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THE RELEASE OF GENETICALLY MODIFIED MICROORGANISMS – REGEM 2

EDITED BY

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The Release of Genetically Modified Microorganisms—REGEM 2

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THE LORD IRONSIDE

FOREWORD

If ripple effect is a measure of greatness in scientific discovery then GEMMOs have a lot going for them and this book dramatically illustrates the risks associated with advances being made by researchers to mobilize and control the power of the microorganism in the world's fight to perfect nature and find remedies for its imperfections.

In the field of genetic science it is abundantly clear that so much more can be achieved through prevention rather than cure and that the indirect kill, by reason of its logic is a much more powerful weapon for winning results. Nevertheless the dilemma facing politicians arises over whether man should tamper with something which is God-given such as Radioactivity and Genetic endowment.

The Roman Catholic church finds difficulty in accepting the proposition that what is God-given can be treated as a product under human control and maybe that is why recently half a century of genetic research on a strain of bees resistant to a devastating parasite at the Buckfastleigh Benedictine Monastery has inexplicably ceased whilst verging on scientific success.(1) The Anglican Community on the other hand does not see the sacrosanctity of Radioactivity and Genetic material as a bar to man-manipulation with appropriate safeguards.

In seeking appropriate safeguards we should consult widely with countries having similar cultural heritage to our own to ensure that we take account of the through life costs and logistics of genetic modification, without constraining the researcher unnecessarily in his quest for knowledge. Regulations drafted more out of fear of the unknown than confidence in the outcome will bring no rewards for research or society.

The advantages to be obtained from the release of GEMMOs are exciting and regulatory constraints must reflect the future benefits likely to accrue to Society. Pressure Groups who have battled over the nuclear industry are now setting their sights on biotechnology and the ripple effects must generate their own harmonic to keep out of trouble. This book helps all readers to understand the implications of the scientific advances being made.

The Lord Ironside
Member of the Parliamentary
and Scientific Committee
House of Lords
London
SW1 0PW, UK

(1) The Sunday Times, London, 1 March 1992.

PREFACE

The potential benefits which could be derived from the use of genetic modification techniques are apparent. However, scientists are aware of their obligations to society and are striving to ensure that the release of modified microorganisms will be safe. The first international conference on release, REGEM 1, was held in Cardiff, UK in 1988. It was a recognized success and there was agreement that 'release' should be discussed regularly . Consequently, under the sponsorship of the Federation of European Microbiological Societies, the Society for Applied Bacteriology and the Society for General Microbiology, REGEM 2 was held in Nottingham, UK in the autumn of 1991. In his message of welcome Sir Hans Kornberg said "I greatly welcome the proposal to hold a second Conference on the release of genetically-modified microorganisms. Not only has much scientific information and experience been gained since REGEM 1 in 1988, but this year sees the implementation in national law of the two European Directives on Biotechnology. The provisions of the 'Deliberate Release' Directive will put in place a Community-wide, harmonized, approach to the regulatory oversight of both small-scale trials and of commercial-scale releases of GEMMOs. It will clearly be of benefit to all who use and intend to release GEMMOs into the environment to familiarize themselves with these important advances, and to attend REGEM 2."

This book forms the permanent record of the Plenary sessions, Workshops and Poster contributions. The text of the camera-ready manuscript was prepared from computer-disk presentations with conversion programmes to Apple Mac. Mistakenly, it was believed this would be straightforward but imagine the reaction to thirty-four pages of the following converted hieroglyphics:

