Journal of Hazardous Materials, 28 (1991) 13–27
Elsevier Science Publishers B.V., Amsterdam

Contribution of molecular biology to bioremediation*

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Abstract

Molecular biology contributions to hazardous waste biodegradation and bioremediation are broad ranging and clearly transcend the issue of genetic engineering of degradative performance. The molecular tools used in recombinant DNA technology can contribute to critical issues in bioprocess monitoring and optimization regardless of whether engineered or indigenous organisms are used in hazardous waste control. The objective of this report is to describe the broader developing applications of molecular biology in hazardous waste biodegradation and bioremediation. These developments and applications can permit the establishment of a new generation of more effective and predictable biotechnology for hazardous waste management.

Introduction

In the past decade new knowledge has been developed on the biochemical and molecular basis for microbial degradation of hazardous chemicals and environmental pollutants. Specific genes and regulated operons for biochemical pathways of degradation have been identified and isolated. Using modern recombinant DNA technology, microorganisms have been genetically engineered to develop or improve biodegradation capacity. Engineered strains have been patented.

For some researchers these successful developments reinforce an optimistic outlook for molecular biology to contribute to enhanced performance and optimization of biological treatment and bioremediation of hazardous wastes. Yet, for some researchers and environmental practitioners major questions exist concerning both the need and practical application of the technology in hazardous waste management.

Both points of view can be justified and, yet, neither can be totally justified on their own frame of reference. Even though a high diversity of biodegradative potential exists in the environment pollutant chemicals do persist for signifi-

*Paper presented at the GCHSRC Third Annual Symposium: Bioremediation, Fundamentals and Effective Applications, Lamar University, Beaumont, TX, USA, February 21–22, 1991.

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cant time periods. To date, relatively few microbial strains and biodegradative pathways have been sufficiently well-characterized to permit broad scale genetic engineering. No microbial strains have been developed to degrade chemicals for which a natural degradative microbial counterpart does not already exist. On the other hand, microbial strains have been developed with increased and expanded ranges of biodegradative activity [1,2]. The potential has been demonstrated for superior process control with engineered strains as compared to indigenous degradative organisms [3].

Numerous meetings and workshops have been held to document the broad degradative capabilities of microorganisms, environmental factors influencing the degradative fate of hazardous chemicals in the environment, current state of knowledge on the biochemistry and molecular biology of biodegradation, and application of biodegradative processes in hazardous waste control [4–8]. Collectively, these past efforts demonstrate; (i) the capacity of microbes to degrade a majority of chemicals considered hazardous, (ii) the potential for modifying the degradative capacity of microbes, (iii) the difficulty of monitoring and controlling biodegradative processes in the environment and (iv) questionable credibility of biodegradation performance and predictability in the field.

In 1987 a National Science Foundation (NSF) research planning workshop attempted to integrate the molecular and environmental perspectives into a framework for environmental biotechnology for hazardous waste biodegradation and bioremediation [9]. Limitations affecting the development and implementation of the technology were identified and have not changed marginally:

(1) The present knowledge on the biochemistry, genetics, and ecology of biodegradative microorganisms and genes is limited to a few well-characterized model systems of unknown relevancy to applications in open environments and engineered systems.

(2) Attempts at strain improvement by either conventional selection or genetic engineering techniques are largely limited in their scope relative to the needs for application to real problems.

(3) Engineering applications for biodegradative microbial populations are generally limited to relatively easy-to- degrade chemicals under relatively ideal conditions.

(4) Specific and quantitative analytical and molecular tools are needed to monitor and control the population dynamics and activity of biodegradative organisms and genes.

(5) Integration of the required disciplines in problem- identification and problem-solving modes has not been achieved.

(6) Available resources to support the needed fundamental research contributing to environmental biotechnology are insufficient and scattered among diverse sources and are not targeted to a strategy that will result in the

timely development and implementation of the technology for the control of hazardous chemicals.

A consensus research agenda was established that would help to overcome these limitations and promote unity in developments of molecular biology with the needs and applications in engineering practice [9]. This agenda is summarized as follows:

(1) Agent and strain development and improvement.
(2) Development and improvement of analytical and molecular monitoring methods.
(3) Environmental and ecological systems analysis.
(4) Engineered systems analysis.

Molecular biology contributions to this agenda are broad ranging and clearly transcend the issue of genetic engineering of degradative performance. The molecular tools used in recombinant DNA technology can contribute to critical issues in bioprocess monitoring and optimization regardless of whether engineered or indigenous organisms are used in hazardous waste control. The objective of this report is to describe the broader developing applications of molecular biology in hazardous waste biodegradation and bioremediation. These developments and applications can permit the establishment of a new generation of more effective and predictable biotechnology for hazardous waste management.

