Review Article

Development of molecular methods for the detection of specific bacteria in the environment

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Introduction

The detection and isolation of a wide range of bacteria from the environment are essential components in the study of microbial ecology (for reviews see Austin, 1988; Grigorova & Norris, 1990). In recent times great emphasis has been placed on the detection and enumeration of groups of bacteria that are indicative of pollution and contamination of terrestrial and aquatic habitats. Accurately determining numbers of pathogens or indicator organisms is imperative for assessing public health and safety (Kasper & Tartera, 1990). The most common microbial indicators of pollution are associated with sewage effluent and include, for example, enteric bacteria such as *Escherichia coli*, *Vibrio* sp. and faecal streptococci. The presence of each can be determined routinely using set procedures and specific bacteriological media. Concerns over the possible consequences of releasing genetically modified micro-organisms (GMMOs) into the environment and the ecosystem effects that may occur as a result have prompted a need to detect and enumerate novel types of bacteria (for example see Brill, 1985; Gillett *et al.*, 1984). So far only limited numbers of micro-organisms have been released or are intended for release. These include strains of *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Rhizobium* sp. and baculoviruses (see Sussman *et al.*, 1988). Sensitive monitoring methods to detect a host and its recombinant DNA in various ecosystems are essential for determining the ability of GMMOs to survive, grow and disseminate, and to assess any likely environmental impact.

Traditional methods for the detection and enumeration of micro-organisms

There are numerous strategies for the detection and isolation of bacteria from environmental samples (for a review see Herbert, 1990). Conventional methods are designed for enumeration either of the culturable population or of the total population *per se*. A count of culturable bacteria is obtained after growth on a suitable medium containing carbon and/or other energy sources (Roszak & Colwell, 1987). Since all media are selective to a lesser or greater extent, and not all bacteria are recoverable, viable counts are rarely quantitative. Modification of recovery procedures can increase the number of viable bacteria isolated from any sample. For example, CPS medium, which comprises low concentrations of casein, peptone and starch, has given the highest counts of aquatic bacteria (Jones, 1970). Incubation temperatures play a significant role in the efficiency of recovery. Temperatures optimized for medically-important bacteria (i.e. those above 30 °C) select against the majority of environmental isolates. However, maximum recovery can be obtained following incubation between 10 and 20 °C for periods of as long as 23 d. Further modifications of isolation procedures such as the use of media incorporating purified agars or gelling agents (such as pluronic polyol F127) that solidify on warming have proved beneficial. Agar substitutes are particularly useful in pour plate methods since bacteria are often sensitive to temperatures prevailing in molten agar (Gardener & Jones, 1984). More accurate counts than those obtained using spread or pour plate methods can be achieved by most-probable-number (MPN) procedures. The MPN technique employs a serial dilution of the sample in appropriate media and a high degree of replication provides statistically significant

Abbreviations: GMMO, genetically modified micro-organism; MPN, most probable number; NCBV, non-culturable but viable; PCR, polymerase chain reaction.

enumeration of culturable organisms (Roszak & Colwell, 1987).

It is not, however, normally possible to enumerate all the culturable bacteria in an environmental sample. Public health regulations focus on detection of known pathogens or specific groups of indicator bacteria and selective media have been developed accordingly. Commercially available media can be used to isolate specific bacterial groups such as enteric bacteria, faecal streptococci and pseudomonads (Lanyi, 1990). Similarly, media have been designed for the isolation of groups of ecologically important bacteria such as fluorescent pseudomonads, methanogens and yellow-pigmented bacteria (including *Flavobacterium* and *Cytophaga*; Schneider & Rheinheimer, 1988). In addition, some media can be used to isolate and identify bacteria to a species level (for example, *E. coli*; Burman, 1967).

Methods for direct counting do not rely on the culturability of bacteria in the sample and consequently are more quantitative than viable counting procedures. Direct counting of bacteria on black membrane filters by epifluorescence microscopy has become the most frequently used method for total bacterial population estimates and permits a rapid quantitative estimation of aquatic bacteria (Hall *et al.*, 1990). It is not always necessary to use membranes since this technique can be applied directly in counting chambers or on natural or artificial surfaces (Hall *et al.*, 1990). Epifluorescence is achieved by using a range of nucleic acid or protein stains that fluoresce when excited by light of a suitable wavelength. The quality of the microscopic image depends on many factors, such as light source, optical characteristics of the instrument used, the type of fluorochrome employed and the nature of the sample under analysis (Fry, 1988). Although problematic, techniques have been developed to count bacteria directly *in situ* (Jones, 1977; Fry & Humphrey, 1978). However, if the only information required is total numbers as opposed to information on community structure and distribution, then standard procedures can be employed to remove bacteria from solid surfaces or particles. The direct count can then be performed on a cell suspension (Goulder, 1987; Fry, 1988). In addition, labour-intensive methods such as electron microscopy have been used for direct counting of bacteria from filtered samples (see Hall *et al.*, 1990).

