CHAPTER 1

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1.1 Microbial ecology

The term ecology is derived from the Greek words oikos (household or
dwelling) and logos (law). Thus, ecology is “the law of the household” or, by its
contemporary definition, the science that explores the interrelationships between
organisms and their living (biotic) and nonliving (abiotic) environments. Microbial
ecology is the science that specifically examines the relationships between
microorganisms and their biotic and abiotic environments (Atlas and Bartha, 1998).
It relates bacteria, fungi, algae, protozoa and viruses to the many types of
environments in which these small organisms inhibit – aquatic environments
(freshwater, seawater, hot spring, marine) and terrestrial environments (soils and
landfill) (Alexander, 1971).

1.2 Soil

Soil represents a highly heterogeneous environment consisting of solid,
liquid and gaseous phases. The dominating solid phase in soil is composed of
inorganic (sand, silt and clay) and organic (humic matter) materials, which are to
varying degrees complexed with one another. The soil biota, including soil
microorganisms such as bacteria, fungi and protozoans, are known to inhabit
different sites in the soil pore matrix. Organisms themselves associate in particular
with soil solid, e.g. clay/organic matter complexes, in soil pores conductive to their
survival. Although a wealth of information is available about many microorganisms
isolated from soil by culturing techniques, the nature of a substantial part of the soil
microbiota is essentially unknown due to their unculturability (van Elsas, 1995). All

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soils and sediments (unconsolidated or 'loose' deposits) are composed of particles with a wide range of sizes. These are generally divided into 3 major groups: gravel (greater than 2 millimetres), sand (0.062 – 2 milimetres) and mud (silt and clay). The mud fraction is further divided into coarse silt (62-15.6 μm), fine silt (15.6-3.9 μm) and clay (less than 3.9 μm). A graded scheme for soils is given by the Wentworth Grade Scale (Table 1.1; Folk, 1974). The proportion of clay, silt and sand, together with the grain size, dictate the permeability (or hydraulic conductivity) of the soil to water, which influences soil salinity and water content. Nutrient status is also affected by the physical composition of the soil, with clay soils generally higher in nutrients than sandy soils.

**Table 1.1  Wentworth Grade Scale (adapted from Folk, 1974).**

<table>
<thead>
<tr>
<th>Materials</th>
<th>Grade limits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm</td>
</tr>
<tr>
<td>Boulder</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Cobble</td>
<td>256-64</td>
</tr>
<tr>
<td>Pebble</td>
<td>64-4</td>
</tr>
<tr>
<td>Granule</td>
<td>4-2</td>
</tr>
<tr>
<td>Very coarse sand</td>
<td>2-1</td>
</tr>
<tr>
<td>Coarse sand</td>
<td>1-0.5</td>
</tr>
<tr>
<td>Medium sand</td>
<td>0.5-0.25</td>
</tr>
<tr>
<td>Fine sand</td>
<td>0.25-0.125</td>
</tr>
<tr>
<td>Very fine sand</td>
<td>0.125-0.062</td>
</tr>
<tr>
<td>Silt</td>
<td>0.062-0.0039</td>
</tr>
<tr>
<td>Clay</td>
<td>&lt;0.0039</td>
</tr>
</tbody>
</table>
1.3 Mangrove soil

Since mangrove soils are typically waterlogged, and hence anaerobic, microbial decomposition takes place through a series of oxygen-reduction (redox) processes. The redox potential (Eh) is a quantitative measure of reducing power which provides a diagnostic index of the degree of anaerobiosis or anoxia (Patrick and DeLaune, 1977). Completely anoxic sediments have redox potentials below -200mV, while typical oxygenated soils have potential of above +300 mV. The measurements of Eh has been used to describe a marine sediment (Pearson and Stanley, 1979). Reliable measurements of redox potential require great care in order to minimize exposure of the soil sample to air.

The acidity of the soil influences the chemical transformation of most nutrients and their availability to plants. Most mangrove soils are well buffered, having a pH in the range of 6 to 7, but some possess a pH as low as 5. Measurement of the acidity or alkalinity of soils using pH must be done with fresh samples to avoid oxidation of iron pyrites (a common constituent of mangrove soils) to sulphuric acid, thus giving a much lower value of pH than normally occur in situ.

1.4 Diversity of soil microorganisms

Generally, ecologists feel that a stable ecosystem would have high genetic diversity that it tolerate environmental fluctuation. Nevertheless, it is by no means clear what level of diversity is required to maintain the stability of a soil ecosystem, or what degree of disturbance will trigger a significant decline in diversity (Atlas, 1998). Although microbial diversity in soil is generally assumed to be high, some soil management practices (e.g. conventional tillage operations) can bring about
both a decline in soil microbial biomass and deterioration of soil physical properties. It would be extremely useful to know if changes in microbial population composition and genetic diversity could serve as an early warning of deterioration in soil properties. In theory, high genetic diversity exists in most soils because immigration rates are high and environmental stresses are neither of sufficient intensity nor sustained for sufficient time to selectively eliminate large numbers of genotypes. Furthermore, nutrients are rarely present in excess for a sufficient period of time to allow selective enrichment of the more adapted genotypes.

