Genetically modified *Pseudomonas* associated with plants: aspects for environmental risk assessment

Katarina Björklöf

Faculty of Science
Department of Biosciences, Division of General Microbiology

and

Finnish Environment Institute
Research Department, Research Programme for Biodiversity

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Supervisors: Docent Martin Romantschuk
Department of Biosciences
Division of General Microbiology
University of Helsinki, Finland

Docent Kirsten S. Jørgensen
Research Department
Finnish Environment Institute, Finland

Reviewers: Docent Kristina Lindström
Department of Applied Chemistry and Microbiology
University of Helsinki

Docent Benita Westerlund-Wikström
Department of Biosciences
Division of General Microbiology
University of Helsinki, Finland

Opponent: Associate Professor Ole Nybroe
Department of Ecology and Molecular Biology
Section of Genetics and Microbiology
Royal Veterinary and Agricultural University, Denmark

Front cover: Double staining of *P. syringae* Cit7sp using FITC and CTC stains. In epi-fluorescence microscopy using 450-480 nm exciter filter and 515 nm barrier filter, cells stained green by FITC and respiring cells contained intracellular yellow CTC-formazan crystals.

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Abbreviations

ANOVA	analysis of variance
Ap	ampicillin
CCD	coupled charged device
CFU	colony forming unit
CLPP	community level physiological profiles
CTC	5-cyano-2,3-ditolyl tetrazolium
DGGE	denaturing gradient gel electrophoresis
EU	European Union
FITC	fluorescein isothiocyanate
GM	genetically modified
GMO	genetically modified organism
Hg	mercury
IPTG	isopropylthiogalactoside
kb	kilobase pair
Km	kanamycin
Nal	nalidixic acid
OECD	Organisation for economic co-operation and development
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorhpism
Rif	rifampicin
Sp	spectinomycin
Tc	tetracycline
UPGMA	unweighted pair group method with arithmetic means
VBNC	viable but non-culturable

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SUMMARY

Bacteria belonging to the genus *Pseudomonas* can be used as biological control agents in association with plants, for protection against plant diseases or frost damage. Biological control is considered to be more environmentally friendly than the use of traditional chemical agents. In planned commercial products, there is however, often a need to improve the used bacterial strains by genetic engineering techniques. Because little is known about the behaviour and impact of introduced genetically modified bacteria in the environment, legislation requires a risk assessment to be performed, proving the strain to be safe to use, before genetically modified bacteria are allowed to be released into the environment. In this work, the survival and impact of introduced *Pseudomonas* bacteria were studied in two habitats likely to be the target for future biological plant protecting agents; the leaf surface and the roots. The bacteria were genetically modified by tagging with marker genes to facilitate monitoring. Experiments were performed in laboratory setups mimicking environmental conditions.

Introduced *Pseudomonas* bacteria survived on the leaf surface regardless of the prevailing humidity. In soil and in the root system of birch, the introduced bacteria did not survive as large populations. In particular, at high soil temperatures, the bacteria disappeared when measured by plating techniques, but were detected using molecular techniques. This indicates that the introduced bacteria had lost their culturability soon after introduction. The live, remaining population could be reactivated in laboratory conditions. On the leaf surface, the introduced bacteria stayed culturable.

A selective marker gene devoid of any antibiotic resistance coding genes was successfully used for monitoring of the introduced strain in soils. The most sensitive monitoring method used was plate counting, but this method records only culturable cells. Methods that monitor non-culturable bacteria should therefore also be used to complete the picture of the survival of genetically modified bacteria in soil. This monitoring was complicated by the low recovery and impurities in DNA- and protein extracts from soil samples. The molecular techniques should be improved to increase sensitivity of the methods and to obtain a more complete picture of the survival of introduced bacteria.

The leaf surface acted as a hot-spot for gene transfer by conjugation in certain conditions and gene transfer to indigenous bacteria was observed. Conjugation was promoted by nutrients and the leaf surface itself, to which bacteria attached in micro-aggregates. The conjugation rate was dependent on the method used for introducing the bacteria, which recipient bacteria were investigated and the prevailing humidity conditions. In soils, observed impacts of the introduced strain included some changes in bacterial and fungal communities, nutrient utilization, and denitrification potentials. The ecological significance of these results is difficult to establish without further research on microbial ecological principles in soil and on plants. In addition, experiments on the likelihood for these events to occur in the environment is needed.

The results presented in this work, demonstrate the behaviour of introduced *Pseudomonas* bacteria in plant associated environments. Monitoring methods using culturing techniques are sensitive, but additional methods are required in environments such as soil, where the introduced bacteria can turn non-culturable. Molecular techniques should then be used in combination with culturing techniques. The results emphasize some possible changes introduced bacteria may pose on the environment. Both gene transfer to indigenous bacteria and changes in microbial communities were observed as a result of introduction of *Pseudomonas* bacteria to plant associated environments. In future evaluations of genetically modified plant protecting agents, the likelihood and impact of these changes should not be neglected.

SAMMANFATTNING

Bakterier som hör till släktet *Pseudomonas* är användbara som biologiska bekämpningsmedel för att förebygga växtsjukdomar och frostskador i växter. Biologisk bekämpning anses vara mera miljövänlig än användningen av traditionella kemiska bekämpningsmedel. För bruk i kommersiella produkter finns det dock ofta ett behov att förbättra de använda bakteriestammarna med gentekniska metoder. Kunskapen om hur genetiskt modifierade bakterier överlever och påverkar omgivningen är liten och därför kräver lagen att en riskbedömning utförs, som visar att stammen ifråga är ofarlig att använda i miljön. I detta arbete undersöktes överlevnaden och inverkan av tillsatta *Pseudomonas*-bakterier i två habitat som är troliga mål för framtida biologiska växtskyddsprodukter; bladytan och rotsystemen. För att underlätta observationen av de tillsatta bakterierna var de märkta med markörgener, som var introducerade med gentekniska metoder. Experimenten utfördes in laboratorieförhållanden som imiterade förhållandena i naturen.

Tillsatta *Pseudomonas*-bakterier överlevde på bladytan oberoende av de rådande fuktighetsförhållandena. I jord och i björkens rotsystem överlevde inte de tillsatta bakterierna som stora populationer. Speciellt vid hög marktemperatur försvann bakterierna mätt med odlingsteknik, men observerades mätt med molekylärbiologiska metoder. Detta tyder på att bakterierna hade förlorat sin förmåga att växa på odlingsmedium snart efter att de tillsatts. Den återstående, levande populationen kunde reaktiveras i laboratorieförhållanden. På bladytan var de tillsatta bakterierna odlingsbara.

Markörgenen, som saknade gener som kodar för resistens mot antibiotika, användes framgångsrikt för observation av den tillsatta bakteriestammen i jord. Den mest känsliga metoden var odling på skål, men denna metod registrerar endast odlingsbara celler. För att erhålla en fullständig bild av överlevnaden av genetiskt modifierade bakterier i jord, måste också metoder som mäter icke-odlingbara bakterier användas. I jordprov försvårades dessa mätningar av den låga utbytet och orenheter i DNA- och proteinextrakt. De molekylärbiologiska metoderna bör vidare-utvecklas för att öka deras känslighet och därmed få en klarare uppfattning om överlevnaden av tillsatta bakterier.

Genöverföring genom konjugation gynnades på bladytan under vissa förhållanden och genöverföring till naturliga bakterier observerades också. Konjugationen stimulerades av näring och av själva bladytan, på vilken bakterierna fäste sig i mikroaggregat. Konjugationsgraden på bladytan var beroende av metoden för tillsättning av bakterier, vilken mottagarbakterie som studerades och de rådande fuktighetsförhållandena. Tillsatta bakterier påverkade både de naturliga bakterie- och svampsamhällena i marken, samt användningen av näringsämnen och denitrifikations-potentialen. Den ekologiska betydelsen av dessa resultat är svår att avgöra utan ytterligare forskningsresultat angående mikrobekologin i marken och växter. Dessutom behövs flere experiment som klargör sannolikheten för att de observerade företeelserna också sker i naturen.

Resultaten som presenteras i detta arbete, demonstrerar hur tillsatta *Pseudomonas* bakterier beter sig i växtmiljöer. Observationsmetoder som baserar sig på odlingsteknik är känsliga, men är inte tillräckliga i habitater som jord, där de införda bakterierna kan bli icke-odlingsbara. Molekylärbiologiska metoder ska då användas kombinerat med odlingsbaserade tekniker. Resultaten påvisar några potentiella förändringar som införda bakterier kan orsaka i miljön. Både gen-överföring till naturliga bakterier och störningar in mikrobsamhällena observerades efter tillsättning av *Pseudomonas*-bakterier till växtmiljöer. I kommande bedömningar av miljöriskerna för biologiska genetisk modifierade växtskyddsprodukter bör sannolikheten för och verkan av dessa förändringar aror inte underskattas.

YHTEENVETO

Pseudomonas- sukuun kuuluvia bakteereita voidaan käyttää kasvien biologisina torjunta-aineina, suojaamaan kasvisairauksilta ja hallavaurioilta. Biologinen torjunta käsitetään yleensä ympäristöystävällisemmäksi kuin perinteinen kemiallinen torjunta. Suunnitteilla olevissa kaupallisissa tuotteissa on kuitenkin usein tarve parantaa käytettyjä bakteerikantoja geeniteknisten menetelmien avulla. Toistaiseksi ei tiedetä tarpeeksi, miten geenitekniikalla muunnetut bakteerit käyttäytyvät ympäristössä ja miten ne siellä vaikuttavat. Siksi, lainsäädäntö vaatii riskinarvioinnin, jossa geenitekniikalla muunnettu bakteerikanta todetaan turvalliseksi käyttää, ennen kuin se voidaan päästää luontoon. Tässä työssä tutkittiin miten lisätyt *Pseudomonas* bakteerit selviytyvät ja vaikuttavat kasvien lehdillä ja juuristossa. Nämä ovat todennäköisiä paikkoja, joihin tulevia biologisia torjunta-aineita tullaan lisäämään. Bakteereihin lisättiin geeniteknisin keinoin seurantaa helpottavia merkkigeenejä. Kokeet suoritettiin laboratoriossa, järjestelyissä jotka matkivat luonnon olosuhteita.

Lisättyjen *Pseudomonas* bakteerien eloonjäänti kasvien lehdillä riippui vallitsevasta kosteudesta. Maahan tai koivun juuristoon lisätyistä bakteereista ei elävänä selviytynyt suurta määrää. Viljelykokein havaittavat bakteerit katosivat viljelmiltä korkeissa lämpötiloissa, mutta tällöinkin bakteerit havaittiin molekyylibiologisilla menetelmillä. Ilmeisesti bakteerit menettivät nopeasti kykynsä kasvaa viljelykokeissa lisäyksen jälkeen. Jäljellä oleva elävä populaatio voitiin aktivoida uudelleen laboratorio-olosuhteissa. Kasvien lehdille lisätyt bakteerit säilyivät viljeltävinä.

Valikoivaa merkkigeeniä, josta puuttui antibioottiresistenttiyttä koodaavia ominaisuuksia, käytettiin onnistuneesti lisättyjen bakteerien seurantaan maaperässä. Herkin määritysmenetelmä oli maljalla kasvattaminen, mutta tämä menetelmä kuvaa vain viljeltäviä soluja. Jotta saataisiin todenmukaisempi kuva geenitekniikalla muunnettujen bakteerien selviytymisestä maaperässä, pitäisi lisäksi käyttää menetelmiä, jotka havaitsevat myös ei -viljeltävät bakteerit. Maaperä hankaloitti tätä seurantaa, sillä DNA- ja proteiiniuuttojen saanto oli huono, ja uutoksissa oli epäpuhtauksia. Molekyylibiologisten menetelmien kehittäminen on tarpeen luotettavan kokonaiskuvan saamiseksi.

Lehtien pinnalle lisätyt bakteerit pystyivät suotuisissa olosuhteissa geeninsiirtoon konjugaation avulla, ja geeninsiirtoa tapahtui myös luonnon bakteereihin. Konjugaatiota edistivät ravinteiden saatavuus ja itse lehden pinta, mihin bakteerit sitoutuivat rykelmissä. Konjugaatioaste riippui bakteerien lisäysmenetelmästä, vastaanottajabakteereista ja vallitsevista kosteusolosuhteista. Lisätyt bakteerit vaikuttivat luonnon bakteeri- ja sieniyhteisöjen koostumukseen, ravintoaineiden käyttöön, sekä denitrifikaatiokykyyn. Tarvitaan kuitenkin enemmän tutkimustuloksia maaperän ja kasvien mikrobiekologiasta sekä lisäkokeita havaittujen tapahtumien todennäköisyydestä luonnossa ennen kuin tulosten ekologisia merkityksiä voidaan arvioida.

