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Molecular methods for environmental monitoring and containment of genetically engineered microorganisms

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Abstract

Plans to introduce genetically engineered microorganisms into the environment has led to concerns over safety and has raised questions about how to detect and to contain such microorganisms. Specific gene sequences, such as *lac*Z, have been inserted into genetically engineered microorganisms to permit their phenotypic detection. Molecular methods have been developed based upon recovery of DNA from environmental samples and gene probe hybridization to specific diagnostic gene sequences for the specific detection of genetically engineered microorganisms. DNA amplification using the polymerase chain reaction has been applied to enhance detection sensitivity so that single gene targets can be detected. Detection of messenger RNA has permitted the monitoring of gene expression in the environment. The use of reporter genes, such as the *lux* gene for bioluminescence, likewise has permitted the observation of gene expression. Conditional lethal constructs have been developed as models for containment of genetically engineered microorganisms. Suicide vectors, based upon the *hok* gene have been developed as model containment systems.

Introduction

When molecular methods developed the capacity for genetically engineering organisms it was clear that a scientific revolution would ensue that would impact human health, agricultural productivity, and environmental quality. The power of recombinant DNA technology to create organisms with novel genetic characteristics offered great promise for solving environmental problems but as an unknown technology also was met with caution within the scientific community and fear among the public (Curtiss 1976; Sharples 1983; Brown et al. 1984). Scientists sought to reassure the public of the safety of recombinant DNA technology and declared a moratorium in 1975 at the Asilomar Conference until host strains could be developed for recombinant research that could not escape from the laboratory. By assuring the containment of genetically engineered microorganisms within the laboratory, molecular biologists reduced the risk of the organisms they created causing any untoward effects on human health or the environment. They, perhaps unwittingly, furthered public fears by fostering an image of harmful 'Andromeda strains' that would cause great harm if they escaped the containment of the laboratory.

Given the atmosphere of peril surrounding the power of genetic engineering, proposals for the deliberate release of genetically engineered organisms to enhance agricultural productivity and environmental quality have been met with great caution. Regulatory constraints have been placed on such releases of genetically engineered organisms to a greater extent than upon nonrecombinant ones (Federal Register 1986). Several symposia have

been held to examine what we know about releases of organisms into the environment, survival of organisms in various environments, rates and processes of natural recombination in the environment, and other factors relating to risks and potential applications of genetically engineered organisms in the environment (Halvorson et al. 1985; Sussman et al. 1988).

The scientific community, having examined the organisms that can be created by recombinant DNA technology and having gained a sense of safety through several years of experience, now feels that there are no greater risks associated with recombinant organisms than with other naturally occurring and genetically modified organisms. Scientists have proposed (a) that risk analysis be used as the basis for establishing the relative safety of deliberate releases and (b) that all genetically modified organisms be evaluated in terms of their phenotypic properties and our familiarity with releases of organisms with identical or similar phenotypes.

Cultural methods for detecting genetically engineered microorganisms

Selective markers

Most genetically engineered microorganisms contain marker genes that can be used for their detection. These marker genes often are essential in the construction of the genetically engineered microorganism as they allow for the selection of recombinants that are likely to contain the desired combination of genes. Frequently, antibiotic resistance markers are employed so that by plating onto media containing the antibiotic, only resistant microorganisms will grow. The problem of using antibiotic resistance markers for microorganisms that are deliberately released into the environment is twofold. First, there is a background of resistant microorganisms in environmental samples, especially in soils, so that the selection of antibiotic resistant microorganisms will include both target genetically engineered microorganisms and nontarget background microbial populations. Second, it is not desirable to increase the prevalence of antibiotic resistance genes that may become disseminated into populations of pathogenic microorganisms. To reduce the risk of creating medical problems with drug-resistant pathogens, only antibiotic resistance markers associated with antibiotics of limited therapeutic use should be employed.

