Effect of Genetically Modified Bacteria on Ecosystems and Their Potential Benefits for Bioremediation and Biocontrol of Plant Diseases – A Review

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Abstract For centuries, microorganisms have served mankind in many ways. Relatively recent developments include the use of bacterial inoculants for bioremediation and agricultural purposes like biological control of plant diseases. Whereas agricultural applications of bacteria have been successful to some extent, improvement of their efficacy is necessary for commercial applications on a large scale. For instance, the remediation of mixed organic and metal-contaminated sites poses problems that may be overcome by introducing metal resistance in the bacteria used for bioremediation. For biological control of plant diseases the efficacy can be improved by combining several mechanisms of antagonism against pathogens in a biocontrol agent. Genetic modification now enables us to construct microbial strains with such novel and enhanced properties. Large-scale introduction of genetically modified strains into the environment poses some challenging questions.

This chapter provides an overview of studies concerning the application of bacteria and their genetically modified derivatives, with the emphasis on their fate and effects on the ecosystem. We limited this chapter to genetically modified microorganisms (GMMs) for agricultural applications, as biosensors, and for bioremediation purposes. Proliferation and survival of the introduced strains in the environment are discussed, but we have mainly focused on recent studies concerning the possible impact of GMMs on microbial communities and ecosystem functioning. Survival and colonization of GMMs is either equal or less when compared to that of the parental strain. The impact of bacterial inoculants (genetically modified or not) on microbial communities is either negligible or small as compared to effects of general agricultural practices and the effects are transient.

Keywords Agriculture · Biocontrol · Biofertilizer · Biosensor · Bioremediation · Ecosystem effects · Field trial · GMMs · Pseudomonads · Rhizobacteria

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List of Abbreviations

ARDRA	amplified ribosomal DNA restriction analysis
DAPG	2,4-diacetylphloroglucinol
DGGE	denaturing gradient gel electrophoresis
2,4-DNT	2,4-dinitrotoluene
GMM	genetically modified microorganisms
GM	genetically modified
PCA	phenazine-1-carboxylic acid
PCB	polychlorinated biphenyls
PCR	polymerase chain reaction
PGPR	plant growth-promoting rhizobacteria
SSCP	single-strand conformation playmorphism
T-RFLP	terminal restriction fragment length polymorphism

1 Introduction

For more than a century, bacteria have been deliberately introduced into the environment for specific purposes. The efficacy of microbial inoculants for remediation and agricultural purposes is, however, in many cases not sufficient to allow large-scale commercial application. Improved degradation of pollutants or resistance to co-pollutants present in contaminated soils, or increased efficacy of disease suppression by combining different mechanisms of antagonism against pathogens, may lead to increased opportunities for applicability. Current knowledge on bacterial genetics allows us to construct new strains with exciting capabilities. Such genetically modified microorganisms (GMMs) have been postulated to be applicable in agriculture for effective control of plant pathogens (biocontrol), and to improve plant growth (biofertilizers) (Amarger, 2002). Moreover, GMMs have been employed for degradation of polluting compounds in the environment (Dutta et al., 2003), or as biosensors to determine levels of pollutants in soil and water (Belkin, 2003).

However, the success of applications of GMMs also appears to be variable and is dependent on the survival and activity of bacterial species and the prevailing environmental conditions. This situation emphasizes our lack of understanding of the ecology of microorganisms in the environment (Prosser et al., 2007). While a number of investigations have dealt with environmental aspects of genetically modified (GM) plants, little is know on the fate of GM bacteria in the ecosystem (Marchetti et al., 2007). Some constraints that currently prevent large-scale application of GMMs is our lack of knowledge on the effects of the introduced strains on the indigenous microflora (Winding et al., 2004), and public concern regarding gene technology. In this chapter, practical and fundamental aspects of GMMs in agriculture and potential effects on the ecosystem will be discussed.

2 Genetically Modified Bacteria for Agricultural Purposes

Many bacterial genera have members that can suppress plant diseases caused by plant pathogens (biocontrol strains) or that can contribute to increased plant growth

by enhancing the availability of nutrients (biofertilizers) (Weller, 1988). These so-called plant growth-promoting rhizobacteria (PGPR) thrive at the interface between soil and plant root (the rhizosphere). The cells can be applied as seed coating or directly to soil. In order to exert their functions, sufficient numbers of the introduced bacteria have to survive in soil and rhizosphere (Raaijmakers et al., 1995). However, the efficacy of PGPR is not always sufficient for commercial applications and strategies to improve their performance include genetic modification (Mark et al., 2006).

2.1 Survival of Genetically Modified Bacteria in Soil

Cells introduced into the environment will encounter a large number of biotic and abiotic factors affecting their survival. The fate of bacteria is determined by an intricate interplay between the environmental conditions and the physiological state of the bacteria. As a reaction to these conditions, bacteria can revert to different physiological states. From being in a "normal" culturable state, cells can become more stress resistant or form dwarf cells (Van Overbeek et al., 1995), they can produce exopolysaccharides for protection (Ophir and Gutnick, 1994), they can enter a viable but non-culturable state (Colwell et al., 1985), and some are able to form spores or associations with plants.

Both biotic and abiotic factors will affect bacteria introduced into soil. Factors such as high clay content, high pH, and relatively high moisture content can have a positive effect on bacterial survival (Da and Deng, 2003; Heijnen et al., 1988; Van Elsas et al., 1986). Factors that may negatively affect numbers of introduced bacteria include dry periods, presence of competing microorganisms, predation by protozoa, and lysis by bacteriophages (Ashelford et al., 2000; Eberl et al., 1997; Heijnen et al., 1988; Johansen et al., 2002; Smit et al., 1996; Stephens et al., 1987; Tan and Reanney, 1976).

