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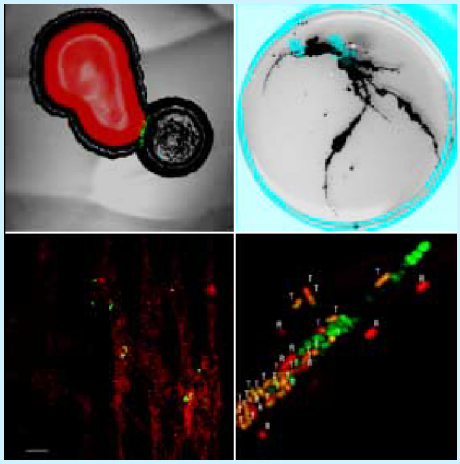
Conjugative plasmids and the transfer of mobile genetic elements among bacteria in plant rhizosphere environments

PhD thesis

Lars Mølbak



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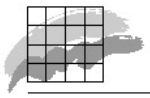

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National Environmental Research Institute
Department of Microbial Ecology and Biotechnology

and

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Abstract: "The thesis is about conjugative plasmids and was the partial fulfillment of the requirements to obtain the Ph.D. degree at the Technical University of Denmark. Plasmids are double stranded pieces of extra-chromosomal DNA that has the ability to replicate autonomously and be transferred to a recipient cell. The traits specified by plasmids include ex. antibiotic resistance, symbiotic and virulence determinants and bacteriocin production. By transferring genetic material, plasmids play a major role in enhancing the genetic diversity and adaptation of bacteria. The first chapter in the thesis is a general introduction to conjugative plasmids with special focus on IncP-1 plasmids, plasmid hostrange and plasmid conjugation in the rhizosphere environment. The next four chapters are manuscripts for international journals. The headlines were: 1) The plasmid genome Database. 2) Host range of a natural barley rhizosphere plasmid amongst bacteria isolated from a barley field. 3) Plasmid transfer from *Pseudomonas putida* to the indigenous bacteria on alfalfa sprouts: Characterization, direct quantification and in situ location of transconjugant cells. 4) Factors affecting the frequency of conjugal transfer in the rhizosphere of barley and pea."

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Abbreviation List

BHR	broad host range
bp	basepair
CBS	Center for Biological Sequence Analysis
CEH	Centre for Ecology and Hydrology
CFB	Cythophaga- Flavobacterium-Bacteroides
CFU	colony forming unit
Conju	image analysis program (this study)
DDGE	denaturing gradient gel electrophoresis
DFI	differential fluorescence induction
DNA	deoxyribonucleic acid
DsRed	red fluorescent protein
DTU	Technical University of Denmark
FACS	fluorescence activated cell sorter
Gfp	green fluorescent protein
Inc	incompatibility
IVET	in vivo expression technology
kb	kilo base
MGE	mobile genetic element
MPF-complex	mating pair formation complex
NHR	narrow host range
ORFs	open reading frames
PCR	polymerase chain reaction
PGD	plasmid genome database
SCLM	scanning confocal microscopy
STM	signature tagged mutagenesis
T/D	transconjugant pr. donor

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Preface

This thesis is made as a partial fulfillment of the requirements to obtain the Ph.D. degree at the Technical University of Denmark (DTU). The Ph.D. scholarship was financed by a grant from the Directorate for Food, Fisheries and Agro Business under the Danish Ministry of Food, Agriculture and Fisheries.

I would like to thank my supervisor at DMU, Niels Kroer for support and fruitful discussions, and Søren Molin for inspiration and to take the time whenever it was needed.

The people at MIMI (MIBI), DMU I would like to thank for good company, and I explicitly would like to thank the old Ph.D-group, Jens Efsen Johansen, Hap Prichard and Bjarne Munk Hansen for help and advices.

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And most important I thank my love Roberta Mustacchi for great help, support and for being.

Vesterbro, May 2003
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Summary

Plasmids are double stranded pieces of extra-chromosomal DNA that has the ability to replicate autonomously and be transferred to a recipient cell. Accessory functions, like antibiotic resistance and degradation of xenobiotic compounds, facilitate rapid adaptation to environmental selective pressure, and are typically located on mobile genetic elements (MGEs) such as genomic islands, conjugative transposons, mobilizable transposons and conjugative plasmids. Resistance genes are often found on plasmids that are transferable, and horizontal transfer by conjugation, or conjugation-like processes, seems therefore to be of particular importance for the spread of these genes under environmental conditions (van Elsas, 1992; Sørensen 1996).

In chapter 1 a new plasmid genome database (PGD), including 422 fully sequenced plasmid genomes (updated on January 6, 2003) with links to structural maps of each plasmid, is presented. The PGD divides the information into categories such as: host taxonomy, accession number, size (bp), plasmid name and date of submission to the database. Systematic interrogation of this comprehensive collection of plasmid genomes should allow for a better understanding of the role plamids have in contributing to the biology of bacteria, and it is important that all researchers have ready access to the information already available on it.

Some of the most promiscuous plasmids, which have been found in diverse geographical locations (usually as agents conferring antibiotic resistance in clinical isolates of Gram negative bacteria), belong to the plasmid incompatibility group IncP-1. Since few studies have directly tested plasmid transfer among indigenous soil (or rhizosphere) bacteria, very little is known about host ranges of plasmids isolated from the soil environment. In Chapter 2 of this PhD-thesis, the ability of an IncP plasmid (pKJK5) - isolated from a barley rhizosphere by Sengeløv et al., 2001 - to spread among bacterial isolates was compared to that of RP4 (a plasmid isolated from a hospital in Birmingham (Thomas and Smith, 1987)). In order to achieve this aim a gene transfer reporter system, based on the green (Gfp) and red (DsRed) fluorescent proteins, was used. By this method donor cells (red cells containing the DsRed and

the plasmid with unexpressed *gfp*) could be distinguised from recipient (colorless cells) and transconjugant cells (green cells containing the plasmid expressing the *gfp* gene). 3% out of the tested 1400 strains were able to take up pKJK5::*gfp* while 4 % were able to take up RP4::*gfp*. The data showed that the strains able to receive pKJK5::*gfp* belong to the following phylogentic groups: *α-Proteobacteria β-Proteobacteria*, *γ- Proteobacteria*, *Cythophaga-Flavobacterium-Bacteroides* (CFB), and to the Gram-positive phylum. Among these, the highest incidence of transconjugants belonged to the *α-Proteobacteria* group. In conclusion this study revealed a very broad phylogentic host range of soil and rhizosphere bacteria able to receive IncP-1 plasmids and underline the potential risk for spread of antibiotic genes among distantly related bacteria.

The work presented in Chapter 3 used the same *in situ* monitoring system as in Chapter 2. In this case, however, the level of genetic exchange among bacteria on alfalfa sprouts was studied. Since bacteria readily colonize the surface of sprouts used for human alimentation, it is important to understand the degree of horizontal gene transfer among these bacterial populations. Because of the small size and ease of cultivation, alfalfa sprouts were used as model system. Two different types of plasmids were tested: a broad host range plasmid (pKJK5::*gfp*) and a narrow host range plasmid (TOL::*gfp*). The donor strain (*Pseudomonas putida* KT2442) was inoculated on the alfalfa seeds. The results showed that indigenous transconjugant bacteria appeared 6 days after onset of germination. Bacteria that were transformed with either plasmid mainly belonged to the genera *Pseudomonas* or *Erwinia.* Scanning confocal laser microscopy (SCLM) revealed that, from Day 6, sprouts were heavily colonized with donor cells, and most transconjugant cells were located around the hypocotyl and the root areas. It was concluded that alfalfa sprouts constituted an environment that allowed considerable plasmid transfer among pseudomonads.

In the forth study (Chapter 4) the importance of bacterial density and distribution on the frequency of conjugal plasmid transfer in the rhizosphere of barley and pea was investigated. By use of fluorescent reporter proteins (similar to the ones described above), it was possible to differentiate *in situ* (at the single cell level on the plant

surface) between donor cells (Gfp tagged), recipient cells (DsRed tagged) and transconjugant bacteria (expressing both Gfp and DsRed). The donor strain (*P. putida* KT2442/pKJK5::*gfp*) was inoculated onto the plant seedlings, while the recipient strain (*P. putida* LM24), was present in the growth medium (soil or vermiculite). The cell-to-cell contact was studied by CSLM and counting of colony forming units (CFU). The study showed that the high transfer frequency measured in the pea rhizosphere relative to the barley rhizosphere was directly linked to an elevated root exudate production which in turn resulted in a higher density of the donor cells and, hence, a higher probability of donor-to-recipient contact.

In order to facilitate the image analysis of future conjugation studies using the same (or a similar) marker system (based on fluorescent proteins) a novel computer program (Conju) was developed.

This program allows a computer-automated quantification of potential cell-to-cell contact between donor and recipient cells and it allows the simultaneous analysis of other useful parameters (i.e. number of micro-colonies, average size of micro-colonies and the densities of donor, recipient and transconjugant cells).

In conclusion the work shown in this thesis has contributed to the understanding of conjugative transfer of plasmids among bacteria and showed the development of new research tools that may be of great utility in future studies in this field.

Dansk sammenfatning

Dansk titel: Konjugerbare plasmider og overførsel af mobile genetiske elementer mellem bakterier i planterhizosphere omgivelser.

Plasmider er dobbeltstrengede stykker af ekstra kromosomalt DNA, der kan replikere uafhængigt og kan overføres til en recipient celle. Ekstra funktioner, som antibiotikaresistens og evnen til at nedbryde fremmedstoffer, medfører hurtig tilpasning til et selektivt tryk fra omgivelserne, og disse gener sidder ofte på mobile genetiske elementer så som "genomic islands", konjugative transposoner, mobiliserbare transposoner og konjugative plasmider. Resistensgener ses ofte på plasmider, der kan overføres og især horisontalt overførsel ved konjugation eller konjugerbare processer ses derfor som værende af særdeles vigtighed ved spredning af disse gener under naturlige forhold.

I det første kapitel bliver en ny plasmidgenom database (PGD) præsenteret. Den har 422 totalt sekventeret plasmidgenomer (opdateret d. 6 januar 2003), med internet links til oversigtskort over hver enkelt plasmid. I PGD opdeles informationerne ind i kategorier så som værtens taksonomi, "accession" nummer, størrelse (bp), plasmid navn og indsendelsesdatoen til databasen. Systematisk interrogation af denne udbytterige samling af plasmidgenomer, bør give muligheden for en bedre forståelse af plasmiders betydning for bakteriers biologi, og det er vigtigt at forskere har adgang til denne information.

Nogle af de mest promiskuøse plasmider, der er fundet i diverse geografiske områder (typisk er det plasmider fra gramnegative isolater, der koder for antibiotika ressistens) hører til plasmid inkompatibilitet gruppe IncP-1. Da det kun er få studier, der direkte har testet plasmid overførsel blandt de naturlige jord (eller rhizosfære) bakterier, er viden omkring værtskabet af naturlige plasmider utroligt begrænset. I kapitel 2 i denne PhD-afhandling, blev et IncP plasmids (pKJK5), (et plasmid isoleret fra en byg rhizosfære af Sengløv et al. (2000)), spredning til forskellige bakterielle isolater sammenlignet med RP4´s (et plasmid der er isoleret fra et hospital i Birmingham (Thomas og Smit, 1987)). For at opnå dette mål blev et reportersystem, der er baseret

på grønt (Gfp) og rødt (DsRed) fluorescerende proteiner, brugt. Ved hjælp af denne metode kunne donorceller (røde celler der indeholder DsRed og plasmidet med det represseret *gfp* gen) blive adskilt fra recipient (farveløs) og transkonjugant celler (grønne celler som indeholder plasmidet der udtrykker *gfp* genet). 3 % ud af de 1400 testede stammer kunne optage pKJK5::*gfp* hvorimod 4 % kunne optage RP4::*gfp*. Disse data viste, at de stammer, der kunne optage pKJK5::*gfp,* tilhørte de phylogenetiske grupper: *α-Proteobacteria β-Proteobacteria*, *γ- Proteobacteria*, *Cytophaga-Flavobacterium-Bacteroides* (CFB), og Gram-positive fylo. *α-Proteobacteria* var den gruppe, der hyppigst kunne optage plasmidet. Konklusionen på dette studie var, at der er et bredt fylogenetisk værtskab iblandt jord og rhizosfære bakterier, der kan optage IncP-1 plasmider, og det underbygger den potentielle risiko for spredning af antibiotika resistente gener blandt fjernt relaterede bakterier.

