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Marichromatium litoris sp. nov. and *Marichromatium chrysaorae* sp. nov. isolated from beach sand and from a jelly fish (*Chrysaora colorata*)

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ABSTRACT

Three strains (JA349T, JA553T, JA439) of phototrophic sulphur bacteria were isolated from marine habitats of India. 16S rRNA gene sequence of the three strains clustered phylogenetically with members of the genus *Marichromatium* of the family *Chromatiaceae* belonging to the class *Gammaproteobacteria*. All the strains shared highest sequence similarity with the type strains of *Marichromatium* spp. (96–99% sequence similarity) and the new strains were characterized based on polyphasic taxonomy. Strains JA349T and JA553T can be distinguished from closest relative species of the genus *Marichromatium* with respect to distinct differences in cellular polar lipids, fatty acids and carbon/nitrogen sources utilization. Both strains were distinctly related (<50% based on DNA–DNA hybridization) with the type strains of the genus *Marichromatium*. Multilocus Sequence Analysis (MLSA) of the concatenated five protein coding genes (*fusA*, *pufM*, *dnaK*, *recA*, *soxB*) along with internal transcribed spacer (ITS; 16S–23S rRNA) had sequence similarity of less than 92% with the type strains of *Marichromatium* spp. Distinct phenotypic, chemotaxonomic and molecular differences allow the separation of strains JA349T and JA553T into new species of the genus *Marichromatium* for which, we propose the names *Marichromatium litoris* sp. nov. and *Marichromatium chrysaorae* sp. nov., respectively.

The genus *Marichromatium (Mch.)* was created based on the distinct 16S rRNA gene cluster from other members of the family *Chromatiaceae* [12]. The genus *Marichromatium (Mch.)* is a taxonomically challenging group of organisms due to poor correlation of phenotypic and genotypic traits [22] thus, making it difficult in the identification and classification of newly isolate strains. Only 2 species names were assigned to the genus *Marichromatium* when members of the family *Chromatiaceae* were reclassified [12], these include, *Marichromatium gracile* DSM 203T and *Marichromatium purpuratum* DSM 1591T. While *Marichromatium indicum* JA100T [4], *Marichromatium bheemlicum* JA124T [3] and *Marichromatium fluminis* JA418T [25] are later additions.

Strain JA349T was isolated from an enrichment culture of beach sand collected at seashore near Pamban bridge, Rameshwaram, Tamil Nadu (GPS positioning of the sample collection site is 9°16′31.61″N, 79°13′34.37″E) on 14 November 2007. Strain JA439 was isolated from an algal sample collected from Gujarat (GPS positioning 22°23′27.86″N, 69°55′01.97″E) on 27 February 2008. Strain JA553T was isolated from a reddish-brown colony of a live striped jelly fish (*Chrysaora colorata*; Supplementary Fig. 1 a–c) near a sea shore at Thirumullaivasal Beach, Tamil Nadu (GPS positioning of the sample collection site is 11°33′10″N, 79°45′17.29″E) on 22 May 2009. Samples were kept for enrichments in fully filled (8 ml) screw cap tubes in a modified Biebl and Pfennig's medium [5] containing (g l^{-1}): KH_2PO_4 (0.5), $MgSO_4 \cdot 7H_2O$ (3), NaCl (20), NH_4Cl (0.6), $CaCl_2 \cdot 2H_20$ (0.15), sodium pyruvate (3), yeast extract (0.3), ferric citrate [5 ml l^{-1} from 0.1% (w/v) stock] and trace element solution SL 7 (1 ml l^{-1}; [5]). The medium was supplemented with sodium bicarbonate (0.1%, w/v), $Na_2S \cdot 9H_2O/Na_2S_2O_3 \cdot 5H_2O$ (1 mM) in light (2400 lux) at 30 ± 2 °C. Cultures were purified as described earlier [25] and the pure cultures were preserved by lyophilization and maintained at 4 °C.

Well grown isolated colony was used for PCR analysis. Primers 5′-GTTTGATCCTGGCTCAG-3′ and 5′-TACCTTGTTACGACTTCA-3′ (*Escherichia coli*, positions 11–27 and 1489–1506, respectively) were used for complete sequencing of 16S rRNA gene as described previously [12]. MLSA of five protein-coding genes *recA*, *fusA*, *dnaK*, *pufM* and *soxB* together with ITS region (16S–23S rRNA genes) were amplified using touchdown PCR (TD-PCR) protocol as described by Serrano et al. [22] with modifications (Supplementary Table 1 [1,8,10,18]). The accession numbers and length of concatenated sequences of all the genes are listed in Supplementary Table 2. Conceptual translation of nucleotide sequences was identified and verified via BLAST [2] searches to GenBank database (http://blast.ncbi.nlm.nih.gov/Blast) and EzTaxon server [7].

