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PCR- RFLP based bacterial diversity analysis of a municipal sewage treatment plant

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Abstract

Bacterial diversity of sewage soil is an essential study to discover novel bacterial species involved in biodegradation. Restriction Fragment Length Polymorphism is one of the most useful molecular technique for diversity analysis in terms of cost effectiveness and reliability. The present study focuses on bacterial diversity of municipal sewage treatment plant in Chennai, Tamil Nadu, India through metagenomic approach. A 16S r DNA clone library was constructed from metagenomic DNA of sewage soil. 200 clones from the library were subjected to colony PCR and RFLP analysis. Upon RFLP analysis, 16 different Operational Taxonomic Units (OTU's) were obtained and a single clone from each OTU was subjected to sequencing. Phylogenetic analysis of sequences revealed the presence of five different groups of bacteria namely Proteobacteria (56%), Actinobacteria (7%), Firmicutes (5%), Bacteroidetes (17%) and Plancomycetes (7%). Three novel and uncultured groups of bacteria (8%) were also discovered. Most of the organisms identified through this study were reported to be efficient degraders of hydrocarbons, aromatic compounds and heavy metals, thereby promoting biodegradation of polluted environment.

Key words

Bacterial diversity, Biodegradation, Municipal sewage, Restriction fragment length polymorphism

Introduction

Municipal sewage is one of the most polluted environments due to accumulation of wastes from domestic, agricultural, medical and industrial sectors. The major components of municipal sewage include aliphatic hydrocarbons, polycyclic aromatic hydrocarbons (PAH's), chlorophenol, chlorobenzene, phthalate esters, pesticides, detergents, heavy metals, halogens, nitrosamines, etc., (Marttinen *et al.,* 2003). Excessive presence of these pollutants can be hazardous to the existing biota and cause depletion of natural resources. However, microorganisms present in such polluted environments play a vital role in maintaining the various bio-geo chemical cycles. They greatly contribute to recycling of various organic compounds and heavy metals (Yun *et al.,* 2004) and possess mechanisms for adaptation and degradation of such toxic substances. A recent study by Huang and Li (2014) reported the bacterial degradation of organic pollutants such as naphthalene, benzene, toluene, ethyl benzene and xylene present in activated sludge. Biodegradation of PAH by anaerobic bacteria like sulfate reducing bacteria, methanogen and eubacteria was reported by Chang *et al.,* (2002). Numerous other studies have also reported the role of aerobic and anaerobic bacteria in waste- water treatment process for removal of organic and heavy metal pollutants (Dhall *et al.,* 2012; Lee *et al.,* 2002; Hawari *et al.,* 2000; Zilles *et al.,* 2002).

Since microbes play a vital role in biodegradation of municipal wastes, it is essential to study microbial diversity of such polluted environment to understand the interaction between these organisms and soil structure and function in order to maintain soil quality (Jennifer *et al.,* 2004). Diversity analysis is the foremost basic research to understand the role of microbial communities involved in degradation of pollutants present in sewage soil. It also helps us to find out depletion of microbes in sewage due to large amount of toxic substances. Earlier, culture-dependent microbiological studies were used for microbial diversity analysis. However, it is estimated that less than 1% of bacteria present in environment can be cultured in the laboratory owing to methodological limitations (Torsvik *et al.,* 2002). Hence, metagenomic molecular-based approaches were developed to

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circumvent the limitation of culture-based approach. Use of these methods reveals a vast diversity of prokaryotes from a single sample (Daniel, 2005), however, these molecular methods have limitations such as large number of sequencing reactions, polymerase chain reaction (PCR) bias, requirement of large amount of DNA, insensitivity, etc., (Kirk *et al.,* 2004). Restriction fragment length polymorphism (RFLP)-based method of microbial diversity analysis remains ahead of all other methods in terms of cost-effectiveness, as it requires less number of sequencing reactions. This method is also reliable and reproducible. Hence, PCR-RFLP-based approach was applied in the present study with the aim of identifying novel bacteria associated with biodegradation. The present study involve construction of 16S rDNA clone libraries, RFLP, sequencing and phylogenetic analysis. The study revealed the presence of many phylogenetic groups involved in bio-degradation and also a few uncultured bacterial groups.