Det studie, der præsenteres i kapitel 3, bruger det samme *in situ* moniterings-system som i kapitel 2. I dette tilfælde blev mængden af genoverførsel blandt bakterier på lucernespirer studeret. Da bakterier med en høj densitet koloniserer overfladen af spirer, der bruges som fødevare, er det interessant at undersøge, i hvor stor en grad der foregår horisontalt genoverførsel mellem bakterier i disse omgivelser. Lucernespirer blev pga. deres lille størrelse og lethed ved dyrkning brugt som modelsystem. To forskellige typer a plasmider blev testet, det ene var et bredt værtskabs plasmid (pKJK::*gfp*) og det andet var et snævert værtskabs plasmid (TOL::*gfp*). Donor stammen (*Pseudomonas putida* KT2442) blev inokuleret på lucernefrø. Af resultaterne fremkom det, at de naturlige transconjugante bakterier først viste sig efter 6 dage. Bakterierne, der havde modtaget enten det ene eller det andet plasmid, tilhørte hovedsageligt familierne *Pseudomonas* eller *Erwinia*. Skannings konfokal mikroskopi (SCLM) afslørede, at fra dag 6 var spirerne kraftigt koloniseret med donorbakterier, og de fleste af transconjuganterne var lokaliseret omkring ”hypocotyl” og roden. Det blev konkluderet, at lucernespirer udgør et miljø, der tillader en hvis mængde plasmidoverførsel blandt pseudomonader.

I det fjerde studie (Kapitel 4) blev vigtigheden af bakteriel densitet og fordeling på frekvensen af plasmid overførslen i rhizosfæren af byg og ært undersøgt. Ved hjælp af fluorescerende reporterproteiner (de samme som beskrevet ovenfor), var det muligt

at differentiere *in situ* (på enkelt celleniveau på planteoverfladen) mellem donor celler (Gfp mærket), recipient celler (DsRed mærket) og transconjugant bakterier (udtrykker både Gfp og DsRed). Donor bakterien (*P. putida* KT2442/pKJK5::*gfp*) blev inokuleret på spirerne, mens recipientstammen (*P. putida* LM24) var tilstede i vækstmediet (vermiculit eller jord). Celle til cellekontakten blev studeret med CSLM og tælling af bakteriekolonier på agarplader (CFU). Dette studie viste, at den høje overførselsfrekvens i ærterhizosfæren sammenlignet med overførslen i bygrhizosfæren var direkte relateret til en højere rodexudat produktion for ært, hvilket igen resulterede i en højere densitet af donorceller og derved en større sandsynlighed for donor og recipient kontakt.

For at kunne foretage billedanalyser af fremtidige conjugations studier, der bruger de samme (eller lignende) markør systemmer (baseret på fluorescerende proteiner), blev et nyt computer program (Conju) udviklet. Dette program tillader automatisk computer kvantificering af den potentielle celle til cellekontakt mellem donor og recipient celler, og det kan samtidig analysere andre brugbare parametre (f.eks. antal af mikrokolonier, gennemsnitlig størrelse af mikrokolonier, og densiteten af donor, recipient og transkonjugante celler).

Det arbejde, der er udført i denne afhandling, har tilført en større viden til området omkring overførsel af plasmider mellem bakterier og udviklet nye forskningsværktøjer, der forhåbentlig kan blive til stor gavn for fremtidige studier på dette område.

1. Introduction

The discovery of antimicrobial drugs to control infectious diseases must be among the greatest achievements of medicine in this century. The therapeutic application of sulfonamides in the 1930´s was one of the pioneering works leading to such dis-covery. Approximately ten years later (in the 1940´s) the group of tetracyclines was discovered (review see Roberts, 1996). During the same period, however, there was an alarming increase in bacterial resistance to these antibiotics. In 1953 the first tetra-cycline-resistant bacteria were isolated from *Shigela dysenteriae*, a bacterium which causes bacterial dysentery (Roberts, 1996).

The first plasmids were isolated and characterised in 1950´s and they become asso-ciated with the acquired antibiotic resistance. As the interest in the processes of gene transfer between bacteria started to grow, it was observed that the massive use of anti-biotics in prophylaxis and therapy created selective conditions for the spread of resis-tance genes within obligate and facultative pathogenic bacterial populations (Fig.1).

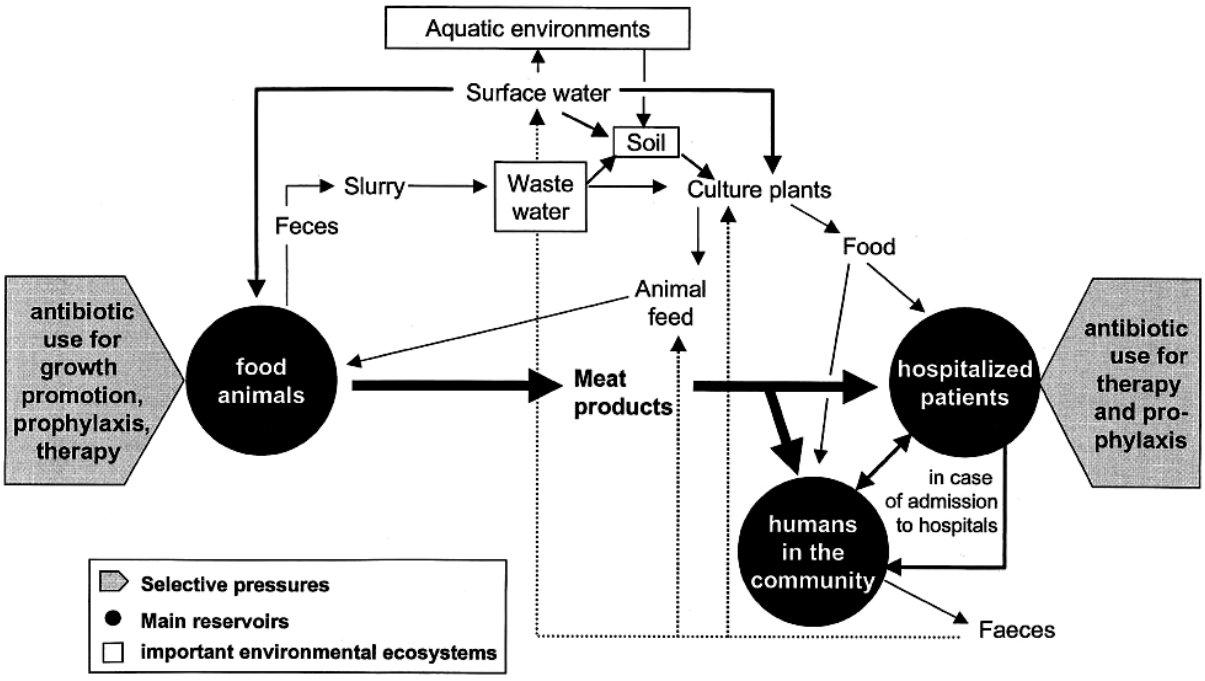

Figure 1. Routes of transmission of genes conferring antibiotic resistance. (Witte, 2000)

Most antibiotics were discovered by random screening campaigns, but over the past 20 years, this strategy has largely failed to deliver a sufficient range of chemical diversity to keep pace with the high rate at which bacteria develop resistance to currently used antibiotics (Allsop, 1998). Antibiotic resistance among microorganism is therefore regarded as a major threat to the future treatment of infectious diseases in both humans and livestock (Kruse and Sørum, 1994).

Development of antibiotic resistant bacteria is mainly based on two factors, the selective pressure coursed by antibiotics, and the presence of resistance genes (Levy, 1993). Humans and domestic animals are the known main reservoirs of antibiotic resistant pathogens resulting from the selective pressure created by the use of anti-biotics (Fig. 1). The clonal spread of resistant bacterial strains from animals to hu-mans is well documented for zoonotic pathogens like *Salmonella spp*. (Bower and Daeschel, 1999). Nevertheless, the extent to which genes for antibiotic resistance transfer between the microfloras from different environmental ecosystems, is not well understood.

Proliferation of resistance genes can take place by vertical transfer (multiplication of cells harbouring a resistance gene), and by three recognized mechanisms of pro-karyotic gene transfer (transformation, conjugation, and transduction). Horizontal transfer by conjugation, or conjugation-like processes, seems to be of particular im-portance under environmental conditions, because resistance genes are often found on plasmids that are either transferable or mobilizable (Van Elsas, 1992; Sørensen 1996). Conjugative plasmids have been identified in bacterial populations inhabiting, for instance, the phytosphere of sugar beet, the rhizosphere of wheat, soils, river epilithon, marine sediments, marine air-water interfaces, marine water, marine biofilm communities, sewage and activated sludge (Review see Dröge et al., 1999; Davison, 1999). Because of the fact that soil can continuously be enriched with antibiotic resistant genes, it is important to investigate the role ecosystems play in the transmission of antibiotic resistant genes. Enteric bacteria from the intestinal flora of humans and animals (often containing antibiotic resistant genes) are spread to the soil through waste waters and the use of manure as fertiliser (Haack and Andrews, 2000; Smalla et al., 2000b). Furthermore, the natural production of antibiotics in soil by the micro-biota can be another potential source of selection for antibiotic resistance genes within the soil environment (van Elsas, 1992; Hansen et al., 2001).

The transfer of conjugative plasmids (potentially containing genes for antibiotic resistance) is more frequent in the rhizosphere of plants than in bulk soil. Plasmid transfer was shown to occur at relative high frequencies in the rhizosphere of wheat (van Elsa et al., 1988), pea (Kinkle and Schmidt, 1991, sugar beet (Lilley et al., 1994), water grass (Kroer et al., 1998), radish (Walter et al., 1991) and barley (Sørensen and Jensen, 1998).

A multitude of factors, such as the density (spatial distribution) and the metabolic activity of the bacteria, may affect the frequency of plasmid transfer in soil and in plant-associated habitats (Dröge et al., 1999; Kroer et al., 1998; MacDonald et al., 1992; and Normander et al., 1998). A detailed knowledge of such factors, combined with an evaluation of potential recipients for conjugative plasmids, is essential for assessing the possibilities and the risk associated both with the transmission of mobile genetic elements in the rhizosphere (when techniques such as bioaugmentaion or biocontrol are applied), and with the evaluation of the flow of antibiotic resistance genes through the terrestrial environment.

