The Coming Together of Sciences: Metagenomics for Microbial Biochemistry

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Abstract

Microbes are essential for the smooth functioning of life as every life process in the biosphere involves the contribution of microbes. Previously, the study of the microbes has been primarily centred over laboratory-based pure-culture techniques due to which the extensive understanding regarding the microbial communities lags far behind. Metagenomics presents a novel strategy for examining the microbial populations that has upgraded the whole scenario of microbiological research. It involves the application of genomic analysis to whole microbial communities thereby clearly avoiding the necessity of isolation and culturing of individual members of the microbial community. It constitutes techniques like high-throughput sequencing, shotgun metagenomic sequencing of amplicon-based assays, gene prediction, metatranscriptomics and statistical studies, thus, making it feasible for the researchers to investigate the functional as well as metabolic diversity of microbiome by combining the power of genomics, bioinformatics and systems biology. It is a novel technique that can accommodate the analysis of genomes of many organisms concurrently.

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2.1 Introduction

The field of microbiology has gone through a considerable transformation during the last few years that has resulted in a complete alteration of the outlook of microbiologists towards microorganisms and the ways to study them. Microbes are an essential part of human life (Stark 2010). Most of the vital processes that occur in the biosphere involve the contribution of the microbes and hence act as the backbone of every ecological system by controlling biogeochemical cycling of numerous elements that are essential for life on the planet. The biogeochemical cycles that convert the key elements of life, i.e. carbon, nitrogen, oxygen, phosphorous and sulphur into the biologically accessible forms are mainly dependent on the microbes (Vieites et al. 2008). Microbial communities are also involved significantly in providing essential nutrients to their hosts that includes plants as well as animals. They perform very vital functions that are needed to maintain life on earth, for example, extraction of energy from the food that we eat, remediation of the toxins that are produced naturally or due to the activities of the humans in the environment and also adds value to the food through fermentation (Young 2017). Earlier, the study of microbes was based on laboratory-based culturing methods that were focused mainly on single species in pure culture. Therefore, most of the information regarding microbes are mainly laboratory-based attained in the conditions of growing them in the synthetic media in pure culture without considering the ecological factor. The understanding that most microorganisms cannot be grown in pure culture compelled the microbiologists to search for other alternative strategies (Riesenfeld et al. 2004). With the development of genomics and molecular biology, this physiological knowledge has got a strong support of its fundamental genetic basis. Moreover, with the emergence of metagenomics, it now become possible to investigate microbial population in their own natural environments which has proven to be a great boon for the research in microbiology as well as in medicine by opening new channels of research through unique analyses of genome heterogeneity and thus providing more access to the microbial diversity and its thorough understanding (Riesenfeld et al. 2004; Sleator et al. 2008).

The term metagenomics is defined as the analysis of a collection of similar but not alike items. Environmental genomics and community genomics are other synonyms of metagenomics (Handelsman 2004). Understanding about metagenomes has been considerably improved by the use of next-generation sequencing and also advances in the construction of clone libraries as well as bioinformatics (Méndez-García et al. 2018). The first experiment of cloning in the phage vector has been reported in the year 1991 (Schmidt et al. 1991). After that, the construction of a metagenomic library with DNA obtained from an assortment of organisms from the laboratory enrichment cultures has also been reported. Clones expressing cellulolytic activity

were found in these libraries, which were referred to as gene libraries, also known as zoolibraries (Healy et al. 1995). Another group of researchers reported the construction of libraries from prokaryotes from seawater where they have identified a 40-kb clone containing a 16S rRNA gene demonstrating that the clone was derived from an archaeon that had never been cultured (Stein et al. 1996). Likewise, many studies based on metagenomics were reported from time to time that have revolutionised the whole avenue of microbiological research. The chapter will cover different approaches of metagenomics analysis and will also throw some light on its contribution towards mankind by exploring the unexplored areas of microbiology.

