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Research Article

Identification of arsenic resistant bacterial isolates from semi arid coal mine tailings of Assam using 16S rRNA phylogeny

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Abstract:
Rhizospheric and non rhizospheric soil samples were taken from the tailings of North Eastern coal mines of Assam state of India to isolate the arsenic resistant bacteria having the ability to transform different arsenic forms particularly the toxic As (III) and As (V) using arsenic bioremediation methods. We describe the isolation and characterization of four strains with high resistance towards arsenic. Isolates were processed and characterized using biochemical and molecular characterization techniques. The 16S ribosomal RNA gene isolated were sequenced and the sequencing data were aligned using the multiple sequencing alignment tool (MSA). The sequences of the strains formed a monophyletic clade with the members of the genus *Bacillus* and *Rhodanobacter* in the The results of phylogenetic tree. The sequence representing *Bacillus subtilis* showed maximum similarity with 99% 16S rRNA gene sequence similarity followed by *Bacillus cereus* (99%) and *Rhodanobacter thiooxydans* with 97% similarity. The phylogenetic tree data suggests that the isolated and identified strains is of the type strains of species belonging to the genus *Bacillus* and *Rhodanobacter* together with the 16S rRNA gene sequence in the Ribosomal Database, small subunit rRNA and large subunit rRNA databases. It is of interest to isolate, identify and characterize the highly resistant strains, which were highly resistant to arsenic from the rhizospheric and non-rhizospheric soils from the tailings of the coalmines of Assam using the 16S rRNA gene sequence phylogenetic analysis. Thus, the four arsenic resistant strains were reported using the 16S rRNA gene sequence phylogenetic analysis. The accession numbers for the sequences are MN706211, MN701565,

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MN706210 and MN706212. A monophyletic clade representing the members of the genus *Bacillus* (*Bacillus subtilis* and *Bacillus cereus*) and *Rhodanobacter thiooxydans* is thus documented.

Keywords: 16S rRNA gene, phylogenetic, arsenic resistant bacteria, ribosomal database, coal mine tailing.

Background:

As per the Northeast Today newspaper 2017, it was reported that arsenic and fluoride contamination was found in around 23 districts of Assam **[1].** Arsenic contamination has become a widely spread problematic issue in the ground water and drinking water contamination. The concentration levels of above two metals were found highly toxic range in many developing countries like India and Bangladesh. Evolution of recent modern technologies for arsenic removal in ground water and in different aqueous systems has become an area of interest in research in recent years. Arsenic is a highly toxic metalloid present in the form of arsenate and arsenite and as sulfides and sulfosalts as insoluble form in rhizosphearic and non-rhizosphearic soil. A study in Italy reported the bacterial isolates that have showed high resistance towards both form of arsenic i.e. As III and As V and therefore it is indicated to be a good source for bioremediation of the polluted sediments near it **[2].** Arsenic exists in the environment in both soluble and insoluble forms. As insoluble form it exists as geological origin (rocks and minerals) and in soluble forms arsenic occurs as trivalent arsenite As (III) and pentavalent arsenate As (V). As III and As V are the most common forms of arsenic in environment. Both are highly toxic forms present in the lithosphear. It should be noted that comparative toxicity of arsenite is greater than arsenate. They can interfere with cellular function of the bacterial system around it in the biological system **[3].** Arsenate i.e. As V a phosphate structural analogue enter bacterial cell through the phosphate transport system and cause toxicity due to the interference in normal phosphorylation process by replacing cellular phosphate. Recently it was revealed that at pH 7, As III enters the cell by aqua glyceroporin i.e. glycerol transport proteins in bacteria, yeast and mammals **[4]**, and thus causes toxicity by binding with sulfhydral group of cysteine residues in proteins and thus they deactivate them. Microbially and biologically As III can be oxidized to As V as it is more toxic from. This process of converting high toxic As III to As V less toxic form has much interest because of its low cost and ecofriendly applications. Microorganisms use either arsenite as electron donor or arsenate as electron acceptor under aerobic and anaerobic growth conditions, respectively and thus they are able to resist in high concentration of arsenic species through various detoxification pathways. For preparing the inoculums for bioremediation of heavy metals or metalloid it is very important to know the resistance and sensitivity of the microorganism. Bacteria have different mechanism within itself to protect them from the oxidative stress caused by arsenic. This process is known as detoxification mechanism. The ability of bacteria to convert more toxic form of arsenic to less toxic could be one of the important plans for bioremediation as an alternative method for removal of arsenic. Some microorganisms take As V via phosphate transporters and then reduce to As III, a more toxic form, subsequent to which extruded from the cell**.** According to Valverde's study in 2011, it was show that there was shift towards the dominance of gram positive bacteria that is Firmicutes in high concentration of arsenic in soil. It was depicted that under aerobic conditions strains were able to tolerate high As III concentration predominated over the strains that were able to tolerate As V that is less toxic form of arsenic present in environment **[5]**.

