Marichromatium bheemlicum sp. nov., a non-diazotrophic, photosynthetic gammaproteobacterium from a marine aquaculture pond

P. Anil Kumar,[1] T. S. Sasi Jyothsna,[1] T. N. R. Srinivas,[1] Ch. Sasikala,[1] Ch. V. Ramana[2] and J. F. Imhoff[3]

Correspondence
Ch. Sasikala
r449@sify.com *or*
sasi449@yahoo.ie

[1]Bacterial Discovery Laboratory, Centre for Environment, Institute of Science and Technology, JNT University, Kukatpally, Hyderabad 500 085, India

[2]Department of Plant Sciences, School of Life Sciences, University of Hyderabad, PO Central University, Hyderabad 500 046, India

[3]Leibniz-Institut für Meereswissenschaften, IFM-GEOMAR, Marine Mikrobiologie, Düsternbrooker Weg 20, 24105 Kiel, Germany

A rod-shaped, phototrophic, purple sulfur bacterium, strain JA124^T, was isolated in pure culture from a marine aquaculture pond, located near Bhimunipatnam, in a medium that contained 3 % NaCl (w/v). Strain JA124^T is a Gram-negative, motile rod with a single polar flagellum. Strain JA124^T has a requirement for NaCl, with optimum growth at 1.5–8.5 %, and tolerates up to 11 % NaCl. Intracellular photosynthetic membranes are of the vesicular type. Bacteriochlorophyll *a* and probably carotenoids of the spirilloxanthin series are present as photosynthetic pigments. Strain JA124^T was able to utilize sulfide, sulfate, thiosulfate, sulfite, thioglycollate and cysteine as sulfur sources. Strain JA124^T was able to grow photolithoautotrophically, photolithoheterotrophically and photo-organoheterotrophically. Chemotrophic and fermentative growth could not be demonstrated. Strain JA124^T lacks diazotrophic growth and acetylene reduction activity. Pyridoxal phosphate is required for growth. During growth on reduced sulfur sources as electron donors, sulfur is deposited intermediately as a number of small granules within the cell. Phylogenetic analysis on the basis of 16S rRNA gene sequences showed that strain JA124^T clusters with species of the genus *Marichromatium* belonging to the class *Gammaproteobacteria*. The highest sequence similarities of strain JA124^T were found with the type strains of *Marichromatium indicum* (98 %), *Marichromatium purpuratum* (95 %) and *Marichromatium gracile* (93 %). However, DNA–DNA hybridization with *Marichromatium indicum* DSM 15907^T revealed relatedness of only 65 % with strain JA124^T. The DNA base composition of strain JA124^T was 67 mol% G+C (by HPLC). Based on 16S rRNA gene sequence analysis, morphological and physiological characteristics and DNA–DNA hybridization studies, strain JA124^T (=ATCC BAA-1316^T=JCM 13911^T) is sufficiently different from other *Marichromatium* species to merit its description as the type strain of a novel species, *Marichromatium bheemlicum* sp. nov.

The genus *Marichromatium* was established to separate the true marine species of the genus *Chromatium* from their freshwater counterparts (Imhoff *et al.*, 1998). At present, the genus *Marichromatium* comprises three described species, *Marichromatium gracile* (Imhoff *et al.*, 1998) (originally described as *Chromatium gracile*; Strzeszewski, 1913), *Marichromatium purpuratum* (Imhoff *et al.*, 1998) (originally described as *Chromatium purpuratum*; Imhoff & Trüper, 1980) and *Marichromatium indicum* (Arunasri *et al.*, 2005). In this communication, we propose a novel species of this genus which has properties distinct from all other *Marichromatium* species based on phenotypic and phylogenetic analysis.

Strain JA124^T was isolated from enrichments of a sediment and water sample from an aquaculture pond near Bheemli, Visakhapatnam (India). The medium of Pfennig (Pfennig & Trüper, 1992) supplemented with NaCl (3 %, w/v), pyruvate (0.3 %, w/v) as a carbon source/electron donor and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JA124^T is AM180952.

