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Full Length Research Paper

Molecular characterization of *Azotobacter* spp. *nifH* gene Isolated from marine source

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The *nifH* gene sequence of the nitrogen-fixing bacterium *Azotobacter* spp. was determined with the use of polymerase chain reaction (PCR). The *Azotobacter* species was isolated from marine source in two different seasons. They were cultivated under laboratory conditions using Nitrogen free Azotobacter specific medium. We observed that they were present in both seasons. The phylogenetic tree revealed that our isolated *Azotobacter* spp. was distantly related to uncultivated and uncultured organisms. They did not form any branch with other *Azotobacter* spp. in the data base.

Key words: Azotobacter, *nifH*, PCR, nitrogenase, biological nitrogen fixation.

INTRODUCTION

Biological nitrogen fixation is an important source of fixed nitrogen for the biosphere. Microorganisms catalyze biological nitrogen fixation with the enzyme nitrogenase, which has been highly conserved through evolution (Zehr et al., 2003). The sea harbors an extensive population of bacteria, varying greatly in numbers and in the variety of their activities. Microorganisms are widely distributed in sea water and on the ocean floor, where they influence chemical, physiochemical, geological, and biological conditions (Zobell, 1946).

Nitrogen fixation can be considered as one of the most interesting microbial activity as it makes the recycling of nitrogen on earth possible and gives a fundamental contribution to nitrogen homeostasis in the biosphere (Aquilanti et al., 2004). Diazotrophs are found in a wide variety of habitats including free-living in soils and water, associative symbioses with grasses, symbiotic associations in termite guts, actinorhizal associations with woody plants, cyanobacterial symbioses with various plants and root–nodule symbioses with legumes (Dixon and Kahn 2004).

Azotobacter is a free-living nitrogen-fixing bacterium, which is used as a biofertilizer in the cultivation of most crops. It has several metabolic capabilities, including interest to scientists, who are working toward a better agriculture. *Azotobacter* spp. has the highest metabolic rate of any organisms. Biological nitrogen fixation is an essential step in the nitrogen cycle in the biosphere and it is a major contributor to the nitrogen available to agricultural crops. Nitrogenases are composed of two proteins that can be purified separately: dinitrogenase and dinitrogenase reductase. Dinitrogenase, also referred to as the MoFe protein or component 1 is a 220- to 240-kDa tetramer of the *nifD* and *nifK* gene products that contains two pairs of two complex metalloclusters known as the P-cluster and the iron molybdenum cofactor (FeMo-co). Dinitrogenase reductase, also referred to as the Fe protein or component 2, is a 60- kDa dimer of the product of the *nifH* gene, which contains a single [4Fe-4S] cluster at the subunit interface and two Mg-ATP-binding sites, one at each subunit (Rubio and Ludden, 2005).

Nitrogenase activity appears to be tightly regulated on both the transcriptional and posttranslational levels, in part due to the energy demands of N_2 fixation (16 molecules of ATP for each molecule of N_2 fixed) and the sensitivity of nitrogenase to O_2 (Church et al., 2005). The *Azotobacter* spp. has a very high respiratory rate, and its ability to fix N_2 in O_2 tensions at and above air saturation levels has intrigued researchers for many years. One mechanism *Azotobacter* species use to protect nitrogenase from O_2 damage is termed conformational protection and involves the association of a FeSII protein with nitrogenase during periods of oxygen stress (Maier and Moshiri, 2000). Protection of dinitrogenase from

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oxygen in *Azotobacter* occurs mainly through two mechanisms: a high respiratory activity that removes oxygen at the cell surface and formation of alginate, which increases the viscosity of the culture broth and reduces the oxygen transfer rate from the gas phase to the aqueous phase (Sabra et al., 2000)

