Enumeration and identification of bacteria from environmental samples using nucleic acid probes

TC Hazen* & L Jiménez

DNA probes are useful for both identification and enumeration of specific bacteria and functional groups of bacteria in environmental samples. Because probes can detect genes, chromosomes, and plasmids, they also promise to be major sources of information about the relatedness of bacteria and groups of bacteria in the environment.
Enumeration and identification of bacteria from environmental samples using nucleic acid probes

TC Hazen* & L Jiménez

DNA probes are useful for both identification and enumeration of specific bacteria and functional groups of bacteria in environmental samples. Because probes can detect genes, chromosomes, and plasmids, they also promise to be major sources of information about the relatedness of bacteria and groups of bacteria in the environment.

Introduction

One of the greatest problems confronting studies of microbes in natural environments has been accurate determination of the composition, activity and function of microbial communities. Isolation and identification of bacteria has its origins in clinical environments; subsequent application of these techniques to natural environments has met with only limited success. Although natural environments quite often have a large number of clinically unknown species, clinically developed identification techniques assume a certain known bacterial community and proven sampling techniques, which can lead to poor or false identifications of environmental isolates. In addition, most natural environments are more dilute, nutrient-poor, and physically extreme than clinical environments. Thus, bacteria in natural environments are quite often found existing concomitantly at different physiological states. Recently it was discovered that even pathogenic bacteria can assume a ‘viable but non-culturable’ state in dilute or stressful natural environments.¹ These discoveries have further emphasized the need to develop enumeration and identification techniques specific to natural environments. Indeed, most current environmental studies of bacteria are employing direct enumeration techniques (e.g. AODC, DAPI), direct measurement of physiological activity (e.g. [3H]-thymidine incorporation, INT-reduction, frequency of dividing cells), and culture techniques which utilize conditions more typical of the environment being studied (e.g. dilute media and low incubation temperatures for microbial isolation and characterization from oligotrophic environments).²

The recent application of nucleic acid probes to bacteria found in environmental samples shows great promise.¹⁻⁴ The ability of these probes to identify specific nucleic acid sequences and determine the relatedness of similar sequences is ushering in a host of new studies that would have been impossible until now. Because nucleic acid probes can code for particular nucleotide sequences, they can be more specific than fluorescent, monoclonal antibodies or fluorochrome stains such as acridine orange. Nucleic acid probes can even identify microorganisms with a particular gene, thus enabling identification and quantification of environmental organisms belonging to a particular functional group (e.g. mercury-resistant, xenobiotic degraders).⁶⁻⁷ This type of identification was previously impossible: indeed, the only techniques that are closely related are those that involve enrichment and tracer substrate utilization. These later techniques, however, are ripe with the inherent difficulties of culture techniques described above. Thus not only are bacterial DNA probes a more sensitive and specific autecological tool, but they also are allowing us to do new types of synecological studies.

What are DNA probes?

DNA probes are sequences of nucleotides, single- or double-stranded, with a label or reporter that gives a readable signal. The probe sequence can be a whole chromosome, a gene, or a plasmid. The sequence can thus recognize a functional ability of all types of bacteria, a species, a strain (e.g. serogroup), or the presence of extrachromosomal DNA (e.g. virulence and antibiotic resistance plasmids). When the probe encounters a complementary sequence of DNA it hybridizes with it forming a stable double strand under stringent conditions. The greater the quantity of the probe that is hybridized, the stronger the signal from the label.

Constructing DNA probes

In constructing a DNA probe, the hypotheses that are to be tested must be carefully considered, i.e., whether the probe is to be used for identification, enumeration, or detection. When constructing a chromosomal probe for identification or detection, DNA is extracted from the bacterial cell, purified, concentrated, labelled and denatured.⁸⁻⁹ The probe is then ready for application. This method is tedious and time consuming, since it requires repeated isolation of DNA from the bacteria. The probes developed in this way usually lack specificity, unless the organism used has very unique nucleotide sequences.⁷ Chromosomal probes are most useful for looking at relatedness of bacterial isolates.⁸⁻⁹

*E. I. du Pont de Nemours & Company, Savannah River Laboratory, Environmental Sciences Division, Aiken, South Carolina 29808-0001, USA.
Received 18 August, 1988.
creating sticky ends which can recombine with any other piece of DNA that has been cleaved with the same enzyme. Since these enzymes cleave DNA at specific sequences of nucleotide bases, they must be carefully chosen to give the desired sequence. Thus, this technique also requires a map of the sequence desired whether it is a gene, a chromosome sequence, or a plasmid. A vector plasmid e.g. pBR322 is cleaved with the same restriction endonuclease, allowing the cleaved sequence to be inserted into the plasmid. The plasmid is then closed with DNA ligase and introduced into a host, such as Escherichia coli, by transformation. The plasmid will then be replicated in the host, and thus the desired sequence is then cloned, producing as many copies as desired and allowing its continuous production. The cloned sequence can then be reisolated from the plasmid, labelled, and the sequence denatured to produce the probe.

