4.0 ISOLATION AND MOLECULAR IDENTIFICATION OF MAGNETOTACTIC BACTERIA FROM MARINE SEDIMENT OF PALK STRAIT

4.1. Introduction

Magnetotactic bacteria (MTB) are the hotspot of research in microbiology with tremendous biotechnological and nanotechnological applications (Schuler, 1999), thereby attracting the interest of researchers from multiple fields. As of now, only few pure cultures of MTB are available, however cultivation of these bacteria is difficult due to their microaerophilic to anaerobic lifestyle. Some workers have also reported the isolation of aerobic magnetic cultures (Jun et al., 2006). Aquatic microorganisms have the ability to orient and migrate or swim along geomagnetic field lines, this behaviour referred to as ‘magnetotaxis’ (Bazylnski and Frankel, 2004). This property is based on the specific intracellular structures, the magnetosomes, which in most MTB are nanometer sized, enveloped, membrane bound, single magnetic domain crystal particles composed of the iron mineral magnetite (Fe₃O₄) and/or greigite (Fe₃S₄) (Bazylnski and Frankel, 2004; Bazylnski, 1999). Magnetosomes are organized in one or more straight chains parallel to the long axis of the cells. Such an arrangement confers a magnetic dipole to the cell, which is sufficiently large so that it will orient the entire bacterium along the geomagnetic field lines at ambient temperature (Schuler, 1999).
Magnetosomes are nanometer sized (25-100 nm), membrane bound magnetosomes. MTB can absorb the element of Fe from outer environment to synthesize intracellular nano sized magnetosomes (Tan and Feng et al., 2000).

The application of genomic approaches to study marine microbes is resulting in rapid advancements in marine microbial ecology. It has long been recognized that, marine biogeochemical cycling is mediated by microbes. However, the complex interplay between tremendously diverse microbes, many of which remain uncultured, has hampered efforts to model function in marine ecosystems. This discovery dramatically shifted the thinking of oceanographers and emphasized the need to understand microbial functional gene complements and their expression in the environment. Progress in this area has come through the application of genomics to marine systems. The value of functional genomic approaches is that processes can be learnt about in the absence of prescribed hunting or previous experimental knowledge. Hence this present study has been undertaken to isolate and identify genetically of the MTB’s from marine aquatic environment.
4.2. Materials and methods

4.2.1. Description of sampling area

Thondi is a small village situated in the Palk Strait region of Tamil Nadu. The study area lies between the latitude of 9° 44’ N and longitude of 79° 02’ E (Fig 1). The rainfalls in Thondi region are mainly due to North East and South West monsoon. Thondi coast has a very minimal wave action. Turbidity of the seawater is moderately low and also they are rich in nutrients hence, it serves as a treasure house for valuable marine resources like sea grass, seaweeds and invertebrates like coelenterates, echinoderms and shell fishes. The major occupation of the people is fishing. This area is also considered to be a one of the good source crab and of sponge diversity in Indian coast.

Fig. 1. Map showing the study area
4.2.2. Collection and preparation of samples

The sediment sample was collected by scooping the mud with a sterilized beaker, scrapping 3 to 6 cm thickness of mud from the bottom of an (marshy intertidal, shore and coral reef regions) aquatic environment. The water was removed from the sediment by filtration through gauze. The sediment was dried at 45°C for 48 hours. After removing the larger particles, samples was pulverized with a mortar and pestle for making it to fine powder and passed through a filter of 250µ mesh size.

4.2.3. Preparation of mud agar-medium

Agar mud mixture was prepared in 1 liter conical flask, heated to boil and was thoroughly mixed and filtered. The warm mixture was poured into test tubes (120x15 mm) up to 2/3 of their height. All the tubes were plugged with cotton and sterilized in an autoclave (15 lbs for 15 minutes, 121°C) and were used after cooling.

