**REVIEW
ARTICLE****Molecular marker systems for detection of genetically engineered micro-organisms in the environment**

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Recent research into the commercial exploitation of genetically engineered micro-organisms (GEMs) in the environment, for bioremediation and improvement of plant growth and protection, has raised concerns regarding associated potential risks. This in turn has led to legislation regarding environmental release of GEMs such that detailed information on their ecology and environmental impact must be provided before approval for release is granted. Assessment of environmental impact and of risks associated with environmental release of GEMs requires knowledge of microbial survival, growth, activity and dispersal within the environment and of the persistence of recombinant DNA and its transfer to the indigenous microflora. It is therefore necessary to detect and quantify concentrations of total, viable, culturable and nonculturable inoculum cells, to measure their actual and potential activities and to determine the presence and expression of recombinant DNA in the progeny of inocula and in indigenous organisms.

Risk assessment cannot rely solely on traditional microbial detection techniques. Many enumeration techniques require cultivation in the laboratory and cannot, by definition, detect nonculturable cells. Growth on laboratory media depends on the choice of media and growth conditions and is affected by competition with other organisms. The efficiency of cell extraction prior to cultivation varies between organisms and is never complete, while extraction procedures may reduce cell viability. It is also difficult to distinguish inocula from indigenous populations. Nonculturable cells may be detected microscopically and fluorescently labelled antibodies enable specific detection of target organisms. They do not, however, distinguish living and dead cells, must be made to the correct specificity and must overcome problems of background fluorescence and autofluor-

escence. They also require expression of genes coding for the antigen and its stability, and quantification is difficult when cell concentrations are low.

Deficiencies in traditional microbial detection techniques have led to research into new methodologies, in particular molecular techniques, with the selectivity and sensitivity required to track GEM inocula and recombinant DNA used in their construction. These techniques fall into three major groups: immunological methods, nucleic acid probing and molecular markers. Traditional immunological techniques have been enhanced by improvements in production of poly- and monoclonal antibodies, development of enzyme linked immunosorbent assays (ELISA) and the potential application of flow cytometry. Probing techniques involve detection of specific DNA and RNA sequences, which may be random or associated with functional genes (Sayler & Layton, 1990), and sensitivity has been increased greatly by amplification of sequences using the polymerase chain reaction (Steffan & Atlas, 1991). Nucleic acid probing can be highly specific and enables tracking of DNA, although the ability to track inocula using this technique depends on the stability of the specific DNA sequence within the original host. Immunological and probing techniques are of most use in providing information on total cell concentrations, but give no indication of the viability or activity of a specific population. They are therefore limited in their ability to predict the environmental impact of an inoculum, as opposed to its mere presence.

The third technique, introduction of molecular markers, is the subject of this review. In its broadest sense the term refers to any molecule specific to an organism, or group of organisms. Here marking or 'tagging' will be defined more closely as the intentional introduction of genes conferring distinctive phenotypic properties which enable 'tracking' of the marked organism after introduction into the environment. The technique provides significant advantages for risk assessment studies in comparison with

traditional methodologies and other molecular techniques. It must be emphasized, however, that no single technique can provide all the information required for risk assessment, and the combined use of traditional and molecular techniques is essential. It should also be remembered that, although the potential commercial application of GEMs has provided the driving force for developments in molecular detection technology, the information required by regulatory authorities is on fundamental aspects of microbial ecology. The application of marker techniques therefore has great potential for increasing our understanding in more general areas of environmental microbiology.

There are a number of general requirements for marker systems. Firstly, the expressed phenotype must not be exhibited by the indigenous population. This presents a challenge for detection in the environment, where the range of micro-organisms is enormous, and prevents realistic application of some markers used in pure culture systems. For example, the presence of bacteria with glucuronidase activity in the environment limits application of *gus* marker systems (Jefferson, 1989). In addition, the marker gene may be present in the indigenous population, but not expressed, reducing the usefulness of probe-based techniques. The efficiency of marker systems requires stable maintenance of marker genes within the host and levels of marker gene expression sufficient for detection. Both maintenance and expression depend on several factors and for any marker system will vary with the host organism, the method of introduction and nature of the marker gene. While many naturally occurring plasmids are stable in the absence of selective pressure, introduced marker genes are generally more stable when integrated into the chromosome than when plasmid-borne. This is particularly important for studies in natural environments where plasmid maintenance by application of selective conditions is often inappropriate or impossible. In addition, there is a possibility that genes may be transferred to non-marked organisms, particularly if the marker is plasmid encoded. A further potential limitation is impairment of the ability of the host to grow and survive, due to the extra metabolic burden imposed by the marker characteristic or maintenance of a plasmid encoding the characteristic. Several strategies have been employed to minimize this risk.

Although a number of marker genes have been proposed, discussion here will be limited to those which have been tested for detection in the environment: antibiotic resistance, *lacZY*, *xylE*, genes for TFD monooxygenase and *lux*.