Microbial biodegradation and strain improvement

The degradative capabilities of microorganisms for a wide variety of environmental pollutants has been well-documented [10–13]. In general, degradative organisms are recovered from batch or static enrichment cultures. These probably represent the majority of currently available microbial agents for candidate use in bioremediation. As indicated by Table 1, batch enrichment techniques are only one of at least six principle methods for generating pure or mixed biodegradative cultures.

Often, the initial indications that biodegradation of a particular chemical is possible is made from observations of environmental fate or treatability studies. From such studies, pure cultures may be recovered that promote partial or complete biochemical conversion of the chemical of interest. In some cases, pure culture metabolism may be incomplete due to the existence of only a partial metabolic pathway or degradation can be co-oxidative in nature due to a secondary metabolism of the pollutant as a non-growth promoting substrate usually at the expense of a primary oxidizable substrate. Often time consortia, stable mixed populations, may be found to collectively promote the complete degradation and mineralization of some otherwise poorly degraded substrates.

Applications for molecular information and techniques in degradative strain isolation and improvement are primarily associated with *in vivo* or *in vitro*

TABLE 1

General approaches in microbial strain development and improvement for degradation of hazardous wastes

Approach	Candidate microbes recovered
Batch enrichment	Pure and mixed cultures capable of growth on specific contaminants
Chemostat selection	Selection of high efficiency pure or mixed cultures for specific contaminants
Gene probe selection	Pure cultures with biodegradative pathways related to existing organisms, but with perhaps greater versatility and fitness
Plasmid assisted molecular breeding	Pure cultures with new biodegradative pathways
Plasmid expansion of catabolic pathway	Pure cultures with broadened range of biodegradation
Genetic engineering and pathway construction	Pure cultures with new and broaden ranges or more efficient and controllable degradation

development or selection of organisms with degradative plasmids, and gene cloning techniques for construction of engineered strains. These techniques may give rise to organisms with broadened ranges of chemical degradation or organisms with new degradative capacity. Gene probe methods have been successfully applied to isolating organisms from the environment. Organisms recovered by gene probe methods may have biodegradative pathways related to existing organisms, but demonstrate greater diversity and, perhaps, versatility.

Plasmids

In organisms for biotechnical use, specific genes associated with pollutant degradation have been localized in both the chromosomes of the organisms and on plasmids. Plasmids are small, circular extrachromosomal strands of DNA which are not normally required by the host microbe for normal maintenance functions. Plasmids may contain a number of genes associated with their own maintenance and transfer to other cells, and frequently contain genes encoding partial or complete degradative pathways for specific chemicals. In some cases such as naphthalene degradation, distinct alternative pathways may be both chromosomally and plasmid encoded. Because of their relative molecular simplicity, ease of characterization, and molecular use in genetic engineering technology, substantial information has been developed on their molecular biology and involvement in diverse modes of biodegradation [16]. A summary of representative plasmids associated with biodegradation is presented in Table 2. Many of these plasmids are associated with metabolism of naturally occurring substrates. However, enzymes associated with these plasmid encoded genes, in particular the oxygenases, may demonstrate rather broad

TABLE 2

Common catabolic plasmid with potential for use in environmental remediation

Reference plasmid	Incompatibility group	Compounds degraded[a]	Original bacterial host	Environmental source
pSS50	IncP1	4-CB	*Alcaligenes* and *Acinetobacter*	sediment
pJP4	IncP1	2,4-D, 3-CBA and MCPA	*Alcaligenes*	soil and water
OCT	IncP2	Octane, hexane and decane	*Pseudomonas putida*	soil
CAM	IncP2	Camphor	*Pseudomonas putida*	soil
pJP2	IncP3	2,4-D and MCPA	*Alcaligenes*	soil and water
Naph	IncP7	Naphthalene	*Pseudomonas*	soil
pWWO	IncP9	Toluene, *p* and *m*-xylene	*Pseudomonas putida*	soil
NAH7	IncP9	Naphthalene	*Pseudomonas putida*	soil
Cresol	IncP9	Cresol	*Pseudomonas putida*	soil

[a]Abbreviations: 4-CD, 4-chlorobiphenyl; 2,4-D, 2,4-dichlorophenoxyacetic acid; 3-CBA, 3-chlorobenzoic acid; MCPA, 4-chloro-2-methylphenoxyacetic acid.

