Microbial Biodiversity Investigation techniques

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Microorganisms represent the foundation of life on earth. They impact life on earth through their activities that effect the biogeochemical cycles to their beneficial uses and their pathogenic properties. The survival, propagation and ability of microorganisms to inhabit a wide variety of environments and habitats demonstrate their evolutionary success. This versatility inspired several scientists to consider how microorganisms could impact life on other planets. There were several restrictions that limited the ability to study and to explore microbial life on earth. The majority of the traditional microbiology techniques and methods that required growing microorganisms on lab based media, isolating them, producing pure colonies to study their physiology and morphology. However recent developments such as direct DNA extraction from water and soil samples coupled with the new sciences of Bioinformatics, Proteomics and Genomics enhanced our ability to study and describe new microorganisms that have not been described previously. The concept that environmental factors influence the functionality of microorganisms and its correlation with the formation of new proteins gave a new insight into microbial evolution and adaptations. This has enhanced the exploration of a vast number and variety of microbial life with a better understanding of their ability to survive and propagate in new environments. This chapter describes the techniques used in the isolation and describing of new bacteria from water and soil samples. These activities include periodical water sampling from selected areas of the West Bridgewater section of the Hockomock Swamp. Microbial identification followed two paths through the traditional cultural isolation procedures that included morphology and biochemistry methods to identify pure cultures of isolated microorganisms. DNA extractions from pure colonies were compared to direct DNA extraction from samples. This added a new dimension of identification of microbial life through non-culturing methods. Other activities include PCR-denaturing gradient gel electrophoresis (PCR-DGGE) analysis, 16S rRNA, phylogenic tree, fatty acids analysis and protein sequencing. Once the 16S rRNA base-pairs were obtained BLAST searches was carried out to compare the nucleotide sequences were conducted against those in the National Center for Biotechnology Information (NCBI) nucleotide database at: (http://www.ncbi.nlm.nih.gov/index.html) and the European Molecular Biology Laboratories (EMBL) (http://www.ebi.ac.uk/embl/) prokaryote database. The chapter will include sequences of new bacteria, their FAME analysis results and phylogenies tree alignments.

Keywords, Biodiversity, Phylogenies, novel species, bacteria, FAME.

1. Introduction:

Biodiversity is the variety of life on Earth at all its levels, from genes to ecosystems, and the ecological and evolutionary processes that sustain it (Gaston, 1996). Biodiversity is not limited to describe the various species living in a certain habitat but it also identifies the genetic variation and the functionality of these species within their ecosystems. These parameters would be useful in the identification of the many unknown and undescribed microbial species living on earth. Microbial Biodiversity is gaining more importance not only to understand their evolution but also to further determine their ecological impact. Microbial biodiversity encompasses the variability of microorganisms. A publication by the Center for Microbial Ecology in 2003 suggests that there may be up to 1 million species of prokaryotes yet only 3,100 are known and have been fully described in Bergey's Manual. Microorganisms are essential for life on earth to function, maintained and continue. They play many roles on land, air and water, including being the first to colonize and transform effects of naturally occurring and man-made disruption to the environment. Nations has committed to protecting the environment, and has included ecological diversity in this goal (Cohen 2002).

Bacteria were the only free living cellular organisms some 3.5 billion year ago. Their survival, propagation and ability to inhabit a wide variety of environments demonstrate their evolutionary success. This remarkable versatility inspired several scientists to consider the ways in which this may impact other planets. There have been discussions that some form of microbial life does exist on some planets (Rummel 1996 & 2000). There have also been discussions on the issue of planetary protection and the affect of possible deposition of bacteria, such as *Bacillus,* on other planets such as Mars (Rummel 2000) reflecting the ability of microorganisms to survive and propagate in any environment.

The fact that there may be microbial life on other planets does signal the need to explore the vast amount of microbial life here on earth. The methods to explore microbial life on earth were limited through the use of traditional microbiology techniques and methods. However, the recent development of new molecular techniques including PCR-denaturing gradient gel electrophoresis (PCR-DGGE) analysis, 16S rRNA, phylogenic tree, fatty acids analysis and protein sequencing have increased our knowledge of the depth of microbial life that exist. We now have a greater understanding of the vast diversity of microorganisms inhabiting the environment from soil to the deep sea, from fresh water to volcanic ash.

