

2. Marine Microbiology: Challenges and Future Directions

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Introduction

The planet earth is believed to have formed about 4600 million years ago and life forms originated at about 3,500 million years on this planet. As we know Antonie van Leeuwenhoek (1632-1723) was the first to observe bacteria (he called them as “animalcules”) under a microscope he invented. He might have thought that scientists who would follow him would discover many more such microscopic organisms from different niches. His dream came true but at a pace, which he would not have thought about. With the introduction of better microscopy, our ability to observe natural samples improved tremendously and we started recognizing the ubiquitous nature of the tiny creatures of the planet earth. However, the introduction of modern molecular techniques in late seventies revolutionized our understanding of these organisms, especially the microorganisms. Now we know that these microbes are present everywhere from the permanently frozen continents, high altitudes (up to 60 km high in the atmosphere), thermal springs, deep-seas to even deep-sea hydrothermal vents. They are the building blocks of any ecosystem and perform varied and all-important tasks that include everything from pathogenesis to fixing atmospheric nitrogen in the soil. An interesting question to be asked, therefore, is: has there been any realistic estimate of these bacteria on Earth? Now, for the first time, a team of researchers from the University of Georgia (USA) led by microbiologist William B. Whitman (1998), has come up with direct estimate of the total number of bacteria on earth. They estimate the number to be five million trillion trillion – *i.e.*, a five with 30 zeroes after it. They also give a picturization of these numbers: If each bacterium is a penny, and when stacked flat one over the other, the stack would reach a trillion light years. These almost incomprehensible numbers give only a sketch of the vast pervasiveness of microbes in general and bacteria in particular, in their natural world.

As the existence of the microbial life was recognized only relatively recently in history, about 300 years ago, the knowledge we have gained is still rudimentary. Microorganisms still represent the largest reservoir of undescribed biodiversity. The recent techniques such as Ribosomal RNA genes -sequencing have helped us survey the biodiversity sufficiently faster and

comprehensively. Eventhough, an impressive number of bacteria (ca. 30,000 species) are represented in “GenBank” our view of the microbial world is far from complete. A complete cataloging of Earth’s microbial biota is needless and of course, impossible. Still, we can only –and should- under take representative survey that would give us clues to understand the unseen. We should also know that only about 13,000 bacteria represented in “GenBank” have been formally described and almost all of them (~ 90%) lie within 4 of the 40 bacterial divisions (DeLong, 2001). A recent estimate suggests that the sea may support 2 million different bacteria (Curtis et al., 2002) but we know that our ability to culture and study them under laboratory conditions is very far from satisfactory. The term “the great plate count anomaly” coined by Staley and Konopka (1985) to describe the difference in orders of magnitude between the numbers of cells that form colony forming units (CFU) on nutrient media and that countable by microscopic examination. Marine ecosystem is a typical example of this phenomenon: only 0.01 to 0.1% of marine bacteria are culturable. There are different explanations for this – the growth state of cells in nature, dormancy, exceptionally high concentration of nutrients and complex organic carbon in laboratory media. Amann et al. (1995) gave a compilation of data on the culturability determined as a percentage of culturable bacteria in comparison with total cell counts:

Habitat	Culturability
Seawater	0.001-0.1
Freshwater	0.25
Mesotrophic	0.1-1
Lake	0.1-3
Unpolluted	1-15
Estuarine water	0.25
Activated sludge, } Sediments, Soil }	0.3

One of the biggest challenges for marine microbiologists today is how to increase the percentage of culturability or in other words how to culture these unculturables. Recently, high-throughput culture (HTC) methods were developed, which allowed large number of microbial isolates to be recovered by dilution to extinction in natural sea-water media