4.2.4. Isolation of magnetotactic bacteria

One liter of sediment sample along with water was collected by using one liter beaker and the mouth was covered from the place where sample was collected. The beaker containing sample was kept at room temperature in dark condition without shaking, to allow the settlement of sediment interphase. The sample containing magnetotactic bacteria was obtained from the bottle by concentrating the bacteria between the two bar magnets. The
The south magnetic pole of first magnet was positioned to touch one external side of the bottle at the height of the sediment water interphase. The North pole of the second magnet was similarly positioned on the other side of the bottle for 15 minutes. After that, 10 ml of water was drawn by means of a pipette from the water sediment interphase around the South magnetic pole. The sample was placed in a 50 ml beaker and magnetotactic bacteria was concentrated between the two bar magnets for 15 minutes. The final inoculum was removed with a capillary pipette from the South pole. The same procedure was followed for the concentration of magnetobacteria in the North pole in order to study their presence in North pole also. The capillary pipette containing the magnetotactic bacteria inoculum was inoculated downward into the test tubes containing the sterile agar-mud medium and its content was released while the pipette was being slowly withdrawn. The tube was loosely plugged with cotton and incubated for 10 days at room temperature in semi darkness.

4.2.5. Pure culture

Twenty days old culture tubes containing the suspended white ring was used for the isolation of pure culture. Up to the level of the ring, the semi solid medium was scooped out with the help of the sterilized spatula and a loopful of culture from the ring was taken and streaked onto the nutrient agar plate. After 24 hours of incubation the isolated individual
colonies were restreaked thrice in a nutrient agar medium. The culture grown on nutrient agar plates were streaked on to the nutrient agar slants and stored in refrigerator for further identification

4.2.6. Confirmation of magnetotactic strains

As raw samples were added to the mud agar medium, the presence of non magnetic bacteria is inevitable in the white ring formed by the magnetotactic bacteria. To differentiate the magnetotactic and non-magnetotactic strains found in the white ring, strains were isolated by the conventional pure culture technique. All the isolated strains were inoculated in to different mud agar tubes and incubated in semi darkness for minimum 10 days. The formation of white rings by the strain confirmed their magnetotactic nature. The strains which is not show any ring formation were noted as non-magnetotactic.

4.2.7. Survivability test

The test tube cultures containing the magnetotactic populations as a white line in 10 days incubation was selected as representative culture tubes of different sampling areas and incubated at room temperature for the period of 6 months in semi darkness condition as such without any protective measures. At the end of the study period, the survivability of the magnetotactic population in mud agar medium was carried out. The
viability was tested by streaking a loopful of culture taken out from the ring onto nutrient agar plate and the growth was observed.

4.2.8. Magnetotaxis test

The motility or non-motility of the isolated magnetotactic strains is generally ascertained by microscopic examination of the culture in hanging drop or wet mount method. In order to confirm the presence of magnetic field, 18-24 hours old pure culture from nutrient broth was transferred to a cover slip and then it was fixed to a cavity slide and observed under a binocular microscope (X1000). A bar magnet was placed on the stage of the microscope in such a way that the South pole of the magnet was in the vicinity of the hanging drop or edge of the mount. Observations were made for every 30 minutes.

4.2.9. Morphological characterization

Morphological characterization of isolated MTB’s were carried out by MTB’s were streaking a loopful of isolated MTB on nutrient agar medium. After a required period of incubation, morphological characters *viz.* colour of the colony, forms, elevation and margins *etc.*, tabulated were observed and tabulated.
Fig. 2 Picture showing the marine sediment collected for the isolation of magnetotactic Bacteria

Fig. 3 Picture showing the sediment concentrate sample with magnetic bars

Fig. 4 Picture showing the sediment sample concentrate with North and South pole magnetic bars

A-North pole B- South pole
4.2.10. Extraction of Magnetosomes

4.2.10.1. Preparation of MTB cell suspension

The culture harvested in exponential phase was centrifuged for 5 minutes at 3000 rpm. Then pour out the supernatant fluid and abandon the precipitate. The supernatant fluid is centrifuged for 10 minutes at 10000 rpm to collect MTB-1 to MTB-5. Then pour out the supernatant fluid and diluted sulfuric acid with pH 2.0 was suspended with the MTB-1 to MTB-5 and shaken well for 5 minutes to dissolve Fe precipitate. Centrifugation, suspension and shaking procedure was repeated for about 10 times to clear the iron ion out of the MTB-1 to MTB-5 cells completely.