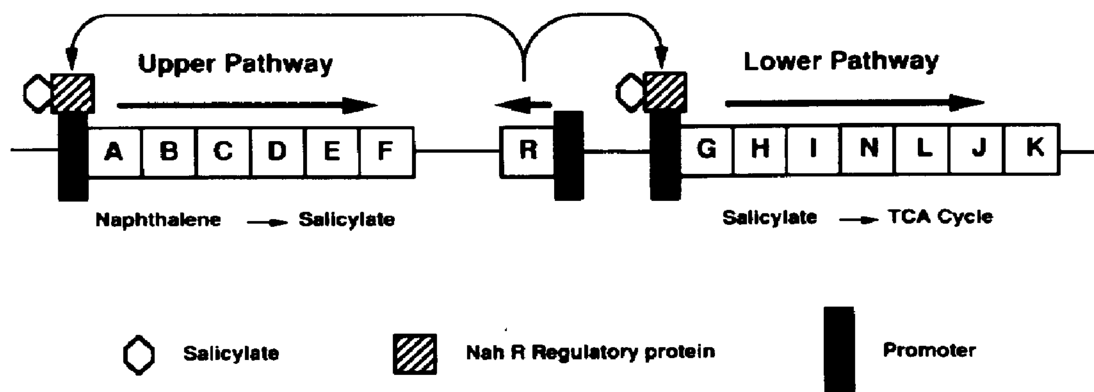


Fig. 1. Dual operon model of catabolic gene regulation for plasmid mediated naphthalene degradation.

substrate specificity. Two of the best characterized plasmids in terms of the degradative genes are the toluene (TOL) and naphthalene (NAH) catabolic plasmids. These two closely related plasmids contain entire genetic operons

for catabolism of xylenes and toluene (in the case of TOL) and naphthalene (in the case of NAH7) to central intermediary metabolites. These metabolites are capable of supporting sole carbon and energy source requirements in some *Pseudomonas* strains. The genetic operon of the NAH7 plasmid is described in Fig. 1. Both plasmids are structured around a dual operon model of an "upper" and "lower" pathway. The upper pathway for naphthalene degradation mediates oxidation of naphthalene to salicylate and the lower pathway the oxidation of salicylate. Both the upper and lower pathways are induced by benzoate. In the case of TOL, the upper pathway is induced by toluene and functions to degrade xylenes and toluene to toluate and benzoate, respectively. The lower pathway is benzoate inducible and is responsible for the meta cleavage pathway of degradation of toluate and benzoate. For both plasmids, the products of specific regulatory genes (R and S, in the case of TOL; and R, in the case of naphthalene) interact with the appropriate inducer to promote expression of the operons.

Genetic constructions for strain improvement

Genetic engineering methods do result in improving the degradation range of individual isolates. An example of this case is the degradation range expansion of *Pseudomonas putida* strain B13 [17].This strain was naturally able to completely degrade 3-chlorobenzoate (and 4-chlorophenol by selection) through a novel modified ortho cleavage pathway for chlorocatechol metabolism. By plasmid introduction of specific toluene degradation genes (D and L) and the regulatory gene S (benzoate metabolism) into this organism, the organism's degradation range was expanded to also include mineralization of 4-chlorobenzoate [2]. Further pathway expansion was achieved by introduction of genes from an *Alcaligenes* strain for 4-methyl lactone isomerization and oxidation to allow B13 to grow also on 4-methyl benzoates and chlorophenols.

A good example of genetic engineering for developing controllable degradation processes is TCE degradation by an *Escherichia coli* strain containing cloned genes for a toluene mono oxygenase (TMO) that normally oxidize toluene to *p*-cresol (in *Pseudomonas*) (3). Cresol normally induces TMO and the enzyme non-specifically oxidizes TCE as a non-growth substrate in *Pseudomonas*. In *E. coli* the cloned TMO gene was placed under regulatory control of a temperature inducible lambda phage promoter (P_l). The resulting *E. coli* strain can be grown on sugars without cresol and the TMO can be temperature induced at 42 °C to degrade TCE. Degradative strains such as these would seem to have immediate applications in confined reactor treatment systems.

These examples of microbial strain development are clearly dependent on substantial basic knowledge of the biochemistry, genetics and molecular biology of degradative pathways. There is a major need for expanded research in this area to exploit more fully applications in environmental biotechnology.

Bioanalytical measurement and environmental monitoring

A critical path to successful biotechnical application of microbial processes in waste control is sensitive and efficient monitoring of the biological process (the responsible organism or genes). This is true regardless as to the engineered or indigenous nature of the organisms involved; although in the case of engineered organisms, adequate genetic monitoring capability contributes substantially to issues of safety assurance.

Contributions to new bioanalytical measurement methods are derived from both the knowledge and the tools of modern molecular biology. There is a broad spectrum of current microbial or molecular monitoring technology [18] and some of these are selected as examples for their relevancy to hazardous waste bioremediation.

Plasmid analysis methods have been employed to determine the prevalence of specific degradative genes in bacterial populations mediating waste degradation. In one example, a *Pseudomonas* strain carrying a TOL plasmid and an antibiotic resistance plasmid was introduced into groundwater aquifer material under laboratory simulation in order to correlate plasmid maintenance with chemical degradation [19]. In both chemically contaminated and clean aquifer materials the introduced plasmids could be re-isolated from bacterial culture of the aquifer material, even after prolonged incubation (8 weeks). Agarose gel electrophoresis techniques were employed to confirm the presence of the specific plasmids during the course of incubation.

