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# Cloning the Metagenome to Access the Biodiversity of Unculturable Bacteria

**Joshi A.**
National Institute of Animal Biotechnology,
Hyderabad, Andhra Pradesh,
India.

**Abstract**:
Metagenomics constitutes the sequence based analysis of the collective microbial genomes (microbiome) in a particular environment. It provides culture independent access to the gene pool of whole microbial community. The variety of laboratory culturing methods when fail to culture the unculturable bacteria, tools of metagenomics are applied to reveal their existence in a given environment. Metagenomics tools deal mainly with the direct isolation of genetic material from a defined ecological habitat (soil or marine) and cloning of the complete genome of the entire microbial population. The present review discuss the culture independent approach to access the diversity of unculturable bacteria using metagenomics tools.

**Keywords: Genome, Bacteria, Unculturable, Metagenomics**

## Introduction

Being the essential part of earth's microbiota and their involvement in chemical, physical and biological transformations, bacteria are considered as a very important group of microorganisms. Those bacteria which cannot be grown easily on artificial nutrient media are referred as unculturables. The evidence for the presence of yet to be cultivated bacteria came from the molecular data. The capability to obtain DNA sequence information from an environmental sample by PCR manipulations and direct sequencing allowed identification of these phylogenetically important groups. When a sample is collected from environment, the total number of bacterial cells within sample is extremely high, which is not appropriate for isolation of uncharacterized bacteria. Few methods can be applied to reduce the number of microorganisms in mixed samples before cultivation. The majority of culture media are nutrient-rich. It is now thought that these conditions may favors the growth of faster growing bacteria at the expense of slow growing species, some of which grow in nutrient poor environments and may be inhibited by substrate-rich conventional media (Deming and Baross, 2000).

There are certain reasons behind the unculturability of microorganisms. It could be that the organism has a low prevalence or is particularly slow growing has been over looked in cultural analyses. Many genetically distinct phenotypes are phenotypically indistinguishable for example few bacteria are resistant to culture on conventional media. Certain bacteria have fastidious growth requirements including the need for specific physical conditions like pH conditions, incubation temperatures or oxygen levels in the atmosphere. There may be competition for nutrients among organisms cultured together (Kopke *et al*, 2005). Growth may also be inhibited by bacteriocins released from other bacteria in a mixed culture or by antibacterial substances present within the culture (Tamaki *et al*, 2005). The bacterial community structure, properties and survival are dependent sometimes on quorum sensing mechanisms involving several signaling molecules (De Kievit *et al*, 2001). Signaling molecules present only

within the natural habitat are thought to be essential for the growth of many bacteria. In the absence of these beneficial interactions and signals, some bacteria may struggle to grow in artificial culture and may be faced with an unfamiliar environment devoid of essential factors (Nichols *et al*, 2008). Significant efforts have been made in recent years to devise culturing methods for unculturable bacteria. If suitable culture conditions are provided it is possible to cultivate them in the laboratory. These culture techniques include the use of extinction culturing technique with low nutrient media (Button *et al*, 1998), use of a diffusion chamber that allowed the passage of substances from the natural environment, use of community interactions and cell–cell communication (Dinsdale *et al*, 2008), use of dilute nutrient media (Hoff *et al*, 2008) and the use of combinations of techniques (Nichols *et al*, 2008). The difficulties of cultivating the unculturable bacteria collected from the specific environmental niche are circumvented today by cloning the metagenome in suitable vectors. The term metagenomics 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). The study of metagenomics involves the manipulation of genetic material, recovered directly from environmental samples. The whole procedure contains a few important steps as described below (Figure 1).

## 1. Categories of unculturable bacteria

The obligatory symbiotic and parasitic bacteria are categorized first. These bacteria include *Prochloron*, *Cristispira*, *Holospora*, *Caedibacter*, *Lyticum*, *Blattabacterium* and *rickettsiae*. These bacteria expand under the host provided conditions but fail to grow on artificial media. The second category include, viable but non culturable. These organisms are viable in natural conditions but fail to undergo cell division on routinely used growth media (Colwell *et al*, 1985).

## 2. Molecular identification of unculturable bacteria using tools of metagenomics

Molecular identification is the characterization of micro-organisms on the basis of their genetic material either DNA or RNA. Metagenomics is a field of biology which deals with the direct isolation of genetic material from a defined habitat (such as soil, marine or estuarine water), followed by cloning of the complete genomes of the entire microbial population (Riesenfeld *et al*, 2004). This technique can be divided into function-driven and sequence-based analysis of uncultured microorganisms (Sanger and Coulson, 1975). Functional metagenomics is the screening of metagenomic libraries for a particular phenotype, e.g. antibiotic production, temperature or salt tolerance, enzyme activity, and then identifying the phylogenetic origin of the cloned DNA (Sanger *et al*, 1977). On the other hand, Sequence based approach, involves screening of clones for the highly conserved 16S rRNA genes for their identification and then sequencing of 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 (Riesenfeld *et al*, 2004 and Hoff *et al*, 2008).