2.2 Metagenomic Approaches for Analysis of Microbial Communities

Metagenomics is a comparatively recent introduction in science that has already generated much information about the uncultured population of microbes. The success of metagenomic approaches is attributed to the availability of high-throughput methods of DNA sequencing and the advanced computing capabilities that are required to analyse the millions of random sequences in the libraries. Metagenomics constitutes amplification, sequencing as well as the study of the hypervariable region of the 16S rRNA prokaryotic gene and other phylogenetic marker genes (Mathieu et al. 2013; Martín et al. 2014). The study by Metagenomics includes techniques like genomic DNA extraction, library construction, taxonomic composition analysis, shotgun sequencing, and statistical analysis. Metagenomics can be divided into two different approaches, namely sequence-based and function-based analysis of the uncultured microorganisms (Kennedy et al. 2011).

Function-based metagenomic approach assesses the biochemical as well as metabolic activities of interest by cloning the random DNA fragments in vectors in order to generate an expression library followed by the screening of the library with a specific substrate for a particular phenotype, e.g. salt tolerance or enzyme activity along with identification of the phylogenetic based origin of the cloned DNA (Courtois et al. 2003; MacNeil et al. 2001). It is a powerful approach to identify clones that express a specific function. Functional metagenomic analysis has recognized numerous novel antibiotics, genes responsible for antibiotic resistance, transporters (Na (Li)/H) as well as degradative enzymes (Healy et al. 1995; Majerník et al. 2001). The ultimate potential of this approach is that it does not rely on the genes of interest to be identified by the sequence analysis, thus has the power to classify completely new classes of genes for new functions. A significant limitation of this approach is the inefficient expression of some metagenomic genes in the host bacteria to be used for screening. For example, during the investigation of lipolytic clones derived from German soil reported that only one among the 730,000 clones displayed lipase activity. From the soil sample collected from North America, only 29 out of 25,000 clones from the DNA library expressed hemolytic activity (Henne et al. 2000). Thus, it can be concluded that there is a dearth of active clones, thus requires the progression of efficient screening and selections intended for finding new functions as well as bioactive molecules. Similarly, as bacterial genetics

depends on selections in order to perceive events of low frequency, the approach of metagenomics will also be enhanced by looking for selectable phenotypes in order to expand the number of biologically active metagenomic clones that can be analyzed and further could also be used to put together a basic structure for function-based metagenomic analyses (Rondon et al. 2000).

Sequence-based metagenomics is based on sequencing of DNA demonstrating the whole environmental sample. With the application of Next Generation Sequencing, it becomes possible to acquire complex information and facts about the microbial organisms present in a sample. It involves large-scale screening of the clones for the highly conserved 16S rRNA genes for the objective of identification followed by sequencing of the entire clone with a gene of interest along with sequencing of the total metagenome on an extensive scale in order to explore phylogenetic anchors within the reconstructed genomes (Hoff et al. 2008). The strategy of Sequence-based metagenomics is highly preferred as it not focussed on a limited set of microorganisms. Instead, it presents extensive information regarding all potential activities of the microbial population represented by the metagenome. However, the addition of new data in genetic databases and advancement of various bioinformatics tools enhances the popularity of this strategy for the search of functional activities of interest. The primary function of this approach in the analysis of microbial population is the reconstruction of metabolic pathways and investigating their services in the ecosystem. For this, genome sequences of microorganisms of a specific community are determined followed by performing the assembly of contigs derived from individual metagenomic sequencing reads by using different algorithms as well as tools of bioinformatics like MetaVelvet and Meta-IDBA (Namiki et al. 2012; Peng et al. 2011). The compilation of phylogenetic markers is increasing, and thus with the rise in the markers diversity, the potential of Sequence-based metagenomics approach will also increase and consequently more fragments of unidentified DNA will be assigned to the particular organisms from which they are derived (Tyson et al. 2004; Venter et al. 2004).

2.3 Role of Metagenomics in Imparting Commercial Perspective to Microbial Biochemistry

The exploration of the microbial world by metagenomics has helped in disclosing the extent of genetic as well as biochemical diversity that exists in the biosphere (Chistoserdova 2009). This has indeed added to our knowledge about the types as well as the statistics of microbes that are accountable for the functioning of various important biological and geological cycles which in turn deeply enhance our understanding about climate change and vigour of the ecosystem. It can not only perform the identification of the species in each population; however, also endow with an insight into the microbial metabolic activities as well as their functional roles (Langille et al. 2013). The applications of metagenomics in the context of microbial biochemistry are given in Fig. 2.1. Besides, growing access to the microbial biodiversity through metagenomic also presents an abundance of potential applications in both biomedicine and industry. Metagenomics also revolutionized the pathogen

