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Thioflavicoccus mobilis gen. nov., sp. nov., a novel purple sulfur bacterium with bacteriochlorophyll *b*

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A novel phototrophic purple sulfur bacterium was isolated from a flat, laminated microbial mat in a salt marsh near Woods Hole, Massachusetts, USA. The cells were monotrichously flagellated motile cocci with internal photosynthetic membranes of the tubular type. The main photosynthetic pigments were bacteriochlorophyll *b* and the carotenoid 3,4,3′,4′-tetrahydrospirilloxanthin. The marine bacterium showed optimal growth in the presence of 2% salts. It was obligately phototrophic and strictly anaerobic. It grew photoautotrophically and photoassimilated acetate, pyruvate and ascorbate as the only organic substrates. In the presence of sulfide, elemental sulfur globules were formed inside the cells. Elemental sulfur was further oxidized to sulfate. The DNA base composition of the new bacterium was 66·5 mol% G+C. The 16S rDNA nucleotide sequence was most similar to strains of *Thiococcus pfennigii*, there being approximately 92–93% sequence similarity. The new bacterium is described as a new species and a new genus, and the name *Thioflavicoccus mobilis* is proposed; the type strain is 8321^T (= ATCC 700959^T).

Keywords: phototrophic purple sulfur bacteria, *Thioflavicoccus mobilis* gen. nov., sp. nov., *Chromatiaceae*, bacteriochlorophyll *b*, tubular membranes

INTRODUCTION

The first purple sulfur bacterium found to contain bacteriochlorophyll *b* as the major photosynthetic pigment and with the main *in vivo* absorption maximum at 1020 nm was described as a *Thiococcus* species (Eimhjellen *et al*., 1967). This bacterium differed from all other photosynthetic bacteria on the basis of the internal photosynthetic membranes of tubular structure. The cells were non-motile cocci, contained sulfur globules, and did not contain gas vesicles. Strains of *Thiococcus* were isolated from sediments of lakes and rivers but also from marine habitats containing sulfide. This bacterium was included, later, in the genus *Thiocapsa* Winogradsky (1888) as *Thiocapsa pfennigii* (Eimhjellen, 1970). However, on the basis of 16S rDNA sequences, it was significantly different from the type species of this genus, *Thiocapsa roseopersicina*, and was reclassified as *Thiococcus pfennigii* (Imhoff *et al*., 1998). Only a few strains of the *Chromatiaceae* containing bacteriochlorophyll *b* have been isolated so far, such as the recently described bacteria from alkaline soda lakes in Siberia, which were described as *Thioalkalicoccus limnaeus* (Bryantseva *et al*., 2000).

In sandy intertidal sediments of the Great Sippewissett Salt Marsh on Cape Cod (MA, USA), flat, laminated microbial mats of unusual thickness regularly developed. During the sampling period in the summer of 1986, these mats were about 10 mm thick and comprised four to five distinctly coloured layers (Nicholson *et al*., 1987). Phototrophic purple sulfur bacteria from the central pink layer and the peach-coloured layer immediately below were analysed. The bacteria of the peach layer consisted of small spirilloid bacteria that were classified as *Rhodospira trueperi* (Pfennig *et al*., 1997), as well as motile and non-motile cocci resembling *Thiococcus pfennigii* (with respect to the presence of bacteriochlorophyll *b* and bundles of tubular internal membranes). Both types of coccoid bacteria were isolated in pure culture. This paper reports on the properties of the new, motile, coccoid,

The EMBL accession numbers for the 16S rDNA sequences of strains 8320 and strain 8321^T are AJ010125 and AJ010126, respectively.

purple sulfur bacterium containing bacteriochlorophyll *b*, and on its genetic relationship to other purple sulfur bacteria.

METHODS

Source of organisms. The coccoid bacteria were isolated from deep-agar dilution series that had been inoculated with a peach-coloured sample of the laminated microbial mats from Great Sippewissett Salt Marsh, Cape Cod, MA, USA. A detailed description of these microbial mats has been given by Nicholson *et al.* (1987).