Phase-contrast and electron micrograph images of cells of strain JA124^T and absorption spectra of a whole-cell extract and acetone-extracted pigments are available as supplementary material with the online version of this paper.

ammonium chloride (0.12 %) as a nitrogen source was used for photoheterotrophic growth under fluorescent light (2400 lx) at 30 ± 2 °C. Purification was achieved by repeated agar-shake dilution series (Pfennig & Trüper, 1992; Imhoff, 1988; Trüper, 1970). Purified cultures were grown in completely filled screw-capped test tubes (10 × 100 mm) for photoheterotrophic growth. Morphological properties (cell shape, cell division, cell size, flagella) were observed by light microscopy (with an Olympus BH-2 microscope). To study the ultrastructure of the flagella, cells were stained with 1 % phosphotungstic acid; ultrathin sections were viewed through a transmission electron microscope (H-7500; Hitachi) to examine intracytoplasmic structures such as the internal membrane system. *In vivo* absorption spectra were measured with a Spectronic Genesys 2 spectrophotometer using sucrose solution for cell suspension (Pfennig & Trüper, 1992). Absorption spectra were also recorded of pigments extracted with acetone from the cell suspension. Cellular components (fatty acids, intracellular and membrane proteins, polysaccharides, photosynthetic pigments and nucleic acids) of strain JA124^T were compared with cells from *Mch. indicum* DSM 15907^T based on information obtained from Fourier-transform infrared (FTIR) spectroscopy as described previously (Ramana *et al.*, 2006).

The total cellular protein profile of strain JA124^T was compared with that of *Mch. indicum* DSM 15907^T as follows. The strains were grown under similar conditions, cells were lysed and protein profiles were obtained by SDS-PAGE (Sorokin *et al.*, 2004). After electrophoresis, the gels were silver stained to visualize the protein bands. The utilization of different carbon substrates and electron donors (0.3 %, w/v or v/v, unless otherwise mentioned) was tested in the medium of Pfennig (Pfennig & Trüper, 1992) containing 5 mM $Na_2S.9H_2O$. Ammonium chloride was replaced with different nitrogen sources in order to test for utilization of nitrogen sources (0.12 %, w/v). Diazotrophy of the culture was determined by growing strain JA124^T under a N_2 atmosphere and was confirmed by repeated subculturing (four times). Cultures of strain JA124^T in the exponential phase grown photoheterotrophically (pyruvate, sulfide and ammonium chloride as carbon, electron and nitrogen sources, respectively) were used to assay for acetylene reduction activity as described earlier (Sasikala *et al.*, 1990). Growth was measured turbidometrically at 660 nm. Genomic DNA was extracted and purified according to the method of Marmur (1961) and the G + C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989). 16S rRNA gene sequence analysis was carried out as described by Srinivas *et al.* (2006). Briefly, sequences were aligned using the CLUSTAL W program (Thompson *et al.*, 1994) and the alignment was corrected manually. The CLUSTAL W alignment file was used as input file to the SEQBOOT program in the PHYLIP package (Felsenstein, 1989) and the output file of SEQBOOT was used as the input file for maximum-likelihood analysis with 100 datasets and five times jumbling. One single tree was generated using 100 trees generated during maximum-likelihood analysis using the CONSENSE program. The distance matrix was calculated on the basis of the algorithm of Jukes & Cantor (1969) with the DNADIST program within the PHYLIP package. The FITCH program in the PHYLIP package was employed to fit a tree to the evolutionary distances using the DIST file as infile and the CONSENSE file as intree. DNA–DNA hybridization was conducted by the Identification Service of the DSMZ (Braunschweig, Germany), using a spectrophotometric method (De Ley *et al.*, 1970; Huß *et al.*, 1983) after chromatographic (hydroxyapatite) purification of DNA (Cashion *et al.*, 1977).

Sediment and water samples were collected on 24 February 2005 at around midday from an aquaculture pond near the sea (Bay of Bengal) at Bheemli, India. GPS positioning of the sample collection site is 17° 51′ 41.03″ N 83° 25′ 16.81″ E. The sample yielding strain JA124^T had a pH of 6.8, a salinity of 2–3 % NaCl and a temperature of 30 °C. Individual cells of strain JA124^T were rod-shaped (Supplementary Fig. S1, available in IJSEM Online), 0.8–1.0 μm wide and 2–4 μm long. The cells were highly motile with single polar flagella and multiplied by binary fission. Electron photomicrographs of ultrathin sections of the cells revealed a vesicular type of internal membranes (Supplementary Fig. S2).