2. Bacterial Plasmids

In biotechnology today most researchers associate plasmids with cloning vectors and tools for genetic manipulation. The truth is that plasmids can be "naturally" present in nearly all bacterial species (Amabile-Cuevas and Chicurel, 1992) ranging in size from a few to more than one thousand kilobases (kb) and can represent a large proportion of the whole bacterial genome (see chapter 1). Screenings from different environments have revealed that the plasmid incidence in the phyllosphere, sugar beet leaves, marine sediments, deep subsurface sediments, lake and marine air-water interface was 18, 18, 32, 34, 50 % respectively (Fredrickson et al., 1988; Kobayashi and bailey, 1994; Hermansson et al., 1987; Sobecky et al., 1997). The fact that the presence of conjugative plasmids in bacteria perhaps not has increased due to modern medicine was demonstrated by a study carried out by Hughes and Datta (1983), which tested the incidence of plasmids within a strain collection of Enterobacteriaceae from year 1917 to 1954, and demonstrated that conjugative plasmids were as common in the tested *Enterobacteria* before the medical use of antibiotics as they are in drug-sensitive strains today.

Plasmids can provide their hosts with growth advantages (Ricci and Hernandez, 2000). The traits specified by plasmids include antibiotic resistance, toxic heavy metal resistance, degradation of xenobiotic compounds, symbiotic and virulence determinants, bacteriocin production, resistance to radiation and increased mutation frequency (Trevors, 1985; Summers, 1996; Snyder and Campness, 1997; Thomas, 2000a). By transferring genetic material, plasmids play a major role in enhancing the genetic diversity and adaptation of bacteria.

Sequencing the complete plasmid or parts of it has become a potential straightforward approach for the characterization of plasmids. As expected most of the plasmid genomes that have been totally sequenced originated from clinically and industrially important strains (Table 1 and chapter 1). It supports the hypothesis, that the majority of well characterised plasmids originate from clinical bacteria and that our knowledge of the prevalence and diversity of bacterial plasmids from non-clinical environments is very limited (Smalla et al., 2000).

Table 1: Distribution of plasmid hosts ranked after amounts of total sequenced plasmids. The data is based on the plasmid genome database (see chapter 1)

	Group name of plasmid host	Number of plasmids total sequenced	Total amount of Bp sequenced
1	Unknown host	32	534962
2	*Borrelia* spp.	25	699548
3	*Corynebacterium* spp.	20	266141
4	*Lactococcus* spp.	20	261800
5	*Staphylococcus* spp.	19	218818
6	*Bacillus* spp.	18	638800
7	*Lactobacillus* spp.	16	130729
8	*Escherichia* spp.	13	288186

Table 2: Distribution of the 10 biggest plasmids there have been total sequenced. The data is based on the plasmid genome database (see chapter 1)

	Strain name of the plasmid host	Name of the plasmid	Size of the plasmid (Bp sequenced)
1	*Sinorhizobium meliloti*	pSymB	1683333
2	*Sinorhizobium meliloti*	pSymA	1354226
3	*Agrobacterium tumefaciens str. C58 (Cereon)*	AT	542869
4	*Agrobacterium tumefaciens str. C58 (U. Washington)*	AT	542780
5	*Rhizobium sp. NGR234*	pNGR234a	536165
6	*Nostoc sp. PCC 7120*	pCC7120alpha	408101
7	*Rhizobium etli*	p42d	371255
8	*Halobacterium sp. NRC-1*	pNRC200	365425
9	*Streptomyces coelicolor A3(2)*	SCP1	356023
10	*Mesorhizobium loti*	pMLa	351911

The host strains of the 10 biggest plasmids that have been totally sequenced so far (with exception of the plasmid from *Halobacterium* sp. NRC-1), have been shown to belong to strains isolated either from soil or plant-associated environments (see table 2). It is striking that this group of plasmids have a much lager size than the plasmids isolated from the clinical important strains. Environmental plasmids isolated from bacteria are often very large (up to 500 kb in size), tend to be transferred by conjugation and are able to replicate in a number of different bacterial hosts. In a study by Lilley et al. (1996) conjugative plasmids (ranging between 60-383 kb) were isolated from the rhizosphere, although due to their large size and instability, these plasmid have not yet been sequenced (Bailey, personal communication 2002).

Plasmid structure

By definition, plasmids are non-essential extra-chromosomal fragments of DNA that replicate with different degree of autonomy from chromosomal DNA. Although plasmids have a mosaic structure (they can be composed of a serried of genetic sequences encoding for different functions. (Fig. 2)), the absolute minimum requirement for a plasmid to exhibit its function is the presence of the origin of replication (which gives the ability to replicate). Consequently, the genes contained within plasmids, have as definition either an essential function (also called the plasmid backbone) or an accessory function (Osborn et al., 2000). The plasmid backbone can contain genes coding for replication, copy number control, multimer resolution, partitionering, post-segregational killing system and genes for transfer (Thomas 2000a). The accessory functions of a plasmid can have different phenotypic traits as mentioned previous.

Plasmids (with exception of a few small plasmids) contain a large number of inserted and/or extra chromosomal mobile genetic elements (MGE's) such as IS elements, transposons, integrons, gene cassettes and conjugative transposons (fig. 2) (Toussaint and Merlin, 2002; Osborn et al., 2000). These accessory genes are effectively "hitch-hiking" on the plasmid backbone, giving the plasmid (and the bacterial host) a selective advantage in exchange for their maintenance, and possible transfer to other hosts (Osborn et al., 2000). Nevertheless, it may be important to consider plasmids as "selfish" DNA elements whose own chance of long-term survival is enhanced by

genes that benefit their host (Lilley et al., 2000). MGE (including plasmids), and the genes they carry, constitute the horizontal gene pool (Thomas, 2000b).

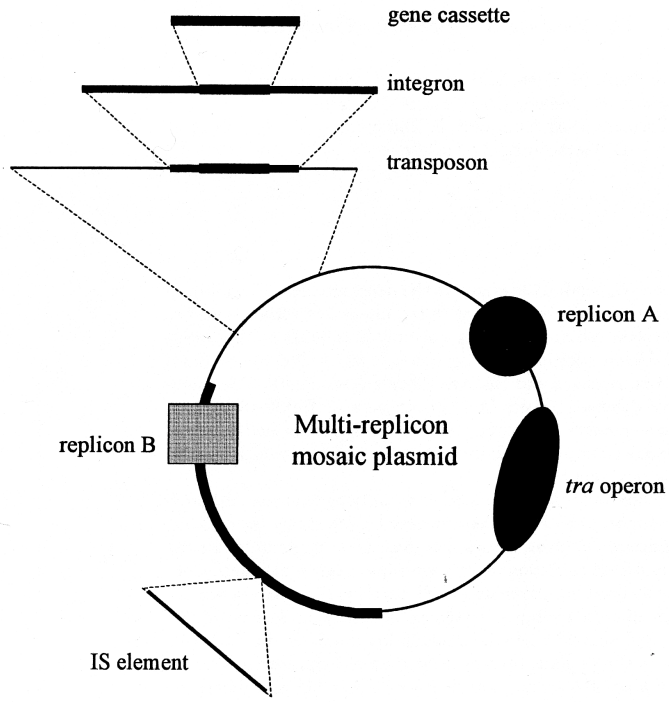

Figure 2. A simplified model to describe the mosaic structure of a circular plasmid. (Osborn et al., 2000)

The unknown gene pool.

Sequence analysis of environmental plasmids shows that plasmids appear to contain large amounts of apparently non-coding, non-functional or "uninformative" DNA. In recently published plasmid sequences, the percentage of open reading frames (ORFs) with no recognisable homology falls between 22%-67% (Le Dantec et al., 2001; Kwong et al; 2000; Scheiker et al., 2001; Galli et al., 2001; Marques et al., 2001; Muurata et al., 2002). The understanding of the functions of the uninformative DNA, is important because such DNA imparts a significant cost of carriage to the host without any off-setting adaptive advantage. This observation announces a fundamental lack in our understanding of plasmids biology: either they contain novel environmentally-specific genes hitherto un-described, or they contain non-functional DNA which is maintained in a bacterial population at a presumable acceptable cost.

Functional genomics and in vivo expression technology

Functional genomics (the field of science that studies the function of genes starting from their DNA sequence) can be used to identify ORFs that encode ecological relevant traits from sequenced plasmids having no known function. Preston and co-workers outlined three principle strategies that enable the identification of genes that are active in both complex and natural environments: signature tagged mutagenesis (STM), differential fluorescence induction (DFI) and in vivo expression technology (IVET; see Fig. 3) (Preston et al., 1998).

In the present work (Appendix I) IVET technology was used to find mating pair formation genes and plant induced genes from the environmental plasmid pQBR103. Nevertheless, the methodological approach used in appendix I differed from the classical IVET strategy (Fig. 3 and Appendix I) because the IVET clones used in the present study where those either containing strictly regulated promoters (genes that are induced only under specific conditions) or containing DNA fragments with no promoter at all. This approach was used to perform high throughput screening tests for the induction of each individual IVET clone, under different conditions (i.e. in the presence of plants, under cell to cell interaction, and in the presence of exudates). This alternative approach generated some positive results which are currently being ultimated.

The recent development of DNA chip technology and proteome technology may be a new and powerful tool to answer some of the questions about the unidentified open reading frames (ORFs) and to improve the understanding of the interaction between the genotype of the plasmid and the genotype of its host. These technologies will in the future hopefully also be able to elucidate the role of the plasmids with respect to their host in a complex environment.

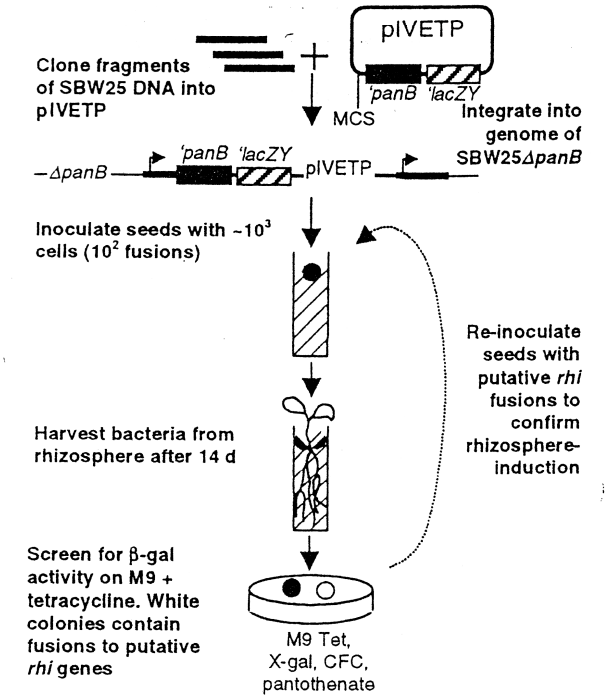

Figure 3. In vivo expression technology (IVET). Selection for genes showing rhizosphere-specific elevation in expression. Genes displaying elevated levels of expression in the expression of a gene that is essential for survival in the rhizosphere. The strategy is based upon random integration of promoterless *panB* (which encodes an enzyme essential for pantothenate biosynthesis) into the chromosome of *P. fluorescens* SBW25 strain carrying a chromosomal deletion of *panB*. SBW25Δ*panB* cannot colonize the rhizosphere because pantothenate is absent or severely limiting, therefore colonization can only occur if the promoterless *panB* gene is inserted downstream of an active promoter. Recovery of strains from the rhizosphere after selection for the ability to synthesize pantothenate results in isolation of promoters that are either constitutive or rhizosphere specific. To distinguish between these, a promoterless marker operon, *LacZY*, is fused to *panB*, enabling the lactose phenotype of the recovered cells to be determined. Lac^+ strains contain fusions to constitutive promoters (dark colonies), whereas Lac^- strains (white colonies) contain fusions to promoters activated in response to rhizosphere signals. (Rainey, 1999).