An important biotic factor affecting the activity and survival of introduced bacteria is the presence of plant roots that provide nutrients to the microorganisms living in their vicinity (Lugtenberg et al., 2001). Many members of the genera *Agrobacterium, Azospirillum, Azotobacter, Bacillus, Erwinia, Pseudomonas, Rhizobium*, and *Xanthomonas* are microorganisms well adapted to the rhizosphere. Cells of these genera can survive very well or might even increase in numbers in the rhizosphere (Bashan et al., 1995; Liu and Sinclair, 1993; Lugtenberg et al., 2001; Rosado et al., 1996). While most reports on the survival of pseudomonads in soil demonstrated that their numbers decline rapidly (Table 1), some *Pseudomonas* strains have been shown to increase in numbers in the rhizosphere (Bailey et al., 2000; Jäderlund et al., 2008; Mavrodi et al., 2006; Raaijmakers and Weller, 1998). There seems to be a continuous succession of different species or genotypes adapted to a certain growth phase of the roots (Duineveld and van Veen, 1999; Ellis et al., 1999; Rainey et al., 1994). Semenov and co-workers (1999) found wave-like patterns in microbial populations along roots, which could result from subsequent root growth and death. Such wave-like distribution patterns were also reported for a green fluorescent protein-marked *Pseudomonas fluorescens* strain that was introduced into the rhizosphere of wheat plants (Van Bruggen et al., 2008).

Table 1 Decline rates of introduced cells calculated as decrease of cell numbers expressed in Log of cells per week and survival characteristics of bacterial species belonging to different bacterial divisions (mean decline rates for each division is presented)

Taxon/Species	Decline rate	Ecosystem	Detection method [2]	References
Proteobacteria				
Alpha subdivision	(x=0.11)[1]			
Rhizobium leguminosarum	0.21	Soil	Cult.	Heijnen et al. (1988)
Rhizobium leguminosarum	0.15	Soil	IF	Heijnen et al. (1988)
Rhizobium leguminosarum RSM2004	<0.01	Soil*	Cult.	Hirsch (1996)
Azospirillum brasilense	−0.10	Rhiz.	Cult.	Bashan et al. (1995)
Azospirillum brasilense	0.46	Rhiz.	Cult.	Bashan et al. (1995)
Bradyrhizobium japonicum	<0.01	Soil	IF	Brunel et al. (1988)
Sinorhizobium meliloti	0.07	Soil*	Luc	Schwieger et al. (2000)
Gamma subdivision	(x=0.34)			
Pseudomonas stutzeri	0.26	Soil	Cult. + Cat.	Byzov et al. (1996)
Pseudomonas stutzeri	0.22	Soil	Cult. + Cat.	Byzov et al. (1996)
Pseudomonas putida WCS358	0.40	Rhiz.*	Cult.	Glandorf et al. (2001)
Pseudomonas fluorescens	0.20	Rhiz.	Cult.	Frey-Klett et al. (1997)
Pseudomonas fluorescens	1.20	Soil	Cult.	Kozdrój (1997)
Pseudomonas fluorescens R2f	0.18	Rhiz.*	Cult.	Wernars et al. (1996)
Pseudomonas fluorescens Q2-87	−0.06	Rhiz.	Cult.	Raaijmakers et al. (1999)

Table 1 (continued)

Taxon/Species	Decline rate	Ecosystem	Detection method[2]	References
Pseudomonas fluorescens CHA0	0.26	Soil	IF.	Mascher et al. (2000)
Pseudomonas fluorescens CHA0	0.39	Soil	Cult.	Mascher et al. (2000)
Cytophaga-Flexibacter-Bacteroides group				
Flavobacterium sp.	2.45	Soil	Cult.	Thompson et al. (1990)
Firmicutes	(x=0.05)			
Paenibacillus azotofixans	−0.2	Rhiz.	MPN-PCR	Rosado et al. (1996)
Paenibacillus azotofixans	0.5	Soil	MPN-PCR	Rosado et al. (1996)
Bacillus megaterium	−0.3	Rhiz.	Cult.	Liu and Sinclair (1993)
Bacillus thuringiensis	0.12	Soil	Cult.	Byzov et al. (1996)
Arthrobacter globiformis	0.14	Soil	Cult.	Thompson et al. (1990)

*Data obtained from field experiment; [1]average decline rate; [2]Cult. = cultivation-based detection method; Luc = luciferase gene used as marker for confirmation; Cat. = 2,3 dioxygenase gene for degradation of catechol was used as marker; IF = immunofluorescent counts; MPN-PCR = most probable number PCR.

It seems logical to assume that GM bacteria would survive in a similar fashion as their wild-type parents. However, expression of the inserted genes requires an extra amount of energy, which could reduce their environmental fitness (Lenski, 1991); moreover, the insertion could have disrupted unknown functions debilitating the competitiveness of the strains. On the other hand, GMMs could evolve and adapt to the prevailing environmental conditions via natural selection. Velicer (1999) showed that evolutionary adaptation of bacteria to degrade the herbicide 2,4-dichlorophenoxyacetic acid could result in increased competitive fitness to use succinate as a substrate. More recently Kishony and Leibler (2003) reported that environmental stresses could alleviate the debilitating effects of mutations. Their results show that organisms may become more tolerant to genetic perturbations under certain environmental stresses. However, GMMs have not often been shown to acquire increased environmental fitness in microcosm studies. In the study of Timms-Wilson et al. (2000), however, chromosomal insertion of the genes encoding for phenazine-1-carboxylic acid (PCA) production in *P. fluorescens* resulted in enhanced ecological fitness in a microcosm system.

