Abstract

Prokaryotic organisms constitute one of the major components of biological diversity not only from the point of view of their abundance but also for their diversity of biogeochemical activities, which sustain life on this planet. Inspite of their immense importance, they have not been given enough attention due to them, because of their invisibility. Moreover, conventional study of prokaryotes requires them to be cultured in the laboratory, but many lines of evidence suggest that most of them (more than 99 %) are not amenable to cultivation. Fortunately, techniques are now available to detect them and to a certain extent tap their gene pool by culture independent approach. Although, such culture independent studies of prokaryotes have highlighted their huge diversity and functions in the last few years, on the whole, their diversity and functions still remains poorly known and understood.

India has a rich diversity of macroscopic life forms (fauna and flora) due to its unique geographical location and possesses two megadiversity "hot spots". One of which is in the North Eastern part and the other is along the Western Ghats. Unfortunately, very little or no systematic study of prokaryotic diversity from any of these hot spots or other regions of this country has ever been taken up.

In this context, the present study was aimed at a systematic study of the bacterial diversity of water of a warm spring located in Assam, a state in the North East of India, using both culture dependent as well as by culture independent approaches. Since, most of the aquatic ecosystems are nutritionally poor (oligotrophic), but still dominated by a huge prokaryotic flora, attempts have been made to understand the molecular basis of oligophily.

The thesis commences with an introductory chapter highlighting the present status of our knowledge about prokaryotic diversity. The chapter also gives a review of the concept of oligophily and copiophily in bacteria.

Chapter 2 describes the materials and methods employed in this investigation.

Chapter 3 presents the data obtained from analysis of the warm spring water sample by culture dependent as well as by culture independent approaches. It also includes some discussions of the results in the context of present understanding of bacterial diversity. Two types of media, a nutritionally rich TSBA (tryptic soy broth agar) and nutritionally poor TSBA100 medium (100 times diluted TSB, solidified with agarose) was selected because the former generally supports growth of many bacteria including *Actinobacteria* and also because

the spring water sample contains very little organic carbon, the latter was expected to help in isolating oligophilic bacteria.

The viable count on TSBA100 medium (4.65 X 10^4 CFU/ml) was almost 5 times more than on TSBA medium (8.8 X 10^3 CFU/ml). In culture based analysis of diversity, a total of 59 morphotypes were initially selected (28 from TSBA plates and 31 from TSBA100 plates) for further study. Based on 10 major phenotypic characteristics, attempts were made to minimize redundancy and the number was reduced to 46. Detailed characterization of these 46 isolates using polyphasic approach including 16S rRNA gene sequencing, was done and identity of most of them were established. In this approach two new genera and six novel species were discovered in this water sample. Atleast 3 more are promising candidates for the status of novel taxa. It was found that some species like those belonging to *Bacillus cereus* group, species of *Aeromonas*, *Hydrogenophaga* and *Pseudomonas*, were recovered from both nutritionally rich as well as from nutritionally poor media. On the other hand, species of *Alishewanella*, *Chitinimonas*, *Curvibacter*, *Zoogloea* and a few novel taxa were recovered from nutritionally poor medium only. In contrast, a few novel taxa and species like those belonging to *Azoarcus*, *Comamonas*, *Paenibacillus*, *Rubrivivax* and *Thauera* were recovered from nutritionally rich media only.

In summary, culturable bacterial representatives in this spring water were found mainly from 4 phyla, namely "*Bacteroidetes*", "*Deinococcus-Thermus*", *Firmicutes* and *Proteobacteria* (β and γ-classes). These 46 bacteria belong to 16 established and 2 novel genera (*Aquimonas* and *Emticicia*), 6 novel species and 25 of which belong to members of already described species. Other than these 46 strains, taxonomic positions of 9 more isolates needs some confirmatory tests but 3 strains belong to the genus *Bacillus* with high probability while one strain is possibly *Vibrio vulnificus* on the basis of phenotypic and fatty acid profiling.

Chapter 3 also describes bacterial diversity from the warm spring water sample by culture independent 16S rRNA gene sequence based approach. Major finding here is the presence of a large number of cyanobacterial sequences in the 16S rRNA gene library. Although attempts were not made to culture *Cyanobacteria*, presence of cyanobacterial sequences is not surprising because this group of bacteria is usually found in most aquatic habitats. Globally, three genera, *Gloeothece*, *Synechocystis* and *Synechococcus* predominate in such habitats but in this hot spring water sample, out of 32 cyanobacterial sequences, 28 showed closest relations (ranging from 87.2 % to 96.7 %) with *Synechocystis* while 4 with *Cyanobacterium* sp. (97-98.8 %). Sequence similarity of some clones was as low as 87.2 % and in the phylogenetic tree two clones (GPENV19 and GPENV123) showed deep branching indicating their higher taxonomic status

