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Full Paper

Prosthecochloris indica sp. nov., a novel green sulfur bacterium from a marine aquaculture pond, Kakinada, India

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A green sulfur bacterium, strain JAGS6^T was isolated from a marine aquaculture pond located near Kakinada on the east coast of India. Cells of strain JAGS6^T were Gram-negative, non-motile, coccoid, 1–1.2 μm in diameter, with prosthecae. Phylogenetic analysis on the basis of 16S rRNA gene sequences showed that strain JAGS6^T clusters with members of the genus *Prosthecochloris* and the sequence similarity with the nearest relative, *Prosthecochloris vibrioformis,* is 96.7%. Cultures of strain JAGS6^T are green in color and the cells contain bacteriochlorophyll *c* and most likely carotenoids of the chlorobactene series as photosynthetic pigments. Strain JAGS6^T is mesophilic, halotolerant (up to 7% NaCl) and is obligately phototrophic, utilizing sulfide but not thiosulfate as a photosynthetic electron donor. Sulfur globules are deposited outside the cells during oxidation of sulfide. On the basis of 16S rRNA gene sequence analysis and its morphological and physiological characteristics, strain JAGS6^T is distinct from described species of the genus *Prosthecochloris* and we propose to describe it as a new species, *Prosthecochloris indica,* sp. nov. The type strain is JAGS6^T (= JCM 13299^T = ATCC BAA1214^T).

Key Words——anoxygenic phototrophic bacteria; aquaculture ponds and 16S rRNA gene based phylogeny; bacteriochlorophyll *c*; *Chlorobiaceae*; green sulfur bacteria; marine bacteria; *Prosthecochloris*

Introduction

Marine ecosystems are natural habitats of anoxygenic phototrophic bacteria and their wide distribution in such environments has led to the recognition of several new species (Imhoff, 2001). It was observed that marine anoxygenic phototrophic bacteria have 16S rRNA gene lineages distinct from the non-marine isolates, which initiated rearrangement and description of new taxa (Imhoff et al., 1998). Marine anoxygenic phototrophic bacteria are known from 3 bacterial phyla. Except for the heliobacteria (phylum *Firmicutes*), marine isolates are well documented in green sulfur bacteria (Phylum *Chlorobi*), the purple sulfur bacteria (*Gammaproteobacteria*), purple nonsulfur bacteria (*Alpha-* and *Betaproteobacteria*) and the green nonsulfur bacteria (Phylum *Chloroflexi*). The majority of these bacteria require salt for optimal growth with NaCl concentrations ranging from 1 to 8% (Imhoff, 2001). The moderately halophilic genera of purple bacteria in-

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clude the genera *Rhodovibrio*, *Rhodothalassium*, *Roseospira*, *Halochromatium*, *Thiohalocapsa* and *Ectothiorhodospira*, while the extreme halophiles include representatives of the genus *Halorhodospira* (Imhoff, 2001). Green sulfur bacteria are widely distributed in brackish and marine waters. Some tolerate or require NaCl for growth with salt optima of 2 to 5% (Imhoff, 2001). *Prosthecochloris aestuarii* and *Prosthecochloris vibrioformis* (formerly *Chlorobium vibrioforme*), are species most frequently observed in marine coastal habitats (Imhoff, 2001). The most halophilic and halotolerant species of the green sulfur bacteria is *Prosthecochloris aestuarii.* This species is widely distributed in estuarine environments and has been found most frequently in various lagoons and estuarine ponds (Imhoff, 2001). It does not grow in the absence of salt, has a salt optimum between 2 and 6% NaCl and can tolerate more than 10% NaCl.

During our study on the diversity of anoxygenic phototrophic bacteria from marine coastal environments of India, we have isolated a number of purple and green bacteria through enrichments. In the present report, we describe a novel green sulfur bacterium, strain JAGS6^T isolated from a marine aquaculture pond located near Kakinada on the east coast of India. On the basis of phenotypic characteristics and the results of a molecular analysis, the novel isolate is classified as a new species of the genus *Prosthecochloris,* for which the name *Prosthecochloris indica* is proposed.

