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SIGNIFICANCE OF MICROBIOLOGICAL DIVERSITY OF THE UPPER CRETACEOUS FOSSIL SOIL

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ABSTRACT

Metagenomics is a habitat-based investigation of mixed microbial populations at the molecular level. It can be used to gather information stored in ancient bacterial genomes. Though sequence analysis of ancient DNA is challenging because of the technological obstacles of degradation and contamination with modern DNA, the only way to study them is to extract collective DNA and analyze it. The present investigation was a product of curiosity to know the type and significance of microbial population that existed in an area that is famous for limestone fossil resources. Two bacterial isolates were recovered by conventional plating; they were characterized and their growth kinetics studied. In parallel study, soil DNA was screened and subjected to PCR. The PCR for 16S rRNA gene amplification was optimized. The future perspective of the investigation is to clone and sequence the amplified PCR products of soil DNA and bacterial DNA so that the evolutionary value could be inferred. Since the area of investigation has evolutionary significance of the surrounding places, the metagenomics approach of the microbial population can reveal some novel organisms and genes.

INTRODUCTION

Microorganisms are ubiquitous and are the 'masters of the biosphere' touching every process in the biosphere by their seemingly endless capacity to transform the world around them. They underpin geochemical cycles and human health conditions that were previously thought to be driven by inorganic processes and stress. Yet, very little is known about their structure, composition and function. A vast majority of the microbial cells (around 99 %) can be seen in a microscope and shown to be alive yet, difficult to culture[1].

Some bacteria may be recalcitrant to culturing for diverse reasons: lack of necessary symbiont, nutrients or surfaces, excess inhibitory compounds, incorrect combinations of temperature, pressure or atmospheric gas composition, accumulation of toxic waste products from their own metabolism, and intrinsically slow growth rate or rapid dispersion from colonies[2].

For identifying and enumerating microbes in the environment that resist being cultured, metagenomics has emerged as a powerful tool. Direct isolation of genomic DNA from an environment circumvents culturing the organisms under study. Also, cloning the DNA into a cultured organism renders it for investigation, manipulation and preservation[3]. Metagenomic DNA is variously known according to its application as environmental DNA libraries[4], Zoo libraries, soil DNA libraries[5], eDNA libraries[6], whole genome treasures[7], community genome, and whole genome shotgun sequencing[8]. Metagenomics is being performed for sequence-based as well as function-driven analysis of uncultured microorganisms. Functional Metagenomics involves screening metagenomic libraries for specific performances such as salt tolerance[9], antibiotic production, or enzyme activity of hydrolysisand bioremediation. This approach quickly identifies clones that have potential applications in medicine, agriculture or industry by focusing on natural products or proteins that have useful activities. Sequence based approach involves screening clones for the highly conserved 16S rRNA genes for identification purposes and then sequencing the entire clone to identify other genes of interest, or large-scale sequencing of the complete metagenome to search for phylogenetic anchors in the reconstructed genomes[10, 11]. Soil is considered a storehouse of microbial activity[12]. Understanding the biodiversity of the soil is difficult because of the heterogeneity of genomic DNA that exists[13, 20]. Texture, temperature, salinity, pH, contaminants and other characteristics allow a unique microbial community to establish a niche[14]. Extreme environments with

variable salinity, temperatures, underground environments (ice and rocks), and deep sea vent (extremely high temperature and pressure) contain specialized microbial halophile communities providing important clues on the origin, evolution of life and geological time scales. They also provide beneficial genomic and protein resources[9].

Ariyalur, of Tamil Nadu state, is famous for limestones and fossils. According to archaeological studies, accidental transformation about 100 million years ago of the sea into land caused burial of biota and subsequent formation of fossil prints of those creatures in the soil layers. The layers hold the same aquatic and terrestrial traces; hence researchers have opined that microorganisms of the ancient times might still be found along with the[15, 16]. Thus the search by traditional and metagenomics approach would offer evidence of the rare microbes and their evolutionary signals.

In the current study, soil samples collected 150 ft below ground off the Ariyalur site were investigated for culturable and unculturable microbial diversity and their possible beneficial genomic and protein fractions.

MATERIALS AND METHODS

A portion of the soil was overlaid with sterile distilled water in a sterile falcon tube and was left undisturbed for about a week. This step allowed the bacterial population to get concentrated in the overlaid liquid.

1) Isolation of single colony

To separate the constituent populations, 100µl of the overlaid liquid was serially diluted (10^{-1} to 10^{-10}). An aliquot of 50µl was spread on Luria-Bertani (LB) agar (pH 7.5). Colonies obtained were quadrant-streaked on LB agar and single colonies were inoculated in LB broth (pH 7.2).

2) Bacterial growth kinetics

One ml of the overlaying aqueous sample was inoculated into 50ml LB broth and incubated at 37ºC in a shaker. The cultures were allowed to grow overnight. This served as the inoculum. A 100µl inoculum was added to 30ml LB broth and kept in a shaker at 37ºC. Sampling was done every 2 hours for 12 hours. Wet cell mass concentration and turbidity were measured.

3) Gram staining

Taking an appropriate smear on a sterile slide, the Gram staining was performed.