Strain JA124^T grows well photo-organoheterotrophically (optimum light intensity is 2400 lx). Strain JA124^T was able to grow photolithoautotrophically [anaerobically in the light (2400 lx) with $Na_2S.9H_2O$ or $Na_2S_2O_3$ (1.0 mM) as electron donors with $NaHCO_3$ (0.1 %, w/v) as carbon source] and photolithoheterotrophically [anaerobically in the light (2400 lx) with $Na_2S.9H_2O$ or $Na_2S_2O_3$ (1.0 mM) as electron donors and organic substrates (0.3 %, w/v) as carbon source]. Chemolithoautotrophy [aerobically in the dark with thiosulfate (1.0 mM) as electron donor and $NaHCO_3$ (0.1 %, w/v) as carbon source], chemo-organoheterotrophy and a fermentative mode of growth [anaerobically in the dark, pyruvate as fermentable substrate (0.3 %, w/v)] could not be demonstrated. Substrates that were utilized as carbon sources/electron donors under photolithoheterotrophic conditions included acetate, pyruvate, succinate, fumarate, glycerol, ethanol, methanol, malate, valerate and caproate (Table 1). Those that could not be utilized included citrate, glucose, mannitol, lactate, tartrate, benzoate and glutamate. Thiosulfate, sulfate, sulfite, thioglycollate, cysteine and sodium sulfide were utilized as sulfur sources under phototrophic conditions. Ammonium chloride, glutamate and glutamine were utilized as nitrogen sources, while nitrate, nitrite, urea and molecular nitrogen did not support growth. Acetylene reduction activity for the enzyme nitrogenase was also not observed in strain JA124^T. Salt is obligatory for growth of strain JA124^T, and growth occurs in 1.5–11 % NaCl (w/v), with optimum growth at 1.5–8.5 % (w/v). The pH range for growth of strain JA124^T is pH 6.0–8.5, with optimum growth at pH 6.5–8.5. The temperature range for growth is 20–35 °C, and the optimum is at 30–35 °C. Yeast extract is required as a growth factor (0.03 %, w/v). In the

Table 1. Differentiating characteristics of species of the genus *Marichromatium*

Organic substrate utilization was tested during photolithoheterotrophic growth in the presence of $Na_2S.9H_2O$ (5 mM). Substrates that were utilized by all of the strains include acetate, pyruvate and malate; those that could not be utilized include glutamate, glucose, mannitol and benzoate. Other similarities include rod-shaped cells, cell division by binary fission, the presence of a single polar flagellum and vesicular internal membrane. +, Substrate utilized; −, substrate not utilized; NR, not reported. Data for reference taxa were taken from Pfennig & Trüper (1989), Caumette *et al.* (1997) and Arunasri *et al.* (2005).

Characteristic	*Mch. gracile* 8611^T	*Mch. purpuratum* 984^T	*Mch. indicum* JA100^T	Strain JA124^T
Cell size				
Width (μm)	1–1.3	1.2–1.7	0.8–1	0.8–1
Length (μm)	2–6	3–4	2–7	2–4
Rosette formation	Absent	Absent	Present	Absent
Sulfur granules per cell	Two or more	Two or more	Single	Two or more
NaCl range (%)	0.5–8	2–7	0.05–13	1.5–11
Optimum NaCl concentration (%)	2–3	5	1–4	1.5–8.5
Optimum growth temperature (°C)	25–30	30–35	30–35	30–35
Optimum pH	6.5–7.6	7.2–7.6	6.0–7.5	6.5–8.5
Growth modes*	POH, PLA, COH, CLA, CLH	POH, PLA, COH, CLA, CLH	PLH, PLA, CLH, POH (with cysteine)	PLH, PLA, POH
Colour of cell suspension	Reddish brown	Purple–red	Reddish brown	Reddish brown
Carotenoids	Spirilloxanthin series	Okenone series	Spirilloxanthin series	Spirilloxanthin series
DNA G+C content (mol%)	70.4 (T_m)	68.9 (T_m)	67.1 (T_m)	67 (HPLC)
Vitamin requirement	None	None	None	Pyridoxal phosphate
Nitrogen sources utilized	Ammonium salts, N_2	Ammonium salts	NH_4Cl, urea, N_2	NH_4Cl, glutamine, glutamate
Utilization of organic substrates/ electron donors				
Propionate	+	+	+	−
Butyrate	+	+	+	−
Valerate	−	+	−	+
Caproate	−	−	−	+
Citrate	−	NR	−	−
Succinate	+	NR	−	+
Fumarate	+	+	−	+
Tartrate	NR	NR	−	−
Fructose	−	−	+	−
Lactate	+	+	+	−
Glycerol	−	−	−	+
Methanol	−	−	+	+
Ethanol	−	−	−	+