Isolation and cultivation. Pure cultures were obtained using repeated deep-agar dilution series (Pfennig, 1978). The basal medium used for isolation and further cultivation of the bacterium contained (per litre distilled water): 0·25 g KH_2PO_4, 0·4 g NH_4Cl, 0·35 g KCl, 20·0 g NaCl, 2·8 g $MgSO_4.7H_2O$, 0·25 g $CaCl_2.2H_2O$, 1·5 g $NaHCO_3$, 0·3 g $Na_2S.9H_2O$, 1 ml vitamin solution (Pfennig & Trüper, 1981) and 1 ml trace-element solution SL 12 (Overmann *et al.*, 1992). The basal medium was sterilized, the pH was adjusted to 7·2–7·3 and then the medium was aseptically distributed into culture vessels as described by Pfennig & Trüper (1981). The purity of cultures was checked microscopically and by using growth tests in AC medium (Difco).

Cultures were grown phototrophically in 100 ml screw-capped bottles with autoclavable rubber seals. Cultures were incubated at 20–22 °C at a light intensity of 300–500 lx from a tungsten lamp. The light intensity was checked in the 400–700 nm range with a Metrolux K light sensor (Metrawatt). The addition of sterile pyruvate solution to a final concentration of 5 mM was used to obtain high cell yields. Stock cultures were stored at 4 °C in the dark.

Growth experiments were performed in duplicate in 20 ml screw-capped tubes with autoclavable rubber seals. Electron donors and carbon sources were aseptically added to each tube, from sterile stock solutions, to the final concentrations indicated. The utilization of organic carbon sources was tested in the presence of hydrogen carbonate and sulfide in the media. Growth was followed by measuring the optical density at 650 nm with a Bausch & Lomb Spectronic 70 photometer.

Electron microscopy. For negative staining, cells were treated with a 3% aqueous solution of uranyl acetate. For ultrathin sections, cells were prefixed in glutaraldehyde, fixed in OsO_4 solution, stained with uranyl acetate in 75% acetone, embedded in Spurr medium, sectioned and then post-stained with lead acetate. Electron microscopy was carried out by Dr H. Lünsdorf (GBF, Braunschweig, Germany).

Pigment and sulfur analyses. Absorption spectra were recorded with a Lambda 2 spectrophotometer (Perkin-Elmer). The identification of carotenoids was carried out by means of TLC, as described by Eichler & Pfennig (1986), using carotenoids of *Thiococcus pfennigii* as a reference (Schmidt, 1978). Sulfide was measured colorimetrically (Trüper & Schlegel, 1964) and sulfate was determined turbidometrically (Dodgson, 1961).

DNA analysis. DNA was isolated using the method of Marmur (1961). The G+C content (mol%) of the DNA was determined by Dr J. Floßdorf (Braunschweig, Germany) according to Floßdorf (1983). Cell material for 16S rDNA sequencing was taken from 1–2 ml well-grown liquid cultures. DNA was extracted and purified by using the QIAGEN genomic DNA buffer set. The PCR amplification and the 16S rDNA sequencing were done as described earlier (Imhoff *et al.*, 1998). Recombinant *Taq* polymerase was used for the PCR, which was started with the primers 5′-GTTTGATCCTGGCTCAG-3′ and 5′-TACCTTGTTACGACTTCA-3′ (positions 11–27 and 1489–1506, 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 position 1381 (according to *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

During the 1986 'Microbial Diversity' summer course at the Marine Biological Laboratory, Woods Hole, MA, USA, samples were taken from the peach-coloured layer of a laminated microbial mat at Great Sippewisset Salt Marsh, Cape Cod, MA, USA. The material was suspended in sterile culture medium and used as the inoculum for a deep-agar dilution series with basal medium plus 3 mM acetate and 1% washed agar. After two weeks of incubation in the light,

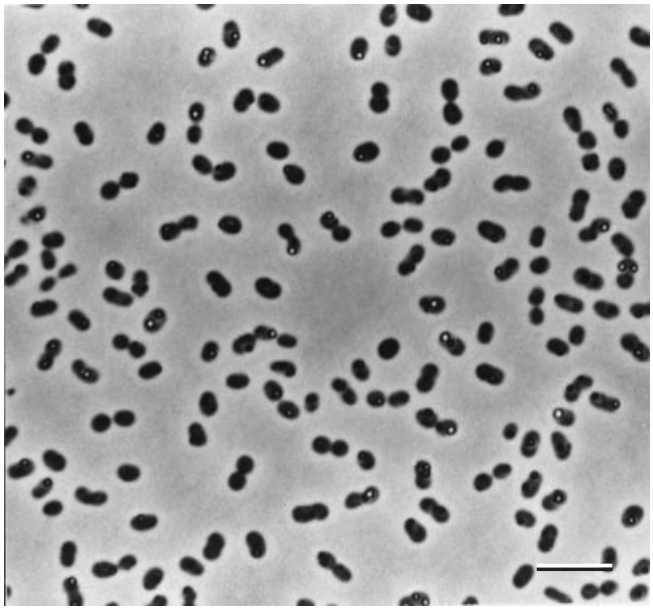

Fig. 1. Phase-contrast photomicrograph of strain 8321^T grown photoautotrophically with sulfide. Different stages of cell division are seen. Some cells contain globules of elemental sulfur. Bar, 5 μm.

