Review

Stress-induced evolution and the biosafety of genetically modified microorganisms released into the environment

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We shall not all die,
but we shall all be changed . . .
1 Corinthians 12, 51

This article is focused on the problems of reduction of the risk associated with the deliberate release of genetically modified microorganisms (GMMs) into the environment. Special attention is given to overview the most probable physiological and genetic processes which could be induced in the released GMMs by adverse environmental conditions, namely: (i) activation of quorum sensing and the functions associated with it, (ii) entering into a state of general resistance, (iii) activation of adaptive mutagenesis, adaptive amplifications and transpositions and (iv) stimulation of inter-species gene transfer. To reduce the risks associated with GMMs, the inactivation of their key genes responsible for stress-stimulated increase of viability and evolvability is proposed.

1. Introduction

The problems of the introduction of the genetically modified microorganisms (GMMs) into the environment are usually discussed in terms of optimistic hopes, uncertain prognoses and pessimistic prophecies. Optimistic hopes are expressed in units of the expected returns and pessimistic prophecies, in the best case, in terms of "common sense and general considerations" (reviewed in Wilson and Lindow 1993; Cook *et al* 1996; Velkov 1996, 2000; Wrubel *et al* 1997; Neilsen *et al* 1998).

There are generalized risk classes of microorganisms, especially of GMMs, designed for the deliberate release into the environment:

Risk class 1: No adverse effects, or very unlikely to produce an adverse effect. Organisms in this class are considered to be safe.

Risk class 2: Adverse effects are possible but are unlikely to represent a serious hazard with respect to value to be protected (human, animal and plant health; air, water, soil quality, biodiversity and others). Local adverse effects are possible, which can either revert spontaneously (e.g. owing to environmental elasticity and resilience) or be controlled by available treatment or preventive measures. The danger of their spreading beyond the application area is highly unlikely.

Risk class 3: Serious adverse effects are likely with respect to the value to be protected, but spreading beyond

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Abbreviations used: AHLs, N-acyl-L-homoserine lactones; CF, cystic fibrosis; 2,4-D, 2,4-dichlorophenoxyacetic acid; GMMs, genetically modified microorganisms; HSP, heat shock proteins; PAHs, polycyclic aromatic hydrocarbons; PHA, polyhydroxyalkanoate; PHB, poly(3-hydroxybutyrate); SSR, starvation-stress response.

the area of application is unlikely. Treatment and/or preventive measures are available.

Risk class 4: Serious adverse effects are to be expected with respect to the values to be protected, both locally and outside the application. No treatment or preventive measures are available (Doblhoff-Dier *et al* 1999).

This article is an attempt to foresee the environmental stress-induced physiological and genetic events which can increase risks of GMMs after their release into the open ecosystems.

1.1 *Will the environmental conditions affect the capabilities of GMMs to survive and evolve?*

Release of GMMs into open ecosystems poses several questions. How will GMMs be affected by severe environmental conditions? Will the GMMs be more vulnerable or more tolerant to the stressful factors? How will the environmental conditions change the physiological and genetic processes in the released GMMs? How will these changes, in its turn, change the environment? In which direction will the genetically modified ecosystem(s) evolve? How to prevent or, at least, to reduce the non-desirable ecological effects of the released GMMs? Environmental stresses could induce a number of physiological and genetic responses, which increase the viability and evolvability of the stressed microorganisms (reviewed in Kolter *et al* 1993; Velkov 1999). The following possibilities could be considered:

1.2 *The consequences of the genetic modifications itself could act as a stress*

In *Escherichia coli* a variety of stress conditions induce the synthesis of more than 20 heat shock proteins (HSPs). Major HSPs are molecular chaperones, including DnaK, DnaJ and GrpE, GroEL and GroES, and proteases (reviewed in Arsene *et al* 2000). The Hsp70 chaperone system from *E. coli* (DnaK-DnaJ-GrpE, the DnaK system) can bind to proteins, prevent aggregation, and promote refolding of chaperone-bound polypeptides into native proteins. It was suggested that: (i) ClpB directly binds protein aggregates, ATP induces structural changes in ClpB, which (ii) increase hydrophobic exposure of the aggregates and (iii) allow DnaK-DnaJ-GrpE to bind and mediate dissociation and refolding of solubilized polypeptides into native proteins. This efficient mechanism, whereby chaperones can catalytically solubilize and refold a wide variety of large and stable protein aggregates, is a major addition to the molecular arsenal of the cell to cope with protein damage induced by stress or pathological states (Goloubinoff *et al* 1999; Diamant *et al* 2000). The possibility is that genetic modifications could act as a stress, is consistent with the observations that chaperone synthesis is induced in some recombinant strains cultivated in non-stressful (optimal) growth conditions.

The induction of chaperones has been observed in *E. coli* in response to overproduction of the insoluble heterologous proteins, for example, α-glucosidase of *Saccharomyces cerevisiae* during glucose-limited fed-batch fermentation. Under the same conditions, a significant but transient increase in the mRNA levels of the heat shock genes *lon* and *dnaK* were observed. Analysis of the soluble cytoplasmic protein fraction 3 h after induction revealed increased levels of the chaperones GroEL, DnaK, and Tig. Host stress proteins, like DnaK, GroEL, IbpA, IbpB, and OmpT, have been found to be associated with the α-glucosidase protein aggregates (Jurgen *et al* 2000). In another case, *E. coli* harbouring heterologous polyhydroxyalkanoate (PHA) biosynthesis genes accumulated unusually large amounts of PHA. The integrated cellular responses of metabolically engineered *E. coli* to the accumulation of poly(3-hydroxybutyrate) (PHB) in the early stationary phase were analysed at the protein level by two-dimensional gel electrophoresis. Out of the 20 proteins showing altered expression levels, three HSPs, GroEL, GroES, and DnaK, were significantly upregulated in PHB-accumulating cells. It was concluded that the accumulation of PHB in *E. coli* acted as a stress on the cells, which reduced the cells' ability to synthesize proteins and induced the expression of various protective proteins (Mee-Jung Han *et al* 2001).

It seems that the stress response that happens in some GMMs could be caused by the increased hydrophobicity of the cytoplasm because of the accumulation of the (insoluble) foreign product(s). Does it mean that at least some GMMs are stressed in non-stressful conditions? Can genetic modification induce physiological changes, which usually happen when "normal" microbes encounter the real environmental stresses and, as a result, acquire general resistance and increased mutability?

1.3 *Are GMMs really predisposed to increased mutability?*

If the GMMs are planned for release, this question need to be answered by conducting the corresponding measurements made in different conditions including those that mimic the environment.

The stress-induced mutability of a recombinant strain was observed during the usage of a novel system constructed to study the evolution of transcription signals in heterologous systems under selective starvation conditions. The system was based on the plasmid-mediated transfer

of the histidine biosynthetic genes from *Azospirillum brasilense* into an *E. coli* mutant lacking histidine biosynthetic ability. It was observed that under highly selective stressful conditions (starvation for histidine), genetic changes in the donor plasmid lead to mutated sequences that are efficiently recognized as promoters by the *E. coli* RNA polymerase (Fani *et al* 1998). Of course, stress can stimulate mutations not only in the foreign DNA, but in the chromosomal DNA as well. This was found when DNA from starving *E. coli* was assayed to determine the influence of osmotic and/or oligotrophic stress on variations in randomly amplified polymorphic DNA banding patterns. Considerable genetic rearrangements or DNA topology variations were detected. Also, a new amplicon generated in bacteria prestarved by an osmotic stress and resuscitated in rich medium was found (Jolivet-Gougeon *et al* 2000). For the released GMMs, such mutagenesis and a following proliferation are highly undesirable.