In one study conducted by Ueda et al. (1995), diazotrophic bacteria associated with rice roots were investigated by the diversity of sequences amplified by PCR from DNA extracted from the rhizosphere. The diversity of nitrogenase genes in a marine cyanobacterial mat was investigated through amplification of a fragment of *nifH* which encodes the Fe protein of the nitrogenase complex. The amplified *nifH* products were characterized by DNA sequencing and were compared with the sequences of nitrogenase genes from cultivated organisms (Zehr et al., 1995). Kirshtein et al. (1991) used polymerase chain reaction and degenerate oligonucleotide primers for highly conserved regions of *nifH*, a segment of *nifH* DNA was amplified from several aquatic microorganisms. The study of genes in the ocean had its start more than a decade ago when the new field of molecular ecology first allowed oceanographers to measure the diversity and distribution of selected protein-encoding genes. Polymerase chain reaction (PCR) amplification and sequencing of *nifH* genes led the way of nitrogen-fixing organisms in marine environments and investigating the factors that limit their activity (Mary and Virginia, 2007).

MATERIALS AND METHODS

Sampling location

Samples were collected from different locations of Rameshwaram (Ramnad district) marine region (Gulf of Mannar) at the depth of 1 m. Water and sediment samples were collected at equal distance from the shore in sterile bottles and sterile test tubes (tubes containing selective media for the isolation of *Azotobacter*) .Totally 28 samples were collected (14 from water and 14 from sediment region) during the month of May and August 2007. The water samples were collected in the slant medium and the sediment NaCl). As the isolates are of marine origin, the media were prepared using 3.5% sodium chloride (Macleod, 1965). Then thesamples were collected in broth medium (Burk's medium with 3.5% collected samples were carefully transported to laboratory for further analysis. Burk's medium with $NaCl_2$ was used for the isolation of *Azotobacter* spp. from marine water and sediment samples. The marine water samples were named as A1, A2, A3, A4, A5, A6 and A7 and the sediment samples were named as B1, B2, B3, B4, B5, B6 and B7 collected during the month of May. The water samples collected during the month of August were named as C1, C2, C3, C4, C5, C6 and C7 and the sediment samples were named as D1, D2, D3, D4, D5, D6 and D7.

Azotobacter reference strains

The following reference strains of *Azotobacte*r spp were used as control. Azotobacter reference strains were obtained from MTCC and NCIM. The strains were sub cultured on Burk's medium.

Azotobacter reference strains were as follows: *Azotobacter* spp. 2632, *Azotobacter vinilandii* 2821, *Azotobacter chroococcum* 2452, *Azotobacter macrocytogenes* 2454, *Azotobacter lactogenes* 2633, *Azotobacter indicus* 2055, *Azotobacter agilis* 2819, MTCC 123 (*A. beijernickii*) , MTCC 124 (*A. vinelandii),* MTCC 446 (*A. chroococcum*).

Amplification of *nifH* gene

All the 28 (A1-A7, B1-B7, C1-C7, and D1-D7) isolates were inoculated in Burk's nitrogen free medium. The DNA was isolated as per the following protocol (Jones and Barlet, 1990). 1.5 ml of broth culture was transferred into micro centrifuge tube and spin 2 min. supernatant was decanted and drained well. Pellet was re-suspended in 467 µl TE buffer by pipetting. 30 µl of 10% SDS and 20 mg/ml proteinase K was added and incubated at 37ºC for 1 h. An equal volume of phenol/ chloroform was added and mixed well by inverting the tube until the phases were completely mixed. The mixer was centrifuged at 10,000 rpm for 10 min. The upper aqueous phase was transferred to a new tube. 1/10 volume of sodium acetate was added and 0.6 volume of isopropanol was added and mixed gently until the DNA was precipitated .The DNA was spooled on to a glass rod and washed by dipping end of rod in to 1 ml of 70% ethanol for 30 s. Then the DNA was resuspended in 100-200 µl TE buffer. After DNA had dissolved, the concentration was measured by diluting 10 µl of DNA in to 1 ml of TE (1:100 dilutions) and the absorbance was measured at 260 nm. Concentration of original DNA solution in µg / ml = Abs x 100 x 50 µg / ml. The quality of the DNA was checked using agarose gel electrophoresis along with Hind III/λ marker. The DNA of reference Azotobacter strains were used as the control. The DNA was amplified using nif H-g1 primers (Helmut et al., 2004). The primers were acquired from Operon Biotechnologies, Germany. The forward and the reverse primers were Forward 5'-GGTTGTGACCCGAAAGCTGA-3', Reverse 5'-GCGTACATGGCCATCATCTC-3'. Helmut et al. (2004) developed new primer sets by constructing consensus sequences from *nifH* genes of phylogenetically related organisms based on these cluster analyses. Primer set nifH-g1 targets *Azotobacter* spp. *nifH* gene. (GenBank accession numbers M11579, M20568, X13519, M73020, X03916). The reaction mixture contained: Template DNA (100 ng), Enzyme: Tag polymerase (3 U/µl), 10 X Tag polymerase buffer contains (100 mM Tris (pH 9), 500 mM KCl ,15 mM $MgCl_2$, 0.1% Gelatin) dNTP mix (10 mM), 10 µm each primers. The reaction conditions were 94°C for 1 min, 94°C for 30 s, 50°C for 1 min, 72°C for 30 s (for 40 cycles), and then 72°C for 10 min.