Direct counting procedures that employ simple fluorescent stains are non-discriminatory by nature. Counts of individual genera or species can be achieved only if the target is morphologically distinct or distinguishable from the rest of the indigenous microflora by some other means. Although bacteria have little morphological diversity it is possible to recognize a limited number of organisms based on cell morphology using microscopic

techniques. Filamentous organisms are an obvious example, especially since they can comprise up to 50% of the biomass in freshwater sediments (Jones & Jones, 1986). Similarly distinct organisms such as *Ochrobium* sp., *Achromatium* spp. and the putative genus *Metallogenium* can be identified easily and enumerated (J. G. Jones, personal communication). However, the majority of bacteria do not possess any distinct morphological characteristic and therefore identification requires additional biochemical or immunological techniques. Immunofluorescence microscopy has been widely applied to the detection and enumeration of particular micro-organisms when conventional techniques have proved difficult. Fluorochromes, such as fluorescein isothiocyanate, can be coupled to an antibody that binds directly with a target antigen on the cell or to a second antibody that recognizes an antibody produced against the micro-organism (for example, see Chantler & McIlmurray, 1988). Immunofluorescence detection has been used for various bacteria that are difficult to culture, for example methanogenic bacteria (Conway de Macario *et al.*, 1982), methane oxidizers (Reed & Duggan, 1978), N_2 -fixing organisms (Renwick & Gar-eth, 1985), nitrifiers in soil and marine habitats (Belser & Schmidt, 1978; Ward & Carlucci, 1985) and acid-tolerant *Thiobacillus ferrooxidans* (Apel *et al.*, 1976). Successful application of fluorescent antibodies can be affected by a range of factors, including specificity and cross-reactivity, autofluorescence (particularly when algae are the target micro-organisms), nonspecific staining, expression of the antigen-coding genes, stability of the antigen under environmental conditions, and the inability of the technique to distinguish viable and non-viable cells and be quantitative (Schmidt, 1974; Ford & Olsen, 1988).

Sampling strategy

One of the major limitations to research into microbial communities, and consequently the detection of bacteria in the environment, is an inability to isolate and grow in culture the vast majority of bacteria. There has always been a discrepancy between cell numbers obtained from direct and viable counts. In the aquatic environment direct counting methods indicate that there are approximately 10^6 bacteria ml^{-1} in lakewater, yet only 10^3 cells ml^{-1} are culturable (Jones, 1977). Studies by Ferguson *et al.* (1984) and Hoppe (1978) concluded that culturable bacteria represented only 0.01–12.5% of the viable bacterial population from the marine environment. Similarly in soil, the greater part of the microbial population cannot be cultured (Atlas, 1983). Furthermore, some bacteria have been shown to become

unculturable but retain their viability after exposure to the environment and have been called 'non-culturable but viable' (NCBV) (Colwell *et al.*, 1985). This complicates both the detection and enumeration of key pathogenic organisms and, potentially, candidates for deliberate release into the environment. Micro-organisms known to achieve the NCBV state include *E. coli*, *Salmonella typhimurium* and *Vibrio* spp. (Colwell *et al.*, 1985; Roszak & Colwell, 1987). There are two other factors which contribute to this discrepancy. The direct count cannot distinguish between cells that are viable, NCBV, or dead. Conversely, media used for the isolation of viable bacteria may actively select against growth because they are too rich in nutrients or do not supply essential co-factors. Methods have been developed that go some way to distinguish viable cells under epifluorescence microscopy (for a review see Roszak & Colwell, 1987). Kogure *et al.* (1979) showed that elongation of cells provided an indication of viability when a nutrient source supplemented with the DNA gyrase inhibitor nalidixic acid was added to environmental samples. Although nalidixic acid is commonly used as an inhibitor, piromidic acid can be used as an alternative in some cases (Fry, 1990). However, it is clear that not all viable cells, for example species that exist as cocci, can react by elongation in this way. Also, as with bacterial isolation procedures, it is clear that all experimental conditions are not suitable for all samples. Despite some limitations, this method represents a bridge between counting culturable bacteria and direct counts and has been termed a direct viable count (DVC; Kogure *et al.*, 1979).

