Conjugal gene transfer between bacteria in soil and rhizosphere

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1. Dat het onderzoek naar genoverdracht in de bodem zich vooral richt op conjugatie wil niet zeggen dat transductie en transformatie geen rol spelen.

2. Het optreden van "plate mating" bij het bepalen van de conjugatie frequentie in de bodem dient voorkomen te worden.

   Hoofdstuk 3, dit proefschrift

3. Plasmiden, virussen, transposons en IS elementen zijn mede gezien hun wijd verbreide voorkomen waarschijnlijk niet alleen parasitair maar ook symbiontisch.

   Biel, S.W. and Hartl, D.L. (1983) Evolution of transposons: natural selection for Tn5 in *Escherichia coli* K12. Genetics 103, 581-592.

   Edlin, G., Tait, R.C. and Rodriguez, R.L. (1984) A bacteriophage lambda cohesive end (cos) DNA fragment enhances the fitness of plasmid containing bacteria growing in energy limited chemostats. Biotechnology 2, 251-254.

4. Selectie voor een specifiek genotype, essentieel voor de overleving van een bepaalde bacteriesoort in zijn eigen niche, verklaart waarschijnlijk het feit dat bacteriesoorten, ondanks het optreden van genoverdracht, toch genetisch verschillend blijven (Young, 1992).

   Young, J.P.W. (1992) The role of gene transfer in bacterial evolution. **In** Genetic interactions among microorganisms in the natural environment. pp. 3-13. E.M.H. Wellington and J.D. van Elsas. Pergamon Press, Oxford.

5. Genoverdracht is een natuurlijk proces en de aard van het gen en de selectiedruk zijn waarschijnlijk belangrijker dan de feitelijke overdracht van het DNA voor risico analyse onderzoek.

6. Onzorgvuldige (spectaculaire) berichtgeving door actiegroepen tegen genetische modificatie maakt in het algemeen meer indruk bij de bevolking dan het zorgvuldig gekozen weerwoord van een terzake kundige onderzoeker.

   Arnhemse Courant 21-01-91; Gelderlander, 18-01-91.

7. Door goed samen te werken kan veel meer (onderzoeks) werk verricht worden dan hetgeen er door de individuen afzondelijk kan worden gedaan.

8. Het voorin plaatsen van niet-rokers in een vliegtuig kan gezien de procentueel lagere overlevingskans bij een crash compenseren voor hun relatief hogere levensverwachting.

9. Het feit dat de kranten alleen in het voorjaar en de zomer veelvuldig melding maken van schennisplegers suggereert dat mensen gedreven worden door instincten.

10. Het door Onderzoeksinstituten centraal beheren van onderzoeksbudgetten leidt tot frustraties bij, en vervroegd uittreden van, hoog gekwalificeerde productieve onderzoekers.

Stellingen behorende bij het proefschrift "Conjugal gene transfer between bacteria in soil and rhizosphere" van Eric Smit.

Wageningen, 18 januari 1995.

CONTENTS

Most of the chapters in this thesis are published before as an article in the following journals (a complete list of publications including title and authors can be found on page 138):

Chapter 2: FEMS Microbiol. Rev. 88, 263-278.

Chapter 3: Curr. Microbiol. 21, 151-157.

Chapter 4: Revised version of the following two articles on methods

a) E. Smit and J.D. van Elsas (1992) Methods for studying conjugative gene transfer in soil. In: Genetic interactions between microorganisms in the natural environment. (E.M.H. Wellington and J.D. van Elsas. eds.) Pergamon Press, London.

b) J.D. van Elsas and E.Smit (1992) Conjugal gene transfer in the soil environment: new approaches and developments. In: Gene transfers and environment. (M.J. Gauthier, ed.) Springer-Verlag, Berlin.

Chapter 5: Appl. Environ. Microbiol. 57, 3482-3488.

Chapter 6: FEMS Microbiol. Ecol. 101, 281-292.

Chapter 7: Appl. Environ. Microbiol. 59, 2257-2263.

Chapter 8: Submitted to FEMS Microbiol. Ecol.

Let your acts be directed toward a worthy goal, but do not ask if they will reach it; they are to be models and examples, not means to an end.

Leo Szilard
Ten Commandments

CHAPTER 1

Introduction

The dramatic increase of agricultural and industrial productivity has created severe environmental problems. Soil and groundwater are often polluted with pesticides, xenobiotics and heavy metals. Introduction of bacteria could in some cases substitute pesticides, or serve as a soil remediation technique. Bacteria have been introduced into soil to promote plant growth (De Freitas and Germida, 1992; Gaskins et al., 1985; Lugtenberg et al., 1991; Schmidt and Robert, 1985), to control pests (Hofte and Whitely, 1985; Lugtenberg et al., 1991; Schroth et al., 1984) or to degrade polluting compounds (Alexander, 1981; Brunner et al., 1985; Pipke et al., 1992). Many bacteria can be used for specific agricultural purposes. For instance *Rhizobium* species have been sprayed to serve as a natural fertilizer (Nutman, 1975) and *Bacillus thuringiensis* species and pseudomonads have been used for biocontrol (Schippers et al., 1987; Weller, 1988). Bioremediation of pollutants in soil and sediment by bacteria has been studied on laboratory scale and seems to offer possibilities (Brown et al., 1988; Focht, 1988; Frick et al., 1988; Kilbane et al., 1983). In some cases, the use of natural isolates seems limited since the strains do not always survive or show the expected activity in the soil ecosystem (NAS, 1989). Nowadays, bacteria isolated from different ecosystems can be genetically modified for specific environmental purposes (Doyle et al., 1991; Duque et al., 1992; Lindow, 1985; Lindow et al., 1989; Obukowicz et al., 1987; Ramos et al., 1991; Skϕt et al., 1990; Van Elsas et al., 1991; Waalwijk et al., 1991; Watrud et al., 1985).

Since it is nessecary to describe the term genetically engineered or modified microorganism (GEM), we adopted the definition given in Article 2 of the Directive of the European Communities (Commission of the European Communities, 1992), which is: "genetically modified organism means an organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination". Most environmental problems can not yet be solved since knowledge on and experience with applying GEMs is mostly limited to laboratory microcosm studies with a few species. The possibility of evaluating the use of these recombinant organisms in large scale field trials is limited, because of a lack of knowledge on the fate of the heterologous DNA and the possible effects of the GEMs on the ecosystem (Alexander, 1985; Covallo and Fiksel, 1985; Griffiths, 1981; Keeler, 1988; Levin and Strauss, 1990; NAS, 1989; Sussman et al., 1988; Suter, 1985; Tiedje et al., 1989; Wellington and van Elsas, 1992).

To obtain basic knowledge on the fate of the organism and its DNA, microcosm studies have to be performed using model organisms under conditions simulating those of the environment. This thesis deals with the extent of transfer of recombinant DNA present in GEMs to other

bacteria. As a general introduction, the soil environment and the different processes involved in gene transfer will be discussed below.

The soil environment

Soil is a structured environment composed of solid, liquid and gaseous phases. All three phases are heterogeneous in terms of the distribution of different compounds within them (Smiles, 1988). In particular, components of the solid phase, i.e. inorganic substances such as clay, silt and sand, and organic matter (humic substances), are distributed unevenly (Van Veen and Van Elsas, 1986) and are often complexed in aggregates. Aggregates important for soil microorganisms are the clay-organic matter complexes, due to their charged surfaces and increased nutrient availability (Hattori and Hattori, 1976; Smiles, 1988). The solid phase is interspersed with the soil pore network containing the liquid and gaseous phases. The relative abundance of pores and their sizes determines the water retention capacity of soil, and thereby to a large extent the availability of water for soil microbes.

Soil not directly under influence of plant roots can be regarded as an oligotrophic environment since it is generally poor in ready available carbon (Williams, 1985). The low amount of available carbon generally precludes bacterial growth and activity, and is estimated to be sufficient for only a few cellular divisions of the soil microorganisms per year (Gray and Williams, 1971; Shields et al., 1973). Nutrients may, however, become available in so-called "hot spots", e.g. in decaying material of plant/animal origin and trigger some microbial growth and activity.

Plant roots represent one of the major sites of carbon input into soil (Curl and Truelove, 1986; Lynch and Whipps, 1990). Both soluble compounds like root exudates and insoluble ones like remnants of root cortex cells are released in the rhizosphere. Root-released carbon is likely to be more available for soil bacteria than carbon present in the bulk soil as it is easier to degrade. Rhizosphere bacteria often show increased growth and activity due to the enhanced availability of organic carbon in the rhizosphere (Foster, 1988). Moreover, the water flow in soil induced by roots may enhance bacterial movement towards them. This may promote cell-cell, cell-bacteriophage and cell-DNA contacts and influence gene transfer processes.

Transformation in soil

Studies on transformation in soil have been done on intact, disturbed and sterile soil systems. Simplified systems such as columns filled with pure sand or tubes with soil extract have also been used (Lorenz et al., 1988; Lorenz and Wackernagel, 1991). In these systems, which do not have much resemblence to natural soil, the occurrence of transformation as a gene transfer mechanism could be detected. First, several reports focused on the persistence of DNA when adsorbed to a mineral surface and its availability for transformation. Although in early studies DNA was found to be rapidly degraded in a (sandy) soil, its persistence has also been shown, in particular when adsorbed to montmorillonite clay (Greaves and Wilson,

1970; Khanna and Stotzky, 1992). DNA added to soil was shown to persist for several weeks (Romanowski et al., 1992) and DNA adsorbed to pure sand was further shown to be resistant to DNase as opposed to its degradation in the unadsorbed state (Lorenz and Wackernagel, 1987). Various studies showed that DNA adsorbed to clay or sand surfaces may indeed be available for transformation (Khanna and Stotzky, 1992; Lorenz et al., 1988; Romanowski et al., 1992).

Development of competence is another factor of importance. Evidence provided by Lorenz et al. (1987, 1988) showed that *Bacillus subtilis*, adsorbed to sand particles, was in an state of enhanced competence.

The first report of transformation in (sterile) soil was that by Graham and Istock (1978). They observed that two strains of *Bacillus subtilis* with different sets of chromosomal markers introduced into sterile potting soil, co-evolved into various different types, with exchange of certain linkage blocks. Transformation was invoked as the transfer mechanism involved, even though the process was insensitive to DNase. The occurrence of transformation rather than conjugation or transduction was further supported by the fact that incubation in soil of one parent strain with DNA of the other strain also resulted in the appearance of transformants. Later, Lee and Stotzky (1990) also reported that bacilli co-introduced into soil were able to exchange chromosomal markers, both antibiotic resistance and auxotrophic genes. Unfortunately, they did not provide conclusive proof since appropriate controls such as the addition of free DNA to soil lacked.

A combination of these results seems to provide sufficient indication that soil may provide conditions conducive to transformation, although transformation in natural soil was only shown with *Bacillus* species (Graham and Istock, 1978; Lee and Stotzky, 1990).

Transduction in soil

Transduction requires the presence of donor bacteria on which bacteriophages are propagated, bacteriophage and recipient bacteria which are in turn infected by phages carrying genes from the donor bacterium.

The chances for transduction in soil are probably enhanced by an increasing abundance of phages in soil following a burst of infected host cells, which act as reservoirs of phages (Reanney et al., 1983; Van Elsas, 1983). Soil nutritional conditions promoting bacterial activity were also shown to promote phage propagation (Van Elsas, 1983), since indigenous bacteriophages of different *Bacillus* spp. drastically increased their titers at 37°C in soil amended with nutrients but not in unamended soil. Recently, Herron and Wellington (1990) also presented data showing initial propagation of the temperate actinophage KC301 on its host *Streptomyces lividans* added to both sterile chitine- and starch-amended and non-sterile soils. Also, Stephens et al. (1987) showed that naturally occurring phage was responsible for the decline of introduced fluorescent pseudomonads in the rhizosphere of sugarbeet. However, the propagation of bacteriophage on hosts present in soil is probably limited to host densities above a certain threshold, estimated to be around 10^4 cfu per ml or g (Wiggins and Alexander,

1985).

Next to phage propagation and abundance, the persistence of phages in soil affects the chances for transduction. Herron and Wellington (1990) showed that the actinophage KC301 steadily declined in soil following sporulation of the host. On the other hand, Germida (1986) and Stotzky (1980) provided data showing that bacteriophages and viruses sometimes persist in soil and survive different physical and chemical stresses for prolonged periods even in the absence of hosts. Persistence of bacteriophages T2 and MS2 was variable in different soils and was affected by soil type (Hurst et al., 1980). Soil temperature and texture was also shown to affect the persistence of two phages, MS2 and PRD-1, in two different soils (Straub et al., 1992); at increasing temperature, the inactivation rate was higher. Further, phage was better protected from inactivation in a clay loam than in sandy soils. Drying of soils resulted in a complete disappearance of plaque-forming units.

The actual potential for the occurrence of transduction in soil has been shown by Zeph et al. (1988) and Germida and Khachatourians (1988). Both groups showed that *Escherichia coli* cells added to soil could be reached by phage P1 carrying selectable markers, and that the markers were stably inherited by the recipient cells. The reported frequency of transduction was 10^{-6} (Germida and Khachatourians, 1988). However, the report of Zeph et al. (1988) described a process more akin to phage conversion than to transduction, since a lysate was used that consisted entirely of phage particles containing the selected marker. Germida and Khachatourians (1988) used a transducing lysate obtained from donor cells marked with auxotrophic markers and Tn*10*. In addition, preliminary evidence for transduction of thiostrepton resistance by bacteriophage KC301 between streptomycetes in sterile starch- and chitine-amended soil was also given by Herron (1991).

Transduction is thus likely to occur in soil, in particular in microsites where conditions are conducive. The frequencies of such transfers measured over a whole soil system may be extremely low. Therefore, it is difficult to assess the impact of transductional gene transfer on soil bacteria, at phage concentrations naturally found in soil.

Conjugation in soil

Conjugal gene transfer depends on close contact between donor and recipient cells under favourable conditions of temperature, pH, and possibly nutrient availability; often, cells adsorbed onto a surface form mating aggregates very efficiently in the presence of nutrients dissolved in an aqueous environment. Self-transmissible plasmids can mediate their own transfer since they carry both an origin of transfer (*oriT*) and the transfer (*tra*) genes which are nessecary for the formation of mating pairs, nicking of the plasmid and transfer of the DNA (Freifelder, 1987). They can also mobilize certain plasmids, carrying *oriT*, from a donor to a recipient bacterium by providing the transfer functions. Recently, the process of retrotransfer was discovered, i.e. the mobilization of a plasmid and even of chromosomal DNA into the (donor) strain containing the self-transmisssible plasmid (Mergeay et al., 1987; Top, 1993). The occurrence of retrotransfer might have implications for transfer of recombinant

DNA from introduced bacteria to indigenous ones, since self-transmissible plasmids with retromoblizing capacity have been found in bacteria in the environment (Diels et al., 1989; Don and Pemberton, 1985; Kelly and Reanney, 1984; Pickup, 1989; Sayler et al., 1990). However, there is still discussion whether retrotransfer is a one- or two-step process (Heinemann and Ankenbauer, 1993; Top, 1993).

Our knowledge on the occurrence of conjugation between various different bacteria in soil has increased considerably in recent years (Henschke and Schmidt, 1990; Krasovsky and Stotzky, 1987; Richaume et al., 1989; Schofield et al., 1987; Van Elsas et al., 1988a, 1988b; Wellington et al., 1990). Circumstantial evidence for conjugal transfer has been obtained retrospectively, using indirect data such as the occurrence of similar plasmids in different chromosomal backgrounds of *Rhizobium* (Schofield et al., 1987). In these studies, however, it is unclear whether the actual transfer event occurred *in noduli* or in the soil. Experiments in which donor bacteria carrying transferable selectable markers, either or not accompanied by recipients, were added to soil, have provided more direct evidence of conjugal gene transfer (Henschke and Schmidt, 1990; Richaume et al., 1989; Stotzky, 1989; Top et al., 1990; Van Elsas et al., 1987, 1988a, 1988b, 1990; Wellington et al., 1990). Both plasmid-encoded (e.g. Van Elsas et al., 1987, 1988a) and chromosomal (Krasovsky and Stotzky, 1987) genes have been shown to be transferable in natural soil. Transfers between introduced Gram-positive donor and recipient cells, e.g. different *Bacillus* spp. (Van Elsas et al., 1987), and between introduced Gram-negative cells, e.g. between differentially-marked *Pseudomonas fluorescens* strains (Van Elsas et al., 1988a, 1988b) or from *Escherichia coli* to *Rhizobium fredii* (Richaume et al., 1989) have been detected in soil. Furthermore, the conjugative transposon Tn*916* which is transferable *in vitro* from Gram-positive to Gram-negative bacteria (Bettram et al., 1991) was recently found to be transferred in nutrient-amended soil from introduced *Bacillus subtilis* to indigenous *Streptomyces* spp. (Natarajan and Oriel, 1992). Heterogramic transfer of this element however, has so far not been observed in soil, even though it is able to express its selectable marker, resistance to tetracycline, in Gram-positive and Gram-negative bacteria.

Conjugal transfer in soil has been shown to be affected by many variable soil factors such as temperature, pH, organic matter and clay content, water availability (Stotzky, 1989; Van Elsas et al., 1988b; Van Elsas and Trevors, 1990). Briefly, the presence of nutrients, montmorillonitic clay minerals and plant roots and the absence of a competing microflora have been shown to promote conjugal plasmid transfer. Transconjugants could be detected in soil after the addition of nutrients (Top et al., 1990; Van Elsas et al., 1988a; Wellington et al., 1990), after soil sterilization (Richaume et al., 1989) and in the presence of plant roots (Van Elsas et al., 1988b). In addition, the presence in soil of montmorillonite clay (Krasovsky and Stotzky, 1987) or bentonite clay (Van Elsas et al., 1988a) greatly enhanced conjugal transfer between introduced bacteria. Other soil variables also affected conjugation. Conjugation rates were highest at moderate pH, and very acid conditions did not permit transfer (Richaume et al., 1989; Stotzky, 1989). Soil organic matter content positively affected conjugal transfer in

a sterile soil (Richaume et al., 1989), but it had a negative effect in the wheat rhizosphere under non-sterile conditions (Van Elsas et al., 1988b).

It was shown that soil poses a barrier to cell-cell contact by Van Elsas et al. (1990), who reported that bacteria initially introduced into different soil portions which were subsequently mixed, were less able to transfer plasmid RP4 than cells added to the same soil portion, at different points in time. The presence of wheat roots alleviated the barrier effect of soil, allowing the detection of transconjugants also in the mixed soil portions.

The oligotrophic conditions in soil pose a second barrier to conjugation, since cellular energy is needed for a successful conjugal transfer event. Most of the bacterial cells in soil are in an almost permanent state of starvation (Williams, 1985) and therefore presumebly unable to serve as donors or recipients in conjugal matings.

Aims and outline of this thesis

The aim of this work was to study the fate of recombinant DNA in bacteria introduced into soil. The main focus was to develop methods to enable the detection of conjugal gene transfer to indigenous soil bacteria, and to study the effect of different locations of the recombinant DNA, such as on a self-transmissible plasmid, on a mobilizable plasmid, or on the chromosome.

The current state of the art and the problems of risk assessment research, as for instance the gaps between regulators, research and industry are discussed in Chapter 2. Of the three known DNA transfer mechanisms, conjugation seems to be an important process in the soil environment, since many selftransmissible plasmids have been found in soil and aquatic environments (Burton et al., 1982; Diels et al., 1989; Don and Pemberton, 1985; Kelly and Reanney, 1984; Pickup et al., 1989; Schmidt and Schlegel, 1989) and self transmissible plasmids have been shown to transfer under ecologically relevant circumstances (Brokamp and Schmidt, 1991; Fry and Day, 1990; Fulthorpe et al., 1991; Lacy and Leary, 1975; McClure et al., 1990; Mergeay et al., 1990; Top, 1993; Van Elsas et al., 1988a, b, 1990). Still several questions remained. The occurrence of conjugation on the transconjugant selective plates after the bacteria were isolated from soil was suspected to occur in donor/recipient experiments in soil. To prevent this, an adequate donor counterselection method had to be developed. The use of nalidixic acid and rifampicin in combination with a resistant recipient was shown to prevent plate matings significantly (Chapter 3). Secondly, there were no data on the occurrence of *in situ* plasmid transfer to indigenous soil bacteria. To detect transfer to indigenous bacteria two problems had to be solved, (a) a different donor counterselection method had to be developed to kill the donor, since the recipients were unknown and could not be selected for, and (b) a specific detection method was necessary to discriminate between transconjugants and naturally resistant bacteria which might grow on the selective plates. This was realized by the use of a phage specific for the donor strain allowing an effective donor counterselection, in combination with an unique target sequence that could be detected by colony filter hybridization with a labelled probe. This way, transfer of a marked derivative of RP4, RP4p, to indigenous soil

bacteria was detected (Chapter 4 and 5). Different soil types could now be studied to assess the applicability of the method and to study the extent of transfer of RP4p to indigenous microorganisms (Chapter 6). Since selftransmissible plasmids were transferred relatively easily, they were not considered to represent the vectors of choice for recombinant genes. Other broad-host-range plasmids such as those of the IncQ group (which lack the *tra* genes) seemed more suitable, and had hardly been investigated in microcosm studies. Therefore mobilization of a recombinant IncQ plasmid was studied, in the presence and absence of the mobilizing plasmid RP4p (Chapter 7). Finally, the effect of different genomic locations of marker genes on stability, transfer and expression was studied in long-term soil microcosms experiments (Chapter 8).

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CHAPTER 2

Risks associated with the application of genetically modified microorganisms in terrestrial ecosystems

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1. Introduction

There are many potential applications of genetically modified microorganisms (GEMs) in agriculture, for instance in biological control of plant pathogens, in the stimulation of nitrogen fixation and in the enhancement of plant growth (Keeler, 1988). One of the obstacles still preventing the full-scale exploitation of the possibilities offered by genetic modification of microorganisms is the fear of unwanted effects on man, animals, plants and on the ecosystem by the release of these microorganisms. In many countries these concerns have led to restrictive measures imposed on releases both at a research scale and at a commercial scale. Whereas the usefulness of some restrictive measures will not be contested, criteria used have often been confusing especially to scientists involved in the field. A gap still exists between regulatory agencies, whose guidelines are usually too vague to provide a firm basis for research and scientists who perform experiments that do not always fit in the scheme of the guidelines of the regulatory agencies. This gap negatively influences progress in risk assessment studies.

This paper will firstly attempt to link risk assessment theory with existing experimental data and techniques used and secondly to establish sound scientific criteria on which decision-making should be based. Emphasis will be on the effect of the genetic modification on GEM fate in the environment, in particular soil, and on possible ecological consequences of a GEM release. Furthermore, experimental bottlenecks such as the quantification of risk, and possible biological containment methods will be discussed. Finally a universal decision-making tree will be presented which could be useful in the case-by-case judgement process adopted by most regulatory agencies.

2. Risk assessment theory and framework

Assessment of the risk of production and use of chemicals and energy has been under development ever since these human activities became predominant in industrial countries. Much of the biotechnology risk assessment which is currently being developed is based on concepts originally put forward by the "chemical" risk assessment. According to Griffiths (1981), risk assessment can be defined as a means to provide an estimate of the risk of an unwanted potentially harmful event in terms of the likelihood of its occurrence and the severity

of its consequences. Risk assessment provides an analytical framework of obtaining and interpreting experimental data, with the objective of providing an estimate of the risk posed by a potentially harmful process or activity (Levin and Strauss, 1990). The most striking characteristics which distinguish the assessment of the risk of microorganisms from that of chemicals are the unique capabilities of microorganisms to grow and multiply, to exchange genes with members of the indigenous flora and potentially to move actively through an ecosystem (Katz and Marquis, 1990). This raises serious difficulties for microbial risk assessment as compared to that of chemicals where physio-chemical laws of dissipation and decay tend to reign. Several books and reviews have described the development of biotechnology risk assessment in recent years (Alexander, 1985; Covello and Fiksel, 1985; Domsch et al., 1987; Ginzburg, 1991; Levin and Strauss, 1989; NAS, 1989; Strauss et al., 1985; Sussman et al., 1988; Suter, 1985; Tiedje et al., 1990; Van Elsas and Trevors, 1991) some of these focused on the potential risk posed on the environment by the deliberate or accidental release of GEMs. Many of the concepts put forward are based on the knowledge of the fate and effects of unmodified organisms released into the environment. However, a large gap still exists between theoretical risk assessment, i.e. the development of concepts and experimental risk assessment, the performance of experiments aimed at estimating risk, ultimately striving to solve the problem how to quantify risk having obtained experimental data. To tackle this problem a risk assessment framework has been invoked. As recently described by Strauss et al. (1985), a reasonable framework for assessing risks of released microbes may be based on the U.S.E.P.A. framework for assessing health effects of toxic chemicals in the environment. This scheme consists of 4 basic stages: (1) hazard identification, the actual identification or estimation of the hazard involved, (2) exposure assessment, the estimation of exposure to the potentially harmful agent, (3) dose-response assessment, the assessment of responses to doses of the agent in case of exposure, and (4) risk characterization, the final attempt to quantify the risk. This conceptualization, for being relatively straightforward and simple, has been adopted by a number of groups working in risk assessment. However, alternative frameworks have been proposed, the most striking being those of Tiedje et al. (1989) and the one of the NAS (1989). The latter one brings in the interesting concept of familiarity as a criterion for judgement, i.e. the more predictable the characteristics and behaviour of a GEM are, the lesser the risk of the occurrence of unpredictable events. The familiarity concept seems to provide a powerful risk management tool. As described by Strauss et al. (1985), "if there is experience with relevant aspects of the microbe, its intended use, and intended location, and no adverse effects have been noted, then no further analysis is necessary, however if any one of these aspects is unknown, then further analysis is necessary". However great the potential for this scheme, it will probably have to be worked out further in practical terms before it can be made operational. This review will further deal with aspects of the former U.S.E.P.A. framework.

The ultimate goal of the risk assessment endeavour would seem to be to provide reliable, quantitative predictions of risks associated with the use of microbes in the environment. The

four-step scheme can be seen as a guideline for the acquisition of such information. Strauss et al. (1985) proposed that the outcomes (in "quantitative" terms) of the exposure and hazard/ dose-exposure assessments, taken together in a probabilistic or deterministic manner, would be able to provide some measure of risk ("risk characterization"). It is clear that any of the conceptual risk assessment procedures require information on the following: (1) survival of the introduced GEM in the environment, (2) the nature of the heterologous element, (3) localization and transfer of the heterologous element, (4) ecosystem effects. These data put in a framework of risk assessment of the type presented in Fig. 1, implies a case-by-case approach for every intended release.

2.1 Hazard identification

Generally speaking, hazard of activities potentially detrimental to man, animals, plants may be classified on a somewhat arbitrary scale which ranges from catastrophical via critical, controlled and marginal to negligible (see Table 1). Activities with both the highest and the lowest classification would seem to be judged easily from a risk assessment perspective. Activities with potentially catastrophical outcomes, such as the release of bacteria severely pathogenetic to man or animals and plants (as was done with *Bacillus anthracis* in the second world war), would obviously have to be severely restricted or forbidden, whereas activities with negligible risk (such as the use of modified *Lactobacilli* in cheese) would probably be liberated. If is in the middle categories, i.e. the activities of which the potential hazard may be rated as anywhere from critical to marginal, where problems emerge, since here judgements will have to be made based on disputable arguments. A precise detection of hazard under all environmental conditions is a difficult task and might require toxicological testing prior to environmental experiments. In addition, a judgement on the hazard of certain ecosystem effects is often difficult given the large natural fluctuations observed and the undefinedness of ecosystems. Criteria on the acceptability of ecosystem effects will have to be developed for each ecosystem based on the naturally occurring fluctuations of defined parameters (see also risk characterization).

2.1.1 *Exposure assessment*

Exposure assessment can be defined as the quantification of the exposure of man, plants, animals and the ecosystem to the effects of the released microorganisms. Exposure is thus linked to the fate of the introduced GEM in the environment which in turn is affected by microbial survival, competition, selection, transport and gene transfer (as will be discussed in

Table 1. Potential hazardous effects following GEM release

Hazardous event	Hazard ranking
1. Pathogenicity to man, plants or animals	+++
2. Disturbance of ecological balance	+++
3. Causing unwanted biochemical reactions	++
4. Mobilizing toxic chemicals	++
5. Affect community diversity	+
6. Dominance over indigenous microorganisms	+
7. Dissemination of heterologous genes	+/-
8. Input of extra C and N	-

+++ : catastrophical hazard
++ : critical hazard
+ : between critical and controlled (depending on duration of the effect) hazard
+/- : marginal hazard (depending on the gene)
- : negligible hazard

practical risk assessment). There is an inherent dilemma in assessing exposure in that "accurate assessment would require testing in the field, but testing in the field would require prior estimation of risk and be therefore discouraged". Exposure assessment of an actual release requires information on the release method, the frequency and quantity in which the microbe is released, and on the climatic, geographic and hydrogeological characteristics of the release site. Monitoring data, i.e. data or models regarding persistence, growth or the transport of the microbe, and data on the identity, size and location of susceptible populations and processes are also needed (Gillet et al., 1985). Given the infeasibility of testing all organisms or processes, the identification of sensitive groups of organisms and ecosystem processes has been advocated (See Table 2). Obviously, methods should be available to convert the ambient exposure concentrations into doses taken in by the organism, population or ecosystem of concern.

2.1.2 *Dose-response assessment*

Microorganisms produce their effect in a dose-related manner which can be seen as a function of hazard and fate. The exact dose which produces an effect, however, will differ between microorganisms and be dependent also on environmental conditions. Some microbes may be neutral (produce no effect) when present in numbers below a certain threshold, whereas others may produce an effect even when only one cell is present.

2.1.3 *Risk characterization*

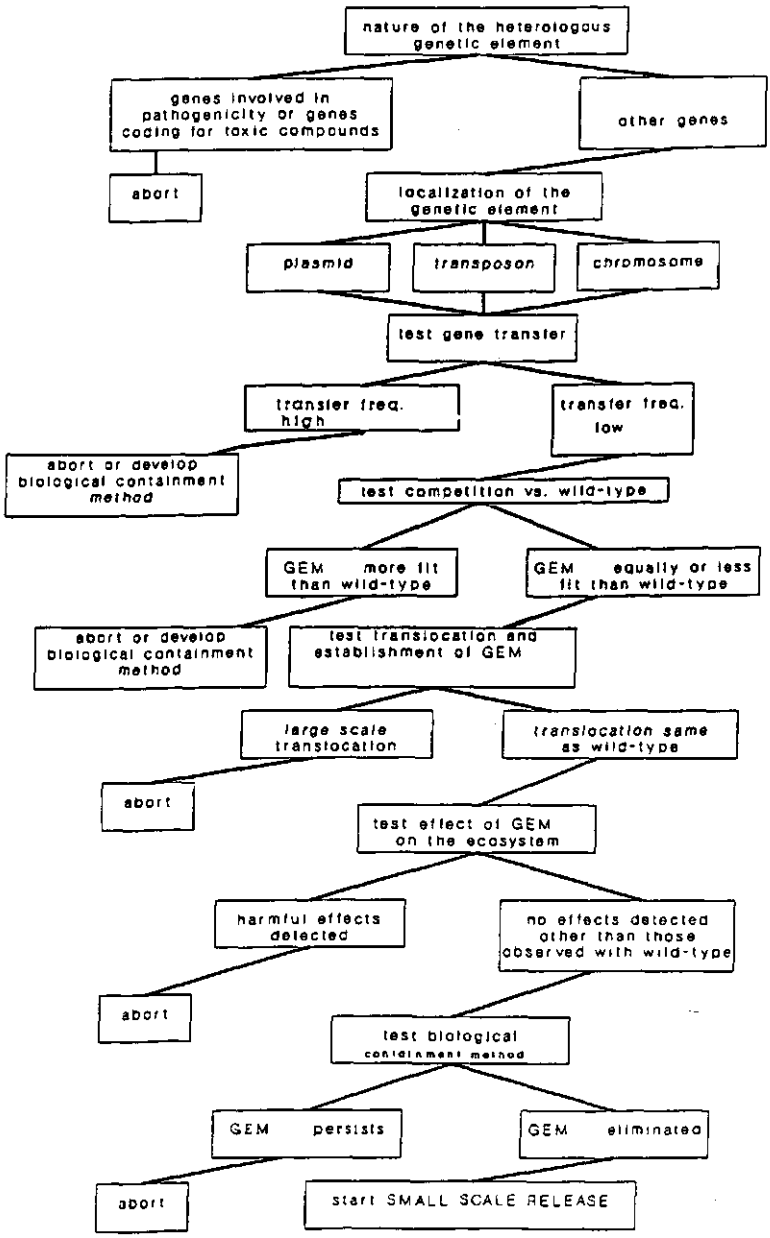
Risk characterization combines the quantitative prediction of the exposure assessment with the quantitative dose-response data for each hazard identified. The outcome would be some quantitation of risk in the form of a number. Interpretation, use and communication of such "quantitation of risk" pose additional problems outlined in Levin and Strauss (1990). An example of final risk characterization is the case of a low-hazard organism of which high doses would be needed to obtain a response, which would represent little exposure of sensitive organisms, systems, or processes. Such an organism could probably be safely released, since its risk can be judged to be negligible. The case of a highly-hazardous organism with high exposure of sensitive organisms, systems, or processes would probably lead to a high risk figure, impeding its release.

