International Journal of Systematic and Evolutionary Microbiology (2007), **57**, 2458–2461 **DOI** 10.1099/ijs.0.64880-0

Two novel species of marine phototrophic *Gammaproteobacteria*: *Thiorhodococcus bheemlicus* sp. nov. and *Thiorhodococcus kakinadensis* sp. nov.

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, J. N. T. 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

Two coccoid phototrophic purple sulfur bacteria were isolated from marine habitats (marine aquaculture pond near Bheemli, Visakhapatnam and marine tidal waters from a fishing harbour, Kakinada) in a medium that contained 3 % NaCl (w/v). Strains JA132^T and JA130^T are Gram-negative, motile cocci with a single flagellum. Both have an obligate requirement for NaCl. Intracellular photosynthetic membranes are of the vesicular type. Bacteriochlorophyll *a* and most probably carotenoids of the spirilloxanthin series were present as photosynthetic pigments. Both strains were able to grow photolithoautotrophically and photolithoheterotrophically. Chemotrophic and fermentative growth could not be demonstrated. There is no vitamin requirement for strain JA132^T, while strain JA130^T requires niacin, biotin and pantothenate as growth factors. Phylogenetic analysis on the basis of 16S rRNA gene sequences showed that both strains cluster with species of the genus *Thiorhodococcus* belonging to the *Gammaproteobacteria*. The DNA G+C contents of strains JA132^T and JA130^T were 65.5 and 57.5 mol%, respectively. Based on the 16S rRNA gene sequence analysis, morphological and physiological characteristics, strains JA132^T and JA130^T are significantly different from each other and from other species of the genus *Thiorhodococcus* and are recognized as two novel species, for which the names *Thiorhodococcus bheemlicus* sp. nov. and *Thiorhodococcus kakinadensis* sp. nov. are proposed. The type strains of *T. bheemlicus* sp. nov. and *T. kakinadensis* sp. nov. are JA132^T (=MTCC 8120^T=ATCC BAA-1362^T=JCM 14149^T=DSM 18805^T) and JA130^T (=ATCC BAA-1353^T=DSM 18858^T=JCM 14150^T), respectively.

Marine habitats are excellent niches for bacterial diversity and more specifically for the anoxygenic phototrophic bacteria (APB). Marine habitats studied so far for the diversity of APB include sediment samples of salterns, man-made and natural lagoons, aquaculture ponds, marine sulfur springs, marine tidal waters, salt marshes and estuarine waters (Imhoff, 2001). Members of the family *Chromatiaceae* isolated from such habitats include: *Lamprobacter*, *Halochromatium*, *Thiohalocapsa*, *Marichromatium*, *Rhabdochromatium*, *Isochromatium*, *Thiorhodovibrio*, *Thiococcus*, *Thiorhodococcus*, *Thioflavicoccus*, *Thioalkalicoccus* and *Thiorhodospira* species (Imhoff, 2001). Currently, the genus *Thiorhodococcus* comprises two species with validly published names *Thiorhodococcus minor* (Imhoff *et al.*, 1998) (originally described as *Thiorhodococcus minus*; Guyoneaud *et al.*, 1997) and *Thiorhodococcus mannitoliphagus* (Rabold *et al.*, 2006) ['*Thiorhodococcus drewsii*' (Zaar *et al.*, 2003) is also a well characterized species but not validated so far] isolated from anoxic sediment of a fishpond (man-made coastal lagoon), a microbial mat of salt marsh and a microbial mat of an estuary, respectively. In this report, we propose two novel species, *Thiorhodococcus bheemlicus* sp. nov., isolated from anoxic sediment from an aquaculture pond and *Thiorhodococcus kakinadensis* sp. nov., isolated from marine tidal waters. Based on phenotypic and

Abbreviation: APB, anoxygenic phototrophic bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains JA132^T and JA130^T are AM282559 and AM282561, respectively.

Supplementary figures are available with the online version of this paper.

phylogenetic analyses these bacteria have properties distinct from all other species of *Thiorhodococcus*.

