Environmental genetic engineering: Hope or hazard

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Genetic engineering, and the pros and cons of using it to 'improve' the environment, is a t of intense debate in both the scientific as well as popular literature these days. The pre article presents a critical discussion of the issue and attempts to assess the advantages disadvantages that might ensue from the intentional release into nature of genetically modi microorganisms (GMMs). The article also discusses the physiological strategies of surviva well as the ecological impact of GMMs released into the environment.

TWENTY years ago a call for a voluntary moratorium on certain genetic engineering experiments amazed the scientific and public community world-wide[1]. This unprecedented action by a group of scientists was caused by concern that an unfettered pursuit of such experiments might engender unforeseen and damaging consequences for human health and the Earth's ecology. The biosafety guidelines for recombinant DNA manipulation which was subsequently promulgated in many countries had two main aims; to minimize the probability of the occasional release of genetically engineered organisms from contained conditions and to ban the deliberate release of such organisms into the environment.

But today other problems are fevering the scientific community: how best to design organisms especially intended for release into environment and how to assess possible risks associated with the intervention of genetically modified organisms into biosphere. Why has the opinion of the scientific community changed so much? The first reason is: 'Literally millions of experiments, many even inconceivable in 1975, have been carried out in last 20 years without incident. No documented hazard to public health has been attributed to the application of recombinant DNA technology'[1]. An attempt to describe and to discuss the second reason is the subject of this article.

As human civilization enters the 21 century, the planet is facing problems that many experts see as intractable. Currently 4.5 million km^2, or 35% of total land of planet, is threatened by desertification[2]. The areas of polluted soils and water are expanding. The greenhouse effect will change the world's climate. A large number of world fisheries are seriously depleted. Irrigation water is in short supply in many parts of the earth and erosion threatens the productivity of agricultural lands. While the amount of land under cultivation cannot be expanded greatly, almost 100 million people are expected to be added to the world population each year for the 30 years[3].

Is it possible that modern biotechnology, base the sophisticated art of genetic engineering, could s all these problems? It is believed that it really has potential, at least, to alleviate many of them[2,3]. Bu many cases, for this alleviation, genetically mod organisms should be released (or introduced) into vironment[4,5].

Benefits and hazards expected from environmental genetic engineering

The benefits expected from the release of genetic modified microorganisms into the environment are s marized in Table 1.

Bioremediation

Bioremediation – the use of living organisms (prim microorganisms) to degrade environment pollutant

Table 1. Benefits from release of genetically modified microorganisms into environment

Protection of environment
– bioremediation of polluted environment
Control of global environmental processes
– reversal of land desertification
– reversal of greenhouse effect
Agriculture
– increasing efficiency of plant nutrition
– pest control (safe bio-pesticides)
– protection of plants from climate stress
– protection of plants from tumour formation and diseases
Food industry
– microorganisms-producing enzymes for food industry
– microorganisms with improved efficiency of fermentation
– improved microorganisms for milk industry
Health care
– microorganisms as live attenuated oral vaccines

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to prevent pollution through waste treatment – is the technology for removing pollutants from the environment, restoring contaminated sites and preventing future pollution. Bioremediation can have global, regional and local applications and both indigenous and genetically modified microorganisms (GMMs) may play important roles. The enormous natural capacity of microorganisms to degrade organic compounds is the basis for bioremediation. This capacity could be improved upon considerably by genetic engineering. GMMs may be constructed for the attack, for example, of complex chlorinated hydrocarbons, such as dioxins, that are recalcitrant to biodegradation by natural microorganisms[2].

Inserting genes that code for enzymes that break down toxic chemicals into microorganisms that are thereby able to survive and grow in very disturbed and hostile environments, would greatly extend the range of compounds that could be treated by bioremediation. A species of *Pseudomonas* has been isolated, that can grow in solvents containing more than 50% toluene (conditions that kill most organisms); cloning of appropriate genes for biodegradative enzymes in such strains has the great potential for creating new ecological niches for bioremediation into non-aqueous solutions[2].

But how will the strains engineered for bioremediation in test tubes work in the polluted field? In a large-scale field test (10 km^2, 30,000 cubic meters of soil treated), three different genetically modified strains of *Pseudomonas putida* were released into soil containing coal tar and phenol. The release led to a decrease of the concentration of polychloraromatic hydrocarbons in the soil by 25–40% (ref. 6); the number of viable cells of the destructor strains decreases with the decrease of the concentration of the pollutant, i.e. they were eliminated from the soil after utilization of pollutants used as substrates[6]. There is no available information about large-scale bioremediation field tests in aquatic systems, but the results of microcosm studies seem promising; strains of *Pseudomonas* sp. engineered for biodegradation of 3-chlorobenzoate and 4-methylbenzoate were efficient in an active sludge microcosm[7]. A genetically engineered strain of *Pseudomonas* sp. was constructed to mineralize the mixture of methylated and chlorinated benzoic acids and phenols[8]. It was shown that in aquatic microcosms containing untreated lake water with aromatics added before the experiment, the strain 'has a very high efficiency in aquatic ecosystems and, thus, clearly has the potential to be used for a field bioremediation experiment'[8].

Environmental improvement

The next step, which, it seems, is concerned with environmental genetic engineering, is a giant jump from bioremediation of the environment to improving the environment. In Japan, academic, industrial and governmental research is tightly coordinated for global application of environmental biotechnology[2,9]. Researchers are exploiting large-scale applications of bioremediation that may have a fundamental impact on desert formation, global climate change and the life cycle of materials. One project attempts to develop microorganisms that can help reverse desert formation; the program tries to develop biological systems and products such as biopolymers that retain water and reverse this phenomenon on large landscapes. The microorganism *Alcaligenes latus* is being used to produce a 'superbioabsorbent' – a polysaccharide, composed of glucose and glucuronic acid – that can absorb and hold more than a thousand times its own weight of water[2]. Another immense problem of environmental genetic engineering is to reverse the global warming by developing bioremediation systems to remove CO_2 already emitted into the atmosphere from the burning of carbon-based fuels[2,9]. The increased CO_2 contributes to a global greenhouse effect. Theoretically, microorganisms could remove enough of this greenhouse gas from the atmosphere that the threat of global warming would be removed. Some strains convert CO_2 to various organic compounds, and new strains could be produced, at least in theory, by genetic manipulation *in vivo* and/or *in vitro*, to do so more efficiently[2]. Simultaneously, another part of the project would develop biotechnology for producing hydrogen and other fuels that do not emit CO_2 and hence do not contribute to the greenhouse effect[2,9].

