Abstract

Dutch microbiologist and botanist Martinus Willem Beijerinck (1851-1931) first discovered the process of biological nitrogen fixation. It is the ability to convert the molecular nitrogen into cellular nitrogen. This process is a unique property of a group of free living, associative and symbiotic prokaryotic microorganisms. During this nitrogen fixation process, an enzyme complex called nitrogenase complex catalyzes an ATP dependent reduction of atmospheric nitrogen to ammonium. This fixed nitrogen is the primary source of available nitrogen in nature. It is very difficult to calculate any direct correlation among nitrogen fixing bacteria, however the process of biological nitrogen fixation and its enzyme system is very similar in all nitrogen fixers. They are distributed among 27 families and 80 genera of Eubateria (including cyanobacteria) and three thermophilic genera of archaebacteria.

The bacterium *Frankia* is an important member of biological nitrogen fixer. This bacterium is a microaerophilic, gram positive to gram variable; sporulating actinomycetes belong to the Darmatophilous group. They produce characteristic sporangia and vesicle. This morphology differentiates *Frankia* from other actinomycetes. *Frankia* make symbiotic association for biological nitrogen fixation with a number of woody dicotyledonous collectively known as actinorhizal plants.

The present work deals with the diversity of this bacterium isolated from coastal region to the Darjeeling hills. For diversity study the bacteria was isolated and characterized. *Frankia* were isolated from the root nodules of *Casuarina equisetifolia* and *Alnus nepalensis* using a newly standardized technique. The isolated strains showed typical *Frankia* culture characteristics in liquid Q_{mod} as well as in nitrogen free defined propionate minimal medium (DPM). The vesicles were produced only in DPM medium. Visible nodule and typical root hair deformation was observed on *Casuarina* seedlings in reciprocal inoculation. However the seed germination of *Alnus* and its survival, in both *in vitro* and *in vivo*, is a serious challenge to scientists as the germination process is very slow. Mature seeds of *Alnus* were collected from the healthy plants of Darjeeling hills. Some of the surface sterilized seeds were overnight soaked in aerated water, three different media WPM, MS and Hoagland in half and full strength was used. For the study of effects of hormones in seed germination, the media were separately supplemented with 1-5mg/l NAA and IBA singly or in combinations. Maximum germination was observed in Hoagland media for both treated and untreated seeds, whereas minimum in ½ MS for treated and ½ WPM for untreated seeds. IBA and

NAA+IBA were found to be more effective than NAA in terms of germination percentage and rooting. Good rooting and its growth were maximum in the media supplemented with IBA(@ 3, 4 & 5mg/L) These experiments showed that germination of *A. nepalensis* seeds have specific hormonal requirements, which is fulfilled by mycorrhizal and associative fungal association.

Frankia strains isolated from both *C. equisetifolia* and *A. nepalensis* were subjected to various concentrations of heavy metal salts to study the heavy metal resistance pattern of *Frankia*. This experiment was a part of physiological characterization of *Frankia* of these regions. *Frankia* strains isolated from *C. equisetifolia* were studied in sensitivity to nickel, copper, lead, cadmium and cobalt salts. They were found to be resistant to high concentration of cadmium and cobalt salts. Heavy metal salt resistant patterns of *Frankia* isolated from the *A. nepalensis* were also prepared. The strains were highly resistant to cadmium chloride and lead nitrate, moderately resistant to cobalt chloride and less resistant to nickel chloride and copper sulphate. The heavy metal resistant *Frankia* colonies were re-isolated from various plates and preserved for further studies. A characteristic heavy metal induced either purple or red or both type of pigment production was noted in *Frankia* strains isolated from the *A. nepalensis* in all the cases except in lead at MIC or higher concentrations. This type of pigment production was totally different from the pigment production of the strains isolated from *C. equisetifolia* growing in North Bengal University campus where a faint red pigment was produced only in lead. It was found that *Frankia* isolated from *C. equisetifolia* could tolerate higher heavy metal salt concentration than *Frankia* strain isolated from *A. nepalensis.*