Any estimation of the numbers of bacteria in the environment, whether they are pathogens, indicator organisms or GMMOs, must allow for the fact that a proportion of the target organisms may have entered the NCBV fraction of the microbial population. The traditional and molecular methods that are available show a dichotomy in the organisms they can detect. Culture techniques impose strict limitations on the amount of sample that can be removed from natural ecosystems and adequately processed in the laboratory. Coupled with the non-representative nature of the population obtained this emphasizes the need for sampling procedures that preclude a requirement for culturing. Detection methods will target either the culturable population or the total population (including viable, NCBV, and dead or dying cells). The sampling strategy therefore determines the route and ultimately the sensitivity of the method being used or developed.

Lakewater is probably the most amenable medium to sample and process for the isolation of micro-organisms. Viable colonies can be obtained by spreading the water sample (1 µl–1 ml) directly on to solid media. In samples

where bacterial numbers are low, or when highly selective media are used, large volumes of water can be filtered and the filter placed directly on to the solid medium. Alternatively, the filter can be washed and the cell suspension spread to give single colonies. In addition, single colonies can be obtained from MPN dilution tubes when cultured on selective media. It is difficult to obtain a representative sample of a bacterial population from solid substrates such as soil or freshwater sediment. Problems arise due to the spatial distribution of the micro-organisms in soil and the strong associations they form with particulate matter (Stotzky, 1985). To obtain viable cells, the soil sample can be converted into a suspension, agitated by vortexing or sonication and the supernatant spread on to solid media to obtain single colonies. Alternatively, the cells can be removed from the soil by using chemical dispersants to dissociate the micro-organisms from soil particles (for example, MacDonald, 1986; Hopkins *et al.*, 1991*a, b*) in single- or multi-stage procedures followed by either the isolation of single colonies or an estimation of biomass.

As with all techniques that require the isolation of viable cells, a vast untapped portion of the population will remain unscreened. Microbial ecologists have recently begun to apply molecular techniques to the detection of bacteria from the environment, obviating the need for cell culture. Like traditional methods, these techniques also rely heavily on the sampling strategy for their efficiency. Detection methods that do not rely on culturability but use immunological and nucleic acid hybridization techniques can employ strategies that either involve isolating total cells and from those obtaining total DNA, or extract total DNA directly from a sample. DNA extraction can be performed with bacterial cells isolated by a bulk method or by direct lysis of the cells followed by DNA recovery. A rapid method for the direct isolation of DNA from the aquatic environment comprises filtration of a large volume of water through a cylindrical filter membrane with DNA extraction occurring within the filter housing (Sommerville *et al.*, 1989). In a similar approach, Ogram *et al.* (1988) used direct lysis followed by ultracentrifugation and hydroxyapatite chromatography to obtain DNA from freshwater sediments. These methods circumvent the need for culturing the organisms yet have limitations in the range of organisms and the size of DNA fragments that can be isolated (Table 1). In the soil environment two strategies have been developed. Firstly, removal and collection of bacterial cells followed by lysis of the cell suspension can be used to maximize the release of DNA from the various cell types (Holben *et al.*, 1988). Secondly, Steffan *et al.* (1988) directly lysed bacterial cells and recovered the DNA using the procedure of Ogram *et al.* (1988). In cases where DNA is to be

Table 1. *Quality and quantity of DNA extracted from environmental samples by various methods*

Method	Substrate	Sample size	Method of DNA extraction*	Cell numbers per g	DNA yield	DNA size (max. kb)	Reference
1	Water	> 1 litre	Direct cell lysis/ethanol precipitation or CsCl centrifugation	10 ⁶ †	1 ng	> 25	Sommerville <i>et al.</i> (1989)
2	Soil	50 g	Cell lysis after dispersion and PVPP treatment/CsCl centrifugation	10 ⁶	ND	> 50	Holben <i>et al.</i> (1988)
3	Soil	100 g	Direct cell lysis/ethanol precipitation or CsCl centrifugation	10 ⁹	350 µg	ND	Steffan <i>et al.</i> (1988)
			Cell lysis after dispersion and PVPP treatment/CsCl centrifugation/hydroxyapatite chromatography/ethanol precipitation	10 ⁹	40 µg	ND	
	Sediment	100 g	Direct cell lysis/ethanol precipitation or CsCl centrifugation	10 ⁹	1.9 mg	ND	
			Cell lysis after dispersion and PVPP treatment/CsCl centrifugation/hydroxyapatite chromatography/ethanol precipitation	10 ⁹	30 µg	ND	
4	Sediment	100 g	Direct cell lysis incorporating glass beads/DNA precipitation/CsCl centrifugation/hydroxyapatite chromatography/ethanol precipitation	10 ⁷	2.6 mg	20	Ogram <i>et al.</i> (1988)

ND, Not determined.