## 3. Procedure in Metagenomics

### 3.1 Collection of samples

The first step of metagenomics is collection of samples, which should represent an entire population of interest. The samples can be taken from environment (soil and water) or from human or insect origin. The size and number of samples is depending on the diversity of a particular object.

### 3.2 Filtering samples

This procedure is also known as sorting of samples. The main goal of this step is to explore and identify the maximum diversity of microorganisms in a given sample. While exploring bacteria samples can be filter out for the smaller viroid particles, larger protists. This process will leave behind the lysogenic phages and prophages which are integrated in bacterial genome and mimivirus particles which are having large size as bacteria. For filtration of sample density gradient centrifugation, elutriation and extinction-dilution or filtration acclimatization method have been used extensively.

### 3.3 Recording samples or Metadata

It is the collection of physical and chemical data and data pertaining to the other environmental character/s from samples. The samples collected form soil and water metadata typically includes sampling date and time, depth, salinity, light intensity, geographical coordinates, pH, soluble gases, etc.

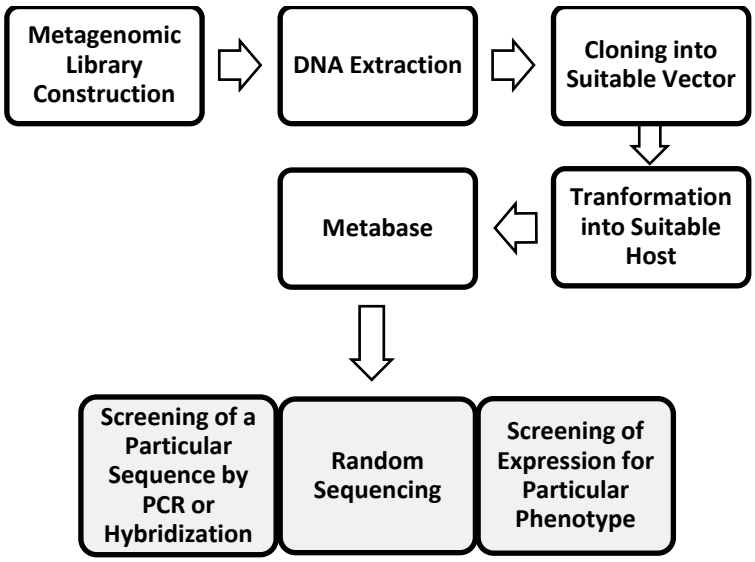


**Figure 1. Construction of Metagenomic Library**

### 3.4 Cloning and Sequencing

Sanger shotgun sequencing is generally used to sequence prokaryotic genomes (Sanger and Coulson, 1975, Sanger *et al*, 1977). The first step involves shearing DNA content of a genome into random fragments. Thus generated fragments are cloned into plasmids and transformed to produce enough genomic material for sequencing. The DNA is sequenced finally using Maxam-gilbert sequencing and chain termination methods. The concept of cloning DNA directly from an environment was initially suggested by (Pace *et al*, 1985). Schmidt et al. (1991) constructed the first $\lambda$ phage library from a sample from seawater and screened for 16S rRNA genes. Other terms to describe the above method include environmental DNA libraries (Stein *et al*, 1996), soil DNA libraries (MacNeil *et al*, 2001), recombinant environmental libraries (Courtois *et al*, 2003) and whole genome shotgun sequencing (Venter *et al*, 2004).

Construction of metagenomic libraries from environmental samples poses many challenges. If seawater contains 200 species per ml, then the metagenome would contain 1 Gbp of unique DNA (Curtis et al. 2002). Strategies for library construction vary and depend on the anticipated study. Libraries containing large DNA fragments are being constructed in lambda phage, cosmid, fosmid and BAC (***B***acterial *A*rtificial ***C***hromosome) vectors. A library having very large size is required to contain sufficient coverage of the metagenome of even the simplest population, which poses a significant screening challenge. In this case Brute-force sequencing is applied which provide a clear picture about the libraries and the communities from which they were derived. (Tyson 2004, Venter *et al*, 2004).

### Conclusions

Amongst bacteria cultured till date, many useful bacteria have been identified which are known to perform many biological conversions in order to provide several useful by products to be utilized by human and animals. As far as bacterial diversity is concerned several bacterial groups have been identified but still ~99% bacteria are yet to be cultivated. Culture-independent microbial diversity analysis in the last decade has revealed previously uncharacterized members in both bacterial and archaeal domains. The *I*nternational *C*onvention on *B*iological *D*iversity (CBD) defines biodiversity as *"The variability among living organisms from all sources including, terrestrial, marine, and other aquatic ecosystems"*. This variability amongst microorganisms is being studied at meta level with the help of metagenomics. Metagenomics represents a powerful tool to access the abounding biodiversity of native environmental samples. It provides the ability to characterize the genetic diversity present in samples irrespective of the availability of laboratory culturing methods. Information from metagenomic libraries can be utilized to enrich the present knowledge as well as for its future applications in industry, therapeutics and environmental sustainability. In order to create a healthy human population that lives in close association with the environment, the information derived from metagenomics can be applied directly to the society. Metagenomics is a fascinating field of molecular biology being exploited as a standard method to explore biological diversity of several yet un-known microorganisms.

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