The importance of soil microbial diversity and community interactions in the degradation of natural and anthropogenic organics is well recognized but not completely understood. Traditionally, the analysis of soil microbial communities has been conducted using viable plate counts or most probable number techniques. However, due to the high degree of selectivity and bias inherent in culture methods, only approximately 5,000 bacterial species have been described (Amann et al., 1994). Direct visualization of stained bacteria suggests that the proportion of culturable cells is usually 65% of the total number of viable cells present in the soil sample (Bakken, 1995). Furthermore, this culturable subset is not representative of and contains significantly less genetic information than the collective genomes (metagenome) of the total microbiota (Rondon et al., 2000). To overcome some of these problems, molecular techniques using DNA or RNA extracted directly from the soil have been employed (Ward, 1990; Torsvik, 1998).
1.5 Molecular microbial ecology

Numerous works that are devoted to the study of the DNA originated from total soil microorganisms are briefly discussed below. The choice of a protocol must be a compromise between a criterion of quantity and quality. On the one hand, the greater recovery of DNA is likely to include more representative for the DNA of the soil microbial community and, on the other hand, the requirements as to the DNA purification step are imposed by the objectives of the study, such as the detection of specific organism, diversity estimation, community differentiation or sequencing. However, each additional step, such as repetitive purification procedures for humic acid removal, that is necessary to have successful PCR reactions, inevitably induces DNA loss.

1.5.1 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) which is a sensitive tool for detecting and characterizing organisms from small amounts of DNA, has been widely applied in the work of microbial DNA extraction from soil. The DNA-based approach often consists of amplification of the ribosomal RNA genes (rDNA) from the whole bacterial or fungal community DNA. The use of the bulk DNA that is a mixture of DNA extracted from diverse microorganisms as PCR template generates rDNA products differing in sequence but generally not in size. The analysis consists of discriminating between these PCR products to obtain a survey of the microbial communities. One way to identify the microorganisms in soil is based on the sequence of the rDNA molecules obtained after cloning (Liesack and Stackebrandt, 1992; Stackebrandt et al., 1993; Ueda et al., 1995; Borneman et al., 1996; Gray and Herwig, 1996; Borneman and Triplett, 1997; Zhou et al., 1997; Øvreas and Torsvik, 1992; Stackebrandt et al., 1993; Ueda et al., 1995; Borneman et al., 1996; Gray and Herwig, 1996; Borneman and Triplett, 1997; Zhou et al., 1997; Øvreas and Torsvik, 1997).
1998). A complete survey of the phylotypes in a particular rRNA gene library would require sequencing all unique clones in the library – several hundreds or thousands of individual clones. Screening of clones in libraries can also be facilitated by restriction fragment length polymorphism (RFLP) technique (Øvreas and Torsvik, 1998). Technique based on 16S rDNA fragment length polymorphisms of restriction digests, such as amplified ribosomal DNA restriction analysis (ARDRA) or terminal restriction fragment length polymorphism (T-RFLP) has recently been used to probe community structure. For the ARDRA technique, DNA is obtained by PCR amplification by using universal primers and the product is digested with restriction enzymes (Massol-Deya et al., 1995; Smit et al., 1997). For T-RFLP method (Liu et al., 1997; Lukow et al., 2000) fluorescently tagged primers are used and are detected by an automated sequencer. The size of only the terminal restriction fragment can be detected and the amount can be quantified using the sequencer.

PCR is now routinely used in molecular microbial ecology, it suffers from some biases. When the nucleic acids used are extracted from natural ecosystems, the PCR reaction can be inhibited by contaminants such as humic substances (Wilson, 1997). Moreover, all the targeted DNA does not have the same chance to be amplified depending on the quantity (competition between DNA template), the quality (sheared or intact DNA) or the nature of the DNA (bases composition). For example, Chandler et al. (1997a) observed that the same clones are not obtained when the PCR is performed with diluted and undiluted DNA solutions. Suzuki and Giovannoni (1996) showed that DNA having the lowest percentages of guanidine and cytosine are preferentially amplified by PCR.
1.5.2 Adaptation of DNA probe technology for use in soil samples

To adapt DNA probe methodology for use in soils, the following features of a protocol needed to be improved or developed. (i) A procedure is needed which would allow processing of more samples simultaneously and in a shorter period of time for analysis of the number of treatments and replicates needed for ecological studies. (ii) The isolated DNA had to be of sufficient purity and size for use in experiments involving digestion with restriction endonucleases, transfer to cellulose nitrate membranes, and hybridization to DNA probes. Humic acids and other contaminants, if not removed, could reduce the efficiency of digestion by restriction endonucleases and reduce the specificity of hybridization. (iii) It is also necessary to develop probes that are both sensitive and specific enough to detect the presence of a particular sequence of low frequency in the complex mixture of DNAs isolated from the soil bacterial community (Holben et al., 1988).