Agriculture

It is believed that in agriculture, released GMMs could be beneficial, for example for increasing agricultural plant nutrition. In fact, two genetically modified strains of the symbiotic nitrogen fixing bacterium, *Rhizobium meliloti*, constructed for inoculation of alfalfa and strain *Bradyrhizobium japonica* engineered for inoculation of soybean have been successfully field-tested for the increasing nitrogen availability in their respective plants[10]. Genetically modified endophytic strains of *Clavibacter xyli cynodontis*, containing the gene encoding an entomopathogenic toxin from *Bacillus thuringiensis*, was field-tested for its ability to express insect-specific toxin and to control European corn borer (*Ostrinia nubilalis*)[10]. A genetically engineered soil bacterium (*Bacillus thuringiensis kurstaki*), containing the same insect-specific toxin, is now under prolonged field testing (from 1990) to verify the ability of GMM to control caterpillars[10]. In model experiments, *Agrobacterium tumefaciens*, with a deleted gene for tumour formation, replaced similar wild type strains and protected the corresponding plants from tumour formation[6]. Five genetically modified viruses have been field-tested during 1989–1995; recom-

binant vaccinia virus containing a gene from the rabies virus, which has been distributed in bats, was field tested to immunize wildlife populations to combat the spread of rabies. This virus was the first, and to date the only live intergeneric microorganism to be approved for commercial use in the USA[10]. In July 1995, a genetically modified insect pathogenic virus containing a gene coding neurotoxin from scorpion venom was released in the USA to field test the efficiency of GMM to control caterpillars[10]. Genetically modified *ICE* nucleating bacteria *Pseudomonas siringae*, with deleted gene of ice nucleation, was field-tested in 1987–1990 for the frost protection for crops (e.g. strawberry and potato)[10].

Very exciting and promising prospects and problems of release of transgenic plants and animals are not discussed in this article; for brief reviews see refs 3 and 11.

Food industry and health care

GMMs can be released into the environment not directly, but, for example, carried in food: in cheese, made with the help of enzymes (chymosin) produced by GMMs; in milk products, fermented with genetically modified lactic bacteria; in beer, prepared with the usage of genetically modified yeasts, synthesizing and secreting barley beta-1,3–1,4-glucanase which decelerates the aging of beer[6].

One of the most promising prospects of the release of GMMs is as live attenuated vaccines constructed on the basis of *Salmonellae* strains. Such GMMs could be used for the control of invasive salmonellosis and as live polyvalent vaccines, carrying cloned heterologous antigens[6].

Although the expected beneficial applications holds great hope and promise, each also may cause harm to human health, ecological systems, or economic and social structures (see Table 2).

Is it possible to assess the degree of these risks by rational methods? For example, is there a real chance that GMMs released into environment will overgrow the natural microorganisms? The answer is no. The main reason for this confidence is the experience of the many releases of natural and GMMs into different soil and water ecosystems. In all the cases studied, according to the enumeration of culturable cells, microorganisms (natural or modified), released from comfortable laboratory conditions into an open environment, could not survive in it[6,12–14].

Physiological strategies of survival of the microorganisms in the environment

Released GMMs will undergo starvation

There are three types of dynamics of elimination of microorganisms released into soil ecosystems[6]: (1) rapid (in three days) disappearance of culturable cells (*E. coli* strains); (2) exponential decrease of cell number (after 7 days up to 10–100 viable cells per gram of soil; *B. subtilis* strains), and (3) comparatively rapid exponential decrease (after 1–14 days the size of population decreases by 1–4 logarithms, then follows rapid decline to the point of disappearance of viable cells (*Rhizobium* strains). Genetically modified strains of *Pseudomonas* were retained in soils; but the rate of their elimination varied from 0.2 to 1 logarithms in 10 days and depended on the nature of the strains and the type of soils[12,13]. A similar trend is observed upon release of GMMs into drainage agricultural water and sea or river water ecosystems[6,15–17].

The primary reason for the inability of released

Table 2. Possible types of risks associated with the release of GMMs

Type	Mechanism	Results
Risk for humans, animals, plants	Transfer of drug resistance (or harmful) genes into clinical and/or symbiotic strains	New forms of known diseases
	GMMs usage for food production	Toxins and/or unwanted physiologically active substances (hormones) in food
Risk for environment	GMMs will overgrow the indigenous strains	Decrease of biodiversity
	Disturbance of the ecological balance	Activation of earlier unknown pathogens
	Massive transfer of foreign genes into indigenous strains	Formation of new pathogens
Social risk	Beneficial usage of the GMMs in industrial countries	Increasing of economical and social difference between industrial and developing countries
	GMM release for military and/or improper purposes	Industrial countries will use territories of developing countries for field tests of GMMs release
Ethical risks	Commercial secrecy concerning information about release of the GMMs	Violation of consumers rights

microorganisms to grow in open soil and water ecosystems is the very poor substrate availability in environment. Substrates accessible to microorganisms in open nature are so limiting that within one year a typical soil microorganism could (on average) go through only 1–36 generations of growth[18]. Under such conditions of nutrient starvation non-differentiating microorganisms respond to this situation of multiple environmental stress by entering in a dormant (or resting) viable but not culturable (VNC) state[19]. Briefly, in early stages of starvation special mechanisms rearrange metabolism for the adaptation to new conditions[18,19]: redox potential deceases drastically, energy status becomes lower, the composition of membrane proteins changes, specific proteinases (their synthesis are induced at this time) degrade all proteins that now become not necessary[18]. In starving cells enzymes of biosynthetic pathways could not be found. But the enzymes and proteins of energy-generating pathways remain intact; this makes non-growing cells prepared to consume a nutrient substrate immediately after it appears[18,19].

