# Diversity of *Magnetospirillum* sp. from the southern coast of India

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**Magnetotactic bacteria (MTB) are ubiquitous in the aquatic environment and have been identified from almost every continent; however, there are only a few reports of MTB from India. This article studies the diversity of cultivable MTB from the southern coast of India. Five strains of MTB have been identified in this study using gradient culturing; three of the five strains were isolated in pure culture. The strains identified are vibrioid to helical in morphology and grow microaerophilically in oxygen–sulphide gradient media. Phylogenetic analysis of isolates based on 16S rRNA gene sequences shows that they belong to the family Rhodospirillaceae, class Alphaproteobacteria, and genus *Magnetospirillum*. The three isolated strains were physiologically characterized and found to utilize a wide range of substrates as electron donors and electron acceptors for metabolism. The autotrophic growth of the strains was confirmed by the detection of type-II RuBisCO (*cbbM)* gene by PCR amplification. The presence of key magnetosome formation (*mam*) genes in the strains confirms the similar mechanism of magnetosome biomineralization among *Magnetospirillum* species. These reports of MTB from the Indian coast would contribute to the study of their evolution and biomineralization.**

**Keywords:** Aquatic environment, biomineralization, *Magnetospirillum*, *mam* genes, phylogenetic analysis.

MAGNETOTACTIC bacteria (MTB) are morphologically and phylogenetically diverse prokaryotes that orient and navigate along the Earth's magnetic field. The presence of intracellular, membrane-bound, nano-sized magnetic particles called magnetosomes assists the bacteria in sustaining an optimal location in redox gradient habitats[1,2]. Magnetosomes are crystals of iron mineral[3] that consist of either iron oxide ($Fe_3O_4$) or iron sulphide ($Fe_3S_4$). A combination of aerotaxis, magnetotaxis and chemotaxis help MTB to move away from oxygen in surface water, thus directing them in maintaining an optimal position in and around the oxic–anoxic interface (OAI)[4,5]. Research on MTB is of great significance in biogeochemistry[6], biomagnetism[7] and biotechnology[8]. Magnetic nanoparticles have now emerged as an important class of functional material with a potential for use in devices with reduced dimensions[9]. Magnetosomes can be widely used in many fields such as biology, electronics and materials science[10].

MTB cells are of diverse morphological types, including rods, spirilla, cocci and vibrios; they are abundant at the OAI in sediments of marine, brackish and freshwater habitats[11]. Due to the fastidious growth requirement of these microorganisms, isolation and cultivation have been a difficult task. Even though some of the identified MTB are available in axenic culture, most of them remain uncultivable in laboratory conditions. Most identified MTB are associated with the classes Alphaproteobacteria, Gammaproteobacteria and Deltaproteobacteria, and phylum Nitrospirae[12].

Magnetosomes are considered as a model to study the cell biology of organelle formation in prokaryotes. However, the processes leading to the biomineralization of magnetite and formation of magnetosome chain remain unclear[13]. Four conserved gene clusters present in the genomic magnetosome island (MAI) primarily participate in magnetosome formation[14,15]. Comparative genomic analyses of *Magnetospirillum* sp. suggest that a 16–17 kb length mamAB operon is essential and sufficient for the biomineralization of magnetosomes[16]. New information on genome sequences and isolates is necessary to understand the phylogenetic diversity and evolution of biomineralization in MTB[17]. In this study, five strains of *Magnetospirillum* sp. were identified, among which three were isolated in pure culture and characterized.

## Materials and methods

### *Sample collection*

Sediment and water samples were collected from different oceanic, estuarine and freshwater sites across the southern coast of India (Table 1). We have sampled from 10 different estuarine and 6 oceanic sites, 3 mangrove swamps and 1 freshwater habitat. The above-mentioned sites were selected based on the previous reports of MTB isolation around the world[18]. Sampling of MTB was done based on the collection of sediment layer that includes and surrounds the OAI. The 20 sites were sampled between March and August 2013. Based on the geographic location and limnological analysis, the sample