There is structural similarity between As V and inorganic phosphate therefore As V can also enter the cells following the same system as inorganic phosphates disrupting metabolic reaction which require phosphorylation and can stop the synthesis of adenosive triphosphate **[6].** When people exposed to arsenic concentration above the permissible limit of 0.05 ppm, it causes various adverse affect on human health among which the common symptoms are birth defects, skin lesions, itching, cancer, growth retardation leading to disabilities, liver and kidney damage, weight loss, loss of appetite and lethargy, chronic respiratory disorder, disorders like anorexia, nausea, pain in abdomen, enlarged liver and spleen, anaemia **[7].** Therefore correct identification of bacteria is important for diagnosing the disease and thus the treatment of infection and therefore tracking back the reason of the disease outbreak related with particular bacterial infection. Arsenate reductase and siderophore production in some isolates is regulated by Ars operon, which plays an important role in the mobilization of As V to As III **[8].** For the aquifers, which are contaminated by arsenic, the isolation, identification and characterization of arsenic resistant bacteria from here can be fruitful in developing arsenic detoxification strategies **[9].** Classical method of bacterial isolates is not so specific as genotypic methods **[10].** The rRNA-based analysis is the central method in microbiology and not only to explore microbial diversity but also to identify new strains. The

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genomic DNA is extracted from isolates and amplified with universal 16S rRNA gene fragment and thus sequenced and then sequences were matched for percentage similarity with other bacteria through blast in ncbi. The tree thus depicts similarity and dissimilarity of the test strain with other bacterial isolates. Comparison of test strain against known sequences of SSU rRNA and Isu rRNA databases is critical.

Pandey and Keshavkant isolated arsenic resistant plant growth promoting indigenous soil bacteria from Centre Eastern region of India in 2019 **[11].** Arsenic rRNA is essential for the survival of all cells and the genes encoding for rRNA are highly conserved in bacteria and other kingdoms. rRNA and protein sequences comprising the ribosome are highly conserved from the time of evolution because they require to maintain protein synthesizing machinery and complex inter and intermolecular interactions **[12-14].** Degenerate primers have permitted us to distinguish different life genes. The ars primer sets were designed originally to amplify regions containing active sides or to cover the most homologous sections of DNA indicated in multiple gene alignment with E. coli R773, R46 and chromosomal ars operons **[15]**. In the present study we have isolated, identified and characterized unknown bacteria from the rhizospheric and non rhizospheric soils of North Eastern semi arid coal mine tailings of Assam, India and characterized the four strains at molecular level based on the universal phylogenetic gene 16S rRNA which are thus used for bioremediation process.

Material and Methods:

Culturing of bacteria:

Soil samples collected from trailing of North Eastern Coal Mines of Assam were diluted and spread on nutrient agar medium followed by incubation at 30°C under aerobic condition. Single colonies of bacterial strains were isoalted and further grown and sub-cultured several times to obtain a pure culture. The protocol for bacterial culture was standardized using various literatures available with minor modifications **[16-18].**

