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METAGENOMICS: AN APPROACH FOR NON CULTIVABLE MICROORGANISMS – A REVIEW

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For approximately 4.5 billion years, the Earth has been evolving from a barren volcanic landscape into the vibrant globe full of life that it is today. The transformation of the microbiological experience during last 25 years has alter the microbiologists view of microorganisms. The total no. of prokaryotic cells on earth has been estimated at 4-6 x 10^{30} (Whitman et al., 1998) and having 10^{6} - 10^{8} separate gene species (Amman et al., 1995). But more than 99% of bacteria in the environment can not be grown readily in pure culture which forced the microbiologists to question their belief that the microbial world had been conquered. (Cowan et al., 2000). In this ensuring years microbiologists dedicated intense effort to describing the phylogenetic diversity of environments - Ocean surface, soil etc. The next challenge was to elucidate the functions of these new phylotypes and determine whether they represent the new species, genera or phyla of prokaryotc life. This challenge produces various techniques including metagenomics, the genomic analysis of environmental sample/organisms. Metagenomics has provided to access for more microbial diversity than has been viewed in the petridish.

HISTORY OF CULTURE DIVIDE

The history of microbiology can be divided into

(a) Early microbiology and microscope

The roots of microbiology are firmly associated with the microscope. Antonic Van leeuwenhoek in 1663 observed bacteria through his home made microscope. He was an outstanding maker of microscopes and his keen observation and illustrations of microbial life influenced many other observers to take an interest in the microscopic world. Next to this Botanist Ferdinand Cohn classified many bacteria and described the life cycle of Bacillus subtilis based on his microscopic observations (Geison et al., 1981). In 1880s Franz Unger divided the microbiological world into the cultured and uncultered.

(b) Modern Microbiology

One of the indicators that cultured microorganism did not represent much of the microbial world was observed great plate count anomaly (Staley et al., 1985) which describes the discrepancy between the sizes of populations estimated by dilution plating and by microscopy. The food industry generated intense interest in “injured bacteria” in food which live organism that can not be cultured in stressful condition such as heat, chilling, or desiccation. The concept of organisms that were viable but not culturable emerged from the work of Colwell and collegues who showed that strains of Vibrio cholerae were alive and virulent when isolated from aquatic environments but did not grow in culture until after passage through a mouse or human intestine (Colwell et al., 2000).

The Paradigm shift

In 1985, an experimental advance changed the way of microbial work. On the basis of work, Carl Woese showed that rRNA genes provide evolutionary chronometers (Woese et al., 1987). Pace and collegeus used directly analysis of 5S and 16S rRNA gene sequencing in the environment to describe the diversity of microorganisms in an environmental samples without culturing (Pace et al., 1986). The next technical breakthrough arrived with the development of PCR technology and the designing of primers that can be used to amplify almost

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the entire gene. The application of PCR technology provided a view of microbial diversity that was not distorted by culturing bias and revealed that the uncultured majority is highly diverse.

Metagenomics

Metagenomics is a new field combining molecular biology and genetics attempting to identify and characterize the genetic material from environmental samples and apply that knowledge. The term metagenomic, coined by Jo Handlesman in 1998 is derived from the statistical concept of meta analysis (the process of statistically combining separate analysis) and genomics (the comprehensive analysis of an organism's genetic material) (Rondon et al., 2000). The most micro organisms in the environment can't be cultured by standard method that stimulated the development of metagenomic which is the key in genomic analysis of uncultured microorganisms. There are so many approaches have been taken to analyze the uncultured microbial world.

Approaches to metagenomic analysis

Metagenomics is also described as “the comprehensive study of nucleotide sequence, structure, regulation and function” of environmental samples. Metagenomics is employed as a means of systematically investigating classifying and manipulating the entire/genome, isolated from environmental samples.

This environmental samples are purely culture independent and recovery the genomes of the environmental samples is multistep processes.

(i) Isolation of genetic material/DNA

First, a sample is collected which represents itself as a representative of the environment which is under investigation because biological diversity is different in different environments. The sample contains many different types of organisms, the cells of which can be broken open either by using.

i. Chemical method; alkaline condition.
ii. Physical method; sonication.