But how will non-growing bacteria overcome possible environmental stresses? Starvation-induced proteins, which will protect non-growing cells from environmental stresses, are preferentially produced in *E. coli* during the first hours of starvation. Their synthesis is controlled by sigma factor-32 and sigma-s, encoded by *rpoH* and *rpoS* (*KatF*) respectively[20,21]. A very important role in the physiology of starvation belongs to unfoldases and chaperones; these proteins (their structures are conserved in evolution of prokaryotes and eukaryotes, which indicates their crucial role in survival) provide correct folding of some proteins and proper assembly of macromolecular complexes[22,23]. Unfoldases and chaperones are capable of solubilization of denatured proteins and so they help in preserving starving cells from damage caused by the presence of denatured proteins. In this state of acute substrate limitation, non-growing cells attain a so-called general resistance to stresses, such as heat shock, oxidative stress and osmotic stress[22,23].

Released GMMs could enter into VNC state

After some days of starvation and after the above-mentioned physiological rearrangements, microorganisms practically do not require energy for maintaining their viability[18]. They then can enter into a VNC state, i.e. do not grow on plates with corresponding media but can be detected by methods based on microscopy, immuno fluorescence assay and hybridization with specific DNA probes, and not on the detection of growth or on any kind of metabolic activity[24]. VNC cells could persist in soils and waters for a long time (years); during this period they may retain plasmids[6,12–14]. According to some estimates, only 10% of the total number of terrestrial microbes and only 1% of aquatic ones are culturable and all others are in a VNC state[25,26]. After prolonged incubation on standard media, VNC cells sometimes regain their ability to divide, but the mechanisms of restoration are far from clear[27]. But there is some evidence concerning the mechanisms of regulation of entry into a VNC state[28]. The VNC response of initially aerobically grown *E. coli* in oligotrophic sea water may be regulated at the translational level and could be related to the efficiency of ribosome translation kinetics; this efficiency might be, at least in part, regulated by the sigma factor *RpoS* (*KatF*)[28].

So, this is in brief, the physiological survival strategy of non-differentiating bacteria in the natural environment. According to this there is a very high probability that GMMs, sometime after their release into the 'wild' environment, will enter into a VNC state. Of course, this is no reason for the over-optimistic confidence that the GMMs will never restore their growth. In theory it is not excluded that under favourable conditions VNC GMMs could reactivate and begin to divide[29].

Is it possible to reduce this uncertain risk? With the aim of increasing the probability that VNC GMMs, persisting in open nature, will not grow again, special suicide strains could be engineered[30]. These strains carry different suicide genes under the control of promoters which could be induced in soil or in water after the released GMMs carry out their beneficial work[30]. Are there any chances that foreign DNAs, released into soil after this suicide, will be capable to transform indigenous strains? This is under investigation; according to the results, DNA released after lysis is as efficient as purified DNA in natural transformation[31], (about transformation in soil and aquatic ecosystems, see below).

Genetical strategies of survival of microorganisms in the environment

What is the genetical strategy of survival and evolution of bacterial populations in open environment? Do significant genetical and evolutionary processes occur only during the very short periods of growth when substrates are available? Are microorganisms, persisting in open environments in a non-dividing state, evolving? How and in which direction? For making the decisions to release bacteria or not, these are the questions of great concern.

Released GMMs could undergo spontaneous and, may be, adaptive mutagenesis

It seems that at least two different types of mutagenesis generate biodiversity of microbes in open environment. First, spontaneous mutagenesis occurring only during the short growth phase and without regard to adaptive

benefit to the cells. The second type of mutagenesis is occurring in non-growing cells. This so-called adaptive mutagenesis leads to mutations induced by potential fitness benefits only[32]. Although the mechanisms of generation of adaptive mutation is not understood yet, there is no doubt now, that adaptive mutagenesis really occurs, and that *recA*[33] and *recBCD* systems of *E. coli*[34], DNA polymerase III[35], and conjugal functions[36], are essential for its activity. Because the methyl-directed mismatch repair system is deficient in nutritionally deprived cells, it might increase the frequency of the mutation realization during starvation[33,35]. Adaptive mutations are realized when: i) cells are non-growing and, ii) potential substrates (or growth factor) are present in the media. Only under these conditions do mutations, which 'adapt' non-growing cells to growth on the potential substrate, arise[32–36].

Although there is no available information about corresponding environmental experiments, it seems that by adaptive mutagenesis non-growing microorganisms could be attempting 'to adapt' to new substrates, potentially present in open ecosystems.

In general, it is reasonable to assume that in populations of microorganisms, growing and persisting in open environments, the spontaneous random mutagenesis is increasing their biodiversity and adaptive mutagenesis 'adapt' the non-growing cells to new substrates potentially present in the environment.

Will the released GMMs undergo all these types of mutagenesis? Whether they do so or not, it would be reasonable to inactivate the genes of GMMs, essential for adaptive mutagenesis. This, it seems, may increase the safety of the released GMMs by decreasing their chances to evolve.

Another important question concerning possible ecological risks of GMM releases: will foreign genes from GMMs spread into indegenous microbes? If 'yes', one cannot exclude the possibility that recombination of foreign genes with genomes (or with plasmids) of natural strains will generate unpredictable and, may be, hazardous combinations.