(Connon and Giovannoni, 2002). Button and colleagues pioneered an approach that has been successful in isolating oligotrophic, heterotrophic cells from marine ecosystems (Button et al., 1993). This method uses un-amended environmental water as the medium and is often referred to as “extinction culturing” to distinguish it from dilution culturing, which also uses natural water, but involves complex microbial communities. Their approach was to dilute natural communities of microorganisms to a known number, ranging from 1 to 10 cells per tube and then examine these potential cultures for microbial growth by flowcytometry. By this method bacterial culturability varied from 2 to 60% in marine waters around Alaska and the Netherlands (Button et al., 1993). Even though new species have been isolated, the method is laborious. If culturing methods are going to be difficult, then what are the other ways of understanding them? Application of molecular-phylogenetic methods to study natural microbial ecosystems without the traditional requirement for cultivation has resulted in the discovery of many unexpected evolutionary lineages. Over the last three decades, researchers have compiled an increasingly robust map of evolutionary diversification showing that main diversity of life is microbial, distributed among three primary-relatedness groups or domains: Archaea, Bacteria and Eucarya. However, understanding the connection between the structure of the bacterial communities and their ecological function, would still be a difficult task without pure cultures to study them under controlled conditions in the laboratory.

In one of the recently published article on, “Microbes, Molecules and Marine Ecosystems” by Azam and Worden (2004) we see that heightened concerns for ocean health and biodiversity, highlighted in the 2003 Pew Oceans Commissions report prompted microbial explorations of the sea with state-of - art tools such as satellite and laser based imaging, as well as massive genomic surveys rivaling the human genome project. As life science research becomes more sophisticated, so does the instrumentation needed to gather quality data and accurately analyze results. For example, the discovery of picophytoplankton (<2-3 microns in size) in late 1970s have contributed enormously to understand the role of these unicellular photosynthetic organisms in the carbon cycle of the oceans. The recent development of genomics of picoplankton species such as *Prochlorococcus*, *Synechococcus* and *Ostreococcus* provide new and fundamental insights. Use of flowcytometry has tremendously contributed to the knowledge of these organisms. Scientists are now exploring new techniques and methodologies that can be applied to improve our understanding.

Microbial ecogenomics (also called environmental genomics or metagenomics) is a new emerging field that utilizes such novel technologies having great potential to understand physiology and role of unculturables in the ocean. Characterization of rRNA genes from environmental DNA extracts has helped us to characterize phylotypes occurring in nature. However this method does not give any information on physiology, biochemistry and ecological functions of uncultivated microorganisms. This is now being tackled by identifying the functional genes (protein-coding genes) through Bacterial Artificial Chromosomes (BAC) and fosmids. The introduction of BAC and fosmid vectors in 1992 has dramatically improved our genomic cloning efforts. The advantage of BAC vectors is that they can stably maintain and replicate inserts larger than 300 kb. Even though the usefulness of this technique was realized late, scientists were excited when the initial results came in. The study using this method gave the first glimpse into the genomic organization and protein composition of the marine Archaea. Subsequently the construction of a marine picoplankton BAC library was achieved only in the year 2000. While reviewing the role of BACs in marine ecogenomics, Beja (2004) highlights the discovery of a novel light-driven proton pump (proteorhodopsin, a bacteriorhodopsin homolog) on a 150 kb BAC fragment coming from the uncultured SAR86 group (gamma proteobacterial group; Beja et al., 2000). This new light harvesting apparatus was new to science and was not expected in a bacterial genome, certainly not in a marine bacterium. Interestingly, since then, proteorhodopsins have been reported from different oceans and divergent bacterial groups. Several BAC and fosmid libraries have now been constructed from different marine environments (Pacific, Red Sea, Mediterranean Sea, Antarctica and deep sea hydrothermal vent organisms; Beja, 2004).

Recently, a new approach was introduced to ecogenomics, the shotgun sequencing of entire marine communities without the need to construct large-insert clone libraries. This technique became popular in the Human Genome project. This approach is now being used by J.Craig Venter, from the Centre for the Advancement of Genomics, in an attempt to sequence and achieve the inventory of the Sargasso Sea (Venter et al., 2004). A total of 1.045 billion base pairs of non-redundant sequence was generated with an estimated 1800 genomic species, including 148 novel bacterial phylotypes. The authors report finding more than 782 new proteorhodopsin-like photoreceptors in their libraries.