4.2.10.2. Extraction of magnetosomes

Being suspended in the 10ml PBS buffer liquid, MTB-1 to MTB-5 was crashed (600 W/cm², 2.5 mins, 30 pluses) by ultrasonic crasher surrounded with ice. The specimen was subjected for freezing and thawing for three times before crushing. The magnet was used at the bottom of the beaker to attract the magnetosomes. When the cell wall had been broken up by ultrasonic crasher (Fu and Jiang, 2004; Ruan and Tan, 1998), the magnetosomes were cleaned by PBS buffer again and again until no ion of iron ion inside. It is important that, the magnetic iron can't leave the bottom of beaker when pour the liquid. Then 0.5 ml PBS buffer was added to suspend the magnetosomes (the raw specimen) in the beaker.
4.2.10.3. Detection of ionic ion in magnetosomes

A few drops of 1 mol HCl in PBS buffer was added to suspend the magnetosomes and shaked the beaker lightly to make the compound of iron dissolved. Two drops of 0.2% 0.5 ml potassium thiocyanide was added to the beaker after the compound of iron dissolved completely. If the solution’s colour turned red which indicates that, the solution contained the ironic which confirms the presence of magnetosomes in MTB’s.

4.2.10.4. Purification of magnetosomes

The magnetosomes was extracted with the above mentioned method again, and was suspended it in the 0.5 ml PBS buffer. It was purified by recovering the magnet particle band after being centrifuged by 40%~80% sucrose gradient centrifugation (6000 rpm, 10~20 min) and being suspended in the PBS buffer. The magnetosomes suspended liquid was scattered by ultrasonic wave and washed by PBS buffer and ethanol to clean the cell fragment which was absorbed on the surface of magnetosomes. These processes repeated for 15 times to clean the magnetosomes completely and the magnetite was used to magnetize the magnetosomes by close up the bottom of beaker.
4.2.11.1. *In silico* analysis of MTB's using 16S rRNA gene sequencing

4.2.11.1. Extraction of MTB’s genomic DNA

A protocol was devised for different magnetotactic bacterial genomic DNA extraction. Culture (MTB 1-5) was grown in 20 ml of Nutrient broth in 100 ml conical flask at 37°C in a rotary shaker (200 rpm) for overnight.

Transfered 1.5 ml of bacterial culture to the eppendorf tube and centrifuged at 8,000 rpm for 2 min. Discarded the supernatant and drained well on to a tissue paper. The bacterial pellet was resuspended in 400 µl of sucrose TE buffer. To this add 32 µl of lysozyme (10 mg.ml⁻¹) and incubated the tubes at 37°C for 30 min. 100 µl of 0.5M EDTA at pH 8 and 60 µl of 10% SDS were added. To this add 1.5 µl of proteinase K (20 mg.ml⁻¹) was added and incubated the tubes at 50°C (in water bath) for 12 hrs. Brought down the tube to room temperature and 250 µl of equilibrated phenol (equilibrated with Tris HCl) was added and mixed well and further added 250 µl of chloroform. The tube was centrifuged at 10,000 rpm for 10 min and extracted twice with phenol chloroform (1:1 ratio). The aqueous phase was extracted once with chloroform isoamylalcohol (24:1 ratio) and collected the supernatant and precipitated with 2 volume of absolute ethanol. Centrifuged it at 10,000 rpm for 10 min and discarded the supernatant and air dried it completely. Washed the pellet with 70% ethanol and allowed it to dry at
room temperature. After complete drying, dissolved the pellet in 20 to 50 µl of sterile distilled water (or) TE and stored at -20°C.