Also in studies with artificial growth conditions, GMMs have been shown to survive better than the wild-type strain (Biel and Hartl, 1983; Bouma and Lenski, 1988; Edlin et al., 1984). However, enhanced survival of GMMs has rarely been observed under field conditions.

Often, numbers of introduced bacterial cells decline rapidly in soil (Table 1), and the survival of GM bacteria is similar to that of non-modified bacteria. There are quite a number of studies in which no difference in survival between GMM and parent strain could be detected (Bailey et al., 1995; Blouin Bankhead et al., 2004; Glandorf et al., 2001; Orvos et al., 1990; Timms-Wilson et al., 2004; Viebahn et al., 2003). Gagliardi et al. (2001) found similar survival rates of the wild-type strain and GM *Pseudomonas chlororaphis* and *P. fluorescens* in five different soils. Schwieger et al. (2000) investigated survival of *rec*A$^-$ and *rec*A$^+$ luc-tagged *Sinorhizobium meliloti* in field lysimeters. Both strains declined from 10^6 to 10^4 colony forming units per gram of soil in lysimeters planted with alfalfa in the first year. However, the *rec*A$^+$ strain survived significantly better.

In other studies GMMs were reported to survive less than their non-modified parent strains (Brockman et al., 1991; Bromfield and Jones, 1979; Da and Deng, 2003; De Leij et al., 1998; Van Elsas et al., 1991; Wang et al., 1991). In the study of Blouin Bankhead et al. (2004) there was no difference in colonization of wheat rhizosphere between *P. fluorescens* Q8r1-96 and its GMM that produces PCA when they were inoculated separately. However, when co-inoculated the GMM was out-competed by the parent strain. Results from a study of De Leij and co-workers (1998) showed that the presence of a number of constitutively expressed marker genes in a GMM had a negative effect on its survival in competition with the wild-type strain. The place of insertion into the chromosome did not affect survival. The evidence suggested that it was purely the metabolic load that was responsible for the decreased fitness, since the study also indicated that this effect did not occur under nutrient-rich conditions.

The method of detection is of crucial importance for interpreting bacterial survival data, since cells that have become non-culturable escape detection with cultivation-based methods.

In a number of studies GMMs introduced into soil were shown to have become non-culturable (England et al., 1995; Kluepfel, 1993). The ecological significance of the presence of viable but non-culturable cells, dead cells, or naked DNA, detected with molecular techniques, remains largely unsolved and definitely requires to be studied further.

Experimental design is of importance to assess the effect of the genetic modification on the fitness of the GMM. Small differences in fitness only become apparent when the GMM and its parental strain are co-inoculated and thus are in direct competition. In most studies in which the GMM was less fit, such a combined inoculation of the parental and GMM strain was used (De Leij et al., 1998; Van Elsas et al., 1991). However, commercial application of GMMs will not include direct competition between GMM and wild-type strain. Therefore, results from such direct competition experiments have to be interpreted with care.

A general conclusion regarding survival of GMMs as compared to their parental strains cannot be drawn. Thus, in each case where colonizing ability and survival of the GMM are of importance, these parameters will have to be determined.

2.2 Ecosystem Effects of Genetically Modified Microorganisms

Possible effects of the introduction of GMMs on natural microbial ecosystems range from the input of organic substrate, displacement of species, changes in population structure, and possible loss of certain functions, to the production of toxic metabolites, which might lead to disturbance of key ecological processes (Smit et al., 1992). However, small changes in community composition are difficult or even impossible to determine, and the relationship between microbial diversity and ecosystem functioning is not well understood (Prosser et al., 2007). Since the number of bacterial species in 1 g of soil is estimated to range between 10,000 and 40,000 (Torsvik et al., 1998), microbial diversity is enormous, and a high redundancy of functions is expected to be present (Degens, 1998; Griffiths et al., 2000). Disappearance of a few species with certain functions will be difficult to detect, since many functions can be performed by a large number of different microbes. Only extreme disturbances might affect soil microbial communities to the extent that certain functions will be reduced (Griffiths et al., 2000).

One of the major problems in microbial ecology is the limited culturability of the indigenous microflora (Amann et al., 1996; Bakken, 1997; Hugenholtz and Pace, 1996; Pace et al., 1986; Schmidt et al., 1991). DNA- and RNA-based techniques, which do not involve cultivation of the microorganisms, are currently used to detect the impact of GMMs on the indigenous microbial community (Torsvik et al., 1998). Methods such as denaturing gradient gel electrophoresis (DGGE) (Fischer and Lerman, 1979; Muyzer and Smalla, 1998), amplified ribosomal DNA restriction