Screening of bacteria for arsenic-resistance: The isolated bacterial strains were screened for their abilities to tolerate high level of As. These strains were grown in NA plates containing different concentration of As (V) (using sodium arsenate) and As (III) (using sodium arsenite). All plates were incubated at 30 °C for 48 h. After screening, bacterial isolate *Rhodanobacter thiooxydans sp.* and *Bacillus sp.* could tolerate the highest As (V). These bacterial isolate that could tolerate the highest As (V) and As (III) concentration were selected for DNA isolation **[16-19].**

DNA isolation of bacteria:

Pure culture of the target bacteria was grown overnight in liquid NB medium for the isolation of genomic DNA using a method described with minor modifications **[19].**

Polymerase chain reaction (PCR) amplification 16S rDNA gene:

Some universal primers sets were selected from previous studies **[7, 20-23]** the suitable primers were selected using standard protocol for PCR primer designing with minor modifications in sequence and PCR protocol. PCR reaction was performed in a gradient thermal cycler (Eppendorf, Germany). The primer set used in present study having the sequence as: (Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-CTTGTGCGGGCCCCCGTCAATTC-3') The reaction mixture of 50 µl consisted of 10 ng of genomic DNA, 2.5 U of Taq DNA polymerase, 5 µl of 10 × PCR amplification buffer (100 mM Tris-HCl, , 500 mM KCl pH-8.3) , 200 µM dNTP, 10 p moles each of the two universal primers and 1.5 mM $MgCl_2$. Amplification was done by initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing temperature o f primers was 55°C for 30 s and extension at 72°C for 1 min. T he final extension was conducted at 72°C for 10 min.

Agarose gel electrophoresis:

Ten microlitre of the reaction mixture was then analyzed by gel electrophoresis using 1.0% agarose with ethidium bromide at 8 V/cm and the reaction product was visualized under Gel doc/UV trans-illuminator.

Purification of PCR product:

The PCR product was purified by Qiagen gel extraction kit using the standard protocol of the supplier.

DNA sequencing of the 16S rDNA fragment:

The 16S rDNA amplified PCR product (100 ng concentration) was used for the sequencing with the single 16S rDNA with above primer sets by ABI DNA Sequencer (Applied Biosystem Inc) and the sequences obtained were submitted to NCBI database and having the accession numbers MN706211, MN701565, MN706210 and MN706212.

Multiple Sequence analysis:

A comparison of the 16S rRNA gene sequence of the test strain against the nucleotide collection (nr/nt) database was completed using BLAST **[22].** A number of sequence of both the genus *Bacillus* and *Rhodanobacter* were selected on the basis of similarity score (<99% and ≥ 95%) of the determined sequence with a reference sequence. Multiple sequence alignment of these selected

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homologous sequences and 16S rRNA gene sequence of test strain was performed using ClustalW **[24].** Subsequently, an evolutionary distance matrix was then generated from these nucleotide sequences in the dataset. A phylogenetic tree was then drawn using the Neighbour joining method**.**

Results and Discussion:

The rRNA based analysis is a central method in microbiology used not only to explore microbial diversity but also to identify new strains **[23-35].** The genomic DNA was extracted from isolated bacterial strains and universal primers 27F and 939R were used for the amplification and sequencing of the 16S rRNA gene fragment. A total of 849 bp of the 16S rRNA gene was sequenced and used for the identification of isolated bacterial strain. Subsequently, a 16S rRNA gene sequence based phylogenetic tree showing the relationships between the test strains and selected representatives of the genus *Bacillus* and *Rhodanbacter* is given in **Figure 1**. It is evident from phylogenetic analysis of 16S rRNA gene that the isolates represent a genomic species in the genus *Bacillus* and *Rhodanbacter*. Comparison of test strain against known sequences of ssu rRNA and lsu rRNA databases showed that the gene sequence of isolates have 99% sequence similarity with 16S rRNA gene sequence of *Bacillus subtilis, Bacillus cereus, Bacillus sp. and Rhodanobacter thiooxydans*. Thus, data shows that the isolates are a member of the genus *Bacillus* and *Rhodanbacter*.Identification of As-resistant bacterial strains is necessary because they can facilitate the accumulation of more As in their cell system and therefore can be used for the exploitation in bioremediation studies **[35-37].** Pennanen and his coworkers **[35]** reported that at long-term contaminated field sites, soil microbial communities have time to adapt to metal and/or metalloid stress. These results clearly demonstrate that microorganisms usually modify or convert in high stress.