These advances have assisted in the identification and classification of bacteria through direct DNA extraction from water and soil samples then sequencing the isolated DNA. This chapter review's both traditional culture dependent techniques that include molecular microbiology methods and the non-cultural that are molecular based in nature. This

would include work carried out on the Hockomock Swamp with its associated wetlands and water bodies that comprise the largest vegetated freshwater wetland system in Massachusetts. Some of these procedures were already used in the isolation and identification of a new species of *Bacillus. Bacillus samanii so nov* that was isolated from snow covered soil.

The identification process involved culture based techniques, 16S rRNA sequencing and Fatty Acid Methyl Ester Analysis (FAME). The phylogenic analysis based on 16S rRNA gene sequences that were deposited with the Genbank (sequence GenBank accession EF036537 indicated that the isolate belonged to the genus *Bacillus*, and the *Bacillus cereus* group. It was closest to *Bacillus cereus* with 0.40% alignments and 0.69% to *Bacillus thuringiensis.* The cellular fatty acid profiles were closest to *Bacillus mycoides* with the iso-C15 making 21.25% of the total cellular membrane fatty acids. On the basis of phenotypic and molecular data, the strain represents a novel species within the *Bacillus cereus* group and it's named *Bacillus samanii*. A culture deposit was made to the Biodefense and Emerging Infections Research Resources Repository (BEI Resources) with the BEI code *(*NR-4056),(Saman etal 2007,2008,20102. Culture based Molecular Techniques:

2. Culture based Molecular Techniques:

2.1 Isolation of Pure Colonies of Microorganisms:

Bacteria could be grown on lab based media that would furnish their required growth factors such as sugars, proteins, vitamins and minerals. Sample must be diluted to levels that would reduce the number of growing colonies to a controllable level. Dilution series of 3 test tubes containing sterile 0.9% peptone water in 9 ml tubes with 1 ml of water samples. For soil & river sediment samples 1 gm of the sample should be added to 9ml of peptone water then the same dilution series will be used for the water samples. Using a mineral solution that would provide sources of nitrogen, potassium, sodium, magnesium, sulfate, calcium and chloride added to basal media providing the resources for the growth of various microorganisms. The sodium requirement would ensure the media would support the growth of halophilic species which may be present in the collect samples. The same procedure of dilution would be used but this time using a vitamin solution that would support the growth of many types of Methanogens and types of *Clostridium* that would not grow other wise. The vitamin solution would contain Pyridoxine, Thiamine, Riboflavin, Vitamin B12, Biotin and folic acids in addition to others. (Apajalahti etal 2002**,** Natakus 2007 & Saman 2010).

2.2 Fatty Acid Analysis:

Short chain fatty acid analysis also known as volatile fatty acids (VFA), could be used for the identification of bacteria as they could serve as a second finger print for bacteria. These fatty acids which are found as part of the cell membrane bilipid layer have a length chain of 9 - 20 carbons. This method was used for genus & species identification that shared most of their fermentative abilities.Gram-positive bacteria had more branched fatty acid chains compared to gram negatives that had short chains of fatty acids as this is related to their lipopolysaccharides that make up most of their cell wall. Cellular fatty acids analysis uses gas chromatography of fatty acid methyl esters (FAME) interpret the results. Isolates should be inoculated onto Trypticase soy agar for 24 hours at their optimum growth temperatures. Four reagents are required to cleave the fatty acids from lipids in a five step process that ends with the GC, (Yang etal, 1993 Sasser, 2006, Saman etal 2010).

2.3 Genomic DNA Extraction:

The process involves the harvesting of cells then suspending them in TE buffer. Lysis of a microbial pellet using the Lysosomes and RNAase solution for 15 - 30 min. Lysate solubilization is to be followed by DNA precipitation. The extracted DNA is washed using - 1 ml 75% ethanol then at the last stage DNA Solubilization with TE buffer, water or 8 mM NaOH,(Bowman 1994, Sogin 2006). The extracted DNA could be amplified through PCR and sequenced to determine relativity to known species.

2.4 DGGE- PCR Amplification and 16S rRNA Sequencing:

Denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting method. PCR polymerase chain reaction of the extracted genomic DNA can generate templates of differing nucleotide sequence that once the 16S rRNA fragments sequenced and the results analyzed through phylogenies a firm identification and comparison could be made. This technique that has proved to be useful is identification of new species. This method is being used to study the microbial biodiversity of the Hockomock Swamp resulting in the identification of more than 12 new previously undescribed microorganisms.