Additional markers have been developed as reporter genes that can be introduced into microorganisms so that they subsequently can be detected. Adding the genes for lactose metabolism permits the use of conventional selective and differential media for the detection of organisms containing this marker gene. The *lac*Z system, which encodes lactose utilization (β-galactosidase production) in *E. coli*, has been used as a marker or reporter of recombinant *Pseudomonas* in studies to determine whether genetically engineered microorganisms will survive and disperse if released at an environmental site (Drahos et al. 1986). This marker system has proved very effective in tracking the movement of genetically engineered pseudomonads in soils and especially within the rhizosphere. *Pseudomonas* strains that incorporate the *lac*Z genes are capable of cleaving the X-gal substrate to produce a characteristic blue color. This trait makes them very distinguishable from the nonrecombinant *Pseudomonas* species that are indigenous to the site.

The *xyl*E gene of the TOL plasmid has been cloned for use as a transcriptional fusion reporter gene. This gene encodes catechol 2,3 oxygenase; a simple aerosol spray technique has been used to find recombinant colonies (Zukowski et al. 1983; Ingram et al. 1989). A similar assay system has recently been described by King et al. (1991) using the 2,4-dichlorophenoxyacetate monooxygenase gene, which codes for the conversion of phenoxyacetate to phenol that then can be reacted with a dye to form a colored compound. The *lux* genes that encode bioluminescence are useful as reporter genes that permit not only detection of microorganisms but also monitoring of their activities (Boivin et al. 1988; Engebrecht et al. 1982). Burlage et al. (1990) described a fusion between a promoter (nah) for the degradation of naphthalene in *Pseu-*

domonas and the *lux* genes. Using this plasmid construction, they were able to demonstrate gene expression on a continuous basis, revealing an unexpected pattern. This work suggests the biotechnological applications that may arise from further use of the *lux* genes. King et al. (1990) used another nah-lux construction to demonstrate naphthalene degradation in soil slurries.

Colony hybridization

Colony hybridization is the simplest molecular approach for the detection of genetically engineered microorganisms that can be integrated with conventional environmental microbiological sampling and analysis (Sayler & Layton 1990). In the colony hybridization procedure bacteria are grown on solidified agar media to form colonies. Gene probes and nucleic acid hybridization are then used to detect colonies with specific target nucleic acid sequences. Bacterial colonies are transferred from primary environmental cultivation media to hybridization filters. The colonies are lysed by alkaline or enzymatic treatment and hybridization is then conducted. These methods are dependent on the ability of the target microorganisms to grow on the primary isolation medium and not be totally overgrown by nontarget populations. Growth on the isolation medium increases the number of copies of the target gene to a level that can be detected by a gene probe.

The original colony hybridization protocol developed by Grunstein & Hogness (1975) is suitable for high density plate screening for pure cultures (Hanahan & Meselson 1980). In cultures containing both nontarget *E. coli* and target *P. putida*, one *P. putida* colony containing a toluene (TOL) catabolic plasmid in a background of approximately one million *E. coli* colonies was detected using a whole TOL plasmid probe (Sayler et al. 1985).

Gene probes can be hybridized with primary isolations from environmental samples (Echeverria et al. 1982; Fitts et al. 1983; Hill et al. 1983; Miliotis et al. 1989). The rationale for direct colony hybridization on primary cultivation includes: (a) avoiding a cultivation bias encountered by selective media which may underestimate total abundance of a given genotype; (b) assuring that a given genotype is represented in the population sampled even if the genes are poorly expressed or are poorly selected; (c) providing optimal permissive growth conditions for stressed organisms that may be unculturable on selective media; and (d) reducing the analysis time for cultivation, presumptive quantification and confirmation of a genotype/phenotype (Atlas et al. 1992).