The availability of complete genome sequences of *Frankia* strains from different bio-geographic locations gives us an opportunity to analyze the codon usage of the heavy metal resistance genes, predict their expression level in comparison to protein coding genes and ribosomal protein genes. CLUSTAL W was employed to the nucleotide sequences of studied heavy metal résistance genes to find out the diversity. All the protein coding genes, ribosomal protein genes, and genes for heavy metal resistances were analyzed by the software CodonW (Ver. 1.4.2) and CAI calculator 2. The heavy metal resistance genes clustered along with the ribosomal protein genes and exhibit strong codon bias. The Nc/GC3 plot was done, which demonstrated an effective technique for investigating codon usage variations among the genes. Genes coding ribosomal proteins that are known to be highly expressed were highlighted in the NC/GC3 plots. Compared to the ribosomal protein genes

the heavy metal resistance genes in the genomes showed some difference. Especially the heavy metal resistance genes of CcI3 were less biased compared to the ACN14a and EAN1pec strains. Most of the ribosomal protein genes and heavy metal resistance gen for all the *Frankia* genomes were found to be clustered at lower ends of the plot suggesting a strong codon bias for these genes. Genes with effective number of codons value <40 had much stronger codon bias than be simply explained in terms of mutational bias. Ribosomal protein genes and those associated with metal resistance had a lower mean Nc value than that obtained for all of the protein coding genes suggesting a higher degree of bias in the former. These values indicated a strong codon bias over mutational bias. This codon bias was aroused due to natural selection for translational efficiency.

Codon Adaptation Index is a simple, effective measure of synonymous codon usage bias. The index uses a reference set of highly expressed genes from a species to assess the relative merits of each codon, and a score for a gene is calculated from the frequency of use of all codons in that gene. In ACN and EAN heavy metal genes and ribosomal protein genes had higher mean CAI values compared to that of the protein coding genes. On an average the CAI values of protein coding genes are high. High CAI values indicate better expression levels. Especially the comparatively higher expression levels for heavy metal resistance indicated the ability of *Frankia* genomes to survive in stressed environments and subsequent adaptability. The result obtained in this study put an additional support to the hypothesis that *Frankia* strain CcI3 is more symbiotic than saprophytic.

The heavy metal resistance genes of *Frankia* evolved in a single major clade and two subclades. Clustering of copper resistance and tellurite resistance genes of the different *Frankia* strains suggests that they have co-evolved as a unit. The Zn-Co-Cd resistance gene has a completely different origin and does not lie in a particular clade. Clustering of copper resistance and tellurite resistance genes of the different *Frankia* strains suggested that they had co-evolved as a unit. The putative Zn-Co-Cd resistance gene which is basically a cation diffusion facilitator family transporter had a completely different origin and did not lie in a particular clade. The study of heavy metal resistance has great significance since the heavy metal resistance is a more suitable marker for slow growing bacteria like *Frankia* as it is more stable than antibiotics resistance.

Eleven *C. equisetifolia* and *A. nepalensis* specific *Frankia* strains were isolated and characterized on the basis of organic acid decarboxilation, protease and β-glucosidase

activity as well as utilization of twelve different carbon sources. Three strains isolated from *C. equisetifolia* and two strains isolated from *A. nepalensis* showed decarboxylation and protease activity. All the strains utilize the Na-propionate, Na-acetate and Na-succinate as carbon source in nitrogen free medium in general. The strains isolated from NBU Campus could also utilize fructose and sucrose in addition. The result thus obtained was analyzed with the software POPGENE to calculate the diversity among the *Frankia* strains by Nei genetic diversity, Shannon index, single locus component and two locus component and Wahlund effect. *Frankia* population present in this area shows very low genetic diversity and the total genetic diversity was intrapopulation in origin and divided into two different physiological groups.

The entire *Alnus* specific strains along with CeSi10, CeSi11 and CeSi12 cluster in one group, and the rest of the *Casuarina* strains made other group. Among the strains all alder and CeSi10, CeSi11 and CeSi12 belong to physiological group A and CeSt2, CeSt5 CeSt9 of *Casuarina* strains belong to physiological group B. Field emission scanning electron microscopy was also performed and *Frankia* specific structures were found.

For the molecular biological work, DNA were isolated and purified both from culture and nodules. A new 16s r-DNA specific primers were developed and a 520 bp long portion of the said region was amplified. The amplicon was digested with *Alu*I, *Taq* I, *Hae* III, *Mbo*I and *Msp*I restriction enzymes. The PCR-RFLP profile was photographed. The results were analyzed with the software POPGENE to calculate the diversity among the *Frankia* strains by Nei genetic diversity and Shannon index. The results again showed that *Frankia* population present in this area had very low genetic diversity and the total genetic diversity was intrapopulation in origin. From the dendogram based on PCR-RFLP profile, a more or less distinguished pattern emerged in case of *Alnus* based *Frankia.* The *Casuarina* based *Frankia* were places in different clades. The probable reason of *Casuarina* based *Frankia* behaving differently from *Alnus* based *Frankia* could be that all the *Casuarina* plantations in West Bengal are exotic and were perhaps brought from different places.