The plasmid atlas

The DNA Structural Atlas is a method of visualising structural features within large regions of DNA. It was originally designed for the analysis of complete bacterial genomes, but it can also be used for analysis of plasmids and DNA regions as small as a few thousand bp in length. The basic idea is to plot the values for different mechanical-structural properties of the DNA helix in a circle (or arc) representing the complete genome (plasmid or chromosome) (Jensen et al., 1999). At the level of whole genomes, mechanical-structural properties or architecturally important regions

can be seen. At the level of individual genes inter-genic regions can be examined. The plots are created using the "GeneWiz" programme. Plot similar to the one shown for the complete plasmid genome of R751 (fig. 4) can automatically be generated from GenBank sequence files. The different features shown by the map are: base composition, curvature, stacking energy, GC-skew and the presence of local and global repeats of various types.
The recent maps include two new features. The first new feature can be used to predict functions of unknown ORF by the use of neural network, and the second new feature will try to find the probability from which organism or phyla of organisms a gene or an operon most likely originated from. (personal communication Ussery, 2003).

The perspective for the plasmid database generated (Appendix II) is that plasmid atlas' in the next months will be generated at Center for Biological Sequence Analysis (CBS) at the Technical University of Denmark for all the total sequenced plasmids in the database. The database can in the future be implemented or build out with information about MGE, plasmid backbone information, phenotypic traits. The database and the plasmid atlas will be a great source that can be used for systematic studies, comparative analysis and it will extend our knowledge of the horizontal gene pool. Furthermore, it will be possible to sort plasmids according to various traits and use this information to compute an alternative classification of all the plasmids that have been totally sequenced, because all the plasmids parameters can be linked in new ways.

Classification of Plasmids

Strategies for classifying plasmids have been phenotypic characterisation, physical characterisation of the plasmid DNA (including restriction pattern), incompatibility testing, replicon typing and whole plasmid sequencing (Smalla et al., 2000a). Incompatibility testing is, at present, the most common way to classify plasmids, and approximately 40 different groups of plasmids have been defined on the basis of incompatibility (Inc) properties (Couturier et al., 1988; Viegas et al., 1997). The classification of plasmids by incompatibility is based on the idea that two different plasmids cannot co-exisit within the same host. Whenever two plasmids are incompatible (thus belonging to the same incompatibility group), one of them is

segregated (Couturier et al., 1988). A scheme to classify plasmids based on incompatibility has been created (Datta and Hedges, 1972). These groupings have been applied for the classification of plasmids originating from Gram-negative and Gram-positive bacteria. In a study by Sobecky and co-workers, (1997) on a marine sediment, it was found that 297 different bacterial types carried plasmids unrelated to the known incompatibility groups. Although incompatibility testing is widely used, there are certain complications associated with it. One example is that of "surface exclusion", which occurs whenever the entry of a donor plasmid is inhibited (Couturier et al., 1988).

Several primers and probes have been developed for plasmid classification based on plasmid regions involved in replication and stable maintenance (replicons)(Couturier et al., 1988; Llanes et al., 1994; Götz et al., 1996; Greated and Thomas, 1999). Replicon typing is technically simpler and faster than testing for incompatibility and its use is especially advantageous for plasmids consisting of more than one basic replicon (such as plasmids belonging to the incI or IncF group) (Couturier et al 1988).

Incompatibility group P plasmids.

Plasmids classified in *E. coli* as IncP and in *Pseudomonas* as IncP-1 are a particularly well studied group of plasmids because they are very promiscuous (they can replicate

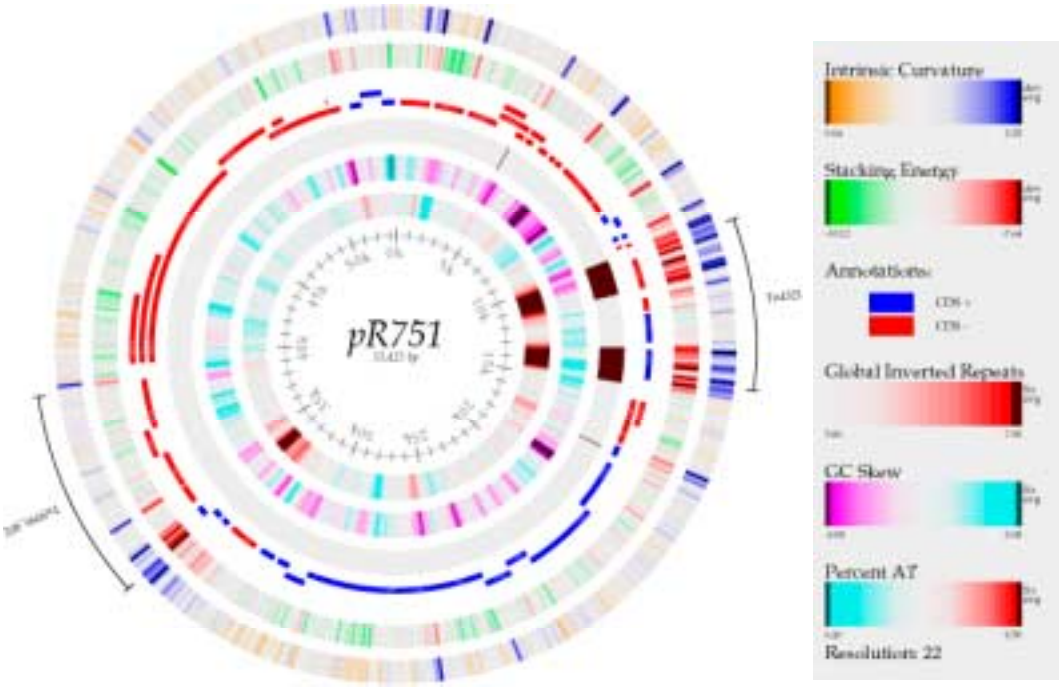

Figure 4. The IncP genome of R751 is consistently interrupted by transposable elements between *ori*V and *trf*A and between the *tra* and *trb* operons. From the atlas it is possible to see that there are inverted repeats clustered around these insertions, especially around the Tn*4321*, which may form the basis of a hot spot for insertion of foreign DNA. The percent of AT, the stacking energy and intrinsic curvature is as well higher in the flanking region of this transposons. This plasmid was generated by the "GeneWiz" programme (see text).

in many different hosts) and have been found not only in clinical contexts, but also in soil and aquatic environments. IncP-1 plasmids carry a variety of phenotypic markers including antibiotic resistance; toxic heavy metal ion resistance and ability to degrade xenobiotics (review Thomas et al., 1998). IncP-1 plasmids have been divided into two subgroups, incP-1α and IncP-1β, which appear to represent major evolutionary branches of the IncP-1 group (Smith and Thomas, 1989). This grouping was primary based on the patterns obtained by Southern blotting *Hae*II digests of 10 IncP plasmids with a probe carrying the *oriT* region of RP4 (Yakobson and Guiney, 1983). The IncP-1α plasmid RP4 is the best studied IncP-1 plasmid and it was totally sequenced in 1994 although, unfortunately, it was not annotated in GenBank (Pansegrau et al., 1994). In 1998 the IncP-1β plasmid R751 genome was also sequenced (fig 4), and compared to the sequence of the RP4 (Thorsted et al., 1998). The comparison between the two sequences confirmed the conservation of the IncP-1 backbone genes for replication, conjugative transfer and stable inheritance functions between the two branches of this family. The typical IncP-1 plasmid backbone is interrupted by 1 or 2 MGE´s (Smith and Thomas 1987). Today more IncP-1 plasmids have been sequenced (i.e. pADP1) and they share the same conservation of the IncP-1 backbone (fig 5).

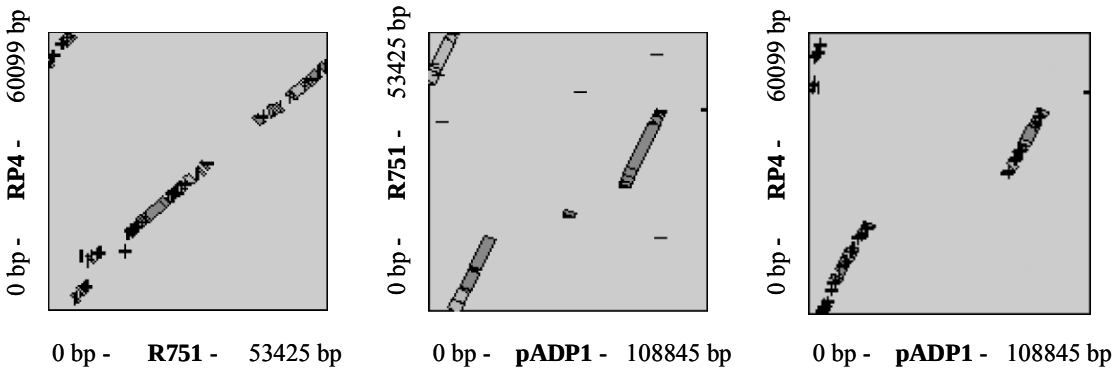

Figure 5. Similarity dot blot of total genome sequence of RP4 (IncP-1α), R751 (IncP-1β) and pADP1 (IncP-1β) was generated by the internet tool "Blast 2 sequences" which align any of the two given sequences using BLAST engine for local alignment. The backbone structure between the IncP-1 plasmids is much conserved and is only split up by 2 transposons. The transposons of these three plasmids did not show any homology. The plasmid backbone homology between R751 and pADP1 is higher compared to the homomology between the 2 IncP-1β plasmids and RP4. (Notice that the axes have relative values.) (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html)

3. Plasmid transfer

Conjugative plasmids are considered to be autotransmissible (or self-transmissible), provided that they contain a self-sufficient conjugative transfer system, and historically the conjugation has been studied most thoroughly through analysis of antibiotic resistance plasmids of Gram-negative bacteria. Conjugation systems were emphasized (on one hand) because of the prevalence and importance of antibiotic resistance plasmids, and on the other because of the ease in the manipulation of *E. coli* (Zechner et al., 2000). The few conjugative plasmids that have been studied in detail are F, RP4, R388 and Ti-pasmids (for reviews see Pansegrau and Lanka, (1996); Thomas et al. (1998); Zechner et al. (2000)). Since the RP4 is the most relevant plasmid for this thesis, its conjugation process is summarized in the next section. Due to the high genetic homology among the IncP-1 plasmids (see previous section) it must be assumed that the transfer of other plasmids from this group is very similar to the transfer of RP4.

Transfer of RP4 and establishment of the plasmid in the recipient cell.

