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RESEARCH ARTICLE

RECENT ADVANCES IN CULTURING THE UNCULTURABLE BACTERIA

Dharmesh Harwani

Department of Microbiology, Maharaja Ganga Singh University, Bikaner-334001, Rajasthan INDIA

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ABSTRACT

The diversity of micro-organisms like bacteria is poorly studied and categorized. Preserving them like plants and animals is extremely difficult as these tiny organisms cannot be observed with the naked eye. Moreover these organisms cannot be classified solely based on their phenotypic characters therefore various methods are being employed to access the diversity of these unculturables. The attempts are being made to understand the biology of the unculturable bacteria by using tools of metagenomics, creating artificial environment in the laboratory, co-culturing in combinations with microfludics devices etc. While metagenomic study provides culture independent access to whole microbial communities based on their genetic material recovered directly from an environment, it is highly difficult to get information about physiology of an organism. The present review discusses the reasons for un-cultivability of these organisms and advances in molecular ecology to culture the same.

INTRODUCTION

Bacteria are always considered to be a very important group of microorganisms not only being the essential part of earth's microbiotabut also due to their involvement in chemical, physical and biological transformations. The term 'unculturable' is used to describe organisms particularly bacteria that are yet to be grown on artificial media. In other wordswe do not have sufficient biological information to culture these bacteria*in vitro*.Thus these bacteria cannot be grown easily on artificial nutrient media and are referred as unculturables. It has been proposed that these microscopic cells are dead and therefore would never grow posed the anomaly without classifying them (Spieckermann, 1912). In fact, many of these cells were shown to be metabolically active, even though they were not able to grow on laboratory media (Roszakand Colwell, 1987).

Recent advances in culturing the unculturables

Significant efforts have been made in recent years to devise culturing methods for unculturable bacteria. The majority of culture media which are used now days in laboratories 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. But if suitable culture conditions were provided it should be possible to cultivate these in the laboratory (Langer et. al. 2006).Epstein and Lewisdesigned a diffusion chamber to create an environment model in the laboratory by enclosing the bacteria within a semi-permeable chamber such that the cells are not able to pass through the membrane barrier but nutrients and growth factors from the environment are able to go through (Kaeberlein et. al. 2002). These chambersafter inoculating with dilute suspensions of cells from marine sedimentwere incubated in an aquarium of seawater on a bed of sand. Analysis of the number of growing colonies with microscopy in the initial inoculums yielded 40% recovery as compared to the 0.05% recovery when the same inoculums were assayed for viable colony on standard petriplates. Ferrariet. al. (2005) also cultured colonies of rare soil bacteria by growing them on filters suspended on soil slurry in the absence of additional nutrients. They primarily identified ubiquitous but uncultured bacterium *Alphaproteobacteria*having important role in primary production in the ocean.*Alphaproteobacteria* is one of the most abundant proteo-rhodopsincontaining organisms in seawater. Proteo-rhodopsin is a light-driven proton pump initiallyidentified in the DNA sequence of an uncultured bacterium(Morris et. al. 2012).Giovannoni and coworkersutilized the natural environment in the form of seawater as a growth medium. They utilized a process whereby a dilution sequence is carried out until only one or a very few bacteria are present in a given culture. In that way they were able to separate the bacteria of *Alphaproteobacteria*into the wells of micro-titer plates (Rappe et. al. 2002). This culture technique has shown path to the culture of new previously un-culturable members of this clade from different marine regions (Song et. al. 2002) and freshwater environments (Connon, and Giovannoni, 2002, Page et. al. 2004, Stingl et. al. 2007, Jang et. al. 2011, Huggett, and Rappe 2012). Keller(Kellerand Zengler, 2004)used a method for encapsulating single bacterial cells in solidified agarose micro-droplets. A marine water sample diluted to extinction series was employed to limit the number of bacteria in each gel micro-droplet and after encapsulation; the droplets were contained in a flow column bounded by membranes that retained the encapsulated bacteria. The seawater was constantly flushed through these columns as a growth medium. After appropriate incubation, the droplets were passed through a flow cytometer and colonies containing droplets were sorted into the wells of a micro-titer plate.Interestingly, phylogenetic analysis revealed that un-amended seawater as a