The major uses for colony hybridization in environmental studies have been for the detection, enumeration, and isolation of bacteria with specific genotypes and/or phenotypes, and for development of gene probes (Ford & Olson 1988; Holben & Tiedje 1988). Colony hybridization has been used to detect *Salmonella* (Fitts et al. 1983), *Shigella* (Sethabutr et al. 1985), *Escherichia coli* (Echeverria et al. 1982; Bialkonska-Hobrzanska 1987; Seriwantana et al. 1987; Nishibuchi et al. 1989), *Listeria* (Datta et al. 1987, 1988; Flamm et al. 1989; Nortermans et al. 1989), *Yersinia* (Hill et al. 1983; Miliotis et al. 1989), *Rhizobium* (Hodgson & Roberts 1983), *Erwinia amylovora* (Falkenstein et al. 1988), *Bacillus subtilis* (Kraus et al. 1986), *Pseudomonas fluorescens* (Festl et al. 1986), 4-chlorobiphenyl degrader (Pettigrew & Sayler 1986), toluene degraders (Jain et al. 1987), naphthalene degraders (Blackburn et al. 1987), and mercury resistant bacteria (Barkay 1985, 1987; Barkay & Olson 1986). Jain et al. (1987) used colony hybridization and gene probes to study the maintenance of catabolic and antibiotic resistance plasmids in groundwater aquifer microcosms. They found that introduced catabolic plasmids or organisms can be maintained in groundwater aquifers without selective pressure. In another study, colony hybridization with a naphthalene gene probe was used to correlate gene frequency with naphthalene degradation by naphthalene degrading bacteria in activated sludge; the gene probe analysis for the catabolic genotype was nearly two orders of magnitude more sensitive than the standard plate assay for naphthalene degradation (Blackburn et al. 1987). Colony hybridization has been used with environmental

samples to enumerate mercury resistant bacteria in contaminated environments (Barkay et al. 1985; Barkay & Olson 1986) and naphthalene degrading bacteria in aromatic hydrocarbon contaminated soils (Jain et al. 1987).

In general, for environmental assays the organism of interest must be relatively abundant in the population so that at least one positive colony on a agar plate of 100 to 1000 colonies can be found. Additional sensitivity can be achieved by plating the isolated bacteria onto selective agar before colony hybridization.

Direct gene probe detection

Molecular analyses can also be performed without culturing microorganisms to detect the presence of genetically engineered microorganisms in environmental samples. DNA can be recovered from water samples by collecting cells using filtration and then lysing the cells to release their nucleic acids (Fuhrman et al. 1988; Sommerville et al. 1989; Bej et al. 1990; Giovannoni et al. 1990). Sommerville et al. (1989) isolated DNA from aquatic samples by filtering large volumes of water through a filter cartridge. Paul et al. (1990) reported that purification by cesium chloride-ethidium bromide (CsCl-EtBr) ultracentrifugation was required for isolation of purified DNA. In other studies multiple phenol or phenol:chloroform extractions have produced sufficiently pure DNA from cyanobacteria (Zehr & McReynolds 1989).

For soils, differential centrifugation can be used to recover cells; the cells can then be lysed by chemical and/or physical description methods (Balkwill et al. 1975; Faegri et al. 1977; Torsvik & Goksoyr 1978; Torsvik 1980; Bakken 1985; Holben et al. 1988; Steffan et al. 1988; Jansson et al. 1989; Steffan & Atlas 1990; Torsvik et al. 1990). The inclusion of a polyvinylpolypyrrolidone (PVPP) decreases humic content prior to cell lysis (Holben et al. 1988; Steffan et al. 1988), simplifying DNA purification.

DNA can also be extracted directly from environmental samples without first recovering cells (Ogram et al. 1987). In this method cells are lysed while still within the soil matrix for example, by incubation with SDS followed by physical disruption with a bead beater. The DNA is then extracted with alkaline phosphate buffer. This method, with some modifications, has been successfully used to recover DNA from soils and sediments (Ogram et al. 1987; Steffan et al. 1988; Pillai et al. 1991; Tsai & Olson 1991; Tsai & Olson 1992). Purification procedures can be any combination of, CsCl-EtBr ultracentrifugation (Holben et al. 1988; Steffan et al. 1988; Paul et al. 1990), hydroxylapatite or affinity chromatography (Torsvik & Goksoyr, 1978; Ogram et al. 1987; Steffan et al 1988; Paul et al. 1990), phenol/chloroform extractions, ethanol precipitations (Fuhrman et al. 1988), dialysis, or repeated PVPP treatments (Holben et al. 1988; Steffan et al. 1988; Weller & Ward 1989; Paul et al. 1990). Significantly higher yields of DNA are recovered with the direct extraction method than with the cell recovery procedure, but the DNA may contain impurities that can inhibit enzymatic manipulation (Steffan et al. 1988).