The RP4 transfer system is incoded by two regions, Tra1(*tra*) and Tra2 (*trb*), which

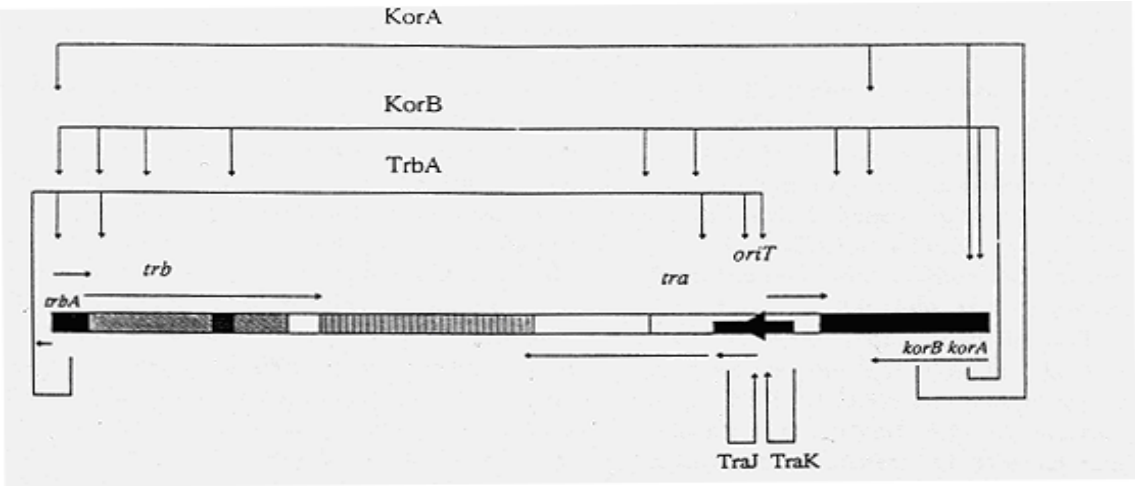

Figure 6. Control of IncP transfer genes. The major control is supplied by three global regulators. KorB and TrbA repress expression of transfer genes directly. KorA is needed to derepress *trbA* expression. Providing a way of shutting down transcription of the *tra* and *trb* genes once the plasmid is established. The assembly of the relaxosome is autogenously controlled by TraJ and TraK which bind to *oriT* and repress the promoters in this region. Regulatory genes, black; DNA processing genes, light grey; mating pair formation, diagonal hatching; white, non-essential or unknown function; vertical hatching, unrelated genes between the two regions. The black arrowhead at *oriT* indicates the direction of transfer. Horizontal arrows indicate proposed transcriptional units (Zatyka and Thomas, 1998)

are separated by transposons and genes coding for housekeeping functions. Tra1 genes encode for DNA transfer and replication, while genes located on Tra2 (*trb* genes) encode proteins involved in mating pair formation complex (Mpf). Tra2 also includes one transfer gene (*traF*) (Guiney, 1993).
IncP transfer genes are regulated both by local autoregulatory circuits and global regulators, which result in coordination of expression of transfer genes with other plasmids functions (Fig. 6) (Zatyka and Thomas 1998).

The *mpf* genes (10 genes of the Tra2 operon) are responsible for assembly, export and placement of the RP4 pili. Pili are rigid and inflexible and RP4 pili have been detected only when cells were grown on semi-solid surfaces, and it was shown that RP4 transfer capacity was several orders of magnitude higher on solid media than in liquid media (Bradley, 1980). A primary requirement for conjugation is close cell-to-cell contact and it has been speculated both that pili (or pilus subunits) further stabilize the mating aggregates and that they can trigger an initial unidentified signal whereby DNA transfer initiates.
The mating aggregates are conjugative junctions consisting of electron dense material between the cell envelopes of the donor and the recipient cell. In a study by Samuels et al. (2000) it was shown that the outer membranes were strictly parallel in the junction and that only 14 nm separated the centers of the bilayers from the outer membranes. The closest these bilayers were in a control system was 35 - 40 nm. The *mpf* genes are sufficient to form conjugative junctions and they can be formed in the absence of pili, although the pilus encoded by the TrbC gene was reported to be required for transfer to take place (Dürrenberger et al., 1991; Samuels et al., 2000 and Haase et al., 1995). Nonetheless, the regulation of the conjugation process is very complex (as mentioned previous) and the initial steps of RP4-mediated bacterial conjugation are still not wellunderstood (Anthony et al., 1999; Panicker and Minkley, 1985, Zechner et al, 2000).

The relaxases (TraI) are also important in the conjugation process. They recognize and bind to super coiled DNA at the origin of transfer (*oriT*) and cleavage at the *nic* site the strand to be transferred. To specify and stabilize this binding, additional proteins are required (TraJ and TraH), and the resulting DNA-protein complex is designated the relaxosome. The *OriT* region is characterized by having a high AT

content compared to the flanking DNA and direct and inverted repeats which results in the formation of extensive secondary structure. (Lanka and Wilkins, 1995; Fürste et al., 1989). Single stranded DNA is generated and transferred from the donor cell to the recipient cell in the 5' to 3' direction by rolling circle replication whereby the 3' –end of the nicked DNA is extended and simultaneously replicated with transfer (Fig. 7). DNA transfer terminates at the *oriT* –region. The TraG protein is believed to couple the nicked DNA processed by the relaxosome to the mating pair formation apparatus, in order to transfer it to the recipient cell (Cabezòn et al., 1994). Nevertheless, it has never been shown that the Mpf-complex directly interacts with the DNA (Zechner et al., 2000).

It has been shown that during the conjugation process a DNA primase (TraC) is transferred to the recipient cell to assist in the establishment of the plasmid (Wilkins and Lanka, 1993). Before the recipient cell becomes a potential new donor (carrying the RP4 plasmid) it is necessary for the transferred plasmid to overcome a number of "obstacles". Firstly, it is necessary for the transferred plasmid to suvive the presence of the restriction enzymes expressed by the host cell, which can potentially cleave the plasmid at functionally essential sites. Secondly, it is necessary for the new host to be able to replicate the transferred plasmid, to have a functional partitioning system, and to be able to express the RP4´s transfer proteins which enable the transfer cycle to be closed.

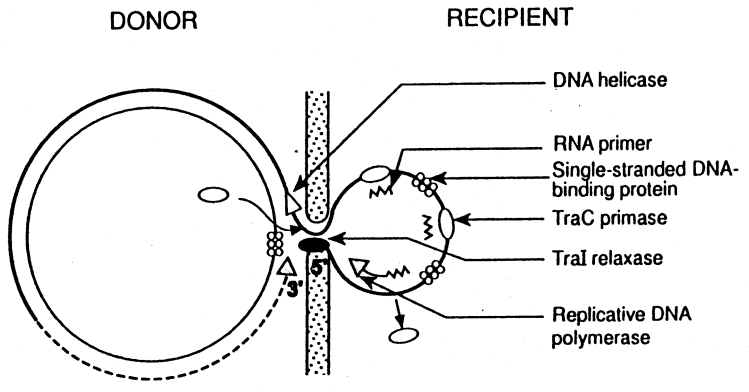

Figure. 7. Scheme of the processing of the RP4 plasmid during conjugation. A central feature of the general model of conjugation is that DNA transfer is coupled to the synthesis of a replacement strand in the donor cell (just like in the rolling circle mode of replication). Wilkins and Bates, 1998)

4. The host range of plasmids

Narrow and Broad host range plasmids

The host range of conjugative plasmids has been divided in narrow host range (NHR) plasmids and broad host range plasmids (BHR), the latter often called "promiscuous plasmids". The original definition of BHR plasmids was used to describe plasmids isolated in species belonging to the *Enterobacteriaceae* that, not only transferred to members of this family, but also to *Pseudomonas* species and *vice versa* (Datta and Hedges, 1972). Since then, many of the isolated plasmids from Gram negative bacteria have been shown to transfer and replicate among members of γ subdivisions of *Proteobacteria.* Consequently, a more stringent definition of BHR plasmids was proposed, which refers to those plasmids transferring and maintaining in bacteria from different phylogentic subgroups (Top et al., 1998).

The question, however is weather a definition differentiating between BHR and NHR plasmids is relevant. If it is relevant, it should be possible to find genetic traits that separate these two types of plasmids. The analysis of the difference between BHR and NHR plasmids showed that BHR plasmids have lower numbers of restriction sites than NHR plasmids, and that, due to this feature, BHR plasmids more easily overcome the restriction barrier of the host cells (Meyer et el., 1977; Guiney, 1984). It will (see plasmid atlas section) perhaps be possible to eventually track the "plasmids history" and predict from which organism the different genes or MGE originates or have passed through. In that way it will be possible to differentiate between NHR and BHR plasmids.

Since there are several examples of plasmid transfer to recipient strains where the plasmid cannot replicate, it is possible that the key difference between BHR plasmids and NHR plasmids is the replication systems (Krishnpillai, 1988). Although some plasmids control by themselves the frequency of initiation of their replication, most plasmids rely extensively on the host replication machinery (Espinosa et al., 2000). This implies an appropriate communication between plasmid and host-specific factors. The narrow host range plasmids of Gram-negative bacteria (ex *Col*E1, F, P1, R6K, pSC101 and R1) have solved this host/plasmid interplay with only a limited number of highly related hosts (Espinosa et al., 2000). The BHR plasmids such as the IncP-1 (i.e. RP4) and IncQ (i.e. RSF1010) incompatible groups have designed dif-

ferent strategies allowing them to be propagated in most Gram-negative hosts and at least in some Gram-positive bacteria. In Gram-positive bacteria, many rolling-circle- and some theta-type-replicating plasmids exhibit a BHR, being able to maintain themselves in several unrelated Gram-positive and negative bacteria (Espinosa et al, 2000). At this moment, there is no software program available to analyze (and link) a totally sequenced plasmid to its host range (BHR or NHR) and to analyze which possible phylogentic groups of recipients the plasmid can replicate in (and transfer to). The only way to achieve information about the possible host range of plasmids, starting from information on the total genome sequence, is to make alignments with already totally (or partly) sequenced plasmids which host range has already been experimental tested.

Plasmid host range

The strongest indication of natural occurrence of gene exchange in natural environments is the finding of the same type of BHR plasmids in bacteria from different genera (Sayler et al., 1990; Ka and Tiedje, 1994). Unfortunately, it can be technically difficult and time consuming to extract plasmids from many diverse strains. As an alternative to extraction, it is possible to use PCR-based detection methods on isolates` total DNA (Götz et al., 1996; Chapter 2). Nevertheless, the characterization of a plasmid is difficult by PCR (Smalla et al., 2000a) because it is necessary to use several primers (or to design multiplex PCR) in order to be sure that the chromosomal DNA of the tested strains does not give false positives (Chapter 2). An additional disadvantage to this technique is that, it is not possible to say anything about the conjugation frequency of the tested plasmid.

The usual way to test the plasmid for its host range is empirically in the laboratory. There are two different lines of approaches which have been used. The first line is the use of *in vivo* studies with optimal cell-to-cell contact between donor and pre-selected recipient strains carried out under controlled physical conditions (typical in filter mating experiments) (Chapter 2). The other line is the use of an indigenous population from an environmental sample. According to this, later method, a known donor strain is inoculated to a test system and subsequently the strains that have received the plasmids are selected (Chapter 3). For both approaches it is necessary to differentiate donor, recipient and transconjugant cells using selective markers (Introduction section

5). The technique is dependent on the culturability of the strains. This may be a major draw back in studies of plasmids isolated from natural environments, because only a minor part (0.01-1%) of the bacteria counted in the microscope is able to form colonies on agar plates (Torsvik et al., 1990 and Amann et al., 1995). To obtain a more realistic picture of the hostrange of plasmids, it will be necessary to develop methods that are independent from the culturability of the host bacteria. (introduction section 5).

There are two important distinctions in host range studies: replication and conjugation based studies. The first, qualitatively distinguishes different plasmids depending on their ability to replicate in the tested strain. The second quantitatively distinguishes different strains depending on how well, and under which conditions, the tested plasmid is transferred to the tested donor. The advantage of filter mating experiments compared to transfer studies to an indigenous bacterial population is in the use of high cell density and in the potentially optimal conjugation conditions. The disadvantage of filter matings is that only a limited amount of pre-selected strains can be tested (you can only find what you are looking for!).