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**Table 1.** Sampling sites and occurrence of magnetotactic bacteria (MTB)

| Sample site | Type | Location | | Presence of MTB | pH | Water temperature (°C) |
|---|---|---|---|---|---|---|
| | | Latitude | Longitude | | | |
| Chavara | Estuary | 8.938956 | 76.549753 | – | 7.78 | 30 |
| Kovilthottam | Estuary | 8.994914 | 76.523713 | – | 7.53 | 30 |
| Ponmana | Estuary | 9.008118 | 76.523112 | – | 7.71 | 30 |
| Cheriyazhikkal | Estuary | 9.059691 | 76.500185 | + | 7.9 | 31 |
| Azheekal | Estuary | 9.126518 | 76.474457 | – | 7.62 | 30 |
| Paravoor | Estuary | 8.812272 | 76.648401 | – | 7.31 | 28 |
| Idava | Estuary | 8.785129 | 76.672433 | – | 7.38 | 28 |
| Kallada | Freshwater | 9.036970 | 76.657155 | + | 6.9 | 31 |
| Cuddalore | Oceanic | 11.771165 | 79.778884 | + | 7.5 | 34 |
| Pichavaram | Mangarove swamps | 11.444907 | 79.784326 | – | 7.61 | 29 |
| Parangipettai | Oceanic | 11.492711 | 79.771932 | – | 7.4 | 27 |
| Nagapattinam | Estuary | 10.753782 | 79.846528 | – | 7.74 | 34 |
| Velamkanni | Oceanic | 10.677945 | 79.851421 | – | 7.45 | 34 |
| Vedaranyam | Mangarove swamps | 10.324180 | 79.874704 | – | 7.3 | 29 |
| Muthupettai | Mangarove swamps | 10.322358 | 79.488347 | + + | 7.28 | 29 |
| Arumbakkam | Estuary | 13.548430 | 80.089363 | – | 6.9 | 31 |
| Irukkam | Estuary | 13.569290 | 80.135540 | – | 6.9 | 31 |
| Rameshwaram | Oceanic | 9.297307 | 79.334550 | – | 7.48 | 32 |
| Danushkodi | Oceanic | 9.160400 | 79.433084 | – | 7.3 | 27 |
| Thoothukudi | Oceanic | 8.776426 | 78.160300 | – | 7.65 | 35 |

+, One strain present or detected. ++, Two strains present or detected. –, Absent or not found.

sites were further divided into groups. Samples 1–7 were collected from different sites of Ashtamudi estuary extending between 76.53° and 76.63° long. and 8.93° and 8.83° lat. along the southwest coast of India (Table 1). Sample 8 was collected from a freshwater lake situated in the plains of the Kallada River, Kerala. Samples 9–20 were collected from the southeastern coast of India, among which samples 10, 14 and 15 were collected from mangrove swamps, while the other samples were either oceanic or estuarine in nature. Because of the logistic constraints, each site was visited only once. Therefore the data represent only a single snapshot. Table 1 provides geographical coordinates of all the sample sites. For limnological analysis, the surface water temperature was measured using a hand-held field thermometer, and pH measurements were made using an Eutech pH tester. Physico-chemical characteristics of sediment samples were analysed using XRD technique (BRUKER, Germany). The sediment samples with interface water in the ratio of 1 : 3 were transferred to screw-capped plastic jars and transferred to the laboratory aseptically. For bacterial enrichment, sediment samples were kept undisturbed at room temperature (27–30°C) in dim light for 6–9 months[19].

### *Magnetic enrichment, enumeration and isolation of culturable MTB*

All chemicals were purchased from Himedia (Mumbai, India), unless otherwise specified. For the separation of MTB from non-magnetic contaminants, we used two-step magnetic collection strategy[19]. This includes magnetic enrichment and capillary race track purification[20]. In the first step, a small ceramic bar magnet was placed on the South Pole near to the sediment–water interface. After 1 h of incubation, 1 ml of water sample was withdrawn from the interface near the magnet. The magnetically enriched samples were purified again using capillary race track method. These purified samples were used as inocula in a semisolid oxygen–sulphur gradient enrichment medium described previously[21]. The mixed culture of microorganisms present in the microaerophilic rings was repeatedly purified by capillary race track method to obtain a dominant fraction of MTB. The cultures were further purified by dilution by extinction in Hungate tubes[22] and sub-cultured in gradient media.