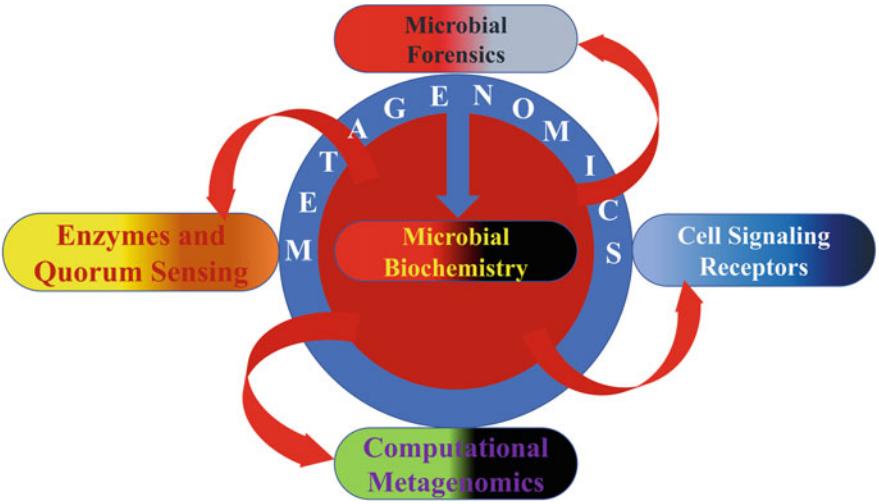

Fig. 2.1 Overview of the scope of metagenomics through microbial biochemistry

detection by allowing the concurrent detection of all microorganisms in a clinical sample by use of next-generation DNA sequencing. It has the potential to identify novel pathogens and find out their specific role in chronic human diseases. With recent studies, it has been shown that with the advancement in DNA sequencing and bioinformatics tools, it has become possible utilizing metagenomics to determine the whole genome sequences of the pathogens. This makes it possible to understand more thoroughly about antibiotic resistance and virulence (Miller et al. 2013). The analysis of the mechanism of action of the xenobiotics (particular the antibiotics) on the human gut microbiome has become very important in order to investigate the underlying mechanism of the drug resistance and also the gene associated with it to combat the problem of drug resistance and also the development of effective drugs that are less prone to pathogens.

Investigation of the mechanisms for resistance against xenobiotics and metabolism in the microbiome of the human gut will provide an insight of interaction between host and microbes, its biochemistry and an explanation to the variation observed in patient to patient response towards drugs. To some extent, this concern has been solved by metagenomics that has facilitated the analysis of microbial community for the aggregate genomes of the gut. Maurice et al. have reported how host-targeted drugs and antibiotics affected the gene expression as well as metabolic activity of a specific set of the active gut microbial population. These results pointed out the need of an assimilated characterization of the host, microbial and environmental factors that are involved in directing the response of the gut microbial community towards the xenobiotics; that could ultimately be used for the invent of various diagnostic tests or for developing therapeutic perspectives (Huttenhower et al. 2012; Maurice et al. 2013). Metagenomics is the most appropriate technology that has been contributing a lot in meeting the huge demand for novel enzymes and biocatalysts. Various industrially important enzymes (Cellulases,

