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MOLECULAR IDENTIFICATION THROUGH 16S rRNA GENE SEQUENCING OF *PARAMAGNETOSPIRILLUM MAGNETICUM* AMB-1 FROM WARSAK, PAKISTAN

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ABSTRACT
Paramagnetospirillum magneticum AMB-1 is a gram-negative, facultative anaerobic bacterium. The aim of current study is molecular identification through 16S rRNA gene sequencing of magnetotactic bacteria (*Paramagnetospirillum magneticum* AMB-1) from freshwater sample. Water sample was collected from the edge of freshwater site near warsak dam and magnets were used for further isolation of magnetotactic bacteria. The ATCC medium 1653 was used for culturing the bacteria. The selected bacterial strain whole genomic DNA was extracted using the phenol/chloroform DNA extraction (SDS-based technique) which was then amplified using PCR. The strain's 16S rRNA sequencing was done by the Centre for Genomic Sciences (CGS). MEGA 11.0 software is then used to perform phylogenetic analysis on these sequences and the other similar sequences. The bacterial culture showed whitish colour and spherical shape. The elevation and edge of culture was convex and entire. Subject's DNA was amplified and aligned onto a molecular ladder, as confirmed by the PCR results, which were visible at 1500 bp DNA molecular ladder. The amplification efficiency of polymerase chain reaction was very almost 100% which is generally considered the best parameter study. The isolated AMB-1 strain of bacteria exhibited 99% and 81% similarity to the magnetotactic bacteria *Magnetospirllum magneticum* and *Magnetospirllum* sp., respectively. The current research contributed to the identification of this bacterial isolate which was discovered for the 1st time in Pakistan's freshwater sample.

Keywords: Bacteria; Extraction; Culturing; PCR; Gene sequencing.

1. INTRODUCTION
Paramagnetospirillum magneticum AMB-1 (Magnetotactic bacteria) is a Gram-negative, magnetotactic bacterium. Magnetotactic bacteria are found in a wide range of habitats in both hemispheres, including freshwater and marine environments.

Certain favorable circumstances are necessary for the presence of bacteria in both marine and fresh water [1, 2]. Magnetotactic bacteria often grow best at the oxic-anoxic transition zone, which is the area between aerobic and anaerobic zones where there is a balance of oxygen and sulphide derivative. As a result, magnetite (Fe_3O_4) and greigite (Fe_3S_4) crystals releases, which significantly contributes to the magnetization of bottom earth sediments [3].

Because of their unique magnetotactic behaviour, magnetotactic bacteria are easily identified in environmental water samples and soil samples. Magnetotactic bacteria can be found via a variety of techniques, the most recent of which is the extension and scaling up of magnetic collection. This process makes use of large "magnetic traps," which may hold many liters of silt particles. The Magnetotactic Bacteria magnetically align with collection tube ends after storage, making collection for further analysis simpler [4].

Magnetotactic bacteria are gram-negative bacteria that are able to produce magnetosomes, which are iron oxide magnetic nanoparticles that bacteria use as a compass in the magnetic field of earth [1]. Magnetotaxis, the capacity of bacteria to move in water in response to a magnetic field, is a characteristic that sets them apart from other types of bacteria. Magnetosomes are the type of organelle seen in magnetotactic bacteria that are in charge of the process.

A substantial portion of the membrane surrounding a magnetosome is made up of glycolipids, proteins, and phospholipids. Magnetosomes are organelles that contain magnetic substances called nanocrystals [5]. Other noble metal and semiconductor magnetosomes can also be produced by magnetotactic bacteria. Since bacterial magnetosomes of iron oxide offer potential therapeutic uses, especially in cancer, they are of primary interest to pharmaceutical researchers [3, 6].

Most strains of Magnetotactic Bacteria that are cultivated in their pure form belong to the genus *Magnetospirillum*.

Magnetotactic bacteria are classified into three classes: alpha-, beta-, and delta-proteobacteria, and they are members of the Proteobacteria and Nitrospirae phyla [7]. The aim of current study was molecular identification through 16S rRNA gene sequencing of magnetotactic bacteria (*Paramagnetospirllum magneticum*) AMB-1 from fresh water sample near warsak dam, Pakistan

2. MATERIALS AND METHODS

2.1. Sample Collection

Sample was collected in a plastic container together with 1/3 to 1/2 of the sediment and silt from a muddy silt layer at Warsak dam (34°09′50″N 71°21′29″E). The screw-top cap was tightened after the bottle had been submerged for some time before water was added.