The special containment systems were constructed to reduce the risk. These systems are based usually on the use of a killing gene and a regulatory circuit that controls expression of the killing gene in response to the presence or absence of environmental signals. It is believed that such systems could be used to contain released microorganisms and ameliorate some of the concerns about their uncontrolled proliferation and about horizontal gene transfer (Ronchel *et al* 1995; Kaplan *et al* 1999). Recently, an active biological containment (ABC) system for recombinant microorganisms that degrade a model pollutant was designed on the basis of the *Pseudomonas putida* TOL plasmid meta-cleavage regulatory circuit. In the presence of the model pollutant, bacterial cells survived and degraded the target compound, whereas in the absence of the aromatic carboxylic acid cell death was induced. But the system had two main drawbacks. First is the slow death of the bacterial cells in soil versus the fast killing rate in liquid cultures in laboratory assays. Second is the appearance of mutants, at a rate of about 10^{-8} per cell per generation, that did not die after the pollutant had been exhausted. The system was reinforced by using *P. putida asd* mutant that is viable only in complex medium supplemented with diaminopimelic acid, methionine, lysine, and threonine. The number of mutants of the reinforced strain that escaped killing was below detection limit ($< 10^{-9}$ mutations per cell per generation) and the strain disappeared in less than 20 to 25 days in soils without the pollutant. The biologically noncontained control strain was detectable in soils after 100 days (Ronchel and Ramos 2001).

GMMs have two avenues in open ecosystems. The preferred way is to carry out the task prescribed by the designer and to be eliminated from that environment. The alternative is to escape from elimination and evolve and proliferate. These aspects have to be considered carefully before they are released into the environment.

2. If the released GMMs proliferate

For biosafety purposes it is important to reduce the GMM's potential for expansions in various ecological niches. In many cases, for such expansions the individual cells must coordinate their actions with each other.

It is now well established that bacterial cells can "speak", or communicate through the secretion and uptake of small diffusable molecules. These signals are often used by bacteria to co-ordinate phenotypic expression and this mechanism of regulation presumably provides them with a competitive advantage in their natural environment. In the environment, microbial activity is mainly associated with surfaces where bacteria form highly structured and cooperative consortia (biofilms). The ability of bacteria to organize structurally and to distribute metabolic activities between the different members of the consortium demands a high degree of coordinated cell–cell interaction.

In Gram-negative bacteria, the most intensively investigated signal molecules are N-acyl-L-homoserine lactones (AHLs), which are utilized by the bacteria to monitor their own population densities in a process known as "quorum sensing". These density-dependent regulatory systems rely on two proteins, an AHL synthase, usually a member of the LuxI family of proteins, and an AHL receptor protein belonging to the LuxR family of transcriptional regulators. At low population densities cells produce a basal level of AHL. As the cell density increases, AHL accumulates. On reaching a critical threshold concentration, the AHL molecule binds to its cognate receptor which, in turn, leads to the induction/repression of AHL-regulated genes. To date, AHL-dependent quorum sensing circuits have been identified in a wide range of Gram-negative bacteria where they regulate various functions, namely: motility, plasmid conjugal, pathogenecity, plasmid transfer and bioluminescence (in marine bacteria). AHLs are involved as intercellular signals controlling a wide range of physiological responses in Gram-negative bacteria. They function especially in *Vibrios*, *Pseudomonads* and *Erwinias* as well as in *Rhizobium agrobacterium* spp, particularly where the bacteria are in symbiotic or parasitic relationships with higher organisms. While the current focus on bacterial signalling centers on AHLs, the quorum sensing signals of Gram-negative bacteria, these are not the only types of signals used by bacteria. Indeed, there appears to be many other types of signals produced by bacteria and it also appears that a bacterium may use multiple classes of signals for phenotypic regulation. Some marine eukaryotes

secrete their own signals which compete with the bacterial signals and thus inhibit the expression of bacterial signalling phenotypes. (reviews Fuqua and Greenberg 1998; Eberl 1999; Rice *et al* 1999; Lazazzera 2000).

2.1 *Functioins associated with AHLs and quorum sensing*

2.1a *Survival under starvation*: The survival of *Rhizobium leguminosarum* under carbon or nitrogen starvation is dependent on cell density at entry into stationary phase and requires the accumulation of an extracellular compound. The effect of this compound is mimicked by AHL (Thorne and Williams 1999). An extracellular compound is also involved in the carbon starvation response of *Vibrio* sp. strain 14, as the addition of stationary phase supernatant extracts to logarithmic phase cells resulted in upregulation of carbon starvation-induced proteins (Srinivasan *et al* 1998). In the case of *P. aeruginosa* the direct genetic evidence have been provided that the *rhl* quorum sensing system is required for expression of the stationary phase Sigma-S (Latifi *et al* 1996).

2.1b *Biofilm formation and colonization*: Biofilms exhibit complex architecture with groups of microcolonies glued together by bacterially-produced exopolymers and separated by aqueous channels. The structure of these microbial communities range from monolayers of scattered single cells to thick, mucous structures of macroscopic dimensions (microbial mats; algal-microbial associations; trickling filter biofilms). Furthermore, these microbial communities are often composed of multiple species that interact with each other and their environment (reviewed in Oleskin *et al* 2000; O'Toole *et al* 2000; Tolker-Nielsen and Molin 2000; Wimpenny *et al* 2000). The cellular properties such as growth differentiation, chemotaxis, and cell-to-cell signalling enable biofilm communities to organize structurally in response to the external conditions and the activities of the different biofilm members. Thereby resource utilization becomes optimized, and processes that require syntrophic relationships or special microenvironments become facilitated. Biofilm-grown cells express properties distinct from planktonic cells, one of which is an increased resistance to antimicrobial agents. Slow growth and/or induction of an *rpoS* mediated stress response could contribute to biocide resistance. The physical and/or chemical structure of exopolysaccharides or other aspects of biofilm architecture could also confer resistance by exclusion of biocides from the bacterial community. Moreover, biofilm-grown bacteria might develop a biofilm-specific biocide-resistant phenotype. Owing to the heterogeneous nature of the biofilm, it is likely that there are multiple resistance mechanisms that work within a single community (review Mah and O'Toole 2001). AHLs have been found in naturally occurring biofilms suggesting that cell–cell communication plays an important role in the colonization of solid surfaces (McLean *et al* 1997; Parsek and Greenberg 1999; Davey and O'Toole 2000; Kuchma and O'Toole 2000). In general, biofilms are of great industrial, agricultural, medical and ecological values due to their prevalence and to get rid of.

2.1c *Motility*: In *P. aeruginosa* quorum sensing systems are involved in the regulation of twitching motility, a flagellum independent mode of surface translocation which requires functional pili (Glessner *et al* 1999). *Serratia liquefaciens* is capable of swarming motility, a special form of surface translocation that involves the differentiation of short motile rods (swim cells) into multinucleate, aseptate, and profusely flagellated swarm cells that are highly elongated. These swarm cells migrate coordinately in groups atop appropriate surface to rapidly colonize all available space. Swarming motility of *S. liquefaciens* is controlled by a quorum sensing system (Lindum *et al* 1998).