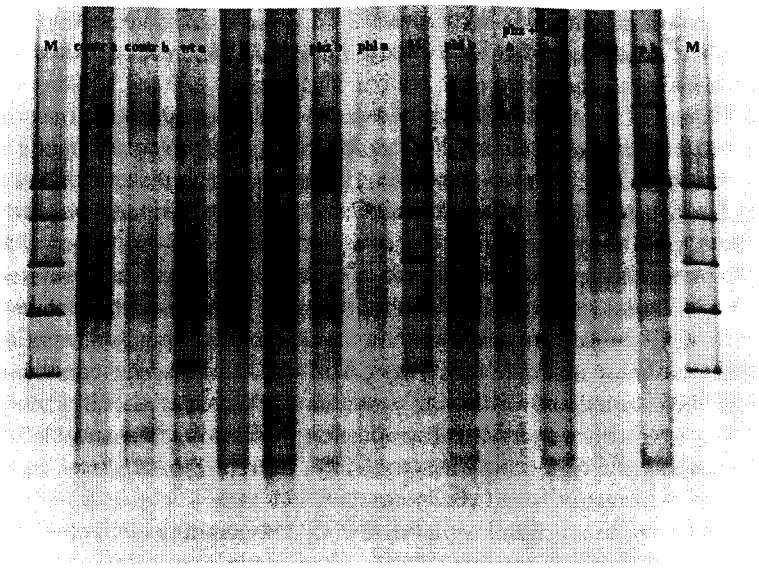

Fig. 1 Denaturing gradient gel electrophoresis (DGGE) profiles showing ascomycete communities of rhizosphere samples of field-grown wheat and potato plants 59 days after sowing. Wheat plants were grown from seeds that were untreated (contr), coated with *P. putida* WSC358r (wt), its phenazine-1-carboxylic acid- or 2,4-diacetylphloroglucinol-producing GMMs (phz, phl), and a combination of both GMMs (phz+phl). rp = samples taken from plots in which potatoes were planted. DNA was extracted from rhizosphere samples and amplified with ascomycete-specific primers. Two replicate plots were analyzed (a, b). Hypothetically, each band in the gel represents one ascomycete species. Note that the potato rhizosphere (rp a, rp b) contains different ascomycete species than the wheat rhizosphere. M = reference marker (Viebahn et al., 2005)

analysis (ARDRA) (Vaneechoutte et al., 1992), terminal restriction fragment length polymorphisms (T-RFLP) (Marsh, 1999), and single-strand conformation polymorphism (SSCP) (Orita et al., 1989) are used to generate banding patterns representing the microbial community in the sample. These methods are more suitable than cultivation-based methods to analyze shifts in community structures. Figure 1 shows DGGE profiles of ascomycete communities of rhizosphere samples from field-grown wheat and potato plants 59 days after sowing, from the study by Viebahn et al. (2005). The wheat plants were grown from control seed and seed treated with wild-type or GM *P. putida* WCS358r. The profiles indicate different ascomycete species in the rhizospheres of potato and wheat and no apparent effects of the bacterial treatments.

2.3 Fate and Effect of Biofertilizer Strains

In this section we will discuss GM derivatives of bacteria that contribute to an enhanced nutrient availability for plants, and thereby increase plant growth.

The most important biofertilizers are bacteria, such as *Azospirillum* and *Rhizobium*, that can fix nitrogen. *Rhizobium, Bradyrhizobium*, and *Sinorhizobium* are plant symbionts, which form root nodules in leguminous plants and fix atmospheric nitrogen. These bacteria have been used widely as plant inoculants to increase yield of leguminous crops. There is a long history of safe use of non-modified rhizobia as inoculants to increase yields of crops (Anon, 1995). However, yield increase is variable (Streeter, 1994), and the success of inoculants seems to be dependent on competition with indigenous strains that are usually less effective (Triplett and Sadowsky, 1992). *Rhizobium, Bradyrhizobium,* and *Sinorhizobium* have been reported to survive in soil for years, in some cases even without the presence of their specific host (Brunel et al., 1988; Diatloff, 1977; Hirsch, 1996, 2004; Schwieger et al., 2000). *Rhizobium* was shown to be able to form nodules when its host plant was planted again after several years (Hirsch, 1996, 2004). This shows that presence of the host plant is not strictly necessary for their survival, but also characteristics of the strain not related to symbiosis play a role in its survival in bulk soil for years. Fast-growing *Rhizobium* species were found to be more susceptible to desiccation than the slower-growing *Bradyrhizobium* (Marshall, 1964). Several *Rhizobium* species have been GM either to improve nitrogen fixation (Birkenhead et al., 1988; Bosworth et al., 1994, Cullen et al. 1998, Hirsch, 2004; Wang et al., 1991), or to study their survival making use of marker genes (Donegan et al., 1999; Hirsch, 2004; Hirsch and Spokes, 1994; Mendum et al., 2001, Van Dillewijn et al., 2002, Watson et al., 1995).

Hirsch and Spokes (1994) studied a Tn*5*-marked *R. leguminosarum* strain introduced into a field as an inoculant for peas and cereals. Cells were enumerated by using a most probable number plant infection test and by direct plate counts. The introduced strain persisted for 5 years in the plots where peas were grown. The persistence of the marked strain was attributed to the soil type, the cultivation of the proper host plants, and the climatic conditions. Potential non-target effects on the microbial ecosystem were not studied (Hirsch, 1996; Hirsch and Spokes, 1994).

Bosworth and co-workers (1994) showed that the use of an improved *R. meliloti* strain, with additional copies of *nifA* and *dctABC*, resulted in an increase of alfalfa yield of 12.9% in a field study. However, at sites with high nitrogen concentrations or native rhizobial populations alfalfa yield did not increase. The plots treated with the recombinant strain target microorganisms were determined. Watson and co-workers (1995) studied the fate of a Tn*903*-marked *R. meliloti* strain introduced into alfalfa-planted field plots. Cell numbers, assessed by polymerase chain reaction (PCR), decreased rapidly after inoculation. One year after introduction, numbers of introduced cells had dropped to below the numbers of indigenous rhizobia.