* Corresponding author: **Dharmesh Harwani**
Department of Microbiology, Maharaja Ganga Singh University, Bikaner-334001, Rajasthan INDIA

medium yielded a higher diversity than seawater with additional nutrients. Using solely seawater as the growth medium, very rare bacteria have also been isolated with gel micro-droplets including lineages of *Planctomycetales* that were previously considered to be uncultured (Zengler, 2002). In an interesting approach Graf used high-throughput sequencing of RNA transcripts to determine that an uncultured *Rikenella* bacterium in the leech gut is utilizing mucin as a carbon and energy source (Bomar, 2011). Utilizing this clue they cultured this isolate on a medium containing mucin which suggests that the RNA sequence information is more useful in this regard than the genomic DNA sequence, as RNA sequence indicated what genes were actually being expressed in the growing bacterium.

Many bacteria exhibit co-culture dependence, bacteria isolated from the chambers developed by Lewis and Epstein would not grow on a petri plate unless they are growing close to other bacteria from the same environment. Similarly *Prochlorococcus* had been cultured from the ocean many times but only two variants had been grown in pure culture (Moore et. al. 2002, Rippka, 2000). The other isolates were all dependent on heterotrophic bacteria for co-culture (Morris et. al. 2008). A number of subsequent efforts to culture other bacteria also revealed plentiful examples of co-culture dependent isolates (Beja, 2000, Kim, 2008, Kim et. al. 2011). In an elegant technique Zinser group set out to separate a dependent strain of *Prochlorococcus* (MIT9215) from its heterotrophic helpers to determine the nature of the help provided (Morris et. al. 2008). They selected for streptomycin-resistant mutants among the abundant *Prochlorococcus* cells in their co-culture, while the smaller population of the helpers was not large enough to contain a spontaneous resistant mutant. They were consequently able to kill the helper population by treatment with streptomycin, resulting in an apparently pure *Prochlorococcus* culture. By maintaining this culture of MIT9215 at high density (minimal dilution on subculture), they were able to propagate it in pure culture. In a subsequent study, it was demonstrated that removal of H_2O_2 is both necessary and sufficient for the helping effect (Morris et. al. 2012). The dependence of *Haemophilus influenzae* on *Staphylococcus aureu*s could be considered as a classic example of co-cuture dependence (Davis, 1921). In this example *H. Influenza* requires an exogenous source of both heme (Fildes, 1921) andNAD (nicotinamide adenine dinucleotide) (Lwoffand Lwoff 1937). Beppu identified a helping factor which is necessary only for laboratory culture of *Symbiobacterium thermophilum*(Suzuki et. al. 1988). This isolate was found to be dependent on a *Bacillus* thermophile, providing carbon dioxide (Watsuji et. al. 2006).

In order to identify further molecular mechanisms of unculturability Lewis, Epstein, and Clardy groups undertook a study to directly isolate bacteria from intertidal sand biofilm that would grow only in the presence of helper organisms from the same environment. The screen was based on the hypothesis that on a "crowded" isolation plate (a petri plate in which the environmental inoculum had been spread at a concentration such that a few hundred colonies would grow), some of the colonies would be growing only because they happened to be close to a helper colony. To identify these isolates, candidate colony pairs were cross-streaked and visually screened for dependent growth of one of the bacteria. Perhaps surprisingly, given the random pairing utilized, up to 10% of the screened isolates showed dependent growth. It was observed that *Maribacter polysiphoniae* KLE1104 is able to grow only in association with a laboratory strain of *Escherichia coli* on a petri plate. Interestingly this group analyzed that *E. coli* mutants that are not able to synthesize enterobactin are unable to induce the growth of KLE1104. Enterobactin is a siderophore, a class of secreted small molecules that are able to solubilize oxidized iron and thereby make this essential nutrient available to cells. Using soluble form of iron, isolated several rare bacteria, includinga member of the *Verrucomicrobia*, a member of the *Parvularculaceae,*and a bacterium distantly related to the *Gammaproteobacteria* (Stewart, 2012).It could be that co-culture dependence of recovered isolates apparently has lost the ability to carry out necessary functions by their own and therefore they need helper/s to grow in that particular environment. Morris group (2012) explained this loss in terms of evolutionary cheating, whereby these bacteria are taking assistance from helpers for necessary functions or it could be that the presence of helper bacteria may act as a signal to begin growth. They also suggest that this dependence on helper organisms could be adaptive which in turn provide additional functionalities and greater fitness for the entire community.