### *16S rRNA gene sequencing of culturable MTB*

The isolated MTB strains were grown in *Magnetospirillum* growth medium (MSGM)[23], and genomic DNA was extracted using the standard technique[24]. PCR amplification of 16S rRNA was carried out using the primers listed in Table S1 (see Supplementary Information online). PCR amplification of genomic DNA was performed with the primer set (1a, 2a) previously determined to amplify 900–950 bp product within 16S rDNA specific to *Magnetospirillum* species[25]. The basic protocol for *Taq* red master mix (Ampliqon, Denmark) includes an initial denaturation step at 95°C for 10 min,

then 30 cycles of 45 sec of denaturation at 95°C, annealing for 60 sec (the temperature varied based on GC% of the primer sets), and extension at 72°C (time varied based on the length of the sequence to be amplified), followed by a final extension step at 72°C for 10 min (Veriti, Applied biosystems, USA). PCR products of partial 16S rRNA gene amplified using *Magnetospirillum*-specific primers (1a, 2a) were sequenced directly (Eurofins Analytical services, Bengaluru, India).

### *Physiological characterization of isolated MTB strains*

Growth experiments were carried out using batch culture on a rotary shaker (120 rpm) with MS1 minimal media[26] supplemented with appropriate concentrations of carbon and energy sources. All the three strains were grown microaerophilically in 100 ml serum bottles with an $N_2$–$O_2$ (80/20%) headspace. Hungate anaerobic technique was used as a standard procedure for bacterial culturing and maintenance[27]. Optimum pH was maintained by adding $NaH_2PO_4$ and $Na_2HPO_4$ in appropriate concentrations to establish stable pH (6.0, 6.5, 6.8, 7.0, 7.2, 7.5, 7.8, 8.0, 8.2 and 8.5). The optimum temperature for growth was standardized by varying the growth temperature from 25°C to 42°C. To analyse the salinity growth optima, NaCl (0.5–2.5%) was added to the culture medium from an anoxic aqueous stock solution. Electron acceptors such as oxygen, perchlorate, chlorate, nitrate, nitrite, $N_2O$, sulphate, thiosulphate, selenate (5–50 mM), and donors such as acetate, propionate, butyrate methanol, ethanol, glycerol, pyruvate, succinate, citrate, lactate, glucose, sucrose (5–50 mM) were added separately from sterile, anoxic aqueous stock solutions and the cell growth was monitored for 7 days by optical density measurement at 600 nm (Table 2).

### *PCR amplification of mam genes and genes involved in $CO_2$ fixation*

The presence of important magnetosome formation (*mam*) genes was determined from the three isolated strains of MTB for further confirmation. Degenerate primers to amplify *mam*A, *mam*B, *mam*C, *mam*D, *mam*F, *mam*J, *mam*K, *mam*M and *mam*Q were used as previously described (Table 2)[19,28]. Degenerate primers for the amplification of RuBisCO type II (*cbbM*) gene of the CBB cycle were used to test for the presence of the gene in the genome of strain. The designed primers were utilized as previously described (see Supplementary Information online, Table S1)[29]. The PCR amplification parameters were similar to the protocol explained above. The PCR products were visualized by 1% agarose gel electrophoresis, and imaged using AlphaImager gel documentation system (Alpha Innotech Corp, USA).

### *Phylogenetic tree construction*

The alignment of 16S rRNA gene was performed using the ClustalW multiple sequence alignment[30]. The phylogenetic tree was constructed using MEGA, version 5 (ref. 31). Bootstrap values were calculated with 1000 replicates.

## Results and discussion

### *Sampling sites, samples and magnetic separation*

Sampling sites were mostly oceanic or estuarine in nature (Table 1). Maximum number of MTB has been