More sophisticated approaches in plasmid analysis use a fingerprinting technique called restriction fragment length polymorphism (RFLP) analysis. This technique uses specific restriction (DNA cutting) endonuclease enzymes to fragment plasmids into linear DNA segments of differing molecular weight to determine identity or dissimilarities. An example of this analysis for a chlorobiphenyl degradation plasmid isolated from bacteria in PCB contaminated sediment is given in Fig. 2. Derivatives of this plasmid can be isolated from the environment for a period over ten years.

While the results demonstrated long-term maintenance of the introduced or natural plasmids and host organism, there is no clear correlation with degradation of test contaminants. Furthermore, plasmid analysis tends to be labor intensive and time consuming (requiring cultivation of the organisms and plasmid purification); hence not routinely practical as a monitoring method, but surely an excellent confirmatory test.

A recent example in state-of-the-art measurement of biodegradative microbial communities is nucleic acid analysis and gene probe technology. DNA probe technology was originally used to detect and quantify degradative bacterial colonies containing catabolic genes (20) or metal resistances genes [21] and relate specific gene frequency with pollutant degradation or metal transformations in the environment. Colony hybridization techniques using specific

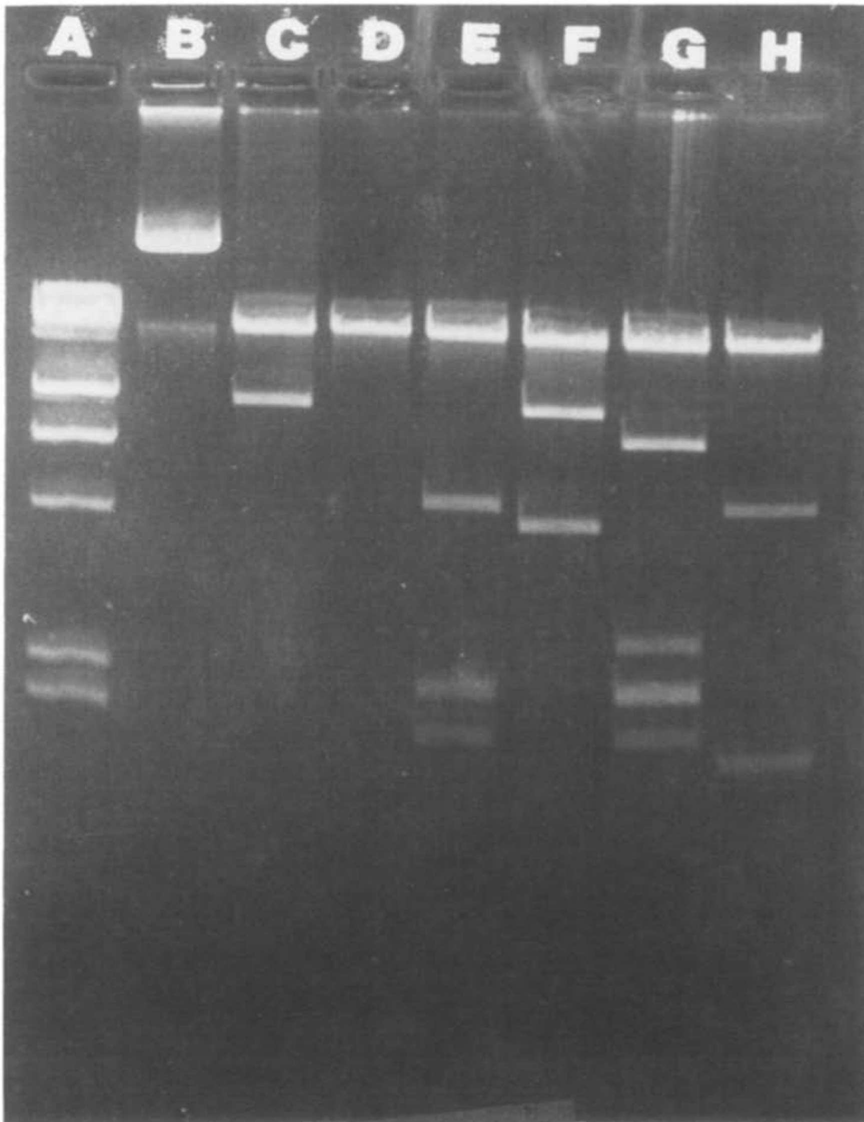
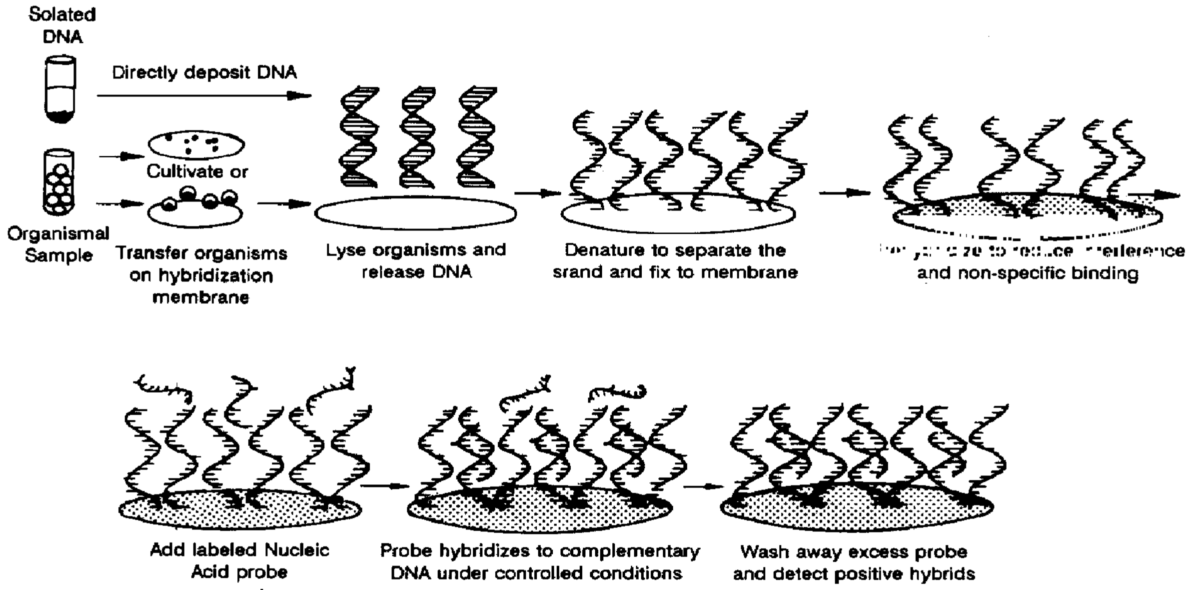