4.2.11.2. PCR amplification of 16S rRNA gene

The 16S rRNA gene was amplified from genomic DNA obtained from MTB 1-5 cultures by PCR with Universal Forward primer-16SrRNA F-5'-TGAGGAAGATAATGACGG-3' and Universal Reverse primer-16SrRNA R-5'-CCTCTATCCTCTTTCAACC-3'. The reaction mixture contained 25 to 50 ng of DNA, Ex Taq PCR buffer, 1.5 mM MgCl₂, 10 mM deoxynucleoside triphosphate mixture, 50 mol of each primer and 0.5 U of Ex Taq polymerase. PCR conditions consisted of an initial denaturation at 94°C for 5 min; 30 cycles at 94°C for 1 min, annealing 58°C for 1 min and 72°C for 1 min and final 5 min extension at 72°C. The amplification products were examined by agarose gel electrophoresis and purified by using a QIA quick PCR clean up kit with the protocol suggested by Qiagen Inc.

4.2.11.3. Sequencing of 16S rRNA gene

The partial 16S rRNA gene was sequenced by using the PCR products directly as sequencing template with above mentioned primers. All sequencing reactions were carried out with an ABI 3730 automated DNA sequencer (Applied Biosystems, Monza, Italy).
4.2.11.4. Sequence and Phylogenetic analysis

Nucleotide sequences were compared to those in the Gene Bank Database with the Basic Local Alignment Search Tool (BLAST) algorithm to identify known closely related sequences. The DNA sequences were aligned and phylogenetic tree was constructed by neighbor joining method by using http://www.ncbi.nlm.nih.gov/blast/tree_view/blast_tree_view.cgi.

4.2.11.5. Prediction of 16srDNA Secondary structure

The 16S rRNA gene sequences of MTB’s isolates were used for the construction of secondary structure models and were folded using Genbee-NEB Cutter (Brodsky et al., 1995). These secondary structures were used to assess the significance of observed differences in 16S rRNA gene sequence data.

4.3. Results

Sediment sample was collected and processed for the isolation of magnetotactic bacteria. Five different MTB strains were isolated from the sediments sample based on the morphological characteristics (Table 1). All the isolated MTB strains were identified as gram negative and rod shaped.

The growth pattern of MTB reveals that, all the MTBs produced very clear ring above the surface of mud agar medium from the South pole concentrated sample. The MTB-3 and MTB-4 strains were produced the maximum distance of ring (0.9 cm) after 10 days and 7 days of incubation.
respectively. The MTB-1 and MTB-5 strains produced 0.8 cm distance ring after 9 and 7 days of incubation respectively. Whereas, MTB-2 strain produced minimum distance of ring (0.7 cm) after 11 days of incubation (Table 2; Fig. 5).

The magnetotaxis test reveals that, the aggregation of bacterial cells towards the magnetic pole was gradual and the aggregation was increased in concentration at different time. Generally, it was observed that a minimum period of three hours exposure to the external magnetic field is required for 100% of aggregation of cells (Table 3).

The present study also carried out for the extraction of magnetosome from all the isolated MTB (MTB-1 to MTB-5) and confirmed by the scanning electron microscopic picture (Fig. 6-10).