Large differences in transfer efficiency were found between pKJK5::*gfp* and recipient strains from the same phylogentic group (chapter 2). This observation is in agreement with a study by Gordon, where he studied the rates of the conjugative R1 plasmid transfer between all combinations of 10 natural isolates of *E. coli* (Gordon, 1992). He found that no strain could be considered either a consistently good (or poor) donor or recipient strain(Gordon, 1992). Strain-specific effects of plasmid uptake are a topic which is not well examined. Stanisich and Ortiz (1976) suggested that a general explanation for strain-specific plasmid uptake can be similar to the response observed in *Pseudomonas aeruginosa* where prophage B3 inhibits IncP replication. An alternative explanation can be that many natural isolates already contain plasmids which can prevent (or reduce) further plasmid transfer, because of plasmid incompatibility.

Plasmid transfer to indigenous soil or rhizosphere bacteria has been performed with several broad host range plasmids i.e. RP4, pJP4, pEMT1k, pEMT3k TOL (Richaume et al., 1992; de Rore et al., 1994; DiGiovanni et al., 1996; Daane et al., 1996;; Top et al., 1998; Chapter 2; Chapter 3). Transconjugants strains, belonging to the α, β, γ subgroups of *Proteobacteria* have been isolated, and it was observed that

Pseudomonas spp. appeared to be a common recipient in all the studies examined. This reflects the importance of this group of bacteria in the tested systems (manuscript 3), and it provides valuable information about the nature of the tested plasmid. That it mostly is *Pseudomonas* related plasmids we find and work with should perhaps also warn plasmid researcher that a big group of plasmids from non culture-able bacteria can be overlooked.

The host range of IncP-1 plasmids

The IncP-1 plasmids (ex. RP4 and R751) are one of the most promiscuous groups of plasmids which are capable both of self transfer and of maintenance within most members of the Gram-negative genera tested so far (Smith and Thomas et al., 1989). *Bacteroides* was the only documented Gram-negative genus that is non permissive for IncP plasmid maintenance (Shoemarker et al., 1986). The IncP-1 plasmids are able to transfer to species in which they cannot replicate (Guiney and Yakobson, 1983). The IncP-1 plasmid R751 was able to mobilize DNA transfer between bacteria and *Saccharomyces cerevisae* (Heinemann and Spraque, 1989) but, unless modified to a shuttle vector, the IncP plasmids are not maintained in species other than *Proteobacteria*.

The IncP plasmid pHH502-1, lacking the *trfA* replication protein, had a reduced host range compared to its wild type (Smith and Thomas, 1987). This is an interesting observation because in Chapter 2 it was found that the *trfA* gene (in plasmid pKJK5) had a very different alignment when compared to the same gene in RP4 (IncP1-α) and R751 (IncP1-β). In Chapter 2, bacterial isolates from both the *Cytophaga-Flexibacter-Bacteroides* phylum (CFB) and one Gram-positive bacterial isolate were positive for both *korA* and *trfA* primers for IncP plasmids, which indicated that these strains contained an IncP plasmid. Nevertheless, from this study, it was not possible to conclude whether the plasmid genes are integrated in the host chromosomal DNA or not. Likewise the transfer of pKJK5::*gfp* (IncP) from *Pseudomonas putida* LM50 to CFB and Gram-positive phyla was observed. This does not again prove that the strains from CFB and Gram-positive phyla can replicate the plasmid (pKJK5::*gfp)* because Breton et al. (1985) reported transfer of another IncP plasmid (RP4) to *Myxococcus xanthus* (Gram positive bacterium) where the plasmid was unable to replicate autonomously but survived by integration into the bacterial chromosome.

5. Conjugation in environmental systems

Differentiating between donor and transconjugant bacteria.

In conjugation studies it is necessary to differentiate between donor, recipient and transconjugant bacteria. Spontaneous antibiotic resistant mutants from different strains are often used as recipients to counter select for the donor and, due to the frequent presence of selective markers on plasmids, selective plating has been used to monitor gene transfer in a broad variety of settings. Although the advantages of this technique stand in its simplicity and sensitivity (detection limit down to 1 cell pr. sample), investigations made on transfer studies to a recipient without a selective phenotype (i.e. or transfer to indigenous population) may be problematic (see also

Table 3. Comparison of different donor counter selection systems and zygotic induction of *gfp* used in plasmid transfer studies. All systems are base on that the plasmid carry a selective phenotypic trait. 1) Could be other fluorescent marker genes. 2) Depending on if the phenotypic trait of the plasmid provides culturabillity or not. 3) Depending on which killing system is used for the donor bacterium.

Systems for differentiation between donor bacteria and transconjugant bacteria in plasmid transfer studies	The assays detection limit of transconjugant cells	Genetic manipulation of donor strain	Transfer to indigenous population	Dependent on Culturability	On-line monitoring
Donor strain poorly adapted to test conditions	High	No	Yes	Yes/No[2]	No
Bacteriophage lysis of donor strain	Medium	No	Yes	Yes/No[2]	No
Auxotrophic mutants as donor strain	Medium	Yes/No	Yes	Yes	No
Donors removed from by immunomagnetic beads	Medium/ High	Yes/No	Yes	No	No
Donor with chromosomal suicide genes	Medium	Yes	Yes	Yes/No[3]	No
Specific antibiotic-resistant recipient	Low	No	No	Yes	No
zygotic induction of gfp[1] (repressor in donor)	Medium	Yes	Yes	No	Yes
zygotic induction of gfp[1] (activator in recipient)	Medium	No	No	No	Yes

Introduction section 4). For these reasons, different selection strategies have so far been made to differentiate between donor and transconjugant cells (table 3). Despite the fact that most of the counter selection techniques are dependent on plating, if the plasmid carries a selective marker which can be detected either by the use of

immunological techniques or by hybridization assays, counter selection techniques can become independent from plating.

In situ markers for gene transfer

The marker system designed by Christensen et al. (1996) allows transfer to be detected by zygotic induction of the *gfp* marker gene, in which expression of the GFP gene is not epressed in the donor strain. This technique removes the need for counter selection and allows transfer to be assessed in a mixed bacterial population. In 1998, Dahlberg et al. (1998a) modified this technique by using a a *LacZ* promoter fused to the *gfp* gene. Their genetic construct allowed the promoter to be repressed in the donor cell by the *LacI* repressor (which was inserted on the donor's chromosome) and allowed the expression of *gfp* in the different recipients strains tested (fig. 8). This approach has since been used for *in situ* transfer studies on biofilms, on activated sludge, on leaves, and on marine bacterial communities. (Christensen et al., 1998; Normander et al., 1998; Dahlberg et al., 1998b; Geisenberger et al., 1999; Hausner and Wuertz, 1999; Lilley and Bailey, 2002).

A zygotic marker system has been developed using the red fluorescent protein DsRed

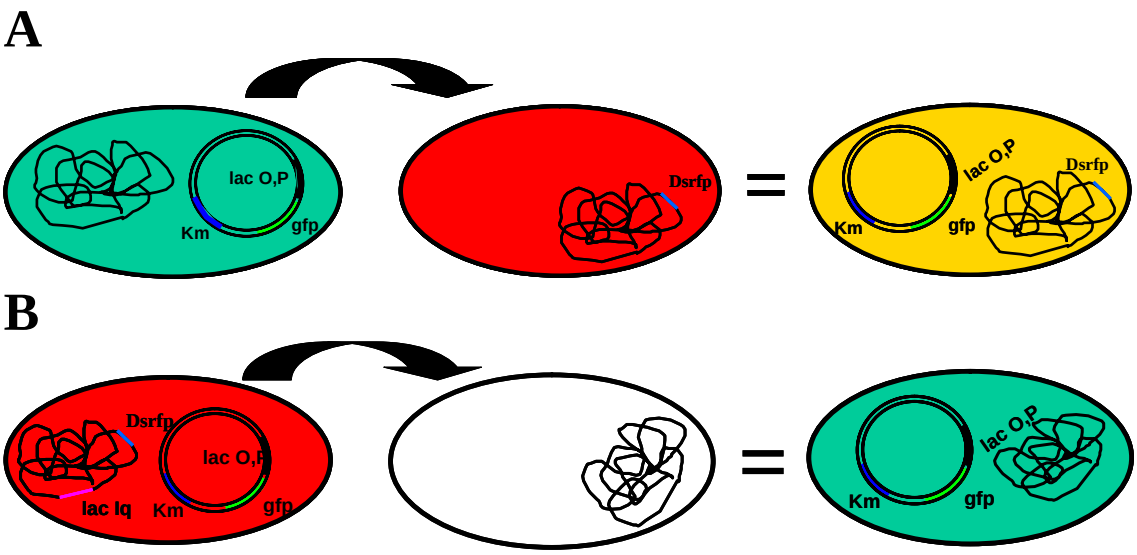

Figure 8. Two different marker systems to visualize gene transfer. In order to monitor transfer to recipient bacteria a specific donor strain with an inserted gene on the chromosome encoding the *LacI* repressor is used in combination with a conjugative plasmid harboring a fusion between the *lacP* promoter and the *gfp* gene. A) A known recipient bacterium is used which is tagged with Dsred on its chromosome, this system allows *in situ* visualization and differentiating between Donor, Recipient and Transconjugant bacteria. B) This system can be used for monitoring of plasmid transfer to the indigenous bacteria. The Donor is tagged the Dsred which allows *in situ* visualization and differentiating from transconjugant cells.

from *Discosoma* in combination with *gfp* (Matz et al., 1999; Haagensen et al., 2002; Chapter 2; Chapter 3; Chapter 4). The use of this protein makes it possible to differentiate donor, recipient and transconjugant cells by *in situ* microscopy and flow cytometry (fig. 8 and fig. 10). Recently, several advances to these systems have been made. Firstly, it is now possible to make *in situ* studies on gene transfer in complex microbial communities, and to simultaneously investigate the composition of a community, its physiological activity, structure and dispersal of plasmids (Haagensen et al., 2002). Secondly, it is possible to make on-line monitoring of plasmid transfer (fig. 9), and to follow donor migration and plasmid spread. Thirdly, it may be possible to see and quantify transfer to non culturable bacteria (fig. 10). In the future it will probably be possible to differentiate also between the actual conjugation transfer rate and the rate of growth of the transconjugant cells at the single cell level.

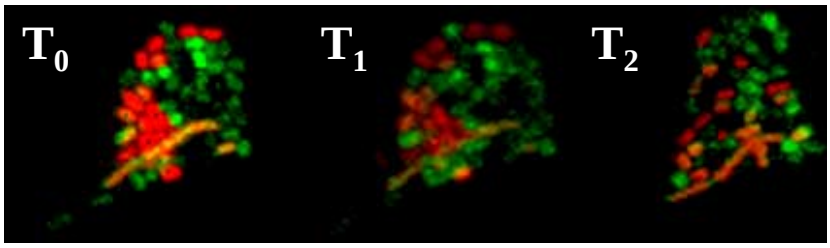

Figure 9. On line pictures of a micro-colony from the rhizosphere of barley. Donor bacteria (KT2442/pKJK5::*gfp*), Recipient bacteria (KT2440::*dsRed*) and transconjugant bacteria (KT2440::*dsRed*/pKJK5::*gfp*). The pictures are superimposed CSLM micrographs and donor cells are green, recipient cells are red and transconjugant cells are orange. T_0); Image obtained after 3 days of inoculation. T_1) Image obtained 6 hours after T_0. T_2) Image obtained 24 hours after T_0.