Sequence alignment and phylogenetic analysis

The product of approx 400 bp fragment was obtained as a result of PCR reaction. Out of 28 amplified *nifH* gene only one was randomly (D6 sample) selected for sequencing reaction. The sequencing reaction was carried out at Bangalore Genei Laboratory. The PCR product (D6 sample randomly selected) was sequenced using ABI applied 31 100 genetic analyzer and big dye terminator software were used. The resulting sequence having 346 nucleotides was BLAST searched and the sequences were retrieved via the World Wide Web and new sequences compared with those held in the databases by using the basic local alignment search tool (BLAST), http://ncbi.nih.gov/cgi-bin/nph-blast?Jform=1. BLAST is likewise extremely useful for comparing query sequences with the greater number of sequences held on-line at EMBL, NCBI and DDBJ (Andrew, 2000). CLUSTAL W was performed for multiple sequence alignment and phylogenetic tree was constructed using neighbor-joining (NJ) method. Bootstrapping analysis using the program SEQBOOT (in PHYLIP) was applied to test the statistical reliability

Table 1. The sample collection data during May 2007.

Sampling location	Depth (m)	Temperature (°C)	Water sample	Sediment sample
At the shore Site 1	0.3 m	28-30	A1	B1
Site 2	1 m	28-30	A2	B2
Site 3	1 m	28-30	A3	B3
Site 4	1 m	28-30	A4	B4
Site 5	1 m	28-30	A5	B5
Site 6	1 m	28-30	A6	B6
Site 7	1 m	28-30	A7	B7

Table 2. The sample collection data during August 2007.

Sampling location	Depth (m)	Temperature (°C)	Water sample	Sediment sample
At the shore Site 8	0.3 m	28-30	C1	D1
Site 9	1 m	28-30	C2	D2
Site 10	1 m	28-30	C3	D3
Site 11	1 m	28-30	C4	D4
Site 12	1 m	28-30	C5	D5
Site 13	1 m	28-30	C6	D6
Site 14	1 m	28-30	C7	D7

of tree branch points.NCIM 2821, Lane 8 = *A. agilis* 2821, Lane 10 = MTCC 446 (*A. chroococcum*), Lane 11 = MTCC 123 (*A. beijernickii*), Lane 12 = MTCC 124 (*A. vinelandii*). Plate 2 shows the PCR product of marine *Azotobacter* spp. A1 to A7 (water sample) and B1 to B7 (sediment sample) samples collected during the month of May. Plate 3 shows the PCR product of marine *Azotobacter* strains that were collected during the month of August; C1 to C7 (water samples) and D1 to D7 (sediment samples).