2.1d *Antibiotic production*: The non-intendent killing of indigenous strains by antibiotic producing released GMMs is undesirable. In many cases, such antibiotic production is associated with quorum sensing. For example, *Pseudomonas aureofaciens* protects wheat from "take-all" decease, caused by an ascomyces fungus, by producing phenazine antibiotics, the synthesis of which is regulated by quorum sensing circuit (Wood *et al* 1997). In *Chromobacterium violaceum* the production of a purple pigment violacein, which possesses antibacterial activity is quorum senescing (McClean *et al* 1997).

2.1e *Production of virulence factors*: In adverse environmental conditions some bacteria became communicative to reach the consensus how to survive together through becoming more aggressive. In fact, in many cases, quorum-sensing regulatory circuits are involved in the regulation of production of extracellular enzymes and virulence factors in human and plant pathogens. In the opportunistic pathogen *P. aeruginosa*, which causes nosomial infections in immunocompromized patients and is associated with chronic infections in cystic fibrosis patients, expression of many virulence factors is regulated by two interlinking quorum sensing circuits (review Van Delden *et al* 1998; Suh *et al* 1999). In *P. aeruginosa* many exoproduct virulence determinants are regulated via a hierarchical quorum-sensing cascade (Eberl 1999; Lazazzera *et al* 2000; Winzer *et al* 2000).

In pathogenic bacteria belonging to the genus *Erwinia* that causes soft root disease in plants, production of

several extracellular cell wall degrading enzymes including pectate lyase, pectine lyase, poligalacturonase, cellulase, and protease are subject to quorum sensing associated regulation (Pirhonen *et al* 1993). In *Pantoea srewartii*, the causal agent of Stewart's wilt and leaf blight of corn, the synthesis of an extracellular polysaccharide capsule which is the bacterium major virulence factor is quorum sensing regulated (von Bodman *et al* 1998).

2.1f *Conjugal transfer of the plasmids*: In *A. tumefaciens* quorum sensing regulates its behaviour while in association with host plants. During pathogenesis of plants *A. tumefaciens* transfers the fragment of its plasmid DNA into plant cells and this DNA fragment integrates into the plant genome, eventually causing the crown gall tumour (reviewed in Tzfira *et al* 2000; Zhu *et al* 2000; Chumakov 2001). This cross-kingdom genetic transfer is an excellent example of naturally occurring genetic engineering, which should be prevented in the released GMMs. Also, the released GMMs should be inhibited for natural transformation by exogenous DNAs.

2.1g *Competence for genetic transformation*: Genetic competence in both *Bacillus subtilis* and *Streptococcus pneumoniae*, as well as virulence in *Staphylococcus aureus*, are regulated by quorum sensing mechanisms that use two component signal transduction systems to respond to extracellular peptide pheromones (reviewed in Tortosa and Dubnau 1999). Recent evidence demonstrates that regulation of competence depends also on a novel *s*-factor, itself controlled by an autostimulatory quorum sensing system that acts through an extracellular peptide signal (Morrison and Lee 2000).

There are at least seven quorum sensing-associated mechanisms providing the establishments and expansion of the microorganisms in different environments. Given the best interests of biosafety, the GMMs planned for release should be deprived of these survival mechanisms.

2.2 *Metabolic activity of GMMs could disturb ecosystems*

A great number of GMMs have been developed for biodegradation of environmental contaminants. It is generally believed that the genetic design of novel metabolic routes offers exciting possibilities in the exploration of the metabolic/evolutionary potential of cells and in the development of innovative applications (reviewed in Holliger *et al* 1997, Keasling and Bang 1998). In spite of the impressive progress, some results of the field's and microcosm's testing of GMMs, designed for bioremediation, evoked considerable ecological concern.

It was observed that: released GMMs can produce toxic and genotoxic compounds because of the incomplete mineralization of the pollutants, especially, if the soil or water contains a complex mixture of pollutants, and some of them were not planned for complete biodegradation. The toxic and genotoxic compounds produced by the GMMs could inhibit the growth of the indigenous organisms (Dorn and Salanitro 2000).

Let us look at a few examples. Usually, during a bioremediation of a polycyclic aromatic hydrocarbons (PAHs) contaminated site, a correspondence between decreasing toxicity and degradation of the easily biodegradable PAHs is indeed observed (Hund and Traunspurger 1994). But in some cases, the results of toxicity screening time courses indicated that under oxygen-limiting conditions, the potential for the accumulation of toxic, acidic metabolites that could adversely affect the rates and extent of bioremediation existed (Long and Aelion 1999). Unfortunately, the GMMs could produce genotoxic compounds also. As usual, the genotoxicity of the PAH polluted sites is reduced as the bioremediation proceeded, but not to undetectable level. The *Tradescantia*–micronucleus test was used to evaluate the genotoxicity of the PAH polluted soil before and after fungal treatment. Soil extracts before fungal treatment exhibited a relatively strong genotoxic effect even at a 1% concentration, and the highest concentration without significant effect was 0·25%. After fungal treatment, the depletion of selected PAH was associated with a reduction of the soil genotoxicity, but the extent of this reduction was not high. In fact, the 2% concentration of the extract from the fungal-treated soil showed genotoxic effects comparable to the 1% soil extract without fungal treatment (Baud-Grasset *et al* 1993).

In another study to evaluate the genotoxicity and identify the mutagens in the soil contaminated with PAHs from creosote before and after various bioremediation treatments, fractionated extracts of five soils were bioassayed for mutagenic activity with a *Salmonella* histidine reversion assay. It was found that mutagenicity of two treatments was significantly greater than the mutagenicity of the untreated soil. The mutagenic fractions contained azaarenes, which are mutagens (Brooks *et al* 1998).

Mutagenicity profiles of the extracts of four differently bioremediated creosote contaminated soils were evaluated by the spiral technique of the *Salmonella* assay. Mutagenic potency (revertants per mg extract) suggested that compost, land treatment, and untreated creosote soil extracts were moderately mutagenic with Arochlor-induced rat liver (S9) but were nonmutagenic without S9. However, the bioslurry extract was strongly mutagenic and the biopile extract was moderately mutagenic either with or without S9. The strong mutagenic activity in the

bioslurry was due by the presence of mutagenic nitro-hydrocarbons (Hughes *et al* 1998)

If mutagenic compounds are produced by GMMs, they will stimulate evolution of ecosystems, if a selection is applied.

2.3 *Evolution of released GMMs: why, how and where?*

It is traditionally believed that released GMMs will perform their intended function and then will be eliminated from the environment due to the inherent fitness disadvantages resulting from their metabolic and physiological load caused by genetic alterations. There are many well-documented cases of GMMs that had a long history of growth in laboratory conditions, which die very rapidly after the release into the wild environments (reviewed in Wilson and Lindow 1993; Cook *et al* 1996; Velkov 1996, 2000; Wrubel *et al* 1997; Neilsen *et al* 1998). But, it seems, that released GMMs could also choose another way: to evolve and survive.

