Mini review

Biosafety issues in biotechnology and engineering of microorganisms

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Abstract
Currently much debate, attention and concern surrounds the use of genetically modified plants or animals. But there has not been much concern about microorganisms, although we all are aware of the place of microorganisms in the circle of life, their abundance and diversity. There are many examples regarding the application of genetically engineered microorganisms (GEMs), however, like other higher organisms, any modification in the natural properties of microorganisms has to be justified and follow certain rules and regulations. Proposal for the construction of an "Iranian GEMs Bank" is another way of preventing unlimited manipulation on microorganisms. Also establishing the "Iran Microbe Zoo" will help governmental and environmental protection organizations to enhance public knowledge and understanding of the role of microorganisms and the significance of their protection.
Keywords: Genetically engineered microorganisms; Microbial release; Risk assessment; Monitoring.

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INTRODUCTION

Genetic engineering has provided useful techniques to modify the genetic composition of microorganisms and hence construct new organisms which have many applications in different scientific branches (Table 1), (Shafiee and Rezaee, 2005). For example, application of indigenous microflora for the removal of environmental hazardous waste is more desirable because of their neutral capabilities. However, the environmental applications of GEMs have been the subjects of many studies that shows the need for more attention to these important entities (Sayler and Ripp, 2000; Moody *et al*., 2001). By means of transmissible plasmids or by the use of other genetic techniques, bacteria have been constructed that have activities different from those of the original organisms. Genetic engineering methods can be used to increase the level of particular proteins, enzymes or series of enzymes in a bacterial cell thus increasing the rate of the reactions that enzyme catalyzes. For example, the levels of the enzyme naphthalene dioxygenase in a recombinant *Escherichia coli* are significantly higher than that measured in the original host strain. However, the rate of : Polychlorinated biphenyl (PCB) degradation by genetically engineered strains is approximately equivalent to those observed with the wild-type strain LB400.

Molecular biology techniques can improve the capability of those natural microorganisms which are not able to use certain compounds below a critical concentration as carbon and energy sources. For example, genetically engineered *E. coli* has been developed to degrade 99.99% of : Trichloroethylene (TCE) at an initial concentration of 20 ppm (Ensley and DeFlaun,

Table 1. Examples of current and planned genetically engineered microorganisms.

Genetically Modified Microorganisms (GMM)	Function or product of introduced gene	Intended use
P. syringae, P. fluorescens	Deletion of ice -nucleating cell membrane protein	"Ice minus bacteria" sprayed on crops to protect from frost
P. fluorescens	Several genes for hydrocarbon degradation and light production	Detect and degrade pollutant (polycyclic aromatic hydrocarbons); fluorescent marker
P. putida	4-ethylbenzoate -degrading enzyme	Degradation of pollutant (benzene derivatives)
Clavibacter xyli	*Bacillus thuringiensis* (*Bt*) crystal protein toxin	Colonize plant vascular tissue to protect plant from insect pests
Baculoviruses	Scorpion neurotoxin; proteases from rat, human, and flesh fly	Biological control of specific insects

This is not a comprehensive list, but rather a sampling of the types of traits under consideration. A few of these organisms have been approved in the USA for commercial production.

1995). Variability of environmental conditions at sites of contamination restricts the application of microorganisms for bioremediation. In addition high concentrations of organic contaminants and presence of toxic heavy metals are other factors which can be detrimental to both the natural micorflora and introduced micoorganisms. For example, bioremediation of sites polluted with fuels such as gasoline is limited due to the sensitivity of microorganisms to toluene, a chemical component of gasoline and many other fuels. Inoue *et al*. (1991) however, isolated a resistant bacterium that could grow in the presence of high concentrations of organic solvents such as toluene, cyclohexane, styrene, and xylene. A strain of *Pseudomonas putida,* has been used in many genetic engineering experiments because broad host range Gram-negative plasmids can be readily transferred into this organism by conjugation with *E. coli* or by electroporation. Further application of such solvent-resistant microorganisms may allow biodegradation even at highly contaminated sites where indigenous microorganisms and, indeed, most microorganisms would be unlikely to survive.