In a contained field experiment a GM *S. meliloti* strain with enhanced competitiveness for nodule occupancy was released in the rhizosphere of alfalfa (Van Dillewijn et al., 2002). Effects of the GMM and the wild type on the indigenous microbial communities were studied by restriction fragment length polymorphism (RFLP) and temperature gradient gel electrophoresis (TGGE). Inoculation of wild type and GMM had only limited effects. It appeared that alfalfa plants had a greater influence on the microbial community than the inoculated strains.

Schwieger and Tebbe (2000) studied both the fate and ecosystem effects of a *luc*-marked *S. meliloti* in a field experiment with *Medicago sativa*. The bacteria were detected up to 12 weeks after introduction. No effects of the strains on carbon and nitrogen concentrations in the soil could be detected, and there were no differences in the total number of colony forming units of indigenous microorganisms (Schwieger and Tebbe, 2000). Over a thousand bacterial isolates obtained from the plots were further studied by ARDRA, and the dominant groups were identified by 16S rRNA sequencing. In the rhizosphere of *M. sativa* numbers of *Alcaligenes* and *Pseudomonas* were reduced as a result of the inoculation. Molecular analysis by studying SSCP banding profiles revealed shifts confirming the effect of the inoculum on the native microbial population (Schwieger and Tebbe, 2000).

In the south of China both wild type and GM *Alcaligenes faecalis* isolates have been introduced into rice fields at a large scale to improve crop productivity (You et al., 1995; You and Zhou, 1991). *A. faecalis*, a non-nodule-forming nitrogen-fixing isolate, was GM by insertion of a constitutively expressed *nifA* regulatory gene (You and Zhou, 1991). Nitrogen fixation appeared to be 15–20% higher and yield was 5–12% higher compared to the non-treated fields (You et al., 1995). Lin et al. (2000) studied possible ecosystem effects of the introduction of this GMM strain by DGGE of amplified 16S rDNA in a microcosm experiment. The introduced GMM survived well in the rhizosphere with cell numbers of about 10^7 per gram of soil throughout the experiment. DGGE banding profiles of samples treated with the modified strain closely resembled profiles of untreated samples throughout the 40 days of the experiment, suggesting that there are no obvious effects on the bacterial community. Overall, the survival of the strain and the increase in crop yield indicate that this derivative of *A. faecalis* is a good candidate for commercial application, while its ecosystem effects seem very limited.

The use of GM strains as biofertilizers seems promising. So far, non-target effects of GM biofertilizer strains that have been reported are small and insignificant compared to natural variations, such as differences between populations of different plant species (Crawford et al., 1993; Da and Deng, 2003; Lin et al., 2000; Schwieger and Tebbe, 2000; Van Dillewijn et al., 2002; Vázquez et al., 2002). The monitoring of spread and survival of inoculants has been facilitated by use of GM strains and has greatly improved our understanding of rhizobial ecology (Hirsch, 2004).

2.4 Fate and Ecosystem Effects of Modified Biocontrol Bacteria

Many bacterial genera have been described to contain members that can suppress plant diseases caused by soilborne pathogens (Cook et al., 1995; Glick et al., 1999). These PGPR include members of the genera *Azospirillum, Azotobacter, Bacillus, Burkholderia*, and *Pseudomonas* (Weller, 1988). Interest in the application of microorganisms for protection of agricultural crops was stimulated by the public

concern regarding environmental effects of agrochemicals. Many biological control agents have been GM to enhance their biocontrol properties including the extension of the metabolic repertoire by the insertion of novel genes and the increase of the levels of active metabolites.

Investigations have mainly focused on fluorescent *Pseudomonas* spp. strains. Pseudomonads are a metabolically diverse group of rhizosphere bacteria, and many strains are able to suppress plant diseases caused by microbial pathogens (Défago and Keel, 1995; Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007). Mechanisms of disease suppression include siderophore-mediated competition for iron (Bakker et al., 1993; Haas et al., 1991), competition for nutrients, induced systemic resistance (Bakker et al., 2007; Van Loon et al., 1998), and antibiosis (Handelsman and Stabb, 1996; Weller et al., 2002). The best-studied antibiotics with respect to biocontrol are 2,4-diacetylphloroglucinol (DAPG) and PCA, which have antibacterial and antifungal properties and play a major role in disease suppression (Raaijmakers and Weller, 1998; Thomashow et al., 1990).

Quite a number of studies describe the potential use, survival or target effects of GM biocontrol strains (Byzov et al., 1996; Choi et al., 2003; Jones et al., 1991; Moënne-Loccoz et al., 1996; Nambiar et al., 1990; Palmer et al., 1997; Sitrit et al., 1993; Van Overbeek et al., 1995; Völksch and May, 2000). However, only a few studies focus on the effects on the indigenous microflora (De Leij et al., 1995; Girlanda et al., 2001), and only some describe field releases of biocontrol agents in the natural environment (Bakker et al., 2002; Glandorf et al. 2001; Jäderlund et al., 2008; Johansen et al., 2002; Schwieger and Tebbe, 2000; Viebahn et al., 2003, 2005, 2006).

