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Microbial population index and community structure in saline–alkaline soil using gene targeted metagenomics

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ABSTRACT

Population indices of bacteria and archaea were investigated from saline–alkaline soil and a possible microbe–environment pattern was established using gene targeted metagenomics. Clone libraries were constructed using 16S rRNA and functional gene(s) involved in carbon fixation (*cbbL*), nitrogen fixation (*nifH*), ammonia oxidation (*amoA*) and sulfur metabolism (*apsA*). Molecular phylogeny revealed the dominance of *Actinobacteria*, *Firmicutes* and *Proteobacteria* along with archaeal members of *Halobacteraceae*. The library consisted of novel bacterial (20%) and archaeal (38%) genera showing ≤95% similarity to previously retrieved sequences. Phylogenetic analysis indicated ability of inhabitant to survive in stress condition. The 16S rRNA gene libraries contained novel gene sequences and were distantly homologous with cultured bacteria. Functional gene libraries were found unique and most of the clones were distantly related to *Proteobacteria*, while clones of *nifH* gene library also showed homology with *Cyanobacteria* and *Firmicutes*. Quantitative real-time PCR exhibited that bacterial abundance was two orders of magnitude higher than archaeal. The gene(s) quantification indicated the size of the functional guilds harboring relevant key genes. The study provides insights on microbial ecology and different metabolic interactions occurring in saline–alkaline soil, possessing phylogenetically diverse groups of bacteria and archaea, which may be explored further for gene cataloging and metabolic profiling.

1. Introduction

Physicochemical and biological processes occurring in soil make it a dynamic and complex ecosystem where microbes link up with each other to live in concert and/or adapt to microhabitats. Microbes are involved in various biogeochemical cycles such as mineralization, nutrient cycling and soil aggregation and thus microbial diversity is an important component for ecosystem functioning. The vast diversity of soil microbial communities has always attracted researcher's interest to study the relationship between microbial communities and their surrounding environment (McGuire and Treseder 2010; Maron et al. 2011). Environmental factors greatly influence bacterial and archaeal abundance, community composition and its dynamics.

Saline–alkaline soil exhibits a specific type of extreme habitat for the development of a halo-alkaliphilic prokaryotic community that grows luxuriantly at high electrical conductivity (EC) and alkaline pH. The arid and semi-arid areas are ingressed by high salinity and alkalinity in local depressions because of their evaporative climate (Foti et al. 2008). The resident microbes therefore, possess unique adaptation mechanism for survival in dual stress at a time, which can be of biotechnological and commercial significance (Margesin and Schinner 2001). The ecological study of microbes from such soil is important in view of *in situ* conservation of biodiversity associated with such niches to sustain delicate ecological processes in the oligotrophic saline–alkaline ecosystem. Microbial survivability and adaptation also increase our understanding of early evolution of the life and biosphere on Earth. The effects of environmental factors on microbial community structure are not well understood so far for saline–alkaline soil microbes. Our study was conducted on the soil of high saline and alkaline nature with EC 23.7 dS/m and pH 9.1. Selected soil is categorized under strong saline and very strong alkaline, according to the soil classification suggested in soil survey manual (Soil survey division staff 1993). Owing inherent complexity of microbial community processes in the environment, a number of processes and key microbes involved in biogeochemical cycles of saline–alkaline soil remain uncharacterized. They are the major contributors in the transformation of carbon, nitrogenous and sulfur compounds with an important role in food webs and nutrient cycling (Oren 2010). Such soil is considered as a good model for studying biogeochemical processes of extreme conditions in which a variety of microorganisms cooperate and interact in complex ways with high extent of phylogenetic conservation of key genes and enzymes.

The key functional genes (*e.g. cbbL*, *nifH*, *amoA*, and *apsA*) involved in different nutrient cycling were considered as conserved

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molecular makers and used to establish the evolutionary relationships (Ueda et al. 1995; Purkhold et al. 2000; Friedrich 2002; Selesi et al. 2005). The phylogenies of *nifH*, *amoA* and *apsA* are (with only a few exceptions) congruent with the phylogenies based on 16S rRNA genes (Ueda et al. 1995; Purkhold et al. 2000; Friedrich 2002), however the *cbbL* phylogeny disagrees with the 16S rRNA gene phylogeny due to lateral gene transfer (Delwiche and Palmer 1996). Carboxylation imparts a base for metabolic pathways (Tourova and Spiridonova 2009) and RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) is well characterized and most abundant protein involved in carbon dioxide assimilation *via* calvin cycle. Form I RuBisCO is the most common in autotrophic bacteria and its catalytic functional unit is encoded by *cbbL* gene (Spiridonova et al. 2006). Nitrogen assimilation is driven by soil diazotrophs and *nifH* gene, encoding dinitrogenase reductase, a key enzyme of nitrogen fixation. Gene *nifH* is extensively distributed among phylogenetically diverse bacteria (Poly et al. 2001) along with *amoA* gene encoding membrane bound protein ammonia mono-oxygenase subunit A (Hooper et al. 1997), involved in rate-limiting step of nitrification. Sulfate-reducing bacteria oxidize a wide variety of low-molecular weight compounds *viz.* short-chain fatty acids, alcohols, alkanes, aromatic compounds, acetate, etc. (Teske et al. 2003). Bacteria and archaea transform sulfate to sulfide under anoxic conditions through unique metabolic pathway of dissimilatory sulfur reduction, catalyzed by adenosine-5′-phosphosulfate reductase, a product of conserved gene *apsA* (Meyer and Kuever 2007).