Tässä työssä selvitettiin lisättyjen *Pseudomonas* bakteerien käyttäytymistä kasvien yhteydessä. Seurantamenetelmät, jotka perustuvat viljelyyn, ovat herkkiä mutta eivät yksistään käyttökelpoisia ympäristöissä, kuten maaperässä, jossa lisätyt bakteerit saattavat muuttua eiviljeltäväksi. Viljelyanalyysien lisäksi tulisi silloin käyttää molekyylibiologisia menetelmiä. Lisätyt bakteerit voivat myös aiheuttaa muutoksia ympäristössä. *Pseudomonas* bakteerien lisäys aiheutti kasviympäristöihin aiheutti geenien siirtymistä ja muutoksia mikrobiyhteisöissä. Näiden muutoksien merkitystä ja todennäköisyyttä ei tule aliarvioida kun geenitekniikalla muunnettujen kasvinsuojeluaineiden ympäristöriskejä arvioidaan.

INTRODUCTION

1. Potential use of genetically modified *Pseudomonas* in the environment

Many bacteria belonging to the genus *Pseudomonas* have properties that are beneficial to growth of plants. These plant growth-promoting bacteria stimulate plant health and growth e.g. by providing the plant with nitrogen, or acting as biological control agents, which means the bacteria can inhibit disease causing micro-organisms in association with plants by expressing antagonistic properties. The interest in using biological control agents for agricultural applications is increasing (Moenne-Loccoz *et al.*, 2001), partly because these are considered to be more environmentally friendly than chemical pesticides and fertilizers. The mechanisms for biological control usually involve production of antibiotics or competition for space and nutrients. The biological control agents are easily degraded, and may be more specific in their action than the corresponding chemical. In addition, resistance against biological control agents is considered to develop more slowly than against chemical control agents.

Bacteria belonging to the genus *Pseudomonas* represent a diverse collection of species that are involved in many important processes in the environment. They use a wide variety of organic compounds as carbon and energy sources and they are among the most significant mineralisers of organic material. Some species are animal or plant pathogens. *Pseudomonas* species inhabit a wide range of ecological niches and are common e.g. in soil and on plant surfaces. Two well characterized *Pseudomonas* species which are frequently associated with plants are *P. syringae* and *P. fluorescens*. *Pseudomonas* species have been used for biological control applications, where large numbers of bacterial cells are released into the environment for agricultural purposes such as biological control of phytopathogenic fungi and bacteria (Sigler *et al.*, 2001). *Pseudomonas* are also important for use in bioremediation, where recalcitrant, often toxic or harmful, compounds are degraded by bacteria. Additionally, natural strains of *Pseudomonas* with ice nucleating capability is used for artificial snow production and a strain lacking this ability is used for plant frost protection (reviewed in Hirano & Upper, 2000).

In planned commercial products, there is often a need to improve the used bacterial strains by genetic engineering techniques. Genetic engineering techniques allow artificial insertion and deletion of genes between genomes of very different organisms, enabling construction of gene combinations that would not be possible to achieve by traditional breeding techniques. Organisms that are man-made by recombinant DNA techniques are called genetically modified organisms (GMOs) or living modified organisms (LMOs). Genetically modified micro-organisms (GMMs) are also referred to as genetically engineered microbes (GEMs). Here, I will use the terms GMO and GM bacteria. Genetic engineering techniques have been successfully exploited in the chemical and pharmaceutical industry for a long time, where recombinant enzymes or drugs are produced by contained use of GMOs. Now commercial biotechnology has inevitably advanced to areas, where living GMOs are planned to be released to the environment. Product development of genetically modified (GM) bacteria is increasing and several field releases are performed each year for experimental purposes. Modifications of *Pseudomonas* strains include addition of genes coding for antifungal compounds to enhance biocontrol activities (Timms-Wilson *et al.*, 2000) and combining catabolic genes from different origins to improve the metabolic pathways in the strain (Haro & de Lorenzo, 2001).

2. Environmental risk assessment of GMOs

Little is known about the behaviour and fate of introduced GMOs in the environment. To avoid unexpected impacts on nature similar to those observed when using some novel chemicals in the 1950's, an environmental risk assessment is needed before the introduction. The risk any GMO pose on nature is a product of possible negative impacts, the hazard, which is the potential of the

organism to cause negative effects, and the likelihood of occurrence, which is related to exposure, fate and persistence of the organism and/or any of its by-products.

2.1. Environmental risks associated with GM bacteria

The most striking characteristics which distinguish risk assessment of GM bacteria compared to that of higher organisms are the vast metabolic diversity of bacterial groups, their important role in biogeochemical processes and their potential for horizontal gene transfer. Further, the removal of introduced GM bacteria from nature is likely to be more difficult than for other GMOs. Some ecologically significant issues for environmental risk assessment of GM bacteria are listed in Figure 1. Potential hazards are related to properties of the bacterium, the genetic traits involved, the combination of these, and the target habitat into which the GM bacteria are released. The hazard can often be predicted from theory, but the likelihood of occurrence in actual environmental conditions are difficult to predict precisely without experiments carefully simulating the environmental conditions or field studies. To make a fair risk assessment of GM bacteria released into the environment e.g. by the use of models (Landis *et al.*, 2000), the potential impacts should be dealt with in a quantitative manner. The final impacts of introduced GM bacteria are difficult to predict, as the natural microbial diversity is so far incompletely described; less than 1 % of the natural bacteria have been isolated and characterized (Amann *et al.*, 1995). In addition, little is known about how species diversity influences ecosystem processes. For these reasons, recently presented equations for risk include a third component: uncertainty (Figure 1). This parameter should describe both incompleteness of the methods used and account for ignorance, the so called unknown unknowns (Hoffmann-Riem & Wynne, 2002).

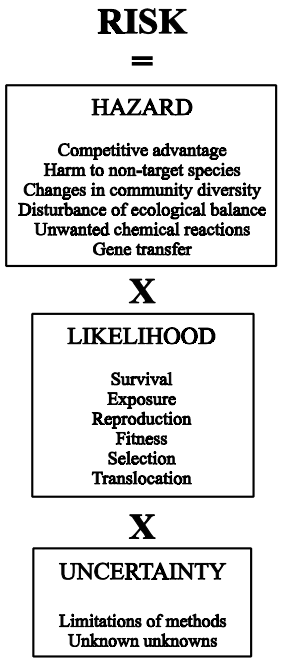


Figure 1. Some ecologically significant issues that should be considered when assessing the safety of introduced GM bacteria in the environment.

2.2. Regulation of use of GMOs in the environment

Potential risks associated with the release of live GMOs into the environment were early realized and at present the release of GMOs to the environment is restricted in most industrialised countries, including the EU. The framework for preparation of risk assessment for GM bacteria was first presented by Tiedje *et al.* (1989) who pointed out that organisms harbouring new combinations of traits are likely to display novel ecological properties. Legislative regulation for use of genetic engineering techniques became a pressing issue when these became standard methods in the 1990s.

The deliberate release of GMOs into the environment in the EU is regulated by the new directive 2001/18/EC (European Commission, 2001), which will be implemented into Finnish national legislation during this year. The directive includes a precautionary principle, which requires appropriate measures to avoid possible, even unlikely, adverse effects on the environment which might arise from the deliberate release of GMOs. Emergency plans to control the GMOs in case of unexpected spread, to isolate and decontaminate areas affected, and to protect humans and nature in case of the occurrence of an undesirable effect are required in the directive. The introduction of GMOs into the environment should be carried out step by step. This means that the containment of GMOs is reduced and the scale of release increased gradually, and only if evaluation of the earlier steps in terms of protection of human health and the environment indicates that the next step can be taken.

According to the directive, a risk assessment of the GMO is required for each case separately. The risk assessment should identify any potentially harmful effects of the GMO on the environment compared to those presented by the non-modified organism. Both direct and indirect impacts as well as immediate and delayed impacts should be considered. Different types of information are required for different cases. Further, the level of detail required in response to each case varies according to the nature and scale of the proposed release. Although the general principles for risk assessments are described in the directive, it does not unambiguously prescribe which questions should be solved and analysed in each case. Further, there is no information of acceptable methods. The organization that is performing the release is preparing the risk assessment, which the competent authorities then accept if it is properly performed. The regulative authorities are therefore in a key position in deciding what a risk assessment should include (Salila, 1999).

3. Plant surfaces as a habitat for microbes

The surfaces of plants provide a diverse environment for micro-organisms to inhabit. The micro-environments differ drastically between the exposed aerial parts of stem- and leaf habitats (phyllosphere), and the protected soil-imbedded root habitats (rhizosphere). The major advantages plant surfaces provide for microbial colonization are surfaces to attach to and available nutrients, that are secreted from inside the plants.

Leaf surfaces are harsh habitats where micro-organisms are subjected to various environmental stresses including strong fluctuations in humidity, temperature, and light. The topography of leaves are dominated by hills and valleys created by epidermal cell contours and different leaf structures such as leaf hairs, water pores and air pores. The epidermal cells of the leaf surface are covered by a cuticule which, due to its lipophilic nature, forms an effective barrier for water and polar substances. Water and nutrients are mainly translocated from the interior of the cells to the leaf surfaces via pores or glands. High humidity on leaves may also passively induce losses of substrates from inside the leaves by leaching.

The rhizosphere is a more protected and therefore more permanent habitat than the phyllosphere. The root surfaces are comparatively smooth and especially young roots are covered by a sheath of mucigel. This layer is composed of plant-produced polysaccharides, exopolysac-

charides of bacterial origin, clay and organic matter particles originating from soil. This medium buffers against changes in temperature and moisture on the root surface, but limits the diffusion of oxygen and carbon dioxide. As the root develops, aggregation of soil, fungal hyphae, and root hairs increase around the roots.

The nutrient status of plant-associated environments is highly dependent on the plant. The abundance of both leachates on the leaf surfaces and exudates in the rhizosphere varies between plant species, with plant age and growing conditions (Morgan & Tukey, 1964; Tukey, 1970; Grayston *et al.*, 1996). Sugars, organic acids, amino acids as well as essential minerals are leached from leaves. Roots exude high molecular weight compounds containing sugars, amino and organic acids, fatty acids and enzymes. In addition, insoluble remnants of root cortex cells can be released and used as a carbon source in the rhizosphere.

3.1. Microbial colonization of the leaf surfaces

Plant leaf surfaces are likely to act as targets for GM bacterial introductions, in applications where large numbers of bacterial control agents are sprayed onto the leaf surfaces of plants to protect against foliar pathogens or to reduce frost damage. The species composition of natural micro-organisms on leaf surfaces varies with plant species and growth conditions. If the physical conditions are permitting, growth of bacteria is generally limited by the amount of carbon compounds (Mercier & Lindow, 2000). Although several species of gram-positive and gram-negative bacteria, fungi and yeasts have been identified on the leaf surface, at least the bacterial populations are normally dominated by only a few genera (Thompson *et al.*, 1993; Jurkevitch & Shapira, 2000). *Pseudomonas* -species are often the dominating bacteria when measured by cultivation on plates. Multiple bacterial traits are involved in survival on the leaf surface, but the only characteristics that has directly been linked to epiphytic fitness is motility, enabling colonization at protected sites (Haeffele & Lindow, 1987). Other traits that have been suggested to increase fitness in the phyllosphere are pigmentation, which protects against UV radiation; attachment, which reduces displacement (Suoniemi *et al.*, 1995), traits related to pathogenicity and the ability to increase the wetting properties of the leaves.

