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REVIEW ARTICLE

PHYLOGENY AND CHARACTERISTICS OF UNCULTURABLE BACTERIA WITH METAGENOMICS APPROACH

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ABSTRACT

Metagenomics is the genomic analysis of bacteria by direct extraction and cloning of DNA from an assemblage of bacteria. Explanation of Metagenomics stemmed from the ineluctable evidence that as-yet-uncultured bacteria represent the vast majority of organisms in most environments on earth. The supporting analyses of 16S rRNA gene sequences would have been direct amplified from the environment that can change the discovery of microbial life. In microbiology, bacteria needed to be cultured in a lab for researchers to understand the organism; those that would not grow in lab conditions are considered Unculturable. The organism was directly taken from environment and has been studied after uncultured organisms. From these Unculturable organisms, the collection and analysis of their genetic material is the study of metagenomics. Two approaches, function-driven and sequence-driven are used to obtain a metagenomic library. The symbiosis relationship of bacteria showed their function which approach while rRNA is primarily used for sequence for functional analysis. Many biochemical techniques are currently used in metagenomics including: stable isotope probing, suppressive subtractive hybridization, differential expression analysis, PCR for amplification, RT-PCR, and microarrays. These experiments have led to many novel discoveries of proteins, organisms, and phylogeny studies. Continued research in the metagenomic field will lead to improved bioinformatics which will in turn allow us to know more about our history and our ever changing environment. Metagenomic sequence used for application part for information and it will be facilitated to design a better strategies for culture to link the genomic analysis with pure culture studies.

INTRODUCTION

Metagenomics is the study of genomic material of bacteria which is directly obtained from the environment, instead of from culture. Meta-analysis means the statistically combining separate analysis. Comprehensive genome analysis of organisms' genetic material in Microbiology has experienced a transformation during the last 25 years that has altered microbiologists' view of microorganisms and how to study them. Most of the microorganisms cannot be directly grown in pure culture forced by microbiologists to question for their belief that the microbial world had been conquered. Replacement of this belief with an acknowledge that are extended of our ignorance about the and organism diversity and metabolic change. That changes occurs a revolution in microbial world. The revolution was the Convincing demonstration that the uncultured microbial world far outsized the cultured world and that this unseen world could be studied (Olsen, J1985, Pace 1985).

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This change in thinking was prompted by another, equally important realization: microorganisms underpin most of the geochemical cycles and many human health conditions that were previously thought to be driven by inorganic processes and stress, respectively. A faint sign of a feeling or quality, especially the influence that microorganisms make an effort on the world drive into particular situations the microbiologists pursue the uncultured world. In 1931, Waksman with approval believed that "a large numebr of information has gather build up that enables us to construct a clear picture of the microscopic population of the soil" (Waksma 1931), and in 1923 as per *Bergey's Manual* postulate flatly state there is not any classification method for organism without being cultured (Smit 2001). In the mid-1980s, microbiologists had lost this confidence, and the language and practice of microbiology changed to adapt the vast unknown of uncultured life. Concepts, acceptance, images, and words needed to be replaced when it became obvious that they were based upon the state that microorganisms did not exist unless they could be cultured. Pace and colleagues highlighted the need for nontraditional techniques to understand the microbial world: "The colony morphology of most of the bacteria provides little

information for their identification; physiological traits are often ambiguous. The microbial ecologist is particularly prevented by obstructing these limitations, since so many organisms resist cultivation, which is an essential event serving as an introduction to something more important to the characterization in the laboratory" (Pace 1986). In the occur afterwards years, microbiologists dedicated intense effort to describing the phylogenetic diversity of exotic and ordinary environments—ocean surfaces, deep sea vents, hot springs, soil, animal rumen and gut, human oral cavity and intestine. Many new lineages were classified based on their molecular signatures alone (Beja O 2002). Phenotypes determine by whether they represented new species, genera, or a phylum of prokaryotic life was to elucidate the function next to the challenges. Various techniques, including metagenomics, the genomic analysis of assemblages of organisms is challenged for deposit eggs. Recent study years back, the study of uncultured microorganisms has become larger beyond asking many questions?" "What are they doing?" The outcomes of the recognition of uncultured microorganisms are worthy of examination. One of these outcomes, metagenomic, is further modifying microbiology. Metagenomics calculate the research findings by enabling never done analysis of evolution and genome heterogeneity in environmental population and providing thoroughfare to far microbial diversity that has been showed in the culture plate. This review confined to explore the origins of metagenomic methods and examine its new era application to microbial ecology (Xu J 2006). Late 17th century, Anton van Leeuwenhoek; was the first metagenomicist who has directly studied microorganisms from pond water and his own teeth. 1920'sCell culture arose moved past away from early stage of metagenomics. If a microorganism could not have ben cultured, it could not be classified.1980's inconsistency observed (1) Number of organisms under microscope in conflict with amount on plates. Aquatic culture differed by 4-6 orders of magnitude from direct observation. (2) Cellular activities in situ conflicted with activities in culture. *Sulfolobusacidocaldarius* in hot springs grew at lower temperatures than required for culture. (3) Cells are viable but unculturable. *Vibirocholerae* unculturable until they pass through human gut.