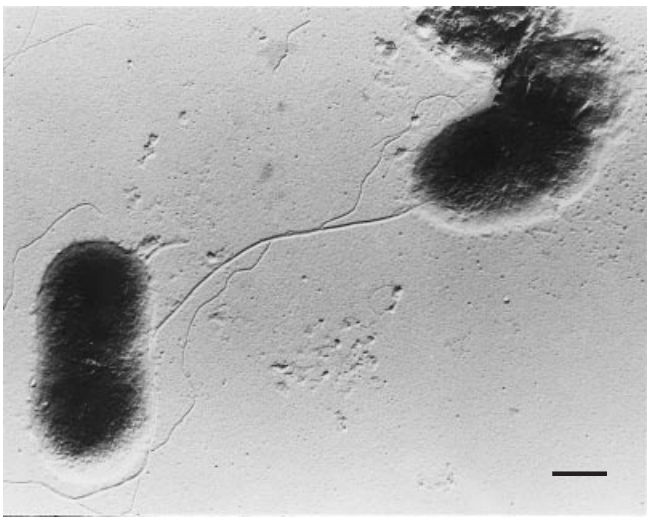

Fig. 2. Electron photomicrograph of negatively stained cells of strain 8321^T showing monopolar–monotrichous flagella and pili. Bar, 0·5 μm. Courtesy of H. Lünsdorf, GBF, Braunschweig, Germany.

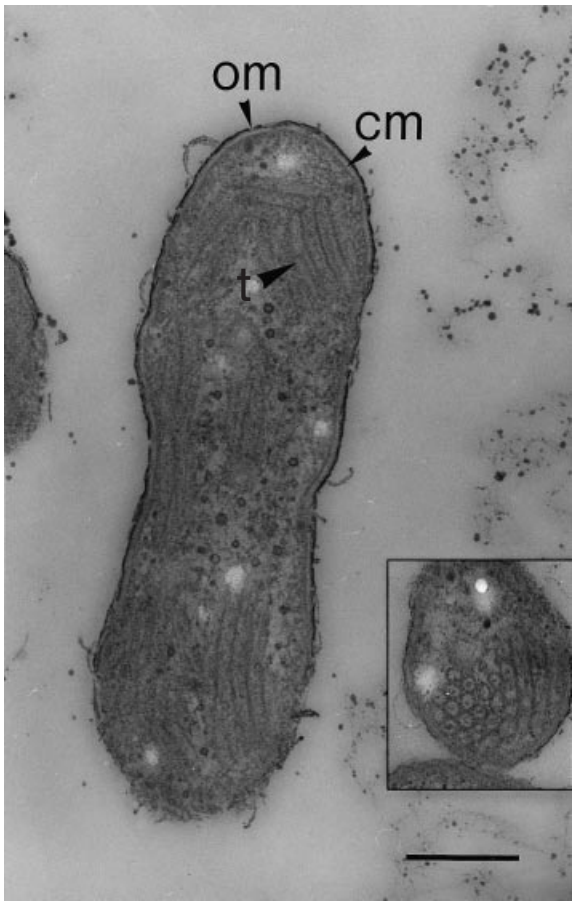

Fig. 3. Electron photomicrograph of an ultrathin section of strain 8321^T showing tubular internal membrane structures in longitudinal section (t) and in cross-section (inset). om, Outer membrane; cm, cytoplasmic membrane. Bar, 0·5 μm. Courtesy of H. Lünsdorf, GBF, Braunschweig, Germany.

various types of pinkish, reddish and yellowish-beige colonies developed. Individual colonies were picked and studied microscopically. The majority of the yellowish-beige colonies contained coccoid non-motile cells. Only a few colonies consisted of highly motile cocci. Single colonies were used as inocula for second and third deep-agar dilution series to obtain pure cultures of strains 8320 (coccoid, non-motile) and strain 8321^T (coccoid, motile), which were maintained as stock cultures and used for further studies. As strain 8320 resembled *Thiococcus pfennigii*, strain 8321^T was studied in more detail.