Ecogenomics, direct environment shotgun sequencing, genomics of cultured microbes and functional genomics, together with different microbial

ecological methods such as microarrays, real-time PCR, FISH (fluorescence in *situ* hybridization) and microautoradiography and new cultivation techniques will contribute significantly to our understanding of the hitherto uncultivated microbes of the sea. However, attempts to develop and refine culturing techniques should continue, as that is the only way to have better background material for further development of molecular techniques and also to enhance the understanding of function and structure. Developing methods that will mimic the environmental conditions, from which the samples are drawn, would be the better option to bring more organisms into culture. We should also remember that there is no single technique available today that can catch the entire diversity of a microbial community.

The challenges for developing appropriate methods to study the animal-microbe interactions are much more complicated as compared to the independent populations of microbes. People have succeeded in isolating several microorganisms associated with the marine animals. But still there are groups that are intimately associated with the animal tissues as in hydrothermal vent organisms: tube-worms and mussels exhibiting unique opportunities for detailed investigations. Many bioactive compounds isolated from higher organisms were traced back to those microbes associated with them and not necessarily to the animal itself. Very recently, it was reported that microbial communities in sponges collected from different oceans were same as per molecular evidences. Let me give more information on this interesting finding (Hentschel et al., 2002). Sponges (Class: Porifera) are evolutionarily ancient metazoans (600 million years old) that populate the tropical oceans in great numbers (~9000 species) but are also known to occur in temperate and freshwater regions. Functionally, sponges share many features with unicellular protozoa. Instead of organs or tissues, sponges possess amoeboid cells that move freely through the three dimensional sponge matrix, termed the mesohyl. Nevertheless, they reach one meter or so in height. Sponges are filter feeders that pump large volumes of water through unique and highly vascularized canal system, leaving the expelled water almost sterile. Nutrients are acquired by phagocytosis of bacteria present in the seawater. However, sponges harbour large amounts of bacteria in their tissues that can account for 40% of their total biomass. Recognizing the fact that sponges have been good source of novel bioactive molecules their intimate association with bacteria calls for more attention. It is most intriguing to note how sponges in different oceans have a uniform structure in their bacterial community. More and more we investigate, more and more surprises may be in store.

Our understanding of the ocean biodiversity, as a whole, still remains, in its infancy in spite of so many modern molecular techniques and advanced instrumentation. Obviously one of the major reasons is the enormity of the system that we are trying to understand – the oceans, which cover 71% of the earth surface. Secondly all these initiatives require massive investment in terms of cost and manpower. Thirdly, more efficient technologies for estimation and identification are needed. Considering this scenario, scientists from different parts of the world, initiated a globally coordinated programme in 2000 called “Census of Marine Life” (CoML) and it is planned to be completed in 2010 (<http://www.coml.org>). Dr.J. Fredrick Grassle, Chairman, International Steering Committee of the CoML explains the strategy as follows: “ Overall, the strategy of CoML is to clarify and make much more accessible what we know, to identify what we do not know. To know much more of what is knowable and also to identify what we may never know or atleast not learn for a very long time, well beyond the life of the research programme. While audacious, CoML humbly recognizes the formidable limits to knowledge. Good reasons including size and the inaccessibility of the deep explain why no one has before tried the task of CoML”. Already more than 300 scientists from 53 countries are involved in this global programme. In 2004, India has joined this programme along with other Indian Ocean countries. As microbes (Bacteria, Archaea and Protista) account for most of the oceanic biomass, a major initiative to understand the ocean’s hidden majority has been started very recently under CoML. As a prelude to the initiation of this programme, a strategic planning workgroup meeting was conducted in November 2003 at the Monterey Bay Aquarium Institute, Moss Landing, CA, USA. During this meeting several fundamental questions were raised which need our attention:

- What accounts for large-scale genetic variation in microbial genomes that share a very recent common ancestry? Is there a cryptic source of genetic information that selectively invades microbial genomes or, are there undocumented mechanisms that can rapidly generate novel coding capacity within a bacterial chromosome?
- What are the roles of the many hypothetical genes of unknown function that are either unique within or shared between nearly all sequenced genomes?
- Do chemical environments select for lineages endowed with particular metabolic capabilities or does the unit of selection correspond to individual genes that can transfer particular metabolic functions between lineages?
- How widespread is horizontal gene transfer? Do viruses mediate this process?