Released GMMs could participate in all ways of gene transfer

There are four ways of gene transfer in microbial ecosystems: transformation by free DNAs[37–40], transduction by bacteriophage DNAs[41], conjugal transfer by plasmids[37,38,42], and conjugal transfer by conjugative transposons[43]. It is believed that conjugational gene transfer is of the most considerable ecological importance[37,38,42,43].

Free DNAs are common in soils[39–40,44] and aquatic[45,46] ecosystems. Such DNAs released from different organisms absorb rapidly to the surface of solid particles (in soils) and become more resistant to nucleases and in the absorbed state, retain the ability of transformation[40,44]. The probability of transformation of chromosomal genes is decreased drastically if there is no homology between transforming DNAs and DNAs of recepient cells. Efficient gene transfer by free DNA transformation occurs only between the same, or closely related species[6,37,38,40] and, it seems, dissemination of foreign genes via transformation could not be common.

In theory, the presence in recepient cells of restriction-modification system could further reduce the low chances of foreign DNA transformation by a factor of 10000 (ref. 38). But there is the evidence that 'stressogenic' factors, at least UV-damage, can alleviate the effects of some restriction enzymes which results in a decrease of the efficiency of restriction[47]. It seems that environmental stress factors will alleviate the efficiency of restriction of foreign DNA transformation, transduction and transfer which will provide the evolutionary advantages for non-growing VNC cells.

Gene transfer by transduction could be realized by bacteriophages containing double-stranded DNAs. Because of the narrow phage-host specificity and because of the necessity of homology between recipient and transducing DNAs (in case of general transduction), efficient transduction can take place only between same or closely related species[37,38,41].

The most ecologically significant flux of genes in the microbial world is realized by conjugative plasmids[37,38,42]. Conjugative broad host range plasmids are widely spread in terrestrial[43] and aquatic microbial ecosystems[45]. In particular, plasmids of the incompatibility class IncP could direct genes transfer across species[37,38,42,43].

The questions of main concern are: i) will GMMs transfer their plasmids into indigenous VNC strains?, ii) will the GMMs receive plasmids from natural (VNC) strains and, after that, iii) will the received plasmids mobilize and spread foreign genes from the GMMs?

According to experiments with an initially sterile soil microcosm in which GMMs and natural soil strains were released, conjugative gene transfer from GMM to soil strains occurred with a frequency of about 3×10^{-6}-3×10^{-5} of transconjugants per donor; addition of glucose and/or tryptone enhances this up to 2×10^{-4} (refs 6, 37, 38, 48–50). In non-sterile soil microcosms, the presence of natural microorganisms inhibits conjugative transfer between two introduced GMMs by two to seven fold[38]. Plasmid transfer in microcosms between released genetically modified strains of *Streptomyces* is considerably effective, 3×10^{-3} transconjugants per donor[51]. In model experiments in the rhizosphere, which is rich in nutrients, effective gene transfer among *Agrobacterium*, *Pseudomonas* and *Rhizobium* was observed[38,52].

There is an abundance of plasmids in aquatic microbial systems[37,38,49,53]. In some lake waters up to 46% of heterotrophs contain plasmids, in river water 10–15%.

The majority of these plasmids are conjugative[38]. The frequency of plasmid transfer in aquatic ecosystems can vary from 5×10^{-8} to 2.5×10^{-3} transconjugants per donor[37,38,45,49]. Very effective plasmid transfer was observed between GMMs and strains of epilithone (aquatic microbial communities associated with stones), 10^{-2} transconjugants per recipient[38,54]. Active sludge is a highly favourable medium for conjugative plasmid transfer[7,55]. A genetically modified strain of *P. putida*, containing the biodegradative plasmid pD10 (utilization of 3-chlorebenzoate) when released into sludge microcosms, initially did not increase the efficiency of xenobiotic destruction. But after the plasmid was transferred into an indigenous strain, which was present in active sludge microflora, the efficiency of destruction of the pollutant increased considerably[6,7,55]

Environmental stress factors could stimulate the participation of GMMs in gene transfer

Will the presence of pollutants in terrestrial and aquatic ecosystems stimulate plasmid transfer from GMMs into natural strains? It seems, it will. Soil and water, polluted with heavy metals and organic xenobiotics, usually are characterized by decreased microbial diversity and by the presence of many strains with plasmids carrying genes encoding resistance to heavy metals and biodegradation[25,26,56]. In such polluted ecosystems, selective pressure could be stimulative for conjugative transfer between GMMs and natural strains[25,26,56].

But are there other environmental factors which will stimulate the efficiency of plasmid transfer in nature? As mentioned earlier, UV damage can alleviate the efficiency of restriction of foreign DNAs[47]. In the case of plasmid transfer, different stress factors, inducing heat, ethanol, acids, bases and sodium dodecyl sulphate can induce a conjugal competence in *Corynabacterium glutamicum*[57]. Possible mechanisms include, stress impairment of the ability of *C. glutamicum* to restrict foreign DNA[57]. It is suggested that stress sensitivity might be a general property of restriction function. Under stress, restriction of incoming DNAs would be alleviated and cells could acquire new genetic information more easily, that may enhance the capability to deal with particular environmental requirements[57]. Of course, the risk of spread of foreign genes by conjugation could be reduced if GMMs do not contain any conjugative (and other) plasmids. But this reduction could not exclude the possibility of spreading by conjugative transposons.