2.5. Nucleotide Sequence Accession Number:

The sequenced obtained will be deposited in the GenBank nucleotide sequence database under accession numbers repeating the steps taken for the *Bacillus samanii* EF036537. There are others that have been deposited at the GenBank such HS 1*Citrobacter hockomockus*- FJ756447, HS 5 II. *Leucobacter mimi*- FJ756448, HS 2 *Serriatia peterii*- FJ756449, HS 5 I. *Bacillus patriciaus*- FJ756450 and YIL *Rhodosporidium hockomockus*- FJ756452.

3. Non-Culturable Technique:

Many of the environmental living bacteria cannot grow on lab based media this had limited the identification of species to about 5% that could grow on lab based media. Non-culture based techniques involves the direct isolation of microbial genomic DNA directly from water and soil samples without the use of any growth media. These methods would enable the identification of microorganisms from various environment and study their interaction and enzymes functionality. This would allow a better understanding of how such microorganisms have evolved and their growth requirements (Mokni-Tlili etal 2009, Inceoglu etal 2010).

3.1. Direct Microbial DNA Isolation from Water & Soil:

In the Hockomock Swamp project WaterMaster DNA Purification kit from EPICENTRE Biotechnologies will be used as one of the methods for direct extraction of DNA from water samples. This Protocol involves filtration of water samples, cell lysis, DNA Precipitation and removal of inhibitors. The other method will again include filtration using multiple Millipore filters that would trap particle-bound microorganisms. Using proteinase K and lysozyme will be used for purification and cell lysis, SDS and chloroform will be used to enhance DNA extraction and ethanol will be used for precipitation. Bovine serum albumin (BSA) will be added before PCR to remove any humic acid that can attach to the Taq polymerase inhibiting the PCR reaction. DNA isolation from soil by technique involves acid washing, suspension in 0.1% (wt/vol) sodium pyrophosphate, and centrifugation. The pellets are to be resuspended in 0.15 M NaCl-0.1 M EDTA (pH 8.0) and used for DNA isolation by a modified Marmur method. This will be compared with a SoilMaster kit for direct DNA extraction from Epicenter Biotechnologies and DNAzol Direct, which is produced, by the Molecular Research Center, Inc for extraction of PCR ready DNA from various sources (Saman etal 2007,2008,2010).

3.2 Guanine and Cytosine (GC) %.:

One of the most important tests that should be conducted is the determination of the GC% in the extracted DNA. The GC ratio is the percentage of guanine and cytosine in the DNA of an organism. This is an important tool that can be used in identification of microorganisms. There are some guidelines that manifest the importance of this process, such as microorganisms with a low-GC content which is more likely to be gram- positive with the exception of *Mycoplasma* which does not have a cell wall and has a low GC content even though it stains as a gram negative. Low GC content could indicate either Non-Endospore forming gram-positive or Endospore forming gram positive or a *Mycoplasmas.*

Gram negative microorganisms will have a GC content of 65% and higher as will filament bacteria such as Actinomycetes. Related groups would only have a 3-5% difference in their G/C content (Tiedje et al. 1999). DNA denaturation will be at 85C followed then by using a UV-spectrophotometer. GC content will be determined at 260nm using gradient fraction which is determined by linear regression analysis of data obtained from control gradients containing standard DNA samples of known G+C. This method may be used for DNA of culturable or directly extracted DNA and it will indicate the type of bacteria that is being isolated and will assist in their identification. A library of all isolated bacteria with their GC content need to be established for use as a reference for future studies.

3.3 Single-Strand-Conformation Polymorphism Analysis & DNA Microarrays:

Single-strand-conformation polymorphism (SSCP) is a mutation analysis method that will be used in the analysis and differentiation of bacteria. 400bp fragments of the isolate or sample 16S or 28S rRNA gene is PCR amplified then they will be selectively digested and separated through electrophoresis. Analysis of the bands and comparison with template mixtures of known microorganisms will be used to distinguish the different types and the presence of any mutations(Atha etal 1998).

DNA-DNA hybridization is used with DNA microarrays to identify microbial species that could not be grown or cultured on lab based media. This method does not only determine the gentic make up of the identified microoragniams but also could determine the level of relativity and their evolutionay pathway. (Cho and Tiedje, 2001). This method coupled with the widely accepted criteria for delineating species in current bacteriology: these state that strains with DNA relatedness values of less than 70% or with more than 3% difference in their 16S rRNA gene sequences are considered to represent different species (Wayne et al., 1987).