Fig. 2. An example of plasmid restriction enzyme fragment analysis for a chlorobiphenyl degradation plasmid, pSS50. A, control; B, uncut plasmid; C-H, DNA bands resulting from enzymatic cutting with mixtures of restriction enzymes.

gene probes for biodegradation is the simplest field application of this technology (Fig. 3).

Recently, techniques have been developed to extract DNA and RNA directly from the environment in order to quantify the frequency and activity of degradative genes without cultivation of individual microbial populations [22]. Such technology currently permits the analysis of an environment or engineered waste treatment system as to its capacity for degradation of specific chemicals and instantaneous activity of degradative genes at the time of sam-



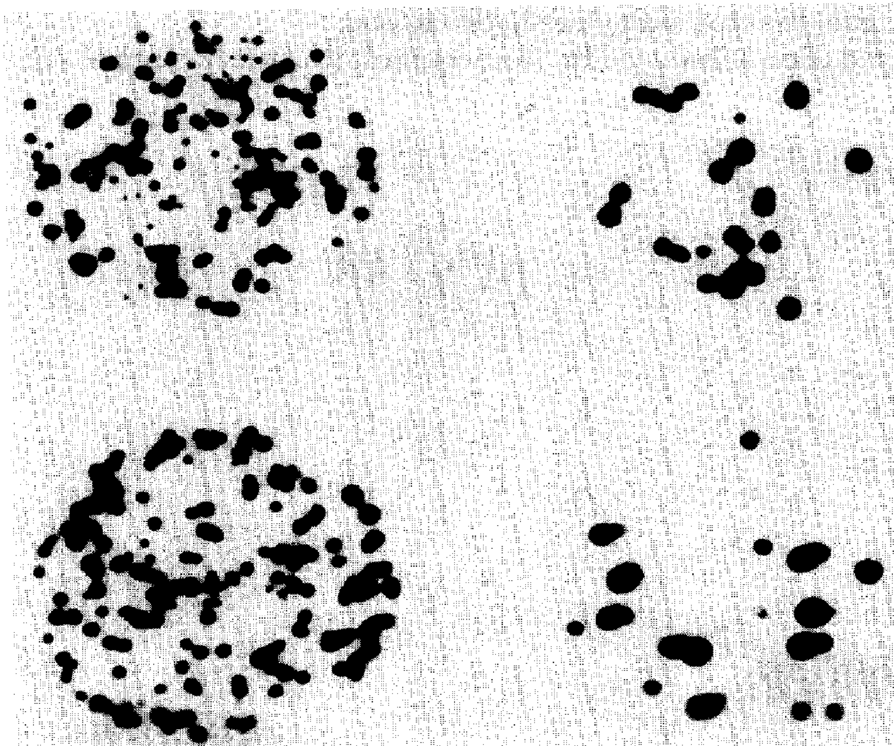


Fig. 3. Gene probe detection technology for biodegradative organisms. Upper panel, molecular mechanism of DNA hybridization; lower panel, autoradiographic detection of bacterial colonies containing genes for naphthalene degradation in soil slurry treatment reactors.

pling. DNA extraction from chemically contaminated, manufactured gas plant soils and gene probe hybridization with naphthalene catabolic genes demonstrates the application of this technology in chemically complex environmental matrices (Fig. 4). This same technology can be applied to the analysis and optimization of waste treatment processes and can provide convincing data that a bioremediation practice is performing as predicted at the biological level.