Materials and Methods

Soil sampling and characterization : Sewage sample was collected from municipal sewage treatment plant situated at Chennai, Tamilnadu, India and refrigerated at 70°C until further use. The soil characteristics, namely texture, pH, organic content and few heavy metals like iron, cadmium, chromium, nickel and lead were estimated as per protocol of American Public Health Association (APHA, 2012).

Metagenomic DNA isolation and assessment of yield and purity of DNA : Metagenomic DNA was isolated from sewage sample in triplicates using Fast DNA SPIN Kit for soil (MPBiomedicals, Santa Ana, CA). Isolated metagenomic DNA was loaded in 0.8% agarose gel incorporated with ethidium bromide and the band was visualized using UVP-MultiDoc-It digital imaging system, CA, USA. Purity of the isolated DNA was determined by measuring the absorbance ratio at 260/230 (DNA/humic acid) and 260/280 (DNA/protein).

16S r DNA PCR amplification and cloning : 16S rDNA region of metagenomic DNA was amplified using 5 pmol of each forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-ACGGCTACCTTGTTACGACTT-3') oligonucleotide primers (Bioserve Technologies, India) as described by Purohit and Singh (2009) followed by addition of 1U Taq polymerase, 5 µl of 10X Taq buffer and 1 µl of 10mM dNTP mix (Bangalore Genei, India). The final mixture was adjusted at 50µl by addition of autoclaved milliQ water. The amplification steps included initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 30 s and extension at 72 °C for 1 min and 30 s with final extension at 72 °C for 7 min. The PCR product (1.5Kb) was visualized on 0.8 % agarose gel and purified using EZ-10 spin column gel extraction kit (Biobasic Inc., Canada). The purified fragment was then ligated to TA region of pGEM-T easy vector and ligated product was transformed in *E.coli* DH10BT^R competent cells. The recombinants were selected by plating the transformed colonies in Luria Bertani media containing ampicillin (50 µg ml^{-1}), IPTG (0.1mM) and X-gal (40µg ml^{-1}). The resulting white colonies were patched in Luria Bertani media supplemented with ampicillin (50µg ml^{-1}).

Restriction fragment length polymorphism analysis : The clones obtained from library were subjected to colony PCR using universal M13 forward (5'-GTAAAACGACGGCCAGT-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers (Bioserve Technologies., India). The resulting 1.5Kb amplified products were then purified using EZ-10 spin column gel extraction kit (Biobasic Inc., Canada). Each product was then digested with 10U of enzyme *Hae*III (Bangalore Genei, India) for 3 hr and the products were visualized on 2 % agarose gel. Clones with similar pattern of digestion and intensity were considered to belong to the same Operational Taxonomic Unit (OTU).

Sequence analysis and phylogenetic tree construction : A single clone was selected from each OTU and sequenced using universal M13 forward (5'-GTAAAACGACGGCCAGT-3') primer with the Big Dye Terminator Cycle sequencing kit on an ABI Prism 3130XL DNA analyzer (Applied Biosystems, USA).The sequences were compared with Genbank and Ribosomal Database Project (http://rdp.cme.msu.ed/index.jsp). Chimeric sequences were identified and eliminated by comparing the alignments at the beginning and the end of each sequence. The obtained sequences were subjected to multiple sequence alignment with Clustal W and phylogenetic tree analysis was performed using PhyML 3.0 with 100 % bootstrap ratio (Guindon *et al.,* 2010). For the construction of phylogenetic tree, all the sequences obtained in this study along with their closest match in the Genbank were taken and uncultured *Cyanobacteria* sp. was chosen as out-group.