2.3a *The released GMMs could evolve to increase their competitiveness for an alternative natural substrate(s)*: Logically, the GMMs used in bioremediation must be foreseen to adapt to growth on the anthropogenic substrate that they are intended to degrade. But if such an adaptation results in improved competitiveness for alternative, naturally occurring substrates, then this will increase the likelihood that the GMM will persist in the environment. In the special study, *b*-proteobacteria *Burkholderia*, capable of degrading the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), were used to test the effects of evolutionary adaptation to one substrate on fitness during growth on an alternative substrate. Twenty lineages of bacteria were allowed to evolve under abundant resource conditions on either 2,4-D or succinate as their sole carbon source. The competitiveness of each evolved line was then measured relative to that of its ancestor for growth on both substrates. Only three derived lines showed a clear drop in fitness on the alternative substrate after demonstrable adaptation to their selective substrate, while five derived lines showed significant simultaneous increases in fitness on both their selective and alternative substrates (Velicer 1999).

2.3b *GMMs carrying the foreign genes will evolve to use them for increasing its own fitness*: An assumption about GMMs is that the introduced plasmid (especially, carrying an overproducing genes) will cause a metabolic burden to GMMs and will decrease their chances for grow in the natural environment even when they provide their hosts with growth advantages over plasmid-free strains (review Diaz Ricciand Hernandez 2000; Velkov *et al* 1999). In fact, a number of studies revealed that plasmid carriage reduces bacterial fitness in the absence of selection for specific plasmid coded functions. But the majority of the experiments were based on the traditional prejudice: these studies have put plasmids into naive bacteria, which had no evolutionary history of association with the plasmid. A crucial question, therefore, was whether bacteria can overcome the cost of plasmid by evolving adaptations that compensate for the harmful side-effects of plasmid genes. And does, for example, the plasmid-carrying drug-resistant strain suffer a cost of resistance (i.e. reduction in fitness) when the antibiotic is absent? (reviewed in Lenski 1997).

In specific experiments, the evolution of an association between a plasmid and its bacterial host was examined. It was shown, in fact, that the non-conjugative plasmid pACYC184 initially reduced the fitness of *E. coli B* in the absence of the corresponding antibiotic. The plasmid-bearing bacteria were subsequently cultured for 500 generations in the presence of antibiotic. The fitness of each combination of host and plasmid, with and without the culture history, was determined by competing it against a baseline strain. The results indicated adaptation by the host genome, but no plasmid adaptation. In another experiment the evolved host, transformed with the baseline plasmid was competed against its isogenic plasmid-free counterpart. The plasmid now increased the fitness of its host in the absence of the antibiotic. During the host-plasmid evolution the host evolves not only to compensate for burdens caused by the plasmid, but also to use it to be more fit (Bouma and Lenski 1988).

It appears from these studies that GMMs proliferating in the environments will evolve to use the man-made genetic modifications for their selfish purposes.

3. If the released GMMs will not grow

If GMMs proliferate in the environment for a number of generation and then stop growing, two different responses will be initiated in the population of non-proliferating cells. The majority of the cells will acquire general resistance and then will come into the stationary phase. However, the minority (the subpopulation) will enter into the risky hypermutable state. The majority will wait for a future improvement of the growth conditions, whereas the minority will mutate to an ability to start the growth in the existing unfavourable environment (reviewed in Velkov 1999) Both responses are controlled, at least in part, by the transcriptional factor *s*-S. The mechanism(s) that facilitate choosing between the two alternatives, “to wait or to mutate”, are not known yet.

3.1 *Released GMMs could acquire general resistance*

In the natural environment, microbial lifestyle in many cases is characterized by long periods of starvation punctu-

ated by shorts moments that allow fast growth. The natural habitats of microorganisms such as soil, sediments, sea and fresh waters could be stressful and usually are characterized by nutrient limitations (review Kolter *et al* 1993). Many (if not all) bacterial species in the severe living conditions rearrange their physiology to increase the chances to survive. The genetic and physiologic changes that the bacterium undergoes in response to starvation-stress are referred to as the starvation-stress response (SSR). The genetic loci whose expression increases in response to the starvation-stress compose the SSR stimulon. The genes of the SSR stimulon are involved in reorganizing and modulating metabolic fluxes, while others consist and integral part of a defence system directed at preventing damaging effects of the environmental stresses. The central player, controlling the SSR stimulon is the transcriptional factor σ-S (reviewed in Kolter *et al* 1993; Velkov 1999).

3.2 *σ-S is responsible for the induction of general resistance*

The expression of a large number of genes involved in cellular responses to a diverse number of stresses, including starvation, osmotic stress, acid shock, cold shock, heat shock, oxidative DNA damage, transition to stationary phase, quorum sensing and virulence are controlled by the transcription factor σ-S, encoded by the *rpoS* gene. A list of over 50 genes under the control of *rpoS* has been compiled. σ-S acts predominantly as a positive effector, but it does have a negative effect on some genes. In *E. coli* the synthesis and accumulation of σ-S are controlled by mechanisms affecting transcription, translation, proteolysis, and the formation of the holoenzyme complex. Transcriptional control of *rpoS* involves guanosine 3′,5′-bispyrophosphate (ppGpp) and polyphosphate as positive regulators and the cAMP receptor protein-cAMP complex (CRP-cAMP) as a negative regulator. Translation of *rpoS* mRNA is controlled by a cascade of interacting factors, including Hfq, H-NS, *dsrA* RNA, LeuO, and *oxyS* RNA that seem to modulate the stability of a region of secondary structure in the ribosome-binding region of the gene's mRNA. As the protein, σ-S is sensitive to proteolysis by ClpPX in a reaction that is promoted by *RssB* and inhibited by the chaperone DnaK. It seems, that the sensitivity of σ S to proteolysis may be the single most important modulator of its levels. The activity of σ-S may also be modulated by trehalose and glutamate, which activate holoenzyme formation and promote holoenzyme binding to certain promoters. In general, σ-S may be regarded as a sigma factor associated with general stress conditions (reviewed in Matin 1996; Loewen *et al* 1998; Hengge-Aronis 2000). The protective role of stress proteins may be due to their ability to rescue misfolded macromolecules (Matin 1991; Kolter *et al* 1993). The majority of loci of SSR stimulon of *Salmonella* are also under the positive control of σ-S. Furthermore, most of the SSR loci identified are also induced during other stresses or environmental conditions; some are induced during P- or N-starvation, in addition to C-starvation; some are induced by extremes in pH or osmolarity; and some are induced in the intracellular environment of epithelial cells, and/or macrophages, and/or medium designed to mimic the intracellular milieu of mammalian cells (ISM). Several SSR loci are required for long-term starvation-survival (core SSR loci). A few of the core SSR loci are also required for stress-specific-inducible and/or C-starvation-inducible resistance to H_2O_2, thermal, and/or acid pH challenge. Interestingly, C-starved cells are resistant to challenge with an antimicrobial peptide, polymyxin B. A link between the SSR and *Salmonella* virulence was hypothesized, since the two major regulators of the SSR, σ-S and cAMP:CRP, are required for full virulence of *Salmonella*. Moreover, the spv (*Salmonella* plasmid-associated virulence) genes, required for *Salmonella* to cause systemic disease, are C (and P and N-)-starvation-inducible (review Spector 1998). In general, *S. typhimurium* also responds to a variety of environmental stresses by accumulating σ-S. The repertoire of σ-S dependent genes that are subsequently expressed confers the tolerance to a variety of potentially lethal conditions including low pH, stationary phase and regulation of virulence in mice (Webb *et al* 1999; Robbe-Saule *et al* 1995).