Materials and Methods

Inoculum, enrichment, and isolation. Strain JAGS6^T was isolated from subsurface water samples were collected from a marine aquaculture pond and collected into 60 ml sterile screw cap bottles. The GPS positioning of the sample was obtained using the Google Earth software. GPS positioning of the sample collection site is 16°57′ N 82°15′ E. Within 3 days of sampling, samples were inoculated into sterile 7 ml screw cap tubes filled completely with the modified medium of Eichler and Pfennig (2001) containing sulfanilate (3 mM) and incubated at 30°C under incandescent light (30 μE m^{-2} s^{-1}) for 1 week. Isolations were carried out in repeated agar shake dilution method (Imhoff, 1988; Pfennig and Trüper, 1981) with the same medium used for enrichment. Shake cultures were placed in the dark at 25°C for 12 h and subsequently incubated at 30°C under incandescent light (30 μE m^{-2} s^{-1}). Culture purity was examined microscopically and by streaking the culture on nutrient agar plates incubated aerobically in dark at 30°C for 3 days. The culture was maintained by repeated sub-culturing into a fresh medium and also by preparing stab cultures. The stabs were preserved in a refrigerator at 4°C. Strain JAGS6^T was deposited into the Japan Collection of Microorganisms, Saitama, Japan as JCM 13299.

Media and growth conditions of pure cultures. Cultures of strain JAGS6^T were grown in 120 ml screw cap bottles completely filled with basal medium under light (25 μE m^{-2} s^{-1}) at 30±2°C. The basal medium contained, per liter deionized water: KH_2PO_4, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; $MgSO_4 \cdot 7H_2O$, 3 g; NaCl, 20 g; NH_4Cl, 0.68 g; yeast extract, 0.5 g; ammonium acetate, 0.5 g; trace element solution SL12, 1 ml; vitamin B_{12} (2 mg/100 ml, w/v), 1 ml; $NaHCO_3$ (10%, w/v), 15 ml and $Na_2S \cdot 9H_2O$ (1 M), 3 ml. $NaHCO_3$ (5 g) was dissolved in distilled water (50 ml) in a 120 ml screw capped bottle and bubbled with CO_2 for 5 min and the bottle was closed tightly under CO_2 atmosphere and autoclaved for 15 min at 15 lbs and added to the basal salts medium after cooling. $Na_2S \cdot 9H_2O$ (2.4 g) was dissolved in distilled water (10 ml) in a 15 ml screw cap test tube and flushed with nitrogen gas for 2–3 min to replace the air in the tube with nitrogen gas and closed tightly, autoclaved and added to the basal salts medium after cooling. Vitamin B_{12} (2 mg/100 ml, w/v) was dissolved in distilled water and filter sterilized by using 0.2 μm pore sized Millipore cellulose acetate membrane filter into a sterile screw cap tube. The final pH of the medium was adjusted to 6.8 with sterile 1 M HCl or NaOH after autoclaving. All physiological tests were carried out in the presence of sodium sulfide and $NaHCO_3$ (0.15%, w/v) unless otherwise mentioned. Photoassimilation of different organic substrates as additional source of carbon was tested in the medium described above except that it contained 5 mM $Na_2S \cdot 9H_2O$ and $NaHCO_3$ (0.1%, w/v). The concentration of these compounds was 0.1%, v/v (formic acid, propionate, butyrate, caproate, valerate, lactate, glycerol, methanol and ethanol), 0.3%, w/v (other organic compounds tested), 1 mM sodium benzoate. For testing sulfur sources, $MgSO_4 \cdot 7H_2O$ was replaced by $MgCl_2 \cdot 5H_2O$; sulfur sources ($Na_2S \cdot 9H_2O$, $Na_2S_2O_3$, sodium thioglycolate, cysteine and $MgSO_4 \cdot 7H_2O$ [1 mM]) and in addition 0.1% $NaHCO_3$ were added to the medium. Nitrogen source utilization was tested by replacing ammonium chloride with different nitrogen sources