Immigration and emigration are important processes for population dynamics on the leaf surfaces. Bacteria are transferred between leaves and removed from the leaf surface by water during rain (Butterworth & McCartney, 1991), and by aerosols during windy, sunny days (Lindemann & Upper, 1985). Inoculated bacteria were also dispersed by invertebrates and viable populations were established on recipient leaves (Lilley *et al.*, 1997). Populations fluctuate diurnally, seasonally and due to environmental changes like rain. Succession occurs during leaf senescence and microbial colonization is generally higher in older leaves (Knoll & Schreiber, 2000). Despite frequent fluctuation in populations, it seems that the community structure is similar at different locations and during proceeding years (Thompson *et al.*, 1995b). Leaves in more protected sites tend to support a larger population of microbes and epiphytic bacterial populations vary by a factor of 1000 between various leaves (Hirano *et al.*, 1982). In addition, bacterial populations within one leaf are not uniformly distributed on the surface. Biofilms containing copious exopolymeric matrices, micro-organisms and debris have been demonstrated on leaves from several plant species (Morris *et al.*, 1997).

3.2. Microbial colonization of the rhizosphere

The root systems of plants are probable sites of action for GM bacterial applications used for biofertilization or protection against soil-borne plant pathogens. Bacterial plant-protecting agents are added to the root system by seed inoculation or by treating the surrounding soil with the inoculant. The high numbers of indigenous bacteria and high microbial activity in the rhizosphere of plant roots are due to mucilaginous material and root exudates. Plants may on the other hand

inhibit micro-organisms by consuming much of the available inorganic nutrients such as ammonium, nitrate and phosphate. Many plants establish symbiotic associations with specialized fungi, producing mycorrhiza. The mycorrhizal mycelium develops an extensive network, which is more effective than the plain root system to provide the plants with nutrients, nitrogen and phosphorous from the surrounding soil. The major part of microbes which colonize the rhizosphere originates from seeds and the surrounding soil (Grayston & Campbell, 1996; Miethling *et al.*, 2000; Marschner *et al.*, 2001). The root tip acts as a slowly advancing point source of carbon, and bacterial colonization patterns have been observed on wheat roots in wave-like patterns that changed with time (Semenov *et al.*, 1999). Although certain dominant bacteria were common on the whole root of barley, other species were found only at distinct root locations (Yang & Crowley, 2000).

Dominance of gram-negative bacteria is commonly assumed in the rhizosphere, and the presence of *Pseudomonas* species is frequently reported. New culturing-independent identification techniques indicate, however, that gram-positive bacteria might be more dominant in the rhizosphere than previously supposed (Smalla *et al.*, 2001). More *Pseudomonas* species were present in soils from birch forest than in soils from spruce or pine forests measured by cultivation on plates (Priha *et al.*, 2001). Further, In the mycorrhizosphere of *Pinus sylvestris* grown in humus, spore-forming bacteria were the dominating type and *Pseudomonas* species were in the minority (Timonen *et al.*, 1998). Traits that promote the colonization of the rhizosphere includes motility, attachment, resistance to low matric potential and ability to sustain oxidative stress and starvation. Genes induced during rhizosphere colonization encoded functions involved in nutrient acquisition and stress response (Rainey, 1999). Immigration and emigration processes play a minor role in the rhizosphere compared to the phyllosphere, due to weaker physical forces. Bacteria are readily leached through soil, but the presence of roots reduce leaching (Kemp *et al.*, 1992). Soil animals, such as Protozoa, Nematoda, Acari, and Collembola feed on soil micro-organisms, and this way microbes may translocate on the surface of these soil animals even tens of centimetres (reviewed in Dighton *et al.*, 1997). Some ingested bacteria have been shown to colonize the gut and this way spread with the feeding animal (Hoffmann *et al.*, 1999).

4. Fate and activity of introduced bacteria into plant habitats

Introduction of specific bacteria into soils and onto plants has been used in agricultural practice for a long time. In general, large population sizes of introduced bacteria decline in natural soils and on leaf surfaces. In soils this is due to predation, presence of bacteriophages, growth inhibitors and competition with the resident microflora for an ecological niche (van Veen *et al.*, 1997). On leaf surfaces, the initial decline of introduced populations reflects death of the cells exposed to the harsh conditions on the leaves. Despite low survival of introduced bacteria, part of the inoculum persists on leaves and in the rhizosphere for considerable periods of time (Thompson *et al.*, 1995a). The method used for introducing the inoculum, including the possible use of carrier material, as well as the physiological state of the inoculum affect survival in soils. If the bacterial strains are adapted to the harsh environmental conditions before inoculation, the survival of introduced bacteria may be improved in soils (van Elsas *et al.*, 1994; Gu & Mazzola, 2001).

The effectivity of biological products often depends on the activity of the introduced strains. The physiological status of the cells is influenced by stress factors frequently encountered in the environment, such as lack of nutrients or water. During stress, such as nutrient limiting conditions in soils, the cell size of inoculated *P. fluorescens* decreased (van Overbeek *et al.*, 1995). Small bacteria in the rhizosphere were shown to contain fewer ribosomes than bacteria grown in optimal conditions in the laboratory (Ramos *et al.*, 2000), indicating some loss of activity. Most

bacteria introduced into soils rapidly loose their culturability in soils, while retaining viability in some form. These viable but non-culturable (VBNC) bacteria can be actively respiring (Heijnen *et al.*, 1995) and able to divide a few times (Kogure *et al.*, 1979; Binnerup *et al.*, 1993). Little is known about the role VBNC cells play in the environment, as the overall physiological status of the VBNC cells remains uncertain. They may be a form of actively produced resting stages that become active when conditions turn favourable or be in a transition state to death. In soil, VBNC cells of *P. fluorescens* did not promote the persistence compared to culturable cells (Mascher *et al.*, 2000). Bacteria exposed to frequently changing conditions, such as conditions encountered on the phyllosphere, are considered to be less likely to turn into a VBNC state (Wilson & Lindow, 1992) but recently, uncultured species was described on the leaf surfaces of potato by comparison of genetical fingerprints by denaturing gradient gel electrophoresis (DGGE) and culturing techniques (Heuer & Smalla, 1999).

5. Tracking the inoculum in the environment

To enable recognition of an introduced bacterial strain among thousands of indigenous bacteria with similar morphology and physiology, it is necessary for the strain to have some unique trait that the natural community is lacking. The trait should be stable in the organism and the signal produced in the cell should be strong. This feature, which can be a structural protein, a DNA sequence or a phenotypic characteristic, may be a natural trait, a so called intrinsic marker, or may be artificially introduced into the strain. Some frequently used marker genes are reviewed in Finnish by Björklöf (1997) and listed in Table 1. Intrinsic marker genes estimate total cell numbers and therefore provide little information on the physiological state or activity of the target organisms (Prosser *et al.*, 1996). The specificity of these markers is often low, because the trait used for selection is indigenous and therefore is likely to be present also in the natural population.

5.1. Introduced marker genes used for tracking GM bacteria

Introduced marker genes are selective or non-selective (Table 1). Selective marker genes are needed primarily in the cloning stage to enrich the tagged clone on culture media and to select against the background population lacking the characteristics of interest. In addition, they provide an easy and inexpensive means to monitor the introduced strain in mixed populations by using culturing based methods with selection. The most frequently used selective marker genes are genes coding for antibiotic resistance. There is however concern that antibiotic resistance genes may spread to unwanted organisms, such as pathogens, in nature (Prosser, 1994). The new EU directive (European Commission, 2001) states that antibiotic resistance genes should be considered as potential hazards in risk assessments for field releases of genetically modified organisms. Alternative marker genes are needed for use in field studies and in commercial products. Alternative selective marker genes have been developed, coding for herbicide- or heavy metal-resistance (Herrero *et al.*, 1990; Sanchez-Romero *et al.*, 1998), or growth on rare substrates such as mannityl opines (Hwang & Farrand, 1997), but these marker genes are not frequently used. Some non-selective metabolic marker genes also require culturing methods for detection. Metabolic marker genes express some specific enzyme activity in the tagged cells, which are determined e. g. by a colour reaction in mixed populations. After culturing the mixed population on plates and adding enzyme-specific chromogenic substrates, tagged bacteria have a distinguishable phenotype. Only in a few cases have metabolic markers been used in the rhizosphere *in situ* (Katupitiya *et al.*, 1995).

Table 1. Some marker genes[a] used for tracking bacteria in the environment.

Type of marker	Method *examples of genes used*	Advantages	Disadvantages
Intrinsic			
	Polyclonal antibodies against surface proteins	No genes inserted	High background
	Monoclonal antibodies against surface proteins	No genes inserted Sensitive	Antigen variation due to growth conditions Tedious to produce
	Probing /PCR of DNA sequence	No genes inserted	DNA extraction
Introduced			
Selective	Antibiotic resistance by point mutation *rif*	No gene technology needed, spontaneous mutation Gene transfer unlikely	Strain may be changed also in other traits during exposure Instability of the phenotype
	Antibiotic resistance by insertion of marker gene *ntpII*	sensitive Easy to perform Inexpensive	Spread of antibiotic resistance genes Instability of phenotype
	Herbicide resistance *aroA, bar* Heavy metal resistance *mer*, *ars*	Non-antibiotic selection Easy to perform	High background Harmful substances for humans
Non-selective	Metabolic markers *lacZY, xylE, gusA*	Frequently used Gene sequence known	Often require culturing techniques High background
	Ice nucleation *inaZ*	No substrate needed No sample processing Sensitive	Seldom used as marker gene
	Fluorescent protein *gfp*	No substrate needed *In situ* detection in plants	Microscopic detection or expensive equipment
	Immunological detection of surface proteins *e.g phoE-caa*	*In situ* detection	Tedious Microscopic detection or expensive equipment
	Luminescence	Measures activity Numerous detection methods	
	luxAB	Substrate freely permeable Genetic load lower than for *luxABCDE*	Substrate addition needed for signal detection

	luxABCDE	Substrate included	Genetic load higher
	luc	High quantum yield	Substrate addition needed for signal detection Substrate not permeable
Non-coding nucleotide sequence		Marker detected also after horizontal gene transfer	DNA extraction No activity measurement
	pat		

[a] Data is collected mainly from Jansson & Prosser (1997) and Prosser (1994).

Many marker genes that do not require culturing techniques can be used for monitoring. These markers report, often quantitatively, both the culturable and the non-culturable part of the population. Introduced genes coding for proteins can be detected e.g. by microscopic cell counts directly if fluorescent (*gfp*), by immunological detection or by a specific reaction like ice nucleation (*inaZ*). Luminescence-based methods on the other hand are often used for studying cell activity (reviewed in Jansson & Prosser, 1997; Prosser *et al.* 2000). The marker genes *lux*, isolated from *Vibrio fisheri*, and *luc*, isolated from the eukaryote firefly, encode for luciferase-enzymes, which catalyse light production of the substrate luciferin in tagged cells. Detection of light-producing bacteria can be done in many ways; by recording light emitting colonies on plates or on plants using X-ray film or a CCD-camera, by luminometry of non-extracted cells, and by lumino-metry of the enzyme fraction of the sample. The *luc* and *lux* genes have been sequenced and the presence of the genes themselves can therefore be monitored by molecular techniques, using marker-gene specific primers. Detection of light producing single cells has been achieved by CCD-enhanced microscopy (Silcock *et al.*, 1992; Rattay *et al.*; 1995).

Introduced marker genes add to the genetic load of a strain, and the added trait is therefore often a burden to the tagged bacterium, causing reduced growth rates or instability of the marker gene itself (de Weger *et al.*, 1991; Amin-Hanjani *et al.*, 1993; Vahjen *et al.*, 1997). The genetic burden of modified bacteria can be reduced by silencing the expression of the marker gene in the environment or by activating the gene only during certain conditions. Corich *et al.*, (2001) used an isopropylthiogalactoside (IPTG)-inducible promotor to regulate the *lacZ* marker gene, which was induced on IPTG-plates containing chromogenic substrate. Wilson *et al.*, (1995) constructed a marker gene which was regulated by a promotor, that was activated during symbiosis. Recently, methods for removal of antibiotic resistance genes in GM plants have been developed (Iamtham & Day, 2000) and of all selective marker genes in microbial systems. This was achieved by using the *parA* gene product, which naturally is involved in the partition of plasmids in daughter cells. The enzyme encoded by *parA* caused removal of selection markers flanked by tandem DNA sequences called *res* sites (reviewed in de Lorenzo *et al.,* 1998). Both the genetic burden of the cells is reduced and the spread of antibiotic resistance genes in nature is avoided by making use of these new methods.