The use of GEMs in hazardous waste treatment is very promising and collaboration between various disciplines to provide a path from initial discoveries in the laboratory to cleaner and safer environments is exciting. In fact the use of recombinant microorganisms for bioremediation will most likely become one of the most important applications of GEMs. Successful application of GEMs for bioremediation depends not only on the construction of metabolic pathways but on the successful introduction, establishment and containment of microorganisms as well.

Risks associated with the applications of GEMs: Environmental application of genetically modified organisms of all plants, animals and microorganisms has been considered in different branches of science since the advent of molecular biology. Consequently intensive debates on ethics and risks of GEMs applications have been taking place in most countries. Scientists have made it clear from the onset of this technology that new recombinant organisms should be handled with caution and experiments involving GEMs must follow stringent guidelines to minimize any potential risks. As a result, new research areas under the general heading “risk assessment” have become very important including issues such as survival, gene transfer, containment and ecological impact of GEMs in the environment. The principle questions concerning risks associated with the construction and use of engineered organisms are ecological and it is not surprising therefore that most research projects within risk-assessment programs are interdisciplinary, involving molecular biologists along with ecologists (Moline *et al*., 1993).

Risk assessment can be defined as the estimation of the risk of an unwanted event, i.e. how often can it be happen and how serious is it. Risk assessment provides an analytical framework for obtaining and interpreting experimental data, the main objective being to provide an estimate of the risk posed by a potentially harmful process or activity. The assessment of the risk of microorganisms can be distinguished from the risk assessment of chemical, with the most striking characteristics of microorganisms such as growth and multiplication, gene exchange and mobility.

In releasing GEMs, instead of being propagated as a monoculture in an optimized, controlled environment, the genetically modified microbe is expected to be released into a diverse biological community where it must establish itself, interact with other bacteria within an unknown environment consisting of uncontrolled parameters (Cases and Lorenzo, 2005). Also microorganisms rather than having a planktonic lifestyle, generally live in biofilms attached to surfaces, hence, many environmental conditions may be unfavorable for GEMs. Therefore novel genetic tools are clearly required for tracking new engineered microorganisms in order to meet the demand of eventual applications in the field. For example the use of stable isotope probing (SIP) has shown that *Pseudomonas* and *Rhodococcus* are less significant under natural conditions (Wackett, 2004). Using molecular approach has revealed that *Deinococcus radiodurnas* endures harsh environmental conditions such as high radiation doses thus making it also suitable for genetic manipulation (Brim *et al*., 2000).

Survival and competition: Bacterial establishment in an evironment depends on the ability of introduced bacteria to survive. There are several environmental factors that can affect bacterial survival in an environment such as soil texture, moisture content, temperature, pH, the presence of plant roots, minerals, organic matter competition and antagonism by other microorganisms and predation by protozoa. In microcosm experiments, these parameters should be as close as possible to the natural situation (Smit *et al*., 1992).

It is often assumed that a GEM carrying extra genes will have a lower ecological fitness than the wild type strain (Tang *et al*., 1995). Some GEMs however have been shown to have a slight growth advantage over wild type strains in chemostat cultures. But chemostat conditions can hardly be compared with environmental ecosystems. Competition experiments showed that *P. fluorescens* containing plasmid RP4 and pRK2501 survived less well in soil than the wild-type, when GEMs were not under selective pressure (Van Elsas *et al*., 1989). However, no differences were observed between survival of the engineered and wild type *Erwinia carotovora* in soil microcosms (Orvos *et al*., 1990).