Delany et al. (2001) compared the naturally DAPG-producing *P. fluorescens* F113 with its two GMMs modified to produce higher levels of DAPG in a microcosm. All strains inhibited growth of *Pythium*, and the GMMs showed enhanced control of damping-off, comparable to that of commercial fungicides. Natsch et al. (1997) studied the effect of a genetically modified derivative of *P. fluorescens* strain CHA0 on the diversity of resident pseudomonads in the rhizosphere of cucumber. The modification consisted of the insertion of an extra copy of a housekeeping gene, resulting in increased production of the antibiotics DAPG and pyoluteorin and improved disease control. Several days after inoculation both the parent strain and the GMM reduced the number of resident pseudomonads in the rhizosphere, but the impact of the modified strain was more significant. Only the effect of the modified strain persisted for more than a month. However, when considering the composition of the *Pseudomonas* population on different developmental stages of the cucumber roots, the effects of both modified and non-modified strains appeared to be small and transient.

In the Netherlands, a number of field trials with GM biocontrol bacteria have been performed to study their possible impact on the indigenous microflora (Bakker et al., 2002; Glandorf et al., 2001; Viebahn et al., 2003, 2005, 2006). The fields were carefully designed (Fig. 2) to meet permit requirements. They were surrounded by reed mats to reduce spread of possibly contaminated soil particles outside the field, and the fence and netting prevented entry of birds and rabbits. Weed-free buffer

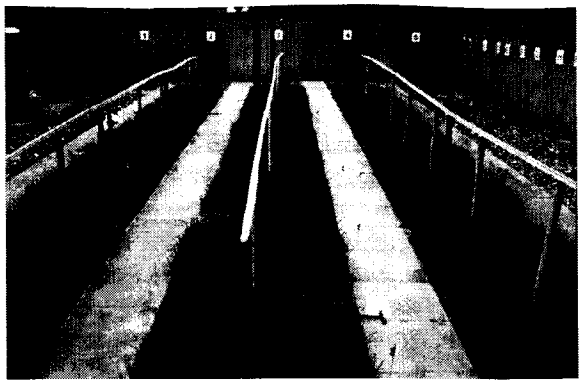

Fig. 2 Experimental field designed to study possible effects of genetically modified biocontrol bacteria on the indigenous microflora of the wheat rhizosphere in Utrecht, The Netherlands. Each treatment was applied to six 1-m^2 plots that were separated by non-planted buffer zones. The fence and netting prevented entry of birds and larger animals, and the reed mats were placed to reduce spread of soil to outside the experimental field (Glandorf et al., 2001)

zones around the experimental plots prevented spread of introduced bacteria by root–root contact.

Possible non-target effects of GM *Pseudomonas* were studied by Glandorf et al. (2001). The biocontrol strain *P. putida* WCS358r was GM with the *phz* locus from *P. fluorescens 2-79*, resulting in constitutive PCA production. Phenazine production in WCS358r resulted in improved biocontrol of take-all of wheat, caused by *Gaeumannomyces graminis* var. *tritici* (Bakker et al., 2002). Two derivatives, one with low and one with high levels of PCA production, were selected for further study in two small-scale field experiments during two subsequent years (Glandorf et al., 2001; Leeflang et al., 2002). Monitoring of various soil ecosystem functions, such as substrate-induced soil respiration, cellulose decomposition, and nitrification potential activity, did not reveal effects of the introduction of any of the strains. Effects of the GMM producing high amounts of PCA on the culturable fungal microflora were transient and were further analyzed using 18S rDNA ARDRA. Introduction of both the wild-type and the GMMs transiently changed the composition of the fungal rhizosphere microflora. However, effects of the GM strains were distinct from those of the parental strain and persisted longer.

In addition to the above-mentioned PCA-producing strain a DAPG-producing GMM was introduced into a wheat field to study long-term effects on the rhizosphere microflora in two consecutive years (Viebahn et al., 2003). In this case, only the DAPG-producing GMM had a transient effect on the structure of the bacterial and fungal microflora, as determined with ARDRA, whereas there was no effect of the PCA-GMM or the wild type. Using DGGE the ascomycete and the bacterial microflora were studied for an additional 2 years (Viebahn et al., 2005, 2006). Whereas the ascomycete communities were not affected by introduction of

the GMMs (Viebahn et al., 2005), the bacterial communities were differentially affected by the parental strain and the GMMs, and especially the DAPG producer had an effect in all 4 years of the field trial (Viebahn et al., 2006). In this field trial the impact of the bacterial inoculants was compared to that of crop rotation between wheat and potato, as an example of agricultural practice. In all cases, the effect of the bacterial introductions never exceeded that of changing the crop (Viebahn 2005, 2006).

Two similar studies on the effects of DAPG- and PCA-producing GM *P. fluorescens* strains were conducted by Timms-Wilson et al. (2004) and Blouin Bankhead et al. (2004). The DAPG-producing *P. fluorescens* strain Q8r1-96 was modified with PCA biosynthetic genes of *P. fluorescens* 2-79, and the resulting GMM was compared to the wild type in pot experiments with wheat. T-RFLP profiles of amplified eubacterial ribosomal sequences demonstrated that inoculation with the wild type or the GMM strain resulted in minimal changes of the bacterial population structure in the rhizosphere (Blouin Bankhead et al., 2004). Chromosomal insertion of the PCA biosynthetic genes in *P. fluorescens* SBW25 resulted in enhanced efficacy of damping-off disease of pea seedlings caused by *Pythium ultimum* (Timms-Wilson et al., 2000). Possible impact of this PCA-producing GMM on bacterial and fungal microbial diversity was studied in pot experiments with pea, wheat, and sugarbeet, and with or without *Pythium* disease pressure. In all these experimental conditions, factors like plant species or development of disease had a greater impact on microbial diversity in the rhizosphere than inoculation with the PCA-producing GMM (Timms-Wilson et al., 2004).