Morphology and fine structure

In well-growing cultures, individual cells of strain 8321^T were 0·8–1·0 μm in diameter. Before division by binary fission, rod-shaped to diplococcus-shaped division stages occurred (Fig. 1). The cells were highly motile and stained Gram-negative. In negatively stained preparations, monopolar monotrichous flagella were observed (Fig. 2). Electron microscopic examination of ultrathin sections revealed an intracellular photosynthetic membrane system of the tubular type (Fig. 3). The membrane system resembled that of *Thiococcus pfennigii* (Eimhjellen *et al*., 1967) and *Thioalkalicoccus limnaeus* (Bryantseva *et al*., 2000). Ultrathin sections also revealed an outer membrane and a cytoplasmic membrane characteristic of Gram-negative bacteria (Fig. 3).

Pigments

Phototrophically grown cultures of strain 8321^T appeared yellowish-beige to orange-brown in colour. The absorption spectrum of intact cells was similar to that of *Thiococcus pfennigii* (Eimhjellen *et al*., 1967). The main absorption maximum at approximately 1025 nm quite clearly indicates the presence of bacteriochlorophyll *b*. In pigment extracts, bacteriochlorophyll *b* was identified by co-chromatography with pigments from *Thiococcus pfennigii*. The *in vivo* maxima at 530, 492 and 462 nm resemble those of the carotenoids of *Thiococcus pfennigii* (Aasen & Liaaen-Jensen, 1967) and indicate the presence of 3,4,3′,4′-tetrahydrospirilloxanthin. Co-chromatography of pigment extracts with those from *Thiococcus pfennigii* allowed identification of the main carotenoid as 3,4,3′,4′-tetrahydrospirilloxanthin (Schmidt, 1978).

Physiological properties

Photolithoautotrophic growth of strain 8321^T occurred under anoxic conditions in the light with hydrogen sulfide and elemental sulfur as electron donors. Thiosulfate was not used. The bacterium was strictly anaerobic and obligately phototrophic. No growth occurred in the dark under oxic or microoxic

Table 1. Substrate utilization and characteristic properties of strain 8321^T in comparison to the most closely related bacteria, *Thiococcus pfennigii* and *Thioalkalicoccus limnaeus*

All strains had tubular internal membranes, bacteriochlorophyll *b*, and 3,4,3′,4′-tetrahydrospirilloxanthin as the main carotenoid; all strains used sulfide, S^0, acetate and pyruvate as substrates; none required vitamins and none was able to use thiosulphate. The following additional substrates were tested (concentrations 5 mM), but were not used by strain 8321^T: arginine, butyrate, benzoate, valerate, glycerol, glycolate, glucose, gluconate, glutamate, caproate, caprylate, crotonate, malonate, mannitol, methanol, ethanol, propanol, tartrate, formate, citrate, 2-oxoglutarate, acetoin, 2,3-butanediol and ethylene glycol.

Property/substrate	*Thioflavicoccus mobilis* strain 8321^T	*Thiococcus pfennigii* strain DSM 226	*Thioalkalicoccus limnaeus* strain A26
Cell shape	Coccus	Sphere	Sphere
Cell size	0·8–1·0	1·2–1·5	1·3–1·8
Motility	Monopolar flagella	Non-motile	Non-motile
Salt optimum (%)	2	0·5–2·0	5
pH optimum	7·2–7·4	7·2–7·4	8·8–9·5
DNA G+C content (mol%)	66·5	69·4–69·9	63·6–64·8
Substrates used:			
Propionate	−	+	+
Fructose	−	+	−
Ascorbate	+	+	−
Peptone	−	(+)	NA
Casamino acids	−	(+)	−
Lactate	−	+	−
Succinate	−	+	+
Malate	−	+	+
Fumarate	−	+	(+)

NA, No data available.

conditions in the presence of organic compounds. In the presence of sulfide and bicarbonate, acetate, pyruvate and ascorbate were photoassimilated. This limited substrate utilization contrasts with the larger substrate spectrum of *Thiococcus pfennigii*. Substrate utilization by both bacteria is shown in Table 1.

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. Growth was dependent on the addition of NaCl, the optimum being at 2% NaCl. Cultures grew well at a light intensity of 500 lx from a 40 W tungsten lamp and in the temperature range 25–30 °C.