The sample was brought to the laboratory after the exterior of the container was dried using a cloth. The cap of the container was loosened and store at room temperature in drawer wrapped in aluminum foil.

The sample was allowed to settle to the container bottom with the sediment and small particles for a period of four weeks without any disturbances. The transparent sides of the plastic bottle confirmed that the particles had settled to the bottom [8, 9].

2.2. Sample Isolation and enrichment

Magnets were placed approximately 1 cm above the sediment-water interface on the outside surfaces of the plastic container.

The sediments in the container's bottom have not moved. Container one side held north side of one bar magnet and the south pole of another bar magnet.
The magnets were positioned to allow for modifications on top of the plastic box. The bacteria were left in this condition for 3 hrs in order to swim towards the magnet.

The capillary racetrack technique was employed for enrichment of sample bacteria that were drawn to south pole of bar magnet after carefully removing water from the container [10].

Magnetotactic bacteria were isolated and then enriched using the racetrack technique [11]. A glass pasteur pipette (146 mm) was prepared using a cutter and melted to create an open and sealed end.

The racetrack was autoclaved and filtered sample water was introduced. Sterile cotton was used to seal the bottom. Magnetotactic bacteria-containing water was filled into the racetrack, and the fluid was collected near the edge.

Light microscope was used to observe the enriched magnetotactic bacteria. Syringe (1 ml) and a needle (25 gauge) were used to remove the fluid that contained enriched Magnetotactic bacteria.

2.3. Culturing of bacterial isolate

Sample was cultivated in American Type Culture Collection (ATCC) medium 1653 (revised magnetic spirillum growth medium) [12].

To prepare the sample for inoculation, 10 ml of ATCC bacterial growth medium 1653 were mixed with 100 µl of ~ 5.108 bacteria. In an incubator kept at 26 °C, the cells were cultivated under micro-aerobic conditions.

The medium was adjusted to a pH of 6.75 using sodium hydroxide solution and supplemented with various chelating agents.

The iron-chelating agent concentration was adjusted to 400 µM to 0.4 µM. The revised magnetic spirillum growth medium (MSGM) included Wolfe's mineral solution (5 ml), Wolfe's vitamin solution (10 ml), sodium acetate (0.37 g), succinic acid (0.05 g), tartaric acid (0.37 g), ascorbic acid (0.035 g), KH_2PO_4 (0.68 g) and $NaNO_3$ (0.12g) respectively.

Nitrilo triacetic acid (1.5 g) was added to water and adjusted to pH 6.5 with KOH. Water and remaining constituents were added one by one along with pH indicator (0.1% resazurin (0.45 ml)) to make up volume 1.0 litre.

Media (12 ml) were dispensed into each 16 × 150 mm screw-cap tube, and the tubes were autoclaved for 15 minutes at 121 ^{o}C. [13].

2.4. DNA Extraction

SDS-based extraction was used to extract DNA [14]. Sample (5g) was mixed with 100 ml proteinase K (10 mg/ml), 100 mM sodium EDTA (pH 8), 13.5 ml of DNA extraction buffer (1.5 M NaCl, 1% CTAB) and 100 mM Tris-HCl (pH 8). Figure 1 depicts the schematic diagram for the SDS-based DNA extraction protocol.