(0.068%, w/v). Vitamin requirement was tested with different vitamins [$\mu g\ L^{-1}$: biotin (100), thiamine (300), niacin (350), *para*-aminobenzoate (200), pyridoxal phosphate (100), pantothenate (100), inositol (meso, myo, 100)], B_{12} (20) both singly and in combinations (number of combinations —8; each combination lacking one of the 7 vitamins). Negative control did not have any of the vitamins or yeast extract. One control was supplemented with yeast extract alone. Chemotrophic growth was determined by growing the cultures in Erlenmeyer flasks placed in an orbital shaker in the dark and at 30°C. Microaerobic growth was tested by growing the cultures in agar dilution tubes without air-tight stoppers. Diazotrophy of the culture was determined as growth under nitrogen atmosphere by four repeated sub-cultures. The acetylene reduction assay (Sasikala et al., 1990) was used for confirming the presence or absence of nitrogenase. Growth was measured turbidometrically at 660 nm in a systronics colorimeter.

Microscopy and pigment analyses. Morphological properties (cell shape, cell division, cell size, flagella) were observed by light microscopy (Olympus BH-2). Cellular ultrastructure was investigated using a transmission electron microscope; for this 5 ml of well grown culture was centrifuged at 4,000 rpm for 5 min and the pellet was suspended in 0.1 M phosphate buffer, centrifuged at 4,000 rpm for 5 min. The supernatant was replaced with 1 ml of fresh phosphate buffer. A small drop of sample was placed on a piece of Parafilm and a carbon coated EM grid was placed on that drop. After 20 min the grid was removed and the excess sample was drained with filter paper. The grid was washed with distilled water and stained with 2% uranyl acetate and allowed to air dry. The grid with sample was observed under transmission electron microscope (Model: Hitachi, H-7500).

In vivo absorption spectra were measured with a Spectronic Genesys 2 spectrophotometer "in sucrose solution" (2.5 g sucrose plus 1.75 ml culture) (Trüper and Pfennig, 1981). Absorption spectra were also recorded from pigments extracted with acetone after eluting the cell suspension with acetone through a 10 × 200 mm column packed with aluminium oxide.

Genetic properties. Genomic DNA was extracted and purified according to the method of Marmur (1961) and the mol% G+C content of the DNA was determined by thermal denaturation (Marmur and Doty, 1962). Cell material for DNA isolation was taken from 1–2 ml of dense liquid cultures. DNA was extracted and purified by using the QIAGEN genomic DNA buffer set. PCR amplification and 16S rRNA gene sequencing was done as described earlier (Imhoff and Pfennig, 2001; Imhoff et al., 1998). Recombinant *Taq* polymerase was used for PCR, which was started with the 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). The identification of phylogenetic neighbors and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007). 16S rRNA gene sequences were aligned using the program CLUSTAL X (Thompson et al., 1997) and the alignment was corrected manually. A maximum likelihood (ML) tree (maximum likelihood criteria, general time reversal (GTR) model constraints, estimated proportions of invariable sites and Gamma distribution parameter) was inferred using PHYML (Guindon and Gascuel, 2003). The resulting tree topology was confirmed by bootstrap analysis with 100 randomly resampled datasets (PHYML).

The 16S rRNA gene sequence of strain JAGS6^T was deposited in GenBank under accession number AJ 887996.

Results

Habitat, enrichment, and isolation

Subsurface water samples were collected from a marine aquaculture pond near Kakinada on the eastern coast of India during August, 2004. The sample had a pH of 6.8, 2.5% salinity and a temperature of 32°C. Green colored enrichments were observed after 1 week of incubation and were used for purification in subsequent agar shake dilution series. Isolation of this organism was done by repeated agar shake dilutions. After 4 days of incubation, small, convex, green-colored colonies were observed in the agar tubes. A single isolated colony was used as inoculum for further agar shake dilution series to obtain a pure culture, designated as strain JAGS6^T.