Gene probes and nucleic acid hybridization permit the detection of DNA sequences recovered by extracting DNA from environmental samples that are homologous to the probe sequence (Holben et al. 1988; Barkay et al. 1989; Sayler & Layton 1990). The stringency of the hybridization is determined by temperature, salt concentration, and the length and concentration of the target and probe sequences (Berent et al. 1985; Britton & Davidson 1985). In most hybridization protocols the target DNA probe is immobilized on a nitrocellulose or nylon filter. Labelled probe DNA is added to the filters so that the gene probe can hybridize to complementary target DNA sequences. The probe often is labelled ^{32}P which is detected by autoradiography. Using gene probes and DNA extraction procedures, it is possible to detect introduced genetically engineered microorganisms from soils (Holben et al. 1988; Steffan et al. 1988; Jansson et al. 1989).

Specific gene sequences, diagnostic of specific genetically engineered microorganisms, can be amplified using the polymerase chain reaction (PCR). PCR is a method for the *in vitro* replication of defined sequences of DNA whereby gene segments can be amplified exponentially (Mullis & Faloona

1987; Mullis 1990; Ehrlich 1989, Ehrlich et al. 1991; Innis et al. 1990; Saiki et al. 1988). One application of this technique is to enhance gene probe detection of specific gene sequences. By exponentially amplifying a target sequence, PCR significantly enhances the probability of detecting rare sequences in complex mixtures of DNA such as those recovered from environmental samples (Bej & Mahbubani 1992; Bej et al. 1991; Steffan & Atlas 1992; Tsai & Olson 1992).

Steffan & Atlas (1988) used PCR to amplify a 1.0-kilobase (kb) probe-specific region of DNA from the genetically engineered, herbicide-degrading bacterium *Pseudomonas cepacia* AC1100 in order to increase the sensitivity of detecting the organism by dot-blot analysis. The 1.0-kb region was an integral portion of a larger 1.3-kb repeat sequence which is present as 15 to 20 copies on the *P. cepacia* AC1100 genome. PCR was performed by melting the target DNA, annealing 24-base oligonucleotide primers to unique sequences flanking the 1.0-kb region, and performing extension reactions with DNA polymerase. After extension, the DNA was again melted, and the procedure was repeated for a total of 25 to 30 cycles. After amplification, the reaction mixture was transferred to nylon filters and hybridized against radiolabelled 1.0-kb fragment probe DNA. Amplified target DNA was detectable in samples initially containing as little as 0.3 pg of target. The addition of 20 μg of nonspecific DNA isolated from sediment samples did not hinder amplification or detection of the target DNA. The detection of 0.3 pg of target DNA was at least a 103-fold increase in the sensitivity of detecting gene sequences compared with dot-blot analysis of nonamplified samples. PCR performed after bacterial DNA was isolated from sediment samples permitted the detection of as few as 100 cells of *P. cepacia* ACl100 per 100 g of sediment sample against a background of 1011 diverse nontarget organisms; that is, *P. cepacia* ACl100 was positively detected at a concentration of 1 cell per g of sediment. This represented a 103-fold increase in sensitivity compared with nonamplified samples.

Chaudhry et al. (1989) also used PCR for detecting genetically engineered microorganisms. They cloned 0.3-kilobase napier grass (*Pennisetum purpureum*) genomic DNA that did not hybridize to DNAs isolated from various microorganisms, soil sediments, and aquatic environments, into a derivative of a 2,4-dichlorophenoxyacetic acid degradative plasmid, pRC10, and transferred the construct into *Escherichia coli*. This genetically altered microorganism was seeded into filter-sterilized lake and sewage water samples (104/ml). The PCR method amplified and detected the 0.3-kilobase DNA marker of the genetically engineered microorganism even after 10 to 14 days of incubation. The PCR method required only picogram amounts of DNA and had an advantage over the plate count technique, which can detect only culturable microorganisms. They concluded that the method may be useful for monitoring genetically engineered microorganisms in complex environments, where discrimination between genetically engineered microorganisms and indigenous microorganisms is either difficult or requires time consuming tests.