During incubation in the oligotrophic environment, *Enterococcus faecalis* became progressively more resistant to the environmental stresses (heat), acidic (low pH), UV irradiation (Hartke *et al* 1998). The nitrogen-fixing bacterium *Rhizobium leguminosarum bv. phaseoli* upon carbon starvation acquires the potential for long-term starvation-survival. The starved cells are cross-protected against pH, heat, osmotic, and oxidative shock. The starved cells are ready to rapidly restart growth when nutrients become available (Thorne and Williams 1997). Thus the mechanisms of general resistance against adversity are widespread in micorbioal world.

Once they exhaust the substances they are supposed to eliminate, GMMs may enter into the state of general resistance. Indeed, the release into the microcosm of the recombinant strain of *B. subtilis*, carrying the plasmid with kanamycin resistance marker and human α-2 interferon gene under constitutive promoter, resulted in generation of its variants with the increased osmotolerance (Boyandin *et al* 2000).

If countermeasures are not taken, the released GMMs will acquire simultaneous resistance to different environmental stresses and will persist in ecosystems for an indefinitely long period of time. To prevent this, inacti-

vation of s-S transcription factor could be tried. There are at least two additional reasons to mutate s-S. First, s-S is also responsible for quorum sensing and, therefore, for its ecological consequences (see above) and, the second, s-S is responsible for activation of the stress induced mutagenesis which could adapt the non-growing starving cells to proliferation in the initially unfavourable environmental conditions (see below).

3.3 *Stasis can induce changes*

Mutation rates are controlled both at the population as well as at the cellular levels. Recent investigations into the adaptive strategies of bacteria to a rapidly changing environment show that the cells with hypermutable characteristics could be selected on a long term basis or could arise as a result of the special physiological transition into a hypermutable state. The mutator strains, which are produced mainly by mutational alterations in the DNA repair genes, are called heritable mutators. Heritable mutators arise because of mutational inactivation of gene(s) responsible for the maintenance of the fidelity of replication; transient mutators arise because of the environmentally programmed upregulation of the error-prone DNA polymerase(s) and downregulation of the DNA repair system.

When starving (and, it seems, in any unfavourable conditions) bacterial cells could follow two ways. The majority of static cells will acquire the general resistance enabling them to persist in the viable state, but the minority (the subpopulation) might enter a risky physiological state and generate multiple mutations (hypermutation). A minor fraction of these hypermutable cells will by chance produce favourable mutations and will therefore survive, whereas the majority of hypermutable cells are going to die.

Under stressful conditions the majority will wait for a future improvement of living conditions, but the minority will mutate to ability to start growth (reviewed in Rosenberg 1997, 2001; Lieber 1998; Velkov 1999; Arber 2000; Metzgar and Wills 2000; Radman *et al* 2000; Sniegowski *et al* 2000; Foster 1999, 2000; Bridges 2001). As was mentioned, the mechanism(s), choosing "to wait or to mutate" is still enigmatic. But the mechanisms of adaptive mutagenesis are better understood.

3.4 *The mechanisms of stress induced mutagenesis*

A temporary state of hypermutation can arise through an increase in the rate of polymerase errors (which may or may not be triggered by template damage) and/or through abrogation of fidelity mechanisms such as proof-reading and mismatch correction. The initial errors are made by various DNA polymerases which vary in their fidelity: several are inducible and are under the control of the SOS system. At present, evolution-accelerating genes could be subdivided into classes according to their response to the stresses and according their location (on the chromosome vs conjugative plasmids). There are: (i) chromosomal stress upregulated genes, (part of the SOS regulon, a gene of the heat shock protein HSP70, and antirestriction defence genes *ard* of the conjugative plasmids) and, (ii) stress downregulated genes (genes of the mismatch repair system MMR), and genes of the restriction-modification system, hsdR-M).

3.4a *Genes controlling adaptive mutagenesis*: *recombination initiates replication; excess of mistakes and lack of corrections*: Adaptive mutagenesis is the generation of genome-wide mutations in the subpopulation of non-dividing cells. It may reflect an inducible mechanism that generates genetic variability in times of stress. The genes orchestrating this type of mutagenesis are: (i) *recABCD*, *ruvABC* and *recG*; homologous recombination and double strands breaks repair; some of them induced during the SOS response; (ii) *dinB* (Pol IV); error-prone mutagenic DNA polymerase IV, induced as the part of SOS response and, (iii) MMR; mismatch repair systems, transiently inactivated in the stationary phase by s-S and HF-1 (Hfq). It is obvious, that mechanisms of adaptive mutagenesis should unite the special interactions between: (i) frequent recombination, (ii) mutagenic replication and, (iii) weak repair (if any).

The simplest mechanism is that, in some static cells, recombination initiates the error-prone DNA synthesis which generates mistakes that are not repaired (Foster 2000). The mechanism of adaptive mutagenesis was studied mainly with the use of *E. coli* FC40, the strain with deleted *lac* operon on the chromosome but carrying a modified *lac* operon with + 1 frame shift mutation in *LacZ* on the F′, a low copy conjugative plasmid.

3.4b *The frequency of transient hypermutators in the static population*: According to the recent estimates, under the lactose selection only 0·001–0·1% of the stressed cells became transient mutators. These cells give rise to about 12% of the singly mutant cells, to 97% of double mutant cells and to all the cells carrying three or more mutations. About 0·1% of the cells in stationary phase culture of FC40 are filamentous, which is an indicator of the SOS induction. The question how many adaptive Lac^+ mutations arise in hypermutating subpopulation remains unsolved (Foster 2000; Rosenberg 2001).

3.4c *The rate of adaptive mutagenesis*: During non-selective growth a mutation rate of FC40 to lactose utilization is about 1 per 10^9 cells per generation. When plated on lactose, Lac^+ revertants arise at 1 per 10^9 cells

per hour and continue to do so for several days. If the rate of mutagenesis in growing cells is expressed in mutations per 10^9 cells per hour, it will give the surprising result: the mutation rate of the subpopulation of static cells is 200-fold (!) higher than that of growing cells. Moreover, adaptive mutation requires recombination functions, especially *recABCD*, but mutation during non-selective growth does not. The rate of adaptive mutation if *Lac* allele is in its own position on the chromosome falls 100-fold, and mutations are no longer dependent on conjugal functions (Foster 2000; Bridges 2001).

3.4d *Activation of conjugal functions, but not conjugal transfer is essential for the high rate of mutations*: F′ contains a special origin of conjugative transfer, *oriT*. Nicking at *oriT* by the nickase *TraI* occurs even if the cells are not mating. This single stranded breaks initiate recombination. F also has vegetative origin from which regular replication bubbles are initiated. Because *Lac*- mutants are leaky, some DNA replication can occur in the starved cells during lactose selection.

3.4e *During adaptive mutagenesis, replication is initiated by recombination*: It was found that successful chromosome replication requires recombination because replication forks frequently stall, and the stalled forks are restarted through recombinational processes (Kuzminov and Stahl 1999; Motamedi *et al* 1999). It is logical, that replication primed by recombination of incoming single stranded DNA primes DNA synthesis and that synthesis produced mutations (Bull *et al* 2000, reviewed in Rosenberg 2001).

3.4f *Molecular nature of adaptive mutations: – 1 base pair deletions in runs of iterated nucleotides*: The Lac^+ mutations that arise during starvation in the presence of lactose are distinguished from those that occur during growth in the non-selective conditions. While insertions and deletions of various types resulting a Lac^+ phenotype occur during non-selective growth, the adaptive mutations that occur during starvation are almost exclusively – 1 base pair deletions in runs of iterated nucleotides. But, in general, the mutagenic mechanisms are not restricted to frameshift mutations, and all types of mutations could be produced.