Other selected clones contained the sequences of *Proteobacteria* (3 α, 16 β and 6 γ) having sequence similarity between 81 to 99 % mostly with uncultured bacterial clones obtained from variety of ecological niches. Only two, GPENV31 and GPENV145 showed sequence similarity of 97.2 % with a culturable bacteria *Janthinobacterium* sp. J 31 and of 83.5 % with *Pantoea* sp. B232 respectively. It is to be noted that none of the culturable bacteria recorded from this spring water (in this study) were found in these clones made by culture independent approach. It is very possible that culturable bacteria are present in low numbers compared to the ones, which could not be cultured and, therefore, low representation in the clone library. *Firmicutes* were altogether not represented in these 25 clones, although, they were a significant component of culturable diversity of this water. This reflects a limitation of 16S rRNA gene sequence based analysis of microbial diversity. The use of atleast 1000 nucleotide sequence length of the clones used in this study makes the detection of uncultured bacteria quite reliable compared to shorter sequences (300-500 nucleotides). However, rarefaction curve, suggested that the number of clones selected for analysis was not sufficient enough to capture complete diversity and more number of clones might have given a better picture of bacterial community structure from the warm spring water sample.

It is clear from this study that a combination of both culture dependent and culture independent approach is essential for assessing microbial diversity of any ecological niche. Bacteria, which are recovered on plates, may be subsequently screened for their potential biotechnological application. Sequences of 16S rRNA gene clones of a community of bacteria [illegible] level (species, genus, family etc.) these bacteria are affiliated to in the hierarchy [illegible]. This information may be very useful not only from diversity point of view but also [illegible] having some idea about the type of media, which may be designed in future to [illegible] any of the uncultured relatives from the same or similar other kinds of habitat.

This study using both culture dependent and culture independent approaches has thrown [illegible] on the diversity of bacterial species in a warm spring. This niche is dominated by [illegible] belonging mainly to the phylum *Cyanobacteria*, *Proteobacteria* and *Firmicutes* with [illegible] representations from members belonging to the phylum "*Bacteroidetes*" and "*Deinococcus-*[illegible]. Although, it may not be possible to say definitely that the diversity discovered in this [illegible] exhausts the total species diversity (due to certain limitations described above) it can be [illegible] concluded that significant component of bacterial diversity of this spring water has been [illegible].

[illegible] study of molecular basis of oligophily in bacteria has been attempted. This [illegible] bacteria, which grow on nutritionally poor media like TSBA100 (TSB

diluted 100 times and solidified with agarose). The isolates that could grow only on TSBA100 medium but not on regular strength media were treated as oligophiles. Strains that can grow only on TSBA were copiophile while the strains that could grow on both TSBA and TSBA100 media were called facultative oligophiles. From the study of growth patterns of all the 59 isolates, 12 were classified as oligophiles and remaining 47 as facultative. On the basis of growth performance on various types of culture media, strain GPTSA100-15 was selected as an oligophile for further study. This was found to be a novel genus and it has been described as *Emticicia oligotrophica* gen nov. sp. nov. This particular strain dies when it is transferred to full strength TSB and drastic reduction in viability follows a typical death curve. In the present study, comparison of total cellular fatty acid profiles from oligophilic, copiophilic and facultative strains were made after growing these bacteria on different concentrations of TSBA medium. Two known oligophilic bacteria (*Sphingopyxis alaskensis* and *Caulobacter crescentus*), one facultative oligophile (GPTSA100-19) and a copiophile (*E. coli*) were taken as control. The analysis could not reveal much drastic shift in cellular fatty acids compositions of the bacteria grown on different nutrient concentrations except monounsaturated fatty acids. Growth at different nutrient concentrations seems to affect monounsaturated fatty acids and the effect is not similar in these strains.

Comparative study of total protein profiles from oligophilic, copiophilic and facultative strains grown in different concentrations of TSBA medium were performed by SDS-PAGE. Although there is an overall similarity in protein profiles of the same organism grown on different concentrations of TSBA, there are quantitative differences in some protein bands, indicating differential expression of certain genes in response to the growth environment. For example a protein of about 32 kDa in strain GPTSA100-19 (facultative strain) and a protein of 27 kDa in GPTSA100-15 (oligophilic strain) are present in relatively higher abundance when grown in TSBA100 medium compared to TSBA or TSBA10 respectively. Although, the 27 kDa protein was hardly produced by the strain GPTSA100-15 when it was grown on TSBA5 (maximum TSBA concentration on which it can grow) but it started appearing with higher dilution of nutrient (TSBA10 and TSBA100).

Since this 27 kDa protein was found in GPTSA100-15, when grown on nutritionally poor media, efforts were made to identify this 27 kDa protein by MALDI-TOF-MS analysis. The results indicated that it might contain other protein(s) albeit in low amount. However, based on different evidences like size conservancy, highest peptide fragment match and highest sequence coverage with transcription regulatory protein (KdpD), it seems that the band may contain protein(s) functionally similar to transcription regulatory proteins (like KdpD). Sequencing of the

protein and identification of the gene(s) encoding this protein will help in assigning its role(s) in oligophily of this bacterium.

Chapter 5, of the thesis presents overall conclusions drawn from the analysis of bacterial diversity of a warm spring water sample of Assam. The chapter also presents in tabular form two lists of bacteria, which showed qualitatively good amylase, protease, lipase and DNase activities.