Once the DNA is free from the cells, by taking advantages of the physical and chemical properties of DNA, they must be separated from the rest of the samples. Some modern advance can be applied to isolate DNA

a. Density centrifugation
b. Affinity Binding
c. Precipitation

ii. Manipulation of Genetic Material

Once the DNA is collected, it is manipulated, so that it can be used in the model organism. Genomic DNA is large in size so by enzyme -restriction endonuclease the DNA is cut up into smaller fragments which are linear in shape. The small fragments are then combined with vectors that can be inserted into cells for replication. Vectors also contain a selectable marker which provides a growth advantage. This advantage is absent from model organisms.

iii. Library construction

The 3rd step is inserting the vector which is having the meta DNA into the model organism. The model organism allows the metagenomic DNA to be grown is not grown under laboratory condition. The metagenomic DNA in vectors are all same sample initially, but the vectors are designed, so that only one kind of DNA fragments from the sample will be maintained in each individual cell.

The transformed cells are then grown on selective media so that only cells carrying vectors will survive. Each group of cell that grows, k/a colony. Each colony contains many cloned cells that originated from one single cell. These samples of cells containing all of the metagenomic DNA are called metagenomic libraries. The classical approach includes the construction of small insert libraries (<10 kb) in a standard sequencing vector and in E. coli as a host strain. However, small libraries do not allow the detection of large gene cluster/operons. To overcome this limitation, researchers have developed large insert libraries, such as cosmid DNA libraries (mostly in the pWE15 vector of stratagene) with insert size ranging 25-35 kb (Entcheva et al., 2001) or bacterial artificial chromosome libraries with insert size upto almost 200 kb (Rondon et al., 2000).

(iv) Analysis of DNA from metagenomic libraries

The fourth and final step in the procedure is the analysis of the DNA from the

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metagenomic libraries. The expression of DNA determines the physical and chemical properties of organism, so there are many potential methods of analysis.

A. Function based analysis/ screening

The function driven analysis is initiated by identification clones that express the desired traits. The clones are selected from libraries and analyzed the active clones. Many enzymes of industrial importance, novel antibiotics, antibiotic resistant genes and Na^{+} (Li^{+})/H^{+} transporters have been identified by metagenomic functional analysis. The best of this approach is that it does not require any sequence analysis. This approach can detect genes with completely novel DNA sequencing may have functions distinct from known biocatalysts.

However, function based screening has several limitations

Lack of efficient transcription of the metagenome derived genes in the host strains.

Weak translation with a poor secretion of the foreign protein by the employed host strain.

Due to absent of required chaperons in the host strain, the desired protein is not folded correctly.

Co-factors might not be synthesized by the foreign host strain

Many genes will not be expressed in any particular host bacterium, selected for cloning

B. Sequenced based screening

It is not dependent on the expression of cloned genes in heterologous host. It relies on the use of conserved DNA sequence to design hybridization probes or PCR primers to screen metagenomic libraries for clones that contain sequence of interest.

However the limitations of sequence based screening are :

DNA consensus must be analyzed and determined which cannot be applied to many biocatalyst.

It does not guarantee in gaining of full length genes or full gene clusters that are necessary for the production of the desired product.

It never screens desired genes with easy expression and completely different sequences or correct folding of the screened gene.

Although both of function and sequence based screening strategies have been applied to isolate novel biocatalyst from metagenomes, but both approaches are laborious due to the low frequency of active clones with desired traits.

For example, lipolytic clones derived from German soil, 1 in 7,30,000 clones showed activity (Henne et al., 2000).

Enrichment strategies to improvethe ODDs

One of the sustained frustration with analysis of metagenomic libraries is low frequency of clones of a desired traits. To increase the proportion of active clones in a library, several strategies have been designed to enrich for the sequences of interest before cloning.

Enrichment strategies

Enriching for genomes from metabiologically active cells from metabolically active cells genome enrichment can be achieved by 3 ways

i. Enrichment for GC content

A simple enrichment is for GC content of the genomes. As many organisms that have a high GC content in their DNA are of particular interest such as Acido bacteria, Actinomycites. DNA can be extracted from the soil organism and then subjected to Ultra centrifugation to enrich for high GC content DNA. Although it is a fairly crude approach and will not provide a complete separation, it will certainly increase the representation of certain genomes in a library.

ii. BrdU enrichment

A more elegant separation method is Bromo deoxyuridine (BrdU) enrichment. The principle of this enrichment is that metabolically active organism will incorporate a labeled nucleotide into their DNA, which can be isolated on the basis of the incorporated label. The BrdU can be fed to a bacteria in soil and the labeled DNA isolated by Immunocapture (Urbach et al., 1999). The addition of selective substrates

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with the BrdU further discriminate among the members of the microbial community enriching metagenomic libraries for those that grow on the added nutrient (Yin et al., 2000). This strategy could be used to enrich for organisms that grow on substrates such as starch, cellulose and protein to find amylase, cellulase and proteases respectively or other enzymes of interest in metagenomic libraries.

iii. Stable isotope probing (SIP)
Another enrichment method is stable isotope probing (SIP) (Radajewski et al., 2000). It involves providing a 13C labeled substrate to soil bacteria. The bacteria incorporate the substrate into their DNA, and making it more dense than normal DNA containing 12C labeled substrate. This SIP has been successfully used for labeling and separating DNA and RNA (Manefield et al., 2000). Density gradient centrifugation cleanly separates the labeled nucleic acid from unlabelled.