- Why do complex microbial consortia retain functionally equivalent but genetically distinct lineages – for example, many different kinds of sulfate reducers or methanogens persist in anoxic settings – rather than selecting for a single “winner” with an optimal suite for metabolic activities?
- When interactions occur between different kinds of microbes, is there intercellular communication at the genome level? What are the emergent functional properties and how do they transcend activities of the individual parts?
- Does the diversity of a microbial guild relate to the stability of the functioning?
- Is there a biogeography for distinct microbial lineages and, if so, what are the principal drivers or restrictors? What genomic changes, if any, are associated with relocation of dormant organisms over large distances?
- How does genotypic diversity shape phenotypic diversity, and how does this diversity influence the functioning of ecosystems?

Finding answer to these questions is a challenge but cannot be ignored. The future research will be concentrating on understanding of the microbial community structure, mostly by *in situ* techniques, and their role in biogeochemical cycles, in which many transformations are catalyzed by consortia and not by single species of microorganisms.

Another very interesting development in the recent past is that the microbiologists are seriously thinking of applying “systems biology” concept to understand and harness complex biological processes in microbial communities. Systems biology has been defined by Ideker et al. (2001) as an approach to study “biological systems by systematically perturbing them (biologically, genetically or chemically); monitoring the gene, protein, and informational pathway responses; integrating these data; and ultimately, formulating mathematical models that describe the structure of the system and its responses to individual perturbations”. Considering the growing relevance of this approach, a workshop was conducted recently on, “Promise and challenges in System Microbiology” (2004; <http://www.nap.edu/>) which summarises as follows: “A system approach, which attempts to use comparative, high-throughput assays and mathematical or computational models, has been used to generate a picture of system-wide activity that can yield insight into processes operating within a single cell. But the concept of integrating advances in genomics, proteomics and metabolomics and incorporating them into mathematical models can also be applied to microbial ecosystems, which typically occur in consortia of related and unrelated organisms. Research on microbial communities using a system based approach could provide a broader perspective on controls on biological processes and

how they operate in and among microorganisms. Although some infrastructure for systems microbiology – such as high-throughput DNA sequencing tools and methods – are similar to those needed for other genome projects, it requires additional tools to address issues specific to microbial communities; for example metagenomics (that is the analysis of the sum of all genomes in an environment) will facilitate studies of microbial communities”.

Marine microbiology has already started contributing significantly to the area of biotechnology – novel enzymes, bioactive compounds (antimicrobial and anticancer), nutraceuticals (PUFA), bioremediation and diagnostics. Researches on nanobiotechnology especially on nanomachines and biocomputing in future may increasingly depend on marine microbes as they have shown their ability to withstand extremely high pressure, very low temperatures and high salt concentrations. Can they also contribute to the development of biological fuel cells that can be used to generate electricity? Recently scientists have shown the method of harvesting electricity (on the order of 0.01 W/m² of electrode) from aquatic sediments by burying electrode in anoxic sediments and making an electrical connection between this electrode and a similar electrode in the overlying aerobic water. The recovery of electricity from these sediments is analogous to that from previously described biological fuel cells. Biological cells use the natural catalytic ability of microorganisms to oxidize a wide variety of substrates, while still producing electrons in a form that can be harvested at an electrode. For example, fuel cells utilizing

sulfate-reducing bacteria have been constructed, with the microbially produced hydrogen sulfide serving to shuttle electrons to the electrode surface. Interestingly there are other bacteria such as *Shewanella putrefaciens*, *Aeromonas hydrophila* and several species of Geobacteraceae are known to transfer electrons from the oxidation of organic compounds directly to the electrode surface without the need for any shuttle. Biological fuel cells are considered much more efficient than the internal combustion engines. So microbial diversity can give very efficient strains for this purpose. Studies with *Desulfuromonas acetoxidans*, a marine representative of Geobacteraceae showed the possibility using them for such fuel cells. Even though many such reports are available, the mechanism of electron transfer and its relevance to cell growth have not yet been fully understood.