* Direct methods refer to the purification of DNA without prior removal of cells from soil or sediments, whereas indirect methods require cell dispersion before lysis is performed. Methods differ within the lysis procedure (see appropriate references). All methods produce DNA of sufficient quality to allow restriction by endonucleases and subsequent hybridization to probes. PVPP, polyvinylpyrrolidone.

† Cells per ml.

extracted directly from soil, the addition of polyvinylpyrrolidone (PVPP) in the extraction procedure removes the humic compounds and results in significantly higher yields and improved quality of DNA (Holben *et al.*, 1988; Steffan *et al.*, 1988). The performance and efficiency of these recovery methods is shown in Table 1.

Detection of specific micro-organisms in the environment

The need to detect GMMOs in the environment has created an impetus for the development of detection methods employing molecular biological techniques. Such techniques have great potential for the study of microbial ecology in general.

A simple way to facilitate identification of a particular micro-organism is to provide it with one or more genetic markers that are unlikely to be widely distributed in the natural environment. Depending on the type of marker employed, a GMMO may be tracked phenotypically, relying on the expression of the marker gene, and/or genotypically, where the organism is identified by the presence of that gene by methods that do not rely on its expression (Jain *et al.*, 1988). In general, detection methods can be classified as either quantitative, which permit the direct enumeration of the GMMO, or

qualitative, where assessment is judged on a presence or absence basis (Ford & Olsen, 1988).

The development of a genetic marker system that distinguishes the released micro-organism from the natural population is the focal point of many monitoring strategies. Marker genes can be divided into three groups: (a) short but unique oligonucleotide sequence signatures or markers; (b) genetic systems that provide a selective characteristic such as resistance to an antibiotic or metabolism of an unusual chemical, or provide a non-selective characteristic in the form of a unique biomarker such as a distinct cell wall protein; (c) chromogenic markers which provide a colour change in the bacterial colonies or cells. In many cases the genetically engineered trait itself may be used. Each marker may be located on a plasmid which is introduced into the release host. However, extrachromosomal marker systems are inherently unstable due to the tendency of many plasmids to segregate at cell division during growth following release into the environment (Morgan *et al.*, 1989; Pickup *et al.*, 1990). In addition, it may not be good practice to use plasmid systems which have the potential to disseminate recombinant DNA efficiently to microbial populations. It is preferable to insert the marker system into the chromosome of the organism, where its stability may be increased, with the further advantage that chromosomally located genes are intrinsically less

Fig. 1. Thermoregulation of *xyIE* by the temperature-sensitive repressor *cI857* and the λ promoter P_R . *Activity of catechol 2,3-dioxygenase (C23O) [mU (mg protein)⁻¹] from the *xyIE* gene in (a) *E. coli* ED8654(pLV1013) and (b) *Pseudomonas putida* PRS2000(pLV1013) at 28 °C (repressed) and at 37 °C (derepressed).

likely than those on plasmids to be rapidly disseminated in the environment by genetic transfer. However, marker gene expression will tend to be reduced in comparison to plasmid-encoded systems, as a result of reduction in gene copy number.

Colorimetric marker systems contain one or more genes whose presence in a bacterial cell may be detected by an ability to produce a colour change in a substrate. Isolation of bacteria on media supplemented with substrates that are capable of being transformed into coloured products has been used successfully to distinguish bacteria carrying *xyIE* (catechol 2,3-dioxygenase; Winstanley *et al.*, 1989) or *lacYZ* (β -galactosidase; Drahos *et al.*, 1986) markers from indigenous bacteria. An alternative which may have potential as a bacterial marker uses the *GUS* (β -glucuronidase gene from *E. coli*) system, which produces a fluorogenic product that can be detected at very low levels (Jefferson, 1989). A further system that uses visual identification is the *lux* operon cassette from *Vibrio fischeri*, in which the bacteria are identified by their ability to bioluminesce (Shaw & Kado, 1986). The *lux* pathway encodes both the two subunits of bacterial luciferase (*luxAB*) and the multi-subunit component of a fatty acid reductase (*luxCDE*). The *lux* operon has been introduced into a range of bacteria, transforming them to a detectable bioluminescent phenotype (for example, Shaw & Kado, 1986; Stewart, 1990). In effect, only *luxAB* need be introduced, as the substrate (long-chain aldehydes) can be supplied in a chemical form and is readily diffusible across the cell membrane. Bioluminescence has been used to monitor the spread of black rot-causing organisms in plants using autophotography (Shaw & Kado, 1986). Rattray *et al.* (1990) were able to detect the presence of bioluminescent recombinant *E. coli* in soil using luminometry. However the applicability of the *lux* system to long-term monitoring of GMMOs in the environment has not been assessed beyond an 8 h sampling period (Rattray *et al.*, 1990).