Morphology and pigments

Individual cells of strain JAGS6^T were spherical to oval, 0.8–1 μm wide and 1–1.2 μm long (Fig. 1). The cells were non-motile, multiplied by binary fission, forming chains of 6–8 cells. Gas vesicles were absent. Transmission electron microscopy of the cells showed the presence of prosthecae (Fig. 2), typical of the genus *Prosthecochloris*. The color of the photosynthetically grown cultures of strain JAGS6^T was green. In vivo absorption maxima and shoulders of intact cells (Fig. 3a) were recorded at 335, 458, 746, 805 nm indicating the presence of bacteriochlorophyll *c*, *a* and carotenoids. Acetone extracts with absorption maxima at 433, 463 and 490 nm (Fig. 3b) indicated the likely presence of carotenoids of the chlorobactene series.

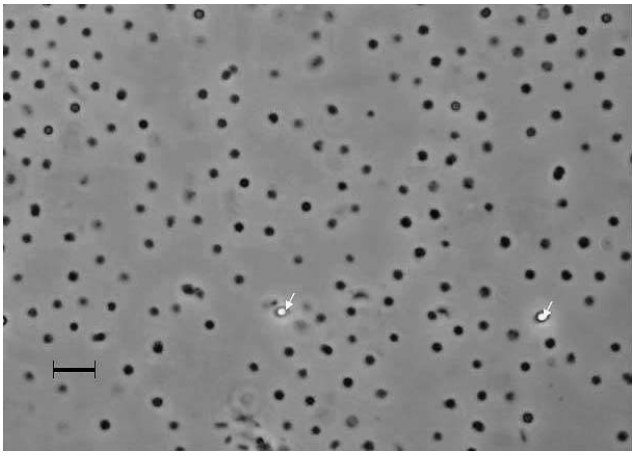


Fig. 1. Phase-contrast micrograph of strain JAGS6^T (Arrow marks indicate external sulfur granules).
Bar, 5 μm.

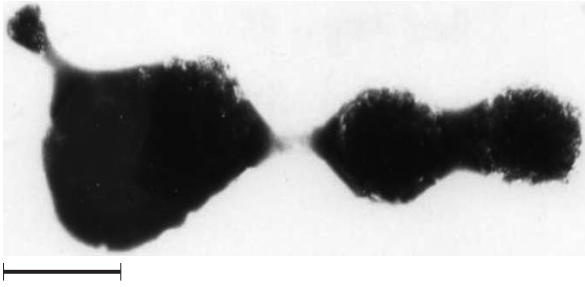
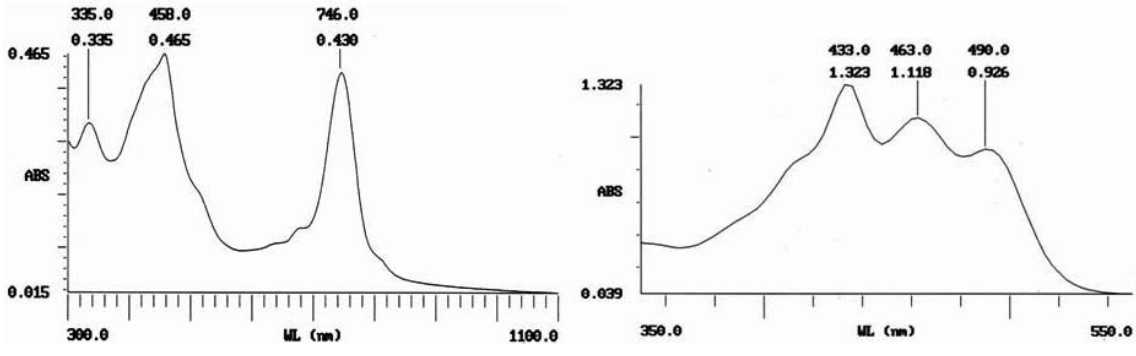


Fig. 2. Electron micrograph of negatively stained cells of strain JAGS6^T showing prosthecae.
Bar, 1 μm.