Genetic properties

The DNA base composition of strain 8321^T was 66·5 mol% G+C. According to 16S rDNA sequence data, the new isolate belongs to a group of bacteria within the *Chromatiaceae* containing tubular internal membranes and bacteriochlorophyll *b*. The sequence of the 16S rRNA gene indicates that the closest relationship is to *Thiococcus pfennigii*, with sequence similarities of only 91·8% for strain DSM 226 (8013) and 92·8% for strain 8320 (Fig. 4). The sequence similarity to strains of *Thioalkalicoccus limnaeus* is below 92%.

DISCUSSION

In deep-agar dilution tubes from the peach-coloured layer of a laminated microbial mat, yellowish-beige colonies were dominant in all dilution steps. In most of the cases, suspensions of individual colonies of this type contained non-motile coccoid cells with internal sulfur globules characteristic of *Thiococcus pfennigii*. Surprisingly, a few colonies contained motile coccoid cells. Therefore, initially the question arose as to whether there are motile variants of the non-motile *Thiococcus pfennigii* or whether the motile cocci represented a separate and new taxon. The results presented in this paper and summarized in Table 1 demonstrated that, apart from motility, many other traits of strain 8321^T are different from those of the typical *Thiococcus pfennigii* strain DSM 226 (8013). Apparent similarities in cell shape, colour of colonies, pigment content and internal membrane structure were counteracted by other significant differences. Only acetate, pyruvate and ascorbate were used as organic substrates by strain 8321^T, whereas *Thiococcus pfennigii* strains were generally able to utilize a number of additional organic compounds. Also, the G+C content (mol%) of the DNA of strain 8321^T was significantly lower than that of *Thiococcus pfennigii*. Furthermore, the genetic relationship between the motile strain, 8321^T, and strains of *Thiococcus pfennigii* (strains DSM 226 and 8320) and *Thioalkalicoccus*