It is possible that introduction of new genetic material into a GMMO will reduce its viability in the environment. Maintenance of the marker system may impose a metabolic burden on the cell. Furthermore, the continual expression of the marker may reduce the ability of the

cell to survive. Locating a marker system on the chromosome may reduce the maintenance budget and controlling the expression of the gene(s) will remove any disadvantages from over-expression. Such a system has been developed for *xyIE*, where the marker gene is expressed from bacteriophage λp_L or p_R promoters under the control of the temperature sensitive λ repressor *cI857* (Winstanley *et al.*, 1989). Any potential metabolic burden imposed on the cell through overexpression is controlled by incubation temperature. Bacteria containing this marker system can be isolated on solid medium with the *xyIE* gene repressed. Expression of *xyIE*, and the subsequent identification of target organisms, requires only that the temperature be raised above 37 °C for 1 h followed by the application of the substrate, catechol, in the form of an aerosol (Fig. 1). *xyIE*⁺ cells are identified by immediate formation of the intense yellow colour of 2-hydroxymuconic semialdehyde (Fig. 2). This type of system has the advantage that it does not require selective media, which have been shown to reduce the efficiency by which organisms are recovered from the environment (Roszak & Colwell, 1987). Furthermore, the presence of cells carrying the *xyIE* marker system can be confirmed directly from lake water by assaying for catechol 2,3-dioxygenase. Its activity is a direct indication of the presence of intact viable cells since catechol 2,3-dioxygenase is inactivated in the presence of oxygen once released from cells (Morgan *et al.*, 1989).

Fig. 2. Conversion of catechol to its coloured product by catechol 2,3-dioxygenase.

The expression and stability of recombinant plasmids containing *xylE* varies depending on the host strain and on the promoter system in operation (Winstanley *et al.*, 1989). Plasmids containing λp_R -*xylE*-cI857 were maintained more stably in *Pseudomonas putida* and *Pseudomonas aeruginosa* than those constructs containing λp_L -*xylE*-cI857. *Acinetobacter calcoaceticus* maintained λp_L -*xylE*-cI857 constructs more stably than λp_R , although stability was low when compared with the other recombinant constructs. Plasmids containing the unregulated λp_L -*xylE* system were less stably maintained than those carrying the temperature-regulated system. The expression of *xylE* from both λp_L and λp_R was tightly regulated by cI857 in *Klebsiella*, *Serratia* and *E. coli* but not in *Pseudomonas* and *Acinetobacter*, where uninduced levels were higher. This was probably due to endogenous species-specific promoter regions fortuitously present in the constructs. Comparative studies between λp_L -*xylE*-cI857 and λp_R -*xylE*-cI857 showed that there was a tenfold increase in catechol 2,3-dioxygenase activity when the λp_R system was operative, except for *A. calcoaceticus*, where the regulation of λp_L was ineffective and λp_R produced only a sixfold increase in catechol 2,3-dioxygenase activity. High levels of catechol 2,3-dioxygenase activity occurred in all hosts containing the λp_L -*xylE* system. This indicates the effectiveness of the λ promoters in Gram-negative bacteria. However, species-specific effects are observed, including differences in thermoregulation of the two promoters, differences in plasmid stability depending on the promoter, and species-specific differences in the regulation or stability of the marker systems. This information suggests that if transfer of recombinant DNA occurs in the environment, the gene(s) carried may not be expressed in a predictable manner. It will be important therefore to treat each candidate release organism as an individual case and to assess the expression, stability and regulation of transfer of released recombinant DNA to potential recipients in model systems prior to release. Furthermore, it seems unlikely that the expression of any one marker-gene-promoter combination could be used as a universal indicator for detection and measurement of microbial activity in all bacterial species.

The above methods share one common strategy in that they require viable cells for the monitoring procedure. It is possible that some organisms chosen for release may enter the NCBV state once they encounter environmental conditions, thus rendering viable cell isolation techniques inoperative. In addition, the expression of the marker gene may not occur efficiently outside the laboratory due to reduced gene expression or interference with the detection system. It is therefore essential that techniques are available that do not rely directly on

expression of the marker gene but allow the organism to be monitored genotypically.