5.2. Advantages and disadvantages of using DNA based methods

Any marker gene for which the DNA sequence is even partly known, can be monitored by hybridization techniques using specific probes or by polymerase chain reaction (PCR) techniques using specific primers. The DNA sequence may be intrinsic; then subtractive hybridization for probe selection can be used to increase specificity (Tas *et al.*, 1996). Further, introduced DNA fragments, such as small non-coding fragments (e.g. *pat,* van Elsas *et al.*, 1991) or artificial junctions between chimeric genes (Zaat *et al.*, 1994) can be used. Isolation of nucleic acids from

environmental samples is a challenge, because both clays and humus compounds have similar properties as nucleic acids. These compounds are therefore difficult to separate during the isolation procedure. Remaining soil-derived substances reduce the sensitivity of analyses by interfering with PCR, restriction enzyme analysis and Southern hybridizations (Saano *et al.*, 1993). Many protocols for isolation of DNA from environmental matrices have been described. The methods, which often are complicated or expensive to perform, differ between various types of matrices, types of DNA and types of bacteria from which DNA is extracted. In general, only a minor fraction of the total microbial DNA from terrestrial environments is recovered, causing low sensitivity and high detection limits in soil samples. For the above mentioned reasons, detection by hybridization without PCR is often not sensitive enough. The specificity and sensitivity of PCR is improved by using nested PCR (Möller *et al.*, 1994).

Extracted DNA can be quantified by comparison to known standards. Quantification of DNA in PCR samples is tedious without expensive equipment, but can be achieved by the use of MPN statistics (Picard *et al.*, 1992) or internal standards (Möller & Jansson, 1997). DNA-derived signals do not reflect the activity of cells and bacteria that are non-culturable or even dead are detected by DNA-based methods. Even if the marker gene has escaped from the host bacterium to indigenous bacteria by horizontal gene transfer, the marker can still be detected. The activity of bacteria can be investigated using nucleic acid-based methods, by specifically isolating the rRNAs or even the mRNAs in environmental samples. The numerous applications for DNA based detections make DNA based methods a useful detection method once the sensitivity problems are overcome.

6. Stability of introduced marker genes

Marker genes can be inserted into the chromosome, onto a vector plasmid or a natural plasmid in the GM bacterium. Chromosomally located genes are more stable than genes carried on plasmids (Thompson *et al.*, 1995c; Sengeløv *et al.*, 2001). Transposons are DNA fragments that can insert themselves into specific recognition sites in the chromosome without homologous regions. Marker genes are often introduced into the chromosome of the host bacterium by using disarmed transposon vectors (de Lorenzo *et al.*, 1990), which insert the marker gene into the chromosome with the help of short inverted repeats of DNA flanking the marker gene. Theoretically, these repeats may act as targets for natural transposons and this way make the inserted gene unstable. A more specific but tedious way of tagging is to use homologous recombination for introduction (Bailey *et al.*, 1995). During homologous recombination, no additional gene sequences are transferred to the genome, and for this reason, this process has been suggested to be more stable than the use of disarmed transposons.

Plasmids harbour their own mechanisms for replication and are therefore maintained as independent entities in the host cells. Plasmid-encoded genes are often present in multiple copies in the cell, resulting in more effectively expressed traits. This poses a burden on the cells and plasmid-containing bacteria which are inoculated into soil frequently have poor survival (Dejonghe *et al.*, 2000). Plasmid-encoded traits are often unstable in the environment in the absence of selection pressure and may be lost. For these reasons, plasmids are generally not considered to be the vectors of choice for constructing GMOs (Smit *et al.*, 1992). Plasmids can persist and spread in the environment, if there is a selection pressure for plasmid-encoded traits. The spread of antibiotic resistance plasmids in medically important bacteria is a consequence of increased selective pressures imposed by the increased use of antibiotics. Similarly, the evolution of degradative plasmids is considered to be a response to the increasing amounts of xenobiotic pollutants in soil and water (Davison, 1999). For biodegradation of pollutants, deliberate dispersal of degradation plasmids to indigenous bacteria in contaminated soils has been suggested (Dejonghe *et al.*, 2000; Sarand *et al.*, 2000). This approach, where highly mobile genes providing

bacteria with competition advantage might, however, be questionable due to the risk this process would cause, if pathogens would receive these traits.

7. Relevance of horizontal gene transfer for risk assessment

Bacteria have effective means to disperse genes to other bacterial species and even to more distantly related organisms such as plants, by processes collectively named horizontal gene transfer. This ability raises much concern that introduced genes in GM bacteria may spread to indigenous bacteria in the environment. To be maintained in the new host cell, the incoming DNA fragment has to integrate into the host genome by homologous recombination, if it is not a plasmid or a transposon. Most genes transferred into new hosts are likely to be neutral or a burden to the host bacterium, but there is a possibility that new gene combinations are created, which provide the bacterium with a selective advantage. These gene combinations may have profound impacts on the environment, e.g. on the nutrient cycling processes. Horizontal gene transfer processes is mediated by conjugation, transformation or transduction (Figure 2).

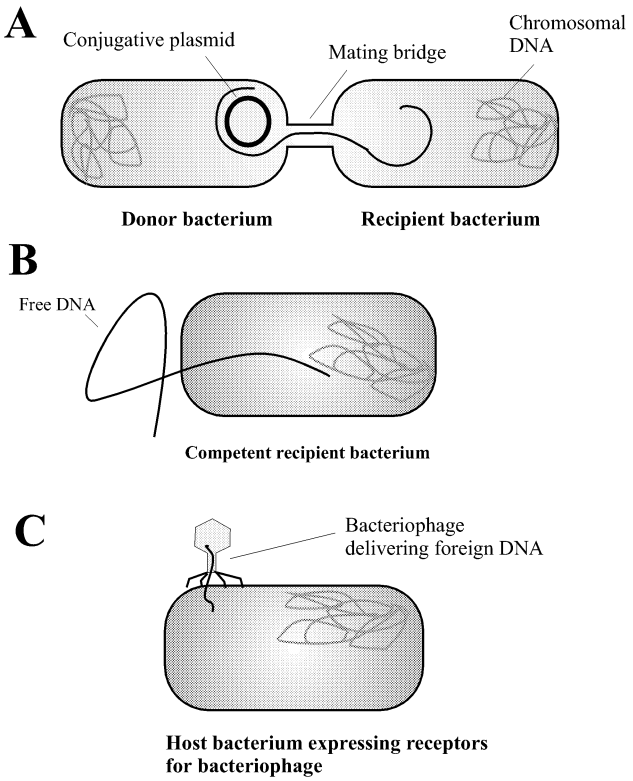


Figure 2. Mechanisms for horizontal gene transfer in bacteria. A. conjugation, B. transformation and C. transduction. Note that the incoming DNA is self-replicating in A. For stable maintenance in the cell, incoming DNA in B and C needs to be either self-replicating or contain a transposon or sequences with homology to the chromosomal DNA in the cell, permitting integration into the chromosome. In C, a transducing bacteriophage is exemplified by a T4-type virus.

Phylogenetic analyses of chromosomal genes in distantly related bacteria indicate that horizontal gene transfer has indeed played a role in evolution (Syvänen, 1994; Suominen *et al.*, 2001). The concerns raised by occurrence of horizontal gene transfer in the environment are of several different types. First, transfer of engineered genes from the GM bacteria to other organisms in nature can occur. If this happens, the modified gene constructs can persist in the environment after the introduced organism itself has disappeared. Plasmids capable of transfer via mobilization were detected in the culturable community in soil even after the introduced GM bacterium itself was not culturable (Henschke & Schmidt, 1990). Secondly, the modified bacterium may act as a recipient for new genes, causing new gene combinations which can behave differently than the originally introduced bacterium. Transfer of indigenous plasmids has been observed from natural bacteria to GM bacteria in the rhizosphere and phyllosphere of sugar beet (Lilley & Bailey, 1997a), showing that released bacteria can indeed acquire new genes from mobile elements at high frequencies in plant systems. For these reasons, information on the possibility of gene transfer is required in risk assessment studies (Tiedje *et al.*, 1989; Smit *et al.*, 1992). Thirdly, horizontal gene transfer causes dispersal of naturally occurring homologous DNA fragments in the environment. Gene transfer mediated by transposons has resulted in spread of the mercury-resistance *mer* operon to gram-negative bacterial species in different parts of the world (Yurileva *et al.*, 1997). The presence of highly homologous sequences in various bacterial species may affect the stability of transgenic organisms by increasing the likelihood for homologous recombination between unrelated sequences.

Because individual events of gene transfer are rare and therefore difficult to observe, experiments are often performed in ways that facilitate gene transfer, e.g. by addition of nutrients, by use of selection pressure, by using unnaturally high DNA concentrations or DNA originating from bacteria of the same species. Reports concerning gene transfer are therefore often worst-case scenarios. On the other hand, studies performed with new marker genes allowing detection of conjugation events without determinations by plating on selective media, indicate that the real *in situ* frequencies really might be several orders of magnitude higher than estimated with plating techniques (Hausner & Wuertz, 1999). The true relevance of horizontal gene transfer seems to be that it is too common to be ignored, and therefore horizontal gene transfer has to be considered in environmental risk assessments.

7.1. Conjugation

Conjugation is an active process where a self-transmissible (conjugative) plasmid in the donor bacterium is transferred to the recipient bacterium via cell-to-cell contact (Figure 2A). The conjugative plasmids contain all the genes needed for independent replication in the host cell as well as genes enabling transfer to new hosts. Plasmids confer context-dependent benefits upon the host that facilitate survival and colonization in field conditions. In terrestrial systems plasmids have been isolated from manure and soil samples (Götz *et al.*, 1996; Smit *et al.*, 1998), from the phyllosphere (Bender & Cooksey, 1986; Lilley & Bailey, 1997a) and from the rhizosphere (Lilley & Bailey, 1997a; van Elsas *et al.*, 1998). On the leaf surface 20 % of the investigated *Pseudomonas* strains carried plasmids (Powell *et al.*, 1993). Similar plasmids have been isolated from both phyllosphere and rhizosphere on the same plant and in the same field during following years even in the absence of selection pressure for the plasmid (Lilley *et al.*, 1996).

For environmental risk assessment, the most troublesome are the conjugative plasmids with broad host range, which often are transferable between a wide range of gram-negative and gram-positive bacteria. The transfer of conjugative plasmids can result in co-transfer of non-conjugative plasmids or even chromosomal fragments. Mobilization of non-conjugative plasmids occurred from inoculated *Pseudomonas* to indigenous bacteria of the same species in soil (Smit *et al.*, 1993). In laboratory conditions, conjugation-mediated transfer of chromosomal markers

from donors to recipients occurred at a frequency of 10^{-3} of the conjugation frequency. In addition, a process called retrotransfer took place, where transfer from recipients to donors was recorded at a frequency of less than 10^{-6} of the conjugation frequency (Mergeay *et al.*, 1987). Retrotransfer in nutrient-amended soil (Top *et al.*, 1995) as well as transfer of chromosomal sequences between fluorescent *Pseudomonas* species in the wheat rhizosphere (Troxler *et al.*, 1997) has also been observed.

The frequency of plasmid transfer is strongly affected by ecological factors (Table 2) and some terrestrial environments are considered to be hot-spot environments for conjugation. These include the rhizospheres in microcosms (van Elsas & Starodub, 1988; Top *et al.*, 1990; Smit *et al.*, 1993) and in the field (Lilley *et al.*, 1994), the phyllosphere (Lacy & Leary, 1975, Nomander *et al.*, 1998), the interface between decaying material and soil (Sengeløv *et al.*, 2000) and the seed (Sengeløv *et al.*, 2001). In environmental conditions, conjugation occurred during a defined period of time (Lilley & Bailey, 1997a), indicating that specific environmental conditions are required also in the field. It is likely that locally high bacterial cell densities in these environments favour gene transfer. In gram-positive bacteria, conjugation seems to be induced by pheromones (Dunny *et al.*, 1995) and recently a quorum sensing signalling molecule was shown to enhance conjugation in *Rhizobium leguminosarum* (Lithgow *et al.*, 2000).