General physiology

Strain JAGS6^T was able to grow photolithoautotrophically [anaerobic, light (25 μE m^{-2} s^{-1}), $Na_2S \cdot 9H_2O$ (5 mM) and $NaHCO_3$ (0.1%, w/v)]. Organic compounds like pyruvate, acetate and glutamate were photoassimilated [anaerobic, light (25 μE m^{-2} s^{-1})] only in the presence of bicarbonate (0.1%, w/v) and sulfide (5 mM). Chemolithoautotrophy [aerobic, dark, $Na_2S_2O_3 \cdot 5H_2O$ (5 mM) and $NaHCO_3$ (0.1%, w/v)], chemoorganoheterotrophy [aerobic, dark, and pyruvate (0.3%, w/v)] and fermentative growth [anaerobic, dark with pyruvate (0.3%, w/v)] could not be demonstrated. Strain JAGS6^T was not able to use thiosulfate as an electron donor for photolithoautotrophic growth. Ammonium chloride and glutamine were used as nitrogen sources while, urea, nitrate, nitrite, glutamate and dinitrogen did not support growth. Acetylene reduction was not observed with strain JAGS6^T under photolithoautotrophic conditions assayed in the absence of combined nitrogen. Salt (NaCl) is obligatory for the growth of strain JAGS6^T in the range from 0.5–7% NaCl (w/v), with an optimum at 2–5% NaCl (w/v). The pH range for growth was 6.3 to 7.7, the optimum at 6.8 to 7.2. The temperature range was 25–35°C; the optimum was at 30°C. Low light intensi-



(a) (b)

Fig. 3. (a) Whole cell absorption spectrum and (b) acetone spectrum of extracted pigments of strain JAGS6^T.