The present study also carried out to identify the isolated MTB (MTB-1 to MTB-5) by using molecular methods. 16S rRNA partial gene sequencing reveals that, all the MTB isolates have unique sequencing (Figs 11, 15, 19, 23 & 27). The BLAST search of the 16S rRNA sequences (850 base pairs) of the MTB-1 showed maximum similarity (100%) with *Bacillus licheniformis* strain BP-WC09009 (GenBank Acc. No. MM006908) (Fig. 12), the MTB-2 (860 base pairs) showed maximum similarity (100%) with *Alcanivorax dieselolei* strain SJV (GenBank Acc. No. GU213911) (Fig 16), the MTB-3 (860 base pairs)
showed maximum similarity (99%) with *Exiguobacterium* sp. 12(2010) (GenBank Acc. No. HU410495) (Fig. 20), the MTB-4 (860 base pairs) showed maximum similarity (98%) with *Alcanivorax dieselolei* strain RUAUMTB-2 (R-Ravikumar, U-Uthiraselvam, AU-Alagappa University, MTB-Magnetotactic bacteria), (GenBank GU339393) (Fig. 24) and the MTB-5 (800 base pairs) showed maximum similarity (98%) with *Vibrio cholera* LMA3894-4 (Gen Bank Acc. No. CB002555) (Fig. 28). Based on the molecular taxonomy and phylogeny the MTB-1 was identified as *Bacillus licheniformis* strain RUAUMTB001 (Fig. 13) the MTB-2 strain was identified as *Alcanivorax dieselolei* strain RUAUMTB002 (Fig. 17), the MTB-3 strain was identified as *Exiguobacterium* sp. RUAUMTB003 (Fig. 21), the MTB-4 strain RUAMTB004 (Fig. 25) and the MTB-5 was identified as *Vibrio cholera* strain RUAUMTB005 (Fig. 29). All the isolated MTB strains (MTB-1 to MTB-5) nucleotide sequencing of 16S rRNA gene partial sequencer were deposited in the GenBank under the Accession No. GU339392, GU339393, GU339394, GU339395 and HM002751 respectively.

The 16s rRNA gene secondary structure of all the isolated MTB strain (MTB-1 to MTB-5) were predicated and represented in Figs 14, 18, 22, 26 & 30 and showed in turn variations among the MTB’s.
Table 1: Morphological characteristics of isolated magnetotactic bacterial (MTB)

<table>
<thead>
<tr>
<th>Isolated strains</th>
<th>Form</th>
<th>Elevation</th>
<th>Margin</th>
<th>Colour</th>
<th>Gram staining</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTB-1</td>
<td>Spreded colony</td>
<td>-</td>
<td>-</td>
<td>Shine blue (Glowing)</td>
<td>Negative</td>
<td>Rod</td>
</tr>
<tr>
<td>MTB-2</td>
<td>Circular</td>
<td>Raised</td>
<td>Entire</td>
<td>Dark orange with dull black</td>
<td>Negative</td>
<td>Rod</td>
</tr>
<tr>
<td>MTB-3</td>
<td>Filamentous</td>
<td>Flat</td>
<td>Undulate</td>
<td>Dull white</td>
<td>Negative</td>
<td>Long rod</td>
</tr>
<tr>
<td>MTB-4</td>
<td>Spindle</td>
<td>Convex</td>
<td>Entire</td>
<td>Dull orange with dull blue</td>
<td>Negative</td>
<td>Rod</td>
</tr>
<tr>
<td>MTB-5</td>
<td>Circular</td>
<td>Raised</td>
<td>Entire</td>
<td>Dull yellow</td>
<td>Negative</td>
<td>Rod</td>
</tr>
</tbody>
</table>

Table 2: Growth pattern of magnetotactic bacteria

<table>
<thead>
<tr>
<th>Isolated strains</th>
<th>Magnetic pole of concentration</th>
<th>Intensity of ring</th>
<th>Distance between the ring and the surface of the medium (cm)</th>
<th>Optimum growth period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTB-1</td>
<td>S</td>
<td>++++</td>
<td>0.8</td>
<td>9</td>
</tr>
<tr>
<td>MTB-2</td>
<td>S</td>
<td>++++</td>
<td>0.7</td>
<td>11</td>
</tr>
<tr>
<td>MTB-3</td>
<td>S</td>
<td>+++</td>
<td>0.9</td>
<td>10</td>
</tr>
<tr>
<td>MTB-4</td>
<td>S</td>
<td>++++</td>
<td>0.9</td>
<td>7</td>
</tr>
<tr>
<td>MTB-5</td>
<td>S</td>
<td>++++</td>
<td>0.8</td>
<td>7</td>
</tr>
</tbody>
</table>