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Concatenated (totalling ∼3600 bp) sequences of housekeeping genes were first aligned by ClustalX and alignments were improved by removing hypervariable regions within each gene sequence by using online program Gblocks [6]. Phylogenetic tree of each of the five protein coding genes, 16S rRNA gene, ITS region or concatenated sequences were constructed using CLUSTAL_W logarithm of MEGA version 4.0 [26], and distance was calculated with default parameters, K2P distance model and the neighbor, algorithm, pair wise deletion procedure [17]. The robustness of tree topologies was evaluated by 100 bootstrap replications. Bootstrap values using minimum evolution (ME) and maximum parsimony (MP) methods in the MEGA4 software as well as by program PHYML were also calculated to compare and validate the tree topologies by using different algorithms.

Genomic DNA of the strains to be tested as well as the reference type strains was isolated by slight modification of the method used by Marmur [14]. DNA–DNA hybridization was performed by the membrane filter method [27]. Each set of experiment was repeated twice in triplicates and standard deviation was calculated. The composition of different bases of the DNA was done by reverse phase HPLC [15] (Shimadzu SPD-10AVP) using a Luna 5 μm C-18 (2) 100 A column (250 mm × 4.6 mm) at a flow rate of 1.0 ml min^{-1} at room temperature.

Fatty acid methyl esters were prepared, separated and identified according to the instructions for the Microbial Identification System (Microbial ID; MIDI; Agilent: 6850) [20] which was outsourced through Royal Research Labs, Secunderabad. Photoheterotrophically grown culture in the presence of $Na_2S{\cdot}9H_2O$ (1 mM) was harvested when growth of the cultures was around 70% of its maximal optical density and were used for FAME analysis. Polar lipids by 2-dimensional TLC [11], carotenoids and quinones by HPLC [16] were analysed from freeze dried cell material.

Organic substrate utilization, vitamin requirement, nitrogen source utilization for all the strains were done in comparison under identical conditions in this study from cells grown photolithoheterotrophically in modified Biebl and Pfennig's medium [5] as mentioned earlier in the presence of $Na_2S{\cdot}9H_2O$ (1 mM) at pH 7.5. Growth was measured turbidometrically after 3–4 days at 660 nm and observation was confirmed after repeated sub-culturing (at least 3 times). *In vivo* absorption spectra was measured with a Spectronic Genesys 2 spectrophotometer in sucrose solution [28].

Morphological properties (cell shape, cell division, cell size, motility) were observed by a phase contrast microscope (Olympus BH-2 microscope). Flagella position and number and the internal membrane structures were also viewed with a transmission electron microscope (H-7500; Hitachi), after the cells had been processed as described by Hanada et al. [9].

Cells of all the three strains appeared as straight rods of size 1–1.3 μm in width and 2–5 μm in length (Supplementary Fig. 2a and b), containing 1–3 sulphur granules per cell, motile with single polar flagellum, multiplication by binary fission and have vesicular type of internal membranes. Phylogenetic relationship of strain JA349^T, JA439 and strain JA553^T to other purple sulphur bacteria as examined by near complete (1305–1450 bp) 16S rRNA gene sequences revealed that the new isolates belong to the genus *Marichromatium* of the family *Chromatiaceae* (NJ tree is shown in Fig. 1; while other [MP, ML] trees have similar tree topologies). The three strains share highest sequence similarity (95.7–99.1%) with the type strains of the genus *Marichromatium* and maintained a separate subclade in the 16S rRNA phylogenetic tree, while strain JA439 along with *Mch. gracile* biotype *thermosulphidiphilum* DSM 21765 [21] formed a distinct microclade with the type strain *Mch. gracile* DSM203^T (Supplementary Fig. 3).