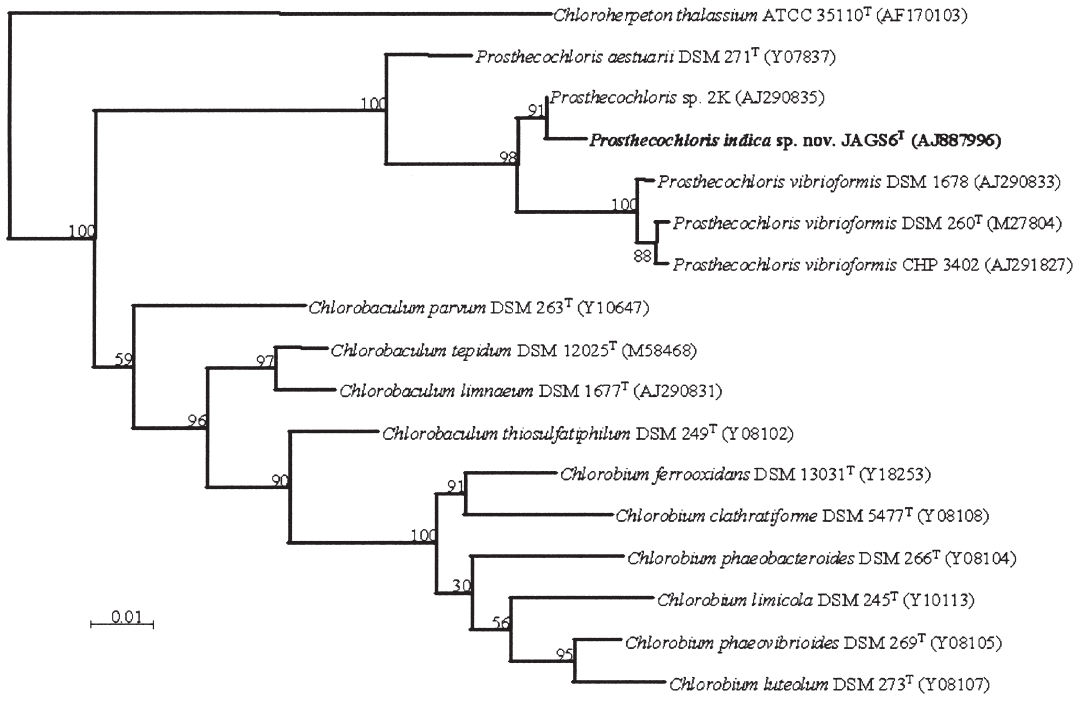


Fig. 4. Phylogenetic tree based on almost-complete 16S rRNA gene sequences showing the relationship of strain JAGS6^T within the family *Chlorobiaceae*.

The tree was constructed by the maximum likelihood method. Numbers at nodes represent bootstrap values (based on 100 resamplings). The GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 1 nucleotide substitution per 100 nucleotides.

ties of 12–25 μE m^{-2} s^{-1} were optimal for strain JAGS6^T. The strain had an obligate requirement for yeast extract (0.03%, w/v) but vitamin B_{12} could not replace this requirement.

Phylogeny

Green sulfur bacteria are a phylogenetically distinct group of anoxygenic phototrophic bacteria grouped under the phylum *Chlorobi* with one family *Chlorobiaceae* (Overmann, 2001). Based on the phenotypic properties, 16S rRNA and *fmo* (Fenna-Matthews-Olson protein) gene sequence analysis, the genera *Chlorobium*, *Chlorobaculum* and *Prosthecochloris* were recognized (Imhoff, 2003). While only a few species have a distinct salt requirement in the genera *Chlorobium* and *Chlorobaculum*, all known representatives of *Prosthecochloris* require >1% salt (Imhoff, 2003).

The sequence analysis of the 16S rRNA genes of strain JAGS6^T and other green sulfur bacteria revealed that strain JAGS6^T had the highest level (95.2–96.7%) of similarity to *Prosthecochloris aestuarii* and *Prosthecochloris vibrioformis*, but formed a separate line from these green sulfur bacteria (Fig. 4). The G+C content of the DNA of strain JAGS6^T was 53 mol% (T_m).

Discussion

Species of *Prosthecochloris* are nonmotile, spherical to ovoid, multiply by binary fission in various directions, possess bacteriochlorophyll (BChl) *c* or *d* as the major bacteriochlorophyll component and chlorosomes (i.e. elongated ovoid vesicles underlying and attached to the cytoplasmic membrane) as antenna structures of the photosynthetic apparatus (Gorlenko, 2001). Strain JAGS6^T also forms prosthecae with only 1 or 2 prosthecae per cell as compared to *Prosthecochloris aestuarii* with 10 to 20 prosthecae per cell. *Prosthecochloris vibrioformis* does not form prosthecae. The spectral properties of strain JAGS6^T are similar to those of *Prosthecochloris aestuarii*. Both have Bchl, while the bacteriochlorophyll and carotenoid composition of *Prosthecochloris vibrioformis* is variable among different strains. The carotenoids of strain JAGS6^T are likely to be of the chlorobactene series, as in *Prosthecochloris aestuarii* and in some strains of *Prosthecochloris vibrioformis*. The main distinguishable physiological trait of strain JAGS6^T is its requirement of yeast extract, while other *Prosthecochloris* species require vitamin B_{12} as a growth factor. Strain

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

Character	*Prosthecochloris indica* JAGS6^T	*Prosthecochloris aestuarii* DSM 271^T	*Prosthecochloris*[a] *vibrioformis* DSM 260^T
Cell shape	Sphere	Sphere	Curved rods
Cell size (μm)	0.8–1 × 1–1.2	0.5–0.7 × 1–1.2	0.5–0.7 × 1–2
Prosthecae (number)	1–2	10–20	None
Bacteriochlorophyll: major (minor)	*c* (*a*)	*c* (*a*)	*d*, *c* (*a*)
Carotenoides	Chlorobactene	Chlorobactene, Rhodopin/Lycopene or their hydroxyl derivatives	Variable
NaCl range	0.5–7%	1–8%	>1%
Optimum NaCl	2–5%	2–5%	>1%
pH range	6.3–7.7	6.7–7.0	6.5–7.3
Vitamin B_{12} requirement	−	+	+
Yeast extract required	+	−	−
Mol% G+C of DNA	53	52.0–56.1	53.5
Nitrogen sources utilized	NH_4Cl, Glutamine	Ammonium salts	Ammonium salts
Photoassimilation of organic substrates[b]			
Pyruvate	+	+	−
Glutamate	+	−	−
Propionate	−	−	+