**Table 2.** Physiological characteristics of strain *M.*VITRJS2, *M.*VITRJS5 and *M.*VITRJS6

| Characteristics | *Magnetospirillum* strains | | |
|---|---|---|---|
| | *M.*VITRJS2 | *M.*VITRJS5 | *M.*VITRJS6 |
| Cell-type | Vibrioid to Spirillum | Spirillum | Spirillum |
| Motility | + | + | + |
| Temperature range (°C) | 25–40 | 25–40 | 25–40 |
| Temperature optima | 31 | 28 | 28 |
| pH range | 5.5–8 | 5–8 | 5–8 |
| pH optima | 7 | 6.5 | 6.8 |
| NaCl tolerance (%) | 1.5 | 1.5 | 1.5 |
| Spore formation | – | – | – |
| Fermentative | – | – | – |
| RuBisCo *cbbM* | + | + | + |
| Magnetotactic | + | + | + |
| Electron acceptors | | | |
| Oxygen | + | + | + |
| Perchlorate | – | + | – |
| Chlorate | – | + | – |
| Nitrate | + | + | + |
| Nitrite | – | – | – |
| $N_2O$ | + | + | + |
| Sulphate | – | – | – |
| Thiosulfate | – | – | – |
| Selenate | + | + | + |
| Electron donors | | | |
| Acetate | + | + | + |
| Propionate | + | + | + |
| Butyrate | + | + | + |
| Methanol | – | – | – |
| Ethanol | + | + | + |
| Glycerol | – | + | + |
| Pyruvate | + | + | + |
| Succinate | + | + | + |
| Citrate | – | – | – |
| Lactate | + | + | + |
| Glucose | – | – | – |
| Sucrose | – | – | – |
| Thiosulphate | – | – | – |
| Iron(II) chloride ($FeCl_2$) | – | – | – |

+, present or with regard to electron donors/acceptors. –, absent or not utilized.

previously reported from the top sediment. Therefore, top ~5 cm of sediment that includes and surrounds the OAI was collected[5,28]. The limnological characteristics of the samples such as temperature and pH were analysed (Table 1). Other characteristics such as salinity, total phosphorus (TP), total nitrogen (TN), chlorophyll (Chl), and suspended solids were not measured because the occurrence of MTB is not dependent on them[5]. The physico-chemical characteristics of sediment samples were analysed using XRD technique (data not shown). From the XRD spectrum, it was found that silicon oxide was the most abundant mineral in the sediments. Ferrosilite ($FeSiO_3$), barbosalite ($Fe^{2+}Fe_2^{3+}(PO_4)_2(OH)_2$), iron tetra thio silicate ($Fe_2S_4Si$), lipscombite ($Fe^{2+}$, $Mn^{2+}$)($Fe^{3+}$)$_2(PO_4)_2(OH)_2$) and rozenite ($FeSO_4{\cdot}4H_2O$) were some of the iron-based silicate minerals detected in the sediments. Other minerals such as sillimanite, bearsite and albite were also present in the sediment samples. Since MTB belongs to the phylum Proteobacteria, the physical and chemical parameters of the water body might influence the magnetosome biomineralization. However, the preliminary analysis (hanging drop) after two-step magnetic collection and 'capillary race track' purification[20] showed no MTB, or bacteria greatly outnumbered by other marine microorganisms. The accumulation of MTB near the magnet was not observed in any of the samples after magnetic enrichment. These findings are contrary to previous reports of MTB isolation[32]. Moreover, the earlier reports mentioned the deposition of brown to greyish cells near the magnet during collection of cells from the sediment[32]. This indicates that the presence of MTB from the Indian coast is insufficient compared to the previous reports of isolation[19]. Also, the techniques previously used for identification of MTB from the sediments, would be inadequate in the Indian scenario.

### *Isolation and diversity of culturable MTB*

MTB is a gradient microorganism, and so redox gradient is crucial for its growth. The redox gradient was provided by culturing MTB in oxygen–sulphide gradient medium. In this study, the semisolid oxygen–sulphide gradient medium[21] was inoculated with magnetically purified cells from the various sample sites. After 8–10 days of incubation at 30°C, evidence of growth was found in most of the inoculated tubes. Microorganisms form sharp and well-defined growing rings according to their oxygen requirement. All microaerophilic growth rings present near the OAI were observed under the microscope. Microscopic examination of microaerophilic cells in samples from Muthupettai, Cuddalore, Kallada and Cheriyazhikkal culture tubes were spiral in morphology and magnetotactic in behaviour (Figure 1). The pure culture was obtained by repeated series of dilution of extinction in anoxic Hungate tubes filled with MSGM medium and culturing in oxygen–sulphide gradient semi-solid medium.