A probe may also be constructed from oligonucleotide sequences synthesized de novo in the laboratory. These probes are quite small, containing fewer than 40 base pairs. This gives them some limitations but makes them quite specific. The use of this probe also requires exact knowledge of the target sequence in terms of its base composition and what it represents in the bacteria to be studied.

**Hybridization and labelling**

The probe is allowed to react with the target sample under conditions of optimal reassociation. The more probe that is reassociated with the sample DNA, the stronger the signal produced from the label on the probe. The degree of reassociation is not only dependent on the complementarity of the sample sequences and the probe, but is also dependent on the stringency of the reassociation conditions, i.e. temperature, pH, and salt concentration of the reaction buffer. High stringency conditions allow fewer mismatches than low stringency conditions. The format of the hybridization reaction will also dictate the type of sample that must be employed and the stringency of the reaction. Environmental samples can be hybridized on a solid support (filter or bottom of a microtitre plate), in solution, in situ, and in a Southern blot. The filter format is the most widely used to date.

Labels that produce signals when bound to the probe can be radioisotopes (\(^{32}\)P, \([\text{H}]\)-thymidine, \(^{35}\)S), biotin-avidin (biotinylated), enzymes (alkaline phosphatase), and fluorescent antibodies (Figure 1). Labelling with \(^{32}\)P by nick translation or oligonucleotide priming are the most common methods and so far have been shown to give the strongest signal per unit probe.

**Sample collection and preparation**

Bacterial identification from environmental samples is done by hybridization of isolates and determining the percent hybridization with the probe (Table 1). This
technique also allows measurement of the degree of relatedness among environmental isolates and to known strains. Since this technique requires starting with a pure culture, it is subject to the same limitations of culture technique from environmental samples (Figure 2). Once a pure culture is obtained, the bacterial cells are lyed and the DNA is extracted and fixed on filters for hybridization with the probe. The signal is detected by scintillation counting or by spectrophotometry, depending on the label used with the probe.

Detection of specific bacteria or functional groups may also require culture enrichment of the original environmental sample. Colony hybridization allows the hybridization to occur on the primary isolates. With this technique, several dilutions of the sample are grown on nitrocellulose or nylon filters on media and replica plated to other filters. The colonies on the filters are lyed, and the DNA is fixed and hybridized with the probe. After hybridization with a radiolabelled probe, an autoradiograph is made with the filter and the positive signal colonies are scored. The MPN (most probable number) method has also been used with this technique by enrichment in microtitre plates. After incubation, contents of microtitre plate wells are transferred to microtitre filter plates and filtered, the cells are lyed, the DNA fixed, and the filter hybridized to the probe. An autoradiograph is made of the hybridized filter and the positive signal wells scored.

 Colony hybridization allows rapid detection of target bacteria or functional groups from soil, sediment, water, shellfish, and food (Table 1). The minimum detectable level (MDL) for colony hybridization ranges from $10^2$ to $10^6$ c.f.u. per gram of sample. The MPN variation is slightly better with a 10 MPN g$^{-1}$ MDL.

Recent studies have also shown that bacterial cells can be extracted from soil, the cells lyed, and the DNA fixed to filters (slot blot). The filter is hybridized and an autoradiograph made (Figure 2). Determination of the smallest amount of soil extract that gives a positive signal allows direct quantification in the original soil sample. This technique does not have the limitations of culturable enrichment; however, it still requires at least $10^4$ c.f.u.g$^{-1}$ in the original sample.

Conclusions

DNA probes have a major application in environmental microbiology, in being able rapidly to detect bacteria with specific functional characteristics, regardless of the activity of that function at the time measured. However, more work is needed to develop direct detection methods that do not rely on cultural enrichment. This will require improved probe sensitivity and improved methods of DNA extraction and concentration from environmental samples.

Probes are also useful in rapid identification of specific bacteria from environmental samples. Indeed these probes can even determine the relative relatedness of isolates from different environments at different times under different conditions. Because of the high specificity of these probes, they have been used for the detection of mercury-resistant bacteria, pathogens, xenobiotic degraders, nitrogen fixers, and genetically engineered bacteria in environmental samples.

Recent studies on probes that use nucleotide sequences from highly conserved regions of chromosomal bacterial DNA, e.g. ribosomal DNA, suggest that these probes could be used for identification of all bacteria present in a sample, and for distinguishing the relative quantities of each one. This technique would involve extracting the bacteria from the sample, lysing the cells, and then cutting the DNA with a series of restriction endonucleases. The cut DNA would then be subjected to electrophoresis, e.g., Southern blotting, and the resulting blot hybridized with the probe. An autoradiograph of the resultant blot could be read by a gel scanner.
and the unique patterns compared to a library of patterns from known organisms. The entire procedure could be automated to give results in less than 24 hours. This type of technology has the potential to change completely the ways in which we initially identify and quantify bacteria from the environment.

Acknowledgements
The information contained in this article was developed during the course of work under Contract No. DE-AC09-76SR00001 with the US Department of Energy.

References