Detection of marker genes using nucleic acid probes

Methodologies involving the detection of target nucleic acid sequences have been reviewed extensively (Hames & Higgins, 1985; Hazen & Jiminez, 1988; Sayler & Layton, 1990). Many applications of nucleic acid hybridization using environmental samples have involved probing immobilized DNA sequences fixed to nitrocellulose or nylon membranes. This approach has the advantage that different types of nucleic acids of varying purity, including those extracted from total bacteria in a community, can be examined, and that multiple samples can be processed simultaneously (Sayler & Layton, 1990). Nucleic acid probes can be labelled either radioactively using ^{32}P -nucleotides or non-isotopically (for instance Hames & Higgins, 1985; Chen *et al.*, 1989). Nucleic acid probes to detect marker genes can be designed to detect a particular genotype or to detect unique sequences inserted into the genome of the target organism (such as a transposon or an oligonucleotide sequence). The probe itself can be double-stranded, comprising either total genomic DNA (Hodgson & Roberts, 1983) or specific sequences of genomic or plasmid origin (see Sayler & Layton, 1990). Similarly, oligonucleotide probes constructed *in vitro* have been used successfully to detect specific 16S RNA sequences (Amman *et al.*, 1990a, b).

If suitable probes are available it is possible to use hybridization to detect the presence of specific nucleic acid sequences ranging from oligonucleotides to functional recombinant genes. This is not only possible in bacteria following culture but also directly from environmental samples, using several available strategies (Hazen & Jiminez, 1988; Sayler & Layton, 1990). Colony hybridization (Hanahan & Meselson, 1980), in which bacterial colonies grown on, or transferred to, a filter are probed for a particular sequence, has been successfully used to detect a range of organisms carrying specific traits (see Sayler & Layton, 1990). Examples of the types of populations monitored by colony hybridization include toluene-degrading bacteria (Sayler *et al.*, 1985; Jain *et al.*, 1987), polychlorinated-biphenyl-degrading bacteria (Pettigrew & Sayler, 1986) and mercury-resistant bacteria (Barkay *et al.*, 1985; Diels & Mergeay, 1990). Frederickson *et al.* (1988) combined DNA hybridization with the MPN method, which permitted the detection of *Rhizobium* spp. and *Pseudomonas putida* that had been genetically marked with Tn5 at approximately 10^2 cells per g of soil. The efficiency of several

Table 2. Limits of various detection systems and methods (adapted from Pickup & Saunders 1990)

Method	Cells per ml or per g	Indigenous background	Target	Medium	Reference
Viable non-selective plating	10 ³	10 ⁶	<i>xylE</i>	Lakewater	Morgan <i>et al.</i> (1989)
Viable selective plating	10 ¹	10 ⁶	RP4-Tol	Lakewater	Pickup <i>et al.</i> (1990)
Bioassay	10 ³	10 ⁶	<i>xylE</i>	Lakewater	Morgan <i>et al.</i> (1989)
ELISA	10 ³	10 ⁶	<i>xylE</i>	Lakewater	Morgan <i>et al.</i> (1989)
Luminometry	10 ³	ND	<i>lux</i>	Soil	Rattray <i>et al.</i> (1990)
DNA hybridization	10 ³	10 ⁶	<i>xylE</i>	Lakewater	Morgan <i>et al.</i> (1989)
Solution hybridization	10 ² –10 ³	ND	2,4,5-T	Soil	Steffan & Atlas (1990)
DNA hybridization MPN	10 ¹ –10 ²	ND	Tn5	Soil	Fredrickson <i>et al.</i> (1988)
Polymerase chain reaction (PCR)	10 ² (100 g)	10 ¹¹	2,4,5-T	Soil	Steffan & Atlas (1988)
Fluorescent antibodies	2 × 10 ¹	ND	<i>Flavobacterium</i>	Soil	Mason & Burns (1990)
Fluorescent oligonucleotides	3 × 10 ⁵	10 ⁸	16S RNA	Mixed suspension	Amman <i>et al.</i> (1990b)

ND, Not determined.