The physiological state of bacteria has been suggested to affect conjugation due to the energy required for pilus-synthesis and replication. Conjugation was not, however, directly affected by the level of protein synthesis of the bacteria inhabiting the interface between decaying material and soil (Sengeløv *et al.*, 2000) or on the leaf surface (Nomander *et al.*, 1998). It has been hypothesised that bacterial conjugation takes place if the protein synthesis is above a certain level, and that further increase in metabolic activity will not further stimulate plasmid transfer (Kroer *et al.*, 1998). Cells starved for up to 15 days retained their conjugation ability for two weeks in aqueous environments (Goodman *et al.*, 1993) and plasmids were maintained in non-culturable cells (Byrd & Colwell, 1990). The conjugation frequency was lowest for exponentially growing *E. coli* cells with large cell size (Muela *et al.*, 1994). It was speculated that all of the energy in the exponentially growing cells was used for biosynthesis processes at the expense of transfer processes such as production of pilin-proteins. The physiological state of the recipients does not affect conjugation frequency (Muela *et al.*, 1994; Sudarshana & Knudsen, 1995), and even non-culturable cells acted as recipients and formed VBNC transconjugants (Arana *et al.*, 1997).

7.2. Transformation

Natural transformation is a process where competent bacterial cells take up free DNA and express it (Figure 2B). The foreign DNA has to be maintained and expressed in the new host bacteria. A requirement is therefore, that part of the incoming foreign chromosomal fragments contain homologous DNA sequences that allows the fragment to be inserted into the chromosome of the host cell by transposition or homologous recombination, if it is not a plasmid. Non-homologous regions can be integrated together with the homologous region. The requirement for homologous DNA sequences ensures that most successful transformations with chromosomal DNA occur between closely related strains.

Table 2. Factors affecting conjugation in gram-negative bacteria colonizing terrestrial environments.

Factor	Observation	Reference
Cell densities	The higher the cell density of donors and recipients, the sooner conjugation occurred.	Ramos-Gonzales *et al.*, 1991
Organic matter- and clay content	Conjugation to indigenous bacteria was highest in soils with high clay and organic matter contents.	Richaume *et al.*, 1992
pH	Highest conjugation frequency at pH 7.5.	Lafuente *et al.*, 1996
	Possibly due to the effect of pH, binding of cells to surfaces and establishment of pilus-mediated cell-to-cell contact.	Dröge *et al.*, 1999
Moisture	Maximum gene transfer at lower moisture than optimum moisture for bacteria growth.	Richaume *et al.*, 1989; Lafuente *et al.*, 1996
Nature of the pilus-structures	Flexibility and length of pilus structure affect conjugation frequencies in wet and dry environments.	Pukall *et al.*, 1996
Temperature	Optimal temperature around 30 °C.	Richaume *et al.*, 1989; Lafuente *et al.*, 1996
Nutrients	Manured soil enhanced plasmid mobilization.	Götz & Smalla, 1997
Rhizosphere	Plasmid transfer frequencies decreased drastically at increasing distance from roots.	van Elsas & Starodub, 1988; Lilley *et al.*, 1994
Earthworms	Earthworms acted as vectors for dispersal and possibly facilitated contact of the conjugal partners.	Daane *et al.*, 1996
Fungi	Absence of fungi enhanced conjugation in the interface between soil and decaying plant material, possibly due to increased bacterial cell densities.	Sengeløv *et al.*, 2000
Selection pressure	Selection pressure for traits coded by the plasmid resulted in higher level of conjugation.	Pukall *et al.*, 1996; DiGiovanni *et al.*, 1996; Smit *et al.*, 1998
	Selection pressure for traits coded by the plasmid resulted in higher level of retrotransfer.	Top *et al.*, 1995
	The observed increase in transconjugants was due to enhanced growth compared to the recipients.	Rore *et al.*, 1994

Nutrient addition clearly enhances transformation in soil (Nielsen *et al.*, 1997b) but humic acid decreases transformation activity *in vitro* (Tebbe & Vahjen, 1993; Nielsen *et al.*, 2000a). Interspecies transformation by DNA originating from cell lysates occurred in soil (Nielsen *et al.*, 2000a), indicating that restriction barriers do not prevent transformation. In addition, transfer of plant DNA to bacteria by transformation has been reported *in vitro* (Gebhard & Smalla, 1998; de Vries *et al.*, 2001), in sterile soil (Nielsen *et al.*, 2000b) and in the field (Gebhard & Smalla, 1999). On the other hand, transformation is not observed in starved bacteria in terrestrial soil systems that are commonly regarded as hot-spots for conjugal gene transfer (Sengeløv *et al.*, 2001). Further, no transformation occurred in natural rivers during winter (Williams *et al.*, 1996).

Competence is the ability of bacterial cells to bind and actively take up DNA in a form that is resistant to digestion by intracellular nucleases. More than 40 bacterial species belonging to gram-negative or gram-positive bacterial groups as well as some members of the *Archaea* have been shown to possess natural transformation systems (reviewed in Lorenz & Wackernagel, 1994). Transformation is induced in pure cultures of gram-positive bacteria by secretion of competence factors, but in gram-negative bacteria competence is induced during transition from exponential to the stationary phase of culture growth. Access to nutrients is critical for development of competence in soil, which is also affected by moisture level and phosphate concentration (Lorenz & Wackernagel, 1991; Nielsen *et al.*, 1997a) but natural competence was even developed in *E. coli* in oligothrophic mineral water (Baur *et al.*, 1996).

Generally, free DNA in the environment is scarce, because the major part of nucleic acids are hydrolysed when released into soil. The half-life of DNA in soil is usually short (Romanowski *et al.*, 1992), but part of the introduced DNA persisted for weeks in soil after its release (Recobert *et al.*, 1993; Romanowski *et al.*, 1993). The persistence and transforming ability of added DNA is dependent on soil type. DNA is released from the bacterial cells passively during cell lysis, but broken cells soon lost their capability to act as a source for transforming DNA in non-sterile soil (Nielsen *et al.*, 2000a). DNA adsorbs to clay, where it is more resistant to degradation by DNAses than free DNA. Clay-absorbed DNA was used to transform competent *Bacillus* (Gallori *et al.*, 1994) and *Pseudomonas* cells (Lorenz & Wackernagel, 1990).

7.3. Transduction

Bacterial genes can be transferred from cell to cell by bacterial viruses, the bacteriophages (Figure 2C). This transduction process occurs when a bacteriophage by mistake takes up bacterial DNA into the virus particle and transfers it to the next bacterium during infection. To persist in the new host any incoming DNA that cannot replicate by itself has to undergo homologous recombination with the DNA of the host cell. This can be a reason why transduction of plasmid DNA is more frequently observed than of chromosomal DNA. Due to their limited host ranges, bacteriophages are not considered to contribute essentially to gene exchange among distantly related bacteria.

Viruses are common in aquatic systems (reviewed in Dröge *et al.,* 1999) and in terrestrial habitats (Germida & Casida, 1981). Transduction has been demonstrated in water, in soil and on leaf surfaces, even when the donor and recipient bacteria were on different plants (Kidambi *et al.*, 1994). Survival of bacteriophages was enhanced by adsorbtion to clays (Vettori *et al.*, 1999), but transduction frequencies were not affected by nutrient- or clay amendments (Zeph *et al.*, 1988). Transduction was enhanced by the presence of particulate matter in water (Ripp & Miller, 1995), indicating influence of host cell density for transduction. Viral infections are most effective in environmental conditions optimal for bacterial growth. Abiotic parameters therefore affect transduction by altering the physiological status of the host cells. The physiological status might affect the production of bacteriophage receptors, which must be expressed on the surface

of the bacterial cell for viral infection to occur. Adsorption of virus particles to bacterial host cells was not, however, influenced by the physiological state of the cells, but replication of bacteriophages was reduced in starved cells (Kokjohn *et al.*, 1991). Despite this, bacteriophages infected and multiplied in bacterial host cells that had been starved for up to 5 years (Schrader *et al.*, 1997). In natural nutrient-poor environments, a majority of the phage infections can be non-lytic, resulting in the bacteriophage genome being inserted into the host DNA as a prophage. Up to 40 % of the culturable proportion of *P. aeruginosa* in different habitats have been shown to contain prophages (Ogunseitan *et al.*, 1992). Environmental strains harbouring prophages can be a predominant reservoir of bacteriophages causing transducing potential in microbial communities. Transduction via lysogenic hosts has indeed been demonstrated in soil (Zeph *et al.*, 1988).

8. Impacts of introduced bacteria on the indigenous microbial communities in the environment

It is expected that introduced bacterial strains will have high impact on populations with similar characteristics to the released strain, due to competition. Indeed, inoculated *Pseudomonas* strains have transiently been shown to displace indigenous populations of natural *Pseudomonas* strains in the rhizosphere (Natsch *et al.*, 1997; Naseby & Lynch, 1998) and on the root surface (Moenne-Loccoz *et al.*, 2001). Studies performed to assess impacts of genetically modified *Pseudomonas* species introduced into soil systems are listed in Table 3.

Evaluation of observed effects of inoculated strains on the natural bacterial communities is difficult because only a part of the total microbes in the environment have been described and the impact of biodiversity on microbial processes in ecosystems are not very well known. It can therefore be difficult to know what impacts to expect or on what level observed impacts should be considered significant. When observed changes due to the inoculant were transient and smaller than temporal changes, these effects were not presumed to harm ecosystem function (Natsch *et al.*, 1997). Similarly, observed changes were considered of minor importance, because the effects were similar to the wild type strain and no obvious effects on plant growth or health was observed (De Leij *et al.*, 1995). Natural biological control strains can also have unintended impacts on the indigenous microbes, which may cause an environmental risk. A natural plant-protecting strain of *P. fluorescens* influenced the readily available nutrients in the rhizosphere and foliage of red clover (Moenne-Loccoz *et al.*, 1998). A better way to determine the usefulness of biological control agents could be to compare them to existing corresponding chemical control agents presently in use. Comparisons of antagonistic *P. fluorescens* to commercial fungicides have so far been done in few cases (Moenne-Loccoz *et al.*, 1998; Thirup *et al.*, 2001).

Table 3. Observed impacts of genetically modified *Pseudomonas* in soil and plant associated environments measured in microcosms or in the field.

Factor	Observation	Reference
Nitrogen cycle	Clay content affected ammonification, dentrification and nitrification more than *P. putida* in soil.	Jones *et al.*, 1991
	P. fluorescens enhanced denitrification in soil.	White *et al.*, 1994
	P. fluorescens enhanced uptake of nitrogen in plants.	Brimecombe *et al.*, 1998
Respiration	The toxic metabolite of the bioremediation strain *P. putida* reduced respiration in soil.	Short *et al.*, 1991
Microbial activity	Antibiotic producing *P. fluorescens* reduced microbial activity in soil.	Brimecombe *et al.*, 1998
Enzyme activities	Non-antibiotic producing *P. fluorescens* affected enzyme activities in rhizosphere.	Naseby & Lynch, 1997
	Antibiotic producing *P. fluorescens* enhanced phosphate and sulphate cycle enzyme activities, but reduced carbon and nitrogen cycle enzyme activities in the rhizosphere.	Naseby & Lynch, 1998
Community level physiological profiles (CLPP)	Lactose utilizing *P. fluorescens* and *P. chlororaphis* affected the CLPP in the rhizosphere as long as they remained culturable.	Garliardi *et al.*, 2001
	P. fluorescens increased utilization of glucose dimers and polymers in the rhizosphere, which the pure culture did not utilize.	Natsch *et al.*, 1998
Culturable populations of bacteria	*P. fluorescens* caused short -term reduction of bacterial diversity in soil.	White *et al.*, 1994
	Antibiotic producing *P. fluorescens* caused a shift towards slower growing strains (K strategists).	Naseby & Lynch, 1998
	P. aureofaciens had transient effects on indigenous *Pseudomonas* on seed, roots and leaves.	De Leij *et al.*, 1994
	P. cepacia (now *Bulkholderia cepacia*) increased taxonomic diversity of culturable populations in soil.	Bej *et al.*, 1991

	P. fluorescens caused transient changes of some culturable populations in the phyllosphere and rhizosphere.	De Leij *et al.*, 1995
Fatty acid profiles	Differences in profiles declined as the culturability of the lactose utilizing *P. fluorescens* and *P. chlororaphis* strains decreased in the rhizosphere.	Garliardi *et al.*, 2001
Community DNA kinetics	*P. cepacia* (now *Bulkholderia cepacia*) increased diversity of the gene pool in soil.	Bej *et al.*, 1991
Fungal population	The toxic metabolite of the bioremediation strain *P. putida* repressed culturable counts of fungi.	Short *et al.*, 1991
	P. putida transiently repressed fungi in the rhizosphere of wheat, measured by plate counts and molecular techniques.	Glandorf *et al.*, 2001
Microfaunal populations	*P. fluorescens* caused increased nematode and protozoan populations in the rhizosphere, which might affect N-mineralization.	Brimecombe *et al.*, 2000