According to the revised standards of describing new species as recommended by the suggestions of ad hoc committee [24], MLSA (Multilocus Sequence Analysis) has been regarded as a promising method to delineate closely related species in bacterial systematics. Simultaneous analysis of a set of protein coding genes (typically seven) as suggested by Serrano et al. [22] were chosen and amplified according to the specifications given in Supplementary Table 1. The individual and concatenated gene sequences of *recA*, *fusA*, *dnaK*, *pufM*, *soxB*, ITS and 16S rRNA (Supplementary Table 2) from the new isolates were compared with those available with the NCBI gene bank and the improved Gblocks concatenated sequences contained 2392 homologous positions (61% of the original 3888 positions). The constructed phylogenetic tree (Fig. 2) clearly shows the separation of two strains (JA349^T, JA553^T) from the type strains with concatenated sequence similarity ranging from 90–93% with the type strains of *Marichromatium* (Table 1). The similarity percentage is well within the recommended limits (<97.5% similarity) to delineate members of the genus *Marichromatium* based on MLSA similarities [22].

DNA–DNA hybridization of the strains JA349^T and JA553^T with the type strains of *Marichromatium* spp. revealed DNA relatedness between 35.7–49.3% and 42.5–55.6%, respectively (Table 1), which is well below the accepted threshold limit for species delineation [23,29]. The interspecies hybridization values however, were in concordance with the 16S rRNA gene sequence similarities of these strains just below 99% and the concatenated MLSA similarity values. The hybridization of the strain *Marichromatium* sp. JA439 with the nearest type strain was above 70% indicating it as a true member of *Mch. gracile* which is also coherent with the phylogenetic position of this strain as shown in Supplementary Fig. 3. Additional genetic markers such as G + C mole content of these strains ranged from 68.4 to 70.5% (as determined by HPLC; Table 2) which is within the range of the mol% G + C content of 67–71.4% of the genus *Marichromatium* [22].

Major differences in whole cell fatty acid profile were observed (Supplementary Table 3). The major fatty acids; $C_{18:1}\omega7c$, $C_{16:1}\omega7c/C_{16:1}\omega6c$, $C_{16:0}$ and $C_{12:0}$ are in common to all *Marichromatium* spp., variations were observed with respect to the minor fatty acids, while strains JA439 and DSM 203^T share a near identical fatty acid composition. Ubiquinone Q-8 (range 67–97%) and menaquinone MK7 (74–100) are the major quinones in all *Marichromatium* spp. (Table 2). Considerable amount of ubiquinone Q-7 (3–33%) was also present. One of the striking differences was the detection of appreciable quantities of menaquinone MK6 (26%) in strains JA349^T and JA553^T, traces in *Mch. purpuratum* DSM 1591^T (8%) delineate these taxa from other type strains of *Marichromatium*.

Diphosphatidyl glycerol (DPG), phosphatidyl glycerol (PG) and phosphatidyl ethanolamine (PE) are the major polar lipids, while minor amounts of ornithine lipid (OL) is present in all the *Marichromatium* spp. tested (Supplementary Fig. 4). Absence of a few unidentified aminolipids (AL1–3) and phospholipids (PL1–4) in the type strain *Mch. gracile* DSM 203^T and strain JA439 clearly differentiates them from other *Marichromatium* spp. (Table 2). While 2 unidentified minor phospholipids (PL3,4) are unique to strain JA553^T, variations in the unidentified aminolipids, phospholipids and other lipids differentiate *Marichromatium* spp.

Strains JA349^T and JA553^T were able to grow photoheterotrophically [anaerobic, light (2400 lux) with pyruvate (0.03% w/v) as carbon sources/electron donor]. Photolithoautotrophy (anaerobic, light [2400 lux], $Na_2S{\cdot}9H_2O/Na_2S_2O_3{\cdot}5H_2O$ [1 mM] and $NaHCO_3$ [0.1%w/v]), chemolithoautotrophy (aerobic, dark, $Na_2S_2O_3{\cdot}5H_2O$ [1 mM] and $NaHCO_3$ [0.1%w/v]), fermentative growth [anaerobic, dark with glucose/fructose/pyruvate (0.3% w/v)] could not be demonstrated in the strain JA349^T and JA553^T. However, strain JA553^T grew chemoorganoheterotrophically (aerobic, dark, with pyruvate [0.3%, w/v] as carbon source/electron donor) and chemolithoheterotrophically (aerobic, dark with pyruvate [0.3%, w/v] as carbon source and $Na_2S{\cdot}9H_2O/Na_2S_2O_3{\cdot}5H_2O$ [1 mM] as