Antibiotic resistance

Antibiotic resistance markers are most frequently used and have formed the basis for development of all the marker techniques discussed below. Their major advantage for viable cell enumeration is their ability to select against background populations, enabling selection of viable populations of marked organisms by plating on medium containing the appropriate antibiotic. In ad-

dition, resistance genes can be detected by probe-based techniques, enabling detection of nonculturable marked cells. Selectivity is of particular importance for detection and enumeration of marked organisms present at relatively low concentrations. Laboratory culture does, however, introduce the disadvantages of traditional viable cell enumeration techniques and many natural environments contain populations with natural resistance to antibiotics. This can limit the sensitivity of these techniques even with organisms marked with resistance to more than a single antibiotic. There is also concern regarding release of drug resistance genes and genes encoding resistance to heavy metals (Bale *et al.*, 1987) and herbicides (Ramos *et al.*, 1991) may be preferable.

lacZY genes

The *lacZY* genes from *Escherichia coli* code for β -galactosidase and lactose permease. Organisms expressing these genes may be detected by growth on solid medium containing the chromogenic substrate X-Gal (5-chloro-4-bromo-3-indolyl β -D-galactopyranoside), cleavage of which gives rise to blue-green colonies. This system has been exploited as a marker system for fluorescent soil pseudomonads which lack the *lacZY* genes and cannot utilize lactose (Drahoš *et al.*, 1986; Hofte *et al.*, 1990; Ryder *et al.*, 1994). Observation of colonies under UV illumination allows simultaneous detection of blue colony colour and green/yellow fluorescence. Initial studies employed strains in which *E. coli lacZY* genes were encoded on broad-host-range plasmids, but plasmid instability in some constructs led to use of more stable chromosomal markers, introduced using a Tn7 transposon system (Barry, 1988; Drahoš *et al.*, 1992; Kluepfel *et al.*, 1991). The marker genes are sequenced and well-characterized, facilitating nucleic acid probing. Discrimination and selection from background populations are provided by growth on minimal medium containing lactose, detection of colony fluorescence and double marking with *lacZY* and natural or introduced antibiotic resistance markers.

Hofte *et al.* (1990) found no difference between parent and chromosomally *lacZY*-marked strains of *Rhizopseudomonas* 7NSK with respect to maximum specific growth rate, siderophore production, survival, promotion of plant growth and root colonization. They achieved a lower detection limit of 10 cells (g soil)⁻¹ and found low but detectable numbers of cells in air-dried soil more than 3 months after inoculation. Drahoš *et al.* (1992) also found no difference in the specific growth rate of parent and *lacZY*-marked strains of *Pseudomonas aureofaciens*, naturally resistant to nalidixic acid and rifampicin. There was no significant difference in colonization and survival kinetics of parent and marked strains introduced separately into the wheat rhizosphere during an 18 month small scale field experiment (Fig. 1). This study also demonstrated similar efficiencies for enumeration based on *lacZY* and on antibiotic resistance and no evidence was found for transfer of the chromosomally encoded marker gene to the indigenous microflora. In contrast, Ryder *et al.* (1994)

Fig. 1. Recovery of parental (Ps. 3732RN) and *lacZY*-marked (Ps. 3732RNL11) strains of *Pseudomonas aureofaciens* inoculated onto roots of wheat. Error bars indicate ± 1 SD. Harvest date was at week 31. (From Drahos *et al.*, 1992; published with the permission of Cambridge University Press.)

found decreased survival of a *lacZY* strain of *Pseudomonas corrugata* in soil. They suggest that this may have been due to the increased metabolic burden associated with constitutive expression of the marker gene or to disruption of host genes following insertion of the marker gene in the host chromosome. This study highlights the need for rigorous testing of marker systems before use in environmental studies.

With the exception of nucleic acid probing associated with gene transfer studies, *lacZY*-marked organisms have been detected by laboratory culture techniques, in particular dilution plate counting. The major advantage of this marker is in selective identification of colonies of marked organisms. Sensitivity is determined by the procedure used for dilution plating and a lower detection limit of 1 cell (g soil)⁻¹ was reported by Drahos *et al.* (1992). Use of liquid medium and a modified most-probable number technique gave a minimum detection limit of 10 cells (g soil)⁻¹ (de Leij *et al.*, 1993). The technique, however, can only be used for marking of *lac* strains, suffers from the accepted limitations of laboratory cultivation and requires selection from indigenous *lac*⁺ organisms.

xyIE gene

The *xyIE* gene is present on the TOL plasmid pWW0 of *Pseudomonas putida* and codes for production of catechol 2,3-dioxygenase (C23O). This enzyme converts colourless catechol to a yellow product, hydroxymuconic semialdehyde. *xyIE* has been used to mark a number of Gram-negative organisms (Winstanley *et al.*, 1989) and the Gram-positive *Streptomyces lividans* (Wipat *et al.*, 1991) for environmental studies. In these organisms, the *xyIE* gene was expressed from the lambda phage promoter *p_L* or *p_R* and was either unregulated or under the control of the temperature sensitive repressor protein *cI857*. In the latter, *xyIE* is expressed at 37 °C but not at 28 °C, the temperature at which dilution plate cultures are incubated. Gene expression is also repressed at temperatures typical

of the natural environments being studied. This inducible system was designed to eliminate any reduction in fitness through additional metabolic burden due to expression of the marker gene.