Results and Discussion

The sample soil was clay loam in texture and alkaline in nature (pH 8.0). It had high organic matter content (69 %), and heavy metals such as iron (36 %), cadmium (2.5 %), chromium (1 %), lead (3 %) and nickel (4 %). The presence of high levels of organic substances and heavy metals in soil is an important factor that could affect DNA yield and purity (Buscot, 2005). The organic substances present in soil are co-extracted along with DNA which in turn, affects yield (Burgmann *et al.,* 2001). The clay like texture of soil is also a factor to be considered, as clay and humus particles absorb free DNA due to their negative charge and lower DNA yields (Roose-Amsaleg *et al.,* 2001). The typical feature of alkaline soils or sediments is the presence of low level of microbial biomass (Verma *et al.,* 2011). So, an efficient method of DNA extraction is necessary for such clay-like, alkaline samples rich in organic content and heavy metals.

Based on soil characterization, Fast-DNA SPIN kit (MP Biomedicals, Santa Ana, CA) was chosen for metagenomic DNA extraction from sample soil. This is a commonly used kit suitable

for soils rich in clay and organic matter. The kit was based on the method of bead beating for cell lysis and purification by silica-based columns. Rojas-Herrera *et al.* (2008) used silica columns to extract high amount of DNA from the coastal marsh on the north coast in Yucatan Peninsula, Mexico. Highly concentrated and pure DNA was obtained from the triplicate samples analyzed in the present study. The average concentration of DNA was found to be 1109 µg µl^{-1}. As reported by Ogram (2000), high absorbance ratios (260/230 > 1.2) and (260/280 > 1.7) are indicative of pure DNA. The average value for purity of protein contamination (260/280) was 1.75 and that of humic acid contamination (260/230) was 1.24. These values indicate purity of the DNA.

The obtained DNA was also suitable for PCR amplification of 16S rDNA region. A total of 200 recombinant clones were obtained on transformation in *E.coli* DH10BT^{R}. Colony PCR resulted in amplification of 1.5Kb fragment from the respective plasmids. RFLP analysis showed that the 200 clones belonged to 16 different OTU's (Fig.1). The individual number of clones belonging to obtained 16 OTU's is given in Table 1. Exclusion of chimeric sequences from clone library was one of the reasons for decrease in OTU's. Bias in PCR and formation of chimeric sequences were the causes for less diversity (Dahllof *et al.,* 2000). The other reason may be the orientation dependence of RFLP pattern, which influences the OTU discrepancy as reported by Lidija *et al.* (2010).

Sequence analysis of 16 clones belonging to different OTU's revealed the presence of five different phylogenetic groups. The most abundant bacteria from clone library belonged to phylum Gammaproteobacteria (31%) followed by Deltaproteobacteria (19%), Bacteroidetes (17 %), Actinobacteria (7%), Plancomycetes (7%), Betaproteobacteria (6 %) and Firmicutes (5 %). Interestingly, about 8 % of the clones from library belonged to uncultured group of bacteria. Five sequences were less than 98 %, similar to the known sequences submitted in the Genbank database (Table 1). Hence, these could be claimed as novel sequences. The results are in accordance with the work of Ramos *et al.* (2010), whose analysis of microbial diversity of UASB reactor obtained four new sequences with overall pair wise alignment, which is less than or equal to 98 % of the sequences deposited in the Genbank.

Phylogenetic tree was constructed for all the 16 sequences with their closely related species in Genbank database. The results are represented in Fig. 2 along with the bootstrap values (%) for each sequence. The boot-strap values represent phylogenetic accuracy of the tree. Majority of clones in library belonged to phylum Proteobacteria, which is subdivided into gamma, delta, and beta subgroups. Significantly, five clones, ASS8, ASS12, ASS13, ASS18, and ASS20, belonged to Gammaproteobacteria. On sequencing. ASS8 was closely related to uncultured *Vibrio* sp. (99 % identity). ASS12 showed 96 % identity to uncultured *Aquicella* sp. ASS13 was related to *Pseudoxanthomons* sp. (99 % identity), which was reported to be an effective strain in degrading many xenobiotic compounds such as phenanthrene and oil in hydrocarbon-rich soil (Patel *et al.,* 2012).