Gene spreading by conjugative transposons the way which could not be controlled

Conjugative transposons are self-transmissible elements that normally are integrated into a chromosome or plasmid but excise themselves and transfer by conjugation to a recipient[43,58]. These recently discovered elements have now been found in a variety of a gram-positive and gram-negative bacteria. They combine features of transposons, plasmids and bacteriophages[43,58]. And what is important, conjugative transposons are capable not only of transferring themselves but also of driving the transmission of other elements. For example, conjugative transposons of *Bacteroides* can mobilize coresident plasmids either in *cis* or in *trans*[58]. The activities of conjugative transposons have broad implication for conjugal transfer of genes in the environment. The very broad host range of the conjugative transposons allow them to move between distantly related hosts, and since they survive by integrating rather than replicating, they can be maintained in strains that might not allow replication of a broad-host-range plasmid[43]. 'The fact that conjugative transposons can mobilize plasmids in *cis* means that there is no such thing as a safe plasmid'[58]. The plasmid that had its *oriT* and *mob* genes removed can become transmissible if a conjugative transposon integrates into it, a fact that should be taken into account in the construction of GMMs that are to be released into environment. Finally, since at least some conjugative transposons are cryptic and since the circular intermediates of the large conjugative transposons may not be visible, 'there is no way to be sure whether a bacterial strain contains a conjugative transposon'[58].

Non-growing and growing microorganisms could participate in gene transfer

Could GMMs released into the environment capture plasmids and conjugative transposons from indigenous strains? Indeed, indigenous strains have an efficient capability to transfer their plasmids into GMMs released in soil[59], or in aquatic[54] ecosystems. This capability of indigenous strains is used especially as the method for isolation of plasmids from aquatic[54] and soil[59] ecosystems. In the last case, this approach was very effective for capturing from the soil, polluted by the herbicide 2,4-dichlorophenoxyacetic acid, the corresponding biodegradative plasmid[59]. In general, it seems that GMMs are able to capture by conjugation transferable plasmids from microorganisms of natural habitat in open ecosystems and, what is important, form putatively VNC cells too[28]. The mechanisms by which VNC cells with blocked protein synthesis could be donors for conjugative plasmid transfer remains mysterious and tantalizing. It seems that a 'selfish' environmental strategy of conjugative plasmids stimulates them to escape from non-growing cells. So the absence of resident plasmids in GMMs will not guarantee that the possibility of foreign genes spreading by conjugational transfer is completely eliminated.

Could plasmid transfer occur in the opposite direction too, i.e. from growing GMMs into non-growing cells? If so, could it increase the chances of non-growing cells to start dividing? As was noted, addition of substrate(s) into soil microcosms (which stimulate growth) increases conjugative plasmid transfer from GMMs into indigenous strains[50]. This does not contradict with the facts that plasmids could be transferred into non-growing cells. Indeed, during the study of the role of conjugative functions in adaptive mutagenesis, it was observed that cells containing the F'*lac* factor with adaptively mutated *lacZ* gene, can transfer the plasmid into non-growing *LacZ*$^-$ recipient cells[36,60,61].

For non-growing strains persisting in an open ecosystem, the capturing of plasmids will enhance the chances of restarting growth. For conjugative plasmids, successfully replicating in growing cells, the transfer into non-growing cells seems to be altruistic. But, in fact, it is not because during the conjugative transfer the initial plasmid copy remains in the donor strain.

The ecological impact of the GMMs released into the environment

Will released GMMs be able to disturb the ecological balance in open ecosystems? For example, could they inhibit or activate the growth of indigenous strains? Soil microbial ecosystems are believed to be disposed to homeostasis and capable of tolerating disturbances[6]. In addition to deep substrate limitation, soil stress factors, both biotic (interaction of GMMs with natural microflora, i.e. antagonism, predation, parasitism) and abiotic (temperature, humidity, pH, etc.), can decrease, in theory, the activity of GMMs and consequently their ecological effects[6].

The available information concerning ecological effects of GMMs released in different microcosms can be summarized as follows:

GMMs can inhibit the growth of natural strains

In fact genetically modified strain of *Klebsiella planticola*, constructed to produce ethanol from the breakdown of agricultural residues (organic wastes) was found (i) to destroy mycorrhizal fungi, which are essential for growth of some important agricultural crops, (ii) to reduce plant growth and, (iii) to increase populations of plant parasitic nematodes[6,10]. A genetically modified strain of *P. putida* pR103 degraded in soil microcosms the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) with an accumulation of toxic metabolite – 2,4-dichlorophenol (2,4-DCP). This intermediate of the incomplete biodegradation of 2,4-D (i) led to a more than 400-fold decrease of the number fungal proplaques and, (ii) markedly reduced the rate of CO_2 evolution, whereas 2,4-D did not depress either fungal proplaques or respiration of the soil microflora. It was concluded that 2,4-DCP, produced by the GMM is more toxic than the initial herbicide 2,4-D (ref. 62).

GMMs are able to replace natural strains from their ecological niche

This ability of GMMs is used in agricultural biotechnology for protection of plants against phytopathogenic strains and from frost injury. To succeed in this, GMMs must compete with oligotrophic microbes in an oligotrophic environment and released GMMs must displace, at least temporarily, indigenous organisms already occupying a niche[6]. As mentioned, a genetically modified strain of *A. tumefaciens*, with the deleted genes of tumour formation replaced the initial wild strain and protected plants from tumour formation[6]. A field test of an *Ice*$^-$ nucleation deficient mutant of *P. syringae* has shown that the number of indigenous ice nucleation active bacteria was reduced on plants colonized with the GMM. An *Ice*$^-$ *P. syringae* strain colonized leaves, flowers and young fruits and significantly reduced the colonization of these tissues by *Ice*$^+$ *P. syringae* and *Erwinia carotovora* as compared with untreated trees[63]. A strain of *pseudomonas* PC1, released in a wheat root system, replaced efficiently the indigenous pseudomonads in their ecological niche[64]. In the field release experiment the strain of *P. fluorescens* containing two reporter gene cassettes resulted in significant but transient perturbation of some culturable components of the indigenous microbial communities that inhabited the rhizosphere and phylloplane of wheat[64].