8.1. Methods used for studying impacts of introduced bacteria

Assessment of unwanted effects of introduced microbes, including GM bacteria, is a pressing issue. Both the structure and the function of the indigenous microbial community might change as a result of introduction of foreign bacteria. For this reason, both these parameters should be measured to be able to determine impacts of GM bacteria. Most indigenous bacteria are unculturable in laboratory conditions. Therefore cultivation-independent methods are required to obtain a complete picture of the total structure of natural communities. The results obtained by these methods often describe a certain parameter of the community, e. g. the fatty acid composition or genetic profiles based on specific DNA sequences. In DGGE, each band in the community profile is represented by a specific group of the microbial community. Denaturing gradient gel electrophoresis has been used to demonstrate transient effects of repeated irrigation of a natural biocontrol strain of *P. aureofaciens* on the bacterial community on leaf surfaces and absence of effect in the rhizosphere of turfgrass (Sigler *et al.*, 2001). Likewise, bacterial community structure in the rhizosphere of potato was not affected by introduction of antagonistic *P. putida* as measured by DGGE (Lottmann *et al.*, 2000). Another genetic profiling technique, PCR-single-strand conformation polymorphism, was used to observe effects of introduced *Sinorhizobium meliloti* on rhizosphere communities (Schwieger & Tebbe, 2000). Additionally, key metabolic processes should be quantified to determine possible impacts on function of the microbial community. Function of community has been characterised through measurement of, for example, rates of ammonification, nitrification or denitrification (Jones *et al.*, 1991), respiration (Short *et al.*, 1991), and overall microbial activity (Brimecombe *et al.*, 1998). Other activity-based approaches include determination of enzyme activities (Naseby & Lynch, 1997)

and community level physiological profiles (CLPP) (Natsch *et al.*, 1998). Community rRNA profiles, reflecting the most active populations of the bacterial community, have been used to study impacts of *Sinorhizobium meliloti* on alfalfa (Miethling *et al.*, 2000).

8.2. Relevance of microcosm experiments

Before releasing GM bacteria into the environment it is essential to study the ecology of the organism using contained facilities. For estimations of environmental risk of GM bacteria without releasing them into the environment, microcosms can be used. Microcosms are systems that attempt to simulate, in part, the prevailing biotic and abiotic conditions of the environment under study. They permit the manipulation of physiochemical variables that are impossible to control in natural environments. However, it is obvious that microcosms fail to totally mimic the natural environment because of the complexity of fluctuating and interacting environmental parameters as well as unknown factors which cannot be mimicked. Microcosms are therefore only an approximation of the natural environment and the results should therefore be viewed within the limitations of their experimental design. They may still provide valuable information for environmental risk assessments (Hood & Seidler, 1995).

A database containing information on field releases of genetically modified organisms performed in the OECD countries is available in the Internet on the OECD home pages. The database can be reached via the following address: http://www.olis.oecd.org/ biotrack.nsf/. Field releases have also been performed with some genetically modified *Pseudomonas* strains. These include release of an *lacZY-aph-xylE* tagged *P. fluorescens* in the rhizosphere and phylloplane of wheat (De Leij *et al.*, 1995) and sugar beet (Thompson *et al.*, 1995c). Further, *P. putida* constitutively expressing a gene for an introduced antifungal compound (Glandorf *et al.*, 2001), a bioremediation strain of *P. fluorescens* tagged with *lux* (Ripp *et al.*, 2001) and a Tn*5*- tagged epiphytic fitness mutant of *P. syringae* (Beattie & Lindow, 1994) have been used in field trials. Although microcosms and greenhouse experiments generally predict the fate of introduced bacteria well, in some cases they have been poor predictors of bacterial behaviour in the open environment. In the field, *P. fluorescens* survived poorer in the rhizosphere of sugar beet than in greenhouse microcosms (Thompson *et al.*, 1995a). On the other hand, the same strain survived better in the wheat rhizosphere than predicted by microcosm studies (De Leij *et al.*, 1995). This might be due to a slightly different inoculation approach, pointing out that the experimental setup influences the results very much. In addition, the reduced fitness of a RecA$^-$ mutant of *Sinorhizobium meliloti* was only detected in the field, but not in greenhouses (Schwieger *et al.*, 2000). Greenhouse grown plants supported larger epiphytic populations of introduced bacteria than field grown plants (Wilson & Lindow, 1993; Beattie & Lindow, 1994) and greenhouse experiments failed to demonstrate plasmid encoded fitness advantages in the phyllosphere observed in field (Lilley & Bailey, 1997b). Interactions of plant pathogenic bacteria was also different in microcosms compared to in the field (Upper & Hirano, 1996).

AIMS OF THIS WORK

The product development of GMOs for environmental applications is increasing, and several field trials using GM bacteria are performed each year in the EU. Potential applications of GM bacteria for plant protection or promotion of plant growth will include introduction of these bacteria into plant associated environments. In this work I wanted to study the survival, activity and possible impacts on indigenous bacteria of genetically modified *Pseudomonas* inoculated to plant associated environments. Especially, I wanted the answers to the following questions:

- Are marker genes useful for tracking introduced bacteria on the leaf surface and in organic soil? Can marker genes encoding antibiotic resistance be replaced with other marker genes ? (paper I and III)

- Is conjugation a relevant process for gene transfer on the leaf surface and what factors might affect conjugation on the leaf surfaces? (paper I and II)

- How do introduced bacteria respond to different temperatures in soil? (paper III)

- Is the survival of introduced bacteria different in soil and in the rhizosphere? (paper III and IV)

- Do high numbers of introduced bacteria affect the indigenous microbial communities in the rhizosphere? (paper IV)

MATERIALS & METHODS

Strains constructed and monitored in association with plants are described in Table 4 and methods used are summarised in Table 5.

1. Microcosm setups

1.1. Leaf surface experiments

outdoors (paper I), were used for leaf surface inoculations. The *P. syringae* cultures used (Table 4) were introduced to the bean leaf surfaces by a brief immersion of the leaves in a bacterial suspension, from which culturing media had been removed by washing the bacterial cells. Control plants were immersed in washing solution alone. For conjugation experiments, donor and recipient bacteria were mixed immediately before inoculation. In one experiment (paper II), only the recipients were added by immersion, and the donor bacteria were added by spraying after a defined time of delay. Inoculated and control plants were kept in high or moderate humidity for up to 2 weeks.

1.2. Soil and rhizosphere experiments

Soil microcosms (paper III) contained sieved humus forest soil from Nastola wilderness area in southern Finland. The washed *P. fluorescens* culture (Table 4) was introduced by mixing into the soil. Control soils were treated with washing solution alone. Inoculated and control soils were incubated at different temperatures for up to six months during which time humidity was kept constant by addition of sterile water.

For rhizosphere microcosms (paper IV), birch seedlings were germinated and grown aseptically for 10 weeks, after which they were planted onto a 3-4 mm thick layer of humus forest soil from the same site as for the soil experiments (paper III) or onto fertilized peat commonly used in plant nurseries. The growth substrates and seedlings were kept between two plastic plates. Six weeks after transplantation, inoculation was performed by spraying washed *P. fluorescens* culture (Table 4) onto below ground parts of the 2-dimensional microcosms. Control microcosms were treated with washing solution alone. Rhizosphere- and bulk soil samples were studied for up to three months.

Table 4. Strains constructed and used in microcosms in this study.

Name	Origin	Reference	Method for tagging (marker genes included)
P. syringae Cit7Sp	Phyllosphere of citrus	(Orser *et al.*, 1985)	Mini-transposon vector[a] (Sp resistance)
P. syringae Cit7*ina::XylE*N	Phyllosphere of citrus	(Wilson & Lindow, 1994)	Mutagenesis (Nal resistance)
P. fluorescens 31K3	Rhizosphere of birch	(Elo *et al.*, 2000)	Mini-transposon vector[b] (*mer/luc*)

[a] Reference for vector construct; de Lorenzo *et al.*, 1990.
[b] Reference for vector construct; Herrero *et al.*, 1990.

Table 5. Methods used in this study.

Parameter	Method	Described and used in paper no. I	II	III	IV
Microcosms					
Bean leaves	Plants grown in pots or in field	X	X		
Bulk soil	Soil in flasks			X	
Rhizosphere of birch	Seedlings in soil				X
Tagging of strains					
	Spontaneous mutation	X			
	Insertion of marker genes	X		X	
Impact of markers on host					
	Growth rate *in planta*	X			
	Growth rate *in vitro*	X		X	
	Antagonism				X
Denitrification of pure culture	N_2O-production in liquid				X
Detection of introduced strains					
Culturing methods	Selective plating	X	X	X	X
	Selective plating + luminescence			X	X
	Luminescence of enrichment cultures				X
Other methods	Microscopic counts of FITC-stained cells		X		
	Luminescence of whole cells			X	
	Luminescence of cell extracts			X	X
	DNA isolation and PCR of environmental samples			X	X
Localization of introduced bacteria on the leaf	Scanning electron microscopy		X		
Survival in non-optimal conditions					
Humidity	40-60 %		X		
Temperature	-20, 4, 15, 20, 30 °C			X	
Observation of non-culturable cells					
Viability testing	Reduction of CTC		X		
Indirect observation	Comparison of culturable population to total population		X	X	X
Gene transfer between added bacteria					
	Conjugation on plates	X			
	Conjugation in liquid	X			
	Conjugation on bean leaves	X	X		
Gene transfer to indigenous bacteria					
	Counter-selection against donor using bacteriophage	X			
Presence of plasmid					
	Hybridization	X			

Identification of isolated strains				
	Biochemical reactions (API)	X		
	Whole-cell fatty acids (FAME)	X		
Impacts on community structures				
Eubacteria	PCR-DGGE		X	X
Fungi	PCR-DGGE			X
Pseudomonas	RFLP			X
Mycorrhiza	Microscopic count			X
Impacts on community functions				
Respiration rate	CO_2-production in soil samples			X
Denitrification potential	N_2O-production in soil samples			X
Carbon utilisation profiles	Biolog- plates			X
Statistical analyses				
	X^2 - test	X		
	Student's T-test	X		
	Simple factorial ANOVA			X
	Clustering method (UPGMA)		X	X
	Regression analysis combined with principal component analysis			X

2. Monitoring of strains

The three strains used for introductions into microcosm systems were tagged by selection of spontaneous mutants or by using the mini-Tn*5* vector systems as described in papers I and III (Table 4). Although many *P. syringae* strains are plant pathogenic, the two strains used here, Cit7Sp and Cit7*ina::xylE*N, are not pathogenic to any plant tested (reviewed in Hirano & Upper, 2000). For conjugation studies on the leaf surfaces, these spontaneous rifampicin (Rif) resistant mutant strains of *P. syringae* were turned nalidixic acid (Nal) resistant or spectinomycin (Sp) resistant respectively; the donor was tagged with the Sp-resistance encoding gene and a spontaneous Nal-resistant mutant of the *ina*$^-$ strain was selected and used as recipient (Table 4). To investigate the usefulness of alternative marker genes to antibiotic resistance genes in soil and rhizosphere studies (papers III and IV), the luciferase producing *luc*- marker gene was combined with the *mer*- gene, encoding resistance towards mercury (Hg), in the same vector (paper III, Figure 1). The resulting construct was used for tagging *P. fluorescens* 31 K3 (Table 4), resulting in a tagged strain devoid of any antibiotic resistance coding genes. The *luc*-marker gene is detectable also by culture independent techniques, because the encoded enzyme catalyses a reaction which results in light production. Essential for light production is energy (ATP) and oxygen. Two different luminescence detection techniques were used in paper III, whole cell luminescence and cell extract luminescence. Whole cell luminescence was measured without ATP addition, reflecting the active part of the population whereas cell extract luminescence was measured after ATP additions.

Inoculated cells were monitored by culturing techniques on selective media, and *luc*-tagged cells in addition by luminometry and PCR (Table 5).