The limitation of using in situ marker systems for monitoring plasmid transfer is that it is very difficult to introduce the donor cells in natural ecosystems and it is therefore only possible to study the tagged bacteria in laboratory model systems. Another potential limitation is that the genetic cassette used to tag the plasmid can influence the plasmid genotype and plasmids functions. For example the two plasmids pKJK5::*gfp* and RP4::*gfp* used in chapter 2, were tested to see if the plasmids transfer efficiency (or plasmid stability) was altered by the presence of the marker genes. The strains harbouring the plasmids with the *gfp* tag did not show any difference in growth rate (in batch experiments) from the strains, without the *gfp* tag, grown under the same conditions. Furthermore, sequence analysis of the region upstream the *gfp* gene (for the RP4::*gfp*) showed that the insertion of the marker was close to the gene

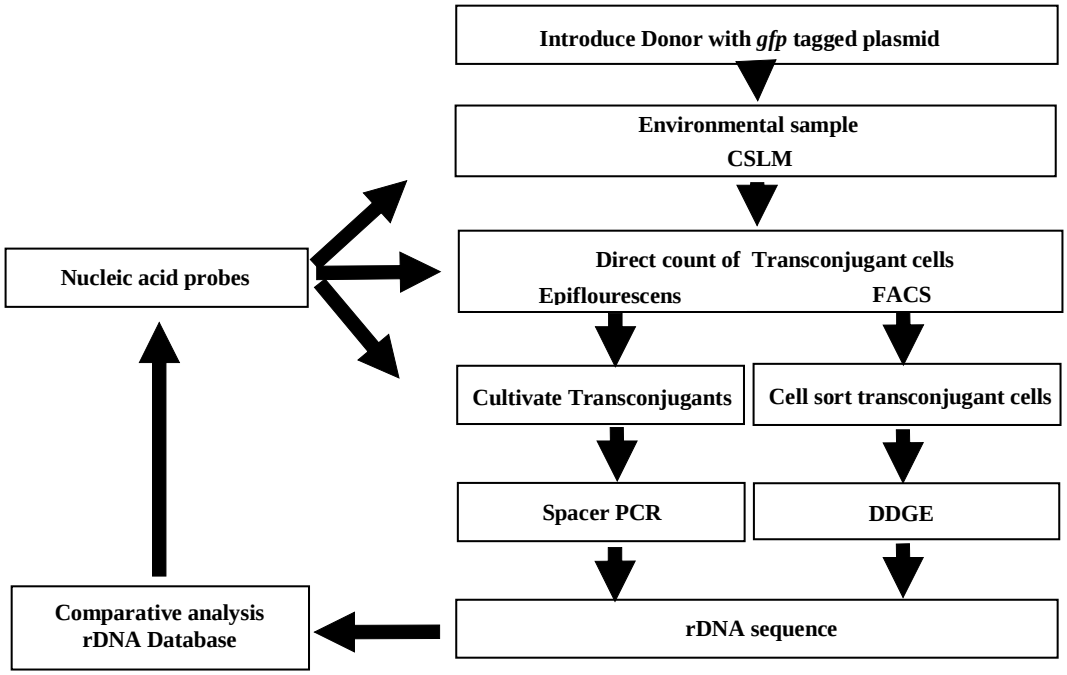

Figure 10. Diagram showing a complete investigation of plasmid transfer from an introduced donor with in situ markers. In this system it is possible to combine quantitative data on plasmid transfer with *in situ* methods for organism identification, in order to estimate the potential of plasmid transfer and map hotspots in the tested community. Part of this approach was used in Chapter 3. To be independent of cuturalble techniques it will be theoretically possible to cell sort green transconjugant cells and make a phylogenetic analysis of the microbial diversity. Such an analysis should always end with the control of the presence transconjugants cells i.e. by in situ hybridization (has been known as a full circle analysis (Amann 1995)). Epiflourescens = Epifourescens microscopy, CSLM = Confocal scaning lasser microscopy; FACS = Fluorescence activated cell sorter.

encoding for the transposase (unpublished data). When the same sequence analysis was carried out for pKJK5::*gfp,* no known homology was found to the sequence upstream of the *gfp* cassette (unpublished data). Despite these experiments, it is very difficult to evaluate if the behaviour of the tagged plasmids will be changed in its natural environment or if it will change in a different host.

The Rhizosphere environment

The soil is composed of particles of different sizes and shapes, surrounded by aqueous and gaseous phases. The amount and composition of these phases fluctuate markedly in time and space, so that a single soil aggregate can contain several micro-habitats (Killham, 1995). Although soil represents, in general, a nutritionally poor (or oligotrophic) environment, nutrients may suddenly become available locally from decaying material of plant or animal. The term rhizosphere was introduced in 1904 by Hiltner and is today used to describe plant roots and the environment closely

surrounding plant roots. Different compartments of the rhizosphere can be identified: endorhizosphere (inside root cells), ectorhizosphere (in close proximity to plants roots) and rhizosplane (at the plant surface) (Lugtenberg and de Weger, 1992). Apart from sloughing of cells and production of mucilage, roots also excrete a wide variety of soluble material and in an experiment a total amount of 3-15 % was excluded of the total dry weight of the root (Campbell and Greaves, 1990). Exudates are typically carbohydrate monomers, aminoacids, and organic aids (Sørensen, 1997). The rhizosphere environment is extremely heterogeneous and depending on plant species, the nutritional status of plants, and the condition of the rhizosphere of roots, its general composition can vary considerably (Marschner, 1992).

Conjugation in rhizosphere environments

High frequency of horizontal gene transfer are often observed in plasmid transfer studies and several field experiments have been performed to evaluate the environmental risks associated to this phenomenon (i.e. Lilley et al., 1994; Lilley and Bailey, 1997; van Elsas and Smit, 1997; van Elsas et al., 1998; Götz and Smalla, 1997; Smalla et al., 2000b). Field studies are often very demanding, both economical and time-wise. For this reason it is necessary to design less complex, and presumably more reproducible systems allowing clearer studies on how the variations of single biotic or abiotic factors affects conjugation among bacterial strains (van Elsas et al., 2000; Hill and Top, 1998). Plant microcosms (controlled environmental conditions) are small systems that contain a plant and where choices must be made depending on the amount of controlled information available. The choices can be substrates for supporting plant growth, sterile versus non-sterile systems, the addition of water and nutrients, the incubation conditions and the age of the plant (Ramos et al., 2001). Over-simplifying a system as complex and dynamic as soil, can give rise to results that are different from natural systems. Therefore, the use of simple microcosms must go hand in glove with the use of more complex, scaled-up soil microcosms, for the validation of the results obtained (Fig. 11) (Hill and Top, 1998).

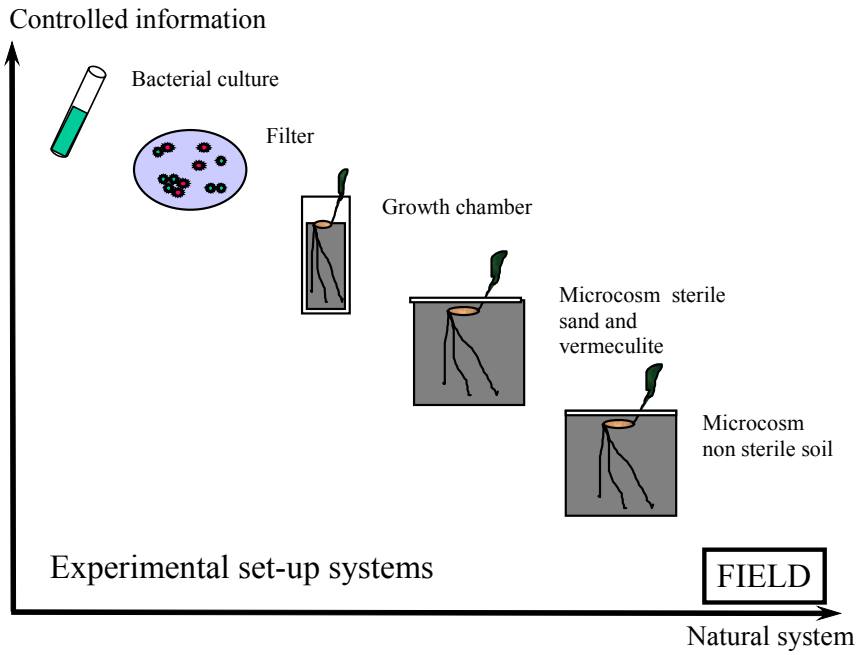

Figure 11. From test tube to field studies. Model illustrating the natural relevance of the system plotted against the amount of controlled information it is possible to obtain.

Many different kinds of soil-plant microcosms have been used to elucidate the effect of key ecological factors affecting plasmid transfer process in these environments (see table 4 and reviews about microcosms see Hill and Top, 1998 and van Elsas et al., 2000). In this thesis different experimental systems were used to study conjugative gene transfer (filter-matings, rhizosphere chambers, sprout-model system and rhizosphere-microcosms with vermiculite or non-sterile soil). The sprout-model system described in Chapter 3 and appendix I is one of the simplest plant systems so far used. With this method, non-sterile seeds of alfalfa were sprouted in a plastic box where only tap water was added. It was shown that in addition to its technical simplicity, the system was surprising reproducible (when the same batch of seed was used) and it provided valuable information about gene transfer. This study showed that there were hotspots of conjugation on the roots which were probably due to cell densities and moisture content.

The use of a rhizosphere chamber filled with sterile vermiculite was also a useful system for non-destructive and on-line microscopic examination. Non-destructive observation of plants is important because the preparation methods for microscopy

may have serious effects on the microbial structures to be examined. Vermiculite is an ideal substrate for fluorescent microscopy because it does not fluoresce.

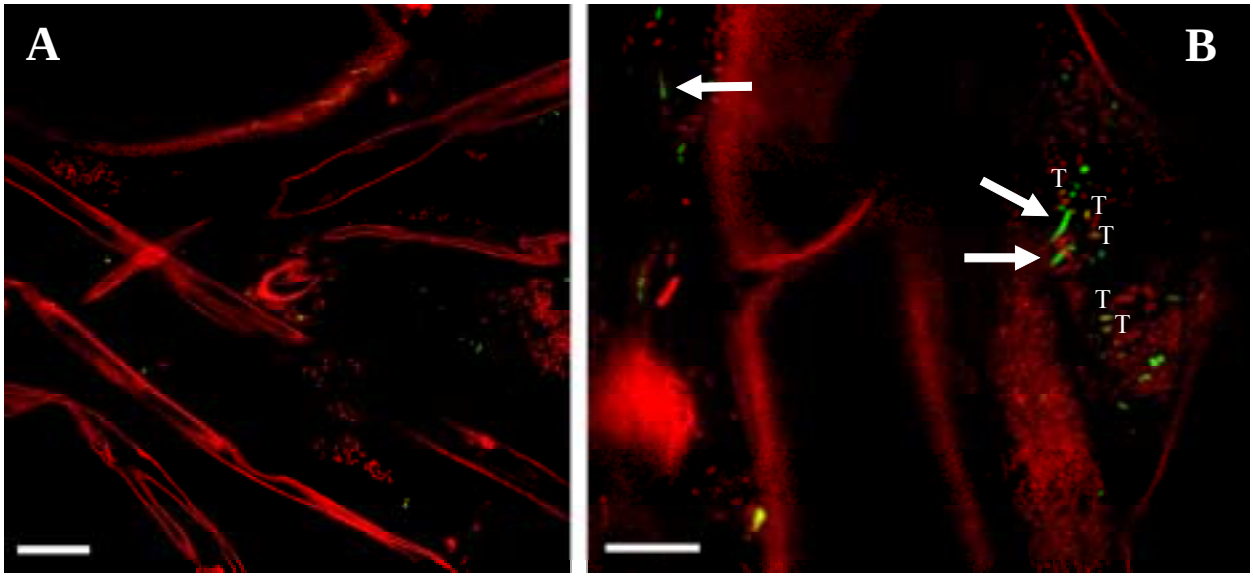

Figure 12. Two CSLM micrographs illustrating hotspots of donor (green) and recipient (red) cells colonizing barley's grown in rhizosphere champers for 3 days (root-hair and root cells fluoresces red). **A)** This picture illustrates the big difference in surface ratio of plant roots compared to bacterial size. In this picture both donor and recipient bacteria are present but there is no cell-to-cell contact. **B)** A local hot spot of donor recipient and transconjugant (T) cells. It is from this picture possible to see motile donor bacteria with a green tail of fluorescent light (see arrows) which can indicate that motility can play a local role in conjugation. Scalebar 20 µm.