Pure Culture is not enough in Modern Microbiology

The depth and detail of modern microbiological knowledge, for a long time the microbiologists ignored challenge to identified and characterized uncultured organisms because culturing of microbes provided the platform for building capacity. The rich source of discovery found in the readily cultured model organisms, they focused instead on and this contributed to the explosion of knowledge in microbial genetics and physiology in the 1960s to mid-1980s. The research of uncultured microorganisms remained in the hands of a little occur over a prolonged time of period that scientist start to accumulate some specific study to find that lightly at the edge of the microbiological perception of something, suggesting that culturing did not capture the full glimmers of microbial diversity. One of the indicators that cultured microorganisms did not represent much of the microbial world was the soft-observed "great plate count method" the similarity between two or more facts of populations determined by dilution plating and by microscopy. This discrepancy is particularly dramatic in some aquatic environments, in which plate counts and viable cells estimated by acridine orange staining can differ by four to six orders of magnitude, and in soil, in which 0.1 to 1% of bacteria are readily culturable on common media under standard conditions (Staley 2002).

Brock and colleagues encountered microorganisms in Yellowstone hot springs that could not be cultured and others whose behavior in culture did not reflect their activities In situ. Many microorganisms could not be cultured on agar medium because their temperature requirements exceed the melting point of the agar. Therefore, need to clearify the physiological function of microorganisms without culturing them required to innovative. Applied the Brock's technique to involved the immersion of microscope slides in the spring for 1 to 7 days, examined by microscopic observation and often staining with fluorescent antibodies raised against cultured members of the taxonomic groups suspected to inhabit the environment. This hypothesis estimated in situ population sizes and growth rates, which indicated, for example, that certain strains of *Sulfolobus* grew in the hot springs at temperatures well below the optima in pure culture. The expanding body of evidence indicating that it was imperative to study physiology in the environment led Brock's group to determine which organisms in the hot spring were responsible for photosynthesis. To do so, they placed an opaque cover over the spring for a week. The pink color lost from spring, leading them to infer that the genus *Synechococcus*, shows pink color in culture, was a major role of constituent to photosynthesis process. (Prasad B 2014) Further evidence that drew attention to the uncultured world accumulated during the 1970s and 1980s. Oligotrophs indicated that incubation times longer than 25 days enhanced the recovery were focused instead on organisms in culture. In food industry the "injured bacteria" in food—live organisms that cannot be cultured following stressful treatments such as heat, chilling, or desiccation but represent a significant risk to human health (Malviya J 2013) The concept of organisms that were viable but not culturable emerged from the work of Colwell and colleagues, who showed that strains of *Vibrio cholera* were indeed virulent and alive when isolated from aquatic environments but did not grow in culture until after passage through a human or mouse intestinal study.