(S=South Pole, ++++ = very clear, +++ = clear)
Table 3: Magneto taxis property of MTB’s at different time period

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Aggregation of bacterial cells towards the magnetic pole (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTB-1</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>90</td>
<td>40</td>
</tr>
<tr>
<td>120</td>
<td>60</td>
</tr>
<tr>
<td>150</td>
<td>80</td>
</tr>
<tr>
<td>180</td>
<td>100</td>
</tr>
</tbody>
</table>
Fig. 5. Picture showing the white ring formation of magnetotactic bacteria

Fig. 6. Scanning electron microscope image (5000X) of magnetic microspheres of MTB-1
Fig. 7. Scanning electron microscope image (5000 X) of magnetic microspheres of MTB-2

Fig. 8. Scanning electron microscope image (5000X) of magnetic microspheres of MTB-3
Fig. 9. Scanning electron microscope image (5000X) of magnetic microspheres of MTB-4

Fig. 10. Scanning electron microscope image (5000X) of magnetic microspheres of MTB-5
Fig. 11. Picture showing 16S rRNA gene amplification and forward DNA sequencing of MTB-1
MTB-1 16S rRNA gene sequences

GTGCCTAATACATGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTT
AGGTCAGCGCGGACGGGTGAGTAACACGTGGGTAACCTGCTGTA
AGACTGGGATAACTCCGGGAAACCGGGCTAATACCAGGTGCTGACTACC
TGAACCGCATGTTCAATCATAAAAAAGGTGGGCTTATGCTACCACCTTAC
AGATGGACCCCGCGGCACCATTAGCTAGTTGGTGAGGTAAACGGGCTCACC
AAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTG
GAACGTGACGACGCGCGCCAGAATCTGAACTGGGAGGAGCAGTAGGGGAA
TCTTTCCGCAAATGGAACGAGAAATCTGACGGAGCAACCCCGCGGCTAGTA
TGAAGGTGTTCGGATCGTAAAATCTGCTGTTGTTAGGGAAGAAACTGA
CCGTTCAATAGGGCGGTACCTTCGACCGTACCTAAACCAGAAAGCCAC
GGCTAAGTACGTGACGACCGCGCTAATACGTAGTGCTGCAAGCGT
GTCCGGAATTTAGGGCGTAAGGCAAGCGCAGCGGTTTCCTTAAAGT
CTGATGTGAAAGCCCTGGCAACCCGGGAGGGTCATTGGAAACTG
GGGAATAGTAGTGGCGAGAGAGTGGAAATCAGTTAGCCG
TGAAATGCGTAGAGATGTGGAGGAACCCAGTGGCGAAGGCGACTC
TCTGGTCTGTACGTGGAGCCTGGCAGCCGGCAAAGCGTGGGGGAGCGAA
CAGGATTAGATACCCCTGGTACGCCCCACGCGCTAAACGATGAGTGCT
AAGTGTATAGGAGGGCTCAGCCTTTAGTGCTGCAAGCAAAGCAGTTAA
GCACCTCGC
Fig. 12. Picture showing the phylogenetic analysis (BLAST) of MTB-1
Fig. 13. Phylogenetic tree of MTB-1
Fig. 14 Secondary structure of MTB-1

Free Energy of Structure = -210.3 kcal/mol
Fig. 15. Picture showing 16S rRNA gene amplification and forward DNA sequencing of MTB-2
MTB-2 16S rRNA gene sequences