3.4g *MMR deficiency results in mostly frame shift mutations*: In *E. coli mutS* strain, the mutations were almost all (> 90%) frame shift mutations, while base substitutions were rare. Most mutations usually are insertions or deletions of one or two pairs in the homopolymeric runs (Levy and Cebula 2001). In fact, there is strong evidence supporting the idea that stationary-phase reversion of a *lac* frameshift mutation is due to decreased mismatch repair capacity. The cellular amounts of MutS and Mut*H* in stationary phase decrease by as much as 10-fold via downregulation by two global regulators, *s*-S and HF-1 (Hfq), which increases adaptive mutagenesis 100-fold (reviewed in Harris *et al* 1999). It seems, that inactivation of *rpoS* could decrease adaptive mutagenesis.

3.4h *DNA polymerase Pol IV generates adaptive mutations*: SOS-controlled *dinB* gene of *E. coli* encodes DNA polymerase Pol IV, which is able to dramatically increase the untargeted mutagenesis. DNA Pol IV mutator activity is independent of *polA*, *polB*, *recA*, *umuDC*, *uvrA*, and *mutS* functions. The mutator activity of Pol IV promotes single-nucleotide substitutions as well as one-base deletions in the ratio of about 1 : 2 (Wagner and Nohmi 2000). Pol V has no role in the adaptive mutagenesis. A nonpolar *dinB* mutation reduces adaptive mutation frequencies considerably but does not affect growth dependent mutations (McKenzie *et al* 2001). In FC40, Pol IV normally accounts for 50% of the adaptive mutations, but for a greater fraction in the absence of Pol II (which is accurate). Thus, at least three if not more DNA polymerases (Pol IV, Pol III and Pol II) appear to compete for the 3′ ends of recombination intermediates (Foster 2000; Rosenberg 2001).

3.4i *SOS response induces adaptive mutations*: It has become evident is that adaptive mutation is regulated by the SOS regulon, which is activated in response to DNA damage or abrupt stop of DNA replication (review Smith and Walker 1998). Ageing in colonies (Taddei *et al* 1997b) and cessation of growth in a complex medium (Dri and Morerau 1994) can also induce the SOS response. It was found that SOS-induced levels of proteins other than RecA are needed for adaptive mutation. Also, RecF is needed for efficient adaptive mutation. Additionally, an SOS-controlled inhibitor *psiB* of adaptive mutation was discovered. These results indicate that adaptive mutation is a response, controlled both positively and negatively by the SOS system (McKenzie *et al* 2000). Are the cells of the hypermutating subpopulation the only ones with the SOS response turned on? If so, why were the majority of cells not SOS-induced?

3.5 *Adaptive amplification*

The origin of genetic complexity is generally thought to be tightly coupled to the reversible adaptation to changed environmental conditions and to the evolution of new gene functions arising subsequent to gene duplication. Usually, gene amplifications arise spontaneously during replication via sequential tandem duplications generated by unequal crossing over. If the selection pressure on the amplified state is not applied the amplified regions

segregates rapidly (reviewed in Velkov 1982, Stark and Wahl 1984). In general, as a mechanism of short term and transient adaptation, gene amplification is more flexible and less risky a genome alteration than point mutations.

As was mentioned above, formation and segregation of duplicated genes is promoted by homologous recombination acting on two newly replicated daughter DNA strains. Stressful conditions, which activate SOS response and inactivate MMR, considerably increase the rate of generation of tandem duplications. In *E. coli* with constitutively activated SOS-response (*recA*730 mutation), there was a ten-fold or greater increase in duplications (Dimpfl and Echols 1989). Interestingly, inactivation of MMR acts in the same fashion, since the formation of large duplications by homeologous recombination in *E. coli* was increased some tenfold by mutational inactivations in the *mutL* and *mutS* genes that encode the mismatch recognition proteins. These findings indicated that the mismatch recognition proteins act to prevent excessive intrachromosomal exchanges, and downregulation of the proteins (especially, in stationary phase) could promote formation of tandem duplications (Petit *et al* 1991).

Adaptive amplification was discovered during the detailed study of adaptive mutations of *lac* operon. Approximately, 20–30% of all Lac^+ adaptive mutations, accumulating during non-lethal selection on the minimal medium without lactose, was shown to be 20 to 100 directs repeats of 10 to 40 kb, the rest 70–80% of Lac^+ cells were point mutations. Additionally, it was found that adaptive amplification is separate from, and does not lead to, adaptive point mutation (Hastings *et al* 2000). Sequencing data confirmed that half of the Lac^+ mutants arising on days 8–10 with no base pair change in the Lac target region, were unstable and exhibited a Lac^- phenotype after successive growth cycles in rich medium (Powell and Wartell 2001).

In general, adaptive mutation and amplification are parallel routes of inducible genetic variability allowing rapid evolution under stress, and escape from growth inhibition. Therefore, it logical to assume that GMMs, stressed by severe environmental conditions, will try to escape from growth inhibition by evolution promoted by adaptive mutations and amplifications. In such an event, the inactivation of *rec* gene(s) of homologous recombination and/or the *rpoS* gene responsible for downregulation of MMR, could reduce this type of risk.

3.6 *Stress increases transposition*

Activation of the SOS-response and inactivation of mismatch repair, which are the two main regulators of genetic stability/variability, results in increased mutagenesis via adaptive mutation and amplification. Accumulating evidence suggest that SOS and MMR could also activate transposition.

3.6a *SOS response can stimulate excision of transposons*: UV irradiation of *E. coli* led to an increase of up to 28-fold in IS10 transposition. UV induction of IS10 transposition was not observed in *lexA*3 and Δ*recA* strains, indicating that the SOS response is involved in regulating UV-induced transposition. IS10 transposition, known to increase the fitness of *E. coli*, may have been recruited under the SOS response to assist in increasing cell survival under hostile environmental conditions (Eichenbaum and Livneh 1998). UV-inducible excision of transposons is a specific SOS-mutagenesis process resulting in deletion formation that involves the direct or inverted IS-sequences of transposons (Aleshkin *et al* 1998).

3.6b *Inactivation of MMR stimulates transpositions*: Excision of the prokaryotic transposon Tn10 is a host-mediated process that occurs in the absence of *recA* function or any transposon-encoded functions. It was shown, that inactivation of *E. coli* MMR genes (*uvrD*, *mutH*, *mutL*, *mutS*, *dam*) increase the frequency of Tn10 precise excision (Lundblad Kleckner 1985). In *S. typhimurium* precise excision of Tn10 is significantly elevated frequencies in *polA7* mutant and is further increased in *polA7 dam*-1 and *polA7 mutB* strains and decreased in a *polA7 mutH* background. (Hafner and MacPhee1991).