Bioluminescent reporter technology is an example of genetic engineering, not for improving degradative strain performance, but to develop an *in situ* measurement technology for the presence of a specific chemical, its bioavailability, and degradation.

In this example, a transposon was used to introduce the bioluminescence (lux) genes (from *Vibrio fischeri*) into the pathway for naphthalene degradation in a *Pseudomonas* strain (Fig. 5). The self mobile genetic element, transposon 4431, has been genetically engineered to carry the bioluminescence genes (courtesy of C. Kado). The transposon inserted itself with the *lux* genes directly into the salicylate hydroxylase gene (nahG) (see Fig. 1) of the lower pathway for naphthalene degradation. This insertion into the nahG gene, inactivates the nahG from making salicylate hydroxylase.

Naphthalene degradation is induced by the accumulation of the partial deg-

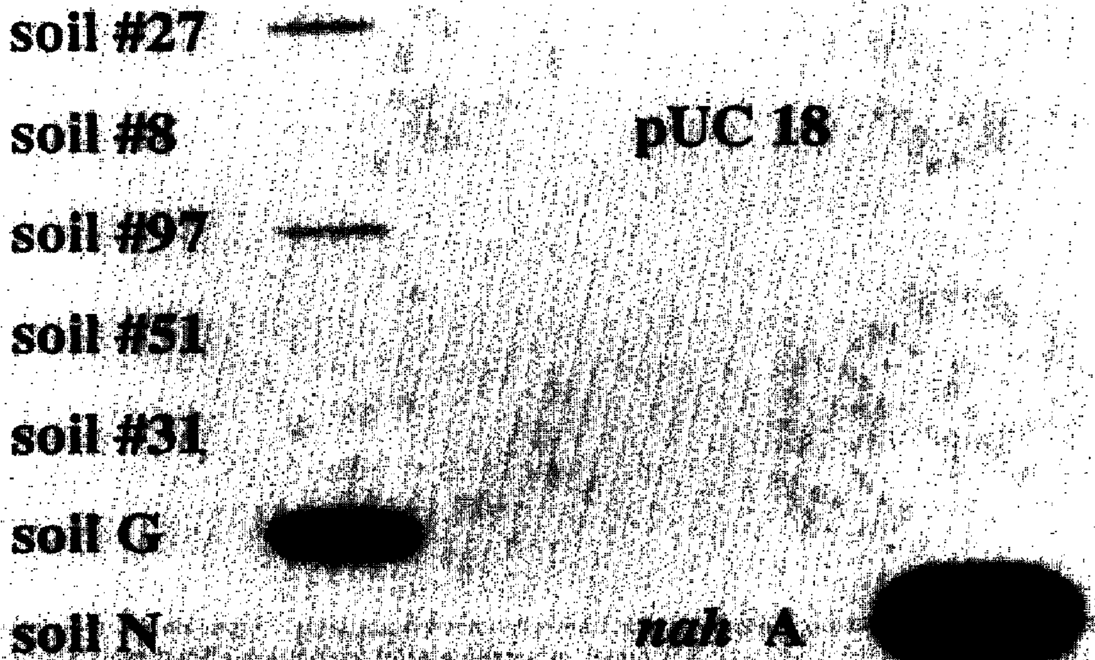


Fig. 4. Detection of naphthalene dioxygenase genes in soil DNA extracts from contaminated manufactured gas plant soils. Plasmid pUC18; negative control, nahA, positive control for naphthalene dioxygenase gene fragment.