3. Practical risk assessment: gathering data

As mentioned, the potential risk of introduced GEMs is controlled by bacterial factors determining their hazard (e.g. pathogenicity, invasiveness), and by exposure related factors such as organism survival and persistence, transport to other sites and gene transfer (enhancing the potential exposure due to expression of the heterologous genes in other organisms). Both types of factors are affected by environmental factors. It is primordial to obtain data on these factors, first in microcosms as long as both hazard and exposure are relatively unknown, and if the anticipated risk is acceptable, in the field. Some methods and procedures useful for exposure assessment will be briefly treated. However, the reader is referred to Wellington and Van Elsas (1992) and Stotzky et al. (1990) for a thorough description of detection methods useful in exposure assessment.

The decision to allow the release of GEMs in a small-scale field trial is dependent on the possible anticipated risks of the introduction, the success of this introduction and the extent of the beneficial effect. The success of the introduction and the extent of the effect depends on the initial cell number, GEM survival, activity, gene expression and possibly even gene transfer. However, an increase of any of these parameters also results in a potentially greater environmental risk since the exposure might be enhanced. In this context it is tempting to allow the use of a GEM more readily when the beneficial effect seems to outweigh the potential risk. However, the decision should be made on scientific criteria based on experimental data (Katz and Marquis, 1990). This leads to the dilemma of the use of GEMs in the environment, since any diminishment of the risk by reduction

Fig. 1. A decision tree for the evaluation of the possible release of GEMs in the ecosystem.



of any of the factors inevitably leads to a reduction of the effect of the GEM introduction. For instance if the number of introduced bacteria is lowered, the effectiveness of the introduction may generally be reduced. While the effect of the introduction can be quantified (e.g. higher crop yields in agriculture, or decreased levels of toxic compounds), risk, composed of hazard, exposure and dose-response relationship, is not easy to quantify experimentally. Nevertheless, an attempt to do so will have to be made. Possible types of hazard are listed in Table 1, but as discussed earlier it is difficult or impossible to predict and assess all these potential hazards in detail. For instance, displacement of previously unknown species from the soil ecosystem upon introduction are undetectable because the soil ecosystem is heterogenous and our knowledge of the ecology and genetics of bacteria in soil is very limited. However, it is reasonable to state that the environmental fitness of GEMs should not be greater then that of the unmanipulated strain, assuming that the strain chosen for manipulation originates from the environment into which the GEM is introduced, if release of the GEM is to be acceptable. This would mean that each engineered strain has to be thoroughly studied in microcosm experiments, which coincides with the current opinion on GEM release that each release should be judged on a case-by-case basis. Comparing behaviour of the GEM with the wild-type organism in a series of experiments mimicking environmental conditions can be a way of quantifying the risk of displacement.

The flow scheme depicted in Fig. 1 gives a basic set of questions and experiments which may lead to a decision whether the organism may be released in a small-scale field trial or not. The microcosm experiments that should be performed and the problems that could arise will be discussed in the next chapters. Although development of microcosms mimicking environmental conditions is very important for risk assessment studies, this subject will not be discussed in this review, since a number of papers on this subject have been published recently (Bolton et al., 1991; Elliot et al., 1986; Greenberg et al., 1988).

In stead of discussing all possible environmental parameters influencing the fate of GEMs, the emphasis will lie on the effect of the genetic modification on the fate of the GEM and its DNA.

3.1 *Survival, competition and translocation*

Bacterial populations introduced into soil often show a progressive decline (Compeau et al., 1988; Liang et al., 1982; Van Elsas et al., 1986; Van Elsas et al., 1989) and bacterial establishment has often been hindered by a lack of success due to poor survival of these bacteria in the environment (Van Elsas et al., 1991). Soil texture, moisture content, temperature, pH, the presence of plant roots, minerals, organic matter, competition and antagonism by other microorganisms and predation by protozoa are all important factors influencing bacterial survival (Van Elsas and Trevors, 1991). These parameters should be as close to the natural situation as possible when performing microcosm experiments with GEMs.

Sometimes it is assumed that a GEM will have a lower ecological fitness then the wild-type strain because of the extra metabolic load of the inserted genes (Lenski, 1991; Tiedje et al.,

1989; Van Elsas and Trevors, 1991; Van Elsas et al., 1991). Given these facts one would expect that GEMs introduced into the environment will decline even more rapidly in numbers than the corresponding wild-type strains without exerting much of the intended effect of their introduction. However, some GEMs have been shown to have a slight growth advantage over the wild-type strain in chemostat cultures (Biel and Hartl, 1983; Edlin et al., 1984), but chemostat conditions can hardly be compared with environmental ecosystems. In competition experiments, GEMs or plasmid bearing strains have been shown to survive less well in soil then the wild-type strain when no obvious selection pressure for the GEM was present (Van Elsas et al., 1989; Van Elsas et al., 1991; Wang et al., 1990). This is thought to be a result of the extra metabolic load of the additional genes and it might ultimately lead to complete displacement of the GEM or loss of the extra genetic element or loss of expression of the extra trait. However, Orvos et al. (1990) did not detect any differences in survival between engineered and wild-type *Erwinia carotovora* in soil microcosms. However, in performing competition experiments one should always be aware that the experimental design, inoculating GEM and wild-type organism in the same soil portion or in separate soil portions may make much difference. Generally, no differences in survival are found when the strains are studied in separate soil portions, but sometimes differences are observed in jointly inoculated microcosms as was outlined by Van Elsas (1992). Williamson and Hartl (1991) studied survival and virulence of genetically modified *Pseudomonas solanacearum* strains with enhanced and reduced levels of both endopolygalacturonase A and endoglucanase, enzymes which are involved in pathogenesis. All modified strains, both with higher and lower levels of enzyme activities showed a reduced virulence and no enhanced survival in non-sterile soil in comparison with wild type organisms. This points to the fact that a lowered survival can be caused by factors other than just the extra metabolic load. In pathogenesis, several genes are often involved and instead of improving the competitivety of the strain by simply increasing the expression of one of the genes, survival was significantly lowered, possibly because of complete disturbance of the concerted gene expression. Strains capable of breaking down xenobiotics, on the other hand, have an advantage over other (non-degrading) bacteria, since many xenobiotic compounds are toxic for the resident microflora and reduce microbial diversity and thus competition (Mergeay et al., 1990). The use of GEMs for bioremediation of polluted soils seems promising since the pollutant itself exerts the selection pressure for the GEM (Brokamp and Schmidt, 1991), often providing a specific carbon source. Upon degradation of the compound the selection pressure will disappear and resident microorganisms may displace the GEM population (Chatterjee et al., 1982; Kilbane et al., 1983).

The extent of active bacterial transport (migration) is generally considered to be limited in soil (Van Elsas and Trevors, 1991). However passive transport by wind, animals or percolating water has been suggested to represent a greater potential hazard. While transport by wind (Knudsen, 1989) or animals is hardly studied, water facilitated-transport has received more attention. Bacterial translocation by percolating water is determined by soil type and structure (Trevors et al., 1990) and by bacterial adhesion properties (DeFlaun et al., 1990;

Huysman, 1991). Genetic modification could alter these adhesion properties and thus influence translocation of the GEM in soil by water movement. DeFlaun et al. (1990) showed that *Pseudomonas fluorescens* mutants with different adhesion properties and different outer membrane profiles could be obtained by Tn*5* mutagenesis. These mutants exhibited an increased mobility with the water flow in soil. It was shown that the integration of Tn*5* had interrupted a gene coding for a 34 kD outer membrane protein which apparently was involved in adhesion. This work shows that chromosomal insertions can potentially influence microbial transport in soil, although in this case the adhesion-minus mutants were specifically selected by an enrichment procedure. Translocation of a GEM should thus be experimentally studied as opposed to that of the wild-type organism (See Fig. 1).

3.2 *The nature of the heterologous element*

Current view among many scientists working in the field of biotechnology risk assessment is that the nature of the heterologous gene which is to be inserted is of great importance with regard to potential risks (Warwick Symposium on GEMs, 1990). The release of a GEM will never be allowed if the cloned gene is involved in pathogenesis of beneficial, or exposed non-target organisms. The same holds for genes coding for compounds toxic to the same groups of organisms. If knowledge about adverse effects is absent, a testing procedure should be performed on non-target organisms which have a likelihood of exposure (Frederick and Pilsucki, 1990). Frederick and Pilsucki (1990) describe that while most *Bacillus thuringiensis* strains produce toxins that are very specific for certain insect genera or species, the ß-endotoxin could have adverse effects in a wide range of organisms when ingested, by inhibiting ATP incorporation.

One of the considerations besides toxicity is that genes from bacterial origin are in general more acceptable for use in bacteria then genes derived from eukaryotes. The more closely related the organism the heterologous gene was isolated from and the potential GEM are, the smaller the change is from an evolutionary point of view. Moreover, there seems hardly to be any barrier for the occurrence of natural gene transfer between closely related bacterial strains inhabiting the same environment. This implies that if the heterologous gene is present in bacteria that reside in the same environment as the strain which is to be manipulated, the gene could also end up in the potential GEM by a naturally occurring process. That this actually can happen was described by Hartly (1984). Hartly (1984) transferred genes of the pentitol operon from *Klebsiella aerogenes* into *Escherichia coli* K12 using a transducing phage, in order to study gene evolution. Almost simultaneously, an *E. coli* C strain was isolated from an other source, which contained the same pentitol operon in the same site on the chromosome. This spot proved to be a specific insertion site for transducing phage DNA. In this case a GEM had been constructed that actually already existed, and which was probably the result of a natural gene transfer (transduction) event. More recently Trieu-Cuot et al. (1991) also showed indirect evidence for natural genetic exchange when they found identical antibiotic resistance genes in various Gram-negative and Gram-positive species. The indirect evidence for the occurrence

of gene transfer events by comparing gene sequences in different organisms is more thoroughly discussed by Van Elsas (Wellington and Van Elsas, 1992).

3.3 *Localization and transfer of the genetic element*

The processes which can be responsible for transfer of genetic elements are transduction, transformation, conjugation and transposition. These processes have been shown to occur in natural environments and each of them, or combinations of them, can mediate the transfer of genetic elements between bacteria under different circumstances and with different probabilities. One of the important factors in this respect is the localization of the genetic element and the way it is cloned and inserted into the genome. The genetic element can be inserted in a plasmid, which can be either self-transmissible, mobilizable or non-mobilizable, or into the chromosome using a transposon, a disarmed transposon (Herrero et al., 1990) or by homologous recombination (Waalwijk et al., 1991). Despite their convenience, plasmids are generally not considered to be the vectors of choice for constructing GEMs for environmental release, because they can potentially be transferred to other bacteria by conjugation at fairly high rates (Curtis, 1988; Van Elsas and Trevors, 1991). Heterologous genes should preferentially be inserted into the chromosome by homologous recombination or by using a disarmed transposon. The use of an intact transposon with the gene is less advisable, since the transposon can still jump to other spots and into plasmids thus enhancing the transferability of the heterologous gene. The localization of the gene should, as a general rule, not increase its transfer frequency as compared to its location in its original host. This is obviously no absolute guarantee for not enhancing the possibilities for transfer of the gene, because other factors such as the host species, cell number and activity, the specific niche of the GEM allowing contact with other cells, differences in restriction system and the susceptibility to transducing phages, all might affect the gene transfer frequency.

At present, experimental data on gene transfer in microcosms mimicking the environment are abundant. Conjugation was found to take place in soil with relatively high frequencies from an introduced donor to an introduced recipient strain when stimulating factors such as nutrient exudating plant roots, certain clay minerals, nutrients or xenobiotics were present (Richaume et al., 1989; Wellington et al., 1990; Mergeay et al., 1990; Van Elsas et al., 1988; Smit and Van Elsas, 1990; Stotzky, 1989). Plasmid transfer in soil from introduced GEMs to indigenous microorganisms, which might be more relevant regarding risk assessment than donor versus recipient experiments was also recently reported (Henschke and Schmidt, 1990; Smit et al., 1991). Plasmid transfer has also been detected in river epilithon (Fry and Day, 1991), in lake water (O'Morchoe et al., 1988), in sewage (McClure et al., 1990) and *in planta* (Lacy and Leary, 1975). Transfer of plasmids carrying genes for the breakdown of xenobiotics and resistance of heavy metals has been reported to occur in environments polluted with these compounds (Brokamp and Schmidt, 1991; Fulthorpe and Campbell-Wyndham, 1991; Top et al., 1990). As a general conclusion regarding conjugal gene transfer in soil it can be hypothesized that any parameter which stimulates activity of the introduced strain (e.g. plant

roots, nutrients, certain clay minerals, lack of competition) also stimulates conjugal plasmid transfer.

Gene transfer mediated by transduction was detected in soil (Zeph et al., 1988; Germida and Khachtourians, 1988; Lorenz and Wackernagel, 1991) and in lake water (Miller et al., 1990) and recently, evidence for the occurrence of transformation in sediments and aquatic systems (Paul et al., 1991; Stewart and Sinigalliano, 1990) has been provided.

These data support the concept that bacterial gene transfer is a common process in nature (albeit with low frequency), and they lead to the prediction that almost any gene could be transferred at a certain time. The fact that all bacterial species are different and adapted to specific niches and that identical genes are not very commonly found in distantly related species seems to contradict this frequent occurrence of gene transfer (Young, 1989; Campbell et al., 1986). Selection probably plays an important role, the majority of the newly acquired genes will be neutral or even disadvantageous for the bacteria and only a very small percentage will improve the competitive ability of the recipient organism. Neutral or disadvantageous genes may disappear or change by mutation, inversion or deletion, since there is no selective pressure for their function (see also competition and selection).

Some self-transmissible broad-host range plasmids can mediate the transfer of chromosomal DNA at frequencies between 10^{-3} and 10^{-8} (Haas and Reimmann, 1989). The presence of conjugative plasmids in environmentally-isolated bacteria has been confirmed (Pickup, 1989; Burton et al., 1982; Don and Pemberton, 1985; Schmidt and Schlegel, 1989; Sayler et al., 1990) and should definitely be taken into account in studies aiming to assess gene transfer risk. On one hand, the presence of these plasmids suggests that plasmid transfer is probably common in nature and that introduction of GEMs is nothing to worry about because gene rearrangement and exchange is a natural process. On the other hand, selftransmissible plasmids present in the environment might be responsible for recruiting and transferring genes from introduced GEMs and thus increase the chances of gene transfer and therefore exposure. Kelly and Reanney (1984) introduced RP1 into several mercury resistant soil bacteria which were not capable of transferring the resistance trait. In laboratory transfer experiments, they observed high frequencies (20 % of the isolates tested) of co-transfer of the mercury resistance gene, and analysis revealed that the gene had integrated into RP1. Some plasmids (e.g. plasmids of the IncP1 group) exhibit retrotransfer capability, i.e. they can mediate the transfer of plasmids or chromosomal DNA from other cells into the cell in which they are present (Mergeay et al., 1987; Diels et al., 1989). The presence of plasmids with retrotransfer capability in indigenous bacteria in the environment may result in the recruitment of chromosomally inserted genes from an introduced GEM at frequencies much higher then expected. Important in this respect is the observation of transfer of chromosomal DNA by the heavy metal resistance plasmid pMOL50, a derivative of plasmid pMOL28 obtained from an *Alcaligenes* species isolated from heavy metal contaminated soil (Diels et al., 1989).

Table 2. Parameters that can be monitored to study the soil ecosystem.

-microbial populations (based on cultivation)
 plating on specific media to enumerate certain groups of
 microorganisms
 enumeration of microorganisms by MPN techniques
-these methods can be combined with the use of specific
 DNA probes
-microscopical counts of
 total bacteria or fungi with general dyes
 specific species or taxonomic groups using antibodies or
 oligonucleotides coupled to fluorescent dyes
-microbial processes
 substrate induced respiration
 respiration
 nitrification
 denitrification
 sulphur oxidation
-enzyme activities
-biomass determinations
-ATP measurements
-DNA (RNA) extraction
 diversity determinations
 specific sequence detection with probes
 or PCR
-LPS extraction and analysis
-muramic acid analysis
-fatty acid and lipid analysis
-food-web interactions

More knowledge about plasmids from environmental bacteria capable of chromosome transfer could lead to a better understanding of natural genetic interactions between bacteria in soil. Assays may be developed to screen the environment for the presence of these plasmids, for instance by applying the polymerase chain reaction (PCR) to specifically amplify *Tra* functions, or by screening for plasmid mobilization by incubating environmental samples with a strain with a mobilizable plasmid. The filter mating assay designed to check the transfer ability of RP4p to a population of indigenous bacteria could in principal also be applied to detect natural mobilization (Smit and Van Elsas, 1989). Such experiments could be performed relatively quickly and give an idea about the chance that transfer of genetic elements might occur in

different ecosystems and under different circumstances without actually introducing a GEM in the environment.

3.4 *Ecological impact of GEM introduction*

GEMs could affect the soil ecosystem as well as non-target organisms which are likely to be exposed to doses producing an effect. These subjects will be treated separately.

3.4.1 *Effects on the soil ecosystem*

The effect of the introduction of GEMs on the soil ecosystem could range from (1) the input of organic substrate, to (2) displacement of certain species, (3) major changes in population structure and function, (4) disturbance of key ecological processes and (5) production of toxic metabolites (See Table 1). Some of these effects could be hard to detect because of a lack of detailed knowledge of the natural soil ecosystem and its structure and functioning. The first problem of detecting ecological changes is to select the parameters to be monitored. Table 2 shows a list of parameters that could be investigated. Each of the parameters has its specific advantages and disadvantages and each will give only limited information about the ecosystem.

Many different parameters have already been used in ecotoxicological studies: for instance the measurement of the overall respiration rate is considered to be useful for estimating effects of pollutants on (soil) ecosystems (Doelman and Haanstra, 1984). The effect of potential soil pollutants is assessed formally by the response of the SIR (Substrate Induced Respiration) method (Anderson and Domsch, 1987) in terms of CO_2 maximum, CO_2 output and the lag-phase. Doyle et al. (1991) measured CO_2 evolution after a genetically engineered *Pseudomonas putida,* capable of degrading 2,4-D, was introduced into soil microcosms. The CO_2 evolution rate was retarded for 35 days in the microcosms amended with both 2,4-D and the GEM (in the presence of glucose) when compared with the unamended soil with the GEM. There was no significant effect on the respiration of the introduction of the GEM without 2,4-D. Wang et al. (1990) observed a short-term increase (5 days) in CO_2 evolution rates when a recombinant *Streptomyces lividans* strain was introduced into soil compared to microcosms into which the wild-type strain was introduced. Although this effect was apparently caused by the nature of the GEM as opposed to the parent strain, the ecological hazard of such a transient enhancement can be debated.

Study of the fate of specific indigenous microbial populations, or certain groups can also be useful. As shown by Doyle et al. (1991) and Short et al. (1991), a decline in fungal propagules in the soil microcosms upon introduction of the GEM and 2,4-D occurred, and numbers of total bacteria, spore-forming bacteria and chitin-utilizing bacteria were transiently reduced. Doyle et al. (1991) concluded that any change in the indigenous populations, as a result of the introduction of a GEM, could affect the functioning of the soil ecosystem. They suggested a broad range of ecological parameters should be studied to monitor unanticipated effects.

Lacy and Stromberg (1991) investigated changes in populations of total bacteria, fluorescent

pseudomonads, actinomycetes and salt-tolerant bacteria by selective plating upon introduction of a GEM into soil and did not detect any significant effect in these parameters. Fluctuations in microbial populations may also be due to changes in temperature, moisture content, nutrient input and plant growth, both in time and space, obscuring possible effects of GEM introductions (e.g. Schurer et al., 1986; Feest and Madelin, 1988). Lacy and Stromberg (1991) state that major effects are predictable and that minor effects which could cause a cascade of events leading to great ecological disturbances are vague, difficult to define experimentally and still only speculation. That hazardous effects on the ecosystem are not always predictable was illustrated by the aforementioned work (Doyle et al., 1991; Short et al., 1991) in which fungal propagule numbers decreased dramatically because of the accumulation of the toxic intermediate 2,4-DCP. This observation highlights the importance of microcosm testing prior to release and shows that it is important to monitor a diversity of ecological factors.

Measuring enzyme activities might be more sensitive then monitoring CO_2 evolution in detecting changes in soil ecosystems, since populations possessing some specific enzymes are smaller then the large microbial community capable of mineralizing organic matter (Doelman and Haanstra, 1986; Burns and Slater, 1982; Burns, 1978). Doyle et al. (1991) for instance monitored phosphatase and arylsulfatase activity in their experiments and only found a suppression of dehydrogenase activity in soil after adding the 2,4-D degrading GEM and 2,4-D. When measuring enzyme activities, it is important to choose enzyme assays that are expected to be sensitive and which correlate with microbial numbers and other parameters (Burns, 1978).

Another factor that can be monitored upon GEM introduction is the diversity of the soil microbial community. This was done recently by Bej et al. (1991). They measured both phenotypical and genetic diversity after introduction of an engineered *Pseudomonas cepacea* able to degrade 2,4,5-T. Surprisingly both diversity indices increased during the incubation period as compared to the control. After six weeks, diversities had declined to the control levels again. The authors explained this increase by an increase of genetic interactions between the introduced strain, harbouring plasmids and a transposon, with the indigenous microorganisms. This method seems promising, however interpretation seems difficult and more data on the relation between ecosystem parameters and these diversity indices are needed.

Although these tests can never be fully standardized, a basic set of experiments should be developed as a guideline for the investigators.

3.4.2 *Effects on non-target organisms*

Potentially hazardous effects on non-target organisms (plants, animals, man) should also be screened for (Frederick and Pilsucki, 1990). A number of potential effects (e.g. those on plants or animals) can be tested in microcosms, whereas others (on man or bigger animals) could be obtained by analogy to known effects of exposure to similar organisms (invoking the familiarity concept) or by toxicological tests. It should be stressed that for intended releases only the effect of the added gene(s) should be taken into account, whereas effects of the carrier

organisms (assuming that they originate from the environment as into which the GEM will be introduced), can be easily judged from common knowledge possible supplemented with additional experimental toxicity data.

3.5 *Biological containment systems for GEMs*

Potential risk can be minimized using genetical systems which could under certain conditions lead to death of the GEM. The biological containment procedure used in industrial fermentors is based on debilitating the organism so that it can not survive outside the artificial environment of the chemostat. Obviously, this method is not suitable for GEMs which are intended to function in the environment. Therefore, Molin et al. (1987) devised a system based on genes which will kill the cell under certain conditions. This conditional killing system is based on the specific induction of the *hok* or the *rel*F or *gef* gene, (Gerdes et al., 1986; Knudsen and Karlstrom, 1991; Contreras et al., 1991) which code for a small polypeptide that is lethal to the cell. The *E. coli trp* promoter was chosen to regulate the expression of *hok*. The system worked in a wide range of bacteria and although a substantial part of the cells was killed (reduction of cell numbers with 10^{-4}) after induction, the system was not 100% effective. Knudsen and Karlstrom (1991) investigated several possible factors limiting the killing efficiency based on *rel*F regulated by a Lac promoter. In culture they found a reduction factor of approximately $5x10^{-5}$ upon induction with IPTG. Surviving cells appeared to possess a non-functional *rel*F gene, suggesting that mutation was responsible for the surviving cells. Duplication of the gene lowered the number of surviving cells to a great extent, but gene duplication does not seem to be the final answer since the presence of identical sequences of DNA can result in homologous recombination and thus reduce the advantage. Bej et al. (1988) studied the dynamics of an *E. coli* strain with the *hok* system fused to the IPTG inducible *lac* promoter on a plasmid in the soil environment. The containment system was shown to function in soil, since cells containing the inducible killing system declined 90 to 99% after induction. The system was, however, not adequate to provide a fail-safe GEM containment. Therefore it was recently suggested to use a stochastic GEM containment system (Molin, pers. comm.). For this purpose, a system containing repeat sequences is used, which upon recombinational excision results in the joining of a functional promoter and the killing gene sequence; subsequent cell death ensues.

Providing the GEMs with these systems can significantly reduce the potential risk of deliberate releases which might lead to an increase in the possibility that small-scale field tests will be allowed by the authorities. This will undoubtedly lead to an accumulation of experimental field data, which is necessary to get a better understanding of the effects of introductions into the open field.

4. Conclusions

It is evident from the theoretical framework and experimental data, that theoretical risk

assessment of GEM releases, and experimental work have developed along separate lines. It is hoped that the present considerations may serve to bridge the gap, e.g. put studies on effects of GEMs in the perspective of hazard identification and those on survival, transport and gene transfer in the perspective of exposure assessment. It is clear that in the extreme cases of no hazard, catastrophical hazard, but also when exposure is excluded, no further assessment is needed, i.e. the planned release may be either permitted without constraints, or should be forbidden. For intermediate cases, further assessment is obviously needed using microcosms, and using biological containment systems in field studies.

Practical risk assessment has been developing along lines addressing mainly exposure. Most studies have focused on bacterial survival and transfer of genetic elements, whereas few publications have addressed GEM transport, competition and GEM impact on the ecosystem. However, some studies describe the fate of GEMs in soil and especially translocation, gene expression and transfer, and the effect on the ecosystem, and show that these processes can not always be predicted. Experiments on genetic transfer seem to confirm more and more that most of the processes which can mediate transfer can occur in the environment, and that while transfer frequencies can be limited by chromosomal insertion, it can probably not prevent transfer. GEM survival and transport seems to be dependent on the strain characteristics and the soil type and genetic modification can potentially influence transport properties and survival.

It is proposed that when the GEM, of which no hazardous effects have been noted in microcosms, behaves similar with respect to survival (or is less competitive than) and transport as the wild-type and when there is no effect on the ecosystem detected other than that caused by the wild-type and when the gene transfer frequency is below the arbitrarily chosen value of 10^{-8}, a small-scale field trial can be performed (See Fig. 1).

Knowledge on the ecological impact of GEMs is scarce and more importantly, it is not clear which ecological parameters should be selected for monitoring. Specialists in the field of microbial ecology and ecotoxicology should present the decision makers and the researchers an experimental framework for monitoring these possible changes. The biological containment systems seem to work reasonable well at present, although high reversion rates are still a problem that will have to be solved, and the application of these methods might accelerate obtaining permission for field testing. It seems clear that the fate and impact of each GEM is dependent on the interactions between the ecosystem and the introduced organism, which should be studied thoroughly in a case-by-case manner.

It should be emphasized, that risk assessment implies the certainty that any risk exclusion does not occur. Therefore, risk assessment should be considered as the determination of the biological safety of released GEMs in connection to the biological effectiveness or beneficial effect. Thus, risk assessment as discussed here should lead to boundaries of risk management, ultimately set by society, what may vary depending on the expected benefits of the use of the GEM.

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CHAPTER 3

Determination of Plasmid Transfer Frequency in Soil: Consequences of Bacterial Mating on Selective Agar Media

E. Smit and J.D van Elsas

Summary

The possible occurrence of bacterial matings on transconjugant-selective plates used in experiments aimed at assessing conjugal transfer in soil was investigated. Matings on transconjugant-selective plates (with rifampicin, tetracycline, and kanamycin) between donor and recipient cells were shown to occur depending on the number of parent cells. Temperature also influenced conjugation frequencies on agar plates, since a lower temperature resulted in a decreased number of transconjugants. The use of nalidixic acid instead of streptomycin in conjunction with rifampicin for donor counter selection inhibited conjugation on selective plates. Conjugation in soil was analyzed by plating on selective media with nalidixic acid or streptomycin. The results indicated that conjugation in bentonite- or nutrient-amended soil was readily detected; however, part of the transconjugants on media without nalidixic acid could be assigned to be the result of matings on the selective plates. Conjugation was also stimulated in the presence of plant roots. Colony hybridization experiments confirmed the presence of plasmid RP4 in the transconjugants.

Introduction

Recently, interest in the detection of microorganisms and gene transfer between bacteria in soil has increased (Bentjen et al., 1989; Halvorson et al., 1985; Holben et al., 1988; Steffan and Atlas, 1988; Trevors and Van Elsas, 1989). The possibilities of using genetically engineered microorganisms (GEMs) to improve agricultural production (Davison, 1988) raised interest in the dynamics of introduced bacteria in soil and the occurrence of gene transfer. Although the use of GEMs seems promising, there is not enough knowledge about the possible unwanted effects, such as persistence of the GEMs or transfer of genes to other microorganisms (Brill, 1985; Collwell et al., 1988; Ford and Olson, 1988; Halvorson et al., 1985). So far, conjugation is considered as the process of highest ecological importance regarding gene transfer in soil (Reanney, 1977; Reanney et al., 1983; Trevors et al., 1987). Some authors even state that gene transfer is part of bacterial communication (Shapiro, 1985) and a means of accelerating bacterial evolution (Reanney, 1977; Sukhodolets, 1988). To study conjugation in soil, usually a donor strain containing a selftransmissible plasmid and a recipient are sequentially introduced. After incubation, the bacteria are extracted from

the soil and plated on agar plates selective for transconjugants. The transconjugants were selected by a combination of chromosomal resistances of the recipient strain and plasmid-encoded determined resistances (Van Elsas et al., 1988a, b, 1989, 1990). Factors such as favourable temperature, the presence of a rhizosphere, the availability of nutrients, or the presence of bentonite clay have been shown to enhance the conjugation frequency (Van Elsas et al., 1987, 1988a, b, 1989, 1990).

It is our aim to use the information derived from these model systems in order to develop a sensitive method to detect gene transfer from GEMs to indigenous soil bacteria. The occurrence of matings on selective agar media (plate matings) could potentially hinder such assays; they would erroneously increase the frequency of gene transfer.

The present work was designed to investigate (a) whether plasmid transfer could take place on selective plates after the bacteria were extracted from soil; (b) to what extent these plate matings would obscure the mating events that actually took place in the soil; and (c) how these plate matings could be minimized.

Materials and Methods

Bacterial strains and plasmids.

A *Pseudomonas fluorescens* strain, denoted R2f, isolated from grassland rhizosphere was used in this study, as described before (Van Elsas et al., 1986, 1988a, b, 1989). To produce the plasmid donor strain, the broad host range plasmid RP4 was introduced into R2f by filter mating (Simon et al., 1983). This plasmid encodes genes necessary for conjugation and the antibiotic resistance genes kanamycin (Km), tetracycline (Tc) and ampicillin (Ap). Two spontaneous mutants, produced by repeated incubation at increasing antibiotic stress, of R2f, one resistant to rifampicin (Rp) and streptomycin (Sm) and the other resistant to Rp and nalidixic acid (Nx), were used as recipients. Donor and recipient strains were cultured in LB broth (tryptone 10 g, yeast extract 5 g, NaCl 10 g, H_2O 1 L; pH 7.2) under the appropriate antibiotic selective pressure (50 μg/ml Km + 50 μg/ml Rp + 50 μg/ml Nx or Sm for the recipient). To determine the number of cfu, cells were plated out on LB agar (LB broth supplemented with 1.5% Difco agar) containing the appropriate antibiotics (Km + Tc for donors, Rp + Sm or Rp + Nx for recipients, and combined antibiotics for transconjugants). Cycloheximide (Ch; 100 μg/ml) was added for enumerations of cfu in samples from soil.

Soil.

The soil used in these experiments, Ede loamy sand, has been described elsewhere (Van Elsas et al., 1986). It was freshly collected, air-dried to about 10% moisture content, and then sieved (4 mm mesh). For some experiments soil amended with 10% (wt/wt) bentonite clay or with LB broth (1 ml/10 g) was used, since in previous work it was shown that plasmid transfer was readily detectable in these amended soil samples

(Van Elsas et al., 1986, 1988a, b, 1989).

Mating studies using bacterial cultures.

In order to study plate matings, two batches of overnight cultures of donor and recipient (Rp^rSm^r and Rp^rNx^r) were washed and diluted in sterile demineralized water (SDW). Separate drops (0.1 ml) of each dilution from both cultures were added in quadruplicate on transconjugant selective plates, left for 5 min, and then mixed by spreading with a Drigalsky spreader. Dilutions of the donor and recipient cell suspension were also plated out separately to determine the number of cfu as well as the mutation frequencies to antibiotic resistance. Experiments were done at different temperatures (4°, 20° and 27°C); plates were kept at these temperatures several hours before plating. Inoculated plates were kept at these temperatures for 3 h before incubation at 28°C for 48 h. After incubation donor, recipient and transconjugant cfu were determined and the mean number of cfu per plate was calculated. Figures represent the mean of two separate experiments. In order to determine whether plate mating could be inhibited, nalidixic acid (Nx), which inhibits DNA synthesis (Lloyd et al., 1980; Sugino et al., 1977), was chosen for donor counter selection, and an Rp^rNx^r mutant was employed as a recipient.

Assessment of plasmid transfer in soil, and the effect of plate mating.

In order to investigate whether plate matings could take place between bacteria extracted from soil, portions of 40 g bentonite-amended soil were inoculated with donor and Rp^rNx^r recipient cells in SDW (recipient added 10 min after donor), establishing a soil moisture content of approximately 20%. Donor and both recipients were inoculated at different cell densities so that their numbers in soil would be about 10^6, 10^7, and 10^8 cells/g soil. Soil was then mixed and added to a vessel to a bulk density of 1.4 (wet weight) and incubated at 20°C for 2 and 8 days. Duplicate vessels were used per treatment. After incubation cells were extracted by shaking 10 g of soil in Erlenmeyer flasks containing 95 ml sterile 0.1% (wt/vol) sodium pyrophosphate (NaPP) and 10 g of gravel (10 min, 290 rpm). As control treatment, donor and recipient cells were inoculated at similar cell densities in separate soil portions, extracted, and plated out together so that the possible occurrence of transconjugants could only be the result of plate mating. The numbers of donor, recipient and transconjugants were determined by plating appropriate dilutions of soil samples in NaPP (0.1%) on selective media. Counts were expressed as cfu/g dry soil; four plates were used per sample dilution.