Conclusion:

We report the isolation and characterization of strains belonging to members of the genus Bacillus (*Bacillus subtilis* and *Bacillus cereus*) and Rhodanobacter (*Rhodanobacter thiooxidans*) from the soils from the North Eastern Coal mines and rhizosphere of Assam, India using phylogenetic analysis of 16S rRNA gene sequences. The data depict the practical implication in addition to increasing our understanding of the metabolic diversity of bacteria **[39-40]**. The recognition of a whole new group of microbes allows their role in AMD remediation to be addressed. This will help in the improvement in design and operation of biotechnologies remediation of AMD. Thus, metal resistant bacteria could prove to be a potential agent for bioremediation of heavy metal pollution. Therefore, the bacterial isolates that were obtained can be exploited biotechnologically for the bioremediation of metal contaminated ecosystem.

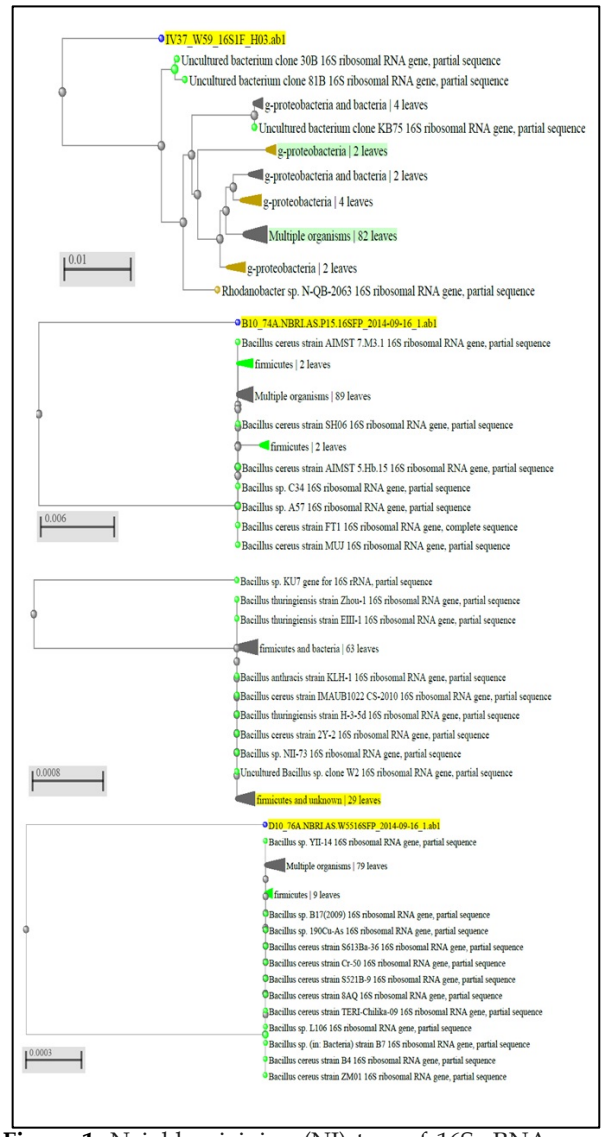

Figure 1: Neighbor-joining (NJ) tree of 16S rRNA gene sequences from the isolated strains with sequences obtained from GenBank shows the characterized strains belongs to the genus *Bacillus* and *Rhodanobacter*. The tree was generated using the rooted RDP Classifier check Program with default parameters

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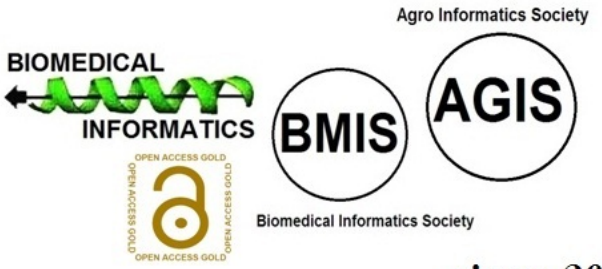

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