ASS18 related to *Psychrobacter* sp. (98 % identity) was reported to degrade petroleum hydrocarbons from oil fields (Malik *et al.,* 2012). ASS20 showed 99 % identity to *Lysobacter daejeonensis strain*, reported to be a potent iron oxidizing and arsenic resistant bacterium (Luo *et al.,* 2012), and is a predominant organism present in clay soils. Two clones (ASS3 and ASS7) belonged to subgroup Deltaproteobaceria which were closely related to sulfur reducing bacteria. ASS3 was closely related to uncultured *Desulfovibrio* (99% identity) and ASS7 was related to *Proteobacterium* sp. (99% identity). These groups of organisms effectively metabolize hydrocarbons and also degrade aromatic compounds such as toluene and benzene (Grigoryan *et al.,* 2008). A single clone, ASS1, belonged to the subgroup

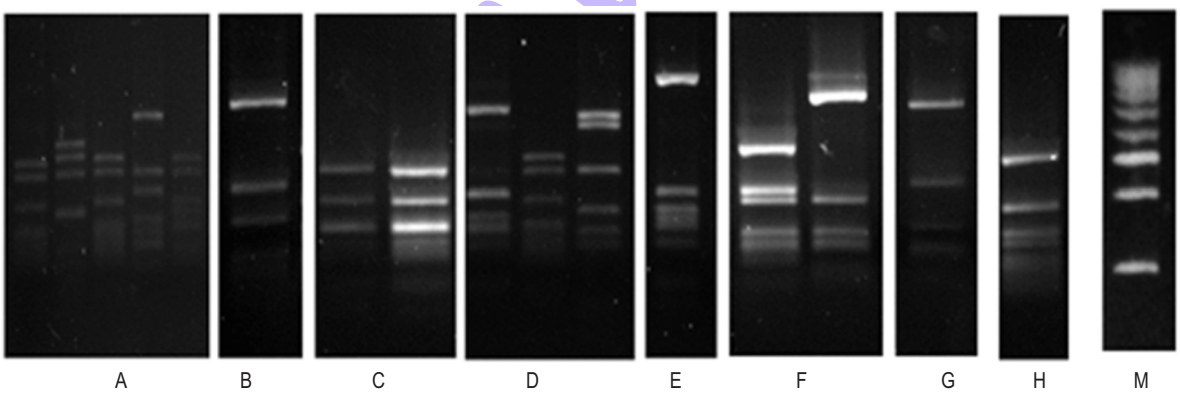

Fig. 1 : RFLP profiles clones belonging to 16 different OTU's after restriction digestion with *Hae*III enzyme in 2% agarose gel. A: gamma Proteobacteria; B: beta Proteobacteria; C: delta Proteobacteria; D: Uncultured bacteria, E: Plancomycetes, F: Bacteroidetes, G: Firmicutes, H: Actinobacteria. M: 1Kb DNA ladder (Merck, India)