A total of five strains of MTB were identified from the sediments collected from the southern coast of India. Among these, *M.*VITRJS2 was vibrioid in morphology, while other four were spiral in shape (Figure 1). Two of these strains (*M.*VITRJS3 and *M.*VITRJS4) originated from Muthupettai sample, whereas *M.*VITRJS2, *M.*VITRJS5 and *M.*VITRJS6 were identified from Cuddalore, Kallada and Cheriyazhikkal samples respectively (Table 1). Based on morphological and phylogenetic data, all new strains were identified as members of the genus *Magnetospirillum*. Further study of the taxonomic status would determine the species of genus *Magnetospirillum* which the new strains belong. The partial 16S rDNA sequences of the isolated MTB strains showed that they phylogenetically belong to the family Rhodospirillaceae, class Alphaproteobacteria, and are members of genus *Magnetospirillum* (Figure 2). The strain *M.*VITRJS5 showed more similarity to *Magnetospirillum beliccus* (VDY), whereas the other four sequences show 99% similarity to *Magnetospirillum gryphiswaldense* (MSR1). Similarities with other *Magnetospirillum* sp. were less than 98%.

### *Growth characteristics*

Strain *M.*VITRJS2 grew between 25°C and 40°C, with optimum temperature of 31°C at pH of 7 (Table 2). Strains *M.*VITRJS5 and *M.*VITRJS6 showed optimum growth at 28°C at a pH of 6.5 and 6.8 respectively. All three strains were found to tolerate up to 1.5% NaCl concentration and grew in oxygen–sulphide gradient medium. The strains utilized a range of compounds as electron donors, including acetate, lactate, pyruvate, succinate, propionate, butyrate and ethanol. Oxygen, nitrate, selenate and $N_2O$ were utilized as electron acceptors. Strain *M.*VITRJS5 utilized perchlorate and chlorate as electron acceptors, other than the substrates mentioned above. Strain *M.*VITRJS2 utilized acetate as a better electron donor when compared with other organic sources, whereas pyruvate and lactate were key electron donors for strains *M.*VITRJS5 and *M.*VITRJS6. All the three strains had higher growth rate on nitrate when compared to other electron acceptors.

### *PCR amplification of specific mam genes*

The genes (*mam*A, *mam*B, *mam*C, *mam*D, *mam*F, *mam*J, *mam*K, *mam*M and *mam*Q) that are responsible for the biomineralization of magnetosomes were studied by PCR amplification. Using degenerate primers, the presence of important *mam* genes was examined to confirm the magnetosome formation and the role of these genes in the biomineralization process. Among the nine genes

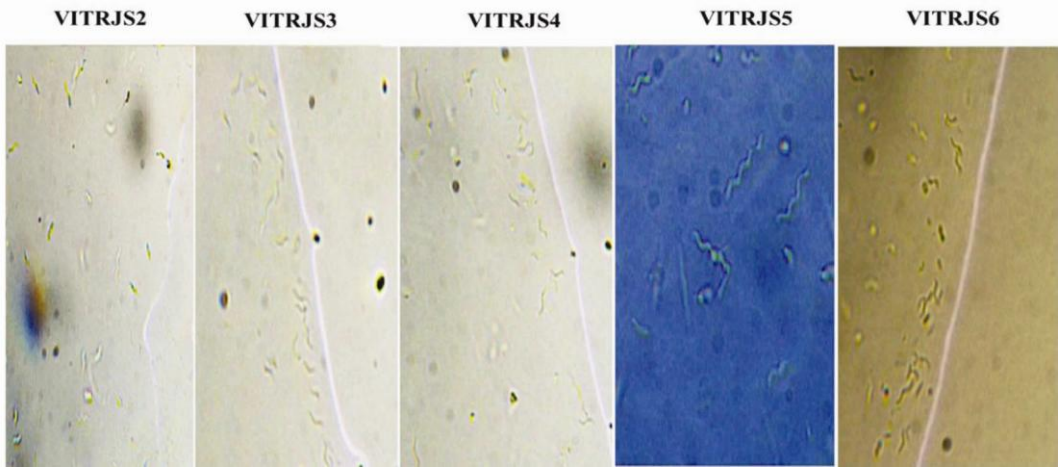


**Figure 1.** Optical photomicrographs of *Magnetospirillum* VITRJS2, VITRJS3, VITRJS4, VITRJS4 and VITRJS6 in a water drop aligned along the magnetic field (100×).