gene probe methods was assessed in freshwater microcosms whilst monitoring the survival of a strain of *Pseudomonas cepacia* that was capable of degrading 4-chlorobiphenyl (Steffan *et al.*, 1989). Although the methods (which included selective plating/DNA hybridization, non-selective plating/DNA hybridization, MPN/DNA hybridization and community DNA extraction/dot blot DNA hybridization) varied in sensitivity and reliability, it was possible to monitor the organism over an 8 week period. The authors concluded that non-selective plating combined with DNA hybridization was the least sensitive method, failing to detect either of the target organisms when the total microbial population was three orders of magnitude higher than the target population (Steffan *et al.*, 1989). Results from methods requiring growth were variable but showed the general decline of the target organism during the course of the experiment. All the gene probe methods were able to detect the presence of the target organisms after 8 weeks (Steffan *et al.*, 1989). However, it was difficult to conclude which method was the most sensitive, emphasizing the requirement for multiple methodological approaches for monitoring GMMOs in the environment.

Total DNA extraction and subsequent probing for a particular trait can be used to monitor that characteristic on a presence or absence basis. The problem is, however, that such samples often do not contain enough of either the target micro-organism or its nucleic acid to make detection possible. This might be circumvented in a number of ways using methods designed to increase the detection sensitivity. One approach is to use high specific activity probes in dot blot procedures to detect sub-picogram levels of DNA (Holben *et al.*, 1988; Somerville *et al.*, 1988). However, this approach is limited when the target DNA comprises a very small fraction of the total. Solution hybridization is another strategy that permits large amounts of total DNA to be screened, and between 100 and 1000 cells per g of sample can be

detected (Steffan & Atlas, 1990). This approach is usually an order of magnitude more sensitive than previous methods (Table 2). Solution hybridization has also been used to examine genetic diversity in environmental samples (Sayler & Layton, 1990).

Another approach to detection is to use the polymerase chain reaction (PCR) to increase the relative concentration of target following extraction of total DNA from an environmental sample. The target nucleic acid sequences are extracted with total DNA from the sample. PCR can then be used to amplify DNA molecules present at essentially undetectable levels to quantities that permit detection of an identifying sequence from which the presence of the target organism may be inferred. Steffan & Atlas (1988) showed that they could detect as little as 0.3 pg of target DNA, which was equivalent to 100 target organisms, in 100 g of soil against a background of 10¹¹ non-target micro-organisms (Table 2). Additionally, PCR may be combined with solution hybridization and high specific activity probes, incorporating the advantages of each method and permitting very sensitive detection of target organisms that are present in extremely low numbers (Steffan & Atlas, 1990). One of the limitations of such methods is that they can be used to detect particular genetic trait (or marker sequence) but not to determine whether the trait was in its original host at the time of sampling or had been transferred to other members of the microbial population.

A further refinement of methods involving probe technology (including PCR) would be to use ribosomal RNA (rRNA) as the target sequence, as it is naturally amplified in the cell and contains sequences that may be specific for the target organism. Oligonucleotide probes based on RNA or on rRNA genes have been developed that are kingdom-, genus-, species- or strain-specific (see Pace *et al.*, 1986). 16S RNA probes are available that can distinguish between eubacteria, archaeobacteria and the eukaryotes using dot-blots, or at a single-cell level using

microautoradiography (Giovannoni *et al.*, 1988). Using 16S rRNA-specific oligonucleotide probes in combination with PCR, Giovannoni *et al.* (1990) investigated the genetic diversity of the microbial flora in the Sargasso Sea. PCR was used to amplify cloned libraries of eubacterial 16S rRNA. Analysis revealed that the microbial population comprised a wide diversity of distantly related bacteria. In addition, Giovannoni *et al.* (1990) identified a novel group of oligotrophic bacteria not previously detected due to their lack of culturability. The value of culture-independent detection techniques was further emphasized by Ward *et al.* (1990), who analysed 16S rRNA sequences from a hot-spring community to reveal a wider diversity of organisms than would be expected from culture techniques.

Fluorochromes can also be attached to oligonucleotide probes for the identification of specific bacteria (Giovannoni *et al.*, 1988; Amman *et al.*, 1990a). Probes specific to 16S RNA that have been coupled to fluorochromes have permitted the identification of single cells of *Fibrobacter succinogenes* and *Methanosarcina acetivorans* in mixed ruminant populations (Amman *et al.*, 1990a). This labelling method has potential in flow cytometric applications due to its specificity (Amman *et al.*, 1990b). Using flow cytometry, Amman *et al.* (1990b) were able to detect *Desulfovibrio gigas* in mixed cultures when the target cells comprised no more than 3% of the total suspension. Fluorescence detection involves fixing the probe into the target cell in a similar way to traditional microscopic staining procedures. The signal can be further increased by applying multiple fluorescent oligonucleotide probes. The invasive nature of labelling with such probes precludes isolating viable cells but will allow enumeration. However, it is likely that the specificity of oligonucleotide probes should allow changes in specific microbial populations in the environment, from kingdom through genera to species, to be monitored (Amman *et al.*, 1990b).

