Thiohalocapsa marina sp. nov., from an Indian marine aquaculture pond

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A spherical-shaped, phototrophic, purple sulfur bacterium was isolated in pure culture from anoxic sediment in a marine aquaculture pond near Bheemli (India). Strain JA142[T] is Gram-negative and non-motile. It has a requirement for NaCl (optimum of 2 % and maximum of 6 % w/v NaCl). Intracellular photosynthetic membranes are of the vesicular type. *In vivo* absorption spectra indicate the presence of bacteriochlorophyll *a* and carotenoids of the okenone series as photosynthetic pigments. Phylogenetic analysis on the basis of 16S rRNA gene sequences showed that strain JA142[T] is related to halophilic purple sulfur bacteria of the genera *Thiohalocapsa* and *Halochromatium*, with the highest sequence similarity to *Thiohalocapsa halophila* DSM 6210[T] (97.5 %). Morphological and physiological characteristics differentiate strain JA142[T] from other species of the genera *Halochromatium* and *Thiohalocapsa*. Strain JA142[T] is sufficiently different from *Thiohalocapsa halophila* based on 16S rRNA gene sequence analysis and morphological and physiological characteristics to allow the proposal of a novel species, *Thiohalocapsa marina* sp. nov., with the type strain JA142[T] (=JCM 14780[T] =DSM 19078[T]).

The genus *Thiohalocapsa* was established to separate species of purple sulfur bacteria from other species of the genus *Thiocapsa* based on their halophilic growth response, lack of gas vesicles, large phylogenetic distance and clustering with marine and halophilic strains (Imhoff *et al.*, 1998). At present, the genus *Thiohalocapsa* comprises only one species, *Thiohalocapsa halophila* (Imhoff *et al.*, 1998; originally described as *Thiocapsa halophila* Caumette *et al.* 1991).

Strain JA142[T] was isolated from photolithoautotrophic enrichments with 2 % NaCl (w/v) of anoxic sediment and water (sample properties: pH 7.0, salinity 2 % and temperature 30 °C) from a marine aquaculture pond near Bheemli, Visakhapatnam, India (17° 54′ N 83° 27′ E). Purification was achieved by repeated agar-shake dilution series (Pfennig & Trüper, 1992; Imhoff, 1988; Trüper,

1970). Polyphasic taxonomic studies and spectral analysis were carried out as described earlier (Anil Kumar *et al.*, 2007a, 2008). Utilization of organic compounds as carbon sources/electron donors for phototrophic growth was tested in the presence of yeast extract (0.03 %, w/v) without any additional carbon source/electron donor. The concentrations of these compounds were 1 mM benzoate, 0.1 % (v/v) for formic acid, propionic acid, butyric acid, caproic acid, valeric acid, lactic acid, glycerol, methanol and ethanol and 0.3 % (w/v) for the other organic compounds tested.

Cells of strain JA142[T] were spherical, non-motile, 1.5–2.0 μm in diameter and multiplied by binary fission (Supplementary Fig. S1, available in IJSEM Online). Electron photomicrographs of ultrathin sections of the cells revealed a vesicular type of internal membranes. Strain JA142[T] was able to grow photolithoautotrophically [anaerobic, light (30 μE m^{-2} s^{-1}), Na$_2$S . 9H$_2$O (2 mM)/ Na$_2$S$_2$O$_3$. 5H$_2$O (5 mM) and NaHCO$_3$ (12 mM)]. Phototrophic growth (Table 1) in the presence of bicarbonate (12 mM) (photomixotrophy) and Na$_2$S . 9H$_2$O (0.5 mM) was observed with acetate, pyruvate, lactate, fumarate, succinate, glucose and Casamino acids.