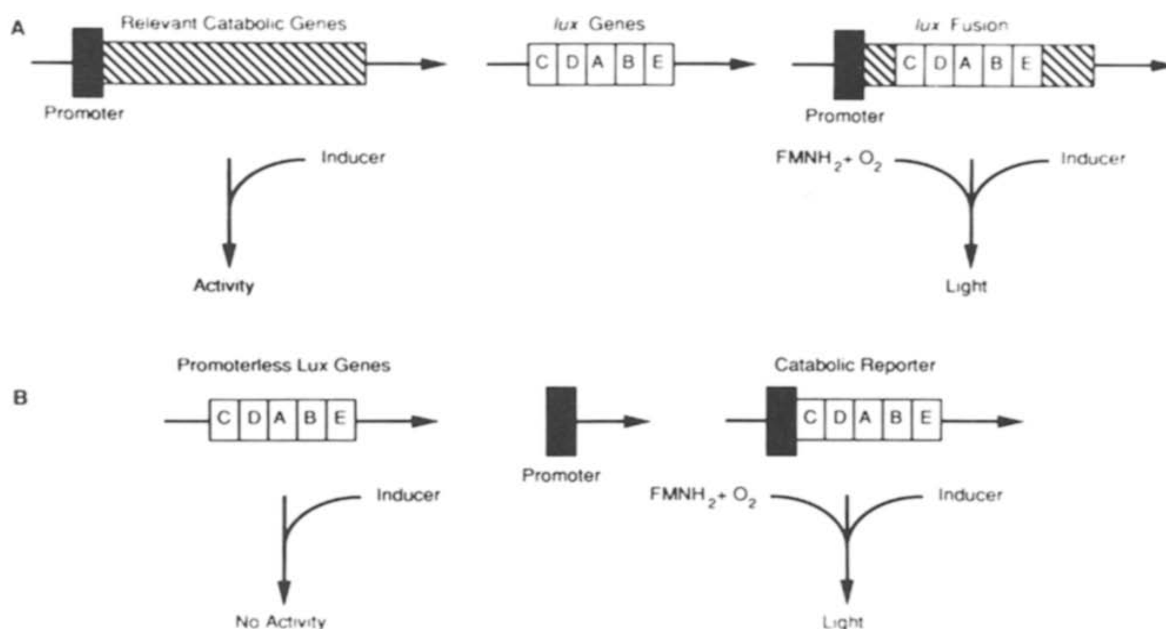


Fig. 5. Molecular strategies for constructing bioluminescent reporter plasmids for naphthalene degradation. Panel A, strategy for transposon TN4431 insertion of *lux* bioluminescence genes naphthalene catabolic operons; Panel: B, alternative promotor cloning strategy.

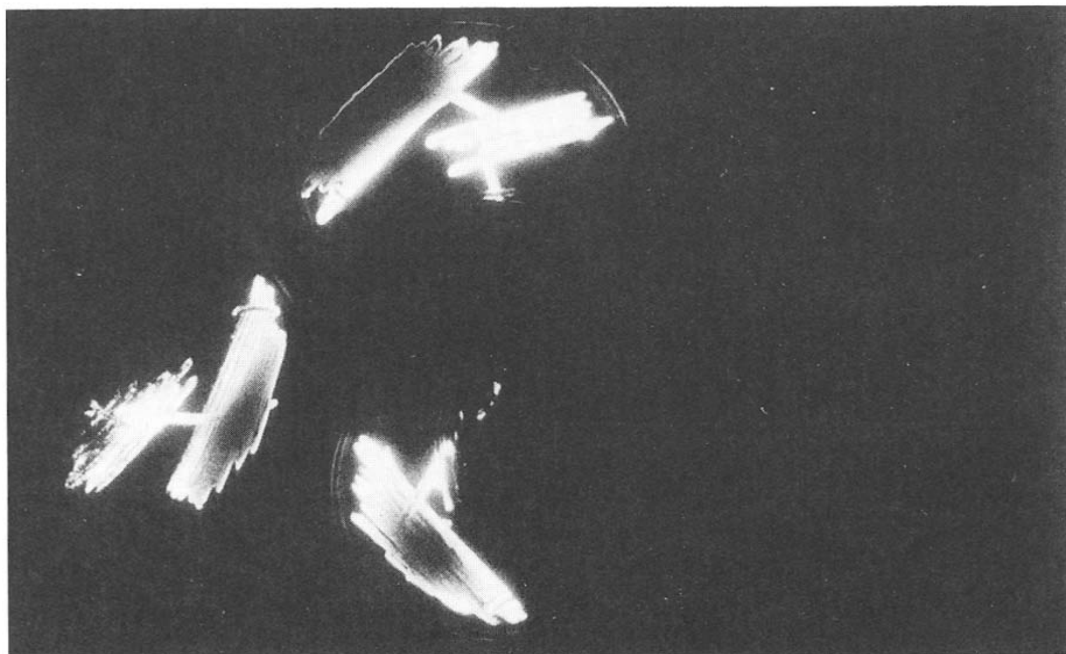


Fig. 6. Representative light emission from engineered bioluminescent reporter bacterial colonies following exposure to naphthalene.