Similar experiments were performed in soil amended with nutrients instead of bentonite clay. Donor and the Rp^rNx^r and Rp^rSm^r recipients were introduced at population densities of approximately $5x10^6$ and $5x10^7$ cells/g soil to compare conjugational behaviour in soil. Soil portions (40 g) were sequentially inoculated with donor and recipient (each in 2 ml LB broth) as described before (Van Elsas et al., 1987) and incubated for 24 at 20°C. Soil portions where then analyzed as described

above.

To screen for spontaneous mutants, donor and recipient cell populations (Rp^rSm^r, Rp^rNx^r) were each introduced into separate soil portions (1 - 10^8 cells/g). Samples of these portions were plated again separately on transconjugant-selective medium after 24 h of incubation at 20°C.

Assessment of plasmid transfer in rhizosphere and non-rhizosphere soil and the influence of matings on selective plates.

Experiments in rhizosphere and non-rhizosphere soil as described previously (Van Elsas et al., 1988a, b, 1989) were performed with both the Rp^rSm^r and the Rp^rNx^r recipients to critically access the obscuring effect plate matings might exert on plasmid transfer frequencies. Soil portions (50 g) were inoculated with about $5x10^6$ and $5x10^7$ cells/g soil and added to soil chambers of the model soil rhizosphere system (Dijkstra et al., 1987) in which wheat plants (*Triticum aestivum* var. *sicco*) were grown (Van Elsas et al., 1988b). The model rhizosphere system was designed to separate the roots from the soil by a nylon membrane so that a rootmat is formed on the top of the soil. The soil adhering to the membrane is regarded as rhizosphere soil, while soil at deeper layers (more than 1 cm from the membrane) is regarded as bulk soil. The system was incubated for 8 days in a climate chamber under a day/night cycle (16/8 h) at 20°/16°C. After incubation, rhizosphere (0-1 mm) and non-rhizosphere ($\geq$ 10 mm) samples were analyzed by shaking the soil ($\pm$ 1 g) in tubes with sterile 9.5 ml 0.1% NaPP and 1 g of gravel. The numbers of donor, recipient, and transconjugants were enumerated as described above.

Molecular analysis of transconjugants.

The presence of RP4 in colonies grown on transconjugant-selective agar medium was confirmed by colony filter hybridization with whole plasmid RP4 DNA as a probe. This method is based on the Grunstein and Hogness protocol described in Maniatis et al. (1982). The colony blots were washed under the most stringent conditions.

Statistics.

Data were analyzed by analysis of variance (ANOVA) at a probability level ($P<0.05$).

Results

Mating studies with bacterial cultures.

To investigate whether conjugation could occur on plates selective for transconjugants and to study the influence of donor and recipient cell numbers on the frequency of conjugation, dilutions of donor and Rp^rSm^r recipient cultures were plated out together on transconjugant selective plates. There is linear relationship between the log number

of donor and recipient cells (expressed as log √DXR) and the log number of transconjugant cells (Fig. 1). The graph suggested that the maximum number of donor and recipient cells per Petri dish that could be plated without conjugation taking place was about 10^5 (20°C curve). When very high cell densities were present (27°C curve), the increase in the number of transconjugants became smaller. The experiments were done at three different temperatures because temperature was thought to influence the conjugation frequency (Van Elsas et al., 1988a). The conjugation frequency is clearly influenced by a change in temperature, as shown in Fig. 1. Regression analysis showed that the effect of temperature was significant ($P<0.05$). Plating at 4°C reduced the number of transconjugants as compared with plating at 20° and 27°C. The number of parental cells that could be plated without conjugation taking place at 4°C was about 10^6, tenfold higher than at 20°C. At 27°C, conjugation frequencies are higher, and the curve is probably levelling off to a certain maximum at very high cell densities.

In order to inhibit conjugation on selective plates, Nx was used (Lloyd et al., 1980; Sugino et al., 1977) instead of Sm (both in combination with Rp) for donor counter selection. The reduction in the occurrence of matings was investigated by plating the donor and either the Rp'Sm' or the Rp'Nx' recipient on the appropriate selective

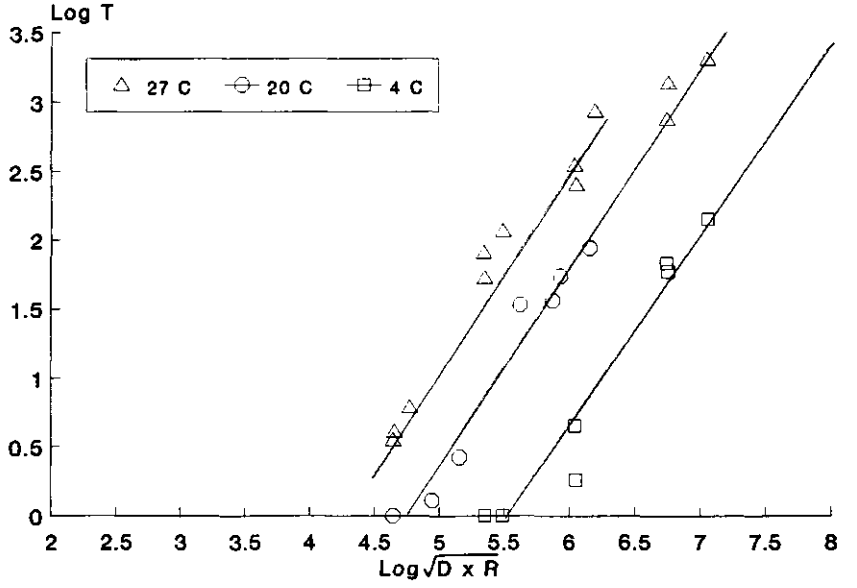

Fig. 1 The effect of temperature on matings on selective plates with freshly grown *Pseudomonas fluorescens* R2f donor (D) and recipient (R), (T: transconjugants).

Table 1. Number of transconjugants when different cell numbers of freshly grown donor and recipient (Rp^rSm^r versus Rp^rNx^r) were plated out on agar medium selective for transconjugants[a].

	Log cfu per plate			
			Transconjugant	
Log cells added	Donor	Recipient	Rp^rSm^r	Rp^rNx^r
6.3	6.32	6.27	2.8[b]	[0][c,d]
5.3	5.32	5.27	1.1[b]	[0][c]
4.3	4.32	4.27	0.4	[0]
3.3	3.32	3.27	[0]	[0]

[a] Results are averages of two experiments.
[b] is significantly higher than [c] ($p>0.05$).
[d] [0] = arithmetic zero.

media. The use of Nx (with Rp) almost completely inhibited matings between freshly cultured donor and recipient cells (Table 1), whereas matings were detectable with Sm (together with Rp). Previous results had indicated that there were no differences in conjugation frequencies between the Rp^rSm^r and the Rp^rNx^r recipient (data not shown).

Mating studies with bacteria from soil.

For studying the extent to which the interpretation of soil conjugation experiments is affected by conjugation on plates, donor and Rp^rNx^r recipient were introduced into the same soil portions and subsequently plated on transconjugant selective media with and without Nx. Donor and recipient were also inoculated in separate soil portions and plated together so that all transconjugants would result from matings on selective plates. The numbers of transconjugants (Table 2) on plates with Nx were in most cases significantly ($P<0.05$) lower than the number of transconjugants on plates without Nx. The differences between these numbers were considered to represent the transconjugants resulting from matings on selective plates. The frequency of matings on the plate appeared to depend on the numbers of donor and recipient cells present in soil, which determine the numbers of parental cells on the selective plates. A relatively low number of surviving parental cells per gram of soil (donor log 4.89; recipient log 6.11; 2 days in soil) produced log 1.88 transconjugants, with and without Nx. These numbers of parent cells resulted in about 10^3 donor and 10^4 recipient cells

Table 2. Numbers of donor, recipient, and transconjugant cfu per gram dry soil in soil versus plate mating experiments

			Log cfu/g dry soil				
					Transconjugants		
Days in soil	Treatment[a]		Donor	Recipient	-Nx	+Nx	% plate mating
2	J[b]	8.0	7.92	8.24	4.32[d]	4.00[e]	50
		7.0	6.88	7.45	3.42[d]	2.62[e]	80
		7.0	7.01	7.02	3.08[d]	1.60[e]	85
		6.0	4.89	6.11	1.88	1.88	0
		6.0	5.69	6.16	[0][f]	[0][f]	0
2	S[c]	8.0	8.06	8.04	0	ND	0
		7.0	6.90	7.61	0	ND	0
		7.0	6.82	7.00	0	ND	0
		6.0	4.69	5.78	0	ND	0
		6.0	5.85	5.77	0	ND	0
8	J[b]	8.0	7.72	7.88	4.13[d]	3.53[e]	75
		7.0	6.79	6.93	1.97[d]	1.80[e]	30
		6.0	5.72	5.74	[0][f]	[0][f]	0
8	S[c]	8.0	7.53	7.43	2.54	ND	100
		7.0	6.20	6.28	0	ND	0
		6.0	5.14	6.61	0	ND	0

[a] Log.number of cells added gram/soil.

[b] J. Donor and recipient cells were inoculated in the same soil portion (joint inoculation).

[c] S. Donor and recipient cells were inoculated in separate soil portions and mixed on the plates (separate inoculation).

[d] is significantly higher than $^{e}P<0.05$.

[f] [0] arithmetic zero; below detection limit (above 1.4 cfu per g).

on the transconjugant selective plate. Mating on the plate is not expected with these cell densities (Table 1); this corresponds with the results (0% plate mating) (Table 2). However, higher surviving donor and recipient cell densities in soil, ranging from log 7 to log 8 per gram soil, resulted in transconjugants. At the expected parent cell numbers (10^5-10^6) on the plates, matings on plates could be predicted (Table 1) and were also found (Table 2). Even after 8 days of incubation in soil, matings between extracted cells on selective plates were detected (Table 2).

It is very striking that conjugation on selective plates was not detected when donor

and recipient cells inoculated in separate soil portions were plated out together, not even at the highest cell densities at which matings on plates were detectable in the joint inoculation treatment (Table 2). Only in one treatment, after 8 days in soil at highest cell densities, was conjugation on plates detected (Table 2).

The obscuring effect of matings on selective plates was also studied with cells from nutrient-amended soil portions. Both the Rp'Sm^r and Rp'Nx^r recipients were used to compare their abilities to act as recipients in soil. The results (Table 3) indicate that a substantial part (78%) of the Rp'Nx^r transconjugants detected could be ascribed to matings on selective plates as evidenced by comparing counts obtained on Nx-amended and unamended agar media; this fraction was comparable to that found in bentonite-amended soil at similar recipient cell densities (Table 2). However the lower number of donor cells (Table 3) makes a comparison with the results in Table 2 difficult to interpret.

Donor and Rp'Sm^r and Rp'Nx^r recipient cells inoculated into separate soil portions were separately plated on transconjugant-selective media to check for spontaneously resistant mutants; no mutants were detected. As an additional test, the colonies suspected to be transconjugants by their resistance pattern and colony morphology, turned out to be R2f type when streaked on King's B agar.

Table 3. The numbers of transconjugants resulting from soil versus plate matings; donor (D) and recipient (R) cfu per gram dry soil in nutrient-amended soil.

	Log cfu/g dry soil					
				Transconjugant		
Treatment[a]	Log.cells/g added	Donor	Recipient	(total) -Nx	(soil) +Nx	% plate mating
D+R1	6	5.37	6.07	3.64	ND[b]	ND
D+R1	7	6.74	7.02	4.43	ND	ND
D+R2	6	5.41	7.35	2.82[c]	2.10[d]	78
D+R2	7	5.69	7.31	3.77	3.18[d]	78

[a] Soil chambers were incubated for 2 days at 20°C. D = donor; R1 = recipient Rp'Sm^r; R2 = Rp'Nx^r.

[b] ND = not determined.

[c] is significantly higher than [d] ($P<0.05$).

Plasmid transfer in rhizosphere and non-rhizosphere soil and the influence of matings on selective agar plates.

Transconjugants were detected only in rhizosphere soil samples and not in corresponding non-rhizosphere samples (Table 4). Donor and both recipient cell numbers were two- to fourfold lower in non-rhizosphere samples than in rhizosphere samples. The Rp^{r}Sm^r and Rp^{r}Nx^r recipients were equally effective in receiving plasmid DNA. A minor fraction (30%-38%) of the transconjugants obtained with the Rp^{r}Nx^r recipient strain could be ascribed to matings on selective plates, whereas the bulk of the transconjugant population was apparently owing to matings in the rhizosphere. However, the differences between the numbers of transconjugants on plates with and without Nx were not significant, as they were in the bentonite- and nutrient-amended experiments (Tables 2 and 3).

Table 4. Numbers of transconjugants resulting from soil versus plate matings; donors (D) and recipients (R) per gram of dry rhizosphere and non-rhizosphere soil[a]

	Log cfu/g soil					
				Transconjugants		
Treatment	Log cells/g added	Donor	Recipient	-Nx	+Nx	% plate mating
D+R1[b]R	6	4.80	5.76	3.65	ND[c]	ND
NR	6	4.41	5.29	0	ND	N
D+R1 R	7	5.60	6.61	3.81	ND	ND
NR	7	5.21	6.10	0	ND	ND
D+R2[b]R	6	4.74	5.04	3.65[d]	3.49[e]	30
NR	6	4.43	4.73	0	0	0
D+R2[b]	R 7	5.60	6.22	3.66[d]	3.45[e]	30
NR	7	5.26	5.85	0	0	0

[a] Soil chambers were incubated for 8 days at 20°/16°C ('cycle'); they then sliced to obtain rhizosphere (R) and non-rhizosphere (NR) soil.

[b] D = donor; R1 = recipient Rp^{r}Sm^r; R2 = recipient Rp^{r}Nx^r.

[c] ND = not determined.

[d] is not significantly different from [e] ($P<0.05$)

Molecular analysis of transconjugants.

Transconjugants were randomly selected from all soil experiments and screened in a colony filter hybridization assay. The plasmid RP4 DNA was used as a probe. The

autoradiograph (not shown) confirmed that all transconjugants produced a hybridization signal with the probe, as did the positive controls (donor colonies) whereas the negative controls (recipients colonies) did not.

Discussion

When donor and recipient cells, freshly grown in LB broth, were plated on an agar medium selective for transconjugants, matings were detected. The antibiotics Rp, Sm, Tc and Km apparently did not inhibit bacteria to the extent at which genetic transfer via conjugation was completely impaired. The expression of the *tra*-genes in the donor cells necessary for RP4 transfer is probably constitutive. Although protein synthesis should be inhibited by Rp and Sm (Davis et al., 1974) present in the medium, conjugation might not be inhibited, since levels of *tra*-gene products present in donor cells might still be sufficient to promote matings. It could also be possible that action of Rp and Sm is delayed owing to relatively slow penetration into the cells, giving the cells time to conjugate. A possible method to circumvent matings on selective agar is by plating at lower temperatures.

The linear relationship between the log number of donor and recipient ($\sqrt{DxR}$) and the log number of transconjugants can be explained if one assumes that the number of transconjugants is dependent on the chance that a donor and recipient cell meet. This chance increases when higher cell densities are plated out up to a certain maximum (Fig. 1), when for instance the conjugation frequency is becoming the limiting factor. This concept also accounts for the fact that there is a minimal density of donor and recipient (between log 4 and log 5 per plate) required for conjugation on plates. Below these numbers, donor and recipient cells possibly are not able to form mating aggregates owing to physical separation.

Dependency of the conjugation frequency on selective plates on the temperature is very clearly demonstrated in Fig. 1. A lowered metabolic activity of the bacteria at lower temperatures might explain the lowered frequency of bacterial matings observed.

A simple method to inhibit matings on selective plates to a greater extent than by lowering the temperature was the use of Nx in transconjugant-selective agar in combination with an Nx-resistant recipient (Lloyd et al., 1980; Sugino et al., 1977), as suggested by S. Walter (personal communication). Nalidixic acid is known to interfere with DNA replication by inhibiting DNA gyrase activity (Davis et al., 1973; Ingraham et al., 1983; Lloyd et al., 1980; Sugini et al., 1977), which is necessary for successful conjugation, and to have a faster effect on the cells than Rp or Sm (Ingraham et al., 1983). The results (Table 1) demonstrated the difference in transconjugant numbers between media with and without Nx. This method was chosen to discriminate between matings on selective plates and soil conjugation in subsequent experiments. Soil conjugation was found in bentonite-amended or in nutrient-amended soil as well as in rhizosphere soil, but matings on selective plates were also detected (Tables 2-4). The

percentage of the total number of transconjugants resulting from matings on selective plates without nalidixic acid was found to vary from 30 to 85% depending on the number of parent cells. Matings on plates did not occur at low donor and recipient cell densities ($>10^4$ cells per plate). The fact that no matings on plates were detected when separately inoculated soil portions were plated out together, whereas cells from jointly inoculated soil portions produced transconjugants on plates (Table 2), was remarkable. Adherence of bacteria to small soil particles might be responsible for this effect, since cells in the joint inoculation might be closer than in the separate inoculation. The fact that donor and recipient cells located on different soil particles cannot make contact as easily as cells that are already existent on the same particles could explain this result.

The time that the bacteria resided in soil under nutrient-deprived conditions might play a role in reducing matings on the plate. After 8 days in soil (Table 2), at the lower cell densities, matings on plates are reduced when compared with the data after 2 days.

The numbers of transconjugants in the rhizosphere experiment attributable to soil matings (Table 4) were high relative to the number of donor and recipient cells when compared with corresponding cell numbers in the experiment with nutrient-amended soil (Table 3). The total number of transconjugants was in the range reported earlier (Van Elsas et al., 1988a, b, 1989, 1990). The difference between transconjugant numbers on plates with Nx and without Nx resulting in 30% mating on selective plates (Table 4) is small and not statistically significant ($P<0.05$). This is undoubtedly owing to the low numbers of parent cells, at which plate mating is not likely to occur. Stimulation of cell-to-cell contact could play an important role in the stimulatory effect of the rhizosphere on conjugation. Cells in the vicinity of roots (a favourable niche) may have the opportunity to grow, form microcolonies, and conjugate. Such small subpopulations could be responsible for the bulk of the transconjugant population. Soil amended with nutrients could potentially yield very high transconjugant numbers, but antagonistic interactions with indigenous soil microorganisms (also stimulated by the nutrients) may reduce this effect (Table 3). Amendment of soil with bentonite clay could also enhance cell-to-cell contact by reduction of the soil average pore size, thereby concentrating the bacteria. Bentonite clay has also been shown to enhance survival of *Pseudomonas fluorescens* R2f (Marshall, 1975; Van Elsas et al., 1986), rendering cell-to-cell contact more likely.

In conclusion, matings on selective plates definitely played a role in these soil mating studies and obscured the actual number of transconjugants caused by conjugation in soil; the use of Nx for donor counter selection was able to circumvent this problem and is, therefore, suggested for further work.

Acknowledgments

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CHAPTER 4

Methods to study plasmid transfer in soil

E. Smit and J.D. van Elsas

Introduction

Gene transfer in the environment has been identified as a process which potentially enhances putative hazards of released genetically engineered microorganisms (GEMs). This contentment has spurred recent research in this area. Much of the current knowledge on environmental gene transfer has been obtained in experiments in which transfer of a plasmid from an introduced donor to an co-introduced genetically marked recipient strain was studied (e.g. Van Elsas et al., 1987, 1988; Richaume et al., 1990; Top et al., 1990; Klingmüller, 1991). These experiments have provided insight in the influence of the environment on bacterial mating processes. However, they have not been *a priori* very predictive of the putative fate of introduced genetic material, i.e. concerning the transfer to the numerous different species present in the indigenous microbial community. In addition, most of these experiments have been focused on the transfer of genes inserted in (often self-transmissible) plasmids, whereas transfer of chromosomal sequences has been rarely studied.

This paper will address development of methods and design of markers for the study of environmental conjugal gene transfer, with a special emphasis on soil.

Transconjugant isolation and enumeration

To isolate bacteria from soil, a soil sample is added to a 0.1% sodium pyrophosphate solution (1:10 (w/v)) supplemented with gravel. After 10 min of vigorous shaking, appropriate dilutions can be plated out (Van Elsas et al., 1986). The agar medium may be chosen according to the purpose. A rich medium will primarily permit growth of copiotrophic soil bacteria (Hattori, 1986), whereas a low-nutrient medium favours oligotrophic organisms. Since any agar medium is selective for certain groups of soil bacteria one should ideally use several media, for instance a rich versus a poor nutrient agar, to detect gene transfer to indigenous soil bacteria.

Transconjugant colonies are generally recovered on selective agar, selecting genetically marked recipient bacteria that successfully received a plasmid. Selection is often based on antibiotic resistances, however other methods can be used (See selectable markers). Moreover, selective plating is necessary for donor counterselection. To study plasmid transfer in soil, both the organism and the DNA should possess unique properties which makes them discernible from the other organisms or from other DNA. Soil may contain between 10^7 and 10^9 culturable bacteria per gram (Alexander, 1977). Assuming

that one colony out of 200 can be isolated from a plate, the detection limit of microorganisms without selectable markers will be between $5x10^4$ and $5x10^6$ cells per gram of soil (using the method described above). Therefore, selection for the introduced organism or, more importantly, for possession of the transferred genetic element is necessary since plasmid transfer in soil is a rare event (Van Elsas et al., 1988a, 1990) and transconjugant numbers are often below the aforementioned detection limit. In genetic transfer experiments with introduced donors and recipients, transconjugant numbers may range between 10^2 and 10^4 cfu per gram of soil (Smit and Van Elsas, 1990), whereas in experiments aiming to detect transfer to indigenous bacteria, transconjugant numbers may be expected to be in this range or lower (Van Elsas and Trevors, 1990).

Colony hybridization

Definitive evidence that colonies enumerated on selective media actually contain the plasmid or gene being studied in gene transfer experiments are required to accurately assess gene transfer. In some studies a random number of colonies was selected and cultured and the presence of the plasmid transferred was confirmed by small-scale plasmid isolations (Van Elsas et al., 1988a, 1988b). However, it is very tedious to investigate large numbers of transconjugants in this manner. The presence of the genetic marker in relative large numbers of bacterial colonies can be screened routinely by using colony filter hybridization techniques (Sayler et al., 1985). As discussed earlier, the use of whole plasmid DNA as a probe could give false positives due to cross hybridization to DNA of indigenous bacteria (Trevors and Van Elsas, 1989; Van Elsas et al., 1989; Sayler et al., 1985). This technique has originally been developed to check for the presence of inserts in *Escherichia coli* in cloning experiments (Sambrook *et al.*, 1989), but has also been shown to be useful for studying colonies from mixed bacterial communities in the environment (Sayler et al., 1985). Briefly, colonies grown on selective plates can be transferred to nitrocellulose or nylon filters by applying the filters directly on the plates (Sayler et al., 1985; Sambrook et al., 1989). Cells can be lysed, and their DNA denatured, neutralized, and finally fixed to the filter by baking or UV treatment (Grunstein and Hogness, 1975; Sambrook et al., 1989). In our laboratory, the lysis procedure was checked by hybridizing colonies of indigenous soil bacteria with a 'universal' probe for prokaryotes, based on a conserved 16S RNA region (Niebel et al., 1987). Over ninety percent of the colonies produced enough DNA to give a hybridization signal (Table 1). Some *Bacillus* strains didn't produce a hybridization signal with this method, which suggested these didn't lyse sufficiently, since chromosomal DNA isolated from such strains did produce a hybridization signal with the universal probe in a dot blot experiment. Therefore, colony filter hybridization of soil isolates may be a suitable detection technique for many bacteria, but insufficient lysis of bacterial species may pose problems.

In addition, for calibration of the hybridization signals it is important to add a

positive (donor) and a negative control on the filters in every hybridization experiment. Sometimes, weak non-specific signals are found that may be due to cross reaction of the probe with cell components such as exopolysaccharides produced by the colonies. A severe limitation of colony hybridization is that it still relies on bacterial (selective) growth, i.e. it is subject to the same limits of detection valid for plating techniques. Therefore, it cannot be used successfully for detection of transconjugants if no adequate separation of donor and transconjugant colonies is achieved.

Donor counterselection

Plasmid transfer in soil can only be adequately studied by employing a donor counterselection method preventing the growth of relative high numbers of donor cells on low-dilution transconjugant selective plates (Van Elsas and Trevors, 1990; Van Elsas et al., 1989). Donor counterselection is usually achieved by using antibiotics and an antibiotic-resistant recipient (See also Chapter 4). Mutants resistant to rifampicin and streptomycin have been used in our laboratory (Van Elsas et al., 1988a, b). However, recent experiments have shown that matings on selective agar plates can occur when these antibiotics are used for donor counterselection (Smit and Van Elsas, 1990). This may result in an overestimation of actual plasmid transfers in the soil or rhizosphere. Since plasmid transfer via conjugation may only take a few minutes, it might occur on plates before rifampicin and streptomycin inhibit donor cell functions. Nalidixic acid, which prevents DNA synthesis, is, in combination with rifampicin, able to prevent these plate matings (Smit and Van Elsas, 1990; O'Morchoe et al., 1988), probably due to inhibition of DNA replication involved in conjugal plasmid transfer. Therefore the use of a nalidixic acid and rifampicin resistant recipient strain could prevent overestimation of plasmid transfer in soil owing to matings on selective agar plates.

If one is interested in plasmid transfer to indigenous soil bacteria, donor counterselection with a resistant recipient is not applicable (Van Elsas and Trevors, 1990). Several solutions have been proposed: (1) an inducible "host- killing"(*hok*) gene (Molin et al., 1987) on the donor chromosome, which will kill the donor on the plates after introduction; (2) a bacteriophage that specifically lyses the donor strain; (3) an auxotrophic mutant as a donor that cannot grow on the transconjugant-selective plates; (4) a donor that will not survive long in soil (Henschke and Schmidt, 1990); (5) a donor that cannot express the selective marker (Top et al., 1990). The use of a host-killing gene that can be induced by a substance added to the transconjugant-selective plates seems a very elegant donor counterselection method. However, it requires tedious cloning work and the extra genes and gene products can have a negative effect on host survival in soil. An auxotrophic mutant may also be less competitive in soil and selection might not be complete when certain substances present in soil end up on the plates. Henschke and Schmidt (1990) used an *Escherichia coli* donor strain which declined very rapidly in soil. This strain is obviously not suited for use as a GEM in

Table 1. Test of the colony filter hybridization method using a number of soil isolates after lysis and hybridization with a 'universal' probe (Niebel *et al.*, 1987).

Strains*	Hybridization signal
Escherichia coli JM 101	+
Escherichia coli S17-1	+
Pseudomonas fluorescens R2f	+
Pseudomonas fluorescens R12t	+
Pseudomonas fluorescens GE1	+
Pseudomonas putida CYM 318	+
Pseudomonas phaseolica BP4	+
Pseudomonas cepacia	+
Pseudomonas paucimobilis	+
Pseudomonas maltophilia	+
Pseudomonas aeruginosa	+
Bacillus subtilis	-
Bacillus cereus FoTC 30	-
Klebsiella aerogenes 418	+
Agrobacterium radiobacter	+
Alcaligenes sp.	+
Flavobacterium sp. F4	+
Flavobacterium sp. F3	+
Arthrobacter sp. C6	+
Arthrobacter sp. C2	+
Corynebacterium C3	+
Enterobacter cloaceae BE1	+

*All strains from the collection at ISFR, Wageningen. The two *Bacillus* species did not produce a hybridization signal, suggesting that the colonies did not lyse substantially, (DNA isolated from these strains hybridized to the probe in a dot blot experiment).

soil. Our own studies and those of others have focused on donor strains isolated from soil or rhizosphere that may compete reasonably well with the indigenous microorganisms. These are likely candidates as GEMs for application in soil, and gene transfer experiments with such organisms might better describe the events that take place after deliberate release.

We isolated a bacteriophage from agricultural drainage water that was specific for our donor strain, *Pseudomonas fluorescens R2f*. Incubation of a crude phage lysate (20 min., 10^9 phages ml^{-1}) with diluted soil suspensions, containing 10^7 donor cells per gram of soil, prior to plating, resulted in donor-free selective agar plates. The effect of the phage is shown in Figure 1. While without phage treatment plates were crowded with

donor colonies, treatment with phage permitted colonies of the indigenous bacteria to develop. Phage-resistant mutants of the donor occurred at a frequency of 10^{-5}, therefore donor cells are rarely found on selective plates when this phage is used. This method also inhibited matings on selective plates; however plate transformation and transduction are hypothetical problems, and proper controls are necessary. So far, we have not obtained evidence of the occurrence of transduction or transformation in our experiments.

Selectable markers

Genes that code for antibiotic or heavy metal resistances can be very useful as indicators of gene transfer events. Addition of one antibiotic to the agar medium often permits the detection of about 10^3-10^4 bacteria per gram of soil out of 10^7-10^9 total culturable bacteria. Not all antibiotic resistance genes are suitable; some (e.g. kanamycin, tetracycline) are more selective than others (e.g. ampicillin) but generally naturally-resistant bacteria are present in reduced numbers in the soil (Stotzky, 1989;

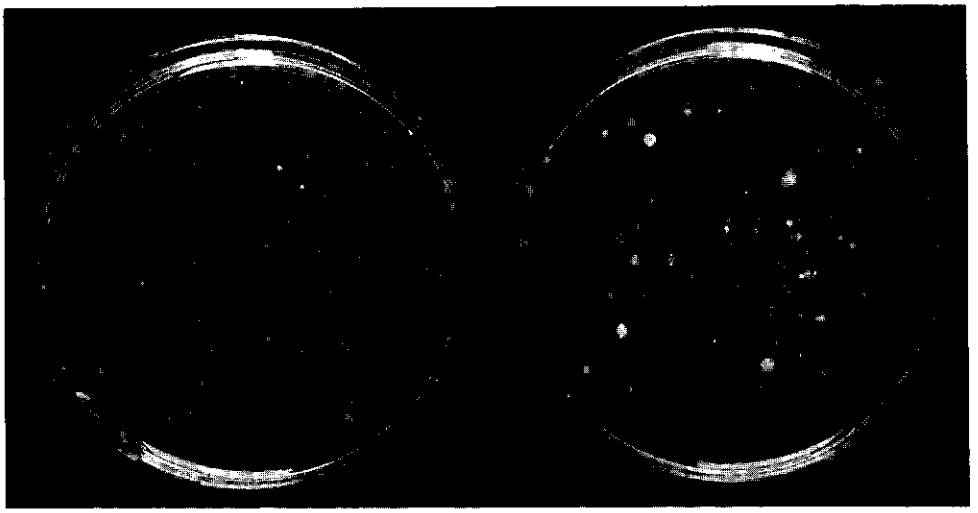

Fig. 1. A 100-fold diluted soil suspension, (soil contained 10^7 *Pseudomonas fluorescens* cells per gram of soil), was plated on 0.1 strength tryptone soya agar supplemented with ampicillin (50 µg/ml), tetracycline (50 µg/ml) and cycloheximide (100 µg/ml). Left plate: no phage treatment; right plate: incubated with a crude phage lysate (10^9 pfu/ml, 20 min.) prior to plating. The left plate is completely covered with small donor colonies (approximately 2000), the right plate shows well-developed colonies of indigenous microorganisms.

Henschke and Schmidt, 1990). Rifampicin, nalidixic acid and tetracycline have often been antibiotics chosen since background resistance levels in many soils were low. Also erythromycin, kanamycin and streptomycin have been used with somewhat higher background resistance levels (Van Elsas and Pereira, 1986; Weller, 1984). The number of antibiotic-resistant microorganisms in soil depends both on the antibiotic and on the type of soil. The levels of bacteria resistant to various antibiotics in Ede loamy sand (Table 1) indicate that a combination of two antibiotics significantly lowered the number of resistant colonies appearing on plates. To obtain optimal sensitivity, 2 or even 3 antibiotics have also been used, in studies with other soils; Liang et al. (1982) were able to lower the detection limit of introduced bacteria in soil to 25 cfu per gram of soil using this approach. The number of naturally-resistant soil bacteria should ideally be negligible when genetic transfer to indigenous microorganisms is studied using antibiotic resistance markers, since plating of low dilutions of the soil suspensions is often required. If the resulting plates are crowded with bacterial colonies, the transconjugants might be overgrown or inhibited in their growth. Also, analysis via colony filter hybridization can only be done with a limited number of colonies per plate.

In addition to selectable markers, non-selectable ones can be very useful. Non-selectable markers may be: (1) so-called "unique" (non-transcribed) DNA sequences, not present in soil bacteria (Devanas and Stotzky, 1986), to be used as hybridization markers; (2) genes that will make the colonies change colour, for instance *xylE* (Mermod et al., 1986; Winstanley et al., 1989) or *LacZ* (Drahos et al., 1986); (3) *Lux* genes that will make the colonies emit light (Shaw and Kado, 1986). Non-transcribed sequences have the advantage that they do not pose an extra metabolic load on the bacteria, which might affect survival and competition in soil (McCormick, 1986). Construction of marker genes with an inducible instead of a constitutive promoter (Mermod et al., 1986), such that the gene will only be expressed on plates containing the inducer, may circumvent this problem. Winstanley et al. (1989) constructed a marker cassette based on *xyl*E with a thermoregulated phage lambda promoter. At moderate temperatures there was no expression of *xyl*E, whereas at elevated temperatures the gene was expressed. The plasmid with the regulated gene appeared to be more stable then the plasmid with the unregulated one (Winstanley et al., 1989), probably because of the difference in metabolic load for the bacteria. The potential advantage of genes that change the appearance of the colony is that transconjugants can be presumptively identified without hybridization to specific probes. However, this identification should be confirmed using hybridization.