3.6c *Stress induced chaperone HSP70 stimulates transposition*: During study of the influence of magnetic fields on *E. coli* it was discovered unexpectedly that, like some naturally occurring environmental stress factors, such as UV irradiation, magnetic field exposure is also stimulatory to transposition activity. It was illustrated by a bacterial conjugation study using the strain that carries Tn5 as the donor. When the donor cultures were exposed to a low-frequency (50 Hz) magnetic field of 1·2 mT, Tn5 located on the bacterial chromosome was stimulated to transpose and settled on the episome, and was eventually transferred to the recipient cell through conjugation. The exciting finding was that such transposition activity stimulation was mediated by increased synthesis of the heat shock protein HSP70 (DnaK/J), overproduction of HSP70 resulted the stimulation of transpositions in the absence of magnetic field treatment (Chow 2000; Chow and Tung 2000). The direct involvement of the chaperones in control of DNA variability becomes even more relevant in GMMs, which could have increased levels of chaperones in normal growth conditions.

4. A hypermutating GMM can spread rapidly

If the released GMMs become transiently hypermutable, what will be the fate of such mutators? Will they rapidly accumulate deleterious mutations or they will have a chance to fix a beneficial one?

4.1 *Hypermutability: dangerous or beneficial?*

Current findings show that in populations of bacteria, "mutator" genes, which increase the mutation rate, can spread rapidly through a population by allowing the bacteria to evolve faster. Moreover, this happens even though mutations produced by the mutator genes, like others, are on the average harmful. This occurs because mutators occasionally arise in individuals that also carry an advantageous gene. In an asexual population, the mutator may then spread with the advantageous gene (hitchhiking effect). A theoretical study predicted that in a changing environment, the faster evolution made possible by mutator genes often outweighs their disadvantage to the individual. In asexual clonal populations, the ability to generate mutator alleles will lead to an increase in mutation rate, so increasingly fitter individuals arise in the population. Such models show that strong mutator genes (such as those that increase mutation rates by 1,000-fold) can accelerate adaptation, even if the mutator gene remains at a very low frequency (for example, 10^{-5}). Less potent mutators (10- to 100-fold increase) can become fixed in a fraction of finite populations (Taddei *et al* 1997c).

The experimental verification of this prediction confirmed that hypermutability is really beneficial and could be a target of positive selection itself. When 12 independently propagated clonal populations of *E. coli* were serially cultured over 10,000 generations in a glucose limited environment and thereby subjected to alternating periods of growth and stasis, most of them retained the ancestral mutation rate, but three populations displayed mutation rates that were between one or two orders of magnitude higher than those in the ancestor. The three strains evolved to a hypermutability which was due to defects in MMR genes. Mutators can get ahead in real populations as well. In three out of 12 bacterial colonies evolving in a new environment, mutator genes swept through the population and became ubiquitous (Sniegowski *et al* 1997).

The data concerning the incidence of mutator strains in laboratory and natural populations could be helpful to decide whether hypermutability really is beneficial or not. If it is, they should occur often.

4.2 *The occurrence of hypermutating strains*

4.2a *In laboratory populations*: The frequency of mutators in a laboratory population of *E. coli* K-12 was estimated to be about $3x10^{-5}$. By and large, this corresponds to a mutation rate from non-mutators to mutators of 5×10^{-6} per bacterium per generation. Also, it was shown that the increase in mutation rates in mutators to be 19- to 82-fold, depending on the test mutation under consideration. The load associated with this increase in mutation rate resulted in a growth inhibition of 1% only. The estimated rate of detrimental mutations in the non-mutators was found to be 2×10^{-4} to 8×10^{-4} (Boe *et al* 2000).

4.2b *In natural populations*: The occurrence of mutators among isolates of pathogenic *E. coli* and *Salmonella enterica* is over 1%. Among 212 strains of *E. coli* 9% were mutators, among 137 strains of *S. enterica* 17 were mutators which displayed at least a 50-fold increase in mutation frequency. Of 9 independent mutators 7 contained defective *mutS* allele (LeClerc *et al* 1996). Mutator strains of *P. aeruginosa* were obtained from 11 of 30 patients (37%) with cystic fibrosis (CF); in total 19·5% of 128 isolates were of the mutator type. This suggests that a single colonizing organism can evolve with the development of different mutator strains. The investigation also revealed a link between hypermutability and the evolution of antibiotic resistance (Taddei *et al* 1997a). Associated with the mutator phenotype were isolates, which were highly resistant to a wide variety of standard antibiotics. In contrast, none of the 75 *Pseudomonas* isolates from non-CF patients (predominantly from blood cultures) showed the mutator phenotype (Oliver *et al* 2000).

Mutation rates change during the experimental colonization of the mouse gut by wild type or mutator strains of *E. coli*. During the course of the ensuing weeks of colonization by the wild type strain, the bacterial population increased its mutability. Individual colonies with an elevated mutation rate were isolated and the increased mutability was attributed to a defect in the MMR genes. These data show that mutator arising spontaneously can quickly become dominant during the course of gut colonization. When mice were inoculated with mutator and non-mutator stains together (1 : 1), the ratio mutator/non-mutator after few days was 800 : 1, showing a clear advantage of the mutator over wild type allele. A high mutation rate was initially beneficial because it allowed faster adaptation, but this benefit disappeared once adaptation was achieved (Giraud *et al* 2001).

In general, studying the incidence of mutators among indigenous terrestrial and aquatic populations, especially occurring in polluted sites, will be of considerable scientific and practical interests. The measurement of mutability of the GMMs, after their introduction in the model environmental systems could be valuable for the risk assessments.

Once adapted, the mutators are expected to return to the non-mutator state if the selection pressure is removed.

Moreover, the state of hypermutability is too risky to be maintained in the long run.

4.3 *How to return from hypermutability?*

There are three known ways how mutator cells can switch off the hypermutability. If hypermutability is transient (not heritable): (i) by restoration of the activity of MMR which was temporarily inactivated by environmental stress and, if mutator state is heritable, (ii) by reversion to normal mutability or, (iii) by recombinational replacement of mutator gene with corresponding wild type gene. The first way is modulated by the resumption of growth (see above), the other two ways could be realized by chance.

If one assumes that a bacterial cell is alive only because it evolved, it should carry the footprint of the times when it was in the hypermutable state. In fact, comparative molecular phylogeny of MMR genes from natural *E. coli* isolates demonstrated that, compared to housekeeping genes, individual functional MMR genes exhibit high sequence mosaicism derived from diverse phylogenetic lineages. This apparent horizontal gene transfer correlates with hyper-recombination phenotype of MMR-deficient mutators (Denamur *et al* 2000). Comparison of *mutS* phylogeny against predicted *E. coli* "whole chromosome" phylogenies revealed striking levels of phylogenetic discordance among *mutS* alleles and their respective strains. These incongruences have been interpreted as signatures of high frequency horizontal exchange among *mutS* alleles, which resulted the replacement of mutators alleles of MMR genes by non mutator ones (Brown *et al* 2001). In general, the sequence mosaicism of MMR genes may be a hallmark of a mechanism of adaptive evolution that involves modulation of mutation and recombination rates by recurrent losses and reacquisitions of MMR gene functions.

5. Risks associated with conjugative plasmids: gene transfer across genetical frontiers and provoked hypermutabiliy

If trans genes are present on plasmids or if they are captured and mobilized from the chromosome by a natural conjugative plasmid, two main undesirable stress induced consequences could be realized: (i) hypermutability of the cloned or captured gene provided by the plasmid and, (ii) introduction of foreign genes into the world wide gene pool.