The confluence of these and many other scientific and technical advances steadily drew an attention to the unculturable microbial world, but two discoveries figured significantly in the sharpened focus. The first was work on the diversity of soil bacteria, which demonstrated with DNA-DNA reassociation techniques that the complexity of the bacterial DNA in the soil was at least 100-fold greater than could be accounted for by culturing. This work suggested that the diversity of the uncultured world exceeded previous estimates. The second discovery was the demonstration that *Helicobacter pylori* causes gastric ulcers and cancer. Although spiral bacteria had been observed in the gastric mucosa of dogs in 1893 and in humans in 1906 and correlations between the appearance of the bacteria and peptic ulcers were noted in 1938, it was not until *H. pylori* was cultured that its role in disease was accepted. Culturing was accompanied by the satisfaction of Koch's postulates on a human volunteer, providing definitive evidence for the causal relationship between the bacterium and ulcers. Ironically, culturing was not that difficult. Plates accidentally incubated for 5 days instead of 3 revealed colonies later shown to be *H. pylori*. The fact that strong microscopic evidence for the role of *H. pylori* long

preceded culturing and might have served as the basis for successful treatment decades earlier, perhaps reducing human suffering and mortality due to ulcers and cancer, did not escape the notice of microbiologists, medical practitioners, and the public. Whereas the studies of the complexity of the soil DNA (Roose-Amsaleg, 2001) demonstrated the diversity of the unknown world, the connection of uncultured bacteria and ulcers provided a striking example of the power of the undetected organisms. These discoveries provided compelling evidence that drew microbiologists to wrestle with the daunting challenge of devising strategies to access these organisms (Bergey's Mannual 1923). Unculturable bacteria were first cultured (Handelsman, 2004) rRNA: "Evolutionary Chronometer:" Very slow mutation rate and 5S and 16S sequences used.

Data Collection Methods

Initially, direct sequencing of RNA and sequencing reverse transcription generated DNA.But it has been progressed to PCR and phylogenetic stains. Phylogenetic staining requires only rRNA from uncultured environmental sample.

Data Storage

Metagenomic Library has two approaches to store the data Function-Driven and sequence driven former focuses on activity of target protein and clones that express a given trait and the latter Relies on the highly conserved sequence of DNA used to design the PCR reverse and forward primers and hybridization to make a probes; that gives the functional information about the microorganism.

Biochemical Test

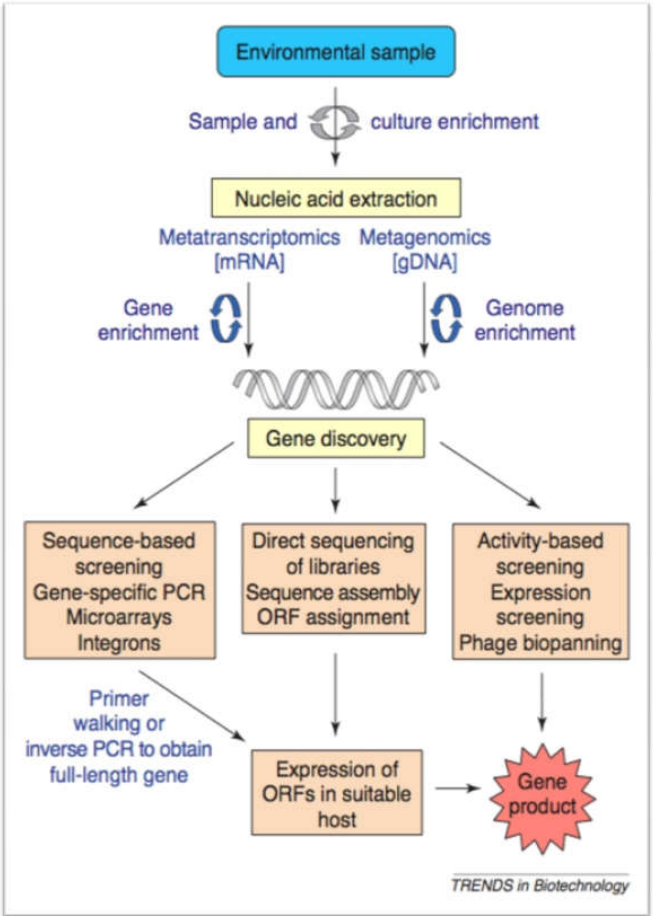
Nucleic Acid Extraction: Cell lysis (by any method chemical, enzymatic or mechanical) is usually followed by removal of cell fragments and nucleic acid precipitation and purification. More oftenit used due to DNA recovery that is a better representation of the entire microbial community within the sample. However, contaminants may also be extracted. There is a compromise between a thorough extraction and the minimization of shearing the DNA. Total DNA extractions from environmental samples must be normalized to get an even representation of a particular genome. RNA recovery is similar to that of DNA except it is modified to minimize single-stranded polynucleotide degradation of mRNA as well as RNAse activity

Genome enrichment: Sample enrichment enhances the screening of metagenomic libraries for a particular gene of interest, the proportion of which is generally smaller than the total nucleic acid content. Enrichment is completed by Stable isotope probing (SIP) and labeling DNA or RNA by 5-Bromo-2-deoxyuridine, which is followed by density-gradient centrifugal separation. Some other methods are also used like; Suppressive subtractive hybridization (SSH) Differential expression analysis (DEA).