4) Biochemical tests

Biochemical tests were carried out to identify metabolic properties of the bacterial species. The following tests were carried:

(i) Indole production test:

Tryptone broth containing 1g peptone in 100ml distilled water was prepared and autoclaved. 5ml of this broth was poured into a test tube. The culture was inoculated and incubated at 37ºC for 48hrs with one tube kept as control. After incubation 1ml of Kovac's reagent was added to the tubes, and the color change was recorded.

(ii) Methyl red test

MR-VP broth was prepared and autoclaved. 5ml was poured into test tubes. The culture was inoculated and incubated at 37ºC for 48hrs. Five drops of methyl red indicator was mixed and observed for the color change after 15 minutes.

(iii) Voges-Proskauer test

Sterile MR-VP broth (5 ml) was poured into a test tube. Culture was inoculated and the tubes were incubated at 37ºC for 48hrs with one tube kept as control. 40% KOH and 5% α-napthol in absolute alcohol was added into the tubes. The tubes were shaken for 30 seconds and observed for color change.

(iv) Citrate utilization test

Simmon citrate agar slant were prepared and autoclaved. The culture was inoculated and was incubated at 37ºC for 48hrs.

(v) Catalase test

A small aliquot of the test organism was smeared on a clean slide. A drop of 3% hydrogen peroxide was added over the smear. The colour change was recorded.

5) Genome extraction and 16S rRNA Amplification

Genome of the isolated cultures and mixed soil populations were extracted using Mo Bio power soil DNA kit. 16S rRNA gene amplification for all the samples was standardized (Pidiyar *et al*., 2004) with repeated PCR using 25pM each of universal primers 16F27 (5′-CCA GAG TTT GAT CMT GGC TCA G-3′) and 16R1525 (5′-TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC-3′). Concentrations of other components are dNTPS (10mM), Buffer (1X), Taq polymerase (1U), sterile milliQ water (15.8 µl/ 25 µl) and finally 1 µl template DNA.

RESULTS AND DISCUSSION

Growth kinetics of culturable population

Two cultivable distinct bacterial populations (species) were obtained through pure culture. They were marked as AFB1, AFB2.

The growth kinetics of all the two isolates followed a similar pattern with maximum growth at 6 hours.

Characterization

Gram staining revealed the nature of cell wall composition. All the cultures AFB1, and AFB 2 took up Saffranin and appeared pink suggesting that they all are gram negative. Among them, Sample AFB 1 was cocci, and AFB 2 was bacilli (Fig. 1 & 2). None of the samples gave a positive result for indole production test which indicated that the test organisms do not produce indole by tryptophan breakdown.

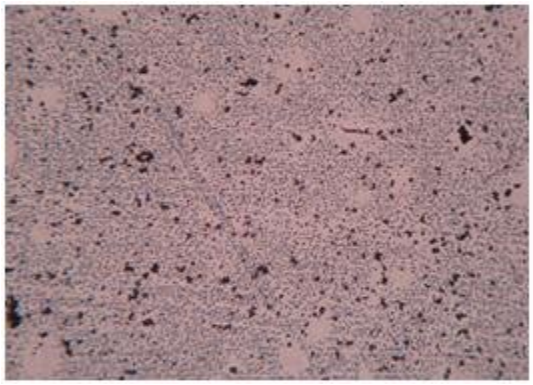

Figure 1 Microscopic view of gram stained slide containing sample AFB 1 which represents the presence of gram negative cocci

Figure 2 Microscopic view of gram stained slide representing gram negative bacilli. AFB2, AFB3 and AFB 4 were gram negative cocci.

Both the strains gave a negative MR test since they convert glucose to non acidic products such as ethanol or butanediol which elevated the pH approximately up to 6. The samples gave a negative result for the VP test too, in which Barrits reagent indicates the presence of acetoin. Sample AFB 2 utilized citrate and hence the medium turned blue. But samples A and C failed to utilize citrate. The two cultures utilized diatomic oxygen, emphasizing the aerobic respiration nature of these cultures.

16S rRNA Amplification

DNA isolation using *Mo Bio powersoil DNA kit* yielded pure DNA within 2 to 3 hours, but the amount was comparatively low. The DNA was used for PCR amplification. After repeated PCR with varying primer concentrations and temperatures, the annealing temperature for the 16S rRNA primers (16F27 and 16R1525) was found to be 55ºC. Thus conditions to amplify 16S bacterial gene from pure culture as well as direct soil sample were standardized.

Cloning and sequencing of amplified

16srDNA PCR products were cloned into vector PDK 101 vector (modified pGEM-2T) within XcmI sites. Using an Applied Biosystems 3100 DNA automatic sequencer, 16srDNA sequencing was carried out and deposited to the Genbank. The accession number for AFB 1 is GU971726- *Bacillus halodurans* and AFB 2 is GU971727-*Stenotrophomonas maltophilia.*

CONCLUSION

The soil sample was found endowed with bacterial diversity of which two strains were cultured, biochemically characterized and subjected to growth kinetics. The metagenomic DNA corresponding to the 16S rRNA was subjected to PCR amplification successfully. The future perspectives of the investigation include cloning and sequencing the amplified PCR products of metagenomic DNA and bacterial DNA so that molecular characterization of isolated colonies and evolutionary studies could be accomplished. Since the area of investigation has evolutionary significance and a vast portion of the area is yet to be investigated, intensive sequencing of the microbial genome may throw novel organisms, genes and properties.

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