In line with these studies a recent paper by Scherwinski et al. (2008) convincingly shows that introduction of active biocontrol agents (*Serratia plymuthica*, *P. trivialis*, and *P. fluorescens*) results in negligible, short-term effects on the indigenous bacterial and endophytic fungal populations.

A different approach is to study the impact of GMMs on specific non-target microbial taxa with an important soil function. Enhanced biocontrol properties of pseudomonads by increased antibiotic production could result in adverse effects on the arbuscular mycorrhizal symbiosis. The hypothesis was tested by Barea et al. (1998) using *P. fluorescens* strain F113 and two GM derivatives: wild-type strain F113, a natural DAPG-producer, and two GM derivatives that either produced no DAPG or had enhanced DAPG-production. Colonization of tomato roots by the arbuscular mycorrhizal fungus *Glomus mossae* was not affected by any of the bacterial strains. Moreover, enhanced DAPG production did not inhibit spore germination and mycelium growth. Apparently, production of DAPG by *Pseudomonas* sp. F113 does not affect the beneficial fungal symbiont *G. mossae*.

From these studies with GM biocontrol strains it can be concluded that effects on non-target microorganisms in soil ecosystems do occur; however, if they occur they are transient and small compared to natural variation. To our knowledge long-term changes in community structure and adverse effects on the functioning of the soil ecosystem after introduction of GM strains have not been reported.

3 Genetically Modified Microorganisms as Biosensors and for Bioremediation

3.1 Genetically Modified Biosensors

An exciting application of GMMs is their use as a sensor for biologically relevant concentrations of agrochemicals, petroleum products, heavy metals, and toxins in environmental samples (Belkin, 2003; Kim et al., 2003; Lei et al., 2006; Shao et al., 2002; Stiner and Halvorson, 2002). GM biosensors are usually not applied *in situ* to soil or water, but samples are taken from polluted sites and incubated in *in vitro* assays. Thus, survival and persistence of the GM biosensor strain is not an issue, since the organism remains contained in the laboratory or in the measuring equipment, and incubation periods are short.

Most of the microorganisms used as biosensors are modified with reporter genes such as *gfp* or *lux* fused to a promoter that induces its expression depending on nature and concentration of the compound(s) of interest. The choice of the promoter determines the applicability of the strain. Some researchers used promoters that respond specifically to certain compounds. For instance, Stiner and Halvorson (2002) used the toluene–benzene transcriptional activator that is only induced by the petroleum products toluene, benzene, ethylbenzene, and trichloroethylene for the detection of contamination in soil and surface water. Miller et al. (2001) used the sucrose repressor gene *scr*R fused to several promoter genes for assessing sugar availability on plant leaves. In order to detect a wider range of toxicants stress-specific promoters such as the SOS and heat shock promoters, *rec*A and *grp*E and *fab*A or *Kat*G, are fused to the reporter gene (Kim et al., 2003; Sagi et al., 2003). Such strains produce signals when they are exposed to substances that exert a stress response. Currently, biosensors are being developed that allow high-throughput monitoring of toxins in waste water (Kim et al., 2003; Philp et al., 2003). Although the future for the application of biosensors is promising (Belkin, 2003), there are a number of problems that have to be solved. Development of highly suitable microbial biosensors is as yet hampered by their long response time, low sensitivity, and poor selectivity (Lei et al., 2006). Non-specific toxic effects of other compounds on the bacteria can influence the measurements, temperature sensitivity of some strains can affect the results, and the need to contain GMMs in the equipment could pose difficulties (Philp et al., 2003, Mirasoli et al., 2002).

3.2 Genetically Modified Microorganisms for Bioremediation

Once the nature and severity of a chemical pollution has been established, GMMs can be applied to clean up the environment by bioremediation. Bioremediation is the reduction or removal of toxic or polluting substances from the environment using microorganisms. Current knowledge on the genetics of metabolic pathways in microorganisms allows the construction of new biochemical pathways to increase

their capabilities to degrade environmental pollutants or herbicides (Dutta et al., 2003; Haro and de Lorenzo, 2001; Lange et al., 1998; Mauro and Pazirandeh, 2000; Mitra et al., 2001; Sriprang et al., 2002; Strong et al., 2000). Although a myriad of different GM strains for bioremediation has been constructed, successful applications in the field are rare (Haro and de Lorenzo, 2001; Morrissey et al., 2002; Van Limbergen et al., 1998). Two factors are crucial for successful bioremediation: the microorganisms have to survive in high numbers and the microorganisms have to be metabolically active. Moreover, difficulties to scale up the laboratory experiments, low bioavailability of the compound, and legislative problems with applying GM strains have precluded wide-scale use. Since soil is often an oligotrophic environment cells usually decline after introduction, and they have a low metabolism. Bacterial survival and degradative activity can be enhanced by choosing the right species and by nutrient amendment. Halden and co-workers (1999) studied the degradation of 3-phenoxybenzoic acid in soil and could increase GMM survival and 3-phenoxybenzoic acid degradation six orders of magnitude by adding phosphate and nitrogen.