1.6 Comparison of molecular and culture techniques

Culture-independent molecular approaches are replacing culture-based methods for comparing the composition, diversity, and structure of microbial communities. Investigations based on these approaches have led to the conclusion that traditional methods of culturing natural populations have seriously underestimated the archaeal and amount of bacterial diversity. Samples of DNA extracted from seawater, soil, and cyanobacterial mats of hot springs appear to represent predominant populations in these ecosystems, while the species that grow on culture plates are numerically unimportant in intact natural communities. These findings are not surprising, since the vast majority of organisms counted microscopically in samples from these environments have not been cultured.
reason for this inadequacy is that cultivation conditions used to isolate organisms do not reflect the natural conditions in the environment examined and thereby select fast-growing prokaryotes that are best adapted to the growth medium (Großkopf et al., 1998; Liesack, 1997; Ward, 1998, 1997). However, greater success in bacterial isolation can be achieved by using culture conditions that closely approximate natural environments (Schut, 1993) or by using novel tools, such as optical tweezers, to physically isolate bacterial propagules (Huber, 1995). There is also molecular evidence that some readily cultivable bacteria are abundant in the environment from which they are isolated (Rhenstam, 1993). These trends suggest that innovative isolation procedures combined with the identification of phylotypes provide a powerful means of addressing the great plate count anomaly.

Relatively few studies have involved a twin-track approach whereby both cultivation and direct recovery of bacterial 16S rRNA gene sequences have been used to gain insight into the microbial diversity of natural bacterial communities (Dunbar et al., 1999; Hengstmann et al., 1999; Suau et al., 1999). Comparative studies such as plating and 16S rDNA cloning (Frostegård et al., 1999) suffer from biases that can distort community composition, richness, and structure. The molecular approaches provide a new perspective on the diversity of prokaryotes in nature but do not yield the organisms themselves. This means that potentially valuable biotechnological traits can, at best, only be inferred from phylogenetic affinities (Amann et al., 1995; DeLong, 1994; Hengstmann et al., 1999). The need to cultivate representatives of phyletic lines of uncultivable prokaryotes for biotechnological purposes poses a major challenge for microbiologists.
A somewhat mixed picture emerges from comparative studies of natural microbial ecosystems. Chandler et al. (1997a) found close correlation at the genus level between the cultivable portion of aerobic, heterotrophic bacteria and data derived from the 16S rDNA approach when examining deep subsurface sediment. However, these correlations were detected after aerobic treatment of sediment samples at the in situ temperature but not with the untreated sediment core. It is possible that the treatments caused a selective shift towards enrichment of specific bacterial groups in the samples analyzed compared with the original sediment core. Studies of hot spring microbial mats highlighted several close matches between the 16S rDNA of organisms obtained by culture methods and directly recovered 16S rDNA, but only after several liquid dilutions of the inoculum were used for cultivation instead of direct enrichment based on undiluted inoculum (Ward, 1997; 1998).

Two major conclusions were drawn from these studies. (i) For the most part, direct enrichment techniques select for populations which are more fit under the chosen enrichment conditions and may not be numerically significant, and (ii) the growth of numerically dominant populations may be favored by using an inoculum diluted to extinction, especially in growth medium which reflects the conditions in the habitat under study. The conclusions drawn by Ward and his colleagues are consistent with the results of a comparative analysis in which bacterial isolates and environmental 16S rDNA clones were recovered from the same sediment sample (Suzuki, 1997). The corresponding data sets showed little overlap, possibly due to direct plating of the undiluted inoculum onto solidified medium with the subsequent isolation of community members that were not numerically significant. In contrast, a close correlation was found between most-probable-number estimates of isolates.
and environmental 16S rDNA clones taken from the bacterial community of rice paddy soil (Hengstmann, 1999). In a comparative study of the bacterial community diversity of four arid soils, similar relationships were found between 16S rDNA results and cultivation, though significant differences were also observed (Dunbar, 1999).