Gene Targeting: PCR is used to probe genomes for specific metabolic or biodegradative capabilities Primer design based on known sequence information. Amplification is limited mainly to gene fragments rather than full-length genes, beacuse the genes undergoes additional procedures to attain the full-length. RT-PCR has been used to recover genes from environmental samples, since RNA is a more sensitive biomarker than DNA

Microarrays are used for various process like to monitor gene expression, to categorize the genes involved in key processes and to quantify environmental bacterial diversity.

Metagenomes sequencing: Entire metagenomes have been sequenced using for large fragments of genomic DNA from uncultured microorganisms. The objective is to sequence the genomic DNA; to identify the thousands of viral and prokaryotic genomes and lower eukaryotic species present in small environmental samples such as a gram of soil or liter of seawater.



Metagenomic Gene Discovery. Courtesy of Cowan, *et al.*

Common metagenomic techniques used in determining the gene for flagellum creation and for many other research projects.

Acquisition of symbiont DNA: Isolated from bacteria that is collected from deep sea thermal vents

Amplification of isolated DNA: PCR techniques used for the amplification of acquired DNA

Creation of cosmid library from symbiont DNA: Cosmid library is used as a collection of sequences which helps to compare the sequence and find similarity/identity.

Phylogentic Analysis of Microorganisms utilizing Metagenomic Methods: Phylogenetic studies with the help of UPGMA neighboring methods to track out origin and findout evolutionary relationships among the species (Cowan

2005).The phylogeny of the unidentified, unculturable bacteria compare with their genetic sequences of known, culturable ones, in order to come to a conclusion about the evolutionary origins of the unculturable bacteria. The 16S rRNA sequence is used for the sequences among species orientation of rRNA's have to be higly conserved in order to preserve sequence for its universal function. Because the main source of genetic material used to study evolutionary relationships with the universal highly conserved sequence of 16S rRNA subunit. These differences or similarities in the rRNA sequence can then be looked at between organisms in sequence alignment software to determine how close there evolutionary origins are (Cowan D 2005). Sulfur-Reducing Bacteria (SRB) is found in sandy marine sediment samples and most of these species are unculturable in lab. The 16S rRNA sequences of the unculturable bacteria and know cultured SRB from the lab can be compiled on sequence alignment software and analyzed. Comparing similarities and differences in sequence in the cultured and unculturable bacteria resultant a phylogenetic tree can be constructed. Many of the marine sediment sequences were found to have 82%-85% similarity to known SRB 16S rRNA sequences. Another useful application for metagenomic phylogenetic is looking at a sampling of the distribution of bacteria populating an environment (Devereux R 1994). The 16S rRNA sequences of the unculturable bacteria in soil were compared to a range of known bacterium. From the sequence alignment data, a general overview of the percentage of different populations of bacteria populating this particular soil sample could be created. A phylogenetic tree of culture and uncultured bacteria was made for this experiment. General conclusions about what type of microfloura populates different regional climates can be made. Divergences in the evolution of cellular mechanisms for dealing with different kinds of environmental changes could also be observed. These methods do have their drawbacks because some of the bacterial populations in a soil sample may be under-represented. Some bacilli are very hard to obtain genetic material from when in spore form.

Conclusion

Metagenomics by now has unblocked alleys of research by opening bizarre analysis of genome based study that has evolved from numerous limitations in phylogeny and genology. Some common techniques applied it can be used to analyzed the genetic material from bacteria to grown in their environment. Crucial symbiotic relationships are more easily studied using metagenomes through allowing the symbiont to grow in its natural environment. Phylogenetic trees can be developed based on the neighboring approaches. Novel pathways will be determined using the metagenomic techniques required for faster analysis of a broader range of organisms

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