Containment of genetically engineered microorganisms

Debilitated strains and vectors

The initial containment strategy for genetically engineered microorganisms was to employ debilitated host bacteria that could not survive outside of a laboratory environment (Curtiss et al. 1977). An *Escherichia coli* strain that obligately requires exogenous diaminopimelic acid and thymine and is very sensitive to bile, detergents, and antibiotics was created as a fail safe containment host; accidental releases of genetically engineered DNA in this and similar microorganisms would be contained because the bacterial host was extremely fastidious. An alternate means of containment would be to create host strains with reduced ATP generating capacities so that they could not compete with wild types in the environment (Curtiss 1988). Such strains would not likely be useful, however, for environmental applications because of their limited survival capacity in the environment.

Vector systems have been proposed and devel-

oped to prevent transfer of genes to other organisms, that is, to contain the recombinant genes to the host organism. One strategy for containing recombinant genes is to construct cloning vectors that will not survive or transfer genes to indigenous microbial populations outside of the laboratory. Vectors carrying mutations that decrease the host range reduce the possibility of transferring genes (Blattner et al. 1977). Plasmid-based cloning vectors that contain only an origin of replication and the desired structural genes lack transfer functions are not readily mobilizable, even by triparental matings, and thus are relatively contained (Levine et al. 1983). Another strategy is to use an impaired transposon delivery system that will insert genetically engineered DNA into the chromosome and is then unable to undergo further transposition (Obukowicz et al. 1986, 1987).

Vectors have also been considered that are suicidal, such that they could not survive transfer to another organism (Curtiss 1988). A plasmid vector with a nonsense mutation in a gene indispensable for plasmid replication and/or maintenance and a suppressor mutation in the chromosome that would allow translational read-through of the message of the gene would be self contained in the host strain (Curtiss et al. 1976, 1977). An alternate strategy would be to endow a plasmid vector with a gene for a restriction enzyme where the ability to synthesize the modification methylase would be specified by a chromosomal gene so that transfer of the plasmid to a recipient cell would lead to the synthesis of the restriction enzyme that would digest the DNA of the recipient and cause death.

Conditional-lethal ('suicide') vectors

One approach for containing genetically engineered microorganisms is to create genetically engineered microorganisms that contain 'suicide vectors' (plasmids with conditionally lethal genes) which could act to prevent movement of genes from the genetically engineered microorganism to other organisms and to contain such organisms within specified bounds (Cuskey & Bourquin 1987; Molin et al. 1987). Organisms could either be designed to remain viable only under the specific environmental conditions of their intended use, for example, only in situations where a particular pesticide or hazardous chemical was present, and to die if they escaped that location or if the environmental condition requirement for their survival changed; alternatively, they could be designed to survive until a specific environmental condition occurred or was introduced that would trigger their death. For industries marketing genetically engineered microorganisms to treat hazardous waste sites, this would be desirable not only because of the reduced potential of environmental risk if the genetically engineered microorganism persisted in the environment, but also because the organisms would have a built-in self destruction mechanism that would lead to new sales for future site bioremediation actions. Thus, creation of suicide vectors may simultaneously meet the economic interest of industry and the public interest for environmental protection.

Model suicide vectors have been constructed using the lethal *hok* gene, which codes for a small polypeptide (Hok) that causes loss of cell membrane potential and rapid death of cells in which it is overproduced (Molin et al. 1987; Bej et al. 1988). We have constructed suicide plasmids containing the *hok* gene under the control of the *lac* promoter. The *lac*Iq gene, which codes for an excess of Lac repressor protein adequately suppresses Hok polypeptide production in strains carrying the suicide vector, permitting the *via*bility of the bacterial cells carrying the conditional suicide vector in the absence of inducer.