Immunological detection

The use of either polyclonal or monoclonal antibodies offers a potentially sensitive and specific means of identifying environmentally important bacteria. Antibodies of either type can be used to identify specific marker gene products or even intact micro-organisms that express an appropriate antigen. There is now increasing interest in producing monoclonal antibodies and polyclonal antisera against ecologically important micro-organisms, particularly pathogens. Enzyme-linked immunosorbent assay (ELISA) has been used for the detection of specific strains (e.g. *Rhizobium*; Martensson *et al.*, 1984) and recombinant bacteria (*Pseudomonas*

putida; Morgan *et al.*, 1989) in the presence of indigenous bacteria. Experience with bacteria of different species marked with *xylE* indicated that the detection limit was around 10^3 cells per ml of lake water using ELISA techniques and polyclonal antisera specific for catechol 2,3-dioxygenase (Table 2).

Immuno-epifluorescence microscopy can be used to enumerate GMMOs and other environmentally significant bacteria in natural samples (see above). Monoclonal antibodies raised against *Flavobacterium* P25, originally isolated from soil and classified as a potential release candidate, were effective in detecting this strain even when the organism encountered starvation conditions; and cell densities as low as 20 bacteria per g soil could be detected by immunofluorescence microscopy (Mason & Burns, 1990). Fluorescent monoclonal antibodies specific for the O1 antigen of *Vibrio cholerae* have been used in conjunction with fluorescence microscopy, providing a more sensitive procedure for assessing water quality than standard culture methods (Brayton & Colwell, 1987; Brayton *et al.*, 1987). The specificity of fluorescently labelled monoclonal antibodies has been exploited to detect micro-organisms such as *E. coli*, *Legionella pneumophila* and spores of *Bacillus anthracis* by flow cytometry (Phillips & Martin, 1988). Two different serotypes of *Nitrosomonas* spp. were enumerated in activated sludge from sewage plants using immunofluorescence and nucleic acid staining (Volsch *et al.*, 1990). Flow cytometry coupled with the use of phylogenetic fluorescent oligonucleotide probes (Amman *et al.*, 1990a, b) may also have enormous potential in detecting specific micro-organisms from environmental samples.

Organisms that are present in low numbers are often difficult to enumerate. The level of sensitivity for many of the methods available may not be sufficient for detecting low numbers of introduced recombinant organisms in the environment. Selective enrichment techniques included as an integral part of any monitoring strategy would increase the proportion of viable target organisms within the total population. One such method has been developed using a monoclonal antibody raised against a strain-specific domain of the flagellin subunits of flagella from a model recombinant pseudomonad (Saunders *et al.*, 1990; Morgan *et al.*, 1991). The surface of polystyrene magnetic beads (10 μ m) was coated with the monoclonal antibody. By mixing the coated beads with lakewater samples containing the target recombinant *Pseudomonas putida*, bead-cell complexes were formed that could be recovered by attraction towards a strong magnet. When re-isolated by standard culture methods, approximately 20% of the initial target population was recovered. This technique represents an initial step in the recovery and detection of specific micro-organisms and it is intended that it would be

followed by other direct or viable detection techniques (Morgan *et al.*, 1991). Immunomagnetic capture has also been used to recover spores of specific recombinant strains of *Streptomyces lividans* with efficiencies which range from 30% recovery from inoculated sterile soil to 4% from non-sterile soil (Wipat *et al.*, 1991). These examples represent new approaches to the detection of bacteria in the environment. In addition, biosensor-based detection systems, for example sensitive measurement of antigen-antibody complex formation using surface plasmon resonance (Saunders & Saunders, 1991), may also find a significant role in the tracking of specific micro-organisms.

Conclusion

It was not intended for this review to list all the organisms that have been detected using molecular techniques but to show the range of both old and new methods that are applicable to detecting bacteria in environmental samples. Due to inherent limitations in the methods developed so far, it is unlikely that any one detection system will be suitable for monitoring GMMOs or other bacteria in all possible habitats (Pickup & Saunders, 1990). Ford & Olsen (1988) and Jain *et al.* (1988) envisage that practical detection and monitoring strategies will extend beyond the issues of monitoring recombinant micro-organisms in the environment. Advances in this area will undoubtedly stimulate further investigations into the microbial ecology of the open environment.

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