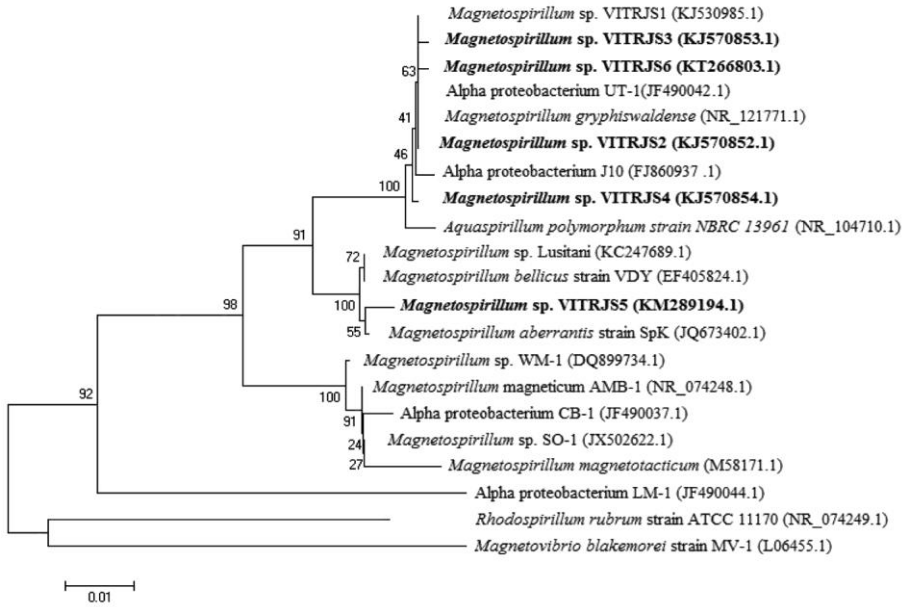


**Figure 2.** Phylogenetic tree based on partial 16S rDNA gene sequences showing the positions of newly isolated *Magnetospirillum* sp. strains. Alignment of 16S rRNA gene was performed using the ClustalW multiple sequence alignment[27]. The phylogenetic tree was constructed using MEGA, version 5 (ref. 26). The bootstrap values at the nodes are percentage of 100 replicates.

amplified, *mam*A, *mam*C, *mam*J, *mam*K, *mam*M and *mam*Q were present in all the three isolates (Figure 3). *mam*B was detected only in strain *M.*VITRJS6; while *mam*D and *mam*F were only present in strains *M.*VITRJS2 and *M.*VITRJS5 (Figure 3). The distribution of *mam* genes in strains *M.*VITRJS2 and *M.*VITRJS5 was found to be similar to the two well-characterized strains *M. gryphiswaldense* (MSR1) and *Magnetospirillum magneticum* (AMB1) and previously isolated strain *Magnetospirillum* sp. VITRJS1, whereas the absence of two important *mam* genes (*mam*D and *mam*F) in strain *M.*VITRJS6 shows the divergence of the evolution of strain from the others[28]. The role of Mam proteins in magnetosome biomineralization is well studied and these putative functions include magnetosome vesicle formation (MamA and MamF), iron transportation (MamB, MamC and MamM), crystal structure formation (MamD), chain formation (MamJ and MamK) and protein sorting (MamQ)[33]. Among the other Mam proteins, the significance of MamC is its unique presence in only MTB strains; no homologs were found in any non-magnetotactic bacteria[34]. The presence of important *mam* genes confirms the magnetosome formation and the role of these genes in the biomineralization process.

PCR-based studies targeting RuBisCO genes have been frequently done to analyse whether carbon dioxide