> ˇÉMˇ icrobiol. 57, 366-373.ÆÆˇÉMˇ cDermott, ˇÉJˇ .B., ˇÉUˇ nterman, R., ˇÉBˇ rennan, ˇÉMˇ .J., ˇÉBˇ rocks, ˇÉRˇ .E., ˇÉMˇ obley, ˇÉDˇ .P., ˇÉSˇ chwartz, ˇÉCˇ .kcC. and ˇÉDˇ ietrich, ˇÉDˇ .K. (1989). Two strategies for ˇÉPˇ CB soil ˇÉrˇ emediation:biodegradation and ˇÉsˇ urfactant extraction. Environ. ˇÉPˇ rog. 8, 46-51.ÆÆˇÉMˇ ileski, ˇÉGˇ .J., ˇÉBˇ umpus, ˇÉJˇ .A., ˇÉJˇ urek, ˇÉMˇ .A. and ˇÉAˇ ust, ˇÉSˇ .D. (1988). Biodegradation of ˇÉPˇ entachlorophenols by the White Rot fungus ˇÅPˇ hanerochoraete ˇÅcˇ hrysosporiumˇ . ˇÉAˇ ppl. Environ. ˇÉMˇ icrobiol. 54, 2885-2889.ÆÆˇÉOˇ livieri, R., ˇÉBˇ acchin, P., ˇÉRˇ obertiello, A., ˇÉOˇ ddo, N., ˇÉDˇ egen, L. and

Sanity was maintained with the constant computer expertise provided in Glasgow by Mulu Gedle — the extent of our gratitude is considerable. Without her contribution this book would have ended up as one of those symposium volumes with different fonts and typefaces of varying shades of grey.

The help provided by Janie Curtis, Nicola Clark and Gregory Safford of Plenum Publishing Corporation is gratefully acknowledged.

Duncan E.S. Stewart-Tull
Max Sussman

May 1992

ACRONYM

You will notice throughout the book that we have changed the acronym for genetically-modified microorganisms, because we were cautioned that GEMS conflicted with the Global Environment Monitoring Service used by WHO since 1975.

During the conference various alternatives were proposed to many of the delegates:

GMO	-	Gēē mō	(a little horse named MO ?)
GAMO	-	Gă mō	(GA, genetically-altered but slightly gaga or a general accident)
GROMs	-	Gr̄r̄ ŏ ms	(may be reorganized but sounds like a gremlin problem)
GEMOMO	-	Gĕ Mō Mō	(too near to Geronimo)

After two days of deliberation there was consensus agreement for:

GEMMO - Gĕ m mō (pl **GEMMOs**) - quite close to the original GEMs and formed from **GE**netically-**M**odified **M**icro **O**rganism. It even sounds a gem when translated literally into Dutch "Edelsteen maaien".

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SUMMARY

ENVIRONMENTAL PRESSURE IMPOSED ON GEMMOS IN SOIL

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This chapter will examine the different types of environmental stress imposed on GEMMOs in soil, and how these stress factors may affect GEMMO establishment and survival. In addition, putative selective advantages or disadvantages to which GEMMOs are subjected in relation to their heterologous DNA are considered. The focus is exclusively on genetically modified bacteria, because these organisms are currently mostly studied with respect to biosafety and effectivity. Evidence is presented which points to an ecological disadvantage of certain GEMMOs compared with their respective parent strains. On the other hand, selective pressure that favours certain GEMMOs, mainly biodegradative bacteria, may enhance their competitiveness in soil. It is concluded that because of the complexity of the soil system, it is virtually impossible to obtain a picture of all potential selective pressures that favour or disfavour introduced GEMMOs.

INTRODUCTION

Genetically modified microorganisms (GEMMOs), in particular bacteria, will certainly be released on a large scale into open-field situations, if regulatory constraints permit. Likely candidates for release world-wide are rhizobia with improved nitrogen-fixing efficiency, genetically modified avirulent derivatives of plant pathogens, for example *Pseudomonas solanacearum* , *Agrobacterium* spp. or *Erwinia carotovora* (Lacy and Stromberg, 1991) and ice-*Pseudomonas syringae* (Panopoulos, 1986). Other organisms developed for biological control purposes, such as pseudomonads and rhizobia that carry *Bacillus thuringiensis* insect toxin genes, have also been proposed for field releases (van Elsas *et al.*, 1991b; Waalwijk *et al.*, 1991; Skot *et al*, 1990; Watrud *et al.*, 1985). In addition, organisms modified to contain functional genes for the degradation of environmental pollutants, such as chlorinated organic compounds, are being developed and tested; these might be used for remediation of polluted soil (Short *et al.*, 1990; Ramos *et al.*,1991).