Table 1. Differential characteristics between strain JA142^T and species of the genera *Thiohalocapsa* and *Halochromatium*

Strain/species: 1, JA142^T; 2, *Thc. halophila*; 3, *Hch. roseum*; 4, *Hch. salexigens*; 5, *Hch. glycolicum*. Data for *Hch. roseum* were taken from Anil Kumar *et al.* (2007b); data for the other reference species were taken from Imhoff (2005). Cells of all taxa studied divide by binary fission. All strains have internal membranes of the vesicular type. Na$_2$S and thiosulfate are utilized by all strains. Organic substrate utilization was tested in the presence of sulfide and bicarbonate. Propionate, butyrate, ethanol, propanol and crotonate were not utilized by any of the strains. +, Substrate utilized or present; −, substrate not utilized or absent; (+), weak growth; NR, not reported.

Characteristic	1	2	3	4	5
Cell shape	Sphere	Sphere	Rod	Rod	Rod
Motility	−	−	−	+	+
Cell diameter/size (µm)	1.5–2.0	1.5–2.5	2.0–3.0 × 3.0–5.0	2.0–2.5 × 4.0–7.5	0.8–1.0 × 2.0–4.0
Gas vesicles	−	−	+	−	−
Colour of cell suspension	Purple–red	Purple–red	Purple–pink	Pink, rose red	Pink, pinkish red
Carotenoid group	(Okenone)*	Okenone	Okenone	Spirilloxanthin	Spirilloxanthin
DNA G+C content (mol%)	64.8	65.9–66.6	64	64.6	66.1–66.5
Vitamin B$_{12}$ requirement	−	−	+	+	−
Chemolithotrophic growth	−	+	−	+	+
pH optimum (range)	7.5 (6.5–8.5)	7.0 (6.0–8.0)	7.5 (7–8)	7.4–7.6 (7.0–8.0)	7.2–7.4 (6.2–9.0)
Temperature optimum (°C)	25–30	20–30	27	20–30	25–35
NaCl optimum (range) (%, w/v)	2 (1–6)	4–8 (3–20)	1.5–2.5 (1–3)	8–11 (4–20)	4–6 (2–20)
Photoassimilation of:					
Hydrogen	−	+	NR	+	+
Sulfur	−	+	−	+	+
Sulfite	−	+	−	+	+
Formate	−	−	−	−	(+)
Acetate	+	+	−	+	(+)
Pyruvate	+	+	+	+	(+)
Lactate	+	+	−	−	−
Fumarate	+	−	+	−	+
Succinate	+	−	+	−	+
Malate	−	−	+	−	−
Fructose	−	+	−	−	NR
Glucose	+	(+)	−	−	−
Glycerol	−	(+)	−	−	+
Glycolate	−	−	−	−	+
Valerate	−	−	−	−	NR
Casamino acids	+	−	+	−	(+)

*According to absorption spectra, the presence of okenone as major carotenoid is likely.

Substrates not utilized included formate, propionate, butyrate, malate, fructose, ethanol, propanol, glycerol and crotonate. Photo-organoheterotrophy [anaerobic, light (30 µE m^{-2} s^{-1}), pyruvate (27 mM)], chemolithoautotrophy [aerobic, dark, Na$_2$S$_2$O$_3$.5H$_2$O (5 mM) and NaHCO$_3$ (12 mM)], chemo-organoheterotrophy [aerobic, dark, pyruvate (27 mM)] and fermentative growth [anaerobic, dark, pyruvate (27 mM)] could not be demonstrated. Na$_2$S.9H$_2$O and Na$_2$S$_2$O$_3$.5H$_2$O were utilized as electron donors under photolithoautotrophic conditions with a minimum concentration of 0.5 mM Na$_2$S.9H$_2$O and a tolerance of up to 4 mM, while sulfite, elemental sulfur and hydrogen did not support growth. During oxidation of sulfide, elemental sulfur droplets were stored inside the cells. Na$_2$S.9H$_2$O and Na$_2$S$_2$O$_3$.5H$_2$O were utilized as sulfur sources by strain JA142^T, while sulfate, sulfite, thioglycolate and cysteine did not support growth.