*CLA, Chemolithoautotrophy; CLH, chemolithoheterotrophy; COH, chemo-organoheterotrophy; PLA, photolithoautotrophy; PLH, photolithoheterotrophy; POH, photo-organoheterotrophy.

absence of yeast extract, pyridoxal phosphate (100 μg l^{-1}) is required for growth of strain JA124^T.

The colour of photosynthetically grown cultures of strain JA124^T is reddish brown. The whole-cell absorption spectrum (Supplementary Fig. S3a) of strain JA124^T showed absorption maxima at 374, 488, 590, 797 and 851 nm, confirming the presence of bacteriochlorophyll *a*, and the absorption spectrum of pigments extracted with acetone (Supplementary Fig. S3b) gave absorption maxima at 475 and 502 nm, indicating the presence of the spirilloxanthin series of carotenoids. FTIR spectroscopic analysis of strain JA124^T in comparison with *Mch. indicum* JA100^T showed no notable spectral differences between the strains (data not shown), indicating little or no difference in the chemical composition of these bacteria. However, significant differences were observed between the two bacteria in protein patterns (Fig. 1).

The DNA base composition of strain JA124^T was 67 mol% G+C (by HPLC). The phylogenetic relationship of strain JA124^T to other purple sulfur bacteria was examined by 16S rRNA gene sequencing. The data obtained revealed that the sequence of the new isolate was included in the cluster of the

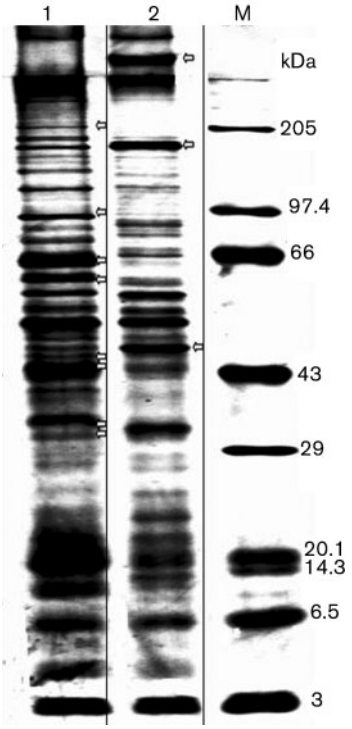

Fig. 1. Protein banding patterns of cell lysates, visualized with silver staining after SDS-PAGE, of *Mch. indicum* DSM 15907^T (lane 1) and strain JA124^T (lane 2). Lane M, molecular mass standards (kDa). Arrows indicate differences.

genus *Marichromatium* (Fig. 2) but was distinct from other species of the genus. The highest sequence similarities of strain JA124^T were found with the type strains of *Mch. indicum* (98 %), *Mch. purpuratum* (95 %) and *Mch. gracile* (93 %). However, DNA–DNA hybridization with *Mch. indicum* DSM 15907^T revealed a relatedness of only 65 % with strain JA124^T. Apart from the 16S rRNA gene sequence dissimilarity and DNA–DNA hybridization studies, strain JA124^T showed clear phenotypic differences from other *Marichromatium* species (Table 1) that justify the description of a novel species, *Marichromatium bheemlicum* sp. nov.