Release of unmanipulated bacteria has a long history of unpredictable and often disappointing results (van Elsas and Heijnen, 1990). One of the major problems has been the often poor establishment and survival of the introduced bacteria. Of the many attempts to establish certain inoculants in soils, in particular those carried out in the Soviet Union in the 1950s and early 1960s (Mishustin and Naumova, 1962), probably the only organism which was introduced with some degree of success, was *Rhizobium.* Nevertheless, the now firmly established *Rhizobium* inoculant industry continues to be plagued by lack of success of inoculation, which is often due to competition for nodulation by the less effectively nitrogen-fixing rhizobia indigenous to the soil (Thies *et al.*, 1991).

Given this recognized difficulty to establish an inoculum in a soil system and the generally poor performance of introduced populations, it is attractive to speculate about the potential

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behaviour of GEMMOs derived from the wild-type organisms. Unless the extra genetic element can provide some predictable or unanticipated ecological advantage for the GEMMO, the general expectation is that the GEMMO will be at an ecological disadvantage as compared with the parent because of the extra metabolic load that leads to decreased fitness (Tiedje *et al.*,1989). However, this should not be regarded as certain, since previous work in chemostat systems on the effects of transposon Tn5 on bacterial fitness has shown unexpected growth advantages for Tn5-carrying strains as opposed to the parent strain (Biel and Hartl, 1983). Also, the presence of the bacteriophage *cos* site on a plasmid in an *Escherichia coli* host increased bacterial fitness (Edlin *et al.*, 1984). Finally, the presence of Tn10 in certain hosts also leads to increased competitiveness (Chao *et al.*, 1983). These examples serve to show that when novel genetic sequences are inserted, unexpected effects on fitness and survival of the host may result. Such effects may work out differently in soil as compared with a laboratory system, or they may be preferentially expressed in a stressed soil environment.

This chapter will focus on the types of stress imposed on GEMMOs in soil and on their differential effects on GEMMOs and parent strains. The soil environment is chosen, because most releases to be dealt with are into this ecosystem. After briefly discussing the dominant stress factors that occur in the soil environment and the overall behavioral response of introduced bacterial populations, the focus will be on specific population effects due to the extra genetic and physiological load carried by the GEMMO. Questions to be addressed will be if and to what extent the extra gene(s) affect bacterial establishment and persistence, and whether environmental stress acting positively on the introduced phenotype, possibly providing selective force, is able to promote its ecological persistence.

STRESS FACTORS IN SOIL

Soil represents an environment dominated by the solid phase, although liquid and gaseous compounds also play a role. Thorough descriptions of the intricacies of the soil environment and of its influences on soil microbial life have been provided elsewhere (Stotzky, 1986, 1989; Stotzky *et al.*, 1991; Oades, 1988) and are not within the scope of this chapter. Perhaps the most important characteristic of the soil environment is its heterogeneity, that is every site in soil may inherently be different from any other site, even at the scale of individual microbial cells (μm). This has serious implications for the life of bacteria present in that system, as well as for any interpretation of experimental data derived from it.

Many different factors in soil can affect the establishment and population dynamics ("fate") of introduced bacteria. These factors can be subdivided into biotic and abiotic factors. Biotic factors are, for example, antagonism, antibiosis, competition and predation or parasitism, brought about by the mixed community present in the soil. In addition, the presence of plant roots may be considered as a biotic factor. Abiotic factors of importance in soil are fluctuations in moisture content, nutrient supply, soil textural type, pH, temperature, presence of charged surfaces (clays) or plant surfaces such as roots. All of these factors may act differentially on various introduced organisms and, in addition, they may constitute a web-like interaction pattern. Stotzky *et al.* (1991) gave some examples of different soil factors acting upon incoming microbes. From this and other information it may be concluded that the effects of such factors on microbial fate can only be described in broad generalizations. This makes any prediction of the fate of incoming GEMMOs inherently difficult. In the following, however, a brief account will be given of well-established dominant soil factors that affect bacterial life in soil and their consequences for establishment and survival of incoming microbes.