Ammonium chloride was utilized as a nitrogen source by strain JA142^T, while nitrate, nitrite, glutamate, glutamine, urea and dinitrogen did not support growth. Strain JA142^T is a true marine strain; growth occurs at 1.0–6.0 % NaCl (w/v) with an optimum at 2.0 % (w/v). The pH range for growth of strain JA142^T is pH 6.5–8.5 with an optimum at pH 7.5. The temperature optimum for growth is 25–30 °C (range 25–35 °C). Strain JA142^T does not require vitamins for growth. The colour of the phototrophically grown cell suspension is purple–red. The whole-cell absorption spectrum of strain JA142^T exhibited absorption maxima at 395, 509, 584, 803 and 845 nm and a shoulder at 878 nm, confirming the presence of bacteriochlorophyll *a* (Supplementary Fig. S2a), and the absorption spectrum for pigments extracted with acetone exhibited maxima at 462, 488 and 516 nm, indicating the presence of the carotenoid okenone (Supplementary Fig. S2b).

DNA was extracted and purified by using the Qiagen genomic DNA extraction kit. The DNA base composition of strain JA142[T] was 64.8 mol% G+C (by HPLC). PCR amplification and 16S rRNA gene sequencing were performed as described previously (Imhoff *et al.*, 1998). Recombinant *Taq* polymerase was used for PCR, which was started with primers 5′-GTTTGATCCTGGCTCAG-3′ and 5′-TACCTTGTTACGACTTCA-3′ (*Escherichia coli* positions 11–27 and 1489–1506, respectively). 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). Nearest relatives and sequence similarities were determined by BLAST search (Altschul *et al.*, 1990) and BLAST 2 SEQUENCES alignment (Tatusova & Madden, 1999). 16S rRNA gene sequences of the closest related type strains belonging to the genera *Halochromatium* and *Thiohalocapsa* were newly determined and the corresponding EMBL database entries were updated prior to phylogenetic analysis. 16S rRNA gene sequences of type strains of representative species of the *Chromatiaceae* and of strain JA142[T] were aligned using the FASTAlign function of the alignment editor implemented in the ARB software package (http://www.arb-home.de; Ludwig *et al.*, 2004) and refined manually employing secondary structure information. For phylogenetic calculations, the PhyML online version (Guindon *et al.*, 2005), MEGA version 4.0 (Kumar *et al.*, 2004) and the PHYLIP DNAPARS program implemented in ARB (Ludwig *et al.*, 2004) were used. For tree calculation, a character-based method [maximum-likelihood (Felsenstein, 1981)], two distance-based methods [neighbour joining (Saitou & Nei, 1987) and minimum evolution (Rzhetsky & Nei, 1993)] as well as a maximum-parsimony method (Eck & Dayhoff, 1966; Fitch, 1971, 1977) were employed. The Tamura–Nei model was determined as the model best suited for phylogenetic calculation using the program ModelGenerator (Keane *et al.*, 2006). The maximum-likelihood tree was calculated using the TN93 model, six rate categories, gamma distribution parameter alpha=0.31 and proportion of invariable sites=0.43 as determined by ModelGenerator. For the maximum-likelihood bootstrap analysis, the non-bootstrapped maximum-likelihood tree was used as the starting tree. The neighbour-joining tree was calculated based on distances corrected by the Tamura–Nei nucleotide substitution model, using sites corresponding to the pairwise deletion option, including transition and transversion substitutions, assuming a heterogeneous pattern among lineages and a gamma-distributed substitution rate (alpha=0.31). The maximum-parsimony tree was calculated using the 'more thorough search' option and a randomized sequence order.