GMMs are able to stimulate the growth of indigenous strains

It seems, GMMs can do it indirectly, by formation of substrate(s) for natural strains. Genetically modified strains of *Streptomyces* strongly stimulate the formation of CO_2 in the soil; it is suggested that peroxidase from the GMM partially cleaves the lignin molecules (contained in the soil), and makes its carbon atoms more accessible to oxidation by soil microflora[66]. The release of a genetically modified strain of the plant pathogen *Erwinia carotovora* affected the indigenous bacterial community; the total bacterial density significantly increased as did the density of bacteria belonging to the proteolytic functional group, indicating intensive protein degradation in inoculated microcosms. The observed increase could be attributed to an inoculum-nutrient effect: inoculated cells of *E. carotovora* died and became a nutrient source for indigenous populations[67].

GMMs are able to increase the diversity of microbial ecosystems

This appears to take place indirectly by formation of substrates which stimulates gene transfer and recombination in natural populations. Metabolic activity of genetically modified *P. cepadia*, the destructor of 2,4,5-trichlorophenoxyacetic acid released in the soil microcosms containing the pollutant, increased significantly taxonomic and genetic diversity of soil microbial communities. The reasons, it was suggested, were that the GMM produced metabolite(s) of the pollutant biodegradation, which stimulated the growth of static cells. This in turn could have led to an increase of taxonomic diversity and a stimulation of plasmid transfer and recombination, ultimately resulting in the increase of genetic diversity[68].

In general; GMMs released in open ecosystems are capable of realizing all possible ecological effects on indigenous microflora: inhibition, replacement, stimulation of proliferation, increasing of taxonomic and genetic diversities. And, what is of serious ecological concern, GMMs are able to produce toxic intermediates from incomplete biodegradation of xenobiotics; GMMs released into the environment are capable of participating in all known processes of evolution of the microbial world.

The principles of the risk assessment of the GMMs released into the environment

Is it possible to assess, in any given situation, the risk of the planned release of GMMs into ecosystem?

Risk assessment – 'step by step'

Microbial ecologists have suggested a staged risk assessment process that starts with the description of the genetics and natural history of the donor and host organisms and moves next to laboratory experiments, and, finally, to field tests[10]. Field testing of GMMs, even at a small scale, presents significant problems. Once released, GMMs may establish and disperse in the environment, becoming nearly impossible to eliminate. The reason for slow progress in developing GMMs planned for large-scale release may have to do with the uncertainty of the risk associated with such field-experiments. Relatively few small-scale field tests have been conducted with genetically modified bacteria (10 strains) and genetically modified viruses (5 strains), in contrast approximately 1000 field tests of genetically modified plants have been permitted[10].

Most European countries as well as the US and Japan, have environmental regulations that ban the deliberate release of GMMs[2]. The US has permitted deliberate release of some GMMs for field tests, but the permitting process is not standardized and is very costly; generally costing millions of dollars for each test[2]. No country has defined what steps will be required to obtain permission for commercial use of GMMs for *in situ* bioremediation. Without this information companies cannot assess the costs and benefits of using GMMs[2].

In the US the administration generally is confident that most uses of biotechnology cause no harm and that, on an international basis, it could be used safely with voluntary and cooperative oversight. Moreover, the US takes the position that binding international protocols governing the use and release of genetically modified organisms would interfere with research and development of the biotechnological industry[3]. In contrast, some European countries are greatly concerned about uses of biotechnology, especially in developing countries. In July 1995, the European Parliament passed a resolution saying that: 'a legally binding international biosafety protocol is necessary and is a matter of urgency and must be immediately negotiated by states partly on the United Nations Convention on Biological Diversity', which was agreed at the Earth Summit in Rio-de-Janeiro in 1992. To justify the needs, the resolution dates: 'Deliberate release of genetically modified organisms are being carried out in many developing countries, which have no legislation or infrastructure to ensure their safe use . . . [and] this situation is putting the entire biosphere of the planet at risk'[3]. An algorithm for the oversight of field trails in economically developing countries is proposed[69].

What is the meaning of environmental genetic engineering for India? India is putting considerable effort into developing national biotechnology[70]. Genetically modified strains are being designed for bioremediation[71–75] and for improving of the efficiency of nutrition of plants[76]. Special genetically modified strains of *Rhizobium* spp. were constructed for ecological studies[77]. Research in progress deals with the construction of biopesticides[78,79] and plant genetic engineering[80–83]. It is obvious that adequate solving of the problems of safety of the environmental genetic engineering is very actual for stable development of Indian biotechnology.

The principal uncertainty of risks associated with the release of GMMs

But will the usage of any algorithms guarantee a rational and definite assessment of risks? Moreover, if the results of all stages of risk assessment will be optimistic, will it guarantee that in open nature, after some time and in some conditions, ecological hazards will never arise? Could it be that environmental genetic engineering will open a Pandora's box of VNC strains which are resting in terrestrial and aquatic ecosystems until released GMMs

disturb their peaceful dormancy? Two principal uncertainties of the assessment of risks associated with possible ecological consequences of GMMs release exist. First, it is believed that only 5% of the total number of all microorganism species actually existing in nature are known to science, the other 95% being unknown[84]. How many potentially hazardous strains are among them? And what factors may activate the resting pathogens? Second, the absence of definite and commonly recognized criteria for the evaluation of similarity between microorganisms poses problems. The main principle underlying the decisions regarding GMMs' field testing is the evaluation of similarity between the GMM planned for release and microorganisms whose release is known to be harmless. There are no clear criteria for the estimation of this similarity; estimates of similarity made by different experts may vary drastically[85].

Will the expected benefits overweigh the high cost of risk assessment experiments, the permanent and indefinitely long period of monitoring of the ecological situation in the place of release and in surrounding areas? And how to estimate the weight of the uncertain risk? Rational answers to these questions will form a part of future scientific knowledge.