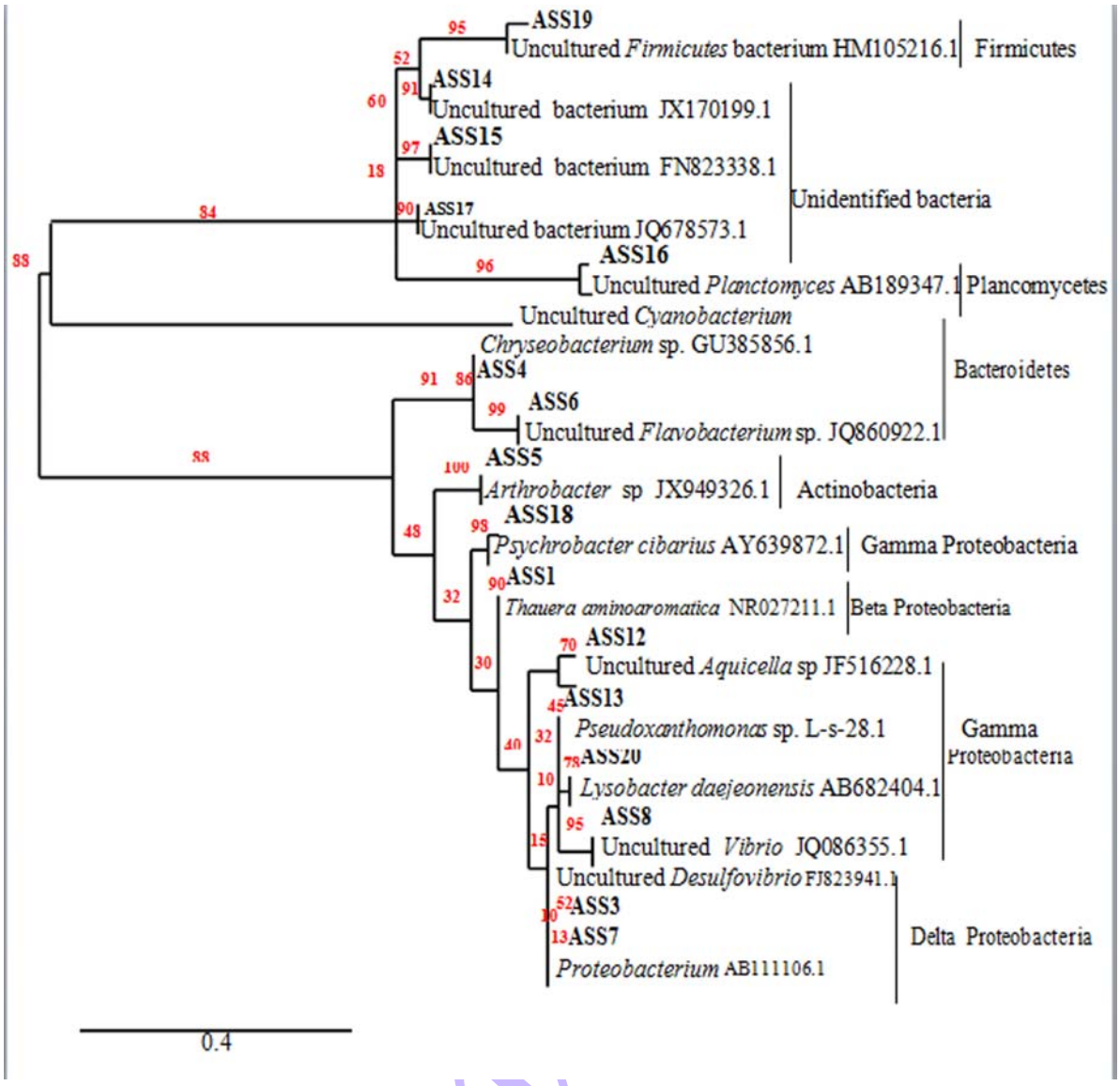

Fig. 2 : RFLP profiles clones belonging to 16 different OTU's after restriction digestion with *Hae*III enzyme in 2% agarose gel. A: gamma Proteobacteria; B: beta Proteobacteria; C: delta Proteobacteria; D: Uncultured bacteria, E: Plancomycetes, F: Bacteroidetes, G: Firmicutes, H: Actinobacteria. M: 1Kb DNA ladder (Merck, India)

Betaproteobacteria which showed 99 % identity to *Thauera aminoaromatica,* a denitrifying bacterium able to degrade aromatic compounds. All the three subgroups were branched closely in the tree, which indicates that they belonged to the same ancestral phylum.