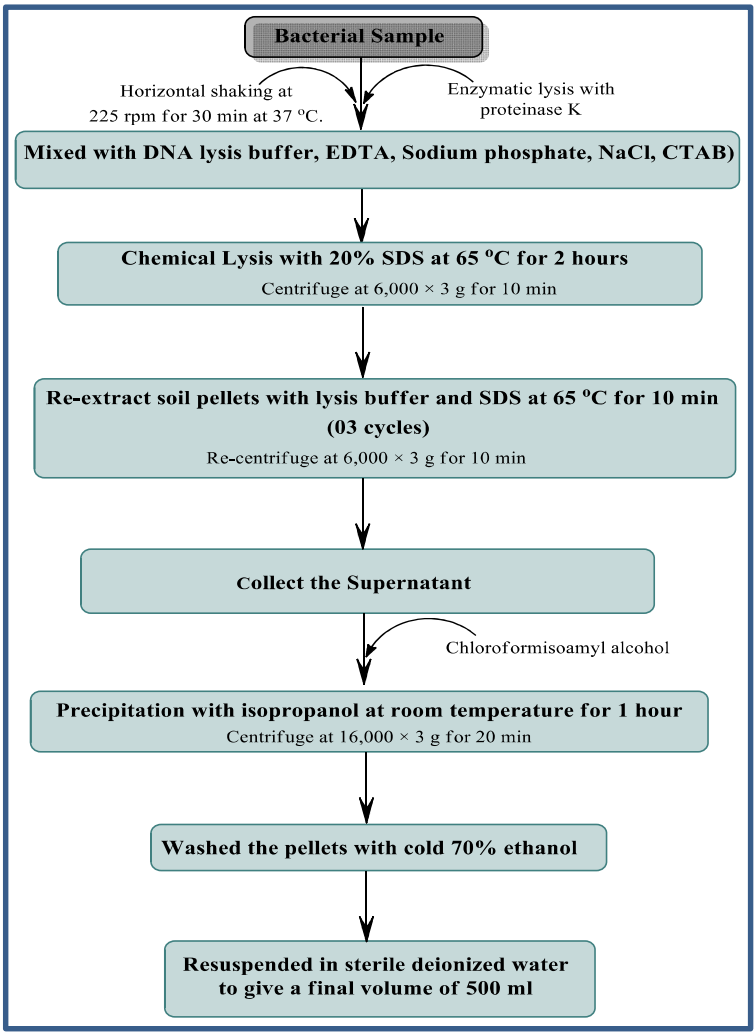

Figure 1. Schematic illustration of an SDS-based DNA extraction technique

2.5. Polymerase Chain Reaction (PCR) amplification

PCR was used to amplify bacterial 16S rRNA gene segments (~1500 bp) using universal primers, as indicated in table 1.

The PCR mixes were made in compliance with the guidelines provided by the manufacturer. Ethidium bromide staining and agarose gel electrophoresis were used to evaluate the PCR result. The PCR was done by following step as shown in table 2. The purified sample was then sent to Centre for Genomic Sciences for sequencing.

Table 1. PCR conditions and primers [15].

S. No.	Gene	Primers	Primer Sequence	Amplicon size
1.	16S rRNA	Forward (27F)	AGAGTTTGATCCTGGCTCAG	~1500 bp
2.		Reverse (1492R)	AAGGAGGTGATCCAGCCGCA	

Table 2. Steps involved in Polymerase Chain Reaction amplification

S. No.	Steps		Temperature	Time
1.	**Denaturation**	**Initial denaturation**	94°C	5 min
		30 cycles of denaturation	94°C	1 min
2.	**Primer annealing**		55°C	1 min
3.	**Extension**		72°C	1 min
4.	**Final elongation**		72°C	10 min

2.6. Molecular identification through 16S rRNA gene sequencing

16S rRNA sequencing for the strain was obtained from the Centre for Genomic Sciences (CGS), which is situated in Peshawar, Pakistan. To compare sequences from different genes with each other in the GenBank databases, the NCBI BLAST tool was utilised.

The MEGA 11.0 program was used to generate a phylogenetic tree using the neighbour joining technique. Phylogenetic tree have been constructed to show how our isolate relates to other closely related strains [9, 15].

3. RESULTS

Paramagnetospirillum magneticum AMB-1 was cultured using American Type Culture Collection (ATCC) medium 1653 (revised magnetic spirillum growth medium). The bacterial culture had a spherical shape and a whitish colour. Culture was convex and entire in its elevation and edge.

PCR results showed that each subject's DNA was amplified and aligned onto a molecular ladder as it was visible at 1500 bp DNA molecular ladder as shown in Figure 2.