4. Construction of Phylogenetic Trees:

BLAST searches to compare the nucleotide sequences obtained against those in the National Center for Biotechnology Information (NCBI) nucleotide database (http://www.ncbi.nlm.nih.gov/index.html) and the European Molecular Biology Laboratories (EMBL) (http://www.ebi.ac.uk/embl/) prokaryote database. The use of this practice is very important and is used in the identification of new previously undescribed species such as the identification of *Bacillus samanii*.

5. Examples of 16S rRNA Sequencing :

5.1. Isolate HS 7 Gram positive non-spore forming rod Bacteria:

TGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACAC
ATGCAAGTCGAACGCTGAAGCCCCAGCTTGCTGGGGTGGATGAGTGGCGA
ACGGGTGAGTAACACGTGAGTAACCTGCCCATCACTCTGGGATAAGCGCT
GGAAACGGCGTCTAATACTGGATACGAGCAGCGACCGCATGGTCAGCTGC
TGGAAAGACTGGTTCGGTGATGGATGGACTCGCGGCCTATCAGCTTGTTG
GTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGT
GACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCAACGCCGCG
TGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGTAGGGAAGAAGC
CTTCGGGTGACGGTACCTGCAGAAAAAGCACCGGCTAACTACGTGCCAGC
AGCCGCGGTA

5.2. Isolate Y1L- Yeast:

AGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAGCACTTTGGA
AAGAGAGTTAACAGTACGTGAAATTGTTGGAAGGGAAACGCTTGAAGTCA
GACTTGCTTGCCGGAGCTTGCTTCGGTTTGCAGGCCAGCATCAGTTTTCC
GGGGTGGATAATGGTGGTTTGAAGGTAGCAGCCTCGGCTGTGTTATAGCT
TTCCACTGGATACATCCTGGGGGACTGAGGAACGCAGCGTGCTTTTTGCG
AAGGTTTCGACCTTTTCACGCTTAGGATGCTGGTGTAATGACTTTAAACG
ACCCGTCTTGAAACACGGACCAAG

6. Conclusion:

The advancement of molecular microbiology techniques would allow the identification of a good number of microbial species have various habitats to live in. These techniques have proven their worth now as they ended the requirement that microorganisms must grow on lab based media so they could be isolated and identified. This would also provide further understanding on the interactive functionality of such microorganisms and their effect on their habitats. This would be a further tool to be used to understand microbial evolution and would provide considerable amount of information on microbial biodiversity. There is however a need to create libraries such as these for GC content and DNA microarrays to enable research a faster more efficient methods of determining if they are dealing with a new or a known species. These molecular techniques would also provide a better understanding to microbial phylogenies and taxonomy that

References

[1] Ash, C., and M. D. Collins. 1992. Comparative analysis of 23S rRNA gene sequences of *Bacillus anthracis* and emetic *Bacillus cereus* determined by PCR-direct sequencing. FEMS Microbiol. Lett. 94:75-80.
[2] Atha,D.H., Wenz,H.-M., Morehead,H., Tian,J. and O'Connell,C.D. (1998) Detection of p53 point mutations by single-strand conformation polymorphism (SSCP): analysis by capillary electrophoresis. *Electrophoresis*, 19, 172–179.
[3] Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, and J. Nienhuis. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. 62:1935-1943.
[4] Bowman J.P. and Sayler G.S. 1996. Nucleic acid techniques in the environmental detection of microorganisms and their activities. Molecular Approaches to Environmental Microbiology, 4, p. 63-97.
[5] Brinkmeyer, R., K. Knittel, J. Jürgens, H. Weyland, R. Amann, and E. Helmke. 2003. Diversity and structure of bacterial communities in arctic versus Antarctic pack ice. Appl. Environ. Microbiol. 69:6610-6619.
[6] Cho JC, Tiedje JM (2001). Bacterial species determination from DNADNAhybridization by using genome fragments and DNA microarrays. Appl. Environ. Microbiol. 673: 677-3682.