Antibiotic or heavy metal resistance markers still provide the best primary selection for transconjugants; the non-selective markers can only be used in addition to these thus distinguishing transconjugants from indigenous naturally resistant bacteria. Therefore most genetic transfer experiments are based on the use of one or several antibiotic or heavy metal resistance genes (Weinberg and Stotzky, 1972; Top et al., 1990; Van Elsas et al., 1988a, b, 1990; Richaume et al., 1989; Henschke and Schmidt,

1989).

Use of marker genes for tracking genetically modified bacteria and their DNA in the soil ecosystem.

To study the fate of genetically engineered microorganisms (GEMs) and their DNA in the soil ecosystem, it is necessary to be able to specifically detect these bacteria and their genes, and to distinguish them from the natural soil population. One of the purposes of our current gene transfer studies in soil has been to study the effect of the localization of a selectable marker in the bacterial genome, i.e. on a self-transmissible broad-host-range plasmid like those of the IncP1 group, on a mobilizable broad-host-range plasmid like IncQ, or inserted into the chromosome, on its mobility.

The IncP1 plasmid RP4 contains three antibiotic resistance genes (tetracycline, kanamycin and ampicillin) that are expressed in a wide range of different Gram-negative bacteria. Plating on double- or triple-selective media, followed by hybridization makes detection of rare transfer events possible. However, when whole plasmid DNA was used as a probe to detect transconjugants, cross-hybridization to DNA of indigenous soil bacteria occurred (Van Elsas et al., 1989; Van Elsas and Trevors, 1990). There are probably DNA sequences present on RP4 that hybridize to (chromosomal) DNA of some soil bacteria. To permit specific hybridization, a unique DNA sequence can be inserted in the plasmid (Devanas and Stotzky, 1986; Chaudry et al., 1989; Van Elsas and Trevors, 1990). We inserted a 0.69 kb sequence of a patatin gene (*pat*) into RP4, resulting in RP4*p* (Van Elsas and Trevors, 1990). Control experiments, plating uninoculated soil on media containing tetracycline and ampicillin failed to produce colonies hybridizing with a radioactively labelled *pat* probe, showing there was no detectable homologous DNA present in the soil bacteria screened.

To study the transfer and stability of non-selftransmissible elements a marker cassette was constructed to insert into an IncQ plasmid and into the chromosome. Main criterion for choice of marker genes was a low probability of occurrence of its phenotype and/or genotype in soil. Antibiotic resistance genes were chosen as prime candidates to make part of the marker cassette, for their capacity to provide the required selectability. A previous study of background resistance to several antibiotics revealed relatively high numbers of bacteria resistant to several antibiotics in the Ede loamy sand soil used in our laboratory. Levels of kanamycin resistant cfu were about 10^4 to 10^5 per gram of dry soil, whereas cfu resistant to a combination of kanamycin and gentamycin occurred at levels of, at most, 10^3 per gram dry soil. Therefore, we opted for the combined use of a gene conferring resistance to kanamycin, *npt*II (Simon et al., 1983), and a gene conferring resistance to gentamycin, *aad*B (Schmidt et al., 1988) in the cassette. The marker cassette should also contain a DNA sequence which is not expressed and which is not commonly found in soil bacteria in combination with the resistance genes. This sequence can be used for hybridization and PCR purposes. As such, part of the *cry*IVB gene, coding for a delta endotoxin, from *Bacillus*

thuringiensis var. morrisoni (Waalwijk et al., 1991), was selected. As will be described in the following, the marker gene cassette was cloned into the broad-host-range (Inc Q) plasmid pSUP104 (Priefer et al., 1985) which can be mobilized into different (soil) bacterial species, and into a disarmed transposon delivery vector (Herrero et al., 1990) to facilitate chromosomal insertion. Both methods were used to mark *Pseudomonas fluorescens* R2f, originally isolated from soil (Van Elsas et al., 1988), to study marker stability, recovery from soil, genetic transfer and expression of the genes in other soil bacteria, and PCR-mediated detection of the cassette from soil DNA extractions.

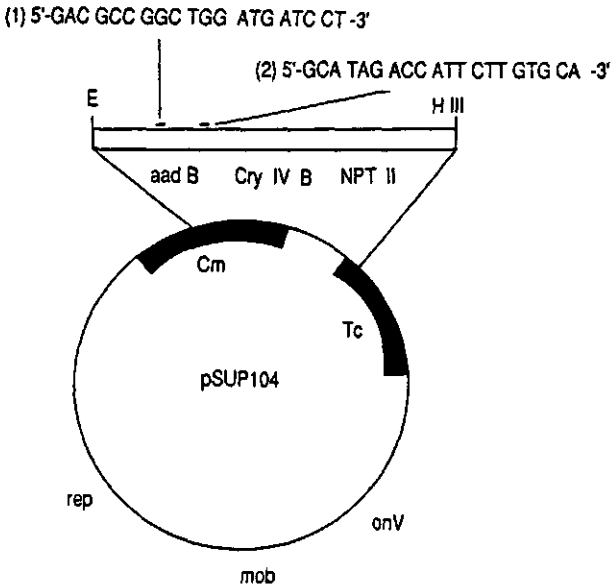

Fig. 2, map of the plasmid pSKTG, the marker cassette inserted in HindIII-EcoRI digested pSUP104 and the primers for PCR.

Introduction of a marker gene cassette into host organisms

Strains used

Escherichia coli JM101 with plasmid pUC19 was used for the cloning work. The *npt*II gene was obtained from plasmid pSUP2021 (Simon et al., 1983). The *cry*IVB gene was kindly provided by Dr. C. Waalwijk (Waalwijk et al., 1991), and the *aad*B gene (in pFL1017) was a gift from Dr. F.R.J. Schmidt (Schmidt et al., 1988). Plasmid pSUP104 was obtained from the Phabagen Collection, Utrecht, the Netherlands. The disarmed transposon delivery system (Herrero et al., 1990) was provided by Dr. B. Zaat, Leiden, the Netherlands. All strains were routinely cultured in LB medium or on LB agar

supplemented with the appropriate antibiotics. The *E. coli* S17.1 mobilizer strain which carries *tra* functions on the chromosome was used to mobilize the marked plasmid pSKTG to different species of soil bacteria. *Pseudomonas fluorescens* R2f wild-type and derivatives were used for soil microcosm studies.

All molecular techniques were performed according to Sambrook et al. (1989).

Construction of the marker gene cassette and its insertion into an IncQ plasmid

The *nptII* gene was obtained from Tn5 by *Hin*dIII-*Sal*I digestion of pSUP2021 and the resulting 1.5 kb fragment was cloned into pUC19 to give pES1. Subsequently, a 1.8 kb *Xba*I-*Xba*I fragment from the *cry*IVB gene was inserted, adjacent to the *npt*II-containing fragment, into pES1, to give pES2. Then, the central 0.47 kb *Pst*I fragment was deleted from pES2, eliminating part of the *cry*IVB gene and some restriction sites interfering with further cloning. This resulted in plasmid pESC1. The 2.9 kb *Hin*dIII-*Bam*HI fragment from pESC1, containing both *npt*II and the truncated *cry*IVB sequence, was then inserted into the RSF1010 derivative pFL1017 (Schmidt et al., 1988), next to the *aad*B gene, giving plasmid pFLm. However, as described further, this rendered the RSF1010-derived plasmid very unstable in *Pseudomonas fluorescens* R2f. In order to obtain a stable plasmid, the 4 kb *Hin*dIII-*Eco*RI fragment from pFLm, containing *npt*II, *cry*IVB (truncated, non-expressed) and *aad*B, was inserted into *Hin*dIII-*Eco*RI linearized pSUP104 (a RSF1010-derived vector constructed by Priefer et al. (1985). This final construct, depicted in Figure 2, was designated pSKTG.

Plasmid pSKTG proved to be stable in *P. fluorescens* and both resistances were expressed, since normal growth was observed on LB agar with 100 mg/l of both kanamycin and gentamycin. Digestion of plasmid DNA with several restriction enzymes also confirmed the presence of the marker cassette (not shown) in the plasmid, and all colonies on selective agar plates hybridized, in a colony hybridization assay, to the *cry*IVB probe.

Insertion of the marker gene cassette into the P. fluorescens chromosome

The strategy for chromosomal insertion of the marker cassette was based on the disarmed transposon delivery system of Herrero et al. (1990). The marker cassette was isolated from pSKTG by *Hin*dIII-*Eco*RI digestion and inserted into helper plasmid p18Sfi (Herrero et al., 1990). The cassette could then be retrieved by *Sfi*I digestion and ligated into pUT/Hg from which the *Hg*^{r} gene had been removed by *Sfi*I digestion. The resulting plasmid, pUT/KTG (Fig. 3), was then transformed into the mobilizer strain *E. coli* SM10 lambda pir (Herrero et al., 1990). Plasmid pUT/KTG cannot replicate in bacteria other then *E. coli* lambda pir. Thus, to obtain bacteria with the cassette inserted into the chromosome, filter matings were performed with *E. coli* lambda pir (pUT/KTG) as the donor and a rifampicin resistant mutant of *P. fluorescens* R2f as the recipient (Smit et al., 1991). Insertion mutants, designated R2fmc were selected on LB

agar supplemented with kanamycin and gentamycin (both 50 mg/l).

Insertion of the cassette into the chromosome of *P. fluorescens* by mating of this strain with *E. coli* SM10 lambda pir(pUT/KTG) occurred with a frequency of 10^{-5}. Southern blotting of genomic DNA digested with *Eco*RI and *Hin*dIII with a *cry*IVB probe (Fig. 3) confirmed the insertion. For comparison, a blot of *Eco*RI and *Hin*dIII-*Eco*RI digested pUT/KTG is given next to similar digestions of genomic DNA of 1 R2fmc strain (Fig. 3). The *Eco*RI-*Hin*dIII cassette sequence was in both cases 4 kb in size (Fig. 3, lanes 1 and 3), since both restriction sites are present in the cassette. Digestion with only *Eco*RI gives a band of similar size in pUT/KTG (Fig. 3, lane 2) due to the presence of an EcoRI site just outside the cassette in the plasmid sequence (Fig. 2, map), whereas it gives a 7 kb band with genomic DNA, thus suggesting that vector sequences are no longer present with the insert.

Stability of the marker gene cassette and expression in other species

To check if the marked plasmid, pSKTG, and the chromosomal KTG insertion were stable in *P. fluorescens* R2f, strains carrying the plasmid and the cassette were cultured in LB broth without antibiotics and plated on selective and non-selective plates. Strains were also introduced into sterile Ede loamy sand and maintained for 7 days at 15°C.

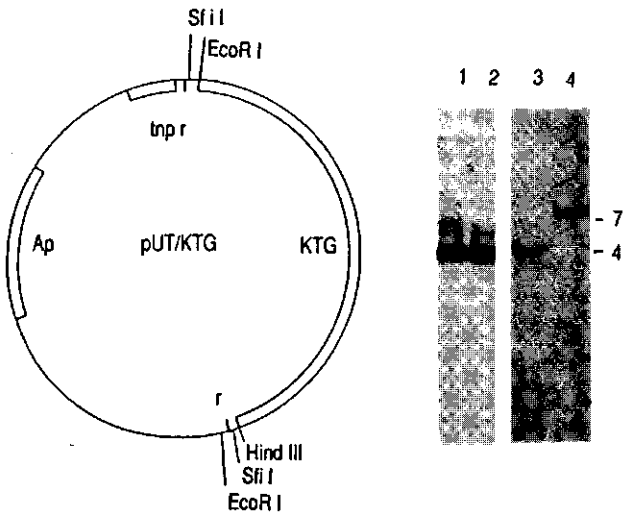

Fig. 3, Left, restriction map of pUT/KTG with some relevant restriction sites, tnp is transposase gene, KTG is the marker cassette, r are the inverted repeats of Tn5; Right, Southern blot of pUT/KTG (Lane 1 and 2) digested with *Eco*RI-*Hin*dIII (lane 1) and *Eco*RI (lane 2) and genomic DNA of R2fmc digested with *Eco*RI-*Hin*dIII (lane 3) and *Eco*RI (lane 4); band sizes in kb are given on the right.

At regular intervals, dilutions were plated on LB agar with or without kanamycin and gentamycin (50 mg/l). Plasmid pSKTG revealed to be fully stable in *P. fluorescens* R2f after overnight culturing in LB (approx. 10 generations, whereas the previous construct in pFL1017, pFLm, was unstable and showed 99% plasmid loss in 10 generations. Also, the chromosomally inserted KTG sequence was 100% stable after culturing (approximately 10 generations). During presence in sterile Ede loamy sand soil for 7 days, we could not find any evidence for loss of plasmid pSKTG or of the KTG sequence inserted into the chromosome, by comparing cfu counts of selective and unselective agar plates, and by probing with the *cry*IVB probe.

Plasmid pSKTG was transformed into the mobilizer strain *E. coli* S17.1, giving *E. coli* S17.1 (pSKTG). Then, filter matings were performed between this strain and several different rifampicin resistant species of soil bacteria to check if the antibiotic resistance genes would be expressed in a wide species range. Whereas *npt*II is known to show broad-host-range expression, less information is available on the expression range of *aad*B. The expression studies were deemed necessary in order to check (a) whether the marker cassette can be used for species other than *P. fluorescens*, and (b) whether transfer of the cassette to other species can be detected by selection on kanamycin and gentamycin. Table 2 shows that in the species tested which belonged to a wide variety of Gram-negative species, *aad*B was expressed (as was *npt*II), and the recombinant plasmid was maintained.

Table 2. Mobilization of pSKTG and expression of the resistance genes in several different bacterial species. Km: kanamycin 50 mg/l; Gm: gentamycin 50 mg/l; ISFR: Institute for Soil Fertility Research; UL: A. Richaume, University of Lyon, France; PD: Plant Pathology Service, Wageningen, the Netherlands.

Strain	Source	growth on Km Gm
Escherichia coli	ISFR	+
Enterobacter cloacea	ISFR	+
Agrobacterium tumefaciens	UL	+
Pseudomonas fluorescens R2f	ISFR	+
Pseudomonas fluorescens P1	ISFR	+
Pseudomonas putida	ISFR	+
Pseudomonas cepacea	ISFR	+
Xanthomonas maltophilia	PD	+

Detection of marked bacteria in soil by selective plating

To check recovery from soil, different bacterial cell numbers of *Pseudomonas fluorescens* R2f (pSKTG) and R2fmc were added to microcosms containing Ede loamy sand soil according to Smit et al. (1991). Cfu were enumerated by plating on King's B agar with gentamycin and kanamycin (50 mg/l) before and 3 hours after addition to soil (Fig. 3). Identical cell numbers added and recovered, resulting in lines with an angle of 45^0 (Fig. 4), indicated 100% recovery of both marked strains from soil, and no short-term loss of expression due to the introduction into soil apparently occurred.

Detection of marked cells in soil by DNA extraction and PCR

The soil portions described above were also used to extract DNA for PCR-mediated detection of the marker cassette of the introduced bacteria. DNA was extracted from soil and purified as described by Smalla et al. (1993). Additional DNA purification was used since no amplification could be achieved initially. Thus, after purification using the CsCl, KAc and spermine-HCl precipitation steps, DNA pellets were dissolved in 200µl of TE buffer. Final purification was as follows. CTAB (20µl) and 5M NaCl (20µl) were added to 100µl of DNA (Ausubel et al., 1987), and the samples were incubated at 65° for 10 min. Samples were then extracted twice with phenol/chloroform-isoamylalcohol after which the DNA was precipitated with 3 vol. ethanol and taken up in 90 µl of TE.

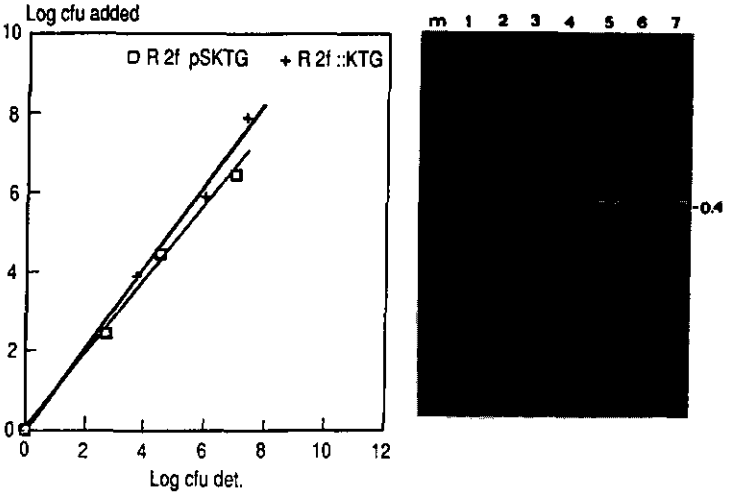

Fig. 4 Left: Log number of cfu per gram of dry soil added against the Log number of cfu recovered on selective plates of *P.fluorescens* R2fmc (square) and R2f(pSKTG) (+); Right: agarose gel of PCR amplified DNA from the samples given on the left.

These solutions were subsequently cleaned with Geneclean II glass milk (Bio 101 Inc., La Jolla, CA, USA). 1 µl was used for amplification using SuperTaq polymerase in a 50 µl reaction mix according to Smalla et al. (1993). Primers used are given in Fig.1. Figure 4 (right) shows the results of the amplification; lanes correspond to the different soil portions with increasing bacterial numbers as indicated in the graph (lane 1 received no cells, lane 2 received Log 2.8, etc). The expected band of 411 bp is identical to the product found after amplification of pure target DNA. The expected band is clearly visible in reaction mixtures of PCR run on soil with the highest inoculum densities, whereas a band is just detectable in the sample with the lowest cell numbers (Log 2.8 per g of dry soil).

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CHAPTER 5

Detection of plasmid transfer from *Pseudomonas fluorescens* to indigenous bacteria in soil using phage ΦR2f for donor counterselection

E. Smit, J.D. van Elsas, J.A. van Veen and W.M. de Vos

Summary

The transfer of a genetically marked derivative of plasmid RP4, RP4p, from *Pseudomonas fluorescens* to members of the indigenous microflora of the wheat rhizosphere, was studied by using a bacteriophage that specifically lyses the donor strain and a specific eukaryotic marker on the plasmid. Transfer of RP4p to the wheat rhizosphere microflora could be observed, and the number of transconjugants detected was approximately 10^3 per g of soil at 10^7 donor cells added per gram of soil; transfer in the corresponding bulk soil was slightly above the limit of detection. All indigenous transconjugants analyzed revealed the presence of a 60 kb plasmid and were able to transfer this plasmid to a Nx^rRp^r *P. fluorescens* recipient strain. The indigenous transconjugants were identified as belonging to *Pseudomonas* spp., *Enterobacter* spp., *Comomonas* spp. and *Alcaligenes* spp.

Introduction

The application of recombinant DNA technology allows the construction of bacteria capable of performing new functions by inserting foreign genes into their genome. The resulting genetically modified microorganisms can be used for different purposes such as the degradation of toxic substances, the promotion of plant growth, and the biological control of plant pathogens in soil (Halverson et al., 1985; Rojo et al., 1987; Sussmann et al., 1988). Recombinant DNA techniques offer the possibility to study the fate of the new gene. One of the key processes of interest is the occurrence of horizontal gene transfer (Smit and Van Elsas, 1990; Van Elsas et al., 1989). So far most of the experiments aimed at assessing genetic interactions in soil have focused on the detection of conjugal transfer, which is regarded as the major process responsible for gene transfer (Reanney et al., 1983; Trevors et al., 1987; Van Elsas et al., 1989). Transfer of selftransmissible broad-host-range plasmids from donor to recipient bacteria has been studied under different ecological conditions (O'Morchoe et al., 1988; Richaume et al., 1989; Smit and Van Elsas, 1990; Top et al., 1990; Van Elsas and Trevors, 1990; Van Elsas et al., 1988a, b, 1989; Wellington et al., 1990). These studies

have been useful for understanding and predicting the conditions in soil favouring genetic transfer by conjugation. Nevertheless, they should be regarded as model studies for transfer to indigenous bacteria, mainly because the recipient is co-introduced in the experiments. Plasmid transfer frequencies to indigenous bacteria could be lower because the indigenous bacterial population is presumably starved, less active and taxonomically heterogeneous (Schilf and Klingmüller, 1983; Van Elsas et al., 1989; Voisard et al., 1988).

To study gene transfer to indigenous bacteria in soil, a suitable donor-counterselection method is required (Henschke and Schmidt, 1990; Smit and Van Elsas, 1992; Van Elsas et al., 1989). Henschke and Schmidt (1990) circumvented this problem by using an *Escherichia coli* donor strain (harbouring the mobilizable inc.Q plasmid pFL67-2), that was not adapted to soil and hence died rapidly after introduction. Members of the indigenous soil microflora that had received PFL67-2 could be detected after 25 days (Henschke and Schmidt, 1990). However, an efficient counter-selection is required in more realistic studies using a soil-adapted donor strain. This can be accomplished for instance by selective killing of the donor population with a phage (Smit and Van Elsas, 1992), the use of a resistance marker that is not expressed in the donor (Top et al., 1990), the use of an inducible *hok* gene on the chromosome of the donor (Molin et al., 1987), or the employment of an auxotrophic mutant as a donor. Plasmid transfer to indigenous bacteria has been detected in various ecosystems with high bacterial densities such as activated sludge (Dwyer et al., 1988; McClure et al., 1990), and *in planta* (Manceau et al., 1986). Recently, transfer of a catabolic plasmid to indigenous bacteria in water and sediment mesocosms, dosed with 3-chlorobenzoate, was reported (Fulthorpe and Campbell-Wyndham, 1991), but it has not been detected in the soil environment with the donor population still present.

We were interested in studying the potential transfer to indigenous microorganisms in soil of the plasmid RP4p, a selftransmissible broad-host-range plasmid (Krishnapillai, 1988; Smith and Thomas, 1989), from a *Pseudomonas fluorescens* strain (originally isolated from soil). Because this *Pseudomonas* strain survives relatively well in soil (Van Elsas et al., 1988a) it was necessary to develop a suitable donor counter-selection method. For this purpose a phage was isolated from agricultural drainage water by enrichment with the host (Goyal, 1987). This phage proved to be specific for the *P. fluorescens* strain (Smit and Van Elsas, 1992). Results of the experiments aimed at studying plasmid RP4p transfer to indigenous bacteria using phage-mediated donor counter-selection are reported here.

Materials and methods

Bacterial strains, plasmids and growth conditions

In this study we used two variants of *Pseudomonas fluorescens* strain R2f, rifampicin resistant mutant R2fR and rifampicin and nalidixic acid resistant mutant R2fN. R2f had

been isolated from the grass rhizosphere (Van Elsas et al., 1986, 1988a, b, 1989; Van Elsas and Trevors, 1990). Other taxa used to assess phage specificity are listed in Tables 1 and 2. The broad-host-range plasmid RP4p, RP4 containing part (0.7 kb) of the eukaryotic patatin gene from potato (*pat*) inserted into the kanamycin resistance gene (Van Elsas and Trevors, 1990), was utilized. RP4p was introduced into R2fR by filter mating (Simon et al., 1983). The plasmid encodes genes necessary for conjugative transfer (Krishnapillai, 1988) and the tetracycline (Tc) and ampicillin (Ap) resistance genes. The donor strain was cultured in LB broth (tryptone 10 g, yeast extract 5 g, NaCl 10 g, H_20 1 l; pH 7.2) under the appropriate selective pressure (50 μg/ml Tc + 50 μg/ml Ap) at 28°C. An overnight culture of R2fR (RP4p) was washed twice in sterile demineralized water before appropriate dilutions were added to the soil.

Phage

A phage specific for the donor strain, ΦR2f, was obtained from agricultural drainage water from a ditch southwest of the city of Wageningen (The Netherlands) by enrichment with the host (Goyal, 1987). The phage was isolated by shaking drainage water samples with equal volumes of an early log phase culture of *P. fluorescens R2f* and F medium (tryptone 10 g, yeast extract 5 g, NaCl 12 g, $MgCl_2$ 0.2 g, $CaCl_2$ 0.15 g, $MnCl_2$ 2 mg, H_2O 1 l). After overnight incubation, samples of 5 ml were taken and treated with 0.2 ml chloroform. Plaques could be obtained by using the agar overlay method (Adams, 1959) in which 0.1 ml of the sample mixed with 3 ml soft agar of 45°C and 0.1 ml freshly cultured R2f cells were poured on F agar (F medium supplemented with 15 g agar per l) plates. The phage was propagated 4 times to ensure purity. High titer phage lysates could be obtained by adding 1 ml of phage lysate (10^9 pfu/ml) to 100 ml of early log phase cultures of strain R2f in F medium and incubating 6 hours at 28°C with intermittent shaking. After centrifugation (10.000 rpm, 10 min.) and filtration (0.2 μ), the phage titer in the filtrate was determined by the agar overlay method (Adams, 1959). The lysate was stored in F medium at 4°C; the phage titer was stable for at least 6 months under these conditions.

The phage formed clear plaques on *P. fluorescens* R2f and transmission electron microscopy revealed an icosahedral head (unpublished results).

Phage specificity

Phage specificity was tested with a number of soil isolates using the agar overlay method (Adams, 1959) (Tables 1 and 2).

To check for the presence of phage-sensitive bacteria in the rhizosphere of wheat, bacteria were extracted (see extraction and enumeration of bacteria from soil) from the rhizospheres of 8 microcosms. The suspensions were either incubated with equal volumes of phage lysate (10^9 pfu/ml) or with inactivated (boiled; 10 min.) lysate. Complete inactivation of the phage by boiling was confirmed by plating on the host. Samples were plated on King's B agar (Van Elsas and Trevors, 1990), Gould's S1 agar

(Gould et al., 1985) and 0.1 strength tryptone soya agar (TSA) to determine differences in cfu counts (Martin, 1975). On King's B and Goulds S1, selective for pseudomonads (Gould et al., 1985), the numbers of fluorescent bacteria were enumerated.

Soil and soil microcosm

Ede loamy sand (Van Elsas et al., 1986, 1988a, b, 1989; Van Elsas and Trevors, 1990) was freshly collected, air-dried to about 10% moisture content, and sieved (4 mm mesh). The soil was inoculated with a suspension of donor cells (10^7 cells per gram of soil) in sterile demineralized water establishing a moisture content of approximately 18%. Portions of about 70 g were compressed in PVC rings (denoted soil chambers, diameter 42 mm; height 30 mm) to a bulk density of 1.4 (wet weight), which were placed on a water tension table to keep the soil moisture content at 18%. Six pre-germinated seeds of wheat (*Triticum aestivum cv sicco*) were planted per soil chamber, while 4 chambers remained unplanted. Microcosms were incubated for 7-10 days in a climate chamber under a light/dark cycle (16/8h) at 20/16°C.

Extraction and enumeration of bacteria from soil

Shortly after introduction, as well as on days 7 and 10 bacterial cells were extracted from duplicate soil microcosms. The plants were gently shaken and the roots were cut off. Roots and adhering soil were considered to represent the rhizosphere sample, whereas the remaining soil was called bulk soil. Samples (10 g) of both were extracted by shaking (10 min., 190 rpm) in Erlenmeyer flasks containing 95 ml sterile 0.1% (wt/vol) sodium pyrophosphate and 10 g of gravel. The resulting suspensions were then serially diluted in 0.1% sodium pyrophosphate and plated on selective agar. To select for possible indigenous transconjugants, samples of undiluted suspensions were incubated with equal volumes of phage lysate (10^9 pfu) for 20 minutes.
Dilutions were then plated on LB agar and 0.1 TSA plates supplemented with 50 µg/ml Ap, 50 µg/ml Tc and 100 µg/ml cycloheximide to prevent fungal growth. The number of donor cells was determined by plating appropriate dilutions on King's B agar containing Rp (50 µg/ml), Ap (50 µg/ml) and Tc (50 µg/ml) (incubation 28°C, 2 days), and total numbers of bacteria were determined by plating appropriate dilutions on 0.1 strength TSA (incubation 28°C, 4 days). Transconjugant-selective plates were incubated at 28 °C for 4 days; after which they were counted single colonies were transferred to duplicate transconjugant-selective plates. After incubation, one of each pair of plates was stored at 4°C while the other was used for colony filter hybridization (Sambrook et al., 1989). The DNA on the filters (Colony/Plaquescreen, Biotechnology Systems, DuPont) was hybridized to the ^{32}P-labelled *pat* probe (nick translation, Sambrook et al., 1989) to distinguish indigenous bacteria naturally resistant to ampicillin and tetracycline from the bacteria that actually had received RP4p. Colony blots were washed according to manufacturers instructions before exposure to X-ray film.

To determine possible plasmid transfer during the sampling procedure or on the

plates, a suspension containing rhizosphere bacteria was obtained from uninoculated microcosms as described above. Freshly-cultured washed donor cells were added to the flasks containing these soil suspensions to a density comparable with the number of donor cells present in the flasks from the inoculated soil samples (indicated by 'donor in suspension' in Table 3). These samples were treated in exactly the same manner as the other soil suspensions, in which the donor had been introduced in soil. The presence of colonies other than putative phage resistant donors, that hybridized with the *pat* probe would indicate that plasmid transfer had occurred in the sampling procedure or on the plates.

Additional tests aimed to show possible transduction or transformation on transconjugant-selective plates were based on addition of DNAase (0.5 mg/ml) to the plates to prevent plate transformation and addition of 0.1 M sodium citrate to prevent transduction by inhibiting phage adsorption. No differences in transconjugant numbers as compared to transconjugant-selective LB plates could be observed in this control experiment, suggesting that transformation and transduction did not take place to a significant extent.

Analysis of transconjugants

All bacterial colonies hybridizing to the *pat* probe in the colony filter hybridization were streaked on LB agar plates, and subsequently tested on King's B agar containing Rp, Ap and Tc to exclude the possibility that they were derived from donor cells that escaped phage lysis. Transconjugants were identified by gas-chromatographically determining whole cell fatty acid patterns, kindly performed by Dr. J. Janse according to Janse and Smits (1990). Additionally, standard taxonomical tests as Gram staining and oxidase tests in conjunction with the commercially available API 20E and 20NE system were done. Randomly-selected transconjugants were analyzed for the acquisition of RP4p by small-scale plasmid isolations according to Trevors (1984). The capacity of these transconjugants to transfer RP4p to *P. fluorescens* R2fN in filter matings was also determined (Simon et al., 1983).

Statistics

Differences in number of cfu between treatments with phage lysate and inactivated phage lysate were statistically evaluated by performing Student's t-test, using the $P<0.05$ level of significance (Table 3). The Student's t-test ($P<0.05$) was also used to determine the significance of the differences in cfu between rhizosphere and bulk samples on day 7, for donor, Ap^rTc^r, transconjugant and total bacteria separately (Table 4).

Results

Phage specificity

The isolated phage proved to be quite lytic, since plate counts of the donor decreased by a factor of 10^5 after addition of ΦR2f to the suspension in a typical soil experiment (not shown). It was further very specific for the donor strain *P. fluorescens* R2fR when tested with a variety of bacterial species isolated from wheat rhizosphere or obtained

Table 1. Bacterial species tested for lysis by phage ΦR2f.

Strain	Source	Lysis*
Pseudomonas fluorescens R2f	ISFR	+
Pseudomonas fluorescens R12t	ISFR	+
Pseudomonas fluorescens P1	ISFR	-
Pseudomonas fluorescens GE1	ISFR	-
Pseudomonas fluorescens 2PS4	ISFR	-
Pseudomonas putida ATCC 12633	ATCC	-
Pseudomonas putida CYM 318	T	-
Pseudomonas putida biotype 1	PD	-
Pseudomonas stutzeri P12	ISFR	-
Pseudomonas maltophilia	ISFR	-
Pseudomonas aeruginosa	ISFR	-
Pseudomonas cepacea P2	ISFR	-
Agrobacterium radiobacter	ISFR	-
Flavobacterium sp. F4	ISFR	-
Escherichia coli MC1061	BR	-

ISFR= Institute for Soil Fertility Research, Wageningen, The Netherlands (soil isolates).
ATCC= American type culture collection,
T= Dr. J. Trevors, University of Guelph, Canada.
PD= Department for Plant Pathology, Wageningen, The Netherlands.
BR=Biorad

from culture collections (Table 1 and 2). Only one isolate, denoted R12t, which is very similar to the host R2f and in fact was isolated from the same rhizosphere sample, was lysed by the phage. Since the number of different bacterial species that can be assayed in this kind of test is limited, we also investigated the percentage of wheat rhizosphere bacteria sensitive to phage lysis. There was no significant difference between the number of fluorescent colonies on Gould's S1 plates between treatments with phage lysate and with inactivated phage lysate prior to plating (Table 3). However, there was a small, but significant, difference in the number of fluorescent colonies on King's B

Table 2. Different groups of soil isolates tested for lysis with phage ΦR2f.

Soil isolates	Number tested	Lysis*
Pseudomonas spp.	12	-
Pseudomonas spp. (fluorescent)	3	-
Alcaligenes spp.	11	-
Flavobacterium spp.	7	-

*+ = detection of plaques; - = no plaques no lysis.

between the two treatments, despite the high variability in the data (Table 3). From these data we concluded that only a small percentage of bacteria of the fluorescent pseudomonads growing on King's B are sensitive to phage ΦR2f.

Table 3. Effect of incubation with phage lysate on bacterial numbers of 3 different groups of rhizosphere bacteria in 8 different microcosms.