RESULTS AND DISCUSSION

The water and sediment samples were collected during the month of May and August 2007 (Tables 1and 2). There were 14 samples, collected in the month of May (A1-A7;B1-B7) at equal distance of 1 m depth. And another 14 samples were collected (C1-C7;D1-D7) in the month of August at 1 m depth. The presence of *Azotobacter* spp was monitored at two different seasons May and August. Vargas and Novelo (2007) hypothesized that N_2 fixation would be influenced by interactions between the periphyton and their environment via seasonal and vegetation changes. Rates of nitrogenase activity varied six fold during the year with the highest rates found during the rainy season. Widmer et al. (1999) collected plant litter and soil cores (2.5 by 20 cm) on 3 days between May 1996 and September 1996. Aquilantia et al. (2004) collected soil samples during spring and autumn 2000 at different areas of central Italy both from cultivated and uncultivated soils. Sediment samples were collected by Katrin et al. (1999) Hornsund off the coast of Spitsbergen, Arctic Ocean, in September and October of 1995. The studies in deep stratified lakes have shown various patterns of *in situ* N_2 fixation, depending on weather conditions and generally showing a sharp decrease with depth. Home and Goldman (1972) noticed major peaks of N_2 fixation in spring and autumn.

In our study we were concentrated only on *Azotobacter* strains. The *Azotobacter* was present both in water and terrestrial environment. An attempt was made to selectively isolate *Azotobacter* spp. in Burk's nitrogen free medium from Marine environment. The colony morphology of *Azotobacter* strains were varying during the isolation in the selective media. The colonies were very clear, large, mucoid, with watery due drops like those initially obtained from the marine source. The mother culture was sub cultured in the same Burk's medium. The colony morphology differs slightly i.e. they were small, circular, and convex in nature. The principal advantage of classical cultivation approach is that organisms are isolated and therefore available for further study (Button et al., 1993). Nitrogen fixation can be considered as one of the most interesting microbial activities as it makes the recycling of nitrogen on earth possible and gives a fundamental contribution to nitrogen homeostasis in the biosphere. Among the free-living nitrogen-fixing bacteria, those belonging to genus *Azotobacter* play a remarkable role, being broadly dispersed in different environments, such as soil, water and sediments. In fact, field trials have demonstrated that under certain environmental conditions, inoculation with *Azotobacter* has beneficial effects on plant yields due to the increase of fixed nitrogen content in soil and to the microbial secretion of stimulating hormones, like gibberellins, auxins and cytokinins (Aquilanti et al., 2004). A combination of isolation, cultivation, and molecular

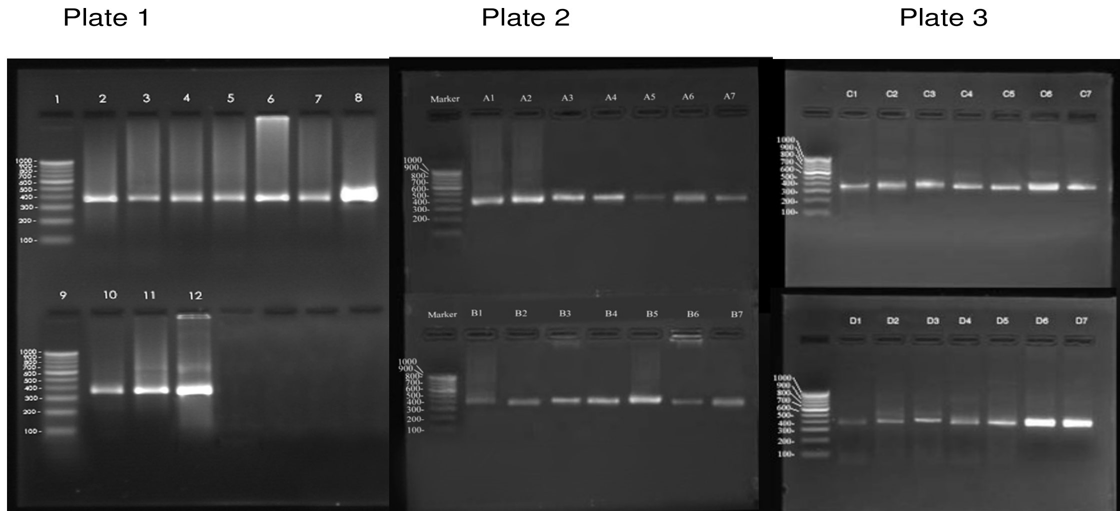

Figure 1. Plate 1 shows the PCR product of reference *Azotobacter* strains as follows Lanes 1 and 9 = 100 bp marker. Lane 2 = *A. lactogens* NCIM 2633, Lane 3 = *A. chroococcum* NCIM 2452, Lane 4 = *Azotobacter* species NCIM 2632, Lane 5 = *A. macrocytogens* NCIM 2454, Lane 6 = *A. indicus* NCIM 2055, Lane 7 = *A. vinelandii*