1. Berg, P. and Singer, M. F., *Proc. Natl. Acad. Sci. USA.*, 1995, **92**, 901–913.
2. Atlas, R. M., *Chem. Eng. News*, 1995, April, 3, pp. 32–42.
3. Hileman, B., *Chem. Eng. News*, 1995, August, 21, pp. 8–17.
4. Wilson, M. and Lindow, S. E., *Annu. Rev. Microbiol.*, 1993, **47**, 913–944.
5. Velkov, V. V., *Russian J. Genet.*, 1994, **30**, 515–524.
6. Stewart-Tull, D. E. S. and Sussman, M. (eds), *The Release of Genetically Modified Microorganisms—REGEM2*, Plenum, New York, 1992.
7. Nuslein, K., Marris, D., Timmis, K. and Dwyer, D. F., *Appl. Environ. Microbiol.*, 1992, **58**, 3380–3386.
8. Heur, H., Dwyer, D. F., Timmis, K. N. and Wagner-Dobler, I., *Microb. Ecol.*, 1995, **29**, 203–220.
9. Ward, M., *BioTechnology*, 1995, **13**, 32–42.
10. Krimsky, S., Wrubel, R. P., Naess, I. G., Levy, S. B., Wetzler, R. E. and Marshall, B., *BioScience*, 1995, **45**, 590–599.
11. Rexroad, C. E., *Chem. Ind.*, 1995, **10**, 372–375.
12. Van Elsas, J. D., Trevors, J. T., van Overbeek, L. S. and Starodub, M. E., *Can. J. Microbiol.*, 1989, **35**, 951–959.
13. Tang, W. Z., Pasternak, J. J. and Glick, B. R., *Can. J. Microbiol.*, 1995, **6**, 445–452.
14. Recordet, G., Steinberg, C. and Faurie, G., *FEMS Microbiol. Lett.*, 1992, **101**, 251–260.
15. Barcina, I. *et al.*, *J. Appl. Bacteriol.*, 1992, **73**, 229–236.
16. Leung, K., Trevors, J. T. and Lee, H., *Can. J. Microbiol.*, 1995, **41**, 461–470.
17. Heur, H., Dwyer, D. F., Timmis, K. N. and Wagner-Dobler, I., *Microb. Ecol.*, 1995, **29**, 203–220.
18. Morita, R. Y., *Can. J. Microbiol.*, 1988, **34**, 436–441.
19. Kjelleberg, S. (ed.), *Bacteria in Starvation*, Plenum, New York, 1993.
20. Hengge-Arinis, R., *Cell*, 1993, **72**, 165–168.
21. Loren, P. C. and Hengge-Aronis, R., *Annu. Rev. Microbiol.*, 1994, **48**, 53–80.
22. Matin, A., *Mol. Microbiol.*, 1991, **5**, 3–10.
23. Rockabrand, D., Arthur, T., Korinek, G., Liver, K. and Blum, P., *J. Bacteriol.*, 1995, **177**, 3695–3703.
24. Roszak, D. B. and Colwell, R. R., *Microbiol. Rev.*, 1987, **51**, 365–379.
25. Lewis, D. B. and Gattie, G. T., *ASM News*, 1991, **57**, 27–32.
26. Atlas, R. M., Sayer, G., Burlage, R. S. and Bej, A. K., *BioTechniques*, 1992, **12**, 706–717.
27. Oliver, J. D., Hite, F., McDougald, D., Andon, N. L. and Simpson, L. M., *Appl. Environ. Microbiol.*, 1995, **61**, 2624–2630.
28. Munro, P. M., Flatau, G. N., Clement, R. L. and Gauthier, M. J., *Appl. Environ. Microbiol.*, 195, **61**, 1853–1858.
29. Colwell, R. R., Brayton, P. R., Grimes, D. J., Roszak, D. B., Huq, S. A. and Palmer, C. M., *BioTechnology*, 1985, **3**, 817–820.
30. Molin, S., Boe, L., Jensen, L. B., Kristen, C. S., Givskov, M., Ramos, J. L. and Bej, A. K., *Annu. Rev. Microbiol.*, 1993, **47**, 139–166.
31. Kloos, D.-U., Stratz, M., Gutter, A., Steffan, R. J. and Timmis, K. N., *J. Bacteriol.*, 1994, **176**, 7352–7361.
32. Cairns, J., Overbaugh, J. and Miller, S., *Nature*, 1988, **335**, 142–145.
33. Foster, P. L. and Cairns, J., *Genetics*, 1992, **131**, 783–789.
34. Harris, R. S., Longerich, S. and Rosenberg, S. M., *Science*, 1994, **264**, 258–260.
35. Foster, P. L., Gudmunsson, G., Trimarchi, J. M., Cai, H. and Goodman, M. F., *Proc. Natl. Acad. Sci. USA.*, 1995, **92**, 7951–7955.
36. Foster, P. L. and Trimarchi, J. M., *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 5487–5490.
37. Levy, S. B. and Mille, R. V. (eds), *Gene Transfer in the Environment*, McGraw-Hill, New York, 1989.
38. Fry, J. D. and Day, M. J. (eds), *Bacterial Genetics in Natural Environments*, Chapman and Hall, London, 1990.
39. Lorenz, M. Z., Aaderma, B. and Wackernagel, W., *J. Gen. Microbiol.*, 1988, **134**, 107–122.
40. Lorenz, M. G. and Wackernagel, W., *Microbiol. Rev.*, 1994, **58**, 563–602.
41. Germuda, J. and Khachtourians, G. G., *Appl. Environ. Microbiol.*, 1988, **54**, 1731–1737.
42. Mazodier, P. and Davis, J., *Annu. Rev. Genet.*, 1991, **25**, 1435–1441.
43. Salyers, A. A. and Shoemaker, N. B., *FEMS Microbiol. Ecol.*, 1994, **15**, 12–22.
44. Paget, E., Monrozier, L. J. and Simonet, P., *FEMS Microbiol. Lett.*, 1992, **97**, 31–40.
45. Frisher, M. E., Stewart, G. J. and Paul, H., *FEMS Microbiol. Ecol.*, 1944, **15**, 127–135.
46. Jiang, S. C. and Paul, J. H., *Appl. Environ. Microbiol.*, 1995, **61**, 317–325.
47. Kelleher, J. E. and Raleigh, E. A., *J. Bacteriol.*, 1994, **176**, 19, 5888–5896.
48. Stotzky, G. and Babich, H., *Adv. Appl. Microbiol.*, 1986, **31**, 73–138.
49. Trevors, J. T., Barkar, T. and Bourquin, A. W., *Can. J. Microbiol.*, 1988, **42**, 717–743.
50. Van Elsas, J. D., Govaret, J. M., Starudub, M. E. and Overbeek, L. S., *FEMS Microbiol. Ecol.*, 1990, **73**, 375–381.
51. Wellington, E. M. H., Cresswell, N. and Saunders, V. A., *Appl. Environ. Microbiol.*, 1990, **56**, 1413–1419.
52. Van Elsas, J. D., Nikkel, M. and van Overbeek, L. S., *Curr. Microbiol.*, 1990, **19**, 375–381.
53. Hermansson, M. and Lindberg, G., *FEMS Microbiol. Ecol.*, 1994, **15**, 47–54.
54. Bale, M. J., Fry, J. C. and Day, M. J., *J. Gen. Microbiol.*, 1987, **133**, 3099–3107.
55. McClure, N. C., Weigthman, A. J. and Fry, J. C., *Appl. Environ. Microbiol.*, 1989, **55**, 2627–2634.
56. Gauthier, M. G., *Gene Transfer and Environment*, Springer-Verlag, New York, 1992.
57. Schafer, A., Schwarzer, A., Kalinowski, J. and Puhler, A., *J. Bacteriol.*, 1994, **176**, 7309–7319.
58. Salyers, A. A., Shoemaker, N. B. and Li, L.-Y., *J. Bacteriol.*, 1995, **177**, 5727–5731.