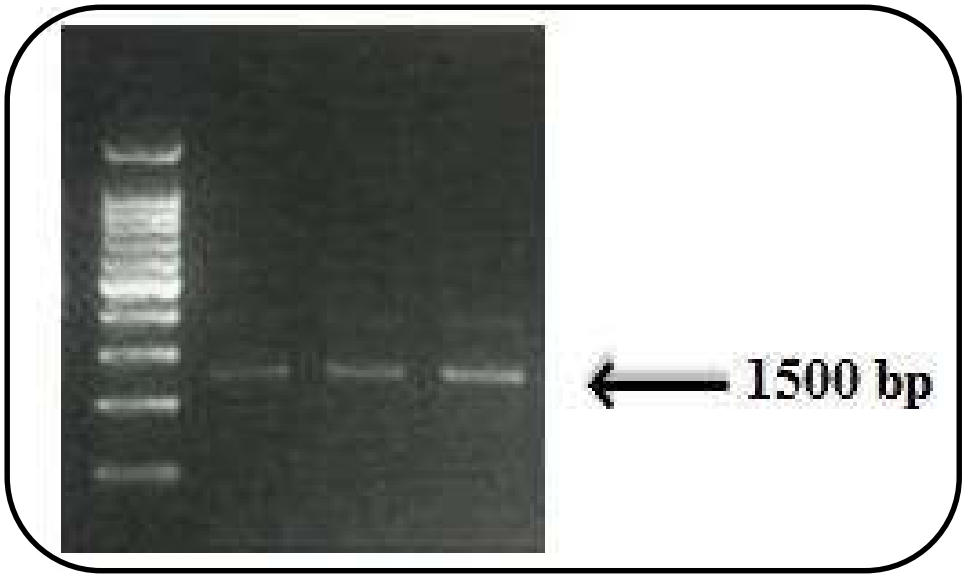

Figure 2. Confirmation of 16S rRNA gene of *Paramagnetospirillum magneticum* AMB-1 by PCR amplification

The strain's 16S rRNA sequencing was provided by the Centre for Genomic Sciences (CGS). Sequences from different genes were compared with one another in the GenBank databases using the NCBI BLAST tool.

According to Figure 3, the magnetotactic bacteria *Magnetospirillum magneticum* and *Magnetospirillum sp.* were 99% and 81% same with the bacterial strains AMB-1.
In order to ascertain the evolutionary history, the Neighbor-Joining approach was applied (Figure 3). The Maximum Composite Likelihood approach was used to calculate the evolutionary distances based on base substitutions per site.

Nine (9) distinct nucleotide sequences were used in the current experiment. For every pair of sequences, pairwise deletion was employed to eliminate any unclear places. Evolutionary analyses

were performed with MEGA-11. The AMB-1 strain we reported has an accession number of PP130205 obtained by NCBI.