Organisms marked with *xyIE* may be detected in a number of ways, the most common being plate counting. Plates are incubated at 28 °C until colony formation, when the temperature is raised to 37 °C or 42 °C for 1 h, to enable expression of *xyIE*. Plates are then sprayed with catechol, colonies of marked cells developing a yellow colouration. C23O activity in intact cells may be measured in environmental samples, although the relationship of such measurements to marked cell number or biomass concentration is not clear. The enzyme may also be detected by ELISA techniques. Data from freshwater systems indicated viable cell enumeration to be the most sensitive of these techniques (Morgan *et al.*, 1989). *P. putida* was originally marked with *xyIE* using IncQ, non-conjugative plasmids (Winstanley *et al.*, 1989), but IncP conjugative plasmids have now been used to facilitate gene transfer experiments (Winstanley *et al.*, 1991).

The efficiency of the inducible *xyIE* system depends on a number of factors and enzyme activity is detectable in most strains even under non-inducing conditions. The level of activity varies with both the lambda promoter used and the host organism, being greater in *Pseudomonas* and *Acinetobacter* than in enteric bacteria. Obviously, the greater the level of expression in the non-induced state, the greater the potential effect on the fitness of the host. The degree of induction also varies considerably in different constructs, with proportional increases in activity ranging from 1 to several thousand. The *p_R* phage system gave greater induction and in *P. putida* constructs used for environmental studies incubation at the higher temperature resulted in 24- to 57-fold increases in activity. Variability in expression of *xyIE* in different hosts affects its usefulness in studying gene transfer from the marked organism. Long-term environmental studies also require plasmid stability which again depends on both the host and the plasmid.

The *xyIE* marker system has been tested in lake water microcosms (Morgan *et al.*, 1989) inoculated with marked strains of *E. coli* and *P. putida*. Lower detection limits for both organisms in sterile and non-sterile lake water were approximately 10³ c.f.u. ml⁻¹ as determined using the ELISA technique. Enzyme activity was lower in sterile lake water and lower for *E. coli* than for *P. putida*, with a minimum detection limit of 5 × 10³ c.f.u. ml⁻¹. Although enzyme activity was correlated with inoculated cell concentration, the relationship was not direct and 10-fold increases in cell concentration in some cases gave less than 5-fold increases in activity. *E. coli* inocula were grown at 37 °C, at which temperature *xyIE* expression will be optimal in this construct. Sensitivity will be reduced for cells which have grown for prolonged periods at non-inducing temperatures.

C23O activity depends on the stability of the enzyme under the conditions prevailing in the environment. In all cases, plate counts were more sensitive than enzyme activity or

ELISA measurements and at low inoculum levels (10^4 c.f.u. ml⁻¹) marked cells could be detected only by using plate counting. The enzyme is inactivated by oxygen and is therefore unlikely to be detected in lysed cells. Comparison of activity with ELISA detection provides a comparison of numbers of lysed and non-lysed marked cells. A disadvantage is that measurement of activity requires purification of the enzyme, which is affected in non-sterile lake water by proteins from the indigenous population.

Winstanley *et al.* (1991) used this system to investigate survival, in sterile and non-sterile lake water, of *P. putida* strains which were auxotrophic or non-auxotrophic for tryptophan, and marked with different *xyIE* encoding plasmids. Numbers of non-auxotrophic strains inoculated at concentrations $< 10^5$ ml⁻¹ initially fell, but then rose to reach about 10^5 ml⁻¹, except where plasmid was lost through instability and growth. Plasmid loss was greatest when cells were inoculated at low concentrations, as these required a greater number of cell divisions to reach final population levels, increasing the probability of plasmid loss. Plasmid instability in some strains was also thought to reflect metabolic load and with some plasmids, enzyme activity measurements in environmental samples indicated expression of *xyIE* even at the temperature used to assess survival. Although plasmids had no detectable effect on survival, stability was greater for low expression plasmids, suggesting 'metabolic burden' as a cause of instability. In non-sterile water all strains declined at similar rates, regardless of auxotrophy, presumably because of competition and predation. Experimental variability was too great to determine whether the marker system affected survival kinetics in sterile microcosms, while inability to distinguish parent and marked strains prevented assessment of differences in their survival in non-sterile lake water. Use of *xyIE*-marked strains of *P. putida* in soil microcosm experiments (MacNaughton *et al.*, 1992) indicated differences in survival patterns in different soils, although again it was not possible to determine whether introduction of the marker gene affected host fitness.

C23O activity in *xyIE*-marked cells of *Streptomyces lividans* is less than that in Gram-negative organisms (Wipat *et al.*, 1991) and induction at 37 °C leads to proportional increases in activity of only 2.8- to 9.5-fold. Variation in activities between plasmids was considered to be due to differences in replicon stability, plasmid copy number and differential regulation of the promoters. Inoculation of *xyIE*-marked and unmarked, streptomycin resistant strains of *S. lividans* into soil, amended with starch and chitin, enabled distinction between survival of parent and marked organisms, and assessment of the effect of the marker on host fitness. This was achieved by plating on media containing either streptomycin or streptomycin and thiostrepton, resistance to thiostrepton being encoded by the *xyIE* plasmid construct. Plasmid stability was generally high but spore counts and the extent of sporulation were less for *xyIE*-marked strains than for the parent. This suggests a significant effect on host metabolism through maintenance of the plasmid or low levels of expression of *xyIE* at the non-permissive temperature.