Perhaps the most important abiotic factor that dominates the life of bacteria in soil is the presence of water, expressed as the soil water activity. With the exception of soils flooded with water, the most commonly found water condition of soil is one in which only certain pores of the soil void are completely water-filled, while other soil pores are merely covered by a water film or are dry. As outlined by Stotzky *et al.* (1991), this limits the possibilities for biological activity, since the many possible modes of bacterial development and activity, including movement and colonization of additional spaces in soil, are limited by spatial constraints. In addition, microbes in such water-depleted soil conditions must cope with water potential stress, that is they need mechanisms to maintain their cellular water content against the forces of soil water suction.

A second factor of importance is the gross lack of available substrate, in particular carbon, in most soils. It is widely accepted that soils are not inherently substrate-poor, but that readily-available bacterial nutrients are generally in short supply. Physical (spatial separation) and/or

chemical (lack of degradability) constraints prevent bacteria from freely utilizing these nutrients. In addition, most carbonaceous compounds may already have been utilized by the indigenous microflora. An obvious implication for invading GEMMOs is that these will commonly enter a state of nutrient deprivation (starvation) in soil.

Soil textural type is another factor that affects bacterial establishment. Bacteria introduced into soils with a higher clay and/or silt content (finer-textured soils) showed better survival than those introduced into coarser-textured soils (Van Veen and van Elsas, 1986). This has been attributed to the capacity of the former soil to preserve a greater biomass, and this in turn has been linked to a higher number of protective microniches for bacteria (refuge sites) because of the larger number of pores with small orifices (Heijnen and van Veen, 1991).

Soil pH and soil temperature may also drastically affect bacterial establishment and subsequent survival in soil (Van Elsas and Trevors, 1991). Studies on the effects of pH on rhizobia in soil showed that organism survival tended to decrease at lower pH (Lowendorf *et al.*, 1981; Thornton and Davey, 1984). However, strains tolerant to lower pH have been selected and these were shown better to withstand soil acidity (Thornton and Davey, 1984). Temperature may act either directly on the introduced GEMMOs, or do so indirectly, via effects on the indigenous microflora. Van Elsas *et al.* (1991a) showed that survival of transposon Tn5 containing *Pseudomonas fluorescens* introduced into soil was higher at lower temperatures (4 and 15°C) than at the higher temperature used (27°C). Similarly, Wessendorf and Lingens (1989) found that survival of *P. fluorescens* in soil microcosms is better at 4 °C than at 25°C. Bolton *et al.* (1991), comparing survival of a psychrophilic *Pseudomonas* strain in soil microcosms, a climate chamber and the field, found survival in the field and climate chamber to be greater than in the soil microcosms. They related this observation to the lower average temperatures in the former two systems. It thus appears that lower temperatures in soil may be favorable for survival of invading microorganisms, possibly due to a reduction of biotic processes which tend to reduce their population size.

Finally, another abiotic factor of potential influence on bacterial establishment and survival in soil is the presence of compounds that exert selective pressure which directly favors the heterologous genes present in the introduced GEMMO. An example of such selection is soil polluted with heavy metals in which a virtual monoculture of *Alcaligenes eutrophus* carrying a heavy metal resistance plasmid developed (Mergeay, M, personal communication).

Biotic factors that exert pressure on introduced GEMMOs are related to biological interactions between organisms of the soil environment and the incoming GEMMO. Such interactions have been grossly classified by Strauss *et al.* (1986) and Stotzky *et al.* (1991), as 'neutral' where there is no advantage to any partner, 'parasitic' where there is exclusive advantage to one partner or 'beneficial' to both partners. The complex web of different biological interactions in the soil is probably responsible for the observed homeostasis of the soil system, that is the tendency of the system to counterbalance disturbances (Strauss *et al.*, 1986). In addition, natural soil has also been termed microbiostatic, that is soil generally does not permit growth of added microbes unless some disturbance to the system is brought about (Ho and Ko, 1985). Soil microbiostasis is probably also largely caused by biological factors, since it is relieved by soil sterilization (Ho and Ko, 1985). Amongst the many microbial interactions which potentially govern bacterial establishment and survival, predation by protozoa and competition for available substrate and space are probably predominant.