The 16S rRNA gene sequence analysis revealed that the new isolate belongs to the family *Chromatiaceae* and is affiliated to a group of marine and halophilic genera including *Halochromatium*, *Marichromatium*, *Thiorhodovibrio*, *Rhabdochromatium* and *Thiohalocapsa*. Highest 16S rRNA gene sequence similarity was shared with *Thc. halophila* DSM 6210[T] (97.5 %) and *Halochromatium glycolicum* 6340[T] (97.2 %). Similarity values of <98.7 % suggest separation at the species level according to Stackebrandt & Ebers (2006). Phylogenetic analyses (Fig. 1) confirmed a close relationship between strain JA142[T] and both *Halochromatium roseum* JA134[T] and *Thc. halophila* DSM 6210[T]. In all cases, *Halochromatium* species and *Thc. halophila* and JA142[T] clustered monophyletically. Additionally, in all trees (Fig. 1 and Supplementary Fig. S3), *Halochromatium* species formed a tight subcluster, strongly supported by bootstrap analysis, that did not include strain JA142[T]. The distance-based trees further indicate a separate clustering of *Thc. halophila* and strain JA142[T]. Detailed comparison of 16S rRNA gene sequences revealed particular sequence differences in a number of characteristic nucleotide positions of strain JA142[T] from both *Halochromatium* species and *Thc. halophila* DSM 6210[T] (Table 2). Overall sequence similarity as well as signature nucleotides demonstrate a closer relationship of strain JA142[T] to *Thc. halophila* compared with *Halochromatium* species (12 nucleotides identical to *Thiohalocapsa* compared with eight identical nucleotides to *Halochromatium*; Table 2). However, nine characteristic nucleotides were different from both *Thc. halophila* and *Halochromatium* species, which indicates an intermediate or borderline position between known representatives of the two genera. This view is supported by phylogenetic relationships, as demonstrated by phylogenetic trees constructed by a variety of different methods. All methods used (neighbour-joining, minimum-evolution, maximum-likelihood and maximum-parsimony; Fig. 1 and Supplementary Fig. S3) demonstrate the clustering of JA142[T] with *Halochromatium* and *Thc. halophila*. Furthermore, all phylogenetic methods strongly support a subcluster of the three known *Halochromatium* type strains that did not include strain JA142[T] or *Thc. halophila*.

Sequences of *pufLM* support the association of the new isolate with the *Halochromatium*/*Thiohalocapsa* cluster (not shown). More specifically, they demonstrate a clear relationship to the *pufLM* sequence of *Thc. halophila*, but not those of *Halochromatium* species (M. Tank and J. F. Imhoff, unpublished results). Similarity of the *pufLM* nucleotide sequence of strain JA142[T] (approx. 1390 bp) to that of the type strain of *Thc. halophila* was 88 %; similarities to several sequences from *Halochromatium* species were 84–85 % and to sequences from *Thiorhodovibrio* species were below 80 %.

A value of 70 % DNA relatedness has been used as a benchmark for separation at the species level for a number of years, and a 16S rRNA gene sequence similarity of 97 % was regarded as borderline for requiring DNA–DNA hybridization data, assuming that this value more or less coincides with 70 % DNA–DNA relatedness. In their critical analysis, Stackebrandt & Ebers (2006) carefully compared 16S rRNA gene sequence similarities with DNA–DNA reassociation values from a great number of
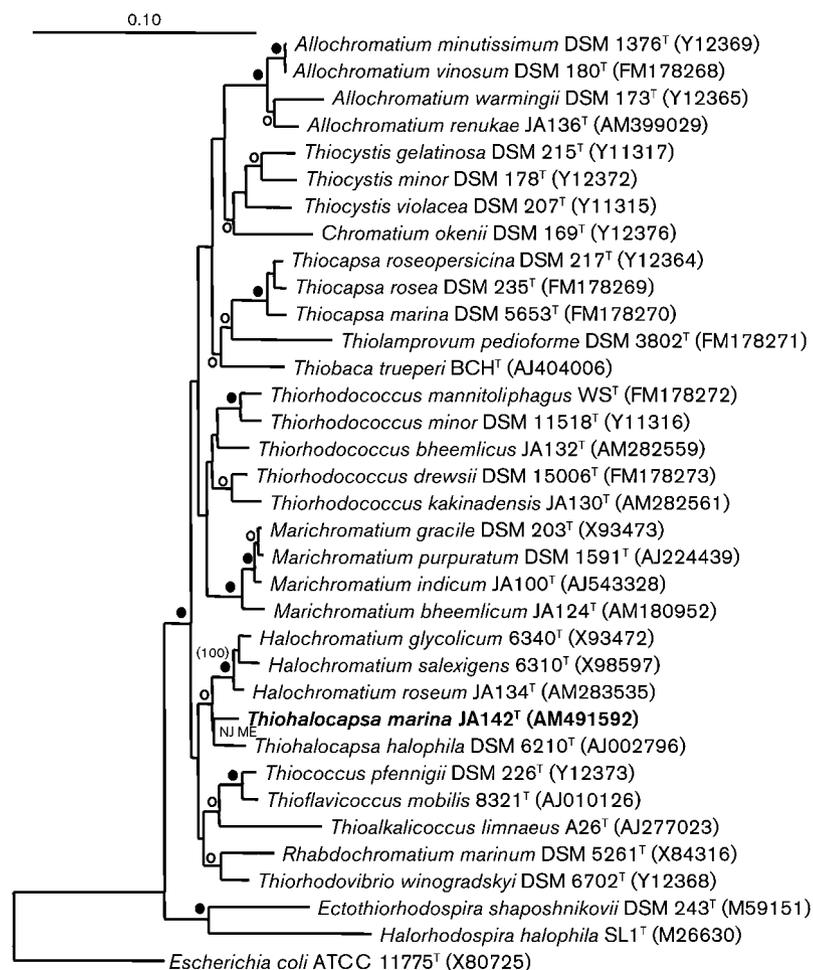
0.10