TFD monooxygenase

A technique which overcomes problems of lack of sensitivity and instability of plasmid constructs in other markers systems is based on conversion of phenoxyacetate (PAA) to phenol by 2,4-dichlorophenoxyacetate (TFD) monooxygenase. King *et al.* (1991) constructed strains of *Pseudomonas aeruginosa* and *P. putida* containing plasmids encoding TFD monooxygenase. In strains in which TFD monooxygenase was deregulated, colonies could be detected by spraying with PAA, incubating for 1 h to allow phenol production, spraying with potassium ferrocyanide and then with potassium ferrocyanide plus 4-aminoantipyrine. Under alkaline conditions, this condenses with phenol to produce a red antipyrine dye, distinguishing colonies from the background population. When applied to samples of suspended cells, this enabled detection of 10^3 cells ml⁻¹ and has potential for enumeration in environmental samples. This system is restricted to cells which are unable to metabolize phenol and, as with previous markers, depends on laboratory culture of cells.

Bioluminescence

Bioluminescence-based marker systems involve the introduction of genes for light emission, originally cloned from the naturally luminescent marine bacteria *Vibrio fischeri* or *Vibrio harveyi*. The molecular biology and biochemistry of bacterial light production have been reviewed by Hastings *et al.* (1985) and Meighen (1988, 1991), and Silverman *et al.* (1989) and Stewart & Williams (1992) recently reviewed the cloning vectors and transposons available and the use of *lux* as a reporter gene, with particular reference to applications in the food industry. A major attraction for environmental applications is the ability to measure light in real time without extraction of cells from the environment or invasion of host organisms which they may be colonizing. The technique is particularly valuable in terrestrial and freshwater environments where naturally occurring luminescent organisms are rare and there is only one report of a luminescent bacterium in soil (Schmidt *et al.*, 1989).

The studies described below all use the bacterial luciferase (*lux*) system but eukaryotic firefly luciferase (*luc*) markers have also been used to detect *Rhizobium meliloti* in root nodules (Palomares *et al.* 1989). Firefly luminescence occurs through ATP dependent conversion of luciferin and provides wider application of luminescence-based techniques to marine environments, where indigenous *luc* gene encoded luminescence is negligible.

Bacterial luminescence results from the activity of the enzyme luciferase and requires a source of reducing equivalents, oxygen and the substrate n-tetradecyl aldehyde, although other long chain fatty aldehydes, e.g. dodecanal or decanal, can act as substrates.

Table 1. Strains marked with *lux* genes for environmental detection

Strain	Reference
<i>Agrobacterium rhizogenes</i>	Shaw & Kado (1986)
<i>Agrobacterium tumefaciens</i>	Shaw & Kado (1986)
<i>Bacillus subtilis</i>	Carni <i>et al.</i> (1987) Karp (1989) Jacobs <i>et al.</i> (1991) Cook <i>et al.</i> (1993)
<i>Enterobacter cloacae</i>	Fravel <i>et al.</i> (1990) Ratray <i>et al.</i> (1992b)
<i>Erwinia amylovora</i>	Shaw & Kado (1986)
<i>Erwinia carotovora</i>	Shaw & Kado (1986) Grant <i>et al.</i> (1991, 1992) McLennan <i>et al.</i> (1992)
<i>Escherichia coli</i>	Shaw & Kado (1986) Ratray <i>et al.</i> (1990)
<i>Lactobacillus</i> spp.	Ahmad & Stewart (1991)
<i>Pseudomonas fluorescens</i>	Shaw & Kado (1986) Amin-Hanjani <i>et al.</i> (1993)
<i>Pseudomonas glumae</i>	Shaw & Kado (1986)
<i>Pseudomonas</i> spp.	de Weger <i>et al.</i> (1991) Beauchamp <i>et al.</i> (1993)
<i>Pseudomonas syringae</i>	Shaw & Kado (1986) Silcock <i>et al.</i> (1992)
<i>Rhizobium meliloti</i>	Shaw & Kado (1986)
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	Shaw & Kado (1986) Shaw (1992)

The structural genes for the luciferase enzyme, *luxA* and *B*, are of size 2 kb. *luxI* and *R* genes are involved in regulation of luciferase production, which operates through an autoinduction mechanism. *luxC*, *D* and *E* code for a fatty acid reductase involved in synthesis of the aldehyde substrate. Additional genes are also associated but with unknown function (Meighen, 1991).