Description of *Marichromatium bheemlicum* sp. nov.

Marichromatium bheemlicum (bheem′li.cum. N.L. neut. adj. *bheemlicum* named after Bheemli, the place from which the type strain was isolated).

Cells are rod-shaped, 0.8–1.0 μm wide and 2–4 μm long, motile and multiply by binary fission. Gram-negative. Growth occurs under anaerobic conditions in the light (photolithoautotrophy, photolithoheterotrophy and photoorganoheterotrophy). Internal photosynthetic membranes are of the vesicular type. The colour of phototrophic cultures is reddish brown. The *in vivo* absorption spectrum of intact cells in sucrose exhibits maxima at 374, 488, 590, 797 and 851 nm. Photosynthetic pigments are bacteriochlorophyll *a* and probably carotenoids of the spirilloxanthin series. The type strain is mesophilic (temperature range 20–35 °C), with a pH optimum at 6.5–8.5, and requires NaCl (range 1.5–11 %) for growth. Photolithoheterotrophy with various organic compounds is the preferred mode of growth. Good carbon sources are acetate, pyruvate and intermediates of the citric acid cycle except citrate. Growth also occurs on glycerol and caproate. Thiosulfate, sulfate, sulfite, thioglycollate, cysteine and sodium sulfide are utilized as sulfur sources under phototrophic conditions. Photolithoautotrophic growth occurs. Diazotrophic growth and acetylene reduction activity are absent.

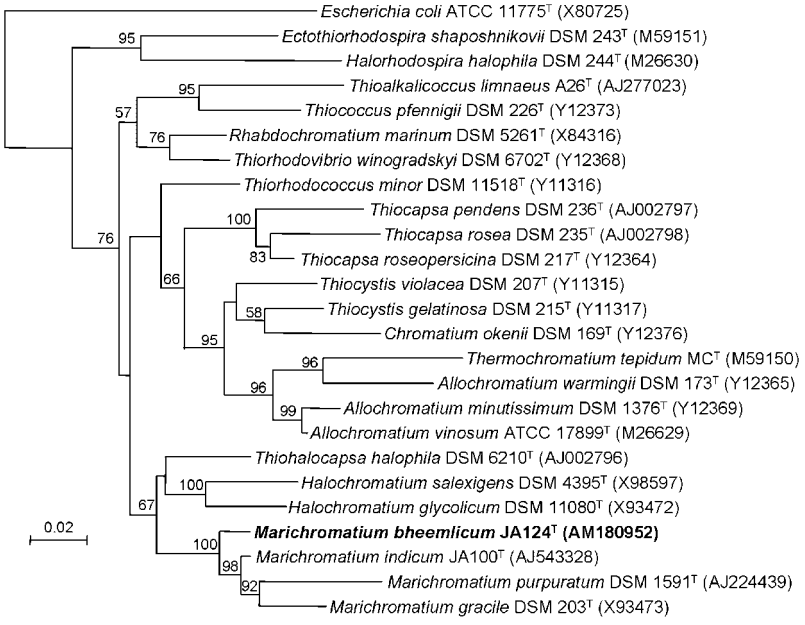

Fig. 2. Dendrogram depicting the phylogenetic relationships of strain JA124^T within the family *Chromatiaceae* determined using 16S rRNA gene sequence analysis. See text for details. Bar, 2 nucleotide substitutions per 100 nucleotides.

Pyridoxal phosphate is required for growth of the type strain. The DNA base composition is 67 mol% G+C (by HPLC).

The type strain, JA124^T (=ATCC BAA-1316^T=JCM 13911^T), was isolated from a marine aquaculture pond exposed to light at Bheemli, on the Bay of Bengal in India.

Acknowledgements

Financial assistance received from Department of Biotechnology, Government of India, is acknowledged. P. A. K. and T. N. R. S. acknowledge the CSIR, Government of India, for the award of JR/SR fellowships. The skilful assistance of F. Lappe (IFM-GEOMAR, Kiel) in molecular analysis and Mercy Rose Stella (JNTU) for SDS-PAGE analysis is gratefully acknowledged. Financial assistance received under the DST-DAAD exchange program (grant 422-PPP-34105) is acknowledged.