Microcosm		1	2	3	4	5	6	7	8
Media*	phage	Log. cfu/g dry soil							
0.1 TSA	+[a]	7.48	7.44	7.50	7.56	7.42	7.56	7.51	7.71
	-[a]	7.45	7.51	7.58	7.61	7.44	7.62	7.50	7.70
King's B	+[b]	5.55	5.80	5.34	5.55	5.13	5.32	5.48	5.52
	-[b]	5.80	6.10	5.29	5.60	5.23	5.58	5.59	5.71
Goulds S1	+[a]	5.15	5.11	4.74	4.93	4.90	5.04	4.86	4.96
	-[a]	5.18	5.41	4.63	5.20	4.83	4.86	4.86	4.90

[a] values are not significantly different ($P<0.05$)

[b] values are significantly different ($P<0.05$)

+ : treatment with active phage

- : treatment with inactivated (boiled 10 min.) phage

1-8 microcosms with wheat plants (10 g roots + adhering soil were sampled, diluted, incubated with phage lysate and plated).

*media TSA: 10% TSA (Martin, 1975); KB: number of fluorescent cfu on King's B (Van Elsas et al., 1988a); S1 number of fluorescent cfu on Goulds S1 (Gould et al., 1985).

Transfer of RP4p to indigenous soil bacteria

Several experiments were performed in the wheat rhizosphere, using the phage-based (Table 4) survived relatively well. After 7 days cell numbers in bulk soil were around Log. 6.1 per gram of soil and cell numbers in corresponding rhizosphere soil were significantly higher (Log. 6.7) than in bulk soil. Also the Ap^r and Tc^r bacterial

population was enhanced in the wheat rhizosphere as compared to the corresponding bulk soil (Table 4). The number of cfu on transconjugant-selective plates was also significantly higher in rhizosphere soil than in bulk soil, as was the total number of bacteria on 0.1 strength TSA. On day 7, indigenous transconjugants harbouring RP4p in the rhizosphere were about 10% of the total number of Ap^r and Tc^r bacteria as determined by hybridization with the *pat*-probe. The numbers of transconjugants were significantly lower in the bulk soil (Log. 1.8), just above the limit of detection, and accounted for only 1% of the Ap^r and Tc^r cells. In unplanted soil microcosms, transfer of RP4p to indigenous microorganisms was not detected (data not shown). On day 10, transconjugant numbers in the rhizosphere declined to Log. 2.9 per g of soil.

Table 4. Numbers of donor cfu, ampicillin (Ap) and tetracycline (Tc) resistant cfu, indigenous transconjugant (itrans.) cfu and total cfu in the rhizosphere of wheat 0, 7 and 10 days after inoculation of the soil with the donor *P. fluorescens* R2f (RP4p).

Treatment	Day		Log.cfu per gram soil			
			donor	Ap^rTc^r	itrans.	total
Donor added	rhiz	0	ND	ND	ND	ND
		7	6.7^a	4.6^a	3.4^a	8.3^a
		10	6.3	4.0	2.9	8.4
	bulk	0	7.3	3.2	[0]	7.6
		7	6.1^b	3.6^b	1.8^b	7.5^b
		10	ND	ND	ND	ND
No donor	rhiz	0	ND	ND	ND	ND
added		7	[0]	4.2^c	[0]	8.3^c
		10	[0]	3.8	[0]	8.2
	bulk	0	[0]	3.3	[0]	7.7
		7	[0]	3.5^d	[0]	7.6^d
Donor in suspension	rhiz	10	6.5	4.0	[0]	ND

Log. 7.7 donor cells per gram of soil were added. As a control on transfer during processing of the soil samples donor was added to uninoculated soil suspension (Donor in suspension).

Numbers are means of duplicate soil samples.

rhiz: rhizosphere; In each data column relevant cfu numbers in rhizosphere and bulk are pairwise compared and ^{a}and^c represent values significantly higher than values marked with ^{b}and^d ($P<0.05$);

itrans. :indigenous transconjugants, detected via Ap Tc selection and colony hybridization to *pat*; ND : not determined; [0] : below the detection limit, approximately Log. 1.5 cfu/g of soil.

Table 5. Identification of the indigenous bacteria that received RP4*p* from *Pseudomonas fluorescens* R2f in rhizosphere soil.

Bacterial species	Number of isolates	60 kb plasmid	Back-* transfer
Enterobacter amnigenus	6	+	+
Xanthomonas maltophilia	11	+	+
Pseudomonas sp.	4	+	+
Comomonas acidovorans	2	+	+
Alcaligenes paradoxus	1	+	+
Alcaligenes sp.	1	+	+
Comomonas sp.	1	+	+
Unknown	2	+	+

*"Backtransfer": transfer of RP4*p* in filtermatings to *P. fluorescens* R2f Rp^r Nx^r, tested with one representative of each species.

In the control microcosms, to which no donor cells were added, cfu hybridizing to the *pat* probe were not detected (both in rhizosphere and non-rhizosphere soil), showing that the indigenous bacteria did not possess DNA sequences homologous to the *pat* probe. Further, no transconjugants were detected in the controls in which donor cells were added to the shake flasks used for bacterial isolation, suggesting that plasmid transfer did not occur during the sampling procedure or on the plates.

Analysis of transconjugants

Most (over 90%) of the colonies producing a positive signal with the *pat* probe were authentic indigenous transconjugants, and did not show growth and fluorescence on King's B supplemented with Rp. Donor cells which escaped lysis by the phage could easily be distinguished from the indigenous soil bacteria by this test. Furthermore, at least 1 representative of each group of transconjugants was tested for its ability to transfer the acquired plasmid RP4p in a second mating to *P. fluorescens* to R2fN. All of the transconjugants were able to transfer RP4p back to R2fN (Table 5). The presence of the plasmid was verified by agarose gel electrophoresis of total DNA isolated from transconjugants (Table 5). In some strains additional plasmids bands were present (not shown). Although RP4p was detected, the method of plasmid isolation may work poorly for some of the indigenous strains as was also found by McClure et al. (1990). Most of the isolates could be identified at the species level (Table 5). All of the species listed were Gram-negative and appeared to belong to

several taxonomical groups. One group is closely related to the donor strain and includes the *Xanthomonas, Pseudomonas* and *Comomonas* isolates. Another group, the *Enterobacteriaceae* is more distantly related to the donor strain. A third group includes the *Alcaligenes* species (Woese, 1987). The transconjugants appeared to consist of groups of identical isolates (Table 5), for instance 11 isolates were identified as *Xanthomonas maltophilia* and 6 as *Enterobacter*. Moreover, the *Enterobacter* strains also showed identical plasmid patterns (not shown).

Discussion

The isolated bacteriophage ΦR2f proved to be very specific for the used donor strain *P. fluorescens* R2f and did not lyse a great part of the bacteria associated with wheat roots (Tables 1, 2 and 3). Among the strains tested it only lysed *P. fluorescens* R12t, which is very similar to R2f. This specificity is not surprising because many bacteriophages have a limited host range, which in some cases is used for strain typing (Ackermann and DuBow, 1987; Bigby and Kropinsky, 1989). Therefore, this phage was considered to be sufficiently specific to selectively kill off the introduced donor cells, leaving undisturbed the majority of the indigenous soil bacteria. Lysis of donor bacteria in soil suspension amended with phage lysate was studied in earlier experiments (Smit and Van Elsas, 1992) and was found to be satisfactory for effective counter-selection. It is, however, likely that any soil bacteria possessing phage receptors similar to those of the donor strain were eliminated by phage-mediated lysis.

The number of indigenous bacteria that received plasmid RP4p in the wheat rhizosphere was rather high (around Log. 3 per g of soil), and was in the same order of magnitude as was found in homologous experiments in which the recipient was co-introduced with the donor (Smit and Van Elsas, 1990; Van Elsas et al., 1988b). However, both donor and recipient cell numbers should be considered if transconjugant numbers are compared. In this experiment, donor numbers were around Log. 7 (Table 4), whereas numbers of potential recipients can only be estimated from total bacterial counts. RP4 is a promiscuous plasmid since it replicates in many Gram-negative bacteria and the antibiotic resistance genes are expressed in a wide range of species, but its host range is limited to the purple bacteria (Krishnapillai, 1988). Schilf and Klingmüller (1983) found that approximately 1.3% of the bulk soil isolates studied were successful recipients of RP4 in mating experiments. We estimate that the potential number of recipients in the wheat rhizosphere in Ede loamy sand is higher than 1.3%. Nijhuis et al. (Selection of bacteria suitable for introduction into the rhizosphere of grass, in prep.) found that approximately 10% of the bacteria isolated from the rhizosphere belonged to the pseudomonad group. The pseudomonads probably represent only a part of the potential recipients, since RP4 can replicate also in other Gram-negative bacteria (Krishnapillai, 1988). The percentage of Gram-negative bacteria in other studies on root associated microflora of grass and wheat range from 18.5 %

(Sperber and Rovira, 1959) to 37 % (Rouatt and Katznelson, 1961) of the total number of culturable bacteria. Very roughly, the mean of these three percentages (22%) might be used to calculate the number of potential recipients in the rhizosphere in our experiment. Hence, the number of recipients will be Log 7.6 (22% of Log 8.3 day 7 in Table 4). Therefore, numbers of potential recipients may actually have been of the same order of magnitude as those of homologous recipients in the earlier experiments of Van Elsas et al. (1988a). The similarity in numbers of indigenous and homologous transconjugants could then be explainable if the overall rates of transfer under the influence of wheat roots and intrinsic transfer rates from R2fR to different groups were similar. However, similarities in transconjugant numbers which possibly could suggest that transfer experiments with donor and recipient bacteria introduced into soil have predictive value for plasmid transfer from introduced donor cells to indigenous bacteria could be just coincidence and more experimental data are needed before such conclusions can be made.

All transconjugants belonged to the group of the purple bacteria (Woese, 1987) and were taxonomically more diverse then the indigenous transconjugants detected in previous plasmid mobilization studies to indigenous bacteria (Henschke and Schmidt, 1990). Recent plasmid transfer studies in water and sediment mesocosms also showed considerable taxonomic diversity among the isolated indigenous transconjugants (Fulthorpe and Campbell-Wyndham, 1991). The fact that the transconjugants include representatives of only a few bacterial groups (Table 5), may suggest that each group originated from one single parent cell that received the plasmid and subsequently proliferated in the rhizosphere. Another explanation may be that these transconjugant cells originated from an indigenous microcolony present on the roots which came into contact with the donor and received RP4p. The plasmid may thus have spread through the cells of the microcolony.

To date most studies on conjugal plasmid transfer in soil have been based on the use of co-introduced donor and recipient cell populations (Richaume et al., 1989; Smit and Van Elsas, 1990; Top et al., 1990; Van Elsas et al., 1986, 1988a, 1989; Van Elsas and Trevors, 1990; Wellington et al., 1990). Transfer to indigenous soil bacteria has been rarely studied, and the few studies reported have been hindered by the lack of an adequate donor counter-selection technique, thus increasing the detection limit. For example Schilf and Klingmüller (1983) studied RP4 plasmid transfer in soil from introduced *E. coli* to indigenous soil bacteria, and found none. Van Elsas and Trevors (1990) also investigated transfer of RP4 from introduced *P. fluorescens* R2f to indigenous bacteria and did not detect transconjugants; they estimated the limit of detection to be in the order of 10^3 cfu per g of soil. Transconjugant numbers found in the present study were around 10^3 cfu per g of soil, consistent with the findings of Van Elsas and Trevors (1990). The application of the phage-mediated donor counter-selection technique enabled us to detect *in situ* transfer of RP4p from introduced *P. fluorescens* to indigenous soil bacteria. The transferred plasmid was shown to persist in

the bacterial population for 10 days. Long-term studies, based on this methodology, are now required to investigate the long-term persistence of broad-host-range plasmids, such as RP4, in the indigenous microflora in soil.

It should be taken into account that the transconjugant numbers detected in this experiment are a reflection of the portion of the bacterial soil population capable of growth on the media used, disregarding putative transfer to cells non-culturable on those media. Detection of transfer to non-culturable bacteria requires different methodologies and are not within the scope of this paper.

Conjugative broad-host-range plasmids (e.g. IncP1) are considered to represent genetic elements with a high frequency of transfer, and as such are sometimes regarded as "worst-case scenarios" of genetic transfer in the environment. Their transfer in soil was confirmed by this work. Although, transfer frequencies of non-selftransmissible plasmids and chromosomally inserted genes are expected to be lower (Lacy and Stromberg, 1991), a study comparing transfer frequencies of these different genetic elements in the soil environment is needed before definite conclusions can be made. On the other hand, transfer studies using conjugative plasmids provide valuable data because they are probably naturally omnipresent in bacteria and they can recruit genes from the chromosome or mobilize other plasmids. These studies therefore provide insight into the ecological role of such genetic elements.

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CHAPTER 6

Influence of soil type on the transfer of plasmid RP4p from *Pseudomonas fluorescens* to introduced recipient and to indigenous bacteria

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Summary

Transfer of plasmid RP4p from introduced *Pseudomonas fluorescens* to a co-introduced recipient strain or to members of the indigenous bacterial population was studied in four different soils of varying texture planted with wheat. Donor and recipient strains showed good survival in the four soils throughout the experiment. The number of transconjugants found in donor and recipient experiments in two soils, Ede loamy sand and Löss silt loam were significantly higher in the rhizosphere than in corresponding bulk soil. In the remaining two soils, Montrond and Flevo silt loam, transconjugant numbers were not significantly higher in the rhizosphere than in the bulk soil.

The combined utilization of a specific bacteriophage to eliminate the donor strain and the *pat* sequence as specific marker to detect RP4p was found to be very efficient in detecting indigenous transconjugants under various environmental conditions. The number of indigenous transconjugants was consistently higher in rhizosphere than in bulk soil. A significant rhizosphere effect on transconjugant numbers was noticeable in Löss soil. Highest numbers of transconjugants were recovered from Flevo and Montrond silt loam; these soils possess characteristics such as high clay or organic matter contents which may be favourable to conjugation.

Introduction

The possible use of genetically engineered microorganisms (GEMs) for agricultural or environmental purposes has raised questions about the possible occurrence of horizontal gene transfer in various environments. Inter- or intrageneric plasmid transfer between introduced plasmid-bearing donor and recipient strains has been demonstrated in different sterile and non-sterile soils (Trevors and Starodub, 1987; Van Elsas et al., 1988b, 1990; Weinberg and Stotzky, 1972; Wellington et al., 1990). The influence of abiotic factors on the transfer rate was demonstrated by modifying soil characteristics, for instance, by adding nutrients, or clay minerals or by changing soil pH or incubation temperature (Bleakley and Crawford, 1989; Krasovsky and Stotzky, 1987; Richaume et al., 1989; Van Elsas et al., 1987, 1988b). Root systems also have been shown to significantly enhance plasmid transfer frequencies (Van Elsas et al., 1988a). For the purpose of assessing the risk of released GEMs in soil, the major interest of gene

transfer studies is the possible transfer between an introduced donor and indigenous microorganisms. Addition of recently cultured, donor and recipient bacteria, in relative high numbers to soil, seems unrealistic for this goal. As a matter of fact, most potential indigenous recipients are generally in a different physiological state than the freshly cultured, metabolically active introduced recipients. Moreover, the location of indigenous bacteria within the soil matrix is probably different from that of newly introduced microorganisms. On the other hand, the indigenous bacteria may be better adapted to the soil environment in contrast to the introduced bacteria which can suffer a severe shock upon addition to soil.

Data on gene transfer to indigenous bacteria in soil are scarce. However, gene transfer to indigenous microbes has been shown in other environments. It was reported by Dwyer et al. (1988) and McClure et al. (1990) in activated sludge, by Manceau et al. (1986) in planta and by Fulthorpe et al. (1991) in water and sediment mesocosms. Only Henschke and Schmidt (1990) and Smit et al. (1991) reported plasmid transfer to indigenous microorganisms in natural soil in direct experiments as opposed to retrospective evidence (Plazinski and Rolfe, 1985).

The main limitation in detecting transfer to indigenous bacteria has been the lack of suitable methods to specifically eliminate the donor strain, which is present in relatively high numbers in the soil, as opposed to the generally low numbers of possible transconjugants. So far, several alternatives for donor counter selection have been proposed such as: (1) the use of specific resistance markers not expressed in the donor (Top et al., 1990); (2) an inducible suicide gene in the donor (Molin et al., 1988); (3) the use of an auxotrophic mutant as the donor; (4) a phage to eliminate the donor. Smit et al. (1991) developed a very efficient method using a specific phage to kill the donor strain. They showed in microcosm experiments that the self-transmissible plasmid RP4p was transferred from *Pseudomonas fluorescens* to various different indigenous bacterial species in the rhizosphere of wheat in a loamy sand soil.

In the present work, transfer of plasmid RP4p from a *Pseudomonas fluorescens* to a co-introduced recipient and the indigenous bacteria was studied in four different soils. The purpose was: (a) to test the applicability of the donor counterselection developed by Smit et al. (1991) in different soils; (b) to determine if the impact of soil characteristics on the transfer; and (c) to determine if the use of model experiments with donor and recipient strains could be a suitable approach to predict transfer to indigenous populations under various natural conditions (Smit et al., 1991).

Material and methods

Bacterial strains, plasmid, phage and growth conditions

In order to study plasmid transfer to indigenous soil bacteria, a rifampicin resistant mutant of *P. fluorescens* R2f (originally isolated from a grass rhizosphere) denoted R2fR, harbouring plasmid RP4p was used as a donor strain (Smit et al., 1991). The

plasmid conferred resistances to tetracycline (Tc) and ampicillin (Ap). The plasmid is marked with a specific sequence, *pat*, which is used as a probe to detect the indigenous transconjugants (Van Elsas et al., 1991).

Experiments on plasmid transfer between introduced donor and recipient strains were performed with an antibiotic-resistant mutant of *P. fluorescens* (R2fN) resistant to rifampicin (Rp) and nalidixic acid (Nx), as the recipient (Smit and Van Elsas, 1990). The wild type *P. fluorescens* R2f strain harbouring RP4p was used as the donor.

Donor and recipient strains were cultured in Luria-Bertani (LB) broth (tryptone 10 g, yeast extract 5 g, NaCl 5 g, H_2O 1 l; pH 7.2) with 50 mg/l of the appropriate antibiotics at 28°C, overnight using a gyratory shaker. Bacterial suspensions were washed twice by centrifugation (7000 x g, 20 min) in sterile demineralized water. Pellets were resuspended in an adequate volume of sterile demineralized water to obtain the appropriate number of cells to be added to the soil.

Phage ΦR2f, a specific lytic phage for the donor strain was propagated and used as described by Smit et al. (1991)

Soils and soil microcosms

Four different soils were used, Ede loamy sand, Flevo silt loam, Montrond silt loam and Löss silt loam. The main physical and biological characteristics for each soil are listed in Tables 1 and 2 respectively. All soils were freshly collected, air dried to about 10% below the soil-field capacity and sieved (4 mm).

Microcosms consisted of soil portions of about 120 g (equivalent dry weight) placed in plastic pots. The soil moisture was maintained constant at pF 2 on a water tension table. For each soil and transfer study, two (to study the transfer to indigenous bacteria in the Montrond soil) or three pots were planted with five pre-germinated seeds of wheat (*Triticum aestivum var. sicco*). Microcosms were incubated for 7 days in a growth chamber under a light/dark cycle (16 h/8 h) at 20/16°C.

To study the dynamics of plasmid transfer to a recipient strain and to indigenous microorganisms, duplicate or triplicate microcosms with the four different soils were inoculated either with the donor strain or with the donor and (after 1 h) recipient strains. The different cultures were suspended in different volumes of sterile demineralized water to obtain 10^7-10^8 cells/g dry soil and a final moisture content corresponding to pF 2 for each soil (see Table 1).

Characterization of the soils. In order to obtain data on the population sizes of potential recipients in rhizosphere and bulk samples of the 4 different soils (Table 2), soil samples were taken as described below. The appropriate dilutions were plated on the following media selective for different groups of microorganisms: Endo agar (Oxoid, London), selective for lactose (coliform) fermenting organisms; Gould's S1 (Smit et al., 1991), selective for fluorescent pseudomonads; King's B (Van Elsas et al., 1988a), also permitting detection of fluorescent bacteria; 0.1 strength Tryptone Soy Agar (1/10 TSA) (Martin, 1975), for total culturable bacteria; 1/10 TSA with 10 mg/l of vancomycin

(Pelczar, 1975), selective for Gram-negative bacteria. The Gram-negative population sizes were also estimated by testing the colonies of the total 1/10 TSA plates with the fast KOH-based Gram-determination method. The sizes of the total (including non-culturable cells) bacterial populations were determined by direct microscopical counts of soil samples using Europium chelate as fluorescent dye (Anderson and Shinger, 1974).

Sampling of microcosms

Separation of rhizosphere and non-rhizosphere soil. After 7 days of incubation, the plants were taken from the pots and were gently shaken to separate roots plus adhering soil (rhizosphere sample) from non-adhering, bulk soil (non-rhizosphere soil). The day 0 sample corresponded to a 3-h incubation of the soil with the donor and recipient strains or the donor strain only.

To determine bacterial numbers, samples (10 g) of soil (rhizosphere or non-rhizosphere) were shaken in Erlenmeyer flasks with 95 ml of 0.1% sodium pyrophosphate (NaPP) and 10 g of gravel (10 min, 190 rpm). The soil suspension was serially diluted in 0.1% NaPP and appropriate dilutions were plated on selective agar media.

Enumeration media. All media were supplemented with 100 mg/l of cycloheximide to minimize fungal growth. The number of recoverable indigenous bacteria was enumerated on 1/10 TSA. The number of donor cells was determined by plating on King's B agar containing 50 mg/l of both Tc and Ap. Recipients and homologous transconjugants were enumerated on King's B containing 50 mg/l of Rp and Nx, and 50 mg/l of Ap, Tc and Rp and Nx respectively.

The putative indigenous transconjugants were selected on LB agar supplemented with 50 mg/l of Ap and Tc and incubated at 28°C for 4 days after phage-mediated donor counter-selection was performed (Smit et al., 1991). Smit and Van Elsas (1992) tested the efficiency of killing of strain R2f by the phage. They reported that phage-resistant mutants occurred at a frequency of 10^{-5}. Therefore donor cells were rarely found on the selective plates when the phage was used prior to plating.

Indigenous transconjugant isolation and hybridization.

Indigenous transconjugants were isolated among viable and culturable soil bacteria. Randomly chosen colonies which had appeared on plates selective for indigenous transconjugants were picked and transferred to two new selective plates. After incubation, one plate was used for colony filter hybridization and the other was kept at 4°C for further strain characterization. The hybridization procedure with the *pat* probe was described by Smit et al. (1991). To determine the number of indigenous transconjugants per gram of dry soil, the percentage of probe-positive (non-donor) colonies among the total number of colonies assayed was calculated. This figure was multiplied with the total number of colonies on the selective plates. We couldn't

exclude the possibility that when no transconjugants were found, (no probe-positive colonies), that a low number, below detection, was still present. That is why we estimated the limit of detection, expressed as cfu per gram of dry soil (as given in Table 3), assuming that 1 colony (from the lowest dilution) was found to hybridize with the *pat* probe among the colonies tested.

Analysis of indigenous transconjugants

Bacterial colonies hybridizing to the *pat* probe were streaked on King's B agar containing Ap, Tc and Rp to determine if they were donor cells which had escaped phage lysis. Bacteria growing on this medium and producing a fluorescent pigment, like the donor strain, were considered not to represent indigenous transconjugants. These bacteria represented about 1/10 of the tested colonies.

The presence of the plasmid was confirmed on some randomly chosen transconjugants which were all Gram-negative by the plasmid-visualisation technique of Eckhardt (1978). The transconjugants were taxonomically analyzed using the Biolog microplate system (Biolog, Hayward, USA).

This testing system permits simultaneous testing of 95 different carbon sources for oxidation by a bacterial strain. Microplates were inoculated with the isolate to be tested according to the manufacturer's instructions. All wells started out colourless when inoculated with the cell suspensions. When a carbon source was utilized, a tetrazolium dye present in the well formed a purple colour. The "metabolic fingerprint" of the tested strain was compared with the Gram-negative data base provided by the manufacturer (Microlog Program Software Biolog, Hayward USA).

Statistics

Differences in numbers of cfu (prior to log transformation) between rhizosphere and non-rhizosphere within each soil were evaluated statistically by Student's *t*-test ($P < 0.05$). The influence the presence of plants (rhizosphere or bulk soils) and of soil type were evaluated by performing a two-way analysis of variance.

Results

Transfer of plasmid RP4p between co-introduced donor and recipient strains in rhizosphere or non-rhizosphere soils

Since donor and recipient population densities are important factors in determining the extent of plasmid transfer, survival in the different soils is an important parameter. As shown in Table 3, survival rate of both inoculants was sightly higher in the wheat rhizosphere as compared to corresponding non-rhizosphere soils. This better survival, however, was not significant ($P<0.05$). All introduced populations survived well in rhizosphere soil, at levels between log 5.2. and log 7.2 cfu per g of soil for introduced donors and log 6.2 and log 8.0 for introduced recipients. Survival was highest in Flevo

Table 1: Physio-chemical characteristics of the soils

soil	Origin	Texture	Particle distribution sand (> 50mm)	silt (2-50mm)	clay (0-2mm)	pH KCl	Organic matter(%)	soil moisture content pF2 (%)
Ede	Eastern part of Holland (van Elsas et al. 1986)	loamy sand	84.8	12.2	3.0	6.2	3.5	18
Montrond	Bas-Dauphine (France)	silt loam	32.2	36.4	31.4	5.5	4.8	40
Flevo	Flevo polder (Holland) (van Elsas et al. 1986)	silt loam	14.3	59.7	26.0	7.5	4.1	42
Löss	Maastricht	silt loam	13.0	71.4	15.6	6.6	1.9	25

Table 2: Characterization of some microbiological populations in rhizosphere (Rhiz.) and non-rhizosphere (Non rh.) of the four different soils that were studied. log cfu/gram dry soil or %

Selection*	Ede		Montrond		Flevo		Löss	
	Rhiz.	Non rh.	Rhiz.	Non rh.	Rhiz.	Non rh.	Rhiz.	Non rh.
Endo	6.84	5.62	6.77	6.55	6.77	6.84	6.75	6.24
Sl. t.	5.82	5.13	6.13	5.79	6.34	6.29	6.05	5.92
Sl. flu.	5.23	1.18	5.29	4.57	5.71	5.23	5.05	4.36
0.1 TSA t.	7.79	7.46	8.19	8.19	8.00	8.00	7.95	7.74
0.1 TSA g⁻ (% 0.1 TSA t)	25.7	12.88	18.6	12.3	20.4	11.5	46.78	22.4
KOH g (% 0.1TSA t)	51.3	33.11	69.18	11.74	50.11	19.05	64.56	44.66
t mic.	9.01	8.48	9.35	9.26	9.22	8.84	9.06	8.92

* Endo: number of cfu on Endo agar plates; Sl t: total number of cfu on Goulds Sl agar plates; Sl flu: number of fluorescent colonies on Gould's Sl agar plates; 0.1 TSA t: number of cFU on 1/10 strengh TSA (total viable counts); 0.1 TSA g⁻: % of gram-bacteria in total on 1/10 strengh TSA with 10mg/ml vancomycin; KOH g⁻ : % of gram- bacteria in total calculated based on KOH method; t mic.: direct microscopical counts of total bacteria using europiumchelate staining; See material and methods for explanation.

TABLE 3: Numbers of donor P. *fluorescens* (R2f RP4::pat), recipient (R2fN) and transconjugants CFU (Transconj.) in the rhizosphere of wheat 7 days after inoculation of soils.

Log CFU per gram dry soil

Soil	Day	Donor	Recipient	Transconj.
Ede loamy sand	Rhiz 0	nd	nd	nd
	7	5.6	6.22	3.45
			nd	
	Non rhiz.0	nd	5.85	nd
	7	5.26		<1.8
Montrond silt loam	Rhiz 0	nd	nd	nd
	7	5.2	6.45	1.93[a]
	Non rhiz 0	5.6	6.9	<1.5
	7	4.9	5.9	1.92[a]
Flevo silt loam	Rhiz. 0	nd	nd	nd
	7	7.2	8.0	4.8[a]
	Non rhiz.0	7.1	7.4	3.65[b]
	7	7.1	7.8	4.45[a]
Löss	Rhiz 0	nd	nd	nd
	7	6.4	7.0	3.6[a]
	Non rhiz.0	7.3	7.4	2.0[b]
	7	6.3	6.6	1.96[b]

About 10^7 donor recipient cells per gram of dry soil were added to Ede loamy sand; 1.6×10^6 donor cells and 1.6×10^7 recipient cells per gram of dry soil were added to Montrond silt loam; 3.1×10^7 donor cells and 8.7×10^7 recipient cells per gram of dry soil were added to Flevo silt loam and to Löss soil.
Rhiz.: rihosphere soil. Non rhiz.: non rhizosphere soil Transconj.: transconjugants, nd:not determined.
For each soil, values with [a] are significantly different from those with [b] ($P<0.05$).

TABLE 4: Numbers of donor (R2f RP4::pat), ampicillin and tetracyclin resistant cells (ApTc^r on LB), indigenous microorganisms (Total) and indigenous transconjugants (Transconj.) in the rhizosphere of wheat 7 days after inoculation of soils

log CFU per gram dry soil

Soil	Day	donor	Total	ApTc^r on LB	Transconj.
Ede loamy sand	Rhiz. 0	nd	nd	nd	nd
	7	6.5	8.0	4.6	3.19[a]
	Non. rhiz. 0	6.51	7.44	4.6	nd
	7	5.9	7.9	4.2	2.36[b]
Montrond silt loam	Rhiz. 0	nd	nd	nd	nd
	7	6.5	8.2	4.9	3.47
	Non rhiz. 0	7.23	8.2	5.2	nd
	7	6.11	8.5	4.3	3.16
Flevo silt loam	Rhiz. 0	nd	nd	nd	nd
	7	7.14	7.87	4.7	3.97 [a]
	Non rhiz. 0	7.4	7.8	3.07	2.47 [b]
	7	7.04	7.66	4.2	3.38 [ab]
Löss	Rhiz. 0	nd	nd	nd	nd
	7	6.7	7.99	3.7	2.96 [a]
	Non rhiz. 0	6.9	7.81	3.3	2.33 [b]
	7	5.75	7.7	3.4	2.17 [b]

About 10^7 donor cells were added to Flevo and Löss soils and 10^8 to Ede and Montrond soils.
Rhiz.: rhizosphere soil, Non rhiz.: non-rhizosphere soil, nd: not determined, nd: not determined
Comparison between the number of transconjugants in rhizosphere or non-rhizosphere, soils: values with [a] are significantly different from those with [b] ($P<0.05$)

silt loam, and lowest in Ede loamy sand and Montrond silt loam.

Counts of total heterotrophic culturable bacteria are not presented for these experiments because they were not statistically different from those presented in Table 4 (see below).

In Ede loamy sand, numbers of donor and recipient cells on day 7 in rhizosphere soil were slightly but not significantly higher than those in the corresponding non-rhizosphere. However, the number of transconjugants was significantly higher in the rhizosphere soil (log 3.45 cfu/g dry soil) than in non-rhizosphere soil (below the detection limit).

P. fluorescens R2f (RP4p) and *P. fluorescens* R2fN introduced into Montrond silt loam showed good survival even in the non-rhizosphere soil. The relatively low number of donor cells at the beginning of the experiment (log 5.6 cfu/g dry soil) resulted in a low number of transconjugants close to the detection limit estimated to be log 1.7 cfu per g of soil even in the rhizosphere soil. No rhizosphere effect on the number of transconjugants was noticeable.

In Flevo silt loam, both donor and recipient strains survived very well. There were no significant changes in their numbers between day 0 and day 7 both in rhizosphere and bulk soil. Transconjugants were already detected after 3 h in soil and an increase of transconjugant numbers was observed throughout the experiment. The number of transconjugants was not significantly higher in the rhizosphere than in the corresponding bulk soil.

Both R2f (RP4p) and R2fN survived relatively well in Löss silt loam and there was no significant difference between their number in the rhizosphere and bulk soil. The number of transconjugants was enhanced in rhizosphere soil: log 2 cfu/g dry soil were found in bulk soil after 0 or 7 days of incubation whereas log 3.6 cfu/g dry soil were enumerated in the rhizosphere soil after 7 days.

Transfer of RP4p from donor to indigenous microorganisms.

In these experiments, the donor was eliminated using phage ΦR2f prior to plating in order to allow enumeration of bacteria resistant to ampicillin and tetracycline. The efficiency of the phage has been tested by Smit and Van Elsas (1992) who reported the appearance of phage resistant at a frequency of 10^{-5}. Transconjugants were enumerated by using numbers of colonies which reacted with the *pat* probe. Results are presented in Table 4.

Survival of donor in the four soils was similar to that in the donor plus recipient experiment (Table 3). The donor survived relatively well in all soils studied. Although the cfu numbers in the rhizosphere were generally higher, we did not detect a statistically significant difference in the total heterotrophic counts, nor in the number of bacteria resistant to ampicillin and tetracycline (between rhizosphere and non-rhizosphere soils).

The number of transconjugants in Ede loamy sand was significantly higher in the

rhizosphere (log 3.19 cfu/g dry soil) than in the bulk soil (log 2.36 cfu/g dry soil). Albeit not significantly, the number of transconjugants was higher in the rhizosphere (log 3.47 cfu/g dry soil) than in non-rhizosphere soil (log 3.16 cfu/g dry soil) in Montrond soil. High numbers of indigenous bacteria harbouring RP4p were recovered from Flevo silt loam. Three replicates of Flevo silt loam and Löss silt loam were sampled, and although we found log 3.97 transconjugants per gram in the rhizosphere soil and log 3.38 per gram in the bulk soil, the difference was not statistically significant. However, the number of transconjugants was significantly higher in the rhizosphere than in non-planted soil after 7 days of incubation (data not shown).