Luminescence-based marker systems are being applied for environmental detection of an increasing number of organisms (Table 1). The basic findings are the same for all host organisms, although maximum levels of light emission per cell vary for different constructs and depend on a number of factors. Emission by plasmid-marked cells increases with plasmid copy number and is generally greater than that from chromosomally marked cells (Amin-Hanjani *et al.*, 1993), although this depends also on the strength of the promoter driving *lux* expression. In strains containing the full *lux* cassette, luciferase production is autoinducible (Eberhard *et al.*, 1981) and luminescence per cell consequently increases with cell concentration. Introduction of the luciferase structural genes (*luxA* and *B*) only, provides constitutive enzyme production and a direct relationship between light output and active cell number or biomass concentration (Ratray *et al.*, 1990). Comparisons of light output by different strains is made difficult by the use of different growth

conditions, different instrumentation and different units for quantification of light emission but Shaw & Kado (1986) found greatest light emission by enteric bacteria and Gram-negative constructs are generally brighter than luminescent Gram-positive strains.

Luminescence-marked organisms can be detected by several techniques. The *luxAB* gene sequences are known, enabling use of gene probing and PCR amplification (Wimpee *et al.*, 1991). Colepicolo *et al.* (1989) demonstrated quantitative immunological detection of luciferase, and its cellular localization, using immunogold labelling based on a polyclonal antibody to luciferase purified from *V. harveyi*. The major advantages of luminescence marker systems lie, however, in the ability to detect light.

Detection of luminescence by eye, photographic film and X-ray film

Levels of light output by many *lux*-marked strains are sufficient for detection of luminescent colonies by the unaided eye in a darkened room, enabling viable cell enumeration by dilution plate counting. Photography can aid enumeration of colonies and long exposures increase sensitivity for less bright strains. Use of this technique has enabled determination of the efficiency of plate counting of marked organisms in the presence of high numbers of colonies on plates (Grant *et al.*, 1991). A range of concentrations of *lux*-marked *Erwinia carotovora* cells was mixed with varying amounts of a non-sterile soil suspension. The mixtures were diluted and plated out and total numbers of luminescent and non-luminescent colonies were determined (Fig. 2). Detection of luminescent organisms was possible even when *lux*-marked cells were significantly outnumbered by the indigenous population, but the efficiency of quantitative detection decreased as the proportion of the marked organism fell below 50% of the total population. The major factor reducing accuracy at low relative concentrations was colony overcrowding.

Fig. 2. Efficiency of detection of colonies of a luminescence-marked strain of *Erwinia carotovora*(pUCD607), inoculated into a non-sterile soil suspension, as a function of the number of colonies per plate. (From Grant *et al.*, 1991.)

This reduced the size and activity of luminescent colonies, and the lower limit of detection was 1 luminescent colony per 3000 non-luminescent colonies. Plates with this many colonies would obviously never be used for enumeration but this provides a measure of the sensitivity of the technique. Similar sensitivity presumably also applies to other marker techniques (e.g. *lacZY* and *xylE*) when used in the absence of selective media.

Luminescence also enables localization of marked organisms *in situ*. Shaw & Kado (1986) followed infection of potato tubers by *lux*-marked *E. carotovora* subsp. *carotovora* by visual detection of luminescing regions and luminometry of tuber samples. Luminescence preceded other visible signs of tissue maceration and light output of tuber samples was directly related to viable cell concentration. They also detected areas of infection of cauliflower leaves by luminescence-marked *Xanthomonas campestris* pv. *campestris* by placing leaves between pre-flashed X-ray film for 6–12 h. Again, luminescence by the pathogen could be detected and localized up to 36 h before visible signs of infection and all areas of light emission subsequently developed disease symptoms. This was the first demonstration of the power of luminescence-based techniques in enabling *in situ* detection and localization of plant pathogens without disrupting the infection process. In a similar study McLennan *et al.* (1992), using photography and luminometry, found highest luminescence activity at the margin of the expanding regions of infection, until tissue disintegration led to increased activity at the point of initial inoculation. Fravel *et al.* (1990) also used photography to identify regions of rhizosphere colonization by plasmid-marked *Enterobacter cloacae* and found a correlation between viable cell concentrations and luminescence.

lux has also been used as an environmental reporter gene for naphthalene catabolism, enabling detection of naphthalene in soil (King *et al.*, 1990) and detection of marked bacteria in the rhizosphere (de Weger *et al.*, 1991). Pseudomonads were marked with either *luxCDABE* or *luxAB* genes. Bacteria were detected, following induction of luminescence by naphthalene, using either autophotography, in which roots were exposed to photographic film for 3–5 h, or an optical fibre light system coupled to a photomultiplier. Detection of the naphthalene inducible strain was possible using both techniques, autophotography demonstrating regions of preferential colonization, e.g. root tips and sites of lateral root emergence. Plasmid-encoded and chromosomally marked constitutive *luxCDABE* strains were not detectable without addition of complex medium. This was believed to be due to energy demand associated with aldehyde production. Strains marked with luciferase structural genes (*luxAB*) only were detectable, following exogenous aldehyde addition, at levels of 10^3 – 10^4 c.f.u. per cm of root, making it 1000-fold more sensitive than detection using *lacZY* markers. This demonstrates the usefulness of inducible systems, operating either at the level of *lux* gene expression or by inducing activity of marker gene products. Induction of *lux* genes by sodium salicylate has also been used to quantify biofilm growth of

P. fluorescens (Mittelman *et al.*, 1992) while O'Kane *et al.* (1988) were the first to demonstrate their use in studying plant–microbe interactions as a reporter for expression of *nif* genes of *Bradyrhizobium japonicum* in root nodules.