The sample that yielded strain JA132^T was collected on 24 February 2005 from a marine aquaculture pond, Bheemli, Visakhapatnam, India (17° 54′ N, 83° 27′ E). The sample that yielded strain JA130^T was collected on 25 April 2005 from a beach of Kakinada, near a fishing harbour, Bay of Bengal, eastern coast of India (16° 54′ N, 82° 14′ E). Both samples had a pH of 6.8, a salinity of 2–3 % NaCl and a temperature of 30 °C. Both strains were isolated from photolithoautotrophic enrichments of these samples. Purification and polyphasic taxonomic studies were carried out as described previously (Anil Kumar *et al.*, 2007). Cell material for 16S rRNA gene sequencing was taken from 1 to 2 ml of well-grown liquid culture. DNA was extracted and purified by using the Qiagen genomic DNA extraction kit. PCR amplification and 16S rRNA gene sequencing were performed as described previously (Imhoff *et al.*, 1998). Recombinant *Taq* polymerase was used for PCR 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 X program (Thompson *et al.*, 1997) and the alignment was corrected manually. The CLUSTAL X alignment file was used as the input file into 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 produced using 100 trees generated during maximum-likelihood analysis using the CONSENSE program. A final dendrogram with evolutionary distances was constructed by taking the alignment.phy file as the infile (input file) and the consensus tree as the intree (input tree) in the maximum-likelihood program within the PHYLIP package (Felsenstein, 1989).

Cells of strains JA132^T and JA130^T were cocci, with a 3.0–6.0 μm diameter. Cells of strain JA132^T are mostly diplococci [Supplementary Fig. S1(a) available in IJSEM Online], while strain JA130^T possesses cells that are arranged as chains or as tetrads [Supplementary Fig S1(b)]. Both strains multiply by binary fission and are motile with a single polar flagellum [Supplementary Fig. S2(a) and (b)]. Electron micrograph of ultrathin sections of both bacteria revealed a vesicular type of internal membrane structures [Supplementary Fig. S3(a) and (b)]. The substrates that were utilized by both the strains are shown in Table 1. Ammonium chloride, urea and dinitrogen were utilized as nitrogen sources for growth by strain JA132^T, while nitrate, nitrite, glutamate and glutamine did not support growth. Strain JA130^T could utilize ammonium chloride and glutamine as nitrogen sources for growth but was unable to utilize nitrate, nitrite, glutamate and dinitrogen. The whole cell absorption spectrum of strain JA132^T showed absorption maxima at 371, 460, 491, 530, 590, 803 and 857 nm, whereas strain JA130^T had absorption maxima at 374, 390, 460, 494, 527, 590, 803 and 857 nm, confirming the presence of bacteriochlorophyll *a*, and most probably carotenoids of the spirilloxanthin series [Supplementary Fig. S4(a) and (b)].

The DNA G+C (by HPLC) contents of strains JA130^T and JA132^T were 65.5 and 57.5 mol%. The phylogenetic relationship of strains JA130^T and JA132^T to other purple sulfur bacteria was examined on the basis of 16S rRNA gene sequences. The data obtained revealed that the novel isolates clustered with the type strains of *Thiorhodococcus* species but were distinct from other genera of purple sulfur

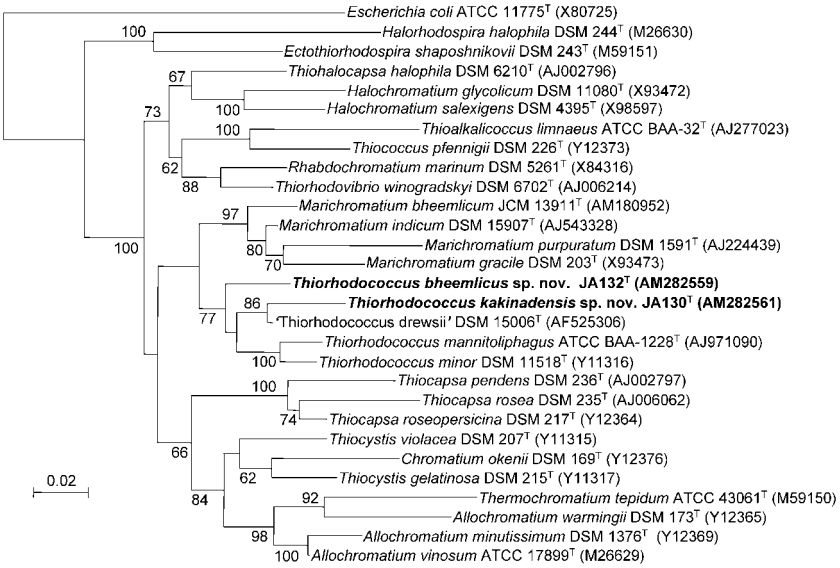

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strains JA132^T and JA130^T within the family *Chromatiaceae*. The tree was constructed using the maximum-likelihood analysis. Numbers at nodes are bootstrap values. Bar, 2 nucleotide substitutions per 100 nucleotides.