Limitations
1.Cross feeding
Any bacterium that is metabolically active will take up BrdU and will therefore be represented in a library that in supposed to be enriched for organism that utilize and added substrate.
ii. Prolonging incubation period
As limitation of the enrichment strategies timing is the additional issue. Prolonged the substrate feeding, the higher the probability the substrate will be recycled in the community and the basis of enrichment will be broken down.
iii. DNA shearing
In enrichment strategies, the advisable methods/technique like immunocapture and density gradient centrifugation may shear the DNA and making it difficult to retrieve pathways encoded on large fragments of DNA.
RNA based SIP reduces the cross feeding problems associated with DNA based SIP, but it has been used only to construct16S rRNA gene libraries (Manefield et al., 2002).
SIGEX 3rd screening method

SIGEX is the abbreviation of “Substrate Induced Gene Expression” coined by Kazuya Watanaba, 2005. In an effort to improve the frequency of screening, SIGEX was developed. Its utility was evaluated by screening the aromatic hydrocarbon induced gene from ground water.

Substrate induced gene expression screening
The design of SIGEX is based on the facts that the expression of catabolic gene induced by substrate/metabolites of catabolic enzymes. SIGEX screens the clones which are harboring desired catabolic gene that are expressed in the presence of substrate.

Protocol of SIGEX
Step - I
To make SIGEX, a high through put process, an operon- trap vector (p/8GFP) was constructed in which the cloning site divides the lac promoter and the gfp structural gene. Metagenomic libraries are constructed using the p18 GFP vector.
Step - II
Self ligated clones and the clones expressing gfp constitutive are removed by IPTG induction in the absence of substrate.
Step- III
The expression of the catabolic genes in cloned meta genomic DNA is determined by gfp expression in the presence of substrate.
Step - IV
Then the positive clones are separated on agar plates and characterized.
Fluorescence activated cell sorting (FACS) is applied to the sorting and separation of GFP expressing clone which are having the desired catabolic genes.

Advantages of SIGEX
1. Efficient and economic : SIGEX has many advantages in metagenomic screening. It provides an efficient and economic way of through put screening because it allows for semiautomation thereby saving time, labour and expense.
2. Catabolic gene detection : It is efficiently used to detect the catabolic genes for which colorimetric or other on plate

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screening are not established. Using this strategies watanabe group screened hydrocarbon induced genes which are difficult to screen under conventional methods.

3. No modified substrate : SIGEX does not require the modified substrate that are often needed in colorimetric screening. This modified substrate are occasionally toxic and cause side effects. Moreover they are generally more expensive than unmodified substrate.
4. Enable deduction of substrate for unknown enzymes: SIGEX enables in deducting the substrate from the induction substrate which is used for screening an unknown enzymes.

Disadvantages of SIGEX

Sensitive to structure and orientation of genes with desired trait.

It can not detect the active clones in which the desired catabolic genes are cloned in the direction opposite gfp.

It is not applicable to metagenomic libraries harboring large insert DNA due to the abundance of transcription terminators.

SIGEX can not utilize the substrate that can not migrate to the cytoplasm.

Ecological inferenec from metagenomics

The urgent questions in microbial ecology highlights on how microorganism forms symbiosis with eukaryotes, compete and communicate with other organisms, and acquire nutrient and produce energy. Thus far, metagenomics has provided insight into each of these areas but in each instance, the challenge is to link the genomic information with the organisms/ecosystem from which the NDA was isolated.

A. Symbiosis

Many symbiont bacteria which are having specialized relationship with their hosts but do not grow readily in culture. Many of them can survive in specialized structure, often in pure or highly enriched culture, in host tissue making them ideal candidates for metagenomic analysis. Because the bacteria can be separated readily from host tissue and other microorganisms.