CCAGCCTACACATGCAGTCGAGCGGAACGATGGGAGCTTGCTCCCAG
GCAGTCCGAGCGCGGAGCGGAGCTGAACACTCGGGAATCTGCCCATT
AGTGGGGGATAACTCGGGGAAACTCGAGCTAATACCGCATAATCCCT
ACGGGGGAAAGCACGGGATCTTCGGACCTTGCTGATGGATGAGC
CCGCGTCGGATTAGCTTGGTGGGTGGTAATGGCCCAACCAAGCGAC
GATCCGTAACCTGGTCTGAGAGGATGCGCAGTCACACCGGGACTGAG
ACACGGCCCGGACTCCTACGGGAGGCGAGCGATGCGGAAATCTTGGAC
AATGGGGCAAGGCGATCCAGCCATGCGCGCGTGTTGAAGAAGGC
CTTCGGGTTTGAAGCACTTTCAGTAGGAGGAAGCTTGGCTTAA
TACCCCTGAGTACTTGACGTGTTACCTACAGAAGAAGCAACCAGCTAA
TCGGTGCCAGCACCGCGGTGTAATACGGAAAGGTGCGAGCCTAACG
AATTACTGGGCGTAAAGCAGCGCGCGTAGGCGGTTGTACAGTGATGT
GAAAGCCAGGGCTACACCTTGGAAATTGCATCGCATACTGGCACGCT
AGAGTGACAGTGGAGGAGTGGAATTTCCGTTGTAAGCGGTAAATGT
CGTACAGGATCGGAAGCACAACCAGTGGCGGAAGCGCGCCTCTGGAC
TGACTAGCAGCTGGAGTGCGAAGCGTGGGGAGCAGAACAGGATTA
GATAACCTGGTAGTCCAGCAGCGTAACCGATGCTACTAGGGCTGGG
GTCCATTAGTCACTTGAGTGCGCAGCTACACGGAATAGTACCGGCC
TGGGGGAGTACGGCGCAAGGTTA
Fig. 16. Picture showing the phylogenetic analysis (BLAST) of MTB-2
Fig. 17. Phylogenetic tree of MTB-2
Free Energy of Structure = $-197.8 \text{ kcal/mol}$
Fig. 19. Picture showing the phylogenetic analysis (BLAST) of MTB-3
MTB-3 16S rRNA gene sequences
CGTGCTATACATGCAGTCGAGCGCAGGAAGCCGTCTGAACCCTTCCGG
GGGGACGACGGTGAATGAGCGGCGGACGGGTGAGTAACACGTAAA
GAACCTGCCCATAGGTCTGGGATAAACCGAGAAATCGGGGCTAAT
ACCGGATGTGTATCGGACCGCATGGTCCGCTGATGAAAGGCGCTCC
GGCGTCGCCCATGGATGGCTTTGCGGTGCATTAGCTAGTTGGTGGGT
AACGGCCCAACCAAGGCAACGTAGCAGCCGCACCTGAGAGGGTGAT
CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA
GCAGTAGGGAATCTCTCCACAATGGAACGAAATCTGATGGAGCAACG
CGCCTCTTTTCCGCTAACTGGGCTTAAGGAAAGAGGGTGATTTGGG
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGAAGCTA
ACGCATATACCTCCGCTTCCG
Fig. 20  Picture showing the phylogenetic analysis (BLAST) of MTB-3
Fig. 21. Phylogenetic tree of MTB-3
Fig. 22. Secondary structure of MTB-3

Free Energy of Structure = -204.3 kcal/mol
Fig. 23 Picture showing 16S rRNA gene amplification and forward DNA sequencing of MTB-4
MTB-4 16S rRNA gene sequences

