cells of strain A26^T grown photoautotrophically. Cells were harvested after complete oxidation of elemental sulfur and treated as described in Methods. Late stages of cell division are shown with the two cells already completely separated. Tubular internal membranes have been developed by the bacteria, which extend through almost the whole cytoplasmic space. Bar, 1 μm.

sibirica, a recently described representative of the *Ectothiorhodospiraceae* (Bryantseva *et al*., 1999) was found. In agar medium, the new bacteria formed large orange-brown lens-shaped colonies. Four strains (A14, A18, A26^T, A31) of the new bacterium were isolated from different lakes (Table 1). All of them had similar physiological properties and absorption spectra. Strain A26^T, isolated from microbial films of lake Verkhneye Beloe, was studied more intensively.

Morphology and fine structure

Individual cells were spherical or ovoid and in the light microscope and appeared indistinguishable from those of *Thiococcus pfennigii*. During the exponential growth phase they were 1·3–1·8 μm in diameter and occurred often in pairs (Fig. 1a). In the stationary growth phase their diameter was less than 1·0 μm. Cells multiplied by binary fission. Rarely, cells with one flagellum (electron microscopy) and weak motility of individual cells (light microscopy) were observed. Electron micrographs (Fig. 1b) indicated the formation of a thin capsule and the presence of a Gram-negative type of cell wall. Tubular internal photosynthetic membranes filled most of the internal cellular space (Fig. 1b). Dense granules of polyphosphate were also found.

Pigments

Phototrophically grown cultures free of sulfide appear orange-brown in colour. Absorption spectra of intact cells were similar to those of *Thiococcus pfennigii* (Eimhjellen *et al*., 1967) and exhibited maxima at 410, 462, 492, 530 and 1030 nm with shoulders at 602 and

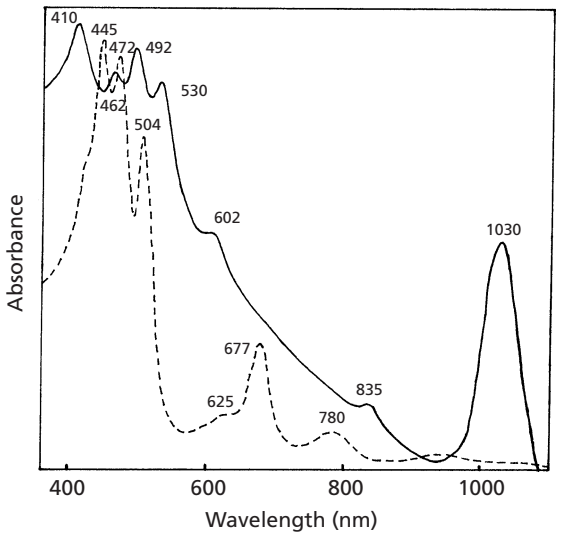

Fig. 2. Absorption spectra of living cells of strain A26^T suspended in 50 % glycerol (continuous line) and of pigments extracted with and dissolved in acetone/methanol (broken line).

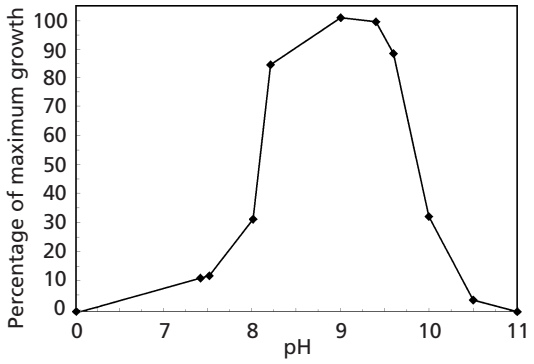

Fig. 3. Growth response of strain A26^T to variation in pH.

835 nm (Fig. 2). The main absorption maximum at 1030 nm quite clearly indicated the presence of bacteriochlorophyll *b*. Maxima at 530, 492 and 462 nm resembled those of *Thiococcus pfennigii* (Aasen & Liaaen Jensen, 1967) and may indicate the presence of 3,4,3′,4′-tetrahydrospirilloxanthin, which has been identified in *Thiococcus pfennigii*.