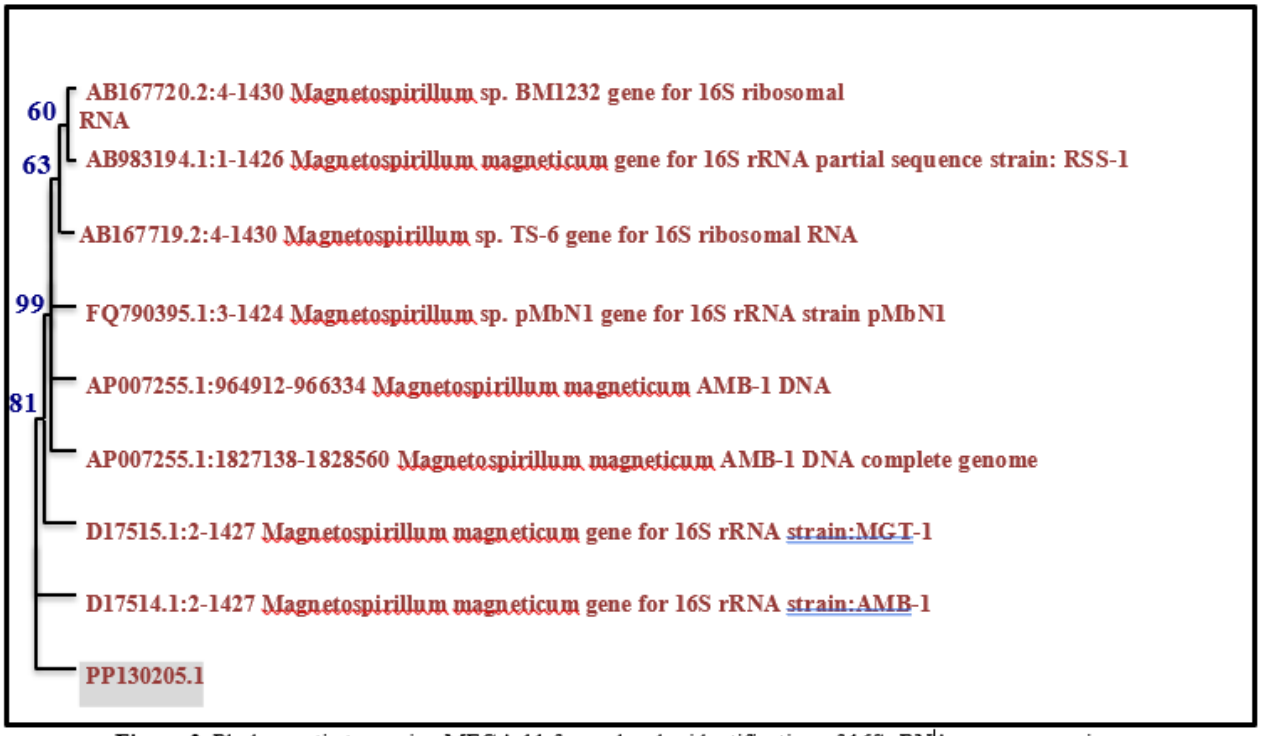

Figure 3. Phylogenetic tree using MEGA 11 for molecular identification of 16S rRNA gene sequencing

4. DISCUSSION

The class α-proteobacteria contains the majority of magnetotactic bacterial species. Blakemore et al. identified the cultivated strains of *Magnetospirillum* [9]. Phylogenetically and morphologically, many species of this genus are also thought to resemble magnetotactic bacteria; however, in the absence of iron, they are unable to biomineralize magnetosomes [7].

Magnetotactic bacteria are found in brackish, hypersaline, fresh, and marine waters. The freshwater source of the magnetotactic bacterial isolate AMB-1 was found near Warsak Dam, which is located in Warsak, Pakistan. The majority of magnetotactic bacteria were found along the oxic-anoxic interface or in water that was chemically stratified [16]. The prevalence of magnetotactic bacteria is directly affected by the amount of oxygen present in the water, according to Christopher T. Lefevre et al. (2011). Magnetotactic bacterial strains are attracted to the magnet at one end of the capillary tube and collected using racetrack technique [17].

Isolation of magnetotactic bacteria was conducted the capillary racetrack protocol [18, 19]. The technique offers the advantage of producing highly pure inoculums of magnetotactic cells in a highly active physiological state quickly. The magnetotactic bacteria were cultivated on Nutrient agar media and Magnetic Spririllum Growth Medium. The [O2] gradient visualization is made possible by the media's resazurin, or redox indicator [20]. Bacteria proliferated throughout an overnight or multi-day incubation period, creating a distinct zone or band along the oxic–anoxic boundary (also known as the pink–colorless transition site).

The magnetism of the sample complied, and the sample's ability to form magnetosomes was ascertained. This work was a promising attempt to highlight the morphological diversity and the cellular diversity of magnetically-property-carrying bacteria in a particular environment.

Magnetotactic bacteria (*magnetospirillum*) are microorganisms that have been identified using various techniques, including the hanging drop technique, Gram stain, and culture [21]. The technique of cultivating microorganisms in a medium suitable for their growth and that replicates their natural environment is termed culture [22, 23]. Sufficient bacteria were growing in media,

according to our findings. The objective of developing a culture medium for any microorganism is to add a mixture of necessary nutrients in a well-balanced amount that promotes healthy growth. The culture medium creates an artificial environment that mimics the natural circumstances required for growth [24, 25]. Our research revealed that semi solid media had a suitable level of bacterial growth.

The amplification efficiency of magnetotactic bacteria was investigated using a standard set of samples and precise calculations based on the nucleotide presence in DNA sequence [26]. The PCR amplification efficiency was remarkably near to 100% in the sample that emerged at 1500 molecular base pairs (bp), which is typically considered the finest parameter research. Using the forward and reverse primers, the gradient temperature was found in this experiment. An amplification efficiency of 90%–105% would be necessary for a pilot study. Primers that are not well-designed or reaction conditions that are not optimal for the PCR's constituent parts could lead to low reaction efficiency [26].

5. CONCLUSION

Paramagnetospirillum magneticum AMB-1, bacterial strain was 1st time isolated from freshwater sample in Pakistan. Further research is needed to improve the diversity and explore further strains of magnetotactic bacteria, as isolated bacterial strains may be helpful in a variety of biomedical applications.

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CONFLICT OF INTEREST

No potential conflict of interest

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