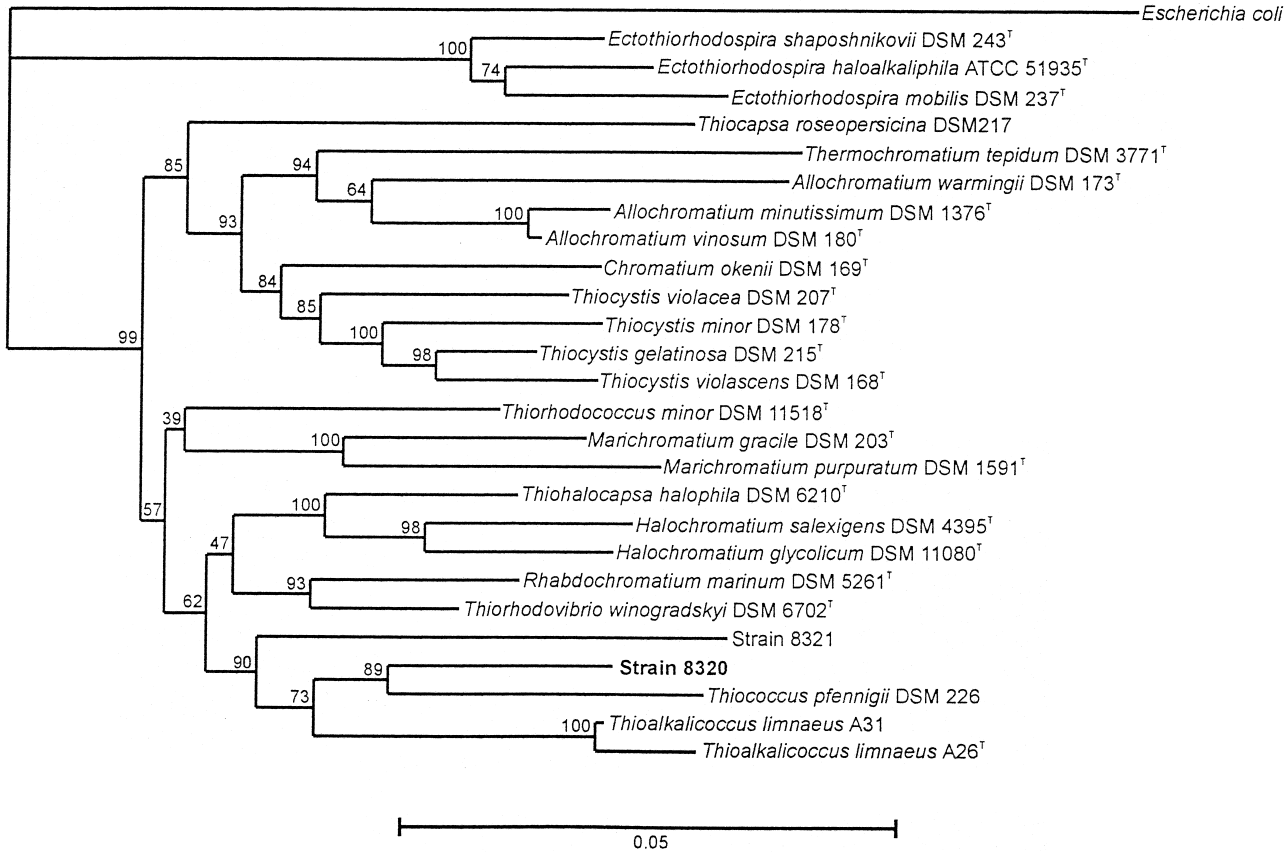

Fig. 4. Genetic relatedness of strain 8321^T to *Thiococcus pfennigii* (strains DSM 226 and 8320) and *Thioalkalicoccus limnaeus* (strains A26 and A31) as well as to other purple sulfur bacteria, based on 16S rDNA sequence similarities. Numbers at the nodes give confidence values of bootstrap analyses with 100 resamplings.

limnaeus (strains A26 and A31), based on the nucleotide sequences of the 16S rDNA (Fig. 4), indicated that strain 8321^T cannot be considered as a motile variant of *Thiococcus pfennigii* or as a new species of the genera *Thiococcus* and *Thioalkalicoccus*. The sequence similarity of strain 8321^T to the closest relative, *Thiococcus pfennigii*, is only 91·8–92·8 %. This is at a level that is generally accepted for differentiation between genera. Therefore we propose that the motile strain 8321^T should be placed in a new genus and species, for which the name *Thioflavicoccus mobilis* is proposed.

Description of *Thioflavicoccus* gen. nov.

Thioflavicoccus (Thi′o.fla′vi.coc′cus. Gr. n. *thios* sulfur; L. masc. adj. *flavus* golden-yellow, beige; L. masc. n. *coccus* sphere; M.L. masc. n. *Thioflavicoccus* beige-yellow coccus with sulfur).

Cells are spherical with diplococcus-shaped division stages, motile by flagella, and multiply by binary fission. Gram-negative; belong to the *γ-Proteobacteria*. Contain internal photosynthetic membranes of tubular shape. Photosynthetic pigments are bacteriochlorophyll *b* and carotenoids. Obligately phototrophic and strictly anaerobic. Photolithoautotrophic growth under anoxic conditions in the light, with sulfide and elemental sulfur as electron donors. During oxidation of sulfide, elemental sulfur is transiently stored inside the cells in the form of highly refractile globules; the final oxidation product is sulfate. In the presence of sulfide and bicarbonate, simple organic substrates are photoassimilated. Mesophilic bacteria, growing well at 20–30 °C and neutral pH (pH range 6·5–7·5) and requiring sodium chloride for optimum growth. The G+C content of the DNA is 66·5 mol% (Bd). The type species is *Thioflavicoccus mobilis* sp. nov.

Description of *Thioflavicoccus mobilis* sp. nov.

Thioflavicoccus mobilis (mo′bi.lis. L. adj. *mobilis* mobile).

Cells are coccoid, short rod-shaped to diplococcus-shaped before cell division. Cocci are 0·8–1·0 μm in diameter. Motile by monopolar monotrichous flagella. Possess an internal photosynthetic membrane system of tubular type. The colour of cell suspensions is yellowish-beige to orange-brown. The absorption spectrum of living cell suspensions exhibits maxima at 410, 462, 492, 530 and 1025 nm, with shoulders at 602

and 835 nm. Photosynthetic pigments are bacteriochlorophyll b and 3,4,3′,4′-tetrahydrospirilloxanthin as the main carotenoid. The metabolism is obligately phototrophic and strictly anaerobic. Photolithoautotrophic growth occurs in the light with hydrogen sulfide as electron donor. Globules of elemental sulfur are accumulated inside the cells. The final oxidation product is sulfate. Thiosulfate is not used. In the presence of sulfide and bicarbonate, acetate, pyruvate and ascorbate are used as organic substrates. Growth factors are not required. Sodium chloride is required for growth. Good growth occurs at 25–30 °C, pH 7·2–7·4, with 1–3 % NaCl (the optimum is at 2 %), and at a light intensity of 500 lx from a tungsten lamp. The habitat is laminated microbial mats of salt marshes. The type strain is strain 8321^T (= ATCC 700959^T).

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