In a similar area of energy survey, gas hydrates in ocean sediments had attracted the attention of scientists as an alternate source of energy in future. Gas hydrates are natural crystalline deposits

consisting of a cage of water molecules surrounding gas molecules (methane). These structures are formed under conditions of high pressure and low temperature as occurring in certain deep-sea locations. Studies have shown that this methane is predominantly of biological origin in marine sediments but our knowledge of bacterial communities responsible for hydrate formation still remains limited. As these deposits are normally seen in deep oceans, sometimes hundreds of meters below the sea floor, locating them with cost effective methods is still a major challenge for the scientists. Most of the studies conducted so far have been limited to shallow sediments obtained from seafloor hydrate deposits near methane sweeps. These studies further gave indirect evidence of methane oxidation by sulfate reducing bacteria (SRB). However we still do not know the fate of methane formed in deeper sediments and the nature of microbial communities in such locations where sulfate becomes limited and bacterial abundance reduces. For most of these studies, technologies to collect samples from remote locations such as deep-sea hydrothermal vents exist today but we do not have appropriate methods to culture all members of the microbial communities. Not all countries have facilities like *in situ* pressure retaining samplers, cultivation tanks simulating deep-sea pressures and sub-surface remotely operable vehicles (ROV) leave alone the human occupied vehicles (HOV).

Another major handicap is the lack of exclusive culture collection centers for marine microbes in many countries. Pure culture is still the most powerful tool of Microbiology to study and exploit microbes. As on November 5, 2004 there are 491 culture collections in 65 countries and hardly 3 or 4 centers deal exclusively with marine microbes. It is true that many of the major centers have marine cultures in their collections but seeing the speed with which new species are being reported especially in last few years, it would be worthwhile to create more exclusive culture collection centers for marine microbes. Moreover, these centers should also develop as reference centers for literature, methodologies and training. It is satisfying to note that under the CoML programme an exclusive information center (Ocean Biological Information System-OBIS) for marine biota (including marine microbes) is being developed (<http://www.iobis.org/>).

As we all know, any attempt to make a new initiative to understand these aspects requires funding support and collaborative research. Many federal governments and private institutions now come forward to support research in marine microbiology. For example, in April, 2004 the Gordon and Betty Moore Foundation's Board (USA) approved a 10-

year, \$145 million Marine Microbiology initiative to generate new knowledge regarding the composition, function, and ecological role of microbial communities in providing the basis of the ocean's food webs and in facilitating the flow of nitrogen, carbon, and energy in the ocean (<http://www.moore.org/>).

Funding strategies for this initiative include:

- The support of research and related costs for selected Marine Microbiology Investigators.
- The support of small interdisciplinary groups including oceanographers, computer scientists, and selected Marine Microbiology Investigators
- The establishment of undergraduate summer intern programs in each supported Investigator's laboratory.
- Grant-making for a limited number of large, high impact research projects that will affect ocean science as a whole.

The GBM Foundation has already approved research projects on the following topics:

1. Ecology and evolution of the marine cyanobacterium *Prochlorococcus*. (Dr. Sallie W. Chisholm, MIT –US\$ 5.5 million –for 5 years).
2. Genetic diversity of planktonic marine bacteria and archaea (Dr. Edward F. Delong, MIT – US\$ 5.3 million – for 5 years).
3. Genetic diversity and physiological capacity of microbes in the ocean.(Dr. David Karl, University of Hawaii- US\$ 3.85 million –for 5 years)
4. Roles of bacteria in the structure and function of marine ecosystems and in biogeochemical cycling (Dr. Mary Ann Moran, University of Georgia – US\$ 2.66 million – for 5 years).
5. Development of remote-sensing probes and sampling procedures for the study of nitrogen-fixing marine microbes (Dr. Jonathan P. Zehr, University of California, Santa Cruz – US\$ 4.19 million – for 5 years).