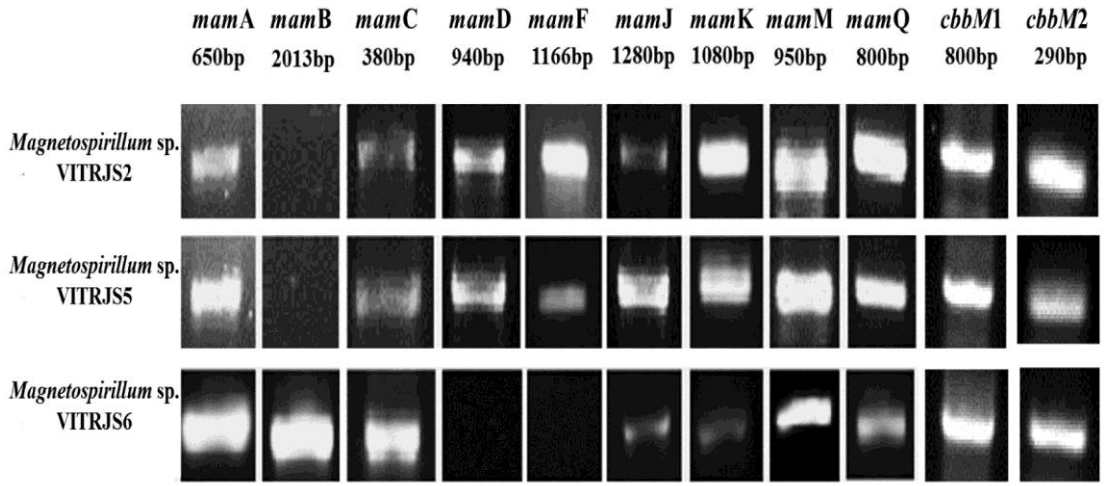


**Figure 3.** PCR amplification of selected *mam* genes from *Magnetospirillum* sp. VITRJS2, VITRJS5 and VITRJS6 on 1% agarose gel. *mam*A, *mam*C, *mam*J, *mam*K, *mam*M, *mam*Q, *cbbM*1 and *cbbM*2 were amplified from all three strains. *mam*J was detected only in VITRJS6, whereas *mam*D and *mam*F were detected only in VITRJS2 and VITRJS5.

fixation is ribulose bisphosphate-dependent. Amplification of genes encoding RuBisCO in the CBB cycle demonstrated the presence of RuBisCO *cbbM* large-subunit (form II) gene and autotrophy pathway in all three strains of *Magnetospirillum* sp. The presence of *cbbM* gene shows that the isolated strains can grow in higher $CO_2 : O_2$ ratio[35]. Magnetotactic bacteria produce magnetosomes only when restricted to grow as microaerophiles with high $CO_2 : O_2$ ratio. During microaerophillic growth and magnetosome formation the three isolated *Magnetospirillum* species rely on form-II RuBisCO enzyme for respiration[35].

The distribution of MTB across the southern Indian coast has been discussed in this study. From the total number of 20 samples collected, 5 MTB isolates were identified from 4 samples (Cuddalore, Muthupettai Kallada and Cheriyazhikkal) by the gradient culturing method. Strains *M.*VITRJS3 and *M.*VITRJS4 were identified from Muthupettai sediment sample, but were not available in pure culture. The other three strains (*M.*VITRJS2, *M.*VITRJS5 and *M.*VITRJS6) were physiologically characterized to study the bacterial metabolism and were found to utilize a wide range of electron acceptors and donors. The presence of form-II RuBisCO gene confirms autotrophic growth in the isolated *Magnetospirillum* sp. suggests the ability of MTB to respire in microaerophilic condition helps the bacteria in magnetosome biomineralization process. A study of the key *mam* genes by PCR amplification shows the similarity in biomineralization pathway of the isolated *Magnetospirillum* strains with characterized strains (MSR-1, AMB-1).

## *Nucleotide sequence accession numbers*

Partial sequences of the 16S rRNA gene determined in this study were assigned GenBank accession numbers. The newly determined sequences of *Magnetospirillum* strains VITRJS2, VITRJS3, VITRJS4, VITRJS5 and VITRJS6 have been deposited under the accession numbers KJ570852, KJ570853, KJ570854 KM289194 and KT266803 respectively.

Thus, we were able to identify five strains of *Magnetospirillum* sp. from the southern Indian coast. Since our sample size is only 20, further sampling would help attain phylogenetically diverse MTB from all over India. Despite being a difficult task, the isolation of MTB from the Indian coast may lead to a better understanding of the evolutionary advantages of producing magnetite minerals. Recently, there have been a number of reports on MTB from all over the world. There are almost 25 axenic cultures of MTB, as reported by Lefèvre and Long-Fei[36]. New reports on MTB can work as the missing link between studies of biomineralization of magnetite, formation of magnetosome chain and evolution studies of the bacteria.

*Conflict of interest.* The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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