5.1 *Plasmids as evolution-accelerating machines*

5.1a *Plasmid-promoted hypermutability*: If genes are located on the conjugative plasmid, (F′ episome), the rate of adaptive mutations of the plasmid-borne genes is 100-fold higher, than of chromosomal genes. Thus, genes carried on F appear to be highly mutable. The genes in F′ plasmids could actively recombine with their homologues on the chromosome, while non-homologous genes could be integrated into the genome by F′. Thus, F factor can be viewed as an evolution machine; it can pick up genes from the chromosome, expose them to a high mutation rate, and transfer the genes into new host (Foster 2000).

5.2 *Plasmids as gene transfer machines*

The issue of horizontal gene transfer has become important in the context of biosafety. Concern has been expressed that transfer of recombinant DNA (e.g. antibiotic resistance genes) from genetically modified organisms, such as transgenic plants, to phytosphere bacteria may occur and thus contribute to the undesirable spread of antibiotic resistance determinants (reviewed in Droge *et al* 1998.)

Nucleotide sequence analysis, and whole genome analysis, shows that bacterial evolution has often proceeded by horizontal gene flow between different species and genera, the recombination between genes on plasmids takes place at a considerably higher frequency than that observed for chromosomal genes (reviewed in Davison 1999; Prozorov 2000).

5.2a *Selective pressure accelerate spreading of plasmid strains*: More than a million metric tons of antibiotics have been released into the biosphere during the last 50 years which, as it is believed, induced an extensive flux of genes in the microbial world. Prior to the introduction and use of antibiotics, antibiotic-resistant microbes were absent from human or animal flora. The majority of bacteria acquired the genes encoding resistance to antibiotics from exogenous and still largely unidentified sources (reviewed in Mitsuhashi 1993; Davies and Webb 1998; Houndt and Ochman 2000). Despite the wide spread of plasmids, the mechanisms responsible for their maintenance in bacterial populations are poorly understood. Indeed, there are indications that some plasmid strains could be found in the sites were the corresponding selective pressure is not obvious (if any). Strains carrying the plasmids of biodegradation occur in the non-polluted pristine lands (Riis *et al* 1995). For example, biodegradation of two chlorinated aromatic compounds was found to be a common capability of the microorganisms found in the soils of undisturbed, pristine ecosystems that had no direct exposure to pesticides or to human disturbance (Fulthorpe *et al* 1996). 2,4-Dichlorophenoxyacetic acid (2,4-D)-degrading bacteria were isolated from pristine environments which had no history of 2,4-D exposure (Kamagata *et al* 1997, Fulthorpe and Schofield 1999).

5.2b *Plasmids transfer under stress*: No doubt, genetic diversity of a microbial community will inevitably be affected by environmental stress. The anthropogenic influences on the environment may be accelerating genetic change within microbiological ecosystems, beyond natural adaptation rates (Ford 1994). Plasmid transfer could occur in a wide spectrum of environmental conditions; as a rule, in nutritionally rich circumstances the transfer is more efficient. However, it was shown that although the absolute number of transconjugants produced during the plasmid transfer in the rhizosphere was increased with increasing substrate concentration, the plasmid transfer frequency actually decreased with the increasing substrate concentration. These observations show that plasmid transfer is either stimulated when growth limiting nutrient is scarce or inhibited when it is abundant (Pearce *et al* 2000). In stationary phase the surface exclusion (mechanism by which F plasmids prevent the redundant entry of additional F plasmids into the host cell) during exponential growth is relaxed and a high level of redundant transfer occurs between non growing cells (Peters and Benson 1995). The rearrangement generated by transposons was shown to increase the stability of the plasmid under starvation conditions (Wyndham *et al* 1994).

5.3 *The decline of the barriers of genetical isolation*

The restriction-modification systems is the first line of bacterial defence against foreign genes (Barcus and Murray 1995), the second is MMR (Matic *et al* 1996).

5.3a *Activation of plasmid coded antirestriction systems*: Restriction endonucleases will degrade invading plasmid DNA that is not appropriately modified. Therefore, many conjugal plasmids produce antirestriction proteins (called Ard) that specifically inhibit type I restriction endonucleases. The genes of Ard family (*ardA, B* and *C*) proteins inhibiting enzymes of chromosomally-encoded type I R-M systems, but not inhibiting plasmid-encoded types II and III R-M enzymes (Chilley and Wilkins 1995; Del'ver *et al* 1998; reviewed in Zavil'gel'skii 2000). Activity of antirestriction genes *ardA* and *ardB* coded by plasmid pKM101 could be controlled by the early stages of mating and by SOS response. The promoter regions of *ard* genes contain the SOS box, to which LexA repressor can bind (Del'ver *et al* 1998).

5.3b *Alleviation of bacterial R-M systems under stresses*: Under certain stress conditions, by induction of the SOS response or by heat shock action, restriction endonucleases can be temporarily inactivated (Kelleher and Raleigh 1994; Edwards *et al* 1999). *EcoK* DNA restriction can be alleviated in wild-type cells, by expression of the SOS response, so that 10^3- to 10^4-fold increases in phage growth and four-fold increases in plasmid transformation is detected with unmodified DNA. Restriction alleviation was found to be a transient effect because induced cells, which initially failed to restrict unmodified plasmid DNA, later restricted unmodified phage λ (Hiom and Sedgwick 1992). *UmuC*-dependent alleviation of *EcoK* restriction is a transient process in which *umu*-dependent mutagenesis plays little part (Hiom *et al* 1991).

5.3c *Stress induced decline of genetic barriers*: Barriers to chromosomal gene transfer between bacterial species control their genetic isolation. These barriers are: different microhabitats, the host ranges of genes transfer vectors, restriction-modification systems, and, the major barrier, genomic sequence divergence. The ability of related DNAs to undergo recombination decreases with increased sequence divergence. On the molecular level MutS and MutL proteins binds to non-homologous regions of heteroduplex formed by the strands of diverged DNAs and prevent homologous recombination. The stress induced transient inactivation of MMR system stimulates interspecies recombination, while natural selection deter-

Table 1. Reduction of the risks associated with released GMMs.

Genes inactivated	Results	Expected effects
rpoS (*s*-S)	No stress tolerances, no quorum sensing	Reduction of viability, reduction of ecological expansion
	Inability to downregulate MMR	Reductions of hypermutability and of inter-species gene transfer
recABCD	No homologous recombination	Reductions of hypermutability and of inter-species gene transfer
recA	No SOS response	Reductions of hypermutability and of inter-species gene transfer
dnaK (HSP70)	No stress tolerances	reductions of viability and of transpositions

mines the effective recombination frequencies (reviewed by Matic *et al* 1996).

An increase in inter-species gene transfer could be promoted by SOS-response. Conjugational crosses trigger SOS induction in *E. coli F(–)* cells mated with *S. enterica* serovar *Typhimurium* Hfr donors. In general, a strong SOS response occurring in a subpopulation of mated mismatch repair-deficient cells eliminates genetic barriers between these two genera (Matic *et al* 2000a, b).

6. Conclusions

Environmental conditions can influence the released GMMs to evolve in the direction of the increased viability. The inactivation of some genes responsible for such escape, may considerably reduce the risks associated with genetic modifications of open microbial ecosystems (table 1). Hopefully, this will reduce at least some risks associated with environmental genetic engineering. But will it diminish its uncertainty?

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I apologize to many authors for not citing them owing to the limitation of space. This article is destined for the wide spectrum of specialists involved in developing and environmental application of GMMs and hopefully, for persons making decisions in these fields. The detailed bibliography could be found in the cited reviews. I thank the referees for helpful criticism and fruitful suggestions.

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