In Löss silt loam, the number of transconjugants was significantly higher in the rhizosphere than in corresponding bulk soil while it remained stable in bulk soil from day 0 to day 7.

Of the total number of colonies on the transconjugant selective plates, the percentage of colonies reacting positively with the *pat* probe ranged from 45-50% in Flevo and Montrond soils and was 25% in the Löss soil and 10% in the Ede loamy sand.

A two way analysis of variance was performed on the numbers of transconjugants from rhizosphere or non-rhizosphere samples for the four soils. The plant (rhizosphere or bulk soil) and the type of soil were controlled variables. It was shown that the plant had a significant effect on the number of transconjugants ($P = 0.004$) as did the soil type ($P < 0.001$).

Analysis of indigenous transconjugant bacteria.

Transconjugants from Ede loamy sand have previously been analyzed and will not be treated here (1991). Among transconjugants recovered from Flevo, Montrond and Löss soils about 10 isolates were analyzed for the presence of the plasmid and were further characterized with the Biolog Gram-negative identification system.

A plasmid of the same size as the RP4p was consistently found in the tested isolates (data not shown).

From the Flevo soil, seven isolates were identified as *Pseudomonas* sp., two as *Xanthomonas* and one as *Enterobacter*. From the Montrond soil, one isolate was identified as *Enterobacter*, one as *Klebsiella*, 9 as *Serratia* and 2 were unknown. From Löss soil, five isolates belonged to the genus *Pseudomonas*, one each to *Comomonas, Yersinia, Xanthomonas* and *Enterobacter* (1 unidentified).

Discussion

In this study we compared plasmid RP4p transfer from an introduced donor strain to a co-introduced recipient or to indigenous bacteria in four soils. Various abiotic factors have been previously shown to affect plasmid transfer (Bleakley and Crawford, 1989; Richaume et al., 1989; Smit and Van Elsas, 1992; Van Elsas et al., 1987). Therefore, we chose soils exhibiting different physico-chemical characteristics such as clay content (ranging from 3% to 31%), organic mater content (from 1.9% to 4.8%) and pH (from

5.5 to 7.5) (Table 1). The soils were microbiologically characterized to estimate the sizes of the possible recipient bacterial populations in the different rhizosphere and bulk soils (Table 2). However, this can only be seen to provide a very rough estimate of potential recipients in soil, since the successful recipients may differ between the soils, both for genetic (e.g. genetic barriers) and for soil microbiological reasons (e.g. varying locations, activity or type of bacteria). Differences in potential recipient populations between the four soils and between rhizosphere and non-rhizosphere soils (Table 2) do not seem to be dramatic enough to explain the differences in the numbers of indigenous transconjugants detected.

Soil texture and structure are recognized as key factors in the control of biological interactions such as competition and predation (Van Veen and Van Elsas, 1986). Van Elsas et al. (1986) reported that the survival of *P. fluorescens* was better in a finer-textured soil (Flevo silt loam) than in coarser-textured one (Ede loamy sand). This may have been due to the protective role of clay minerals against predators (Heijnen et al., 1988).

Plasmid RP4p transfer was detectable between donor and introduced recipient as well as from introduced donor to indigenous microorganisms in all soils. The combined utilization of bacteriophage ΦR2f to eliminate the donor strain and the *pat* sequence as specific marker of RP4p was found to be very efficient to detect indigenous transconjugants among culturable bacteria in these soils. No indigenous bacteria harbouring the *pat* sequence were found in Ede loamy sand soil in previous studies (Van Elsas et al., 1991). In this study, homology to *pat* was also not detected in bacteria of three other soils, unless the bacteria had acquired plasmid RP4p. The number of indigenous bacteria that harboured RP4p after 7 days of incubation was rather high. Those in Ede loamy sand were in accordance with the numbers recently reported by Smit et al. (1991).

Numbers of transconjugants detected in homologous transfer experiments between introduced donor and recipient cells (Table 3) were not similar (usually somewhat higher) to those obtained in experiments on transfer to indigenous bacteria (Table 4), but they were in the same order of magnitude, except for those in Montrond soil. In the latter soil, low numbers of introduced donor and recipient cells resulted in low numbers of transconjugants.

The highest number of indigenous transconjugants was recovered from Flevo and Montrond silt loam soils which also have the highest clay and organic matter contents. The addition of clay minerals has been shown to enhance plasmid transfer due to the beneficial effect on bacterial survival and/or activity (Heijnen and Van Veen, 1991; Stotzky, 1986; Van Elsas et al., 1987) and possible because of the increase of specific surface area in soil. Conjugal transfer of IncP1 plasmids occurs preferentially when bacteria are adsorbed onto surfaces (Wilkins, 1990). Furthermore, Flevo silt loam has a neutral pH which has been demonstrated to be favourable for IncP1 plasmid transfer while low pH values decrease conjugation frequencies (Richaume et al., 1989; Stotzky

and Krasovsky, 1981). Both from theory and practice, it seems that conditions for conjugal plasmid transfer are optimal in the Flevo silt loam soil.

Löss soil, with a clay and organic matter content of 15.6% and 1.9% respectively and Ede loamy sand containing 3,5% organic matter and only 3% clay, showed a significant effect of plant roots on the number of both indigenous transconjugants and introduced recipient transconjugants.

Several studies clearly showed the enhancement of transconjugant numbers in Ede rhizosphere soil compared with bulk or unplanted soil (Van Elsas et al., 1988a, b). The exact mechanism for such an effect has not been clearly demonstrated but one can assume that root exudates stimulate bacterial activity increasing nutrient availability, and that root surfaces and movements as well as water flow induced by the roots could favour cell-to-cell contact. In other soils, the numbers of transconjugants was consistently higher in rhizosphere soil, although statistical analysis did not permit the conclusion that the effect was significant. It has to be noted that the difference in transconjugant numbers in Flevo silt loam between non-rhizosphere and non-planted soil (as mentioned in Results) suggests that the so called non-rhizosphere soil is still influenced by the roots, maybe because of the relatively small volume of the microcosms.

Studying RP4p transfer in four different soils, it became evident that the impact of soil characteristics on plasmid transfer under natural conditions cannot be predicted from simple concepts. However, the results indicate that plants, clay minerals, organic matter content and pH play a role. Roughly, the 4 soils studied can be divided into two classes: (a) Flevo and Montrond silt loam soils, which have high clay and organic matter contents and in which relatively high transfer rates occur; (b) Ede loamy sand and Löss silt loam, which have lower organic matter and clay contents (Löss somewhat higher), and in which initial transfer rates were relatively low. The latter two soils showed significantly increased transfer rates in the presence of plant roots while transfer rates in Montrond and Flevo soils were hardly affected by the plants.

Study of individual environmental characteristics separately might provide information on their relative importance in the conjugation process. But, under natural conditions, physico-chemical and biological environmental factors exert their influence in concert. That is why, even if a characteristic was *a priori* not favourable for the transfer, its effect could have been masked or reduced by the others parameters. This might have been the case for the Montrond soil whose pH was quite low whereas high numbers of indigenous transconjugants could have been detected. The number of transconjugants is the net result of known and probably still unknown factors. However, an exogenous factor such as the presence of plant roots seems to be important since it acts as a positive factor if natural conditions are not optimal for the transfer (for instance in the Löss soil).

We raised the possibility of using 'model' (donor-to-recipient) transfer studies to provide data predictive for gene dissemination to indigenous microorganism for

assessing the risk of deliberately released GEMs. Although from our study the use of introduced donor and recipient strains to measure plasmid transfer seems to give a reasonably good picture of the transfer to indigenous bacteria, differences observed between soils are difficult to interpret only in terms of environmental characteristics. At present, it seems a case-by-case approach as recommended by Klingmüller (1991) would be the most appropriate for risk-assessment purposes. Nevertheless, studies on a wider diversity of soils under standardized conditions (microcosm, donor bacteria, plasmid type) should eventually allow accurate predictions for the occurrence and frequency of gene transfer events between introduced GEMs and indigenous bacteria in soil.

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CHAPTER 7

Mobilization of a recombinant IncQ plasmid between bacteria on agar and in soil via cotransfer or retrotransfer.

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Summary

Mobilization of a genetically engineered IncQ plasmid, pSKTG, was studied *in vitro* and in sterile and non-sterile soil. In biparental and triparental filter matings, mobilization frequencies of pSKTG were identical, and the plasmid was only mobilized in the presence of the self-transmissible plasmid, RP4p. In sterile soil, mobilization was probably limited by reduced cell-to-cell contact, as frequencies were approximately 100-fold lower than in the filter matings.

The transfer frequency of pSKTG in sterile soil, when RP4p was present in the same strain, was about 100-fold higher than when RP4p was present in a separate strain. In microcosm experiments using non-sterile soil to study transfer to indigenous bacteria, pSKTG was transferred to indigenous bacteria only in the presence of RP4p.
However, natural mobilization by genetic elements present in the indigenous soil microbiota could not be detected, although *in vitro* studies suggested the occurrence of such genetic elements in soil bacteria.

Introduction

The fate of recombinant DNA of genetically engineered microorganisms (GEMS) introduced to soil is being extensively studied (Henschke and Schmidt, 1990; Richaume et al., 1989; Selvaratnam and Gealt, 1992; Smit and Van Elsas, 1990; Stotzky, 1992; Van Elsas et al., 1988). Although some attention has been given to transduction (Germida and Khachatourians, 1988; Zeph et al., 1988) and transformation (Khanna and Stotzky, 1992), most of the research in this area is focused on conjugative transfer. Knowledge about the dissemination of self-transmissible broadhost-range plasmids in the soil ecosystem is increasing (Richaume et al., 1989; Smit et al., 1991; Van Elsas et al., 1988) but the fate of other vectors has received less attention. Non-self-transmissible plasmids, such as those of the IncQ class, are probably less frequently transferred (especially the Mob$^-$ vectors) and, thus, safer for use as vectors in GEMs that are to be released to the environment than the conjugative IncP plasmids, which have been shown to be efficiently transferred between bacteria in soil and rhizosphere (Richaume et al., 1989; Richaume et al., 1992; Smit and Van Elsas, 1990; Smit et al., 1991; Van Elsas et al., 1988). Henschke and Schmidt (1990) showed that 25 days after introduction of a Tra$^+$ *Escherichia coli* strain, containing the mobilizable IncQ plasmid, pFL67-1, into soil, the

strain had completely died out, but transconjugants could be detected among the indigenous population. Thus, the recombinant DNA persisted despite the fact that the host organism was not longer detectable. However, it is not clear if the frequency of transfer of a mobilizable plasmid in a strain with all *tra* functions is in the same order as that of a self-transmissible plasmid, and not much is known about the possible transfer of an IncQ plasmid by self-transmissible plasmids present in other soil bacteria, e.g., indigenous ones.

IncQ plasmids can be mobilized by plasmids of different incompatibility groups, e.g, IncP, IncIa, IncM and IncX (Fry and Bagdasarian, 1989), but it is unknown to what extent these plasmids are present in the soil bacterial community. The presence of an IncP plasmid in an introduced strain with a mobilizable plasmid or in another, separately introduced, strain could simulate the presence of conjugative plasmids in bacteria in the environment. Bacteria in the environment commonly possess plasmids, some of which are conjugative (Bender and Cooksey, 1986; Diels et al., 1989; Sayler et al., 1990), and some are able to mobilize other plasmids (Hill et al., 1992; Mergeay et al., 1987). Except for the study by Henschke and Schmidt (1990), not much is known about the dynamics of transfer of IncQ plasmids in soil. Moreover, knowledge about the presence of mobilizing plasmids in soil bacteria, as has been acquired in plasmid isolation studies in the epilithon (Fry and Day, 1990; Hill et al., 1992), could provide information on the transfer potential of the soil bacterial community. IncQ plasmids in introduced GEMs could be transferred by triparental mating, depending on the number, proximity, and activity of cells containing Tra^+ plasmids. Such triparental matings would require transfer of the self-transmissible plasmid into the GEM and mobilization of the recombinant plasmid to a third, recipient, strain. The probability of such a two-step process occurring seems small. However, retromobilization, which requires only contact between the GEM and an indigenous microbe bearing a conjugative plasmid (Mergeay et al., 1987), might make the dissemination of a mobilizable vector to indigenous bacteria more likely.

An IncQ plasmid was chosen to serve as a model of a non-conjugative mobilizable plasmid. To detect low numbers of organisms containing this plasmid, a marker cassette was inserted into a derivative of RSF1010, pSUP104, giving plasmid pSKTG (Smit and Van Elsas, 1992b). The marker cassette contains two antibiotic-resistance genes flanking a nonexpressed sequence (part of *cry*IVB) as the molecular marker (Smit and Van Elsas, 1992b; Waalwijk et al., 1991). The results of transfer studies performed on filters and in soil are reported here. Emphasis was placed on the putative role of naturally occurring selftransmissible plasmids capable of mobilizing IncQ plasmids.

Materials and methods

Bacterial strains, plasmids, phage, and growth conditions

Strains of *Pseudomonas fluorescens* R2f, either containing different plasmids or

without plasmids, with different antibiotic resistances were used in this study (Table 1). The soil isolate, *P. fluorescens* R2f, and plasmid RP4p, RP4 containing a 0.7 kb eukaryotic DNA sequence (*pat*) to serve as molecular marker, have been described before (Smit and Van Elsas, 1990; Smit et al., 1991; Van Elsas et al., 1988; Van Elsas et al., 1991). Plasmid pSKTG, constructed to study gene transfer in soil (Smit and Van Elsas, 1992b), contains the replication functions and the *mob* site of pSUP104, a derivative of RSF1010 into which sequences of pACYC184 were cloned (Priefer et al., 1985), and a marker cassette. This marker cassette contains two antibiotic-resistance genes, *npt*II from Tn*5* and *aad*B from Tn*4000*, which confer resistance to kanamycin and gentamycin, respectively (Smit and Van Elsas, 1992b). The antibiotic-resistance genes flank part of the sequence of the *cry*IVB gene from *Bacillus thuringiensis* (Waalwijk et al., 1991), which was used as a molecular marker. This marker cassette, flanked by *Eco*RI and *Hin*dIII sites, was cloned into pSUP104, which was linearized with *Hin*dIII-*Eco*RI, to give pSKTG. The construction of pSKTG and its properties has been described (Smit and Van Elsas, 1992b). The *pat* and *cry*IVB (Van Elsas et al., 1991; Waalwijk et al., 1991) fragments were used as probes to detect RP4p or pSKTG, respectively. Both had been cloned into pUC18 and were obtained from this vector by agarose gel electrophoresis after restriction with *Hin*dIII for *pat* and *Xba*I for *cry*IVB.

Phage ΦR2f, which was used for donor counterselection, was obtained from agricultural drainage water and is specific for *Pseudomonas fluorescens* R2f (Smit et al., 1991). ΦR2f was propagated and maintained as described (Smit et al., 1991).

All strains were maintained at -80°C in 20% glycerol and cultured in Luria Broth (LB; 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl and 1 l H_2O; pH 7.2) containing 50 mg/l of the appropriate antibiotics.

Experimental design

To study cotransfer and retrotransfer of the mobilizable plasmid, different combinations of donor strains were used (Table 1). The experiments were done in a step-by-step approach, i.e., matings on filters on agar plates were followed by experiments in sterile soil, and, in the last phase, in non-sterile soil planted with wheat plants. Filter matings were also performed to screen the indigenous soil bacterial community for the presence of plasmids capable of mobilizing pSKTG: an extract representing the soil microbial community in suspension was mixed with either a donor strain containing pSKTG or with both a donor and a recipient strain on filters on agar plates.

Filter matings with donor and recipient

Filter matings were done to test the maximum mobilization frequencies of plasmid pSKTG in different combinations of donor and recipient strains (Tables 1, 2, 3). Autoclaved (121 °C, 15 min.) cellulose membrane filters (Millipore, 0.22 μm, ϕ 47 mm) were placed on LB agar (1.5%) plates. Overnight cultures of the strains were washed

twice with sterile demineralized water to eliminate the antibiotics. 50 µl of each cell suspension was pipetted onto the filters and mixed with a pipette tip. The filters were incubated at 28°C for 4 or 24 h. After incubation, the filters were vortexed in 3 ml of LB to dislodge cells, the suspensions serially diluted, and aliquots of 100 µl were plated on LB agar containing 50 mg/l of each antibiotic, donor cells with pSKTG were enumerated on agar with kanamycin (Km) and gentamycin (Gt), donor cells with RP4p on agar plates with tetracycline (Tc) and ampicillin (Ap). Recipient cells were counted on agar with rifampicin (Rp) and nalidixic acid (Nx). Transconjugants were selected using Rp and Nx with either Tc and Ap or Km and Gt. Plates were incubated for 48 h at 28°C before the number of colony forming units (cfu's) was determined. Transfer frequencies, which actually should be regarded as occurrence of transconjugants, were calculated both as the number of transconjugant cfu/number of donor cfu and as number of transconjugant cfu/number of recipient cfu.

Plasmid transfer in sterile soil between introduced donor and recipient bacteria

Transfer of pSKTG in sterile soil was studied using the same donor and recipient combinations as in the filtermatings. Ede loamy sand (Van Elsas et al., 1988) was air-dried to 10% moisture content and sieved (4 mm mesh). A ^{60}C source was used to (gamma) irradiate the soil in 1 l plastic flasks which were rotated during sterilization (4 MRad). Soil was considered sterile since no colonies appeared on 0.1 strength Triptone Soya Agar (Martin, 1975) after 5 days of incubation at 20°C. 50 g aliquots were added, at a bulk density of 1.4 (g soil/volume on wet weight basis), to sterile plastic cups. Overnight cultures of donor and recipient were washed twice in sterile demineralized water and added to soil, establishing a soil moisture content of 18% (-10^{-2} MPa) and a population density of approximately 10^7 cfu each of donor and recipient/g soil. The soil was thoroughly mixed with a spatula. Strains were introduced with at least a 1 h interval to avoid matings in the liquid phase during processing. The microcosms were incubated for 2 days at 25°C in moist chambers to avoid drying, after which they were sampled and processed as described below.

Plasmid transfer in natural soil planted to wheat

Mobilization of pSKTG to indigenous bacteria was studied using *P. fluorescens* R2fR containing either pSKTG or RP4p as donor strains. The chromosomal resistance of R2fR to Rp was used as an extra criterion with which donor cells could be differentiated from indigenous transconjugants (see below).

Freshly collected Ede loamy sand, air-dried to about 10% moisture and sieved (4 mm mesh), was inoculated with washed overnight cultures of different combinations of the two donor strains, with 1 h intervals between introduction of each strain. Populations of each strain at about 10^7 cfu/g soil were established, and the soil moisture content was brought to 18%. Approximately 70 g of soil was compressed into (30 x 42 mm) polyvinylchloride rings, to a bulk density of 1.4 (wet weight basis), and these rings were

placed on a water tension table to keep the soil moisture content constant at 18%. Six pre-germinated seeds of wheat, *Triticum aestivum* cv. *sicco*, were planted/ring, and the rings were incubated for 7 days in a climate chamber under a cycle of 16 h of light and 8 h of dark at 20°C and 16°C, respectively.

Extraction and enumeration of bacteria from soil

Both sterile and non-sterile soils were sampled as described below. In both systems, cells were isolated from soil shortly (3 h) after inoculation to assess initial plasmid transfer and population levels of donor and recipient. The sterile unplanted microcosms were sampled on day 2, and the non-sterile microcosms were sampled on day 7, when the plants had a sufficiently developed rhizosphere. Plants were gently removed from soil, shaken, and the roots were separated from above-ground parts. Roots plus adhering soil were designated as the rhizosphere sample. The remaining soil and soil from unplanted systems was designated bulk soil. Portions of 10 g of both samples were shaken in Erlenmeyer flasks containing 95 ml of sterile 0.1% sodium pyrophosphate and 10 g of gravel (Smit et al., 1991). The suspensions were then serially diluted and plated onto selective agar plates as in the filtermatings (Table 1).

Donor cells from non-sterile soil were enumerated on King's B agar (Van Elsas et al., 1988) containing the appropriate antibiotics. To select indigenous transconjugants from non-sterile soil, suspensions were incubated with equal volumes of ΦR2f lysate (10^9 pfu/ml), to eliminate the donor strain (Smit et al., 1991), and 100 µl was plated on LB agar containing 50 mg/l of Tc and Ap, to select for transconjugants containing RP4p, or 50 mg/l of Km and Gt, to select for transconjugants containing pSKTG. 100 mg/l of cycloheximide was added to all media to minimize fungal growth. The total number of culturable bacteria was determined by plating on 0.1 strength Tryptone Soya Agar (TSA) (Martin, 1975). Plates were incubated for 2 to 5 days at 28°C.

Filter matings with indigenous soil bacterial populations to detect plasmid mobilizing capacity

To isolate plasmids from soil bacteria, filter matings were done (Fry and Day, 1990; Hill et al., 1992). Indigenous cells were extracted from soil as described above, and 100 µl of the cell suspension was mixed with 30 µl of a washed overnight culture of *P. fluorescens* R2fR (pSKTG) or of both *P. fluorescens* R2f(pSKTG) and R2fN. Bacteria extracted from rhizosphere and bulk soil from Ede loamy sand (10^7 to 10^8 cfu) were mixed with donor cells (10^7-10^9 cfu). The mixtures were pipetted onto a filter, as described before. Incubation times from 4 to 24 h at 28°C were used. After incubation, filters were vortexed in LB, and dilutions were plated. To enumerate indigenous bacteria containing pSKTG, dilutions were treated with ΦR2f to eliminate the donor before plating on LB plates containing Km and Gt. To detect plasmids with mobilizing activity, dilutions were plated on recipient-selective plates (containing Rp and Nx) supplemented with Km and Gt. Colonies on transconjugant selective plates were

verified as described below.

Analysis of potential indigenous transconjugants

After incubation, colonies on transconjugant-selective plates were transferred to two selective plates. One plate was stored at 4°C, and the other was used for colony filter hybridization (Sambrook et al., 1989), to distinguish naturally resistant indigenous bacteria from those that had acquired one of the recombinant plasmids. Membrane filters (Colony/Plaque screen, Biotechnology Systems, Du Pont) were placed on the surface of each agar plate, and colonies were lifted, lysed, and the DNA fixed to the filter. The 0.7 kb (*pat*) and 1.4 kb (*cry*IVB) fragments were used as probes. The sequences were excised from their vectors (see above) and the appropriate bands were cut from agarose gels and purified by electro-elution. Fragments were labelled with [^{32}P]-α-dATP by nick translation. Hybridization was overnight at 65°C. The colony blots were washed at highest stringency (according to the manufacturer's instructions) before exposure to X-ray film. Colonies reacting positively with the probe were verified, to discriminate between indigenous transconjugants and donor cells which had escaped phage lysis by ΦR2f, by plating onto King's B agar containing Rp. Transconjugants that reacted with the *cry*IVB probe were verified for the presence of RP4p by plating on LB with Tc and Ap. The presence of plasmids was assessed by small-scale plasmid isolations according to Kado and Liu (1981). The transconjugants were also verified with a specific antiserum for the donor strain (Trevors et al., 1990), to exclude the possibility that they were donor cells. The mobilizability of pSKTG from the transconjugants was further verified by triparental filter matings with donor (RP4p) and recipient strains. Transconjugants were identified using the BIOLOG (Biolog Inc. La Jolla, CA) system.

Statistics

All experiments were performed in duplicate. The soil experiments were repeated once. The Student's t-test ($P<0.05$) was employed to determine the significance of difference between counts of cfu's.

Results

Mobilization of pSKTG in donor x recipient filter matings

It must be noted that most of the results are reported as transfer frequencies, whereas they are actually the result of transfer and possible growth and death of donor, recipient and transconjugants.

pSKTG was transferred from *P. fluorescens* R2f with mobilizing plasmid RP4p present, either in the same strain or in different strains, to the homologous recipient *P. fluorescens* R2fN (Table 2). No transconjugants were detected when the recipient was mixed with the donor strain containing only pSKTG. However, a transfer frequency of

approximately 10^{-2} per donor was found when the recipient and a donor strain containing both pSKTG and RP4p were added. This was equal to the frequency of transfer of RP4p. Similar transfer frequencies were found in triparental matings, with RP4p and pSKTG initially present in different donor strains. However, when frequencies were calculated per recipient pSKTG was transferred a factor 10 lower then RP4p (Table 2). The retro-mobilizing activity of RP4p was demonstrated by adding *P. fluorescens* R2fN(RP4p) as a recipient, together with R2f(pSKTG) and plating on LB agar supplemented with Rp, Nx, Km and Gt (Table 3). The frequency ranged from $5x10^{-4}$ to $2x10^{-3}$ per donor, depending on the incubation time. Spontaneous mutations of either donor or recipient, which might result in growth on transconjugant selective plates, was below 10^{-9} (not shown).

Table 1. Strains used

Strain	Plasmid(s)[a]	Chr. markers[b]	Pl. markers[c]
Pseudomonas fluorescens R2f	RP4p	-	Ap, Tc
Pseudomonas fluorescens R2f	pSKTG	-	Km, Gt
Pseudomonas fluorescens R2f	RP4p, pSKTG	-	see sep.[d]
Pseudomonas fluorescens R2fR	RP4p	Rp	Ap, Tc
Pseudomonas fluorescens R2fR	pSKTG	Rp	Km, Gt
Pseudomonas fluorescens R2fN	-	Rp, Nx	-
Escherichia coli MC1061	-	-	-

[a])See Materials and Methods.

[b])Chr.:chromosomal; Rp:rifampicin; Nx:nalidixic acid; R2fR and R2fN are spontaneous mutants resistant to Rp and Rp and Nx, respectively.

[c])Pl.:plasmid; Ap:ampicillin; Tc:tetracycline; Km:kanamycin; Gt:gentamycin

[d])See sep.: See separate plasmid descriptions.

Plasmid transfer in sterile soil

The experiments in sterile soil were performed with the same donor and recipient combinations as in the filter matings (Table 4). The introduced recipient populations increased slightly (counts on day 2 were significantly higher than initial counts, $P<0.05$), while the donor populations remained roughly constant (no significant differences between initial and day 2 counts, $P>0.05$). Transfer of pSKTG was not detected in the absence of RP4p. When both plasmids were present in the same strain, transfer frequencies of 2.10^{-4} per donor were found for both plasmids (not significantly different, $P>0.05$). When RP4p was present in a different strain, the transfer frequency of pSKTG decreased to $2x10^{-6}$, while the transfer frequency of RP4p was still $6x10^{-4}$; these figures were significantly different ($P<0.05$).

Table 2. Transfer of plasmids RP4p and pSKTG between donor and recipient strains of *P. fluorescens* on filters on agar plates (4 h).*

	Log cfu/ml									
Tr		Donor		Rec	Tran		freq/d	freq/r	freq/d	freq/r
	RP4p	pSKTG	both		RP4p	pSKTG	RP4p	RP4p	pSKTG	pSKTG
A	7.6	6.7	-	7.8	6.0[a]	5.1[b]	$2.8x10^{-2}$	$1.2x10^{-2}$	$2.3x10^{-2}$	$1.9x10^{-3}$
B	-	-	7.9	8.2	ND	6.2[c]	ND	ND	$2.0x10^{-2}$	$1.0x10^{-2}$
C	-	8.8	-	8.5	-	[0]	-	-	[X]	[X]

*Tr=treatment, A: *P.fluorescens* R2f(RP4p), *P.fluorescens* R2f (pSKTG); B: *P. fluorescens* R2f (RP4p, pSKTG); C: *P. fluorescens* R2f (pSKTG); Rec:recipient (*P. fluorescens* R2fN) cfu; Tran:transconjugant cfu; freq/d:frequency per donor; freq/r: frequency per recipient.
[0]:below 10 cfu/ml; ND:not done; -:not relevant; [X]:below 10^{-8}
[a] is significantly different from [b] and [b] is significantly different from[c] (P,0.05).

Table 3. Retromobilization of pSKTG from *P. fluorescens* R2f(pSKTG) to *P. fluorescens* R2fN(RP4p) strain on filters on agar plates.

Time (h)	Donor pSKTG	Recipient RP4p	Transconjugant	
	Log cfu/ml			Freq/d
4	7.7	7.5	4.4[a]	$5x10^{-4}$
24	9.0	8.9	6.3[b]	$1.9x10^{-3}$

Freq/d: frequency per donor;
[a]is significantly different from [b] ($P<0.05$).

Plasmid transfer in non-sterile soil planted to wheat

The purpose of these experiments was to study transfer of pSKTG to indigenous bacteria; ΦR2f was employed for counter-selection of the donor (Smit et al., 1991). Only absolute bacterial numbers are presented in Table 5 rather than transfer frequencies because differences in numbers of donor and recipient after the incubation period were rather high, which would influence frequencies. A comparison of donor numbers on day 0 and day 7 showed that the strains carrying either pSKTG or RP4p survived relatively well. The donor with RP4p declined from about log 7.5 to log 6.5 cfu /g dry soil, and the donor with pSKTG declined from log 6.8 to log 6.4 cfu /g dry soil. No significant effect of the roots on bacterial numbers was noted. The strain with both plasmids declined rapidly in rhizosphere and bulk soil from log 6.8 to log 5 and from log 6.8 to log 4 cfu /g dry soil, respectively. No transconjugants were detected on day 0, i.e. after 3 h in soil.

No indigenous transconjugants were found on day 7 when a strain carrying pSKTG without RP4p was introduced, indicating the scarcity of natural mobilizing agents in the soil/plant system. Mobilization of pSKTG to indigenous bacteria was detected when RP4p was present either in the same strain or in the co-introduced strain (Table 5, treatments A and B). The number of transconjugants obtained with the introduced donor containing both plasmids was significantly higher in the rhizosphere than in the corresponding bulk soil. This "plant effect" was less pronounced when RP4p was present in the separate donor strain (A), although it must be noted that transconjugant cfu counts in treatment A are just above detection limit and not very reliable for statistic analysis. Surprisingly, when both plasmids were present in the same donor strain (B), the number of transconjugants containing pSKTG was approximately 10-fold

Table 4. Transfer of RP4p and mobilization of pSKTG between *P fluorescens* donor and recipient strains in sterile soil after 2 days*).

Tr	Donor			Rec	Tran		Freq	
	RP4p	pSKTG	Both		RP4p	pSKTG	RP4p	pSKTG
			log	cfu	/g	soil		
A	8.1	7.7	-	8.8	4.9^a	2.2^b	$6.4*10^{-4}$	$2.7*10^{-6}$
B	-	-	7.8	8.7	4.1^c	4.2^c	$2.0*10^{-4}$	$2.0*10^{-4}$
C	-	7.3	-	8.9	-	[0]	$4.2*10^{-4}$	-
D	7.9	-	-	8.8	4.5^a	-	-	-
E	-	-	-	8.8	[0]	[0]	-	-

*Explanation: Rec: *P. fluorescens* R2fN cfu; Tran: transconjugant cfu; both: plasmid RP4p and pSKTG present; Log numbers of cells per g of dry soil; Tr: treatment, A: *P.fluorescens* R2f(RP4p), *P. fluorescens* R2f(pSKTG); B: *P. fluorescens* R2f(RP4p, pSKTG); C: *P. fluorescens* R2f(pSKTG); D: *P. fluorescens* R2f(RP4p); E: no donor, *P. fluorescens* R2fN was included in all treatments.
log cfu counts on day 0 (3 h after inoculation) were A: RP4p 7.8, pSKTG 7.7, rec. 8.4; B: both 8.2, rec. 8.5; C: pSKTG 7.6, rec. 8.6; D: RP4p 7.6, rec. 8.5; E: rec. 8.5.
[a] is significantly different from [b] and [c] ($P<0.05$); same letters are not significantly different ($P<0.05$).

Table 5: Log numbers of indigenous transconjugants containing RP4p or pSKTG and donor cells 7 days after introduction of *P. fluorescens* R2f with these plasmids in non-sterile Ede loamy sand planted to wheat in soil microcosms*.

Treatment		Donor			Indig. transconjugant	
		RP4p	pSKTG	both	RP4p	pSKTG
		log cfu/g soil				
A	R	6.3	6.5	-	2.7[a]	2.2[a]
	B	6.4	6.4	-	[0]	2.1[a]
B	R	-	-	5.0	3.0[c]	4.2[d]
	B	-	-	4.0	2.2[a]	3.0[c]
C	R	-	6.5	-	-	[0]
	B	-	6.4	-	-	[0]
D	R	6.3	-	-	4.3[e]	-
	B	6.0	-	-	1.7[f]	-
E	R	-	-	-	-	[0]
	B	-	-	-	-	[0]

* R: rhizosphere, B: bulk soil; Treatment A: *P. fluorescens* R2fR(RP4p), *P. fluorescens* R2fR(pSKTG); B: *P. fluorescens* R2fR(RP4p, pSKTG); C: *P. fluorescens* R2fR(pSKTG); D: *P. fluorescens* R2fR(RP4p); E: no strain added; log cfu/g soil on day 0: A: Donor R2f(RP4p) 7.7, donor R2f(pSKTG) 7.9; B: Donor R2f (RP4p; pSKTG) 6.7; C: Donor R2f(pSKTG) 7.5; D: Donor R2f(RP4p) 7.1; E: no donor added; [0] = below detection limit (approximately 10^2 cfu/g soil).
Values labeled with different letters were significantly different from each other ($P<0.05$).

higher than that of those with RP4p. When nine transconjugants containing pSKTG were analyzed further, none grew on King's B agar supplemented with Rp, and they did not react with the R2f antiserum, indicating that they were not derivatives of the introduced donor strain. Further, none was resistant to Tc and Ap, suggesting that they did not contain RP4p, which might indicate that only pSKTG was transferred or that RP4p was not stably maintained. Plasmid analysis on agarose gels confirmed that only pSKTG was present, since RP4p could not be visualized (not shown). All nine transconjugants were similarly identified by the BIOLOG system as *Pseudomonas fluorescens* subgroup B. They differed from the donor strain in eight traits in the BIOLOG system.