It can be concluded that both innovative cultural procedures and culture-independent methods have a role to play in unraveling the full extent of prokaryotic diversity in natural habitats, especially since there are a number of instances where taxa have only been detected using cultural methods (Suau et al., 1999; Wilson et al., 1996). Although the two approaches sometimes provide different assessments of relative community diversity, the discrepancies may be attributed to sampling different subsets of the microbial community and to limitations inherent in each of the two approaches. In addition, highlighting consistent relationships between environments based on the dual approach may be highly habitat dependent due to the limited ability of a single cultural method to survey the full extent of the bacterial communities and the influence of bacterial physiology in situ on the success of cultivation in the laboratory.

1.7 Recovering bacterial cells from soil

The forces involved in the binding of bacterial cells to soil surfaces include bonding between extracellular bacterial polymers and humic colloids, electrostatic forces, hydrogen bonding, and other surface interactions. These interactions are different for clay and humus, as well as different bacterial types. The bacteria may also be physically entrapped in soil aggregates. The fractionation efficiency is dependent on how efficient bacteria can be released from soil aggregates and soil
surfaces. Optimal procedures for separating bacteria from soil particles and humic colloidal material should be rapid and simple. They must provide purified samples with reasonably high yields without being selective. Faegri et al. (1977) described a differential centrifugation technique for the separation of bacteria from fungi and particles in organic soil. It has later been used by several investigators with some modifications. In the presence of clay, the bacterial cells are not readily released by mechanical cell lysis, and the fractionation efficiency has been improved by using chemical desorbing agents (Torsvik, 1996).

1.8 Recovering nucleic acids from environment

An ideal procedure for recovering nucleic acids from environmental samples should meet several criteria; (i) the amount of nucleic acid recovered should be high and not biased toward certain populations so that the extracted nucleic acids are representative of the total nucleic acids within the naturally occurring microbial community, (ii) the size of DNA fragments should be as large as possible so that molecular studies, such as community gene library construction and gene cloning, can be carried out, (iii) the purity of DNA should be of high grade for reliable enzyme digestion, hybridization, reverse transcription, and PCR amplification, (iv) the DNA should be extracted simultaneously from the same sample so that direct comparative studies can be performed. This will also be particularly important for analyzing samples of small size, (v) the extraction and purification protocol should be kept simple as much as possible so that the whole recovery process is rapid and inexpensive, (vi) the extraction and purification protocol should be robust and reliable, as demonstrated with many diverse environmental samples.
The extraction and analysis of total microbial community DNA from soil is useful for several reasons (Trevors et al., 1989). Firstly, it provides insight in the prevalence of specific genes within microbial communities in the soil ecosystem, which could result in a better understanding of natural selection of specific microbial groups under the influence of soil conditions. Secondly, by using 16S/18S or 23S/25S ribosomal DNA sequences as 'signature molecules' (biomarkers), overall community DNA analysis may assist in describing microbial communities population structure. This can be achieved by applying temperature or denaturing gradient gel electrophoresis (TGGE or DGGE) to PCR products generated with sets of conserved primer, resulting in a type of community structure that were not previously obtainable. Finally, the ability to obtain microbial community DNA allow investigations on the nature of non-culturable cells, which are known to bound to soil particles.

Analysis of DNA extracted from environmental samples has shown that genetic diversity is much greater in natural habitats than was previously recognized (Embley, 1997; Head, 1998; Olsen, 1986; Pace, 1986; Ward 1992, 1990). Such studies show that there are many microbial taxa to be discovered and isolated in pure culture. Despite the inherent problems faced in selectively isolating and characterizing microbes from environmental samples, steady progress continues to be made, as exemplified by advances made in unravelling the systematics of extremophiles (Goebel, 2000; Jeethon, 2000; Kristjansson, 2000), lactic acid bacteria (Axelsson, 2000), legume nodule nitrogen-fixing bacteria (Coutinho, 2000), rhodococci (Goodacre, 1998), sphingomonads (Ederer, 2000), microbial pathogens of insects (Humber, 2000; Priest, 2000), and protozoa (Coombs, 1998).
Nevertheless, substantial difficulties remained in sampling and characterizing representative members of the microbial populations found in natural habitats.

1.9 Objectives of the study

With the development of molecular biology techniques, it is now possible to study specific components of microbial communities in environmental samples directly especially from soil (Roose-Amsaleg et al., 2001). The power and utility of molecular biology, however, depend on the ability to extract and purify nucleic acids from soil sample (Chandler et al., 1997b). Thus, the objectives of this study were to:

1. Evaluate and compare the most common elements of DNA extraction and purification protocols and to use the information obtained to develop a method for obtaining whole-community DNA from tropical mangrove soil samples.

2. Improve DNA extraction and purification methods for tropical mangrove soil and optimize and control each step of the protocol, including lysis of bacteria, DNA purification, and PCR amplification.

3. Develop a rapid and easy-to-use PCR protocol for directly detecting and enumerating microorganisms in tropical mangrove soil.