Physiological properties

Photolithoautotrophic growth occurred under anoxic conditions in the light with hydrogen sulfide and elemental sulfur as electron donors. Sulfide was required for growth and sulfate assimilation was absent. High growth yields under autotrophic conditions can be obtained by repeated feeding with hydrogen sulfide. Thiosulfate was not used for phototrophic growth. The bacterium is strictly anaerobic and growth under aerobic or microaerobic conditions

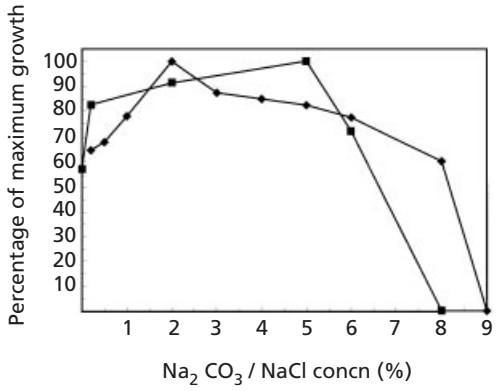

Fig. 4. Growth response of strain A26^T to concentrations of NaCl (■) and Na_2CO_3 (◆) as determined by the absorption at 470 nm of pigments extracted with acetone/methanol (7:2). Growth experiments with varying concentrations of sodium chloride were performed in the presence of 0·5 % sodium carbonates, and with varying concentrations of sodium carbonates concentrations in the presence of 0·05 % sodium chloride.

in the presence or absence of organic compounds was not possible. In the presence of sulfide and sodium bicarbonate, acetate, malate, propionate, pyruvate and succinate were used as organic substrates for phototrophic growth. Growth with yeast extract and fumarate was weak. Ascorbate, arginine, aspartate, butyrate, benzoate, valerate, Casamino acids, glycerol, glycolate, glucose, gluconate, glutamate, caprylate, caproate, lactate, malonate, mannitol, methanol, sorbitol, tartrate, formate, fructose, citrate and ethanol were not assimilated.

Growth factors were not required. During growth on sulfide, sulfur globules accumulated inside the cells and were oxidized further to sulfate as the final oxidation product. Optimum growth was observed at 20–25 °C (range 15–35 °C). The pH range was from 8 to 10 with an optimum at pH 8·8–9·5 (Fig. 3). Slow growth was observed at pH 7·5 with concomitant alkalinization of the medium to pH 8·0. The new bacterium showed good growth over a broad range of salt concentrations without exhibiting a strong salt optimum (Fig. 4). Good growth was observed up to 6 % NaCl (in the presence of 0·5 % sodium carbonates) and up to 8·5 % sodium carbonates (in the presence of 0·05 % NaCl).

Genetic properties

DNA purified from strains A14, A18, A26^T and A31 had a G+C content of 63·6–64·8 mol %, as determined by thermal denaturation (Table 1). DNA–DNA hybridization showed that the level of DNA homology between the new isolates was between 70 and 96 % (data not shown) and indicates that all isolates can be regarded as strains of a single species. The phylogenetic position of the strains relative to that of other purple