GTGTAGCGGTGAACGGCGTAGATTTCTGAGAAACACCCGGTG
GGCGGAAGGGCGGTCCCTCTTTGGACATATACTGACACTCAGATGCGA
AAGGCGTGAGGGAGCCACCCAGGATTAGACCCTCTTCGGCTTTTC
GGAGCTAACCACGTGTAAGTAGAACCCCTGAGGGGAGTACGGTCGAC
ATTAAAACCTAAATGGGACGCGGAGCAGGACAGGGAGTACGGTCGAC
CATGTGGTTTAATTGGAAGCAGCGAAGAAGCCATTACCTACCTTTTGAC
ATCCCAGAGATTTTCCGAGAAGGTGTGTCCTTTTCGAGCTCTGA
GACACGCCGCCGAGCTGGCTTTTGTCAAGCTCTTCTGAAAAATTG
TTAATCTCCCAAGCGAGCCCTCTTCTCTTTTTTTCCCGCACTTAAT
GGTGGAACCTCCAGGGAGACTGCGGCTGACAAACCGGAGGAAAGTTG
GGGATGACGTCAAGTCAATCATGGCCCTTACGAGTAGGGCTACACG
TGCTACAAGTGGCTGATACAAAGGGCAGCCAAACCGCGAGGTGGAGC
GAATCTCACAAAACCGTCTGATCTCCGGATTGAGCTGCAACTCGA
CTCCATGGAAGTCGGAATCGCTAGTAATCGCAAAACGAGAACCGCTG
TGAATACGTTCCCAGGGCCTGTACACCGCCCGTCACACCATGGGA
GTTGCGCTGCTCAAGGAGAGGCTAGGTATTTAACCCTAAGACAGCCACCGCCT

T
Fig. 24 Picture showing the phylogenetic analysis (BLAST) of MTB-4
Fig. 25. Phylogenetic tree of MTB-4
Fig. 26. Secondary structure of MTB-4
Fig. 27. Picture showing 16S rRNA gene amplification and forward DNA sequencing of MTB-5
MTB-5 16S rRNA gene sequences
TGCTAAAAACATGCAGCTGAGCGGAAACGATGGGAGCTTGCTCCCAAGGC
GTGGAGCGCGGAGGGGTACAAACGTGGGAATTCCGACCACGCTGAAATCCCTAC
GGGGGAAAGCGGGGATCTTCCGACCTTCGATCTGATGAGATGATCCCC
CGTCGGATTAAGCTTGGTTGGTTGGAATCTGCCCACCACAGGGGAGCAT
CCGTAACTGCTGTCGGAGGGATGGCAGTCAGTCAACCCGGGACTGAGACA
CGGCCCCTACTCCATCGGGAGGACGACAGTGGGAATCTGGCCAATTTTGGAG
GGGCGAAGCTGATCCATCCATCGCAGCTGCTGTGAGAGAAGGCTCTTC
GGTTGTAAAAGCACTTTTCAGTAGGAGGAAGGCTTTTGGGCTAATACC
CTGAGGTACTTACGGTTACCTACAGAAGGACACGGGCTAATTTCCGT
GCCACACACCCGCGGTAATACGAAAGGTCGAGCGTTAATCTTGAATT
ACTGCGCGTAAAGCGCGGTACGGCGGGTGTGGTAAGTCGGATGGAAG
GCCAGGGCTCAAACCTTGGAAATTGCTACCGATATCGCAGCTAGAG
TGCAGTACGGAGGAGGTGAATTCCCCGGTATCGCGGAAATCGCAG
GACATCGGAAGGAAACACAGTGCGGAAGGGCGGCTCTCCGTGACTGAC
ACTGACGCTGAGGTCCGAAAGCGTGGGGAGCAACACAGGATAGATA
CCCTGTTGTACCCCCAGGGTAAACGATGTCTACTCAGAGGCTTG
CTTACGTACTGCGGAGACTAC
Fig. 28. Picture showing the phylogenetic analysis (BLAST) of MTB-5
Fig. 29. Phylogenetic tree of MTB-5
Fig. 30. Secondary structure of MTB-5

Free Energy of Structure = -212.5 kcal/mol