Predation by protozoa is an ubiquitous stress factor in soil and is probably most frequently responsible for the decline of introduced bacterial populations (Habte and Alexander, 1975, 1977; Heijnen *et al.*, 1988). Predators, however, do not usually completely eliminate their prey but do so only to a certain level. This has been attributed to an energy-expenditure phenomenon, on the assumption that at low prey densities it becomes too energy-expensive for predators to graze (Alexander, 1981). Competition between microbes for the sparse nutrients or available space -"ecological niches"- in the soil is a second biotic factor of utmost importance. That competition plays a large role has become apparent from experience with *Rhizobium* inoculants, which are often outcompeted by indigenous, less effectively nitrogen-fixing, strains (Thies *et al.*, 1991). For biosafety evaluation, it is equally important to obtain information about the competitive ability of the GEMMO *versus* the correponding wild-type or parent organism, since this determines possible hazards of GEMMOs outcompeting wild-type bacteria in soils.

It is evident that both the abiotic and the biotic factors mentioned, and their interactions, affect the fate (establishment, survival) of introduced bacteria, regardless of whether these are genetically modified or not. For a given soil it is inherently difficult to predict which factors

will predominantly control the persistence of an inoculant. However, it seems safe to suggest that the homeostatic and microbiostatic nature of the soil environment represents a natural mechanism that controls the size of bacterial populations, including those released into the environment. In addition, released microbial populations will often be at an ecological disadvantage as compared with indigenous ones, since they tend to occupy less protected niches in the soil environment.

PATTERNS OF BEHAVIOR OF INTRODUCED BACTERIAL POPULATIONS

The population dynamics of an introduced GEMMO depends on its initial establishment and subsequent growth and/or survival, which in turn are heavily affected by the aforementioned stress factors. Measurements of bacterial dynamics in the heterogeneous soil environment provide overall pictures, which represent the net results of all the different forces acting on the population.

The gross patterns of behavior of microbial populations upon introduction into the environment have been classified into the three following categories Gillett *et al.*, 1984):

1. Rapid disappearance, to levels below the limit of detection, in a short time, for example 3 days or less.
2. Exponential decline for about 7 to 10 days, followed by persistence of a surviving population at a low population density,for example 10 to 100 cells per g of soil or ml liquid.
3. Relatively rapid exponential decline of 1 to 4 logs in 1 to 14 days, followed by a slow progressive decline to extinction.

The response of each introduced population will obviously also depend on the ecological hardiness of the organism, for example *Escherichia coli* introduced into soil, in which it is not a native organism, may react according to the first pattern (Henschke and Schmidt, 1990), *Bacillus* added to soil according to pattern 2 (van Elsas *et al.*, 1986), and *Rhizobium* spp. according to pattern 3 (Postma *et al.*, 1988). Fluorescent pseudomonads, genetically modified or unmodified, are widely developed for application to soils (Van Elsas *et al.*, 1991b; Waalwijk *et al.*, 1991; Kluepfel and Tonkyn, 1991). A survey of the behavioral patterns of different fluorescent pseudomonads upon introduction into soils revealed that, without exception, all decayed (Table 1). Decay rates differed widely, ranging from roughly 0.2 to 1 (Log decrease per 10 days), and probably depended on the type of organism and soil. The behavior was difficult to attribute to any of the patterns described above, and the major problem associated with this was the question of detection and eventual resuscitation of low numbers of surviving bacteria. However, the general response was inevitably a decline to low numbers, confirming the consensus statement about the fate of introduced bacteria in soil.