proteases, xylanases, lipases, amylases) have been produced through metagenomics from various natural environments like soil from cold regions or samples from any marine source by the construction of metagenomic libraries and further by the screening of biologically active clones (Lorenz et al. 2002). A novel lipase that is alkaline stable has been isolated by constructing a metagenomic library from the samples collected from marine sediments and has concluded with the results that the specific lipase could be used to impart a characteristic flavour as well as the odour in milk (Peng et al. 2014). Likewise, several papers have reported the isolation of lipases from different metagenomic libraries with novel characteristics and have great potential utility in the industrial sector (Hårdeman and Sjöling 2007; Selvin et al. 2012; Fu et al. 2013). Another alkaline pectate lyase isolated from the metagenome showed various properties like alkalophilic, high specific activity, and thermostability. These observations suggested that it can be used in many industrial prospective as well (Wang et al. 2014). Isolation of a novel amylase from the soil metagenome has been reported that showed 90% of its maximum activity even at low temperature (psychrophilic) which could prove their use very beneficial in various industrial applications (Sharma et al. 2010). It has been reported that screening of salt-tolerant microbial genes has been achieved by using the approach of functional metagenomics that could be used to produce various bioactive compounds in order to enhance the rate of crop production amid high saline conditions (Ahmed et al. 2018). These methodologies can provide new bits of knowledge into the ecologically important microbial networks and exercise that direct matter and energy transition on Earth. Such information in hand will be very beneficial in interpreting the relationship between biogeochemical cycles and human activities that collectively form the fortune of our planet. A detailed review of the enzymes discovered through metagenomics has been given in Chap. 7. Metagenomics has also provided insight into the various symbiotic associations as how microorganisms form symbiotic associations, communicate as well as acquire nutrition and produce energy with other organisms. Shotgun sequencing and reconstruction of metabolic pathway depicted that the symbionts are sulphur-oxidizing and sulphate-reducing bacteria which are proficient of carbon fixation and consequently capable of providing nutrition to the host organism (Woyke et al. 2006).

2.4 Quorum Sensing and Quorum Quenching Through Metagenomics

Bacteria communicate within their populations using quorum sensing (QS). The process of quorum sensing occurs through small signalling molecules called autoinducers (AI). Via these autoinducers, the microorganisms regulate luminescence, production of antibiotics, pathogenicity and growth patterns. The autoinducer molecules are of four types viz. acylhomoserine lactones (AHLs), alpha-hydroxyketones (AHKs), furanosylborate diesters (FBDE or AI-2) and autoinducer peptides (AIPs). One of the best studies mechanisms of quorum sensing mechanisms is the lux system of *Vibrio fischerii*. The QS system of *Vibrio fischerii* is regulated by the binding of the AHL to the intracellular receptor protein called luxR. luxR is a

response regulator of transcription which is activated by binding of AHL. Activated luxR causes transcriptional activation of the luxI gene, which is responsible for production of the AHLs. This signalling works in a cell-density-dependent manner (Fuqua et al. 1994). The knowledge of the mechanism and gene products of the AHL-modulated QS systems is utilized for searching for the homologs of these genes in a microbial population. This is where the metagenomic approach finds significant applications. Hao and colleagues used the biosensor strain HC103 of Agrobacterium tumefaciens transformed with pJZ381 as a recipient strain for the metagenomic library. The metagenomic libraries was constructed from various soil and activated sludge samples (Wang et al. 2006). The metagenomic clones were transferred to the biosensor strain using conjugation method with the help of helper *E. coli* strain DH5a (pRK600). The Ti-plasmid of the sensor strain contained the traC-LacZ fusion, whereas the plasmid pJZ381 contained a pLac(lac promoter)-traR fusion. The screening principle was dependent on the presence of an autoinducer synthesizing gene in the metagenomic clones, which would lead to production of the AI. The AI would bind to the traR protein (response regulator). The activated traR would activate transcription of traC-LacZ fusion, causing production of blue coloured colonies on the agar plate. As a result, twenty-two blue colonies were identified from the screen that produced the quorum sensing molecules (Hao et al. 2010).

Another study on metagenomic approach was reported for the bioremediation application. Anammox (anaerobic ammonium oxidation) bioreactors are nitrogen recycling and removal systems that rely on conversion of ammonium ions to nitrites and nitrates without aeration. This leads to the removal of excess nitrogen without significant emission of greenhouse gases. Anammox bacteria comprise of genera like *Candidatus Anammoxoglobus*, *Candidatus Anammoximicrobium*, *Candidatus Brocadia*, *Candidatus Jettenia*, *Candidatus Kuenenia* and *Candidatus Scalindua* (Chu et al. 2015). These bacteria can grow symbiotically with ammonia and nitrite-oxidizing bacteria in anammox bioreactors. In the anammox bioreactors, unique biofilms are seen, which comprise of bacterial consortia. QS molecules, including N-octanoyl and N-hexanoyl homoserine lactones have been detected in such anammox systems (Tang et al. 2015). Biofilm formation in bacterial is a well-established concept and the mechanisms are also known (Dickschat 2010). Metagenomic approach was applied to the anammox bioreactor biofilms. The team extracted the metagenomic DNA from the biofilm samples and amplified the 16S rRNA genes. They sequenced the amplicons using the Illumina high-throughput sequencing platform and assembled the operational taxonomic units (OTUs). The phylogeny was studied using the EggNOG database. The sequence analysis revealed that apart from the anammox bacteria genera, other species of *Nitrospira*, and *Lautropia* were also detected. The details of these methods are explained in Chaps. 3 and 8.