Mobilization of pSKTG by plasmids from indigenous soil bacteria

As no *in situ* mobilization of pSKTG was detected when the mobilizing plasmid RP4p was not added, the number and/or activity of indigenous bacteria possessing genetic elements with such capacity was apparently too low to enable detection. In an attempt to detect natural mobilizing genetic elements in the indigenous microbiota, filter matings were performed, to detect indigenous soil bacteria capable of taking up pSKTG by retrotransfer or triparental mating, and to assess putative mobilization of pSKTG from an introduced donor by natural genetic elements to an introduced recipient. In three independent experiments, a considerable background of indigenous bacteria resistant to Km and Gt was encountered. A thorough screening of numerous colonies that reacted with the *cry*IVB probe revealed that all were donors that had escaped lysis by ΦR2f. Thus, indigenous transconjugants containing pSKTG could not be detected in these initial studies (detection limit was 10^{-4}).

The triparental approach with an introduced selectable recipient strain was then attempted to eliminate the background, thus allowing lower dilutions to be plated. In the control treatment (donor x recipient without the soil bacterial population) no colonies developed on plates containing Rp, Nx, Km, and Gt. On plates from the donor x recipient x indigenous biota (from unplanted Ede loamy sand soil) treatment, two different colony types were found: one representing the recipient, which acquired resistance to Km and Gt, and one of a strain naturally resistant to Rp, Nx, Km, and Gt. Plasmid analysis (not shown) revealed the presence of pSKTG in the recipient. No other plasmids which might have been responsible for the mobilization could be observed, which could indicate that they were either present but not visible using this extraction method, or that the elements had integrated into the chromosome. The mobilization of pSKTG in a second filter mating to an *E. coli* recipient confirmed the presence of Tra functions in the transconjugant *P. fluorescens* R2fN(pSKTG). These Tra functions were absent before mating with the indigenous microbiota.

Discussion

The experiments described in this work were aimed at answering questions concerning the possible spread of recombinant DNA located on an IncQ plasmid, a versatile vector for purposes of genetic engineering. Advantages of the use of such vectors are the enhanced copy gene number (10 copies/genome) as compared with chromosomal inserts or IncP1-derived vectors, leading to enhanced gene expression, and, possibly, enhanced biosafety as the result to the absence of Tra functions present in self-transmissible plasmids.

Conjugative plasmids are commonly found in bacteria isolated from the environment (Bender and Cooksey, 1986; Diels et al., 1989; Fry and Day, 1990; Hill et al., 1992; Lilley et al., 1992; Sayler et al., 1990; Shoemaker et al., 1992). In addition, stress applied on the soil bacterial community may result in an enhancement of plasmid incidence (Burton et al., 1982; Schmidt and Schlegel, 1989; Wickham and Atlas, 1988). Such genetic elements could potentially be responsible for the dissemination of recombinant Mob^+ plasmids such as pSKTG, present in GEMs introduced in the environment. However, not much is known about the distribution of plasmids with transfer functions in bacteria in natural soil.

To simulate the occurrence of Tra^+ plasmids in soil, plasmid RP4p was added to soil either in the strain carrying pSKTG or in a separate strain. The step-by-step approach used, from matings on membrane filters to those in sterile or in natural soil, gives insight into the extent to which the soil environment affects cell-to-cell interactions and dynamics of mobilization.

The mobilization of pSKTG on membrane filters (Table 2) in a triparental mating was as efficient as direct mobilization in a biparental mating. Under these conditions, optimal for growth and cell-to-cell contact, the supposed 2-step transfer in the triparental mating was apparently not limiting as compared to the one-step direct mobilization. A comparison of these results with those obtained in sterile soil showed that soil acted as a barrier to conjugative transfer, as the transfer frequencies of RP4p and pSKTG (with RP4p present in the same cell) were approximately 100-fold lower then those on filters (Table 4). Similarly, Selvaratnam and Gealt (1992) found a 100-fold reduction in plasmid transfer when comparing liquid and soil matings. The chance of bacterial cell-to-cell contact was apparently smaller in soil than on filters. The triparental mating in soil resulted in a mobilization frequency of pSKTG of $2x10^{-6}$ /donor, about 100-fold lower than in the biparental mating. This mobilization involved contact of three different cell types and two separate transfer events, which combined with a lowered cell-cell contact reduces mobilization in soil even more. Moreover, the lack of nutrients in natural soil might also limit growth of transconjugants as compared to matings on rich media.

Successful conjugal plasmid transfer in soil depends on donor and recipient population densities and on the availability of nutrients, clay or pH, being optimal

(Richaume et al., 1989, 1992; Smit et al., 1991; Stotzky, 1992; Van Elsas et al., 1988). The low frequency of transfer in soil is probably largely the result of infrequent bacterial contact, rather than the possibly suboptimal conditions of pH and nutrient availability, since the number of microsites is relatively large as compared to bacterial cell dimensions and numbers. Further, after transfer, the growth of transconjugants in natural soil is probably limited (Richaume et al., 1992). There is apparently an upper limit for the population sizes of similar bacteria present in the same soil microsites.

Although results obtained with homologous donor and recipient strains indicate the possibility for transfer (Tables 2,3,4), their predictive value for the mobilization of pSKTG to indigenous bacteria in soil was uncertain, as it was unknown if an IncQ plasmid could be mobilized to indigenous bacteria by conjugative plasmids present in the same or in other strains.

The detection of pSKTG transfer to indigenous bacteria in soil planted to wheat, with RP4p present in a separately inoculated strain (Table 5 A,B,C), indicated mobilization in a triparental mating event. Transfer of pSKTG by naturally occurring genetic elements could not be detected in microcosms. The higher number of transconjugants containing pSKTG than those with RP4p that originated from an introduced donor with both plasmids (Table 5 B) indicated that pSKTG was transferred more efficiently then RP4p itself.

Naturally mobilizing elements, if present, were apparently scarce in soil and rhizosphere bacterial populations, as they were not detected in matings between the donor and the indigenous microbiota in non-sterile soil and on filters. The use of a triparental mating involving a selectable recipient, *P. fluorescens* R2fN, enabled the detection of transconjugants, indicating the presence of *tra* genes in the soil microbiota. Although no clear profiles of the element on which such genes were localized after plasmid extractions were obtained, the mobilization of pSKTG to an *E. coli* recipient strain in a second mating showed the presence of Tra functions in these *P. fluorescens* R2fN transconjugants. Use of a PCR detection system using specific primers for the *trf*A gene, indicative of the presence of IncP1-type plasmids, provided evidence for the presence of this gene in one of the *P. fluorescens* R2fN transconjugants (not shown). No PCR product was observed with a control *P. fluorescens* R2fN strain. Recent studies in the human colon (Shoemaker et al., 1992) and on the surface of sugarbeets (Lilley et al., 1992), provided evidence for the occurrence of gene transfer via natural mobilization. However, thorough studies will be needed to evaluate further the natural transfer potential in soil.

In summary, the data indicated that in the absence of added mobilizing plasmids transfer of the IncQ vector, pSKTG, could not be detected. Mobilization could only be shown in the presence of the self-transmissible IncP1 vector, RP4p. However, an IncQ plasmid can potentially be mobilized via the activity of genetic elements present in indigenous soil bacteria.

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CHAPTER 8

Genetic stability, conjugal transfer and expression of heterologous DNA inserted into different plasmids and the genome of *Pseudomonas fluorescens* in soil.

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Summary

The influence of the location of heterologous DNA in *Pseudomonas fluorescens* R2f on gene stability, expression and transfer following introduction into soil was studied. Three strains with markers on different genetic elements were used, i.e a selftransmissible plasmid (RP4p), a mobilizable plasmid (pSKTG) and a chromosomally inserted marker gene cassette (KTG). *In vitro* filter mating experiments showed that the selftransmissible plasmid was transferred with high frequencies (about 10^{-2}) and that this plasmid could mobilize pSKTG with similar frequencies. The chromosomally inserted marker cassette could be mobilized by RP4p to a recipient strain with low frequency (10^{-8}). In sterile soil, transfer of the chromosome could not be detected in the presence of RP4p. When the 3 strains were introduced into 2 soils of different texture planted with wheat, they showed poor survival in Ede loamy sand and good survival in Flevo silt loam. Moreover, a partial, but significant loss of expression of the gentamycin resistance gene *aad*B was observed in Ede loamy sand, but not in Flevo silt loam.

RP4p was found to be transferred from an introduced donor to indigenous bacteria in both non-sterile soils planted to wheat, whereas transconjugants harbouring the mobilizable plasmid pSKTG or the chromosomally inserted marker cassette were not detected when using a donor strain without IncP plasmid.

Introduction

The deliberate or accidental release of genetically engineered microorganisms (GEMs) into the environment has led to an increase in gene transfer studies in different environments (Fry and Day, 1990; Levin and Strauss, 1990; Levy and Miller, 1989; Reanney et al., 1983; Wellington and Van Elsas, 1992). Gene transfer by conjugation is an important process in an environment like soil (Van Elsas et al., 1988a, b, 1987; Ramos-Gonzales et al., 1991; Richaume et al., 1989; Richaume et al., 1992; Smit et al., 1991; Top et al., 1990; Wellington et al., 1990). Most research has focused on the transfer of selftransmissible plasmids (Van Elsas, 1988a, b, 1987; Ramos-Gonzales et al., 1991; Richaume et al., 1989; Richaume et al., 1992; Smit et al., 1991; Top et al., 1990; Wellington et al., 1990), whereas conjugal transfer and genetic stability of non-selftransmissible plasmids (Henschke

and Schmidt, 1990) and chromosomal insertions have rarely been evaluated in environmental studie. Krasovsky and Stotzky (1987) detected conjugal transfer of chromosomal DNA by introducing *Escherichia coli* Hfr donor strains together with *E. coli* recipients into soil microcosms. However, Hfr strains exhibit high mobilization frequencies and are unlikely to be used as GEMs for release.

Besides transfer of recombinant DNA, more attention should be given to the environmental stability and expression of recombinant DNA on non-selftransmissible plasmids or the chromosome (Smit et al., 1992). The mobilizable IncQ plasmid pSKTG (Smit and Van Elsas, 1992), consisting of RSF1010 replication and mobilization (*mob*) functions and a marker gene cassette, was constructed to serve as non-selftransmissible plasmid. Moreover, the marker gene cassette, which contained the neomycin/kanamycin phosphotransferase gene *npt*II and the aminoglycoside adenylating gene *aad*B, conferring resistance to respectively neomycin/kanamycin and gentamycin, and part of *cry*IVB (Smit and Van Elsas, 1992) as a molecular marker, was also inserted into the chromosome.

The present work focused on the stability, expression and possible transfer of chromosomally located heterologous DNA in a *P. fluorescens* strain introduced into soil. Both the fate of the DNA and of the strain was studied for 28 days in one kg microcosms planted with wheat. Chromosomal DNA can potentially be mobilized by IncP plasmids (Barth, 1979; Pemberton and Bowen, 1981; Sistrom, 1977); *in vitro* studies have provided extensive data on chromosome mobilization by such plasmids, with frequencies ranging from 10^{-3} to 10^{-9} (Haas and Reimmann, 1989). IncP or other mobilizing plasmids might be naturally present in soil bacteria and be responsible for the dissemination of chromosomally inserted genes (Hill et al., 1992; Lilley et al., 1992). Gene stability and expression was also investigated, since these parameters have hardly been studied in soil. Both soil type and the presence of plant roots might influence the stability and expression of genes in soil.

Materials and methods

Bacterial strains, plasmids, phage and growth conditions

Strains of the soil isolate *Pseudomonas fluorescens* R2f (Van Elsas et al., 1988a) harbouring either different plasmids or a chromosomally inserted marker gene cassette, were used in this study (see Table 1). The use of plasmid RP4p, RP4 marked with a *Solanum tuberosum* cv *bintje* sequence, *pat*, was described before (Smit et al., 1991). Plasmid pSKTG was constructed from RSF1010 sequences (replication functions and the *mob* site) and a gene cassette composed of *npt*II and *aad*B and part of *cry*IVB, coding for a *Bacillus thuringiensis* endotoxin, as a molecular marker (Smit and Van Elsas, 1992). The same marker cassette was also inserted into the chromosome of *P. fluorescens* R2fR by using the disarmed transposon delivery system of Herrero et al. (1990). This system consists of the transposase (*tnp*) gene and 19 bp of the inverted repeats of Tn*5* cloned into a mobilizable suicide vector (pUT). The marker cassette was cloned into a helper plasmid (p18Sfi), allowing it to be excised again using *Sfi*I. The marker cassette was cloned into the *Sfi*I site

in the pUT plasmid (giving pUT/KTG) and transformed into *E. coli* SM10 lambda *pir* (Herrero et al., 1990; Smit and van Elsas, 1992). Transfer of pUT/KTG to *P. fluorescens* R2fR resulted in *P.fluorescens* R2fmc, resistant against rifampcin (Rp), kanamycin (Km) and gentamycin (Gm). This strain was checked by probing with *cry*IVB and *tnp* (Herrero, et al., 1990), followed by Southern blotting of total genomic DNA. Furthermore, tests were done on the stability of the inserted genes by culturing this strain under non-selective conditions and comparing selective and non-selective plate counts (Smit and Van Elsas, 1992).

Phage ΦR2f, obtained from agricultural drainage water, was used for donor counterselection because of its specificity for *P. fluorescens* R2f, it was propagated and maintained as described before (Smit et al., 1991).

All strains were cultured in LB (10 g Difco tryptone; 5 g Difco yeast extract; 5 g NaCl; 1 l H_2O; pH 7.2) with 50 mg/l of the appropriate antibiotics.

Soils

The two soils used, Ede loamy sand and Flevo silt loam were described before (Richaume et al., 1992; Van Elsas et al., 1988a). Portions of freshly collected Ede loamy sand and Flevo silt loam were air-dried to about 10% (Ede loamy sand) or 20% (Flevo silt loam) and sieved (4 mm mesh) before use.

Chromosome mobilization in filter matings

To test the maximal mobilization frequency of the chromosomally inserted marker cassette, filter matings between *P. fluorescens* R2fmc(RP4p) and *P. fluorescens* R2fSN were done (Table 2). Aliquots of 50 μl of the washed cell suspensions were pipetted onto the filters, either separately or jointly, and mixed. After 24 h of incubation, filters were vortexed in 3 ml LB broth and the resulting suspensions were serially diluted and plated on LB agar with antibiotics as follows: *P. fluorescens* R2fmc (RP4p) was enumerated on LB with 50 mg/l of Km and 50 mg/l of tetracycline (Tc), *P. fluorescens* R2fSN recipient cells were selected on 100 mg/l streptomycin (Sm) and 100 mg/l nalidixic acid (Nx), and transconjugants were selected on LB agar containing 50 mg/l each of Nx, Sm and Km. Also, donor and recipient were plated on plates containing Sm plus Nx, and Km, to check for mutations. Plates were incubated at 28°C for 4 days before the number of cfu was determined. Transfer frequencies were calculated as transconjugant cfu per donor cfu.

Chromosome mobilization in sterile soil

Mobilization of the chromosomally inserted marker cassette was also studied in sterile soil, using the same donor/recipient combinations as in the filter matings (Table 3). The soils, Ede loamy sand and Flevo silt loam were sterilized by irradiation (4 Mrad) prior to use. Portions of 50 g were compressed to a bulk density of 1.4 (wet weight basis) in sterile plastic cups. Washed overnight cultures of donor and recipient strains were added to soil to establish population densities of 10^8 cfu per gram of soil. In order to get a pF value of 2, water was added to the soils to 18% for Ede loamy sand and 38% for Flevo silt loam. Soil

portions were then thoroughly mixed using a spatula. Strains were introduced with at least 1 hour intervals to avoid matings in the liquid phase. The microcosms were incubated for 7 days at 20°C in moist chambers to avoid drying, after which they were sampled and processed as described below.

Bacterial survival, genetic stability and transfer in non-sterile soil planted to wheat.

Overnight cultures of P. fluorescens R2fR (RP4p), *P. fluorescens* R2fR (pSKTG) and *P. fluorescens* R2fmc were transferred to 500 ml LB to which 50 mg/l of the appropriate antibiotics had been added. After incubation at 28°C at 200 rpm for 16 hours, cells were harvested washed and separately introduced into both soils. Then, demineralized water was added to establish a pF 2 in the soils. Portions of 1 kg of inoculated soil were packed in plant pots (14 cm height, 14 cm diameter) to a bulk density of 1.4 (wet weight) and planted with 1 pregerminated (2 days, 20°C) wheat (*Triticum aestivum cv. sicco)* seedling. To limit evaporation, pots were covered with transparent lids with a small hole to allow the wheat plant to grow through. Pots were incubated in a climate chamber using a cycle of 16 h light and 8 h dark at 20°C and 16°C, respectively. Every other day pots were weighed and water was added up to the original weight.

Determination of expression level of aadB in cells from soil

Since a considerable loss of expression of resistance to Gm of cells of *P. fluorescens* R2fmc extracted from Ede loamy sand was suspected to occur, a separate experiment was done to determine the minimal level of resistance to Gm in cells from soil or starvation culture. *P. fluorescens* R2fmc was added to 50 g portions of Ede loamy sand as described above. The strain was also grown to early log phase, washed three times and suspended in 1/4 Ringers solution (NaCl 2.15 g, KCl 0.075 g, $CaCl_2$ 0.12 g, $NaS_2O_4.7H_2O$ 0.5 g, 1 l H_2O) and incubated in a shaker (100 rpm) at 20°C.

After 10 days in soil or 1/4 Ringers, samples were plated on King's B agar supplemented with 50 mg/l Km plus 0, 10, 20, 30, 40 or 50 mg/l Gm. The number of cfu's was determined and the percentage of the population growing on the plates with different Gm concentrations was calculated and compared to the number on plates with only Km.

Extraction and enumeration of bacteria from soil

Both sterile and non-sterile systems were sampled as described below. In both systems cells were isolated from soil shortly (three hours) after introduction to determine initial population levels. The sterile systems were further sampled at day 7, while the non-sterile planted systems were sampled at day 10, day 17 and day 28. Plants were gently removed from soil, shaken, and the roots were separated from aboveground parts. Roots plus adhering soil were designated as the rhizosphere sample. The remaining soil and soil from unplanted microcosms was designated bulk soil. Portions of 10 g of both rhizosphere and bulk soil samples were treated as described before (Smit et al., 1991). To enumerate the introduced

strains in non-sterile planted systems, King's B agar (Van Elsas et al., 1988a) was used; cycloheximide (100 mg/l) was added to minimize fungal growth. For *P. fluorescens* R2fR (RP4p) Rp (50 mg/l) and Tc (50 mg/l) were added, for *P. fluorescens* R2fR (pSKTG) and *P. fluorescens* R2fmc Km (50 mg/l) and Gm (50 mg/l) were added in addition to Rp. Furthermore, samples were plated on King's B with either Rp (50 mg/l) or Km (50 mg/l) to assess loss of marker genes or expression.

To obtain indigenous transconjugants, suspensions from the Erlenmeyer flasks were first incubated with equal volumes of lysate of phage ΦR2f to selectively eliminate the donor strain (Smit et al., 1991). Aliquots of 100μl were then plated on LB agar plates containing 50 mg/l of both Tc and Ap to select for transconjugants harbouring RP4p, or 50 mg/l of both Km and Gm to select for transconjugants harbouring pSKTG or the marker cassette. The total number of culturable bacteria was determined by plating on 0.1 strength TSA (Smit et al., 1991). Plates were incubated for 2 to 5 days at 28°C.

Analysis of introduced bacteria and transconjugants

Non-selective plates (total counts) and selective plates (with Rp or Km, for the enumeration of introduced bacteria) were randomly used for colony filter hybridization (as described below) to check for the presence of the molecular marker. Colonies (>200 per treatment and sampling) on transconjugant-selective plates (for RP4p, pSKTG and ::KTG transfer) were transferred to duplicate selective plates. One plate (master) was stored at 4°C, and the other used for colony filter hybridization (Sambrook et al., 1989). The *pat* and *cry*IVB fragments were used as probes to detect RP4p, pSKTG or the chromosomal marker cassette KTG (Smit et al., 1991; Smit and Van Elsas, 1992). Both sequences were obtained from their cloning vectors by restriction with *Hin*dIII (*pat*) or *Xba*I (*cry*IVB). The 0.7 kb (*pat*) and 1.4 kb (*cry*IVB) fragments were cut from agarose gels and purified by electro-elution. The colony blots were washed at highest stringency before exposure of X-ray film. Colonies reacting positively with the probes were further checked, to discriminate between indigenous transconjugants and surviving donor cells, by plating on King's B agar containing Rp. Since in previous work indigenous transconjugants which received RP4p or pSKTG have been identified to genus/species level (Richaume et al., 1992; Smit et al., 1991, 1993), this was not done here.

Statistics

Differences in colony forming unit counts between treatments were statistically analyzed using ANOVA at a level of significance of $P < 0.05$, using the Genstat-5 programme. The overall LSD value for the whole experiment is given in the Figures.

Results

Chromosome mobilisation on agar and in soil

In previous experiments RP4p was transferred in filter matings with a frequency of 10^{-2} and mobilization of plasmid pSKTG by RP4p occurred with similar frequencies (Smit et al., 1993). In the present filter mating experiments, RP4p was transferred from donor to homologous recipient with similar frequency (Table 2). RP4p present in *P. fluorescens* R2fmc could mobilize the chromosomally-inserted marker cassette with a low frequency ($4.5\ 10^{-8}$), comparable with values found in litterature for RP4 (Haas and Reimmann, 1989). Parallel controls on mutation of donor and recipient were negative. The presence of KTG in the transconjugants was confirmed by colony blotting and antibiotic resistance testing.

Table 1. Strains used in this study

Strain	Plasmid	Markers (resistance)	chr.markers (resistance)
Pseudomonas fluorescens R2f	-		-
P. fluorescens R2fR	-		Rp
P. fluorescens R2fSN	-		Sm Nx
P. fluorescens R2fmc	-		Rp Km Gm
P. fluorescens R2fmc (RP4p) RP4p	Ap Tc		Rp Km Gm
P. fluorescens R2fR (pSKTG) pSKTG	Km Gm		Rp
P. fluorescens R2fR (RP4p)	RP4p	Ap Tc	Rp

chr. marker = chromosomal marker

Ap: Ampicillin, Tc: Tetracycline, Rp: Rifampicin, Sm: Streptomycin, Nx: Nalidixic acid, Km: Kanamycin, Gm: Gentamycin.

Previous work has shown that RP4p was transferred between introduced donor and recipient pseudomonads in sterile Ede loamy sand at a frequency around 10^{-4} (Smit et al., 1993). Mobilization of the chromosomally-inserted marker cassette under the influence of RP4p present in the donor was studied in sterile Ede loamy sand and Flevo silt loam soils.

Table 2. Transfer of RP4p, pSKTG and the chromosomally inserted KTG sequence between *Pseudomonas fluorescens* R2fR donor (D) and *P. fluorescens* R2fSN recipient strains on membrane filters on LB agar plates.

Donor	Transfer frequency per donor of (genetic element):		
	RP4p	pSKTG	KTG
D. (RP4p)	$6\ 10^{-2}$	-	-
D. (pSKTG)	-	[0]	-
D. (RP4p; pSKTG)	10^{-2}	$2\ 10^{-2}$	-
D. (KTG)	-	-	[0]
D. (KTG; RP4p)	$4\ 10^{-2}$	-	$4.5\ 10^{-8}$

- : not performed

[0] = below 10^{-9}

soils. The introduced donor and recipient strains survived well (Table 3) in both soils. RP4p was transferred at a significantly higher frequency in Flevo silt loam soil as compared to its transfer frequency in Ede loamy sand soil. However, no mobilization of the KTG cassette was detected in either soil. The few colonies from Flevo silt loam appearing on the transconjugant-selective plates were, via extensive testing, shown not to represent genuine transconjugants, suggesting that the putative transfer of KTG was below detection.

Survival and transfer studies in non-sterile soils planted with wheat

P. fluorescens R2fR (RP4p) showed a slow but progressive decline in Ede loamy sand over the time course of the experiment (Fig. 1A). In Flevo silt loam, up to day 17, cfu numbers did not decrease, and from day 17 until day 28 the cfu numbers decreased with 1 order of magnitude. Differences in survival rates in the two soils were very distinct; in Ede loamy sand (bulk soil), cfu counts dropped 4 orders of magnitude, while cfu counts in Flevo silt loam decreased only 1 order of magnitude during the 28 days. Cfu counts obtained from plates containing Rp were similar to those on plates containing Ap and Tc (difference < 0.1 Log unit), suggesting RP4p was stably maintained and the Tc and Ap resistance markers were expressed.

Transfer of RP4p to indigenous bacteria (Table 4) had been detected previously in the rhizosphere soil of wheat in short term experiments (Richaume et al., 1992; Smit et al., 1991).

Table 3: Numbers of donor (D), recipient (R) and transconjugant (T) cfu 7 days after the strains were introduced into sterile Flevo silt loam (FSL) and sterile Ede loamy sand (ELS).

	Donor	Recipient	Transconjugant cfu	
treatment	cfu	cfu	RP4p	::KTG
FSL, D	$2.3\ 10^8$	-	[0]	[0]
FSL, R	-	$1.8\ 10^8$	[0]	[0]
FSL, T	$1.9\ 10^8$	$0.5\ 10^8$	$2.4\ 10^6$	[0]
ELS, D	$1.3\ 10^8$	-	[0]	[0]
ELS, R	-	$0.9\ 10^8$	[0]	[0]
ELS, T	$1.5\ 10^8$	$1.1\ 10^7$	$2.1\ 10^5$	[0]

FSL, D: Flevo silt loam with donor *P. fluorescens* R2fR (*chr*::KTG; RP4p) added at 10^8 cells per gram; FSL, R: Flevo silt loam with *P. fluorescens* R2fSN (recipient) added at 10^8 cells per gram soil; FSL, T: Flevo silt loam with both strains added at 10^8 cells per gram soil. ELS, D: Ede loamy sand with donor added at 10^8; ELS, R: Ede loamy sand with recipient added at 10^8; ELS, T: Ede loamy sand with both strains added at 10^8.

In Ede soil transconjugants were found at day 10 and 17, whereas at day 28 none were detected. In Flevo silt loam, around 10^3 transconjugants per gram of soil were found throughout the experiment in rhizosphere and bulk soil.

Numbers of *P. fluorescens* R2fR (pSKTG) in Ede loamy sand (detected on plates with Gm) decreased in the first 10 days (Fig. 2A), after which cell numbers were relatively stable. The introduced strain showed a significantly enhanced survival in the rhizosphere as opposed to the bulk soil. Fluorescent cfu counts on plates containing only Km or Rp were slightly, but not significantly lower than counts on plates with Gm, suggesting that pSKTG was stably maintained and that the markers remained present and were expressed. Moreover, hybrization assays with the *cry*IVB probe of colonies appearing on Rp- or Km-containing agar plates confirmed the presence of the unselected marker in all colonies (50-100 per sampling time) tested. In Flevo silt loam, cfu numbers hardly declined, and loss of plasmid or expression was not detected (Fig. 2B). A rhizosphere effect on bacterial numbers was not apparent in this soil.

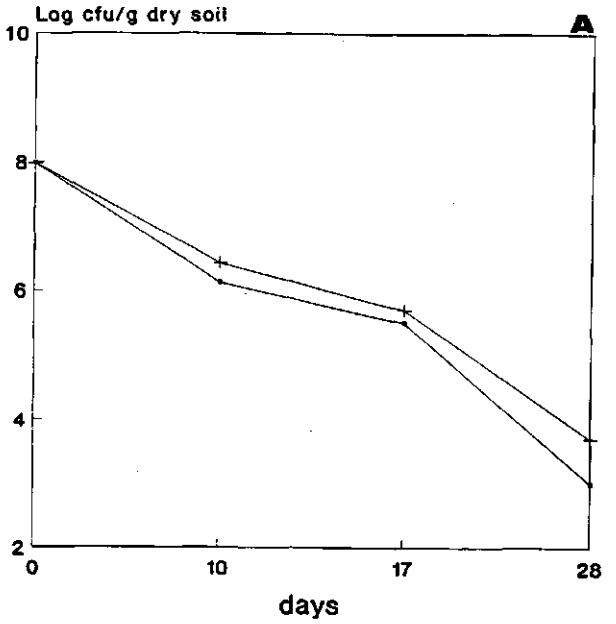
Indigenous bacteria containing the recombinant plasmid pSKTG were not detected in any of the 2 soils either in rhizosphere or bulk soil samples; the calculated limit of detection was slightly below 10^2 cfu per gram of soil.

Table 4. Log number of indigenous transconjugants harbouring RP4p, pSKTG or the chromosomal KTG cassette, detected in a long-term soil experiment with 3 different *Pseudomonas fluorescens* strains with these elements as donors (for donor survival data see Figures 1 through 3).

Genetic element	Soil	Log cfu per g dry soil on (day): 0 B	10 R	10 B	17 R	17 B	28 R	28 B
RP4p	ELS	[0]	3.1	[0]	3.3	[0]	[0]	[0]
	FSL	2.3	2.6	3.3	ND	ND	3.7	3.6
pSKTG	ELS	[0]	[0]	[0]	[0]	[0]	[0]	[0]
	FSL	[0]	[0]	[0]	[0]	[0]	[0]	[0]
::KTG	ELS	[0]	[0]	[0]	[0]	[0]	[0]	[0]
	FSL	[0]	[0]	[0]	[0]	[0]	[0]	[0]

ELS: Ede loamy sand, FSL: Flevo silt loam, R: Rhizosphere, B: Bulk.
[0]: below detection (~10^2 cfu per g of dry soil).

Fig. 1. Survival of *P.fluorescens* R2fR (RP4p) introduced into Ede loamy sand (A) and Flevo silt loam (B), planted to wheat. Cfu counts from plates containing Ap and Tc are given since these were identical to cfu counts on Rp-containing agar medium. LSD = 0.37. —•—ApTc bulk; —+—ApTc rhizosphere.



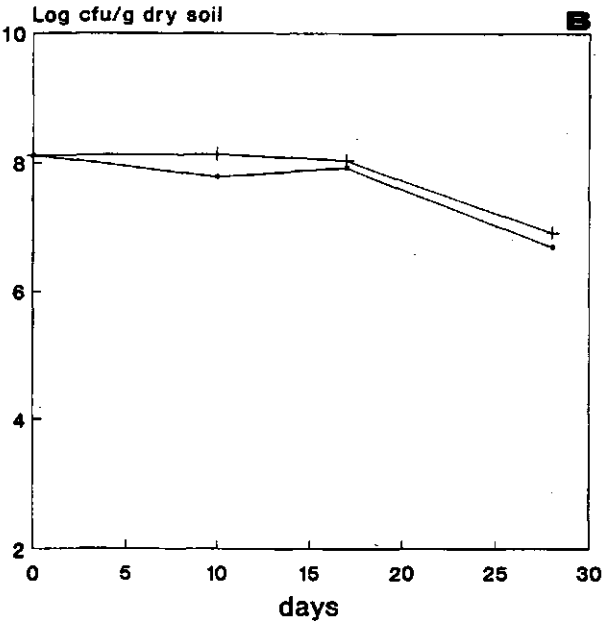


Fig. 2. Survival of *P. fluorescens* R2fR (pSKTG) in Ede loamy sand (A) and Flevo silt loam (B), planted to wheat. In Ede loamy sand, loss of expression of the Gm resistance gene is shown by the differences in cfu counts on media with and without Gm. In Flevo soil differences were within 0.1 Log unit. LSD = 0.37.
-*-Rp/Km bulk; -□-Rp/Km rhizosphere; —KmGm bulk; -+-KmGm rizosphere.