[7] Cohan, F.M. 2002. What are bacterial species? Annual Review of Microbiology, 56, p. 457-487.
[8] Crawford R.L , Microbial Diversity and Its Relationship to Planetary Protection. Applied and Environmental Microbiology, August 2005, p. 4163-4168, Vol. 71, No. 8.
[9] Dunbar, J., L. O. Ticknor, and C. R. Kuske.2000 . Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Appl. Environ. Microbiol. 66:2943-2950.
[10] Foght, J., J. Aislabie, S. Turner, C. E. Brown, J. Ryburn, D. J. Saul, W. Lawson. 2004. Culturable bacteria in subglacial sediments and ice from two southern hemisphere glaciers. Microb. Ecol. 47:329-340.
[11] Gaston, K.J. 1996. What is biodiversity? pp. 1-9 in Gaston, K.J. (ed.). *Biodiversity: A Biology of Numbers and Difference.* Blackwell Science, U.K. 472 pp.) .
[12] Inceoglu, O., Hoogwout, E. F., Hill, P., van Elsas, J. D. (2010). Effect of DNA Extraction Method on the Apparent Microbial Diversity of Soil. *Appl. Environ. Microbiol.* 76: 3378-3382.
[13] Mokni-Tlili, S., Jaoua, L., Murano, F., Jedidi, N., Hassen, A. (2009). Study of the effects of urban organic residues on the distribution of culturable actinomycetes in a Tunisian agricultural soil. *Waste Manag Res* 27: 224-232.
[14] Nakatsu C.H. Soil Microbial Community Analysis Using Denaturing Gradient Gel Electrophoresis. 2007. Soil Sci Soc Am J 71:562-571.
[15] Priorities for Microbial Ecology-Microbial Biodiversity Research Centre- University of Michigan- 2003.
[16] Rummel, J. D. 2000. Implementing planetary protection requirements for sample return missions. Adv. Space Res. 26:1893-1899.
[17] Rummel, J. D., and M. A. Meyer. 1996. A consensus approach to planetary protection requirements: recommendations for Mars lander missions. Adv. Space Res. 18:317-321.
[18] S. Saman, Sudad Saman & Patty Slattery. (2010) Isolation, Description & Novel Species *Bacillus samanii* sp nov. From Snow. Accepted with revisions- Microbiology Insight.
[19] S. Saman, P. Slattery & S. Saman- (2009) Isolation, Description & Sequencing of a number of Novel microorganisms isolated from the Hockomock Swamp in Massachusetts. 3rd ASM Conference on Prokaryotic Development.
[20] S. Saman, Sudad Saman & Patty Slattery. (2008) Isolation & Characterization of protein toxins produce by A Related *Bacillus anthracis* & cereus Novel Species *Bacillus samanii* sp nov. 2008 ASM Biodefense and Emerging Diseases Research Meeting, Washington DC.
[21] S. Saman, Sudad Saman & Patty Slattery. (2007) Isolation, Description & Sequencing Of A Related Bacillus anthracis & cereus Novel Species Bacillus samanii sp nov. From Snow.2007 ASM Biodefense and Emerging Diseases Research Meeting, Washington DC.
[22] Sasser, M. 2006. Identification of bacteria by gas chromatography of cellular fatty Acids. MIDI Labs Incorporated Technical Note #101, p. 1-6.
[23] Schmidt, T. M., E. F. deLong, and N. R. Pace.1991 . Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. 173:4371-4378.
[24] Seung Seob Bae, Jung-Hyun Lee and Sang-Jin Kim, 2005. *Bacillus alveayuensis* sp. nov., a thermophilic bacterium isolated from deep-sea sediments of the Ayu Trough International Journal of Systematic and Evolutionary Microbiology (2005), 55, 1211–1215.
[25] Sogin M, Morrison H, Huber J, Welch D. M, Huse S, Neal P, Arrieta J, & Herndl G. 2006. Microbial diversity in the deep sea and the underexplored ‘‘rare biosphere’’. PNAS: 103(32) :12115–12120.
[26] Tiedje JM, Asuming-Brempong S, Nusslein K, Marsh TL, Flynn SJ(1999). Opening the black box of soil microbial diversity. Appl. Soil Ecol. 13: 109-122.
[27] Torsvik, V., F. L. Daae, R. A. Sandaa, and L. Ovreas.1998 . Novel techniques for analysing microbial diversity in natural and perturbed environments. J. Biotechnol. 64:53-62.
[28] Wang L, Lee F, Tai C, and Kasai H. 2007. Comparison of *gyrB* gene sequences, 16S rRNA gene sequences and DNA–DNA hybridization in the *Bacillus subtilis* group Int J Syst Evol Microbiol, 57: 1846 - 1850.
[29] Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol 37, 463–464.
[30] Zhang, X. J., X. J. Ma, T. D. Yao, and G. S. Zhang. 2003. Diversity of 16S rDNA and environmental factor affecting microorganisms in Malan ice core.Chin. Sci. Bull. 48:947-957.