Luminometry

Luminometry enables quantification of light emitted by *lux*-marked organisms. If all organisms are fully, or at least equally active, luminescence is directly proportional to cell number or biomass concentration over several orders of magnitude. This is achieved in constructs bearing only the *luxA* and *luxB* genes, where luciferase production is constitutive. Luminescence from such strains requires addition of dodecanal at a level which is sufficiently high to avoid substrate limitation and low enough to prevent inactivation or cell death. Maximization of light output from a particular strain therefore requires optimization of incubation conditions, but in practice variation between constructs is not significant. Direct proportionality has been demonstrated in several *lux*-marked strains in cell suspensions and following inoculation into soil. Lower detection limits depend on the brightness of the strain used and background luminescence associated with the luminometer. For plasmid-marked strains of *E. coli* (Rattray *et al.*, 1990), *Erwinia carotovora* (Grant *et al.*, 1992) and *P. fluorescens* (Amin-Hanjani *et al.*, 1993) lower detection limits in cell suspensions were 200, 100 and 1700 cells ml⁻¹ while chromosomally marked *P. fluorescens* could be detected at 8.9×10^4 cells ml⁻¹.

Amin-Hanjani *et al.* (1993) compared the properties of chromosomal and plasmid markers in *P. fluorescens*. Although the chromosomally marked strain was less bright, light output was more closely linked to biomass concentration during growth in liquid culture. This may have been due to variation in plasmid copy number or other aspects of cell metabolism during batch growth. Introduction of *lux* genes on a plasmid led to a reduction in maximum specific growth rate during batch culture, in comparison with that of the parent strain, but chromosomal marking had no effect. Reduction in specific growth rate may result from increased expression of *lux* genes, due to their greater copy number, or to metabolic load associated with plasmid maintenance. It is not due to energy required for light output as this was inducible, after sampling, by addition of the aldehyde substrate. In terms of specific growth rate, therefore, host fitness was not affected by the chromosomal marker, but this is only an indication of the effect on fitness in the environment. A more relevant study is that of Rattray *et al.* (1993), in which no difference was found between survival of parent and *lux*-marked strains of *P. fluorescens* during starvation in soil for several weeks. The major advantage of the chromosomally marked strain of *P. fluorescens* was stability of the marker gene, with no detectable loss of luminescence phenotype after continuous subculturing for 200 generations. The plasmid was lost with a half-life of 11.5 generations. Instability is, however, only a problem when growth occurs. Plasmid loss was not detected in

Fig. 3. Luminescence (a) and dehydrogenase activity (b) following inoculation of a luminescence-marked strain of *Pseudomonas fluorescens* into sterile soil with addition (at 3 h) of phosphate buffer (○) or double strength 523 medium (*). (From Meikle *et al.*, 1992.)

unamended or amended sterilized soil inoculated with relatively high cell concentrations (10^7 – 10^8 cells g^{-1}) and was only evident after inoculation of amended soil with low cell concentrations (10^3 cells g^{-1}), allowing several rounds of cell division before exhaustion of nutrients.

Luminescence by marked soil inocula may be determined in soil suspensions but the presence of soil particles reduces sensitivity by approximately one order of magnitude. For example, the lower detection limit for plasmid-marked *E. coli* decreases from 200 cells ml^{-1} to 6×10^3 cells $(g \text{ soil})^{-1}$ (Ratray *et al.*, 1990) due to quenching and masking of light by soil particles.

As indicated above, light output per cell, and consequently lower detection limits, vary between strains. Improvements in cloning techniques are now providing significantly brighter strains, and lower detection limits, and there is considerable scope for development of luminometers with increased sensitivity to enhance detection further. Measurements of luciferase activity do not require enzyme purification, greatly reducing the time and expense of sample analysis, which can be completed in less than 5 min. This also avoids problems associated with contamination in environmental samples and extraction of cells from particulate material is not required, removing a major source of error in cell enumeration techniques. Luminometry is therefore sensitive, easy and rapid and measures accurately and reliably the biomass or cell concentration shortly after inoculation into the environment. The technique, however, measures population activity which will change following inoculation of cells into the environment. Direct measurement of luminescence from unamended environmental samples therefore provides a measure of the *in situ* metabolic activity of the marked organism. Luminescence following incubation with substrate (potential luminescence) enables quantification of marked biomass, by analogy with other measures of potential activity, e.g. the substrate induced respiration (SIR) method of Anderson & Domsch (1978).