The above funding strategy clearly indicates the areas of concern and support. The European Science Foundation (association of 67 major national funding agencies devoted to scientific research in 24 countries) has also evolved similar strategies to support research in the area of Marine Biotechnology (<http://www.esf.org/>).

Directions for India

Marine microbiology, as a research area, has been attracting the attention of scientists since 1966 (Chandramohan, 1997; 1999) and researchers have worked in various areas depending on their

competence and resources. However, during the last two decades, we saw very significant contributions to this branch of science in many areas including bioremediation, sulfate reducing bacteria (SRB), fungal parasitology, coastal heterotrophy and molecular biology. Interestingly there have been many contributions to understand the microbiology of Antarctic Sea and deep sea (Indian Ocean; Loka Bharathi, et al., 2001; Pradeep Ram, et al., 2001). Extensive studies on the occurrence, distribution and species composition of luminous bacteria in the Arabian Sea were carried out over a period of four years (Ramaiah and Chandramohan, 1992a-c). The utility of some of these bioluminescent bacteria in pollution monitoring was also demonstrated (Ramaiah and Chandramohan, 1993; 1994). Very recently scientists have shown interest in understanding of the structure and functioning of the microbial communities in coastal waters and estuaries (Pradeep Ram, et al, 2003a, b; Maria-Judith B.D. De Souza, et al., 2003). Advances made in this field in India and in other parts of the world have been compiled by LokaBharathi,et.al. (2003) in the book, "The Indian Ocean - A perspective". Even after working for more than three decades, we see that we have missed out several other groups of microorganisms and failed to study their functions that may contribute significantly to the changing global climate.

Considering the developing global scenario and our current problems we should pool our resources and invest in the following areas in the years to come:

1. We have not yet started any serious work on construction of metagenomic libraries of marine environmental samples. As the tropical seas are so rich in diversity, many new species or novel genes may be discovered if we systematically study different niches.
2. Development of new methods for culturing the uncultivated species. As we have seen, there are only a few success stories but not enough.
3. Deep-sea microbiology – We know little about the microbes of the deep-sea for want of infrastructure facilities such as hyperbaric culture facilities, pressure-retaining samplers etc. So far we never had any opportunity to work with deep-sea hydrothermal vents as they were known to be present mostly in Atlantic and Pacific. We have located one good area in the Indian Ocean, and we should improve our capabilities to harness this resource.
4. Microbiology of the anoxic regions of the coastal seas (Arabian Sea).
5. Understanding the microbial communities and their functions – coastal seas and estuaries, submerged surfaces and animal associations.
6. Development of early warning systems for toxic algal blooms in the EEZ of India.
7. To date a very large amount of data has been gathered on picophytoplankton. Information on ecological distributions and taxonomic diversity has come from novel approaches such as flowcytometry and molecular biology. In India we have not yet used these techniques and once we gather information on these prochlorophytes, our concept on carbon budget may even require a revision.
8. Studies on marine Archaea have to be strengthened and more research is necessary with sulfate reduction and methanogenesis.
9. New initiatives in nanobiotechnology and biological fuel cells.
10. It is high time to start new programmes to study the role of marine viruses, especially bacteriophages, in ecosystem functioning. It is true that viruses were not studied until 1989 even though they are reported to be the most abundant biological entities in the oceans (often their numbers reaching 10^7 ml⁻¹). There are reports to show that they influence the carbon cycle significantly and enhance the upper ocean respiration.

There can be many more areas of interest that may become more relevant in the days to come. For example, the role of bacteria in the production of greenhouse and anti-greenhouse gases (DMS), known to influence our climate will attract more attention and funding. We should also explore the possibilities of introducing the approach of "systems biology" involving mathematical or computational models in our research programmes. More centralized facilities and development of appropriate specialized courses in Universities (capacity building) in our country would go a long way in making these new initiatives a great success.

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