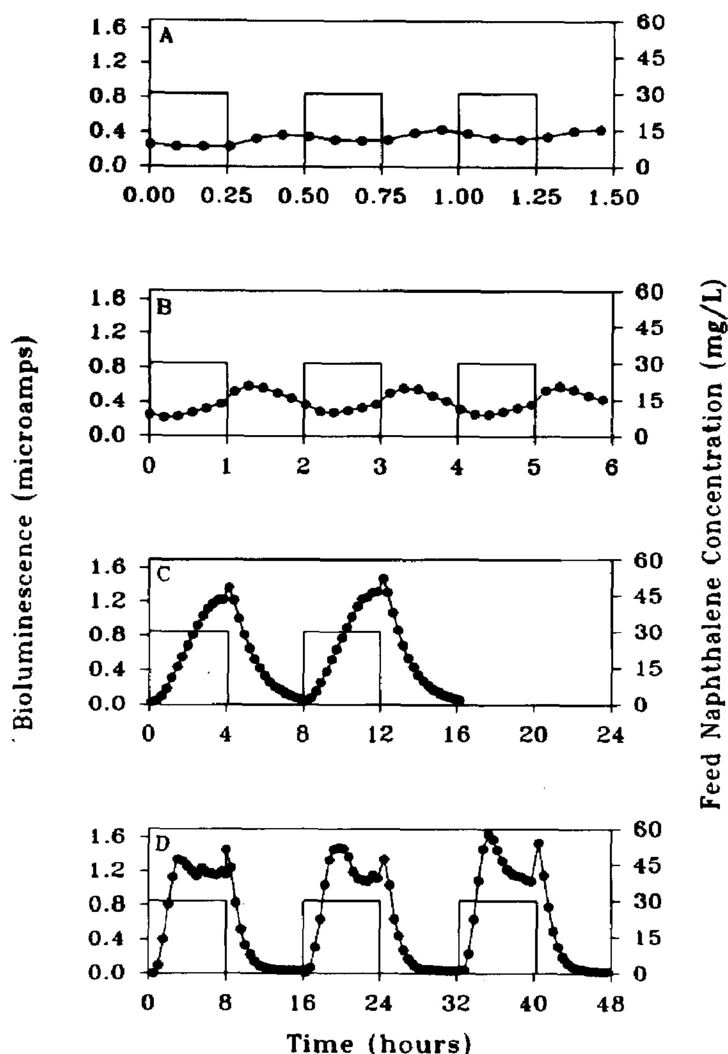


Fig. 7. Dynamic bioluminescence response of reporter strains to naphthalene feed concentration in a continuous flow reactor system. Solid line, square wave perturbation of naphthalene feed; dotted line, bioluminescent response.

radation product, salicylate as previously mentioned (see Fig. 1). In the presence of naphthalene or salicylate the naphthalene degradative genes are expressed (transcribed at the appropriate promoter) as are the *lux* genes. Expression of the *lux* genes results in enzyme (luciferase) activity and the production of visible light that can be measured with relative ease (Fig. 6). This technology has already been used to measure, with remote sensing, naphthalene biodegradative activity in contaminated Manufactured Gas Plant (MGP) soil [23].

The naphthalene degradative *Pseudomonas fluorescens* straining containing the *lux*-naphthalene bioluminescent reporter plasmid produced sufficient light

in response to cyclical naphthalene exposure for quantitative measurement of dynamic degradative gene activity. Bioluminescence as a measure of naphthalene biodegradative activity was found to be directly responsive to dynamic fluctuations in naphthalene feed to a continuous bioreactor system (Fig. 7). Extension of this work to naturally contaminated MGP soils demonstrated the potential of this technology to detect *in situ* naphthalene, its bioavailability and biodegradation [23].

Conclusions

Applications are numerous for knowledge and techniques from molecular biology in hazardous waste treatment. Potential improvements in current bioremediation and *in situ* treatment technology are anticipated from molecular contributions include:

(1). Selection and recovery of novel microorganisms and consortia from natural environments capable of more versatile degradation of hazardous wastes.

(2) Improving the biochemical performance and versatility of microbial strains involved in biodegradation.

(3) Developing microbial strains and technology for environmental process monitoring and control.

(4) Selection and development of microbial strains with increased robustness and environmental fitness for application in suboptimal environments.

These contributions are primarily focused on the organic constituents of hazardous waste matrices. However, it should be noted that resistance, transformation and sequestering of inorganic materials including metals and radionuclides is in parallel development by many investigators.

Currently, the molecular technologies offer the field of environmental biotechnology the promise of diagnostic, and process monitoring and control technology analogous to clinical diagnosis and therapeutics. During the decade of the 90's it is entirely feasible to envision molecular/diagnostic capability to rapidly determine if an environment or waste treatment process is genetically competent for biodegradation and whether those genes are active in that environment. Armed with that information, protocols for introduction of needed biodegradative genes, and induction and control of those genes or those of indigenous microbes will be possible. Process analysis, optimization and control technology will likely be available to provide a front end prediction of the bioavailability of contaminants and site specific variables impeding bioremediation and ultimate process performance. It remains unlikely that bioremediation or biodegradation processes will replace other physical, chemical treatment technologies. However, it is not overly optimistic to predict that biotechnologies for hazardous wastes will be an integral part of an overall waste management program.

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