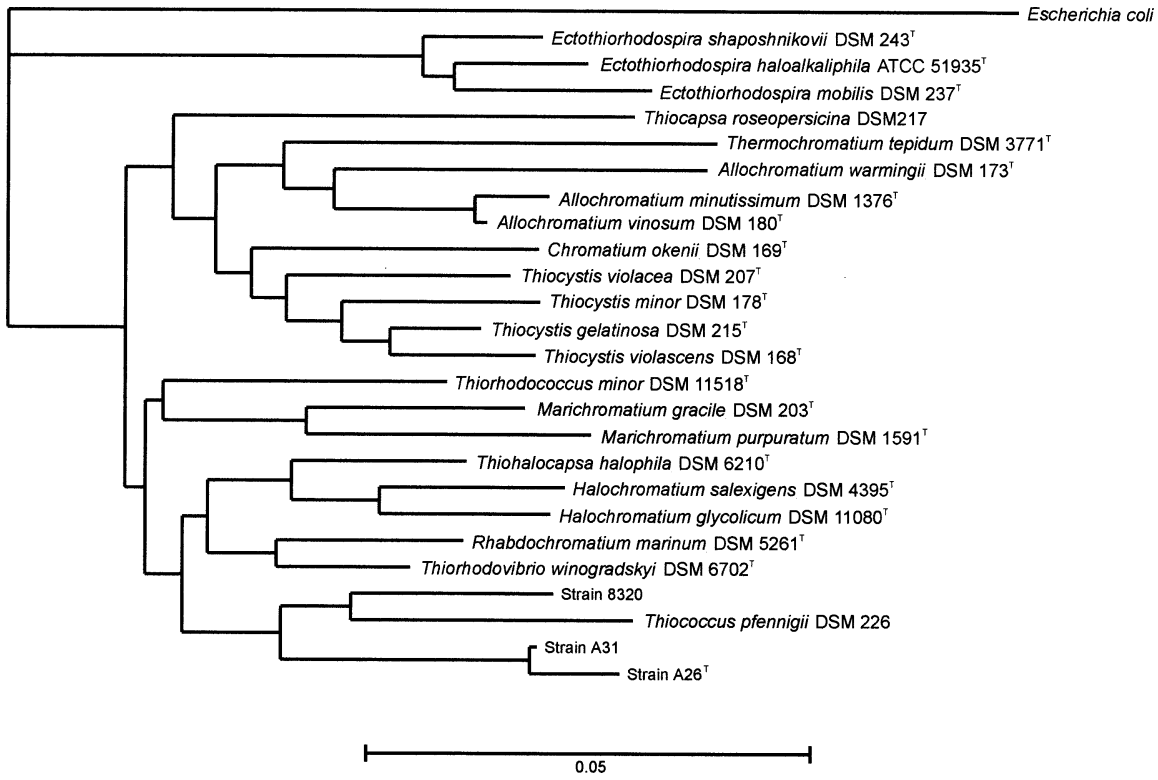

Fig. 5. Phylogenetic relationships between isolates of *Thioalkalicoccus limnaeus*, *Thiococcus pfennigii* and other purple sulfur bacteria, as revealed by 16S rDNA sequence similarity determined on the basis of almost complete 16S rDNA sequences (alignment from position 29 to 1381).

bacteria was examined by 16S rDNA sequencing (Fig. 5). These data revealed 99 % sequence identity between isolates A26^T and A31, thereby proving that they can be regarded as strains of a single species. Because sequence similarity to *Thiococcus pfennigii* was only 92·1–92·6 %, they should be considered as a new purple sulfur bacterium and the name *Thioalkalicoccus limnaeus* gen. nov., sp. nov. is proposed.

DISCUSSION

Thioalkalicoccus limnaeus has a distinctive tubular internal membrane system, which has so far only been found in *Thiococcus pfennigii*. The photosynthetic pigments of the new bacterium are bacteriochlorophyll *b* and carotenoids. As the *in vivo* absorption spectra of *Thioalkalicoccus limnaeus* and *Thiococcus pfennigii* are quite similar, the presence of similar pigments may be assumed, i.e. bacteriochlorophyll *b* and 3,4,3′,4′-tetrahydrospirilloxanthin in *Thiococcus pfennigii* (Eimhjellen *et al.*, 1967; Schmidt, 1978). *Thioalkalicoccus limnaeus* is a physiologically specialized species, strictly anaerobic and obligately phototrophic, uses hydrogen sulfide and elemental sulfur, but not thiosulfate for phototrophic growth and photoassimilates only a limited number of organic substrates. Reduced sulfur sources are required and sulfate can not be assimilated.

The new purple sulfur bacterium *Thioalkalicoccus limnaeus* is a typical member of benthic microbial phototrophic communities developing in low salinity (6–16 g l^{-1}), alkaline (pH 9·5–10·1) soda lakes in the steppe of south-east Siberia, Russia. *Thioalkalicoccus limnaeus* prefers to grow in alkaline environments with pH 8–10 and it appears to be an obligate alkaliphile. Only slow growth was observed at pH 7·5 under alkalinization of the medium to pH 8·0. In contrast, *Thiococcus pfennigii* is found in fresh water and low-salinity environments with hydrogen sulfide and slightly acid pH. No growth of *Thiococcus pfennigii* occurred at pH higher than 7·5.