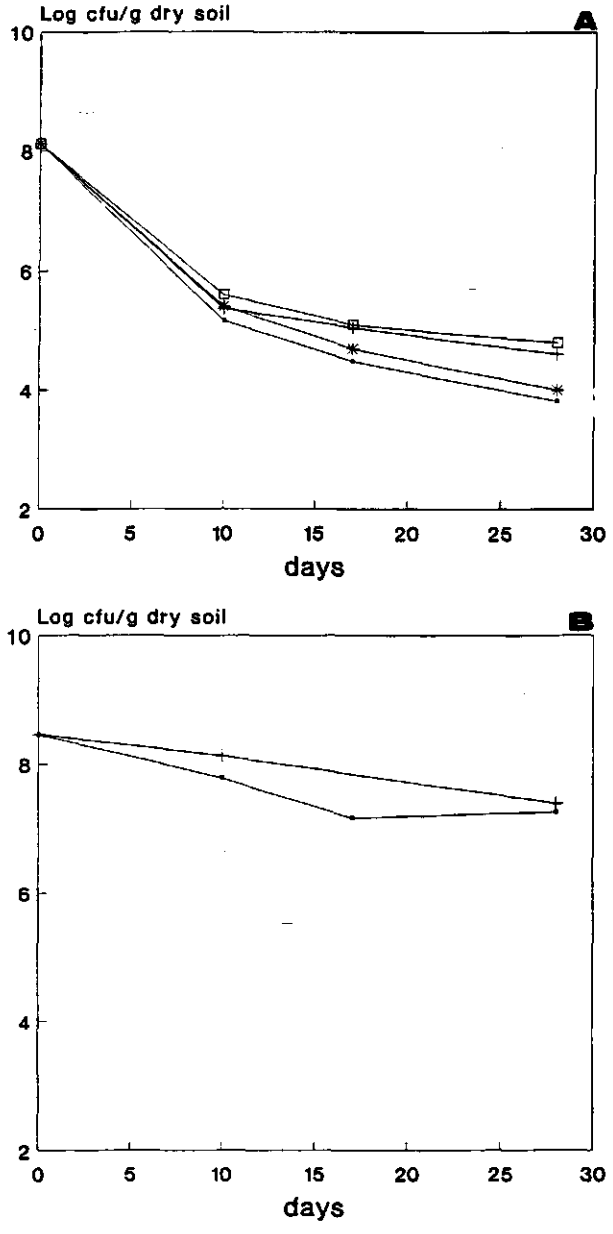
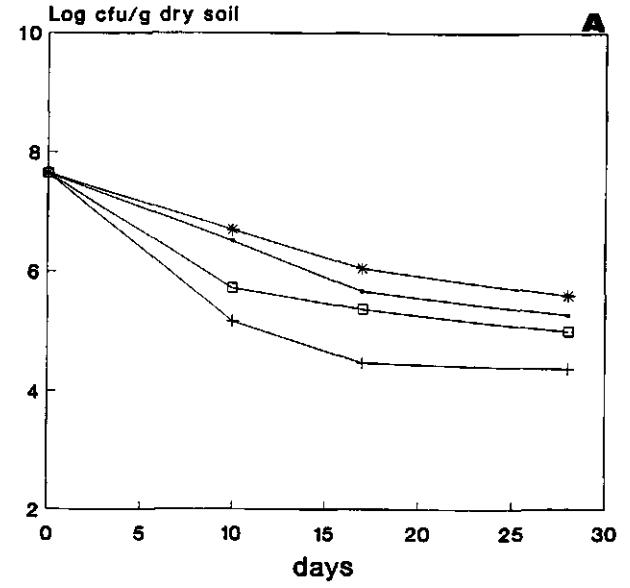


Fig. 3. Survival of *P. fluorescens* R2fmc introduced into Ede loamy sand (A) and Flevo silt loam (B), planted to wheat. Differences between cfu counts on plates with and without Gm in Flevo silt loam were below 0.1 Log unit. LSD = 0.37.
—Rp/Km bulk; —*—Rp/Km rhizospere; —+—KmGm bulk; —⊟—KmGm rhizosphere.



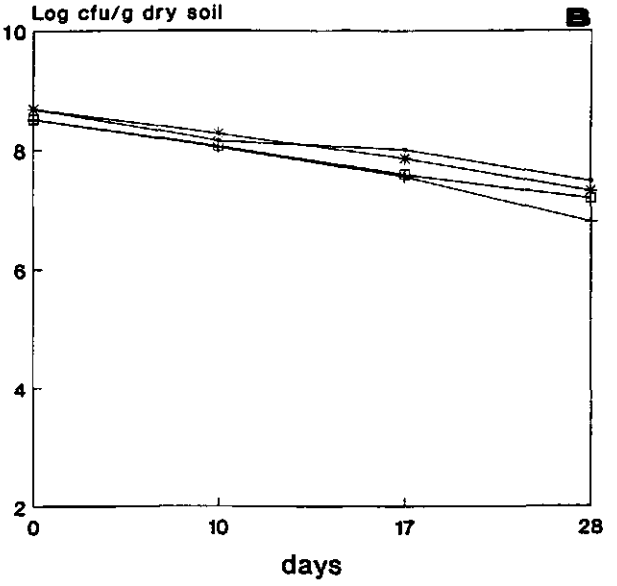
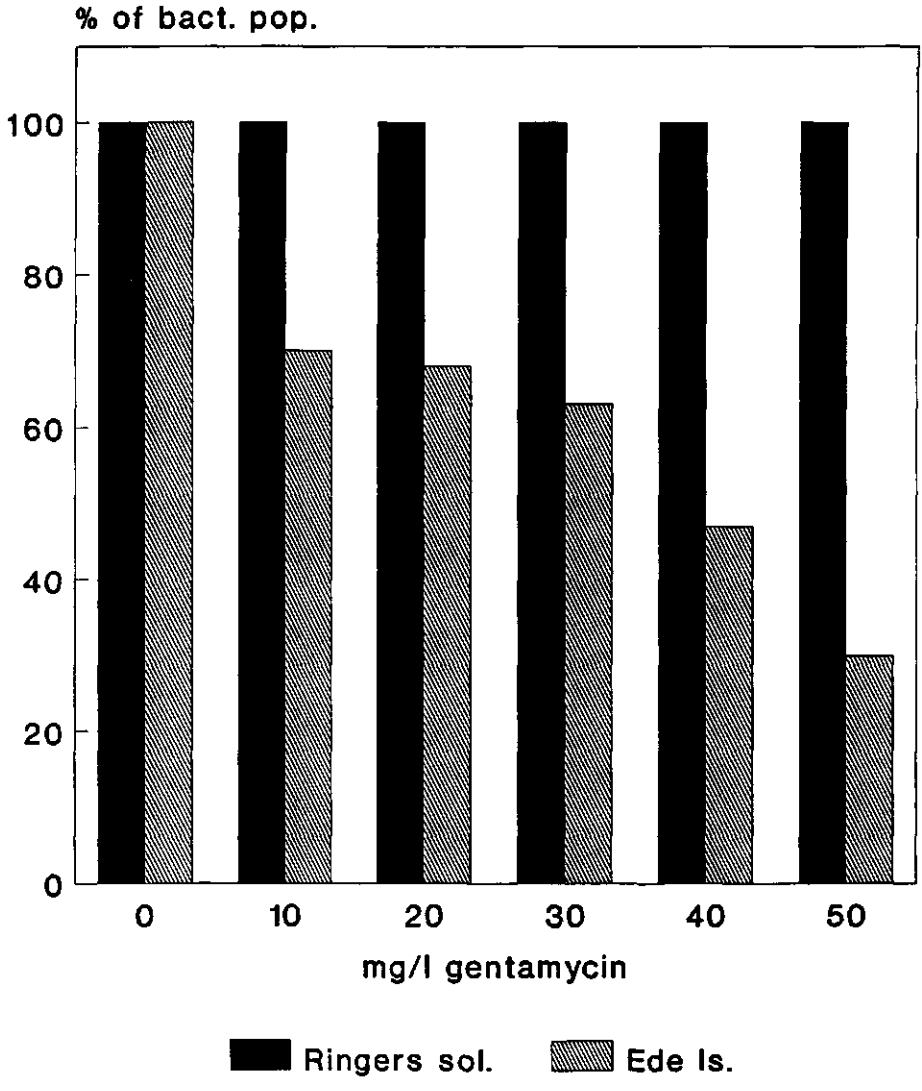


Fig. 4. Percentage of cfu counts of *P. fluorescens* R2fmc on King's B medium supplemented with increasing Gm concentrations, 10 days after introduction into Ede loamy sand or in 1/4 strength Ringers solution.



Significant lower counts of fluorescent cfu were found on Gm-containing plates as compared to counts on Km- or Rp-containing plates, both in rhizosphere and in bulk soil samples, which could indicate loss of expression of the Gm resistance gene *aad*B. On day 28, this loss appeared to be significantly greater in bulk soil (87%) then in rhizosphere soil (25%), suggesting that the presence of plant roots diminished putative stress factors which affected the expression of *aad*B. There was only a slight decline in cfu counts in Flevo silt loam bulk and rhizosphere samples (Fig. 3B) and cfu counts from Km- plus Gm-containing plates did not differ significantly from those on Rp- or Km-containing plates, indicating that loss of expression of *aad*B did not occur in this soil.

Indigenous bacteria containing the marker gene cassette were not detected in either soil in rhizosphere and bulk soil samples, at a limit of detection of around 10^2 cfu per gram of soil.

Determination of expression level of aadB

To determine the level of loss of expression of the Gm resistance gene in Ede loamy sand, *P. fluorescens* R2fmc was plated 10 days after introduction (10^8 per gram of soil) onto King's B agar supplemented with an increasing amount of Gm (Fig. 4). Counts from plates containing Km without Gm were considered to represent 100% of the population. The percentage of the population able to grow in the presence of Gm was lower with increasing Gm concentrations, ranging from 70% at 10 mg/l to 30% at 50 mg/l. However, colonies first obtained on Km-supplemented plates without Gm were upon subculturing again able to grow in the presence of Gm (50 mg/l). *P. fluorescens* R2fmc which had been starved for 10 days in 1/4 strength Ringers solution did not show any loss of *aad*B gene expression (Fig. 4), suggesting that starvation alone was not responsible for the phenomenon.

Discussion

In filter matings, RP4p transferred with a frequency of 10^{-2} from a donor to a homologous recipient strain as found before (Smit et al., 1993). The frequency with which RP4p could mobilize the IncQ plasmid pSKTG appeared to be similar, at least in triparental matings on filters (Smit et al., 1993). This work revealed that the frequency with which RP4p could mobilize the chromosomally-inserted marker gene cassette was around 10^{-8} per donor. Mobilization of chromosomal DNA by conjugative plasmids has been described before (Barth, 1979; Haas and Reimmann, 1989; Pemberton and Bowen, 1981), and gene transfer frequencies ranging from similar to ours to much higher, i.e. 10^{-3}, have been reported (Haas and Reimmann, 1989). However, the chromosome mobilization frequency of RP4 is always around 10^{-8}-10^{-9} (Haas and Reimmann, 1989). The rate-limiting step with RP4-mediated transfer of the chromosome probably is the concatenation (cointegration) of chromosomal and plasmid DNA. Thus, soil-borne plasmids of IncP or other mobilizing Inc groups might transfer chromosomally-inserted genes at frequencies in the range reported here (Hill et al., 1992; Lilley et al., 1992).

In sterile Ede loamy sand soil, RP4p had previously been shown to be transferred at a frequency of 10^{-4}, whereas mobilization of pSKTG by RP4p occurred at frequencies of 10^{-4} or 10^{-6}, depending on the initial localization of the mobilizer plasmid RP4p (Smit et al., 1993). However, soil type may drastically affect gene transfer frequencies (Richaume et al., 1992). Here, we found that transfer of RP4p in sterile Flevo silt loam was 1000-fold higher than in sterile Ede loamy sand (Table 3), at similar donor and recipient cell numbers. Similarly, studies on the transfer of RP4p in non-sterile soil revealed highest numbers of indigenous transconjugants in Flevo silt loam (Richaume et al., 1992). Soil parameters such as pH, clay minerals and organic matter content have been shown to influence conjugal gene transfer (Richaume et al., 1989). Flevo silt loam might be a soil conducive to conjugal gene transfer due to its neutral pH and high clay content. However, environmental factors affecting conjugal transfer seem to interact in a complex way, both in soil (Richaume et al., 1992) and artificial wastewater (Khalil and Gealt, 1987), often obscuring the elucidation of the effect of each factor separately. In none of the 2 sterile soils, mobilization of the chromosomally-inserted marker gene cassette could be detected, not even in the presence of the mobilizer plasmid RP4p. As expected, the detection limit of roughly 50-100 cells per gram of soil in these experiments did not allow for chromosome mobilization in sterile soil to be detected. Transfer of chromosomal markers in sterile and non-sterile soils has been detected (Kraskovsky and Stotzky, 1987; Weinberg and Stotzky, 1972) by using an *Escherichia coli* Hfr donor strain and a co-introduced *E. coli* recipient strain. However, the rates of transfer reported by these authors are not indicative of frequencies which might be expected with non-Hfr strains. RP4-mediated chromosomal transfer in soil might take place at a maximal rate dictated by the rate-limiting step (frequency 4.5×10^{-8}) as argued above. Therefore, provided optimal cellular growth and contact possibilities in soil, chromosome mobilization might be expected to result in, at maximum, 1 transconjugant cell per gram of sterile (Flevo silt loam) soil with the donor and recipient population densities applied.

In both non-sterile soils planted to wheat, RP4p was transferred to indigenous bacteria, as found before (Richaume et al., 1992; Smit et al., 1991), indicating the rates at which cell-to-cell contact between the introduced and indigenous microorganisms was possible under given conditions in the soils. Conjugative plasmids occurring in bacteria in the environment (Hill et al., 1992; Lilley et al., 1992) might promote natural chromosome mobilization, probably at low frequencies. Assuming a frequency of occurrence in soil bacterial populations of such mobilizing plasmids of 10^{-6} per cell, a transfer frequency of 10^{-4} (indicating possibilities for successful cell-to-cell contacts; Smit et al., 1991) and a chromosome mobilization frequency of 10^{-8} (Table 2), a total frequency of 10^{-18} per recombinant cell entering the soil might be expected.

The population dynamics of both *P. fluorescens* R2fR (RP4p) and *P. fluorescens* R2fR (pSKTG) in non-sterile Ede loamy sand exhibited a gradual decline in numbers and both plasmids seemed to be stably inherited, as found before for RP4p (Van Elsas and Trevors, 1990). *P. fluorescens* R2fmc revealed better survival in this soil as compared to the former strains (Fig. 3A). The metabolic load due to the extra genetic information was presumably

smallest in the case of the chromosomal insert.

In Flevo silt loam soil, containing a high amount of clay minerals, all introduced strains survived markedly better then in Ede loamy sand, which confirms earlier work (Heijnen and Van Veen, 1991; Richaume et al., 1992; Van Elsas et al., 1986). The enhanced survival in clay soils as well as in Ede loamy sand amended with bentonite clay (Heijnen and Van Veen, 1991) has been attributed to an increase in numbers of small pores in which introduced bacteria are protected from predation (Heijnen and Van Veen, 1991). However, other factors, e.g. differences in pH, cannot be completely ruled out.

A significantly lower number of *P. fluorescens* R2fmc cfu's was obtained from Ede loamy sand on plates with Gm than on those without Gm. In all likelihood, temporarily reduced expression of the *aad*B gene caused this effect, since the gene was still present as shown by colony blots, and after growth on media without antibiotics, resistance was restored. Such apparent loss of expression of antibiotic resistance genes has been found before with several strains and antibiotic resistance genes in soil and aquatic environments (Caldwell et al., 1989; Devanas et al., 1986; Genthner et al., 1990; Griffiths et al., 1990). Strains reisolated from the environment might be so weakened (injured) due to stressful environmental conditions that antibiotic resistance genes used as markers are temporarily not operational. Caldwell et al. (1989) also showed that expression could be restored after resuscitation of the starved cells. We have no conclusive evidence that the lowered resistance against Gm is caused by loss of expression. For instance, Genthner et al. (1990) found the lower numbers of cfu's on plates with certain antibiotics to be caused by a hypersensitive reaction of the bacteria after starvation. In our experiments, the loss of expression was prominent in *P. fluorescens* R2fmc, and it occurred to an insignificant extent in the pSKTG-containing strain. The higher copy number of the plasmid might have counteracted the loss of expression due to a gene dosage effect. Since loss of expression was not detected in cells from Flevo silt loam or in cells starved in Ringers solution, it was apparently caused by conditions specific for the Ede loamy sand soil other than starvation stress. These conditions might have caused a stress-induced response, invoking regulatory proteins in the introduced strain (Van Elsas and Van Overbeek, 1993) which possibly affected the *aad*B promoter.

The results obtained here confirmed earlier suggestions that transfer of chromosomally inserted genetic elements (or even of those present on non-selftransmissible but mobilizable plasmids) does not detectably occur under soil conditions conducive for the transfer of a selftransmissible element, RP4p. Therefore, when GEM release is cogitated, placement of the beneficial genes on the chromosome seems inherently safer than on mobilizable or selftransmissible plasmids. However, chromosomal inserts might be mobilized, at low frequencies, by selftransmissible plasmids, as shown here in the filter matings, and the experiments performed here with the non-sterile soils did not permit detection of these low-frequency events. It is conceivable that environmental pressures in soils selecting for specific (chromosomal) genes might favour bacteria harbouring these elements after transfer. Future work should focus on aspects of putative selection for the products of such low-frequency transfer events.

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Summary and concluding remarks

The extent of possible conjugal transfer of recombinant DNA present in genetically engineered microorganisms (GEMs) was studied. Occurrence of transfer of recombinant DNA is only one of the concerns regarding the use of GEMs (Chapter 2). Other potential hazards preventing the application of GEMs for agricultural purposes are (1) possible pathogenicity, (2) disturbance of ecological balance, (3) unwanted biochemical reactions, and (4) negative effects on diversity and specific populations (Levin and Strauss, 1991). The fate of GEMs introduced into soil is not very predictable, since knowledge on most of the aforementioned aspects is scarce. In most countries strict regulations limit large scale field studies with GEMs, which slows down research.

Research was focused on (1) development of sensitive detection techniques to study GEMs in soil, (2) detection of conjugal plasmid transfer to indigenous bacteria in soil, (2) assessing the fate of recombinant DNA present in different genomic locations. The soil isolate *Pseudomonas fluorescens* R2f was chosen as model microorganism. All studies were done *in vitro* and in soil microcosms which were planted whith wheat.

Studying conjugal transfer between homologous donor and recipient strains introduced into soil we found that matings could occur on the transconjugant selective plates, thus obscuring the real number of transconjugants in soil (Chapter 3). The use of nalidixic acid instead of streptomycin (in conjunction with rifampicin) to select for recipients which received the plasmid and to counterselect the donor, was shown to prevent these plate matings. The use of nalidixic acid to prevent plate mating was later confirmed by Walter et al., (1991). Employing this donor counterselection, it was shown that the number of transconjugants decreased with decreasing numbers of introduced donor and recipient cells. However, transconjugants could only be detected when the soil was amended with nutrients or bentonite clay or when plant roots were present. The fact that plasmid transfer between *P. fluorescens* R2f strains was enhanced in the rhizosphere was not entirely surprising since this organism was originally isolated from the rhizosphere. Stimulation of plasmid transfer by addition of nutrients or the presence of plant roots was also found by others (Stotzky, 1989; Edwards, 1993).

Experiments in which both donor and recipient are introduced, might be indicative for factors affecting conjugal gene transfer, however they do not necessarily predict transfer to indigenous bacteria. Most potential indigenous recipients are generally in a different physiological state than the freshly cultured, metabolically active, introduced recipients. To detect transfer to indigenous bacteria, another donor counterselection method was developed (Chapter 4). A phage specific for the donor strain, ΦR2f, was isolated, which could be used to lyse the donor prior to plating on transconjugant-selective plates. The selftransmissible plasmid RP4p which containes suitable antibiotic resistance genes for selection, had been marked previously with an eukaryotic DNA fragment for hybridization purposes, giving RP4p. RP4p transfer to indigenous bacteria was observed in the rhizosphere of wheat

(Chapter 5). The number of indigenous transconjugants detected was around 10^3 cfu/g of soil, while transconjugant numbers in the corresponding bulk soil were just below 10^2 cfu/g of soil. All indigenous transconjugants analysed contained the plasmid, and all were able to transfer RP4p to a *P. fluorescens* recipient strain in control filter matings. The transconjugants were identified as belonging to the genera *Pseudomonas, Enterobacter, Comamonas* and *Alcaligenes*. These genera fit very well into the known host range of RP4 (Thomas, 1989).

Transfer of RP4p to indigenous bacteria was also studied in four different soils (Chapter 6). Highest numbers of transconjugants per g of dry soil were found in Montrond silt loam and Flevo silt loam (10^3-10^4), whereas in Ede loamy sand and Loss silt loam transconjugant numbers were around 10^2. The presence of plant roots affected transconjugant numbers to a significant extent in Ede loamy sand and Loss silt loam but not in the other soils. High clay and organic matter contents as present in Flevo silt loam and Montrond silt loam might be favourable for conjugal transfer and obscure any stimulatory effect of the rhizosphere.

Since selftransmissible plasmids are not likely to be used as vectors for inserting recombinant DNA in GEMs, possible transfer of a marked IncQ plasmid was studied (Chapter 7). A marker cassette was constructed based on two antibiotic resistance genes, *npt*II and *aad*B conferring resistance against kanamycin and gentamycin, and part of a *Bacillus thuringiensis* endotoxin gene, *cry*IVB, was used as molecular marker. This marker cassette was cloned an IncQ plasmid (a RSF1010 derivative) wich resulted in plasmid pSKTG. Mobilization of the non-selftransmissible plasmid pSKTG was studied in filter matings, in sterile soil and in natural soil. In filter matings, pSKTG was only mobilized in the presence of RP4p. Transfer frequencies in bi- and tri-parental matings were in the same range, indicating that apparently cell-to-cell contact was not a limiting factor. In sterile soil, mobilization frequencies of pSKTG with RP4p present in the same strain were 100-fold lower then on filters (10^{-4}). When RP4p was present in a separately introduced strain, (triparental) transfer was found to occur with a frequency of 10^{-6}. In microcosms with non-sterile soil planted with wheat, mobilization of pSKTG to indigenous bacteria was detected only in the presence of RP4p. The results obtained in filter matings using using the total soil bacterial community suggested the occurence of genetic elements capable of plasmid mobilization. Such elements have been found by Hill et al. (1992) and Top (1993), elements with mobilizing capacity are probably present at low levels in natural soil, since pSKTG could not be shown to be mobilized *in situ* in the absence of RP4p.

The influence of the location of heterologous DNA in *Pseudomonas fluorescens* R2f on gene stability, expression and transfer following introduction into Ede loamy sand and Flevo silt loam was studied (Chapter 8). Three strains were used with markers on different genetic elements, i.e a selftransmissible plasmid (RP4p), a mobilizable plasmid (pSKTG), and a chromosomally inserted marker gene cassette (KTG). *In vitro* filter mating experiments showed that the selftransmissible plasmid was transferred with high frequencies (about 10^{-2}) and that this plasmid could mobilize pSKTG with similar frequencies. The chromosomally

inserted marker cassette could be mobilized by RP4p to a recipient strain with low frequency (10^{-8}). In sterile soil, transfer of the chromosome could not be detected in the presence of RP4p. The three strains showed poor survival in Ede loamy sand and good survival in Flevo silt loam. Moreover, a partial, but significant loss of expression of the gentamycin resistance gene *aad*B was observed in Ede loamy sand, but not in Flevo silt loam.

RP4p was found to be transferred from an introduced donor to indigenous bacteria in both non-sterile soils planted to wheat, whereas transconjugants harbouring the mobilizable plasmid pSKTG or the chromosomally inserted marker cassette were not detected when using a donor strain without IncP1 plasmid.

A lot of data concering gene transfer in soil have been published since this project started in november 1988. At that time however, most knowledge on conjugal gene transfer had been obtained using selftransmissible plasmids and introduced donor and recipient strains. In this work we showed that care should be taken to avoid matings on selective plates when such experiments are performed. The experiment which showed that RP4p could transfer to indigenous bacteria in soil and rhizosphere was one of the first reports on this phenomenon. Later, we confirmed that RP4p could also transfer to indigenous bacteria in soils of different type and texture. This indicated that the soil environment is not a barrier for gene transfer. The observation that an IncQ plasmid could be mobilized *in situ* to indigenous bacteria by RP4p present in a different strain, showed that tri-parental matings could take place. Transfer of an IncQ plasmid, or markers present on the chromosome could not be detected when RP4p was not added. However, indications were found for the presence of selftransmissible genetic elements in soil bacteria which could mobilize an IncQ plasmid.

Transfer frequencies of recombinant DNA present on either a selftransmissible, a mobilizable plasmid, or on the chromosome can be compared (some are based on estimations) using the data obtained (See Table 1). Multiplication of the figures with the number of introduced donor cells (per gram of soil) gives, depending on the conditions in soil, the number of transconjugants per gram of soil. Since IncQ plasmids and chromosomal inserts cannot propagate their own transfer, we assumed the presence of selftransmissible plasmids in 10^4 indigenous bacteria per g of soil (total 10^8 cfu/g of soil) in soil, with the capability of plasmid and chromosome mobilization. Frequencies clearly indicate that transfer of both the IncQ plasmid and the insert by naturally occurring genetic elements is below the detection limit (which is between 10 and 100 bacteria per gram of soil) and that transfer of those elements is a very rare event. It is difficult to indicate up to what level transfer of heterologous genes is acceptable. We therefore propose that if bacteria containing heterologous genes are to be applied for agricultural purposes, the recombinant DNA will be inserted into the chromosome to prevent unnecessary transfer.

Table 1. Estimated frequencies of events related to the possible transfer of recombinant DNA present on different plasmids or in the chromosome of GEMs to indigenous bacteria in soil (ind. = indigenous; rec. = recombinant)

Event	seltransmissible plasmid	mobilizable plasmid	chromosome insert
GEM and ind. cell collide	10^{-2}-10^{-3}	10^{-2}-10^{-3}	10^{-2}-10^{-3}
plasmid is transferred	10^{-2}-10^{-4}	-	-
GEM acquires ind. plasmid	-	10^{-4}-10^{-8}	10^{-4}-10^{-8}
ind. plasmid mobilizes rec. DNA	-	10^{-1}-10^{-3}	10^{-5}-10^{-10}
GEM and ind. cell collide	-	10^{-2}-10^{-3}	10^{-2}-10^{-3}
rec. DNA is transferred	-	10^{-1}-10^{-3}	10^{-1}-10^{-3}
rec. DNA is expressed	10^{0}-10^{-1}	10^{0}-10^{-1}	10^{0}-10^{-1}
total	10^{-4}-10^{-8}	10^{-10}-10^{-21}	10^{-14}-10^{-28}

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Samenvatting

Het gebruik van genetisch veranderde microorganismen in de bodem biedt grote mogelijkheden voor het optimaliseren van de gewasproductie en het saneren van vervuilde bodems. Er is echter weinig bekend wat betreft de mogelijke overdracht van het veranderde genetische materiaal naar de natuurlijke bodembacteriën. Het doel van dit project was het bestuderen van het lot van recombinant DNA in bacteriën geïntroduceerd in de bodem. Het onderzoek was voornamelijk gericht op bestudering (1) van plasmide-overdracht naar autochtone bodembacteriën in rhizosfeer en bulkgrond, en (2) van het effect van de plaats van insertie van het DNA op de overdracht, waarbij we denken aan insertie van het recombinant DNA in een zelfoverdraagbaar plasmide, in een mobiliseerbaar plasmide en in het chromosoom. Daarnaast was voorgenomen het effect van een aantal bodem parameters, zoals klei of organische stof gehalte en pH, op genoverdracht te belichten. De bodembacterie *Pseudomonas fluorescens* R2f, geïsoleerd uit de rhizosfeer, werd als modelorganisme gekozen.

Uit plasmide-overdrachts-experimenten met homologe donor- en recipientstammen bleek, dat er plasmide-overdracht op het transconjugant-selectieve medium kon plaatsvinden, hetgeen een vertekend beeld gaf wat betreft het werkelijke aantal transconjuganten in de bodem. Het gebruik van nalidixinezuur in plaats van streptomycine, in combinatie met rifampicine, bleek deze overdracht op selectieve platen te verhinderen. Wanneer contra-selectie met deze antibiotica werd toegepast in bodemexperimenten waarin plasmide overdracht in Ede lemig zand werd onderzocht, bleek dat transconjuganten alleen aangetoond konden worden in de rhizosfeer va tarwe of in grond waaraan nutriënten waren toegevoegd en niet in onbeplante grond. Tevens werd aangetoond dat het aantal transconjuganten afhankelijk was van het aantal geïntroduceerde donor en recipient bacteriën.

Plasmide-overdracht tussen homologe stammen in de bodem is niet per definitie representatief voor overdracht van een geïntroduceerde stam naar de autochtone bodembacteriën, die waarschijnlijk in een andere fysiologische staat verkeren. Om overdracht naar autochtone bodem bacteriën te detecteren moest een andere donor contraselectiemethode ontwikkeld worden. Hiertoe werd een bacteriofaag, ΦR2f, geïsoleerd om specifiek de donor stam te lyseren. Met behulp van deze methode kon overdracht van het zelfoverdraagbare plasmide RP4p, RP4 gemarkeerd met 0.7 kb van het patatine gen van aardappel, worden waargenomen in de rhizosfeer van tarwe. Door toepassing van een selectief medium en kolonie-filter-hybridisatie met een probe voor het patatine gen werd het aantal transconjuganten vastgesteld. Het aantal autochtone transconjuganten was rond 10^3 per g grond, terwijl het aantal in de bulk grond net onder 10^2 per g grond was. De transconjuganten werden geïdentificeerd als behorend tot de genera *Pseudomonas, Enterobacter, Comomonas* en *Alcaligenes*. Deze stammen bleken allen in staat het plasmide te kunnen overdragen naar een recipient in *in vitro* overdrachts-experimenten.

Overdracht van RP4p is ook onderzocht in vier gronden met verschillende pH, klei gehalte en organische stof gehalte, om een indruk te krijgen welke parameters genoverdracht

beïnvloeden. De hoogste aantallen transconjuganten werden gevonden in Flevo zavel en Montrond zavel (10^3-10^4), terwijl de aantallen in Ede zwak lemig zand en Loss zavel rond 10^2 lagen. De aanwezigheid van plantenwortels had alleen in Ede en Löss grond een significant stimulerend effect op het aantal transconjuganten. De hoge klei en organisch stof gehaltes in de Flevo en Montrond grond zijn waarschijnlijk verantwoordelijk voor de verhoogde overdrachts frequenties.

Aangezien zelfoverdraagbare plasmiden waarschijnlijk niet gebruikt zullen gaan worden als vectoren voor recombinant DNA in bacteriën, die in het milieu worden gebracht, is de overdracht van het niet zelfoverdraagbare IncQ plasmide pSKTG bestudeerd. Een markercassette is geconstrueerd gebaseerd op twee antibioticum resistentie genen, die voor kanamycine en gentamycine resistentie coderen, en een deel van een *Bacillus thuringiensis* endotoxine gen als moleculaire marker. Deze marker cassette is in een IncQ plasmide (RSF1010) gecloneerd, hetgeen resulteerde in pSKTG. Mobilisatie van pSKTG was onderzocht in overdrachts experimenten op filters, in steriele grond en in natuurlijke grond beplant met tarwe. Uit de experimenten op filters bleek dat pSKTG alleen overgedragen werd wanneer RP4p aanwezig was. Cel-cel contact was waarschijnlijk niet limiterend aangezien de overdrachts frequenties van di- en tri-parental matings in dezelfde orde van grootte lagen. Overdrachts frequenties in steriele grond (met RP4p en pSKTG in dezelfde stam) waren een factor 100 lager (10^{-4}) dan op filters, en wanneer RP4p in een andere apart geïntroduceerde stam aanwezig was (tri-parental), werd een frequentie van 10^{-6} gevonden. Vermoedelijk zijn deze lagere frequenties te wijten aan een afname van het cel-cel contact in vergelijking met filter experimenten en de relatief oligotrofe omstandigheden in de bodem. Mobilisatie van pSKTG naar autochtone bacteriën in niet steriele grond beplant met tarwe kon alleen gedetecteerd worden wanneer RP4p aanwezig was in dezelfde of in apart geïntroduceerde cellen. Voorlopige resultaten van *in vitro* experimenten leken erop te wijzen dat er in de bacteriële bodem populaties genetische elementen aanwezig waren die pSKTG konden mobiliseren.

Om het effect van de plaats van insertie van het DNA op overdracht in de bodem te onderzoeken werden de volgende stammen en constructen gebruikt, (a) *P. fluorescens* R2f (RP4p), (b) *P. fluorescens* R2f (pSKTG), (c) *P. fluorescens* R2f KTG (de marker cassette geïnserteerd in het chromosoom). Uit overdrachts-experimenten op filters bleek dat de chromosomaal geïnserteerde markercassette met een frequentie van 10^{-8} gemobiliseerd kon worden. Echter, in steriele grond kon dit niet gedetecteerd worden, waarschijnlijk omdat frequentie beneden de detectielimiet lag. Het bleek dat de aantallen bacteriën van de drie stammen na introductie in Ede lemig zand snel afnamen, hetgeen niet het geval was in Flevo zavel. Tevens werd een significant verlies van expressie van het gentamycine resistentie-gen aangetoond in Ede grond en niet in Flevo grond.

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Curriculum vitea

Eric Smit werd geboren op 26 maart 1960 in Haarlem. Al vroeg had hij interesse in de natuur en was hij vaak te vinden in de Waterleiding duinen. Na het behalen van het HAVO diploma aan het Lorenz lyceum begon hij aan een opleiding voor botanisch analist aan het Van de Broek Instituut in Amsterdam. De opleiding stelde hem in de gelegenheid slechts even te ruiken aan de vele onderwerpen, maar zijn interesse voor de Biologie was definitief gewekt. Voor hij echter kon beginnen aan zijn biologie studie aan Universiteit van Amsterdam moest hij in dienst. In 1988 had hij zijn studie afgerond en bleek hij een dankzij een curieuze vakkencombinatie van ecologie, populatie biologie, fytopathologie en moleculaire biologie een zeer goede kandidaat voor de functie van AIO bij de Bodembiotechnologie groep op het ITAL in samenwerking met de vakgroep Microbiologie van de Landbouw Universiteit Wageningen. Rond 1990 werd het ITAL opgeheven en de groep werd ondergebracht bij het Instituut voor Bodemvruchtbaarheid, overigens zonder fysiek van plaats te veranderen. Thans werkt hij in het kader van een EEG-BIOTECH project nog altijd bij de groep Bodembiotechnologie van Dick van Elsas, die nu echter bij het Instituut voor Planteziektekundig Onderzoek is gevoegd.

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