Microbial distribution pattern and population dynamics under different environmental conditions have been investigated to decipher the role of specific microbial operational taxonomic units (OTUs) or population in a given environment (Pernthaler and Amann 2005). However, a large number of microbial processes and key microbes, involved in biogeochemical cycles of saline–alkaline soil, are not yet well characterized. A number of molecular markers are available to determine the microbially driven ecosystem processes, however individually none of the markers is adequate to reveal complete understanding of ecological functions. It is quite difficult to establish meaningful results for microbial indices or community structure and dynamics using a single gene based analysis due to extensive genetic diversity (Iwai et al. 2011). Microbial population index and molecular phylogeny can be integrated with functional gene diversity using gene targeted metagenomics to understand microbe–environment interaction. Gene targeted metagenomics reveal more comprehensive database, provide extensive microbial diversity and also reveal functional, ecological and evolutionary patterns (Iwai et al. 2011; Suenaga 2012). In this study, bacterial and archaeal 16S rRNA genes were employed for the profiling of community structure, population index and molecular phylogeny along with functional genes implicated with carbon (*cbbL*), nitrogen (*nifH*, *amoA*) and sulfur (*apsA*) metabolism from a high saline and alkaline soil. Gene sequence analyses were coupled with Quantitative real-time PCR (qPCR) which is specific, highly sensitive, and rapid method to determine copy number of targeted genes (D'haene et al. 2010). The abundance of specific phylogenetic groups of microorganisms in soil can be assessed rapidly and quantitatively (Fierer et al. 2005). We investigated the diversity and abundance of resident bacteria, archaea and key genes of the potential metabolic processes of saline–alkaline soil.

2. Materials and methods

2.1. Sampling site and soil characteristics

The soil samples were collected from Una (20°50.123′N, 71°03.571′E) located at the coastal region of Gujarat (India). The samples were collected from barren land to avoid the interference of vegetation impact on soil microbial composition. An area of about 1 m^2 was delimited and soil samples were taken from a depth of 0 to 10 cm with a sterile scoop from three random spots. Soil samples were grounded and sieved through a pore size of 2 mm. Equal amounts of the three subsamples was pooled to obtain a composite soil sample replicate. All samples were brought to laboratory in polyethylene bags under refrigerated condition. Metagenomic DNA extraction was performed immediately and remaining samples were stored at 4 °C for physicochemical analyses. Soil sample was analyzed for pH, salinity, carbon, nitrogen, hydrogen, sulfur and total organic carbon contents. Soil pH and electrical conductivity were measured in air dried soil using specific electrodes in a 1:5 soil:water slurry. Total carbon, nitrogen and sulfur contents were determined using a CNS analyzer (Perkin Elmer series II, 2400, USA), while total organic carbon was determined using a carbon analyzer (Elementar, Liqui TOC, Germany).

2.2. DNA extraction, primer selection and PCR amplification

Microbial cells were lysed and metagenomic DNA was extracted from approximately 0.5–1 g of the fine triplicate soil samples using the Fast-prep instrument and Fast DNA spin kit (MP Biomedicals, USA) according to manufacturer's instructions. Bead beating was performed at two different speeds in the Fast-Prep instrument (4.5 and 5.5 m/s) for efficient extraction of both completely as well as partially lysed cells. Metagenome extractions were done at both conditions and pooled for further analysis. In the extraction process, metagenome was purified by a silica-based gene-clean procedure using spin filters which removes humic acids/polyphenols and other potential PCR inhibitors. Metagenomic DNA was quantified using Nanodrop (Nanodrop, Peqlab, Germany) and qualitatively analyzed on 0.8% (w/v) agarose gel. DNA from the three replicate subsamples was combined and stored at −20 °C to be used for PCR amplification of gene(s). Inhabiting microbial communities were identified by 16S rRNA gene sequences amplified by universal primers (Table S1 Supplementary material). Microbial population involved in bio-geochemical cycles specifically of carbon, nitrogen and sulfur were analyzed by gene targeted amplification of key functional biomarkers. Genes *cbbL*, *nifH*, *amoA* and *apsA* were amplified using gene specific primers and PCR conditions (Table S1 Supplementary material). Each PCR mix comprised of 1× PCR buffer, 0.2 μM each primers, 250 μM of dNTPs, 2 U *Taq* DNA polymerase (Sigma, USA) and template DNA (50–100 ng). A low cycle number was used in 16S rRNA gene amplification to avoid PCR biasness. The PCR products were evaluated on a 1.5% (w/v) agarose gel.

2.3. Clone library construction and sequencing

Amplicons were purified using QIAquick spin columns (Qiagen, Germany) and cloned in pCR 2.1 vector using TOPO TA cloning kit (Invitrogen, USA). Clone libraries of each gene were constructed by transforming in *Escherichia coli* cells as per manufacturer's protocol. Plasmids were screened for the correct-size insert by PCR amplification using vector specific M13 forward and reverse primers and all positive clones were sequenced (M/s Macrogen, South Korea).

2.4. Phylogenetic analysis

Clone sequences were chimera checked using Bellerophon (Huber et al. 2004). The chimeric sequences were eliminated; nonchimeric sequences were further analyzed and aligned to rule out overlap sequences using BIOEDIT (Hall 1999). All processed sequences were analyzed for similarity search using on line BLASTn algorithm of NCBI. Closely related sequences were