Clones ASS4 and ASS6 belonged to phylum Bacteroidetes (97 % identity to *Chryseobacterium* sp. and 99 % identity to uncultured *Flavobacteria* sp.). They were rooted separately from the phylum Proteobacteria indicating a significant difference between both the phyla. *Chryseobacterium* sp. is reported to be a potent proteolytic and lipolytic strain (Hantsis *et al.,* 2008). *Flavobacteria* sp is widely used in phenol and heavy metal bioremediation (Whiteley *et al.,* 2000). Clone ASS5 belonged to the phylum Actinobacteria (99 % identity to *Arthrobacter* sp.); closely related to the phylum Bacteroidetes and also Proteobacteria. *Arthrobacter* sp. is reported to reduce high levels of chromium and also agricultural pesticides (Camargo *et al.,* 2003). Clone ASS16 belonged to the phylum Plancomycetes (98 % identity to uncultured *Plancomycetes* sp.). They had less

Table 1 : Sequence analysis of the clones from 16 OTU's present in the 16S rDNA clone library of the municipal sewage soil

Phylogenetic classification (No. of sequences)	Clone	Nearest neighbour in Genbank database	% of nucleotide similarity	Accession number	No. of clones with same RFLP pattern
γ Proteobacteria (5)	ASS13	*Pseudoxanthomonas sp.*	99	KC333640	24
	ASS12	Uncultured *Aquicella sp.*	96	KC333635	11
	ASS20	*Lysobacter daejeonensis*	99	KC333646	5
	ASS18	*Psychrobacter cibarius*	99	KC333644	8
	ASS8	Uncultured *Vibrio sp.*	99	KC333632	14
Unidentified (3)	ASS15	Unclture bacterium	100	KC333642	7
	ASS17	Uncultured bacterium	99	KC333643	4
	ASS14	Uncultured bacterium	95	KC333641	5
β Proteobacteria (1)	ASS1	*Thauera aminoaromatica*	99	KC333637	12
δ Proteobacteria (2)	ASS3	Uncultured *Desulphovibrio*	99	KC333628	24
	ASS7	*Proteobacterim sp.*	99	KC333631	14
Planctomycetes (1)	ASS16	Uncultured *Plancomycetes*	98	KC333636	13
Firmicutes (1)	ASS19	Uncultured *Firmicutes*	96	KC333645	10
Bacteroidetes (2)	ASS4	*Chryseobacterim sp.*	97	KC333629	16
	ASS6	Uncultured *Flavobacterium*	99	KC333630	17
Actionobacteria (1)	ASS5	*Arthrobacter sp.*	99	KC333638	16

similarity to both Proteobacteria and Bacteroidetes phyla and were rooted in an entirely different branch. Clone ASS19 belonged to the phylum Firmicutes (96 % identity to uncultured *Firmicutes* sp.). Firmicutes phylum was closely associated with the phylum Plancomycetes. *Firmicutes* sp. was reported to be an efficient degrader of naphthalene, pyrene and other aromatic compounds (Simarro *et al.,* 2013). Clones ASS14, ASS15 and ASS17 belonged to uncultured bacteria. They were closely branched to the phylum Firmicutes in the tree indicating that the strains may have strong evolutionary relationship with the Firmicutes phylum.

The municipal sewage sample used in the present study contained a large amount of organic matter (69 %) and heavy metals like iron (36 %), cadmium (2.5 %), chromium (1 %), lead (3 %) and nickel (4 %). Since it was a collection of wastes from various sectors, it was also likely to be polluted with many other hazardous substances. Thus, diversity of bacteria identified in the present study has potential for degradation of heavy metals, aromatic hydrocarbons, xenobiotic compounds and pesticides. These organisms are mainly responsible for bioremediation of the environment. The study draws the conclusion that PCR-RFLP is a powerful tool for bacterial diversity analysis, among other molecular techniques, mainly on the basis of cost-effectiveness and reliability; and municipal sewage is one of the best environments to be explored for identification of novel bacteria involved in biodegradation.

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