References

Arunasri, K., Sasikala, C., Ramana, C. V., Süling, J. & Imhoff, J. F. (2005). *Marichromatium indicum* sp. nov., a novel purple sulfur gammaproteobacterium from mangrove soil of Goa, India. *Int J Syst Evol Microbiol* **55**, 673–679.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

Caumette, P., Imhoff, J. F., Süling, J. & Matheron, R. (1997). *Chromatium glycolicum* sp. nov., a moderately halophilic purple sulfur bacterium that uses glycolate as substrate. *Arch Microbiol* **167**, 11–18.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Felsenstein, J. (1989). PHYLIP (phylogeny inference package), version 3.5.1. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.

Huß, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.

Imhoff, J. F. (1988). Anoxygenic phototrophic bacteria. In *Methods in Aquatic Bacteriology*, pp. 207–240. Edited by B. Austin. Chichester: Wiley.

Imhoff, J. F. & Trüper, H. G. (1980). *Chromatium purpuratum*, sp. nov., a new species of the *Chromatiaceae*. *Zentralbl Bakteriol Parasitenkd Infektionskr Hyg Abt 1 Orig* [*C*] **1**, 61–69.

Imhoff, J. F., Süling, J. & Petri, R. (1998). Phylogenetic relationships among the *Chromatiaceae*, their taxonomic reclassification and description of the new genera *Allochromatium*, *Halochromatium*, *Isochromatium*, *Marichromatium*, *Thiococcus*, *Thiohalocapsa* and *Thermochromatium*. *Int J Syst Bacteriol* **48**, 1129–1143.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Pfennig, N. & Trüper, H. G. (1989). Purple bacteria. In *Bergey's Manual of Systematic Bacteriology*, vol. 3, pp. 1637–1653. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore: Williams & Wilkins.

Pfennig, N. & Trüper, H. G. (1992). The family Chromatiaceae. In *The Prokaryotes. A Handbook on the Biology of Bacteria. Ecophysiology, Isolation, Identification, Applications*, 2nd edn, pp. 3200–3221. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. Berlin, Heidelberg, New York: Springer.

Ramana, Ch. V., Sasikala, Ch., Arunasri, K., Anil Kumar, P., Srinivas, T. N. R., Shivaji, S., Gupta, P., Suling, J. & Imhoff, J. F. (2006). *Rubrivivax benzoatilyticus* sp. nov., an aromatic hydrocarbon-degrading purple betaproteobacterium. *Int J Syst Evol Microbiol* **56**, 2157–2164.

Sasikala, K., Ramana, Ch. V., Raghuveer Rao, P. & Subrahmanyam, M. (1990). Photoproduction of hydrogen, nitrogenase and hydrogenase activities of free and immobilized whole cells of *Rhodobacter sphaeroides* OU 001. *FEMS Microbiol Lett* **72**, 23–28.

Sorokin, D. Y., Tourova, T. P., Antipov, A. N., Muyzer, G. & Kuenen, J. G. (2004). Anaerobic growth of the haloalkaliphilic denitrifying sulfur-oxidizing bacterium *Thialkalivibrio thiocyanodenitrificans* sp. nov. with thiocyanate. *Microbiology* **150**, 2435–2442.

Srinivas, T. N. R., Anil Kumar, P., Sasikala, Ch., Ramana, Ch. V., Süling, J. & Imhoff, J. F. (2006). *Rhodovulum marinum* sp. nov., a novel phototrophic purple non-sulfur alphaproteobacterium from marine tides of Visakhapatnam, India. *Int J Syst Evol Microbiol* **56**, 1651–1656.

Strzeszewski, B. (1913). Beiträge zur Kenntnis der Schwefelflora in der Umgebung von Krakau. *Bull Acad Sci Cracovie* [*B*], 309–334 (in German).

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Trüper, H. G. (1970). Culture and isolation phototrophic sulfur bacteria from the marine environment. *Helgol Wiss Meeresunters* **20**, 6–16.