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

Species/strains: 1, *Thiorhodococcus minor*; 2, '*Thiorhodococcus drewsii*'; 3, *Thiorhodococcus mannitoliphagus*; 4, strain JA130^T; 5, strain JA132^T. Na_2S is utilized by all the strains. Organic substrate utilization was tested during photoheterotrophic growth. Pyruvate, fumarate and aspartate were utilized by all the strains. Caproate, caprylate, tartrate, methionine were not utilized by any of the strains. Cells of all strains were motile. Cells of all strains were coccoid in shape. The major carotenoid in all strains is rhodopin and the carotenoid group is spirilloxanthin. +, Substrate utilized or present; −, substrate not utilized or absent; (+), weak growth; (i), decrease of growth up to 50 % of control; i, decrease of growth up to more than 50 % of control; ND, not determined.

Characteristic	1*	2†	3‡	4	5
Cell size (μm)	1.0–2.0	2.0–3.5	1.5–2.0	3.0–5.0	4.0–6.0
Colour of cell suspension	Brown–orange	Brown–red	Purple–violet	Purple–violet	Purple–violet
DNA G+C content (mol%)	60–64	64.5	61.8	57.5 (by HPLC)	65.5 (by HPLC)
Vitamin requirement	−	−	B_{12}	n,b,p§	−
Sulfate assimilation	−	+	−	−	−
Chemolithotrophic growth	+	ND	−	−	−
pH optimum (range)	7.0–7.2 (6.0–8.0)	6.5–6.7 (5.2–8.5)	7.0–7.5 (7.0–8.5)	7.2 (6.5–7.2)	7.0–7.5 (6.5–8.0)
Temperature optimum (°C)	30–35	30–35	25–30	25–30	25–30
Salinity range (%)	0.5–9.0	0.0–8.0	0.1–3.0	0.5–5.0	0.5–6.0
NaCl optimum (%)	2.0	2.4–2.6	0.5–2.0	1.0–2.0	1.0–3.0
Carbon source/e^- donor:					
Hydrogen	+	+	ND	ND	ND
Thiosulfate	+	+	+	−	+
Sulfur	+	+	+	−	−
Sulfite	−	+	+	−	−
Formate	−	+	−	−	−
Acetate	+	+	+	(+)	+
Propionate	+	+	+	+	−
Butyrate	−	+	−	−	−
Lactate	+	+	+	(+)	+
Succinate	+	+	+	+	−
Malate	(+)	+	+	+	−
Fructose	+	+	(+)	−	−
Glucose	−	+	+	−	+
Ethanol	+	+	i	−	−
Propanol	+	+	(i)	−	−
Glycerol	−	+	−	−	+
Glycolate	+	+	(i)	−	+
Crotonate	−	+	−	−	−
Valerate	−	+	(i)	−	(+)
Casamino acids	−	+	+	−	+

*Data from Guyoneaud *et al.* (1997).
†Data from Zaar *et al.* (2003).
‡Data from Rabold *et al.* (2006).
§n, Niacin; b, biotin; p, pantothenate.

bacteria. The highest sequence similarities of strain JA132^T and JA130^T were found with the type strains of *Thiorhodococcus mannitoliphagus* (94.3 and 93 %), *Thiorhodococcus minor* (94.5 and 93.3 %) and '*Thiorhodococcus drewsii*' (96 and 97 %). The sequence similarity among the two strains JA132^T and JA130^T was 93.5 % (Fig. 1). Apart from 16S rRNA gene sequence dissimilarity, strains JA132^T and JA130^T also show phenotypic differences to other *Thiorhodococcus* species (Table 1) that justify the description of these strains as novel species *Thiorhodococcus bheemlicus* sp. nov. and *Thiorhodococcus kakinadensis* sp. nov., respectively.

Description of *Thiorhodococcus bheemlicus* sp. nov.

Thiorhodococcus bheemlicus (bhee′mli.cus. N.L. masc. adj. *bheemlicus* named after Bheemli, the place from which the type strain was isolated).