3. Conjugation and impacts of introduced bacteria on community function and structure

Gene transfer by conjugation of the large (60 kb), selftransmissible, broad host range plasmid RP1 (Burkardt *et al.*, 1979), originating form a clinical isolate of *Pseudomonas* and conferring

resistance to kanamycin (Km), ampicillin (Ap) and tetracycline (Tc), was recorded by screening for Rif, Nal and Km resistant *P. syringae* Cit7*ina*::*xylE*N cells on bean leaves by cultivation. For detection of gene transfer to indigenous bacteria, only donor cells were inoculated to field grown bean plants, and transconjugants were detected by Km, Ap and Tc selection in the presence of viral counter selection against the donor strain. Obtained transconjugant colonies were verified by DNA-DNA hybridization.

Changes in the structural biodiversity of natural communities in soil systems were studied using PCR based molecular techniques (DGGE and RFLP), using group specific primers for eubacteria (paper III and IV), fungi and *Pseudomonas* (Paper IV). Changes in function of the natural microbial communities were studied by community level physiological profiles (CLPP) and by respiration rates and denitrification potentials of microbes in soil.

RESULTS & DISCUSSION

1. Introduction of marker genes and their use for tracking *Pseudomonas*

Tagging of strains by selecting for spontaneous mutants (paper I) or by introduction of marker genes (paper I and III) for tracking of introduced *Pseudomonas* did not generally cause changes in growth rates of the tagged strains in laboratory conditions and the chromosomally encoded traits were stably maintained in the cells, measured by comparative plate counts. The antagonistic properties of the tagged *P. fluorescens* strain remained unchanged (paper IV). Only in one case (paper III) did integration of the marker genes cause reduced growth rate of a clone. As observed in many other studies, introduction of the RP1 plasmid into the bacterial cells reduced the growth rate of the bacteria in laboratory conditions in this study. Further, plasmid encoded features were unstable in liquid cultures and on the leaf surface in absence of selection (paper I, Figure 1). It has earlier been shown that 2 % of Tn*5*-tagged epiphytic *P. syringae* had lower epiphytic fitness on the leaf surface than the wild type (Lindow *et al.*, 1993) but spontaneous antibiotic resistant mutants, on the other hand, retained their fitness in the environment (Völksch & May, 2001). Another non-conjugative plasmid related to RP1, pMP92, has also been shown to be highly unstable in the rhizosphere of potato and wheat, compared to other groups of plasmids tested (van der Bij *et al.*, 1996).

Simultaneous use of several marker genes coding for antibiotic resistance were successfully used for monitoring introduced *P. syringae* on the leaf surface by plate counts (papers I and II). Indigenous bacteria resistant to some of the antibiotics used as selective agents for the introduced strains on the leaves were often found (Table 6), but were occasionally absent (paper II). Another selective marker gene *mer,* lacking traits coding for antibiotic resistance, was useful for monitoring *P. fluorescens* in soil (paper III and IV) but a background of indigenous Hg-resistant bacteria was found both in humus soils and in peat (paper IV; Table 1). This may not be uncommon, since the indigenous Hg-resistant community of the peat used as carriers for inoculates has been reported to be 2 x10^6 cfu per g (Corich *et al.*, 2001). Due to the background, colonies on plates had to be further characterized for non-selective traits. When Hg-resistance was combined with luminescence, reliable identification of the *P. fluorescens* inoculum in soil systems was achieved (paper III and IV). Actually, this method for monitoring the *P. fluorescens* inoculum was the most sensitive detection method in soil systems (paper III). This *luc/mer* construct would be feasible to use in field releases, since no antibiotic resistance genes were needed for detection. Luminescence from extracted whole cells or cell extracts correlated directly in a linear fashion to the number of early stationary phase cells (paper III, Figure 3). Therefore luminescence could be used for estimations of cell numbers in the samples.

The high organic matter content in the humus soil (paper III and IV) and in peat (paper IV) reduced the sensitivity of enumeration methods that were not based on culturing techniques. Re-isolation of added DNA or enzymes resulted in 10 % recovery (paper III). These recently introduced molecules probably behave very differently from molecules resident or present for longer periods in soil, for which the recovery was possibly even lower. The total DNA yield was 5 µg per g of soil (paper III). DNA yields from humus soils have not often been reported, but the yield from a soil containing 30 % organic matter was 35 µg per g of soil (van Elsas *et al.*, 1997). Furthermore, the resulting DNA- and enzyme-extracts form organic soils were not pure. This resulted in a 10 % quenching in luminometry and inhibition of PCR in non-diluted samples (paper III). The detection limits for luminometry and PCR were therefore high in humus soils (paper III, Table 1) and luminometry could be used for monitoring of *P. fluorescens* only in the beginning of the experiments (paper III and IV). Quenching of luminescence in sediment samples is a recognized problem (Möller *et al.*, 1995) and humic compounds are well known to inhibit PCR reactions. Knowing the limitations of the method, PCR was useful for estimation of the

Selective agent[a]	CFU / g fresh weight			
	Peat vermiculite mixture	Agricultural soil	Bean leaf surface grown indoors	Bean leaf surface grown out doors
None	2×10^7	1×10^6	2×10^5	4×10^5
Streptomycin	8×10^5	5×10^4	$< 10^2$	8×10^4
Kanamycin	9×10^5	5×10^3	4×10^2	3×10^4
Tetracycline	3×10^4	8×10^4	2×10^2	1×10^4
Nalidixic acid	nd	nd	nd	6×10^4
Rifampicin	nd	nd	nd	3×10^3
Chloramphenicol	1×10^4	5×10^2	$< 10^2$	nd

[a] Concentrations used (μg ml^{-1}): Streptomycin, 50; Kanamycin, 25; Tetracycline; Nalidixic acid, 40; Rifampicin, 75; Chloramphenicol, 35.

inoculum in a semi-quantitative manner. The high inoculum in soil samples was also recorded both by DGGE and RFLP soon after inoculation (paper IV).

2. Survival of *Pseudomonas* in plant environments

2.1 Survival of *Pseudomonas syringae* on the leaf surface

In high humidity conditions, introduction of *P. syringae* bacteria onto the leaf surface of bean resulted in establishment of a population of 10^7 cells per g fresh weight of leaves (paper I and II). At low humidity the introduced *P. syringae* stabilized at 10^6 cells per g fresh weight of leaves (paper II). In scanning electron micrographs, inoculated bacteria were seen unevenly distributed on the dry leaf surfaces and located mainly at leaf veins or around air pores (paper II; Figure 1). This distribution has been reported also for indigenous bacteria (Nomander *et al.*, 1998), and therefore this distribution it is not likely to be affected by the inoculation method or the incubation conditions used here. The introduced bacteria were embedded in mucoid matrix (paper II; Figure 2) after 4 days of incubation at high humidity. Native micro-organisms produced copious exopolymeric matrices, that resulted in formation of biofilms on the leaf surfaces (Morris *et al.*, 1997). It is expected that similar matrices were formed also at low humidity conditions in this study.

Turning into a VBNC state is a general physiological response to stressful conditions and *P. fluorescens* cells turned VBNC during osmotic stress (Jørgensen *et al.*, 1994). Respiring cells, that do not grow on plates, are VBNC. Here, the redox dye 5-cyano-2,3-ditolyl tetrazolium (CTC) was used with fluorecein isothiocyanate (FITC) to determine the respiring portion of the introduced bacteria on the leaf surfaces. In overnight cultures, the respiring portion of the cells measured by CTC reduction was many times only 50 %- 70 % of the culturable cells. According to Garland *et al.* (2001), this can be explained by the lag-phase which cells need, before they respond to favourable conditions in cultures. Although *P. syringae* turned VBNC in liquid cultures, VBNC cells were not detected during 2 days of incubation on the leaf surface of bean

(paper II, Table 2). During constant conditions at high humidity, 95 % of the introduced cells on the leaf surface were respiring after 4 days of incubation. The length of the cells was reduced to half of the original size during 4 days of incubation on the leaf surfaces (paper II; Table 1). At low humidity conditions, the introduced cells were longer compared to cells grown in high humidity (paper II; Table 1) and only 5 % of the introduced cells were respiring after the first day of incubation. At the same time, the culturable counts were 3 % of the introduced cells (paper II; Table 3), indicating that VBNC cells were few or absent. The few remaining respiring cells, measured by intracellular CTC crystals in FITC-stained cells, were frequently very short 4 days after inoculation (Table 7).

A reduction in cell size has often been connected to formation with VBNC cells in gram-negative bacteria (Jiang & Chai, 1996). After a 2-week incubation, all *Escherichia coli* cells in nutrient-poor lake water were VBNC and coccus-shaped (Signoretto *et al.*, 2002). In the work reported here the respiring cells were short, but apparently remained culturable during low humidity conditions. It seems that the reduction in cell size and the production of VBNC cells were different responses to environmental changes and the processes were not directly connected. It is possible, that the rapid change in physical parameters during inoculation, did not allow for adaptation of the cells by reduction in cell size during cell division, but instead killed the cells. The few surviving cells were able to adapt by changing the morphology of the cells. Other inoculated *P. syringae* on sterile plants have been reported to stay culturable for more than 3 days in high humidity and desiccation of the leaf surface did not produce VBNC *P. syringae* cells during the first 10 h (Wilson & Lindow, 1992).

Translocation of *P. syringae* between totally dry leaf surfaces and establishment of these bacteria on the new leaf was demonstrated (paper II). Others (Thompson *et al.*, 1995a), have been unable to detect translocation between dry leaf surfaces pressed together for 5 min. It is possible, that moisture is not relevant for the translocation itself, but is needed for establishment of the translocated cells in suitable places for colonization. In our work colonization niches were found, due to the rubbing together of the surfaces.

Table 7. Length of cells incubated on the leaf surface at low humidity, measured from micrographs of FITC and CTC stained cells.

	Cell length (μm)[a] ± SE	
Time of incubation (days)	**All cells (from paper II; Table 1)**	**CTC reducing cells (unpublished data)**
0	4.1 ± 0.4	4.0 ± 0.1
1	3.6 ± 0.5	2.2 ± 0.1
4	3.3 ± 0.5	1.9 ± 0.1

[a] The result is the mean of six replicate leaves, from which 18 separate microscopic fields were counted.

2.2. Survival of *Pseudomonas fluorescens* in soil and in the rhizosphere of birch

The survival of high numbers of *P. fluorescens* cells was dependent on temperature in humus bulk soil (paper III; Table 1). The *luc* marker gene was detected by PCR during the whole time period up to 6 months at all temperatures except 30 °C (paper III; Table 1), but the introduced population did not establish at any certain level in the soil, measured by other methods. One percent and a hundred percent of the added DNA remained during a half year incubation at 4 °C and -20 °C, respectively. It is noteworthy that some culturable cells were observed even after a 6 month incubation period at 4 °C or below (paper III, Figure 4). Cell numbers, calculated from whole cell luminescence, were one tenth lower compared to other detection methods immediately after inoculation (paper III; Table1), and decreased faster during prolonged incubation than cell numbers measured by other monitoring methods. This indicates that the energy level of the cells introduced into soil diminished compared to laboratory grown cells. Ten days after inoculation, whole cell activities were detected only in soils incubated at 15 °C (paper III; Figure 5a). Part of the *P. fluorescens* cells stayed culturable at 15 °C up to 42 days (paper III, Figure 4). Also other introduced bacteria in soil were found to persist better at low temperatures (Vahjen *et al.*, 1997) and the best survival of another *P. fluorescens* strain, isolated from the rhizosphere of wheat in the temperate region, was at 15 °C (Vandenhove *et al.*, 1991). In addition, genetically modified *P. fluorescens* was observed to survive over winter and transiently colonize plants during the following season (Thompson *et al.*, 1995c).

Plate counts and cell extract luminescence measurements at first corresponded to similar numbers of cells, but on day 10, plate counts from 30 °C were lower than cell extract luminescence (paper III). This may be an indication of VBNC cells. Because respiration assays by CTC-reduction were not performed, it is not known whether these cells had changed to a VBNC state or were dead, but retained their proteins intact. The formation of VBNC of *P. syringae* and *P. fluorescens* has been shown to be temperature dependent, and occurred faster at high temperatures, but plasmid-containing cells turned VBNC faster at low temperature (Oliver *et al.*, 1995).