Bacteria capable of degrading xenobiotics may have an advantage over other (non-degrading) bacteria because many xenobiotic compounds are toxic to indigenous microflora and reduce microbial diversity (Erb *et al*., 1997). Thus, application of GEMs for bioremediation of polluted soils may be advantageous because (1) the pollutant serves as a specific carbon source, while exerting selection pressure for GEMs and (2) selection pressure on soil microorganisms will be removed after degradation of toxic compound, allowing resident microorganisms to displace the GEM population.

However it has to be emphasized that the success of using artificially engineered microorganisms will depend principally on the maintenance of introduced genes, for example in the form of artificially transconjugated plasmids. Yoon (2005) attempted to evaluate the activity of artificially transconjugated multiple plasmids in "designer biocatalysts" for the bioremediation of cocontaminated sites under noselective conditions. They observed profound losses in the precent survivals of artificially transconjugated plasmid activity in reconstituted *Pseudomonas* sp. KM12TC. Such unpredictable high losses of this particular plasmid appeared clearly to be a deleterious effect. Otherwise for the purpose of metal clean up, genetic engineering allows the introduction of desired genes to selectively remove the target metals. *Escherichia coli* has been genetically engineered by introducing the *nixA* gene into JM109 cells to simultaneously express a Ni(II) transport system and overexpress pea metallothionein (PMT) as a carboxyl-terminal fusion to glutathione S-transferase (GST-PMT) (Krishnaswamy and Wilson, 2000). The resulting strain could accumulate 15 µm of Ni (II) per g from a 10 µm Ni(II) solutions.

Ecological impact of the introduction of GEMs: An ecosystem can be affected by introduction of GEMs. Both biological and non-biological parameters might be changed by the presence of GEMs. Investigations have shown that CO_2 evolution rates increase in the short term (5 days) in the soil compared to microcosms in which the wild-type strain of *Streptomyces lividans* are introduced (Wang *et al*., 1991). Hazardous effects of GEMs on the ecosystems are not always predictable. Doyle *et al*. (1991) have shown that fungal propagules in soil microcosms decrease upon introduction of genetically engineered *P. putida* PP0301, and 2,4-Dichlorophenoxyacetic, total bacterial populations, spore forming bacteria and chitin-utilizing bacteria are also transiently reduced.

Another parameter that can be monitored upon

GEMs introduction is the diversity of the soil community. Researches have found that both phenotypic and genetic diversity indices increase during incubation of soil microcosms inoculated with an engineered *P. cepacea* compared to the control (Bej *et al*., 1988). This can be explained as an increase in genetic interactions between the introduced strain, harbouring plasmids and a transposon, with indigenous microorganisms. Effects of GEMs on other organisms such as plants, animals and man should also be considered. A number of potential effects (e.g. those on plants and small animals) can be tested in microcosms while others (on man or large animals) must be investigated by drawing analogies from the known effects of exposing model organisms to GEMs or by toxicological tests.

Genetically engineered microorganisms can influence the fate of other organisms by transferring their novel genes to them. There are three main processes responsible for transfer of genetic elements- conjugation, transduction and transformation. Genetic elements can be introduced into a host bacterium by a plasmid which can be either self-transmissible, conjugative or non-conjugative, or into the chromosome through recombination as well as transposons (Herrero *et al*., 1990).

There are several factors which might affect gene transfer, including host species, cell number and activity and the environment in which GEMs can contact other cells. Lafuente *et al*. (1996) observed maximum plasmid transfer frequencies at: 20% of moisture content, pH 7-8 and 30°C in different donor and recipient bacteria. However, the immediate loss of plasmid encoded catechol 2,3-oxygenase phenotype was shown to exceed 99% after freeze drying, with additional loss occurring during storage, in Alcaligenes eutrophus and P. putida (Lang and Weber, 1995). Stuart-Keil *et al*. (1998) found that a plasmid was a mobile genetic element responsible for transferring naphthalene-catabolic genes among bacteria in coal-tar contaminated sites. In conclusion, any parameter which stimulates activity of the introduced strain (e.g. plant root and exudates, nutrients, certain clay minerals, lack of competition) may also stimulate conjugal plasmid transfer. Gene transfer by transduction has been detected in both soil and lake water and there is also evidence for gene transfer by transformation in sediments and aquatic systems. These data support the concept that bacterial gene transfer is a common process in nature, and lead to the prediction that almost any gene could be transferred at a certain time.