Microorganisms should be chosen depending on the environmental conditions in which they have to function. Lange and co-workers (1998) constructed a recombinant *Deinococcus radiodurans* expressing toluene dioxygenase to clean up toxic solvents in radioactive waste sites. *D. radiodurans* is extremely radiation resistant, the strain can degrade chlorobenzene in a highly radioactive (60 Gy/h) environment. However, *D. radiodurans* can only grow up to 39°C, and since many radioactive waste sites have high temperatures, there was need for a bacterium that could function at higher temperatures (Brim et al., 2003). *Deinococcus geothermalis* is a remarkable organism since it cannot only thrive in radioactive environments, but it is also resistant to high temperatures. Brim and co-workers (2003) constructed a *D. geothermalis* strain, which is able to reduce ionic Hg(II) to the less toxic elemental Hg at elevated temperatures and in the presence of high radiation levels. Such applications have great value for future remediation, since it is estimated that 40 million cubic meters of soil and 4 trillion liters of groundwater are contaminated with radioactive and toxic waste in the US alone (Brim et al., 2003).

So far, field applications of GM strains for bioremediation are scarce. An example of a field-scale remediation is the study of Strong et al. (2000). *P. fluorescens* HK44 with a *lux* gene fused to a naphthalene-degradative pathway was the first GM strain approved for field release in the US (Ripp et al., 2000; Strong et al., 2000). The strain survived for 660 days, and degradation of hydrocarbons was detected with a fiber optic-based biosensor. Strong and co-workers (2000) also applied chemically killed recombinant *Escherichia coli* to remediate atrazine by bioaugmentation. The addition of phosphate and the chemically killed cells resulted in a 77% reduction of atrazine levels as compared to the controls, in which no significant reduction took place.

A combined inoculation of microorganism and its host plant by providing extra nutrients could enhance microbial survival and activity (Brazil et al., 1995). Such use of a dual microorganism–plant system, in which the plant supports microbial growth and the microorganisms perform the bioremediation, is known as rhizore-

mediation (Kuiper et al., 2004; Yee et al., 1998). This strategy was adopted by Dutta et al. (2003), who used a GM *S. meliloti* to degrade 2,4-dinitrotoluene (2,4-DNT) in soil. This symbiotic nitrogen-fixing bacterium can nodulate leguminous plants such as alfalfa. Seeds of alfalfa were coated with the engineered strain and seeded in soil contaminated with 2,4-DNT. At moderate pollution levels plant growth was stimulated by the bacterium, and the level of 2,4-DNT was reduced by 60%. At higher levels, plants could not grow, but application of the bacteria to the soil reduced 2,4-DNT levels up to 90%. *P. fluorescens* strain F113 is a good colonizer of rhizospheres of different plant species and could be exploited for rhizoremediation. A polychlorinated biphenyl-degrading derivative of F113, F113rifPCB, was constructed (Brazil et al., 1995). Heavy metals and metalloids are serious co-contaminants of polluted soils (Ryan et al., 2005) and can hamper the success of rhizoremediation by inhibiting both the rhizobacterium and the plant. A recombinant derivative of F113rifPCB containing the arsenic resistance operon *arsRD-ABC* was constructed, resulting in a strain that degrades biphenyl in the presence of arsenate and that protects the plant to arsenic as well (Ryan et al., 2007). Furthermore, strain F113 was modified with the *bph* operon from *Burkholderia* sp. strain LB400, under the control of a nodbox cassette (Villacieros et al., 2005). This GM derivative of F113 (F113L::1180) had a high level of BphC activity, and in combination with willow plants is expected to degrade a large spectrum of PCB congeners in soil (Rein et al., 2007). In a 168-day microcosm experiment the possible impact of F113L::1180 on bacterial communities in soil and in the rhizosphere of *Salix viminalis*× *schwerinii* was investigated. The modified strain did affect community structure of the eubacteria and specific bacterial populations (α- and β-proteobacteria, acidobacteria, and actinobacteria) in the *Salix* rhizosphere, but not in the bulk soil (de Cárcer et al., 2007).

Bioremediation of pollutants in soil and water using GMMs has been accomplished with success on a laboratory scale; however, field applications have been limited. Long-term field studies are now needed to evaluate their effectiveness. Concerns have been made regarding persistence of GM strains and possible evolutionary adaptation that could increase its environmental fitness (Velicer, 1999). Impact on the natural microbial community by GM bioremediation bacterial strains is a complex issue, since the breakdown of pollutants itself will most likely affect microbial communities.

4 Conclusion

Whereas only a limited number of field trials with GMMs have been conducted, it appears that they can have improved performance in agricultural and bioremediation applications. GM biocontrol bacteria seem to be a suitable alternative for agrochemicals used for crop protection, and modified biofertilizers have been used with success. The success of the introduction is partly related to the survival and activity of the inoculum, which is dependent on the environmental conditions. While the

questions related to field introductions of GMMs have helped to develop tools in the area of molecular microbial ecology, our knowledge on the benefits, fate, and effects of GMMs in the environment is still limited and fragmentary. For sensible applications this knowledge needs to increase rapidly, focusing on specific areas. Questions to be addressed include, how and in which physiological state do bacteria survive in soil, and what effects do they exert on the natural microbial community. More knowledge on microbial community structure and functioning is a prerequisite for optimizing the use of GMMs in agriculture.

Only very few studies have focused on ecosystem effects of GMMs, and in most of those cases the GMM used was modified to express certain markers. More recently studies have been performed using GMMs expected to have an impact on non-target organisms, for instance through the production of antimicrobial metabolites with a broad-spectrum activity. Also in this last category of experiments, effects on the microbial ecosystem were found to be small and transient or were not detected.

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