characterization was used by. Olson et al. (1999) to determine the presence, diversity, and ubiquity of hetero-heterotrophic N_2 fixers in cyanobacteria-dominated microbial mats obtained from geographically diverse sites. They designed internal *nifH* primers from the diazotrophic isolates to examine the potential impact of these bacteria on N_2 fixation rates. Aquilantia et al. (2004) studied the comparison of different strategies for the isolation and preliminary identification of *Azotobacter* from soil samples. In their study they observed all members of the genus *Azotobacter* produced slimy, glistening, smooth, whitish, weakly convex, 2–10-mm in diameter colonies.

The polymerase chain reaction due to its high sensitivity, is finding many practical applications is the detection of microorganisms in nature. It has recently been used to detect genetically engineered microorganisms in the environment (Steffan and Atlas, 1989). The DNA was isolated from all the 28 cultured *Azotobacter* spp. Their banding pattern of DNA was compared with that of reference *Azotobacter* strains. The result showed that there was no significance difference in banding pattern (Figure 1). The PCR reaction was carried out for all the 28 isolated cultures in order to check the presence of *nifH* gene. PCR products were about 400 bp. Helmut et al. (2004) developed the new primer sets that were designed by constructing consensus sequences from *nifH* genes of phylogenetically related organisms based on the cluster analyses. Primer set nifH-g1 targets *Azotobacter* spp. *nifH* sequences (GenBank accession numbers M11579, M20568, X13519, M73020, X03916). They optimized PCR protocols on pure culture of DNA and subsequently analyzed the soil DNA extracts. The primer sets nifH-g1 allowed the amplification of sequences closely related to their intended target sequences but also allowed the amplification of a number of clones belonging to other phylogenetic group. For example nif -g1 targets γ proteobacteria groups. For evolution of nitrogen-fixing population in the environment, analysis of *nifH*, the gene encoding nitrogenase reductase, has been used with various PCR primers that amplify this gene from both microorganisms and environmental samples (Widmer et al., 1999). *nifH* encoding dinitrogenase reductase is evolutionarily conserved and has often been used to detect nitrogen-fixing microorganisms in natural microbial communities (Satoko et al., 2002). Numerous researchers have employed various PCR primers specific for segments of *nifH* sequences from diazotrophic pure cultures and from various environmental samples, including marine plankton, termite hindguts, microbial mats and aggregates, terrestrial soils, and the rhizoplanes of rice (*Oryza sativa*) and of shoal grass (*Halodule wrightii*). These studies have yielded a diverse array of *nifH* sequences representing many, mostly unknown, lineages of diazotrophic *Bacteria* and *Archaea* (Charles et al., 2000). Nitrogenase has been found in phylogenetically diverse groups of prokaryotes and has been highly conserved through evolution (Lei, 2004). The implementation of molecular techniques such as PCR amplification has greatly facilitated the study of diazotrophs in bacterial communities. Numerous studies have employed various PCR primers specific for segments of *nifH*, the structural gene encoding dinitrogenase reductase, to amplify partial *nifH* sequences from various environmental samples and from diazotrophic pure cultures (Betancourt, 2002).

The BLAST search was performed in order to identify closely related species. The BLAST result showed that the *Azotobacter* strain was most similar to unidentified and uncultured bacterial *nifH* gene. In BLAST search the maximum identity values for our sequence was between