Data for *Prosthecochloris aestuarii* DSM 271^T and *Prosthecochloris vibrioformis* DSM 260^T were from Gorlenko (2001).

[a]*Chlorobium vibrioforme* was reclassified as *Prosthecochloris vibriformis* (Imhoff, 2003).

[b]Photoassimilation of organic substrates in the presence of $NaHCO_3$ and $Na_2S \cdot 9H_2O$.

Acetate was utilized by all the strains. Lactate, malate, fumarate, fructose, mannitol, casamino acids, citrate and succinate were not utilized by any of the strains. Symbols: +, substrate utilized or present; −, substrate not utilized or absent.

JAGS6^T was slightly halotolerant with an obligate requirement for NaCl, similar to *Prosthecochloris aestuarii*. Both strains require NaCl for growth with an optimum at 2–5% NaCl and have a tolerance of up to 7–8% NaCl w/v, while strains of *Prosthecochloris vibrioformis* require >1% NaCl (Table 1). Strain JAGS6^T was non-diazotrophic. This could be an adaptation to the conditions in its habitat, the fish ponds which are rich in ammonium salts and other combined nitrogen sources and thereby do not allow the expression of nitrogenase. Only sulfide served as electron donor for photoautotrophic growth of strain JAGS6^T. Based on the 16S rRNA gene sequence data, strain JAGS6^T clusters between both of the known *Prosthecochloris* species (Fig. 4). The position of strain JAGS6^T in the dendrogram was identical in both distance- and character-(data not shown) based methods and was supported by a high bootstrap value. This suggests that strain JAGS6^T could be regarded as a new species of the genus *Prosthecochloris.* In addition to sequence data, phenotypic properties also assign the new isolate to the genus *Prosthecochloris* and also differentiate it from the known species (Table 1), which also supports the description of a novel species.

Species description of *Prosthecochloris indica* sp. nov.

Prosthecochloris indica (in' di.ca. L. fem. adj. *indica* named after India, the country in which the type strain was isolated).

Cells are spherical to oval, measuring about 1.0–1.2 μm in diameter. Multiply by binary fission and are non-motile. Color of the cell suspensions is dark

green. Pigments include bacteriochlorophyll *c* and most probably carotenoids of the chlorobactene series. Obligate requirement of yeast extract. Growth modes: photolithoautotrophy with sulfide as electron donor. Thiosulfate is not oxidized. Organic substrates photoassimilated in the presence of $Na_2S \cdot 9H_2O$ and $NaHCO_3$ include acetate, pyruvate and glutamate; those which could not be metabolized include lactate, fructose, mannitol, malate, citrate, casamino acids, propionate, melanoate, glycolate, asparagine, valerate, caproate, fumarate, succinate, tartrate, glucose, mannitol, glycerol, ethanol, methanol and benzoate. Ammonium chloride and glutamine are used as nitrogen sources. Optimum growth temperature is 30°C (range is from 25–35°C). The pH range is 6.3–7.7, the optimum is at 6.8 to 7.2. Sodium chloride is obligatory for growth, optimum growth is 2–5% NaCl (w/v), up to 7% NaCl (w/v) is tolerated. The G+C content of the DNA is 53 mol% (T_m). The type strain JAGS6^T (=JCM 13299^T=ATCC BAA1214^T) is from marine aquaculture pond water.

Acknowledgments

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