EFFECT OF THE GENETIC ALTERATION ON THE BEHAVIORAL PATTERN OF THE GEMMOS

Probably the most urgent question pertaining to the biosafety issue is the putative effect of the GEMMO on the ecosystem due to its genetic alteration, that is any effect different from the effect brought about by the wild-type organism. The establishment and survival of the GEMMO as opposed to that of the wild-type, affect the extent of these effects and are therefore of interest. In particular, the possibility of a GEMMO displacing its wild-type counterpart from a natural system, thereby effectively taking over the niche occupied by the wild-type and possibly establishing its novel trait permanently, has been of concern. At the same time, in such a situation, the effectiveness of the application would obviously be greatly increased.

To find a suitable "niche" and establish itself in a soil system, a GEMMO will probably have to compete with oligotrophic strains in a nutrient-deprived environment. It will thus rely on the availability of niches occupied by indigenous strains similar to itself, which use the same space and nutrients. The possible outcome of the competition of invading GEMMOs with both oligotrophic organisms and organisms with similar trophic behavior, and thus the likelihood of inoculant establishment, may be estimated by examining the available information on the behavior of the GEMMO *versus* the wild-type in natural (nonsterile) soil. These studies have been performed according to two different strategies, one in which the parent strain and

Table 1. Decay rates of different fluorescent pseudomonads introduced into soils in soil microcosms or in field microplots[a]

Soil	Experimental system	Introduced strain/marker	Decay rate[b]	period (days)	Reference
Loamy sand	Field microplot	*P. fluorescens* (chr::Tn5)	0.5-0.6	60	Van Elsas *et al.*, 1986
Silt loam	Field microplot	*P. fluorescens* (chr::Tn5)	0.3	60	Van Elsas *et al.*, 1986
Loamy sand (wheat rhizosphere)	Field microplot	*P. fluorescens* (chr::Tn5)	0.8	60	Van Elsas *et al.*, 1986
Silt loam (wheat rhizosphere)	Field microplot	*P. fluorescens* (chr::Tn5)	0.2	60	Van Elsas *et al.*, 1986
Loamy sand	Microcosm	*P. fluorescens* (chr::Tn5)	0.8	55	Heijnen *et al.*, in prep
L. sand + bentonite	Microcosm	*P. fluorescens* (chr::Tn5)	0.2	55	Heijnen *et al.*, in prep
Loamy sand	Microcosm	*P. fluorescens* (RP4)	0.9	60	Van Elsas & Trevors, 1990
L. sand + bentonite	Microcosm	*P. fluorescens* (RP4)	0.4	60	Van Elsas & Trevors, 1990
Sandy loam	Microcosm	*P. fluorescens* Pf1-2 Rp^R	0.2-0.4	36	Compeau *et al.*, 1988
Sandy loam	Microcosm	*P. fluorescens* Pf1-8 Rp^R	0.8	30	Compeau *et al.*, 1988
Silt loam	Microcosm	*P. fluorescens* R1 Rp^R	1.1	29	Wessendorf & Lingens, 1989
Sandy loam	Microcosm	*P. putida* Pp1-2	0.7	30	Compeau *et al.*, 1988
Clay loam	Field microplot	*P. putida* N-1R Rp^R	0.5	60	Dupler & Baker, 1984
Sandy loam	Field microplot	*P. putida* N-1R Rp^R	0.5	60	Dupler & Baker, 1984
Silty clay loam	Microcosm	*P. aeruginosa*	0.8	49	Zechman & Casida, 1982

[a] From: Van Elsas *et al.* (1991c). Initial cell numbers added were in the order of 10^7/g soil in most cases. Cells were added from washed fresh cultures without using carrier materials.

[b] Defined as the overall Log^{10} decline in cfu counts per 10 days (calculated over the experimental period).

chr::Tn5: chromosomal insertion of transposon Tn5 encoding kanamycin resistance;

RP4: plasmid encoding kanamycin, tetracycline and ampicillin resistance.

Rp^R: resistant to rifampicin.