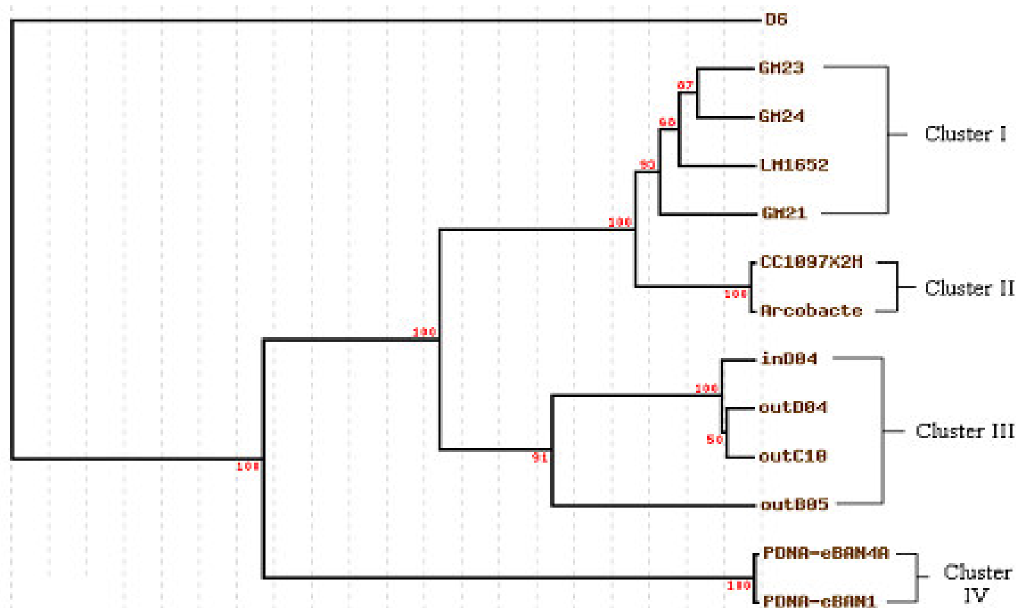

Figure 2. Phylogenetic tree with bootstrap values. Tree constructed using Neighbour- Joining (NJ) method. The botstrapping values were indicated at the node. Our D6 sequence was distantly related to other clusters.

76-86%. The bootstrap values (Figure 2) ranged from 55-100% (denoted at the node). The representative of our D6 *nifH* was diverged from other clones. Our D6 sequence was directly related to cluster 4 having 100% bootstrap value and distantly related to cluster 1, 2, 3. Other studies reported that the analysis of partial *nifH* gene sequences provide information on the phylogeny and composition of diazotroph natural communities (Franck et al., 2001). Many microorganisms have multiple copies of nitrogenase genes or homologues of nitrogenase genes. *Azotobacter* was the first genus to be shown having alternative nitrogenases, *vnfH* and *anfH* (Lei, 2004). Based on pure-culture isolation methods, *Azotobacter*-like organisms have been reported as abundant diazotrophs in vegetated salt marsh sediments (Charles et al., 2000). The *nifH* sequence database is rapidly expanding and is currently composed of over 1500 sequences, most of which have been obtained from environmental samples (Zehr, 2003). Recently, nitrogenase gene sequences (*nifH*) have been amplified and sequenced from a number of environments, including rice roots, soils, and oceans, and invertebrates, such as zooplankton and termites (Sabino et al., 2000). The lack of a sufficient number of *nif* gene sequences from cultured micro-organisms makes the interpretation of results obtained in environmental studies difficult and limits the clarity of the conclusions that can be drawn from phylogenetic treeing analyses (Svetlana et al., 2004).

Burns et al. (2002) studied PCR amplification of *nifH* gene from DNA extracted from whole sediment samples that yielded several unique sequences. None of the sequences were identical to *nifH* genes from known microorganisms. Several sequences cluster with unidentified bacterial sequence from marine microbial mats.

Helmut et al. (2005) carried out experiment on effects of model root exudates on structure and activity of a soil diazotroph community. They amplified *nifH* gene from soil microbes using various group specific primers including nifH-g1 primer. The nifH-g1 primer set was designed to amplify *nif* H genes of *Azotobacter* species. Phylogenetic analysis of the actively *nifH*-transcribing phylotypes detected with nifH-g1 RT-PCR confirmed that these *nifH* sequences were all similar to published *nifH* sequences of *Azotobacter* species. This finding is in agreement with the known ecology and physiology of *Azotobacter* strains, which are frequently found in soil. We expected that our sequence would be related to any of the Azotobacter *nifH* gene in Database. But unexpectedly our sequence did not form any branch with known *Azotobacter* strains, since this may be the new species or further analysis will be required.

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