To demonstrate measurement of *in situ* activity, Meikle *et al.* (1992) compared dehydrogenase activity and light output from sterile soil following inoculation with chromosomally *lux*-marked *P. fluorescens*. Dehydrogenase was chosen as one of the most sensitive *in situ* activity

assays and required incubation of samples with a chromogenic substrate for 6 h. Following incubation for 24 h, soil was amended with complex medium (Fig. 3), leading to parallel increases in dehydrogenase activity and luminescence. Both techniques measure *in situ* activity but luminometry provides four major advantages. The first is speed, as luminometry provides results within 5 min of sampling, rather than 6 h. The second is convenience, as luminometry merely involves addition of aldehyde substrate to a soil suspension prior to measurement, as opposed to addition of several reagents, incubation and centrifugation or filtering. Thirdly, luminometry is at least four orders of magnitude more sensitive than the dehydrogenase assay, enabling detection of *in situ* activity of approximately 10^4 cells g^{-1} , as opposed to 10^8 cells g^{-1} . The most important advantage is selectivity as luminometry measures the activity of the marked organism only, while enzyme assays and other activity techniques, e.g. radiorespirometry, measure the activity of the whole microbial community. This is particularly important for tracking GEMs, where the activity of the organism is of greater significance for environmental impact than its presence. An example of its use is provided by Ratray *et al.* (1992a) who studied the effect of matric potential on the survival and activity of *lux*-marked *E. coli* in soil and found significant differences between changes in viable cell concentrations and activity measured by luminometry and SIR.

Potential luminescence involves measurement of luminescence during incubation of samples with complex medium (Meikle *et al.*, 1994). Any cells capable of activation emit light during this period, typically 2 h. The kinetics of light output provide information on the time taken to recover activity, while the final luminescence value is a measure of the marked population capable of activation within the incubation period. In the SIR technique this is considered to be equivalent to biomass concentration. Again this technique is of particular relevance to studies on risk assessment associated with environmental release of GEMs. The environmental impact of an organism will depend on the speed with which it can react to favourable environmental conditions after periods of, for example, starvation. Activation may also be required for resynthesis of luciferase if protein

Fig. 4. Bright-field image (a) and CCD image-enhanced dark field image (b) of cells of a luminescence-marked strain of *Pseudomonas syringae* inoculated into non-sterile soil. In (a) cells cannot be distinguished from soil particles whereas bright regions in (b) result from the activity of single luminescent cells or microcolonies. Bars, 25 μm . (From Silcock *et al.*, 1992; published with the permission of the American Society for Microbiology.)

Fig. 5. Bright-field image (a) and CCD image-enhanced dark field image (b) of a wheat root colonized with a luminescent strain of *Enterobacter cloacae*. Bars, 10 μ m. Figure provided by E. A. S. Rattray, Department of Plant and Soil Science, University of Aberdeen, UK.

turnover has significantly reduced intracellular levels of the enzyme. Traditional techniques do not enable assessment of the response times of particular organisms, but this is essential for risk assessment. Following inoculation

of *lux*-modified *P. fluorescens* for periods of more than several days, activation does not occur during incubation for 2 h in the presence of complex medium. This has two implications for risk assessment. It suggests that

such populations would have little environmental impact, as substrates would be readily utilized by the indigenous population, preventing growth of the inoculum. Secondly, it indicates that in the absence of any selective advantage the inoculum would be unable to perform the function for which it was originally designed. The information from luminometric techniques is therefore of value for both risk assessment and development of more efficient microbial inocula.

Luminometry has been used by Shaw *et al.* (1992), in conjunction with viable cell enumeration and charge-couple device (CCD) imaging, to assess survival and dispersal of *lux*-marked *Xanthomonas campestris* inoculated onto cabbage plants and into surrounding soil. The construct contained the *luxCDABE* genes, encoding luciferase and aldehyde synthesis, avoiding the need for exogenous addition of the latter. Bioluminescence in samples of inoculated soil was followed during incubation in complex medium for 90 h. Maximum luminescence values were independent of inoculum size, but the time taken to reach maximum luminescence was related to cell concentration. As with the technique described above, this does not require extraction of bacteria from soil. The system was as sensitive as plate counting with respect to detection but requires development before use for quantification and enumeration.

A major problem in ecological studies, and risk assessment in particular, is the formation of viable but nonculturable cells which are not detected by traditional plating techniques. Viability or activity of such cells cannot be detected in non-sterile, environmental samples because of difficulties in distinguishing their activity from that of the background population. Luminometry, however, enables selective quantification of the activity of *lux*-marked cells and it has been shown to be preferable to traditional activity techniques, even in pure culture systems where established viability and activity techniques may be used (S. Duncan and others, unpublished). In comparison with the changes in cell length using the direct viable count method (Kogure *et al.*, 1979), detection of activity of viable but nonculturable forms of *E. coli* and *V. harveyi* was significantly better with respect to speed, convenience and lower detection limits, and may be used in natural samples. This offers, for the first time, a non-extractive technique for quantification of activity of viable but nonculturable cells in natural environments.

CCD microscopy

CCD image-enhanced microscopy enables detection and quantification of single photons from which an image may be constructed. Maximum sensitivity is provided by nitrogen-cooled systems, which reduce thermal noise. The technique provides very sensitive detection of luminescing organisms and the ability to study their spatial distribution in environmental samples. A simple application of CCD microscopy is in detection of luminescing colonies on agar (Waterhouse *et al.*, 1994). Colonies may be detected which cannot be seen by eye, either during early stages of colony development, when insuf-

ficient numbers of cells have been formed, or through reduced activity due to competition for nutrients on crowded plates. It is also possible to detect light emitted by colonies covered by spreading colonies of non-luminescing organisms. Thus CCD imaging increases the speed and sensitivity of viable cell enumeration of *lux*-marked organisms.