Allochromatium minutissimum DSM 1376^T (Y12369)
Allochromatium vinosum DSM 180^T (FM178268)
Allochromatium warmingii DSM 173^T (Y12365)
Allochromatium renukae JA136^T (AM399029)
Thiocystis gelatinosa DSM 215^T (Y11317)
Thiocystis minor DSM 178^T (Y12372)
Thiocystis violacea DSM 207^T (Y11315)
Chromatium okenii DSM 169^T (Y12376)
Thiocapsa roseopersicina DSM 217^T (Y12364)
Thiocapsa rosea DSM 235^T (FM178269)
Thiocapsa marina DSM 5653^T (FM178270)
Thiolamprovum pedioforme DSM 3802^T (FM178271)
Thiobaca trueperi BCH^T (AJ404006)
Thiorhodococcus mannitoliphagus WS^T (FM178272)
Thiorhodococcus minor DSM 11518^T (Y11316)
Thiorhodococcus bheemlicus JA132^T (AM282559)
Thiorhodococcus drewsii DSM 15006^T (FM178273)
Thiorhodococcus kakinadensis JA130^T (AM282561)
Marichromatium gracile DSM 203^T (X93473)
Marichromatium purpuratum DSM 1591^T (AJ224439)
Marichromatium indicum JA100^T (AJ543328)
Marichromatium bheemlicum JA124^T (AM180952)
Halochromatium glycolicum 6340^T (X93472)
(100) *Halochromatium salexigens* 6310^T (X98597)
Halochromatium roseum JA134^T (AM283535)
Thiohalocapsa marina JA142^T (AM491592)
NJ ME *Thiohalocapsa halophila* DSM 6210^T (AJ002796)
Thiococcus pfennigii DSM 226^T (Y12373)
Thioflavicoccus mobilis 8321^T (AJ010126)
Thioalkalicoccus limnaeus A26^T (AJ277023)
Rhabdochromatium marinum DSM 5261^T (X84316)
Thiorhodovibrio winogradskyi DSM 6702^T (Y12368)
Ectothiorhodospira shaposhnikovii DSM 243^T (M59151)
Halorhodospira halophila SL1^T (M26630)
Escherichia coli ATCC 11775^T (X80725)

Fig. 1. Phylogenetic consensus tree based on 16S rRNA gene sequences showing the relationship of strain JA142^T within the family *Chromatiaceae*. Phylogenetic trees were calculated by the minimum-evolution and neighbour-joining methods as well as by the maximum-parsimony and maximum-likelihood methods. Tree topology of all four methods was compared and shared nodes are marked in the minimum-evolution tree as follows: nodes supported by all four methods are marked by open circles (○); nodes shared by all four methods and supported by bootstrap values >95 % or 100 % are marked with filled circles [● and (100)●, respectively]; a node supported only by the distance-based methods is indicated by NJ ME. Bar, 0.1 substitutions per alignment position.