Biological containment systems for GEMs: Potential risk of GEMs can be minimized using genetic systems which could under certain conditions, lead to death of the GEMs. These genetic systems are called "biological containment systems" consisting of a killing element designed to induce cell death and a control element which regulates expression of the killing function (Molina *et al*., 1998). Gerdes *et al*. (1986) devised a system where duplication of the "killing genes" lowered the number of surviving cells to a great extent. Gene duplication does not seem to be the final answer since the presence of identical sequences of DNA can result in homologous recombination and thus reduce the advantage.

Detection and monitoring of GEMs in the environment: The introduction of GEMs into the environment and their effect on natural ecosystems has necessitated the need for detection and enumeration of novel types of bacteria. There are numerous methods for the detection and isolation of bacteria from environmental samples. However, determining whether a specific microorganism is present in an environmental sample is not an easy task.

Sensitive monitoring methods are required for detection of a host bacterium and the recombinant DNA in different environments to determine the ability of GEMs to survive, grow and spread within the environment and to assess any likely environmental impact (Pickup, 1991). The successful application of a method for monitoring any foreign substrate in the environment, either toxic chemicals or introduced organisms, depends on the important criteria of a technique such as sensitivity, specificity, reproducibility and practicality.

Culture techniques: Conventional methods can be used for enumeration of the culturable population or the total population. A suitable medium containing carbon or other energy sources is required for the enumeration of culturable bacteria. Commercial media are available for isolation of specific bacterial groups such as enteric bacteria, faecal streptococci and pseudomonads. Media have also been designed for isolation of ecologically important bacteria, such as fluorescent pseudomonads, methanogens and yellow-pigmented bacteria (including *Flavobacterium* and *Cytophaga*). It is possible to monitor a limited number of organisms according to their cell morphology using microscopic techniques, but because bacteria have limited morpho-

logical diversity some other techniques such as biochemical or immunological techniques will be required for more precise identification (Pickup, 1991).

Immunological methods: Environmentally important bacteria can be identified and monitored using sensitive and specific immunological means such as polyclonal or monoclonal antibodies. Either polyclonal or monoclonal antibodies can be used to identify specific marker genes, products or microorganisms that express an appropriate antigen.

The detection of specific strains (e.g. *Rhizobium*) and engineered bacteria (*P. putida*) is possible in the presence of indigenous bacteria using enzyme-linked immunosorbent assay (ELISA) (Morgan, 1989). Although fluorescent antibody detection systems can be used for identification and localization of components of bacterial and viral pathogens, this monitoring system is not able to distinguish between viable and nonviable cells. This is particularly true for GEMs because non-viable cells may acquire intact r-DNA sequences from other organisms in the environment through transformation. Therefore immunological techniques are unable to give sufficient information about the viability or activity of specific population such as introduced GEMs (Prosser, 1994).

Gene probes and sequencing: It is possible to use hybridization when appropriate probes are available to detect the presence of specific nucleic acid sequences, from oligonucleotides to functional recombinant genes. This can be carried out in both environmental samples and laboratory cultures without first having to culture target bacteria. Several hybridization strategies are available. In colony hybridization, bacterial colonies are grown on a filter which is probed to detect a particular gene. This method has been used to detect a range of organisms carrying specific traits, such as toluene and PCB-degrading and mercury resistant bacteria (Diels and Mergeay, 1990). A combination of DNA hybridization and the most probable number (MPN) method has allowed monitoring of *Rhizobium* sp. and *P. putida* genetically marked with the transposon Tn*5* down to approximately 10^2 cells g^{-1} of soil (Fredrickson *et al*., 1988).