IPTG was found to rapidly induce the hok gene in *E. coli* JM101 carrying the suicide plasmid growing *in vitro*. Compared to controls of *E. coli* JM101 lacking the suicide vector, which continued exponential growth after IPTG addition *in vitro*, cultures containing the suicide vector initially ceased to grow and showed evidence of death, both by optical density determinations and *via*ble cell counts between 1 and 2 h after IPTG addition. After 2 h, though, cultures inoculated with *E. coli* JM101 containing the suicide vector resumed expo-

nential growth at a rate comparable to the control cultures. However, by 4 h after IPTG addition no colonies were observed on selective media containing carbenicillin. Moreover, no *hok* genes or suicide vector plasmids could be detected by colony hybridization or Southern blot analyses from cells growing on nonselective media. These results indicate that induction of the *hok* gene had killed all cells containing the suicide vector and that the cells growing after IPTG addition had lost this plasmid. The percentage of *hok*-carrying cells decreased from 97% to 0% within 4 h of IPTG induction of the *hok* gene.

In contrast to these results, in separate *in vitro* experiments in which carbenicillin was added as a selective pressure against loss of the suicide vector plasmid, Hok-resistant cells developed before all *hok*-carrying cells were eliminated from the cultures; this resulted in the development of a significant population of Hok-resistant-*hok*-carrying cells. After an initial period of significant decline during which the proportion of cells carrying hok decreased from 97% to 14%, the proportion of cells carrying the hok gene increased to 87%; thus, after 8 h the proportion of cells with the suicide vector was only 10% lower than prior to addition of IPTG. Transformation of fresh *E. coli* JM101 cells with the suicide vector from the *hok*-resistant cells resulted in *hok*-sensitive cells that died, indicating that the hok gene was still functional.

Apparently, the presence of antibiotic represented a sufficient selective pressure against loss of the suicide vector such that Hok-resistant mutants, still containing the suicide vector, were selected for. Addition of IPTG to soil microcosms containing *E. coli* JM101 with the suicide vector led to a 90% decline in colonies detected on the selective media, whereas numbers of cells did not decline in soil microcosms where IPTG was not added. The surviving cells were debilitated and not capable of normal growth or prolonged plasmid maintenance even in liquid broth culture. It is likely that the persistence of suicide vector-containing cells in soil was the result of the development of Hok-resistance, which protected the cells against Hok-induced death, but also rendered them unable to generate sufficient energy for long-term survival in the environment or long-term maintenance of the suicide plasmid.

Thus, the model *hok* suicide vector, while not yet an adequate fail safe system for containing genetically engineered microorganisms in environmental samples, appears to have real potential for limiting the spread of genetically engineered microorganisms. Conditional lethal strains of pseudomonads, designed as models for containment of genetically engineered microbes, were also constructed by cloning the *hok* gene into plasmids under the control of the *tac* promoter. *Pseudomonas putida* mt-2 was transformed with this conditional lethal plasmid and growth and survival of the host organism and retention of the suicide vector were examined. *Hok* induction caused elimination of greater than 60% of the cells carrying the suicide vector within 5 h of induction compared to 100% retention in uninduced controls. Resistance to *hok*-induced killing developed in *P. putida*, which may have been due to a mutation or physiological adaptation that rendered the membrane 'resistant' to Hok. Constructs were also designed using a *hok* homolog, *gef*, also under control of the *tac* promoter. These constructs were highly effective in *P. putida*, when inserted into the chromosome or on a stable RK2-derived plasmid.

Knudsen & Karlstrom (1991) investigated factors in *E. coli* which reduced survival after induction of the *E. coli relF* gene, which is homologous to *hok* and *gef*. They found that the use of more tightly-controlled promoters to repress the suicide gene improved the efficiency of their system by preventing selection of cells mutated in the killing function. An additional improvement was observed when the conditional lethal system was introduced into the test organism in multiple copies; in such cases mutation rates were reduced. Our results suggest that an additional factor in containment using such conditional lethal constructs may be the stability of the vector for introducing and maintaining these constructs in the host bacteria. Improved stability (via stably-inherited plasmids or via chromosomal insertion) combined with tightly-controlled promoters and duplicated systems pro-

vide a measure of containment for genetically-engineered microbes which could be deliberately released.

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