59. Top, E. M., Holben, W. E. and Forney, L. J., *Appl. Environ. Microbiol.*, 1995, **61**, 1691–1698.
60. Radicella, J. P., Park, P. U. and Fox, M. S., *Science*, 1995, **268**, 418–420.
61. Galitski, T. and Roth, J. R., *Science*, 1995, **268**, 421–423.
62. Short, K. A., Doyle, J. D., King, R. J., Seidler, R. J., Stozky, G. and Olsen, R. H., *Appl. Environ. Microbiol.*, 1991, **57**, 412–418.
63. Lindow, S. E., *Appl. Environ. Microbiol.*, 1987, **53**, 2520–2527.
64. Bolton, H. Jr., Fredericson, J. K., Thomas, J. M., Li, S. W., Workman, D. J., Bentjen, S. A. and Smith, J. L., *Microbiol. Ecol.*, 1992, **21**, 175–189.
65. DeLeij, F. A. A. M., Sutton, E. J., Whipps, J. M., Fendon, J. S. and Lynch, J. M., *Appl. Environ. Microbiol.*, 1995, **61**, 3443–3451.
66. Wang, Z., Crawford, T. S., Magnunson, T. S., Bleakely, B. H., Heretel, G., *Can. J. Microbiol.*, 1991, **37**, 287–294.
67. Scanferlato, V. S., Orvos, D. R., Cairns, J. and Lacy, G. H., *Appl. Environ. Microbiol.*, 1989, **55**, 1477–1482.
68. Bej, A. K., Perlin, M. and Atlas, R. M., *FEMS Microbiol. Lett.*, 1991, **86**, 169–176.
69. Miller, H. I., Altman, D. W., Bartin, J. and Huttner, S. I., *Bio-Technology*, 1995, **13**, 955–959.
70. Ramachandrans, S. and Sharma, M., *Proc. Natl. Acad. Sci. India, Section B, (Biol. Sci.)*, 1993, **63**, 139–148.
71. Sahasrabudhe, A. V., and Modi, V. V., *Appl. Microbiol. Biotechnol.*, 1991, **34**, 556–557.
72. Johri, S., Qazi, G. M. and Chopra, C. L., *J. BioTechnol.*, 1991, **20**, 73–82.
73. Mahamood, S. K. and Rao, P. R., *Bull. Environ. Contam. Toxicol.*, 1993, **50**, 486–491.
74. Bhagat, R. and Srivastava, S., *Indian J. Exp. Biol.*, 1993, **31**, 590–594.
75. Bevinakatti, B. G. and Ninnekar, H. Z., *World J. Microbiol. BioTechnol.*, 1993, **31**, 590–594.
76. Nasudev, S., Lodha, M. L. and Sreekumar, K. R., *Curr. Sci.*, 1991, **60**, 600–604.
77. Sharma, P. X., Anand, R. C. and Lakshiminarayana, K., *Biol. Biotechnol.*, 1991, **23**, 801–805.
78. Mohan, K. S. and Gopinathan, K. P., *J. Biosci.*, 1992, **17**, 421–430.
79. Suresh, G., Radhika, C. G. and Jayaraman, K., *BioTechnology*, 1992, **17**, 1–30.
80. Biswas, B. B., *Subcell. Biochem.*, 1991, **17**, 1–30.
81. Mukhopadhya, A., Arumagan, N., Nandakumar, P. B. A., Pradhan, A. K., Gupta V. and Pental, D., *Plant Cell Rep.*, 1992, **11**, 506–513.
82 Gupta, V., Lakshmi-Sita, G. and Shaila, M. S., *Plant Cell. Rep.*, 1993, **12**, 418–421.
83. Viegas, P. M. and Notani, N. K., *J. Genet.*, 1993, **72**, 35–42.
84. Microbial Diversity 21: Proposals of IUMS/IUBS, *Uspekhi Sovremennoi Biologi* (Russ)., 1992, **5**, **6**, 807–810.
85. Goodfellow, M., Jones, D. and Priest, F. G. (eds), *Computer Assisted Bacterial Systematics*, Academic Press, London, 1985.

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