Quorum quenching (QQ) or quorum-sensing inhibitors (QSIs) may include enzymes or other small molecules that inhibit the binding of AIs to the response regulators or cell-surface receptors (Dong and Zhang 2005). These inhibitors can also inhibit the process of biofilm formation in microbial communities.

Weiland-Bräuer and co-workers isolated nine QQ enzymes from the metagenome libraries (Weiland-Bräuer et al. 2016). These enzymes repressed the AHL and FBDE-mediated communication between *E. coli*, *Klebsiella oxytoca*, *P. aeruginosa*, *S. aureus* and *Bacillus subtilis*. The QQ enzymes also showed reduced biofilm formation in *Candida albicans and Staphylococcus epidermidis*. The QQ enzyme coded QQ7 also reduced the expression of transcriptional regulator icaR (Weiland-Bräuer et al. 2019).

Yaniv and colleagues used the BAC-metagenomic library constructed from the red sea samples (Sabehi et al. 2004) for screening of quorum sensing inhibitory molecules. The screening strategy was to check for violacein production by *Chromobacter violaceum*. The metagenomic clone with QSI activity could inhibit the production of violacein. Further screening for inhibition of biofilm formation was performed using the crude extracts from the positive clones identified from the violacein production assay. The team showed that the positive clones inhibited the biofilm formation in a dose-dependent manner (Yaniv et al. 2017). Thus, metagenomics likely holds the key to unlock the potential of microbial communities for providing beneficial QS molecules as well as QSIs.

2.5 Ion-Channels and Pumps Through Metagenomics

The ion-channels, pumps and sensors are important cell surface macromolecular machines, regulating the cell signalling and communication. Metagenomics has revealed the potential for the screening and identification of specific pump proteins called rhodopsins. The bacterial rhodopsins can function as light-driven H^+-pumps, sodium ion pumps and chloride ion pumps. Metagenomics provides access to these rhodopsins using the function as well as sequence-based approaches for screening. The screening strategy for a rhodopsin is a simple plate agar-based assay. The metagenomic clone that express a rhodopsin gene, would show orange-red pigmentation when cultured on agar-based media supplemented with retinal. The research carried out by Martinez and colleagues showed two prominent cloned expressing the light-operated proton pump (Martinez et al. 2007). More such studies have been carried out very recently. A unique group of bacterial rhodopsins was identified from a metagenomic screen in Israel. The team constructed and screened a fosmid-library to identify one positive clone using the all-trans-retinal screening (Pushkarev et al. 2018). The knowledge of these unique cell surface proteins can be obtained using the metagenomic approach.

2.6 Conclusion

Metagenomics has changed the approach of microbiologists towards the microbial research by redefining the whole concept of a genome and contributed immensely in the acceleration of the rate of gene discovery. Metagenomics has gained much success in analysing the vast microbiome of a given environmental sample. Various

novel enzymes have been discovered through it that find ample applications in different areas. It has also led to the production of antibiotics and biosurfactants, which has given strength to the address of the issues like drug resistance, oil leakage, among others. Besides, issues of degradation of synthetic compounds which is very important for environmental protection from pollution has also been solved by the metagenomics. Therefore, it can be concluded that it provides a way to analyse the structural as well as functional genomics of the whole microbial diversity and hence plays an essential part in discovering novel genes for obtaining industrially valuable bioactive molecules and enzymes. Using the metagenomic approach, the quorum sensing molecules and the inhibitors of quorum sensing have been characterized. These applications enable the profiling of microbial communities in terms of their biochemical pathways as well as their physiology. Also, the discovery of bacterial rhodopsins through metagenomic approach can provide valuable insights into the biochemistry of microorganisms and microbial communities. Thus, we can say that metagenomics makes it possible to approach the massive diversity of the microbial population and has contributed a lot in understanding as well as gaining from the unculturable microbes.

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