Techniques such as the polymerase chain reaction (PCR) in combination with other molecular biology techniques have been used to detect low numbers of target organisms among numerous non-target microorganisms in the soil. However this method will not indicate whether the trait is in the original organisms or exists in others as well.

Molecular markers: The introduction of marker genes into organisms has been one of the most common applications of molecular genetic techniques to detect and monitor the marked strains from the background strains according to their phenotypic differences (Lindow, 1995). Direct selection of marked strains can be achieved using antibiotic resistant genes, such as amino glycoside phosphotransferase (*aphII*), chloramphenicol acetyltransferase (*cat*), puromycin acetyltransferase (*pac*), and tetracycline resistance (*tet*). Luminescent markers (luc, lux and gfp genes) have been used as reporte genes for the large microbial ecological studies (Mashreghi and Prosser, 2006; Mashreghi, 2005; Mashreghi and Prosser, 2004; Prosser *et al*., 1996; Chalife, *et al*., 1994). Other genes including *xylE*, *lacZ*, *gusA*, *mel* and *phoA* produce unique phenotypes for monitoring of the marked strains.

Conclusion

Genetic manipulation and the release of GEMs can bring benefits as described above. However, the process also involves some risk. Risk assessment has become an important issue in the release of GEMs partly because the technology is frequently updated and new methods are invented. In addition, scientists or their employers may be held responsible for negative effects of releases. It is possible that unwanted or unpredicted site effects of the release on non-target organisms could occur. Risks unique to GEMs include: transfer of foreign genetic material to other microorganisms, the possibility that a bacterium which has been modified to be a superior competitor in the environment may transfer those genes to deleterious organisms, and the likelihood that the modified organism will survive better than the parent strain in some environments, leading to unknown consequences. Therefore, after assessing the benefits and risks for a series of examples of the potential use of genetic manipulation, a scale from highly desirable and relatively safe uses to less desirable and unsafe uses of the technology could be constructed. It should be emphasized that risk assessment implies the certainty that total risk exclusion does not occur. Therefore, risk

assessment should be considered as the determination of the biological safety of released GEMs in connection with ecological effectiveness or beneficial effects.

Suggestions

Although Iran has signed the biosafety protocols, biotechnology laboratories have to be well informed about many risks associated with the application of GEMs and the subsequent ethical effects that they might have on society. Conferences, seminar, workshops could provide additional information for scientists and researchers who work with those microbes. Regional and international conferences are regularly being held in different parts of the world where recent researches on bioethics are presented and discussed. An example of these gatherings include: The international congress of Bioethics 2005, Tehran, Iran; Construction of the "IGEMB" will be useful in many ways, including the limited use of such microorganisms, handling of GEMs only by certified scientific organizations, world wide and regional safety, etc. IGEMB can provide further information on particular microorganisms in which a researcher could better deal with those microbes. Also this kind of bank can increase their activities to a wider regional organization and cooperate with other countries in the region to build up a safe area in which all activities on GEMs are kept under control and clearly determined. Most developed countries have built their own national and international collections of microorganisms, either natural or genetically engineered. Also, in such countries research projects in large scales have been set up to investigate the rate of distribution of microbial species in different areas. These kinds of activities can be used as criteria for better organization and construction of such a microbial bank.

As mentioned above, microorganisms have many applications, but majority of the community are not well informed about the advantages of microorganisms in human life. Therefore, besides several botanical institutions, animal zoos and national natural museums, establishing an "Iranian Microbe Zoo" will increase understanding of many application of these microorganisms. Construction of such a zoo requires cooperation of several research and institutional bodies. Having such basic knowledge will then provide a suitable background for majority of people to further understand the applications of GEMs.

Finally all threats arising from application of GEMs can be minimized not only by a particular method but also with correct and logical composition of different programs and pathways. To take such a path requires appropriate knowledge and management in applying suitable methods to related problems.

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