The roots of the birch seedlings used in microcosms, developed very fast (paper IV; Figure 2) and appeared to influence the whole microcosm during the experiment. Bacterial communities in the close vicinity of birch roots were often similar to those in soils more distant from the roots. As observed earlier in other studies involving allochthonous introductions, the high inoculum density did not assist in the establishment of *P. fluorescens* in the birch rhizosphere, which was inhabited by active indigenous microbial communities. The inoculum was unable to compete for niche colonization and this was reflected in the rapid decrease in inoculum cell numbers in both media during the experiment (paper IV; Table 2). Part of the introduced *P. fluorescens* turned non-culturable after 20 days in humus, but not in peat. The viability of these cells was not tested. A reason why the cells turned non-culturable in humus but not in peat might be found by comparing the two soils. The major bacterial groups revealed by DGGE differed between peat and humus (paper IV; Figure 1), and indigenous *Pseudomonas*-strains were present in peat, but absent in humus. Further, higher numbers of culturable indigenous cells were recorded in peat, and the indigenous community utilized a higher number of different carbon substrates compared to humus soil (paper IV; Table I). Similarly to this study, the viable but non-culturable state in *P. fluorescens* cells in the rhizosphere of cucumber was soil-type dependent (Hase *et al.*, 2000). The bacteria stayed culturable in soils with high fertility status for the host plant, but became non-culturable in soils with low fertility status. In this work, the fertilized peat and forest humus soil most likely represented high and low fertility statuses. After 3 months of incubation, the introduced strain was detected only by luminescence of enrichment cultures. This indicates that a small residual population of viable cells was present by the end of the experiment. These cells were reactivated in favourable laboratory conditions but were not detected by PCR, due to the low sensitivity of the method. The sensitivity of nucleic acid based methods can be increased by

using larger masses of soil, form which the nucleic acids are extracted. This complicates handling of the samples and increases the cost of the methods. Further development of molecular biology techniques is needed to increase sensitivity of these methods and obtain reliable estimations of the persistence of non-culturable populations. Although high inoculation of *Pseudomonas* is often successful using fresh cultures of bacteria (Natsch *et al.*, 1997; Lottmann *et al.*, 2000), it is possible that a better colonization rate would have been achieved if the inoculum would have been starved overnight or introduced on the sterilized seeds in the absence of competing bacteria. Peat is a commonly used carrier for *Rhizobium* species in agricultural applications, but similar applications using *Pseudomonas* are limited. The results presented here, indicate peat might be a good carrier also for *Pseudomonas*. The better survival in bulk soil (paper III), as compared to the rhizosphere (paper IV), may have been due to the presence of less competitive indigenous bacterial populations away from the immediate vicinity of roots. Alternatively, inoculum survival was minimal to non-existent in both experiments but detection was different due to lower degradation rates of the target DNA in bulk soil than in the rhizosphere.

3. Conjugation of the broad host range plasmid RP1 on the leaf surface

Despite the instability of the RP1-plasmid, it did not disappear completely from the *P. syringae* population on the leaf surface (paper I; Figure 1). In high humidity, the conjugation rate was high enough for the plasmid to be maintained in 0.8 % of the tested epiphytic population (paper I; Figure 2). During the first 3 days new transconjugants continued to appear (paper II, Table 3). In liquid cultures, conjugation was not detected in buffer, but the presence of a leaf enhanced conjugation, even when net growth of donor and recipient bacteria was absent (paper I; Table 3). RP1 encodes a rigid pilus structure, which requires presence of surfaces for effective conjugation. It is probable that the leaf provided this surface, where bacteria also were in close contact with each other in aggregates. Additions of peptone or leaf wash (paper I; Table 3), caused net growth of the donor and recipient populations, but the addition of leaf wash did not enhance conjugation compared to the addition of a leaf. By comparing numbers of transconjugants on plants kept in light or darkness, it was concluded that conjugation on the leaf surfaces was affected by photosynthesis of the plant (paper I; Figure 3).

Intra-species conjugation occurred in low humidity, in conditions where introduced *P. syringae* donor and recipient populations decreased. After one day of incubation on the leaf surfaces, similar numbers of transconjugants were detected regardless of the prevailing humidity conditions (paper II; Table 3). The conjugation rates were therefore higher in low humidity than in high humidity during the first 2 days and the transconjugants transiently rose to 40 % of the introduced bacteria in low humidity on day 2. This observation is in line with the theory about adaptive mutagenesis, where genetic instability increases during stressful conditions (Foster, 1993). This is thought to enhance the formation of more fit genotypes. After three days of incubation, the numbers of transconjugants were 200-fold lower in low humidity than in high humidity. Similar to this, conjugation of the TOL-plasmid was 8000-fold lower in low humidity on the leaf surface, during four days of incubation (Nomander *et al.*, 1998).

The broad host range plasmid RP1 can replicate in many gram-negative bacteria. A most interesting result with regard to possible environmental hazard of GM bacteria, was that conjugation to indigenous bacterial species occurred on the leaf surfaces (paper I; Table 2). The isolated transconjugants belonged to the β- and γ-subgroups of *Proteobacteria* and one isolate, *Sphingobacterium spirituvorum*, belonged to the *Bacterioides*/Flavobacteria kingdom. Indigenous bacteria of the β- and γ-subgroups of *Proteobacteria* acted as recipients for the RP4 plasmid also in soil (Thimm *et al.*, 2001). These results raise concern for risk assessment considerations, because populations of the indigenous transconjugants possibly do not disappear over time in a similar way as the introduced bacteria usually do. It should be observed that indigenous transconjugant strains, that are not culturable, were not detected with the methods used here.

Therefore, the true conjugation rate might have been higher. Others have indeed observed higher conjugation rates between known donors and recipients determined with microscopic observation than with plating techniques (Hausner & Wuertz, 1999).

The results indicate that the locally high density of introduced cells, as well as presence of nutrients caused high conjugation rate in the phyllosphere. The spatial aggregation of bacteria into microhabitats on the leaf surface caused high transfer rates in another study (Nomander *et al.*, 1998). In the work presented here, the way of introducing cells to the leaf surface highly influenced the results of the conjugation experiments. Introducing donor and recipient cells separately on different days, resulted in reduced conjugation rates (paper II, Figure 3) in low humidity conditions. Although the leaf surface caused aggregation of cells, which facilitated the cell-to cell contact needed for conjugation, it seems that the compartmentalization of the separately introduced cells and production of mucus limited conjugation between separately inoculated cells. This shows that the experimental setup can affect the results very much. Worst case scenarios similar to the one presented here, emphasize possible hazards and should therefore be recognized for environmental risk assessments, but in addition information on the likelihood of these processes is needed. To achieve a complete picture of what is probable to happen in environmental applications, it is extremely important to design experiments that resemble true environmental conditions e.g. by performing field trials.

4. Effects of *P. fluorescens* on natural communities in bulk soils and in the rhizosphere of birch

The introduction of the *P. fluorescens* strain did not drastically affect major eubacterial populations in bulk soil, regardless of the incubation temperature (paper III; Figure 6). The seldom encountered high temperature in the soil (30 °C) on the other hand, reduced biodiversity in the samples and increased variability between parallel samples, indicating that the molecular method applied (DGGE) was appropriate for detection of large changes in the communities (paper III; Figure 6).

The inoculation of *P. fluorescens* to the rhizosphere had a positive effect on birch seedlings, which was noted by better growth of the shoots (paper IV; Figure 1; and paper IV; Table 3). In humus soils, some changes in specific functions of the natural community were observed as a consequence of introduction of the GM bacteria. First, a reduction in the denitrification potential in inoculated humus soils was observed (paper IV; Table 4). Reduced denitrification potential may affect the nitrogen content in soil and in plants, but in this study no differences in the nitrogen content of inoculated and non-inoculated plants were found (data not shown). High denitrification potentials are found in soils from natural birch stands, compared to spruce or pine stands (Priha & Smolander, 1999). Others have reported changes in the nitrogen cycle due to introduction of *Pseudomonas* species (Table 3). Interestingly, introduction of *P. fluorescens* in the rhizosphere of pea enhanced nitrogen uptake by pea (Brimecombe *et al.*, 1998). This was thought to depend on enhanced mineralization of organic compounds due to the inoculum. Secondly, inoculated humus soils utilized some carbon compounds, which were not utilized in non-inoculated humus soil. A similar observation was reported for another introduced *P. fluorescens* strain (Natsch *et al.*, 1998). The overall changes in CLPPs both in inoculated humus and peat were small and PCA grouped the samples more closely at the last sampling (paper IV; Figure 4), indicating that the impact of the inoculum was transient. Inoculated *P. chlororaphis* caused detectable changes in the CLPP in the rhizosphere of wheat as long as the inoculum was detected on plates (Garliardi *et al.*, 2001). In this work, the observed changes were small when the inoculum was not detected on plates.

Some effects of the introduced *Pseudomonas* on the structural biodiversity in humus were detected. The eubacterial community in humus soil, measured by PCR-DGGE, was slightly reduced by the inoculation (paper IV; Figure 1b). In peat, the community varied more with time

than with treatment. Using another set of eubacterial primers, DGGE patterns did not reveal changes in the rhizosphere of potato after introduction of antagonistic *P. putida* (Lottmann *et al.*, 2000). In the work presented here, the fungal biodiversity measured by DGGE remained constant regardless of inoculation (paper IV; Figure 5), but microscopic counts of mychorrhizal root tips were reduced on inoculated birch seedlings in humus soil (paper IV; Table 5). It is possible, that the antifungal characteristics of our strain were not specific for only plant pathogenic fungi, but inhibited also beneficial fungi-root associations. Antifungal *P. putida* has been reported to transiently reduce fungal populations in wheat roots, measured by plate counts and molecular techniques (Glandorf *et al.*, 2001). Other antifungal *Pseudomonas* strains were reported not to affect the culturable proportion of the indigenous community in the rhizosphere of pea (Naseby & Lynch, 1998) and did even stimulate formation of arbuscular mycorrhiza in the rhizosphere of tomato (Barea *et al.*, 1998).

Comparisons of impacts of the GM bacterium to the wild type strain was not performed in this work. Therefore it is not possible to separate impacts resulting directly from the genetically modified traits and impacts caused by the inoculation. The introduced markers did not provide any competitive advantage in the introduced strains for the conditions used. Therefore it is probable, that the introduced markers did not have any direct impacts the natural communities. Genetically modified bacteria developed for commercial products are likely to exhibit GM traits that provide advantages to the GM strain. The impact of these strains may for this reason be significantly more pronounced than observed in this study.

The microcosms used for studying impacts on natural communities contained thin soil layers often used for studying microbial interactions in the rhizosphere. The physiological conditions in these microcosms differed significantly from natural systems; the oxygen concentration were probably higher and the temperature and humidity different than in natural conditions. These dissimilarities to natural conditions most likely affected both the survival of the introduced strain and measurable impacts on natural communities. In addition, studies reflecting bulk soil conditions were not possible to perform, as the whole microcosms were influenced by the birch roots. Despite these limitations, some indication of colonization capacity and possible impacts on a part of the microbial communities was observed.

CONCLUSIONS

- Antibiotic resistance coding marker genes were successfully used for tracking introduced *Pseudomonas* on the leaf surface and a selective marker gene construct devoid of any antibiotic resistance coding genes was successfully used for monitoring the introduced strain in the rhizosphere of plants grown in soil with high organic matter content.

- Introduced bacteria survived on the leaf surface well. The leaf surface acted as a hot-spot for gene transfer by conjugation in certain conditions and gene transfer to indigenous bacteria was observed. Conjugation was promoted by nutrients and the leaf surface itself, to which bacteria attached in micro-aggregates. The conjugation rate was dependent on the method used for introducing the bacteria, the recipient bacteria investigated and the prevailing humidity conditions.

- Survival of introduced *Pseudomonas* bacteria in soil and in root systems of birch was poor. In particular at high soil temperatures, the bacteria disappeared when measured by plating techniques, but were detected using molecular techniques. This indicates that the introduced bacteria had lost their culturability soon after introduction. In peat the introduced bacteria retained their culturability for a longer time.

- The most sensitive monitoring method used was plate counting, but this method records only culturable cells. Therefore, additional methods that monitor non-culturable bacteria are required in environments such as soil, where the introduced bacteria can turn non-culturable. Molecular techniques should then be used in combination with culturing techniques. To obtain a complete picture of the survival of the introduced bacteria, the molecular techniques should be improved to increase sensitivity of these methods in high organic matter content soils.

- In soils, observed impacts of the introduced strain included changes in bacterial and fungal communities, nutrient utilization, and denitrification potentials. The ecological significance of these results is difficult to establish without further research on microbial ecological principles in soil and in plant associated environments. In addition, experiments on the likelihood for these events to occur in natural conditions in the environment is needed.

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