Cells are coccoid in shape and mostly diplococci. Cells are 4.0–6.0 μm in diameter, motile with a single flagellum and divide by binary fission. Growth occurs under anaerobic conditions in the light (photolithoautotrophy/photolitho-heterotrophy). Internal photosynthetic membranes are of vesicular type. The colour of phototrophic culture is

purple–violet. The *in vivo* absorption spectrum of intact cells in sucrose exhibits maxima at 371, 460, 491, 530, 590, 803 and 857 nm, confirming the presence of bacteriochlorophyll *a* and most probably carotenoids of the spirilloxanthin series. The type strain is mesophilic (30 °C), with a pH optimum at 7.0–7.2 and has an obligate requirement of NaCl for growth (1–3 %, w/v). Photolithoheterotrophy with organic compounds is the preferred mode of growth. Good carbon sources for growth are acetate, pyruvate, fumarate, glucose and Casamino acids. Growth on lactate, glycerol, glycolate and valerate is also possible. Chemotrophic (aerobic) growth could not be demonstrated. Fermentative growth is not possible in the presence of pyruvate as a fermentable carbon source. No growth factors are required. The DNA G+C (by HPLC) content is 65.5 mol%. Natural habitats are anoxic sediments of marine aquaculture pond waters.

The type strain, JA132^T (=MTCC 8120^T=ATCC BAA-1362^T=JCM 14149^T=DSM 18805^T), was isolated from a marine aquaculture pond, Bheemli, Visakhapatnam, India.

Description of *Thiorhodococcus kakinadensis* sp. nov.

Thiorhodococcus kakinadensis (ka′ki.na.den′sis. N.L. masc. adj. *kakinadensis* named after Kakinada, the place from which the type strain was isolated).

Cells are coccoid and are arranged as chains or tetrads. Cells are of 3.0–5.0 μm diameter, motile with single flagellum and divide by binary fission. Growth occurs under anaerobic conditions in the light (photolithoautotrophy/photolithoheterotrophy). Internal photosynthetic membranes are of vesicular type. The colour of phototrophic culture is purple–violet. The *in vivo* absorption spectrum of intact cells in sucrose exhibits maxima at 374, 390, 460, 494, 527, 590, 803 and 857 nm, confirming the presence of bacteriochlorophyll *a* and most probably carotenoids of the spirilloxanthin series. The type strain is mesophilic (30 °C), with a pH optimum at 7.2 and requires NaCl for growth (optimum 1–2 %, w/v). Photolithoheterotrophy with organic compounds is the preferred mode of growth. Good carbon sources for growth are pyruvate, propionate, fumarate, succinate and malate. Growth on acetate and lactate also occurs. Chemotrophic (aerobic) growth could not be demonstrated. Fermentative growth is not possible in the presence of pyruvate as a fermentable carbon source. Niacin, biotin and pantothenate are required as growth factors. The DNA G+C (by HPLC) content is 57.5 mol%. Natural habitats are marine tidal waters.

The type strain, JA130^T (=ATCC BAA-1353^T=DSM 18858^T=JCM 14150^T), was isolated from a beach of Kakinada, near a fishing harbour, Bay of Bengal, eastern coast of India.

Acknowledgements

Financial assistance received from the Department of Biotechnology and Department of Ocean Development, Government of India is acknowledged. P. A. K. and T. N. R. S. acknowledge the CSIR, Government of India, for the award of SR fellowships. T. S. S. J. acknowledges JNTU for the award of JR fellowship. Financial assistance received under DST-DAAD exchange program (grant 422-PPP-34105) is acknowledged.

References

Anil Kumar, P. A., Sasi Jyothsna, T. S., Srinivas, T. N. R., Sasikala, Ch., Ramana, Ch. V. & Imhoff, J. F. (2007). *Marichromatium bheemlicum* sp. nov., a non-diazotrophic photosynthetic gammaproteobacterium from a marine aquaculture pond. *Int J Syst Evol Microbiol* **57**, 1261–1265.

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

Guyoneaud, R., Matheron, R., Liesack, W., Imhoff, J. F. & Caumette, P. (1997). *Thiorhodococcus minus*, gen. nov., sp. nov., a new purple sulfur bacterium isolated from coastal lagoon sediments. *Arch Microbiol* **168**, 16–23.

Imhoff, J. F. (2001). True marine and halophilic anoxygenic phototrophic bacteria. *Arch Microbiol* **176**, 243–254.

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.

Rabold, S., Gorlenko, V. M. & Imhoff, J. F. (2006). *Thiorhodococcus mannitoliphagus* sp. nov., a purple sulfur bacterium from the White Sea. *Int J Syst Evol Microbiol* **56**, 1945–1951.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463–5467.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1997). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Zaar, A., Fuchs, G., Golecki, J. R. & Overmann, J. (2003). A new purple sulfur bacterium isolated from a littoral microbial mat, *Thiorhodococcus drewsii* sp. nov. *Arch Microbiol* **179**, 174–183.