According to 16S rDNA sequence data, the new isolates quite clearly belong to the branch of marine and halophilic species of the *Chromatiaceae*. The low sequence similarity to strains of *Thiococcus pfennigii* (approx. 92 %), which is the most closely related known purple sulfur bacterium, supports their classification within a new genus. These conclusions are supported by differences of the G + C content between the new isolates (63·6–64·8 mol %) and *Thiococcus pfennigii* (69·4–69·9 mol %; Mandel *et al.*, 1971).

Description of *Thioalkalicoccus* gen. nov.

Thioalkalicoccus (Thi′o.al′ka.li.coc′cus. Gr. n. *thios* sulfur; Arab. n. *al kali* potash, soda; L. masc. n. *coccus* sphere; M.L. masc. n. *Thioalkalicoccus* sulfur sphere from soda).

Cells are spherical or oval, typically form diplococcus-shaped cells during cell division, multiply by binary fission and are Gram-negative. Internal membranes are of the tubular type. Photosynthetic pigments are bacteriochlorophyll *b* and carotenoids. The metabolism is strictly anaerobic and obligately phototrophic. During photolithoautotrophic growth with sulfide as electron donor, globules of elemental sulfur are accumulated inside the cytoplasm. The final oxidation product is sulfate. In the presence of sulfide and bicarbonate organic substrates are photoassimilated. Mesophilic, obligate alkaliphilic bacterium with optimum growth at 20–25 °C. Optimal development is dependent on sodium salts in low concentrations and on alkaline conditions. Habitat is the surface of sediments rich in organic matter and microbial mats of soda lakes containing hydrogen sulfide and exposed to light. The DNA G+C is 63·6–64·8 mol% (thermal denaturation). Type species is *Thioalkalicoccus limnaeus*.

Description of *Thioalkalicoccus limnaeus* sp. nov.

Thioalkalicoccus limnaeus (lim.nae′us. Gr. fem. n. *limne* lake, pond, swamp; Gr. adj. *limnaios* pertaining to, living in lakes, swamps; N.L. masc. adj. *limnaeus* living in lakes and swamps).

Cells are spherical or oval in shape, multiply by binary fission and are Gram-negative. Cells are cocci of 1·3–1·8 μm diameter, usually non-motile and surrounded by a thin capsule. Occasionally cells with a single flagellum are observed. Internal photosynthetic membranes of tubular type form by invagination of the cell membrane and fill most of cytoplasm. Colour of cell suspensions is yellowish to orange-brown. The absorption spectrum of intact cells exhibits maxima at 410, 462, 492, 530 and 1030 nm with shoulders at 602 and 835 nm. Photosynthetic pigments are bacteriochlorophyll *b* and carotenoids of similar absorption properties as tetrahydrospirilloxanthin. Metabolism is strictly anaerobic. Photolithoautotrophic growth occurs in light with hydrogen sulfide and elemental sulfur as electron donors. Thiosulfate is not used for phototrophic growth. During growth with sulfide as electron donor, globules of elemental sulfur are accumulated inside the cells. The final oxidation product is sulfate. In the presence of sulfide and sodium bicarbonate, acetate, yeast extract, malate, propionate, pyruvate, succinate and fumarate are used as organic substrates for phototrophic growth. Growth factors are not required. Mesophilic, obligate alkaliphilic bacterium with optimum growth at pH 8·8–9·5 (range pH 8–10) and 20–25 °C. Development is dependent on sodium salts in low concentrations and good growth occurs over a broad range of salt concentrations without exhibiting a strong optimum, up to 6% NaCl (in the presence of 0·5% sodium carbonates) and up to 8·5% sodium carbonates (in the presence of 0·05% NaCl). Habitat is the surface of sediments rich in organic matter and microbial mats of soda lakes containing hydrogen sulfide and exposed to light. The DNA G+C content of the type strain is 64·0–64·5 mol% (thermal denaturation). The type strain, A26^T, has been deposited at the American Type Culture Collection, Manassas, VA, USA, as ATCC BAA32^T.

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