Buchnera-Aphido symbiosis

Buchnera aphidicola an obligate symbiont of aphids, an insect (Abbot et al., 2001); The relation between organism and insects is ancient, leaving each partner unable to function independently of the other. By isolating the bacterial DNA it has been suggested that genus Buchnera contains a reduced genome. Upon comparison with the reconstructed ancestral genome, 1,906 genes appear to be lost and most of the functions are associated with biosynthetic pathway contributed by the host. This genome shrinkage is the result of the symbiotic life style (Daubin et al., 2003).

B. Competition and communication

Competition for resources among community members selects the diverse survival mechanism including antagonism, and metabolism among the members. Understanding this mechanism advances the definition of principles that govern microbial community structure function and robustness. Genes for competition and co-operation are hard to recognize based on sequence alone because the utility of their functions is entirely dependent on ecosystem context and the nature of resources that are limiting.

C. Antibiotic and signal molecule

A surprising result from the Davies group indicated that sub-inhibitory concentrations of many antibiotics induce quorum sensing though no resemblance in structure to the acylated homocerine lactones that appear to be the natural inducer (Davies et al., 2002). This result presents a propitious opportunity that a single screen might capture molecules that are quorum sensing inducer as well as antibiotics. The sensor is comprised of the Lux R promofer which is induced by acylated homoserine lactone and linked with gfp. The lux R promoler resides on a plasmid in an E. coli strain that did not induce quorum sensing. It an inducer of the lux R-mediated transcription of gfp is expressed from the metagenomic DNA, the cell fluoresces and can be captured by fluorescence activated cell sorting or as a colony observed by fluorescence microscopy.

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D. Biogeochemical cycles

Acid mine drainage

Analysis of specific functions across all members of the community can generate integrated models about how organisms share the workload in maintaining the nutrient and energy of the community. Microbial community forms a pink biofilm that floats on the surface of the mine water. Below the biofilm the drainage water has a pH of between 0 to 1 and high levels of Fe, Zn, Cu and As (Berry et al., 2003). There is no source of carbon and nitrogen other than the gaseous forms in the air. The community is dominated by leptospirillum, sulfobacillus, acidomicrobium. the mine is rich in sulfied minerals such as pyrite which is dissolved as the result of oxidation, catalyzed by microbial activity. The metagenomic sequence substantiated a member significant hypothesis.

It appears that leptospirillum group III contains gene with similarity to those known to be involved in nitrogen fixation which suggests it provided fixed nitrogen to the community. Ferroplasma type I and II genomes contain many transporters that indicate that they likely import amino acid and other nitrogenous compound from the environment.

All of the genomes in the acid mine drainage are rich in genes associated with removing potentially toxic elements from the cells, proton efflux systems are responsible for maintaining the neutral intracellular pH and metal resistance determinants pump metals out of the cell maintaining non toxic level in the interior of the cells (Tyson et al., 2004).

E. Microheterogenicity

Metagenomic analysis has revealed that even apparently uniform population contain substantial microheterogenecity with in the population like Cenarchaeum symbiosum (Schleper et al., 1998). It is also found that genome of the species in acid mine drainage varied in their uniformity (Tyson et al., 2004). They also found a high frequency of single nucleotide polymorphism among strains of the same species. Ferroplasma type II group appears to contain a composite genome with segments derived from three source. This studies point out the importance of conducting genomic analysis of mixture strains to obtain the portrait of heterogenicity with in the species.

CONCLUSION AND FUTURE DIRECTIONS

Metagenomic has changed the way by which scientist can approach to solve the many problems, redefined the concept of a genome and accelerated the rate of gene discovery. It has proved itself effective for isolating novel biocatalysts from environment. The potential application of metagenomics to biotechnology seems endless. Functional screening have identified new enzymes, antibiotics and other reagents in libraries from diverse environment. Though no standard protocol exists for isolating sufficiently purified metagenomic DNA from environmental samples, yet robotic automation has been developed to construct and screen metagenomic libraries. SIGEX is also effectively good way to overcome this botteneck.

The potential metagenomic discovery is dependent on the advancement of the methods that are library construction and analysis. Therefore future advance will facilitate the managements and analysis of large libraries.

Functional analysis will require more innovation in method development to improve heterologous gene expression and approach for efficient screening.

Myriad environment on earth have not been studied with cultured independent methods other than PCR based 16 rRNA gene analysis which invite further analysis.

An efficient expression other than E. coli should be developed and settled.

More funding should be emphasized in this field, metagenomics.

Scientists should adopt with new innovative thinking to expose the culture the culture independent organisms and their advantages to identify new problems and solved old one. Therefore to exploit the enormous genetics resources in the environment in more efficient ways, these problems should be solved and improved technologies should be developed.

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