publications. Their convincing result was that, below 98.5 % 16S rRNA gene sequence similarity, there was not a single case where DNA–DNA reassociation was more than 70 %, and these authors argued that, with high-quality sequences (as used in this study), 99 % sequence similarity almost excludes reassociation values of 70 % or more. They recommended a 16S rRNA gene sequence similarity threshold range of 98.7–99 % as the point at which DNA–DNA reassociation experiments should be mandatory for testing the genomic uniqueness of new isolates. Therefore, the 97.5 % 16S rRNA gene sequence similarity between strain JA142^T and *Thc. halophila* DSM 6210^T indicates their separation into different species. This is supported by differences in the G+C content of the two bacteria of 1.1–1.8 mol%, by different salt responses and by a number of differences in substrate and electron donor utilization, including the ability to grow chemolithotrophically (Table 1).

Because of the closer association of the novel bacterium with *Thc. halophila* in terms of both sequence information and phenotypic properties, strain JA142^T is recognized as a member of a novel species of the genus *Thiohalocapsa*, for which the name *Thiohalocapsa marina* sp. nov. is proposed.

Description of *Thiohalocapsa marina* sp. nov.

Thiohalocapsa marina (ma.ri′na. L. fem. adj. *marina* pertaining to the sea, marine).

Cells are spherical, 1.5–2.0 μm in diameter, non-motile and divide by binary fission. Growth occurs under anaerobic conditions in the light under photolithoautotrophic conditions. In addition, several organic substrates can be photoassimilated. Internal photosynthetic membranes are of the vesicular type. Colour of the phototrophically grown cell suspension is purple–red. The *in vivo* absorption spectrum of intact cells in sucrose exhibits maxima at 395, 509, 584, 803 and 845 nm, indicating the presence of bacteriochlorophyll *a* and carotenoids of the okenone series as photosynthetic pigments. The type strain is mesophilic (30 °C), with a pH optimum at 7.5 (range pH 6.5–8.5). Salt is required for growth of the type strain; growth occurs at 1.0–6.0 % NaCl (w/v) with an optimum at 2.0 % (w/v). Photolithotrophic growth is possible in the presence of bicarbonate (12 mM) and $Na_2S.9H_2O$ (0.5 mM). A few organic substrates can be photoassimilated in the presence of sulfide and bicarbonate, including acetate, pyruvate, lactate, fumarate, succinate, glucose and Casamino acids. Photo-organoheterotrophy and chemo-

Table 2. 16S rRNA signature nucleotides for *Thiohalocapsa* and *Halochromatium* species

Positions are given according to the sequence of *E. coli*. Shared nucleotides are highlighted in bold.

Position	*Halochromatium* (*n*=7)	Strain JA142[T]	*Thiohalocapsa* (*n*=5)
144	**G**	**G**	A
148	A	**G**	**G**
223	**G**	A	**G**
250	M (U/G)	**A**	**A**
269	**U**	C	**U**
381	**A**	**A**	C
444	**G**	A	**G**
454	**A**	U	**A**
457	**C**	U	**C**
473	**U**	**U**	C
490	**C**	U	**C**
589	U	**C**	**C**
590	**G**	U	**G**
653	**U**	**U**	C
658	A	**C**	**C**
660	**G**	A	**G**
745	**C**	U	**C**
748	U	**G**	**G**
838	**U**	**U**	C
839	**C**	**C**	U
1001	U	**C**	**C**
1007	–	**U**	**U**
1010	U	**G**	**G**
1021	A	**U**	**U**
1022	–	**U**	**U**
1256	**U**	**U**	C
1257	C	**U**	**U**
1265	**C**	**C**	A
1424	C	**U**	**U**

trophy are not detected. No growth factors are required. The DNA base composition of the type strain is 64.8 mol% G + C (by HPLC).

The type strain, JA142[T] (=JCM 14780[T] =DSM 19078[T]), was isolated from a marine aquaculture pond near Bheemli, Visakhapatnam, India.

Acknowledgements

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