In an attemptto quantify the role of the culturable and unculturablemembers of the gut microbiome, the group of Gordon used germfreemice as hosts for transplanted human intestinal microbialcommunities (Goodman, 2011). Fecal samples from donors were either directlyinoculated into germfree mice or first passed through a petriplatebasedculturing step before introduction into mice. Interestingly,Mice colonized with bacteria that passedthrough the cultivation step showed equivalent fat pad weight gainto those that were directly colonized, a finding which led the authorsto conclude that the uncultured component may not becritical for at least some aspects of host health. However, theirresults on how the composition of the microbiome responded todietary perturbation showed that mice that received a direct transferof bacteria had a stronger response than those who received thecultured bacteria, indicating that some functionality of the microbiomemay have been lost in the cultivation step. Therefore, thequestions of how much of the gut microbiome can be cultured inthe laboratory and what role the remaining fraction plays in hosthealth remains mostly unresolved. Buttongroup (1998)described an approach, successfully employed in isolating novel oligotrophic, heterotrophic cells from marine ecosystems (Button et. al. 1993). It uses unamended environmental water as the medium and technique is referred as "extinction culturing" to distinguish it from dilution culturing (Ammerman et. al. 1984, Li and Dickie, 1985, Carlson and Ducklow, 1996). The approach was to dilute natural communities of microorganisms to a known number, ranging from 1 to 10 cells per tube, and then analysis by flow cytometry so as to count dilute populations of microbial cells(Button et. al. 1993).

Promising approaches for the future

Efforts are being made to culture the unculturable bacteria by furnishing important growth factors required for growth, by creating an artificial environment in laboratory and by making use of microfluidics.Tsuneda developed acapillary-based culturing system based on porous hollow-fibermembranes (Aoi, 2009). The fiber-based system cultivated a higher proportion ofnovel isolates (<97% 16S rRNA gene sequence similarity to culturedstrains) than petri plates containing media designed tomimic each environment. Epstein and Lewis groups developedthe isolation chip for high-throughput cultivation(Nichols, 2010). It contains 384 holes creating

chambers of 1mm in diameter, tested on soil and seawater samples. This allowed cultivation of greater total numbers ofcells as well as greater total diversity of taxa compared to standard petri plate method. Kohler developed a segmented flow chip, in which short intervals of water have been separated with alkane which flow through micro-channels in a silicon wafer chip (Grodrian, 2004).This technique allowed the parallel cultivation of pure cultures in a small volume. Similarly Ismagilov used chemistrodeand segment-based microfluidics in which bacteria multiply within the aqueous compartments. These segmentscould be further split to allow similar processes on single isolate to be carried out(Liu et al. 2009). Lin used auxotrophic mutant strains of *E .coli* in microfluidics in 1-nl microdroplets for which growth was observed when both variants were presentin the same drop (Park et. al. 2011).

Conclusions and Future Perspectives

Culture-independent microbial diversity analysis in the last decade has revealed previously uncharacterized members in both bacterial and archaeal domains. These novel unculturable bacteria represent an unexplored and unexploited vast gene pool. Development of new culture techniques and more innovative tools in molecular biology related to genomic library construction of culturable members of various bacterial groups are expected to revolutionize the field of biocatalysts and drug discovery. Further decrease in sequencing cost is expected to increase efforts for microbial community genome sequencing in an attempt to understand community structure and ecosystem function. Availability of community genome sequences will help in development of gene expression profiles and physiological studies will provide a comprehensive approach to environmental microbiology. In addition to that an access to the neglected bacteria that reside on us may provide new avenues to improve overall health through an enhanced understanding of the yet unknown functionalities of these microbes. The results of the next upcoming years of cultivation efforts will likely exceed those of the last many years, with a similar extent of health, ecology, science and technology benefits.

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