CCD microscopy also enables detection of active single cells and microcolonies. Silcock *et al.* (1992) demonstrated detection of single cells of *lux*-marked *Pseudomonas syringae* in soil suspensions (Fig. 4) and E. A. S. Rattray (personal communication) demonstrated localization of *lux*-marked *Enterobacter cloacae* colonizing wheat roots (Fig. 5). Quantification of light output by individual cells of *P. syringae* demonstrated reduced cell activity in non-sterile soil, in comparison with sterile soil, due to competition for nutrients by indigenous micro-organisms. As with luminometric methods, CCD imaging can be carried out with or without nutrient addition, enabling distinction between active and potentially active cells. Coupled with immunological techniques, which cannot distinguish active and inactive cells, CCD microscopy enables detection and discrimination between active, potentially active and inactive cells, with obvious implications for detection of viable but nonculturable forms. CCD microscopy has also been used by Shaw *et al.* (1992) to detect *lux*-marked *X. campestris* infecting cabbage plants. Light intensity was directly proportional to cell number, with a minimum detection level of 1.5×10^4 c.f.u. per leaf. Beauchamp *et al.* (1993) combined CCD imaging, luminometry and dilution plate enumeration to demonstrate differences in colonization of luminescence-marked rhizobacterial pseudomonads on a range of plant roots. Luminescence was detected in the crown regions but generally not in the tip or mid-root regions. CCD imaging was the least sensitive technique but had the important advantage of enabling true *in situ* detection of the marked population.

Conclusions

All the marker systems described enable viable cell enumeration. Antibiotic resistance markers are particularly useful in selecting against indigenous populations but selection is frequently incomplete in natural environments and marking with several antibiotic resistance genes may be necessary. Other marker systems can achieve such selection through traditional methodologies, e.g. use of selective media or growth conditions, or through combined marking with antibiotic resistance. The use of CCD imaging increases sensitivity of detection of colonies of luminescence-marked cells in the presence of background populations, and colonies may be detected even when overgrown by non-luminescent organisms. In other respects, the sensitivities of the different marker systems for viable cell enumeration are similar and depend on factors such as volume of inoculum per plate, ability to extract cells from the environment and choice of correct media, cultivation conditions and incubation period. The advantage which they provide over traditional plating

techniques is the ability to distinguish culturable cells of the marked organism from those of the indigenous population. They do, however, suffer from the well-accepted problems of traditional culture-based enumeration methods.

The *xyIE* markers have been developed with inducible gene expression systems. Such induction systems reduce metabolic load and are potentially applicable to any marker gene. The indications are that this may not be a problem, particularly for chromosomally marked strains. No differences in growth and survival have been detected between parent and chromosomally marked strains (Ratray *et al.*, 1992b; Drahos *et al.*, 1992) but this must be tested carefully before use in environmental studies (Ryder *et al.*, 1994). Care must be taken in relating effects on growth rates of plasmid-marked strains in laboratory culture to fitness in the environment. Yeung *et al.* (1989) marked cells of *Pseudomonas aeruginosa* and *Pseudomonas putida* with the *pglA* gene, encoding an α -1,4-endopolygalacturonase to determine the effect of galacturonase synthesis on growth and survival of this organism in bulk soil and in the rhizosphere. Selectivity between parent and plasmid-marked strains was achieved by simultaneous marking with antibiotic resistance. Although the specific growth rates of both species were unaffected by high levels of galacturonase production, there was some indication of poorer long term survival in plasmid bearing strains.

Total cell concentrations may be determined by probing for marker genes, most of which have been sequenced. Gene probing coupled with PCR amplification of DNA extracted from natural environments is possible (Steffan & Atlas, 1991) and provides essentially the same sensitivity for any marker system (and for non-marker gene sequences). In this respect, therefore, the marker systems show no differences but it is obviously necessary for the marker gene to remain in the host cell. For this reason, chromosomally marked strains are preferable because of reduced risk of gene loss or transfer. Total cell concentrations may also be determined by immunological detection of marker gene products, which is possible for *lux* and *xyIE* systems.

All marker systems provide the ability to determine viable and total cell concentrations. The most important advance provided by marker systems is the ability to measure marked cell activity without the requirement for extraction of cells and the need to culture organisms. Measurement of activity of *xyIE*-marked cells involves measurement of cell protein, following protein extraction and purification. Real time, *in situ* metabolic activity can be measured using luminescence from *lux*-marked organisms and provides for the first time the ability to quantify and localize the activity of a specific organism in the presence of indigenous microbial communities. The sensitivity of detection of light output, particularly using CCD imaging technology, and the speed of measurement of luminometric techniques make this a powerful technique which is now being applied to ecological studies of a range of organisms. The information now being

obtained is of enormous value for risk assessment studies for environmental release of GEMs, in determining activity and potential activity of culturable and nonculturable cells. Of equal importance is the ability of these techniques to increase our understanding of the environmental factors controlling microbial growth, activity, survival and interactions in natural environments.

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