The gap between sterile vermiculite and non-sterile soil is large. Although auto-claving soil has been shown to cause loss of soil structure (Cresswell and Wellington, 1992), several gene transfer studies using sterile soil having been successfully used to evaluate the influence of competition and predation (Richaume et al., 1989; Top et al., 1990).

Since the efficiency of bacterial conjugation in natural settings depends both on the plasmid and on the donor and recipient cells involved, it may be influenced by numerous biotic (i.e. grazing, predatory, antagonistic, competing or syntrophic organisms and plants) and abiotic parameters (i.e. temperature, pH, moisture content, micro and macronutrient avalilability, presence of sufaces, O_2) (review see van Elsas et al., 2000; van Elsas and Bailey, 2002; Hill and Top, 1998; Dröge et al, 1999). Since the rhizosphere was reported to be a hotspot for conjugation when compared to non-rhizosphere soil, several microcosm studies have been performed since the 1980´s in order to elucidate which factors are important for the conjugative process in

plant environments (table 4). In these studies, it has typically been the same type of strains and plasmids which have been used, as it can be seen from table 4. The results of some of these studies suggested that enhanced nutrient input and water fluxes in the rhizosphere stimulate conjugative plasmid transfers between *Psedomonads* and other organisms (van Elsas et al., 1988; Smit et al., 1991; Lafuente et al., 1996; Kroer et al., 1998) presumably by promoting cellular activities and cell-to cell contacts. The water content along the root, which can vary considerable, is a key factor which influences the availability and distribution of nutrients, oxygen, etc. and it affects the physiological status of bacterial cells (Dröge et al., 1999). In addition, the moisture content on plant roots affects the active and passive movement of bacteria, thus indirectly influencing the probability of mating partner formation and the stability of mating-pair formation (fig. 12).

The influence of the physiological status of donor and recipient cells on conjugative gene exchange has been addressed in different studies (for review Dröge et al., 1999). Whilst in some systems the increase in the metabolic activity was related to the increase in plasmid-transfer frequencies, no such correlation was found in other systems. Hausner and Wuertz carried out a biofilm experiment where *Alcaligenes eutrophus* was used as recipient for mobilization of a small Gfp-tagged RP4 plasmid deriving from *E. coli*, and it was found that direct visualization of transconjugant cells indicate much higher transfer rates than those obtained by plate counts. It was also found that there was no direct correlation between transfer efficiency and the concentration of nutrients in the medium (Hausner and Wuertz, 1999). Different results were obtained in a biofilm experiment by Haagensen and co-workers, where KT2442 lacI^{q} carrying the TOL::$P_{A1\text{-}04/03}$::*rfp* was used as donor strain and KT2442, which carries a chromosomal insertion of a *rrnB*P1-gfp[AGA] fusion expressing unstable Gfp which only will be expressed in actively growing cells was used as recipient strain (Haagensen et al., 2002). The result from this study showed that transconjugant cells were entirely associated with the growing cells and the authors hypothesized that efficient plasmid transfer mainly occurs with growing cells as recipients. The different plasmids used in these studies can explain the different observations because it was shown that the transfer of TOL plasmid is more affected by starvation of the donor cells than the RP4 plasmid (Lambertsen et al., 2001). An alternative explanation for why only the outer layer of a biofilm receives the plasmid

can be that the cells in these biofilm micro-colonies were fixed by extra-cellular polymeric substances, which inhibited the direct cell-cell contacts required for plasmid transfer (Molin personal communication, 2003). In this study (Chapter 4) we found that the use of an increased amount of exudate from the pea rhizosphere and pea spermosphere compared to the amount used with barley increased the total amount of cell-to-cell contact and thereby enhanced the numbers of T/D.

Image analysis

There has been a lot of debate and mathematical modeling about how plasmids are maintained in bacterial population, because plasmids persistence requires one or both of two basic mechanisms: infectious transmission (maintenance as genetic parasites) and selection on hosts for the genes that the plasmids carry (Bergstrom et al., 2000; Stewart and Lewin, 1977; Simonsen, 1991). Although it is not in the scope of this introduction to consider the evolutionary maintenance of plasmids, it is important to note that the application of Gfp (facilitating the *in situ* tracking of plasmid transfer to recipient cells) and the analysis of plasmid transfer at the single cell level, is a powerful tool for the experimental test of some of these models. The use of image analysis programs as a tool for analyzing fluorescence data from bacteria either labeled with fluorophore-tagged oligonucleotide probes or tagged with Gfp have already been shown (Møller et al., 1995; Bloem et al., 1995, Ramos et al., 2000). The program COMSTAT written as a script in MATLAB 5.1 has already been used to analyze biofilm structures (Heydorn et al., 2000). In this thesis a program CONJU (appendix II) written as a script in MATLAB 5.1 can be used to analyze a single cell layer of donor, recipient and transconjugant cells marked with two different reporter genes. The main requirement for using the program is 2 pictures of high resolution that displays bacteria at a single cell level. The aim of the program is to separate the factors influencing the numbers of cell-to-cell contacts from factors influencing the transfer kinetics once cell-to-cell contact has occurred. The program can also be used to analyze time sequenced pictures by detecting which parameters are changing in time. Figure 13, describes shortly the flow in the program, however in appendix 2 a link to the program and a further descriptions of the program features can be found. Unfortunately, time constraints have permitted any experiments using the program to be performed as yet. Nevertheless, CSLM-micrographs were taken in order to control and optimize the program.

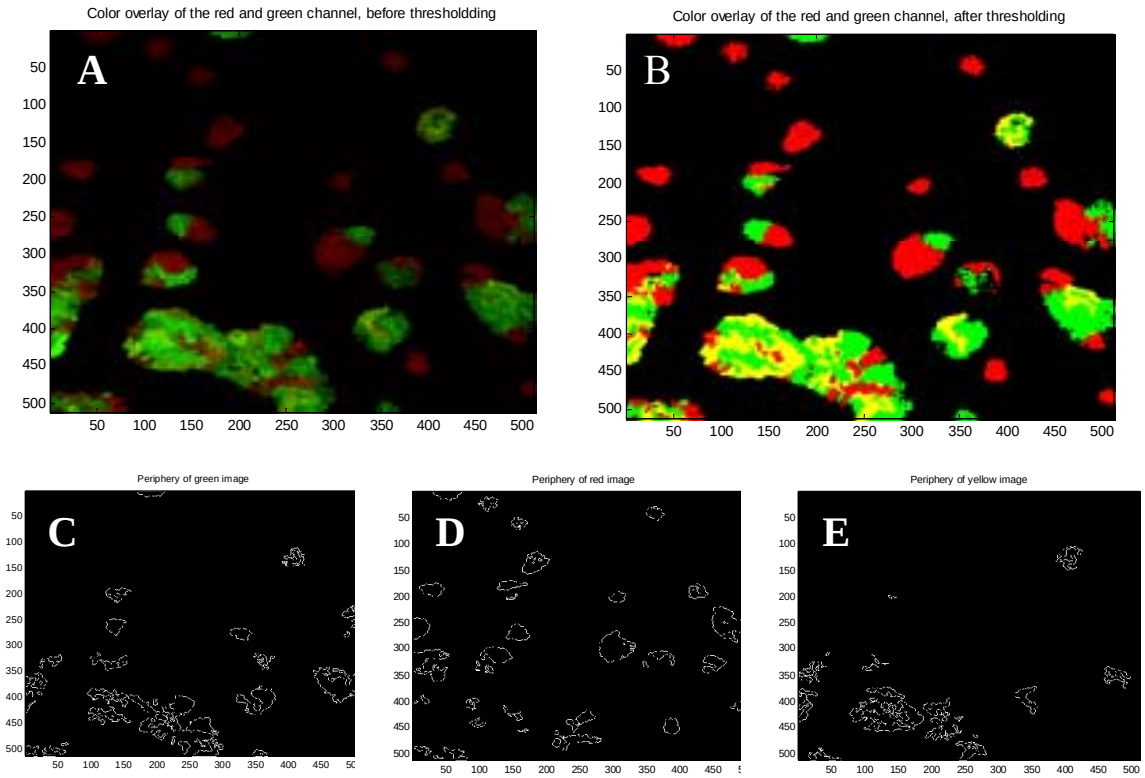

Figure 13. **A)** The image analyze program Conju (appendix 2) superimposed 2 CSLM micrograph of single layered micro-colonies composed of a donor strain (*P. putida* KT2442/RP4::*gfp*), and a recipient strain (*P. putida* KT2440::*dsRed)* incubated on membrane filters placed on PBS buffer for 24 hours. Donor cells are green, recipient cells are red and transconjugant cells are yellowish (mix between red and green colour). Picture A was threshold in **picture B** and transformed into three new images of donor, recipient and transconjugant cells respectively. These three new pictures were used for all the calculation the program performed such as measuring i.e. total amount of cells, total amounts of micro-colonies, sizes of micro-colonies and average size of micro-colonies. The next step in the program is to calculate the peripheries of donor **(C)**, recipient **(D)** and transconjugant **(E)** micro-colonies respectively. The program measured then the shortest distance from each single pixel in the peripheries of C (donor) to the closest peripheries of D (recipient) and the shortest distance from each single pixel in the peripheries of E (transconjugant) to D (recipient). The idea was that with these parameters it will be possible to find the "operational distance" a donor cell needs on a membrane filter for conjugation can take place to a recipient cell. If it is possible to estimate such a distance it can be used in future experiments to measure the total potential for that conjugation can take place (the total contact length between potential donor and recipient microcolonies). The program can as well be used for on line studies and as a control that the parameters between different setups of strains and plasmids are the same (see text for further details).

Table 4a. Plasmids transfer in plant environments

Year	Plasmid type and Inc-group	Environment	Plant	Non sterile Soil (+ yes; - no)	Donor	Recipient	Transfer to Indigenous population (+ yes; - no)	Reference
1988	RP4 (IncP1)	Soil Rhizosphere	Wheat	+	*P. fluorescens* R2f	*P. fluorescens* R2f		Van Elsas et al., (1988)
1990	RP4 (IncP1)	Soil Rhizosphere	Wheat	+	*P. fluorescens* R2f	*P. fluorescens* R2f		Van Elsas et al., (1990)
1991	pJB5JI	Bulk soil Rhizosphere	Pea	+	*Sinorhizobium fredii* USDA201	*Rhizobium Leguminosarum* 6015		Kinkle and Schimidt., (1991)
1991	RP4::*pat* (IncP1)	Bulk soil Rhizosphere	Wheat	+	*P. fluorescens* R2f		+	Smit et al., (1991)
1992	RP4::*pat* (IncP1)	Bulk soil Rhizosphere	Wheat	+	*P. fluorescens* R2f	*P.fluorescens* R2fN	+	Richaume et al., (1992)
1993	R68.45 (IncP)	Soil (nutrients + mercury) Rhizosphere	Soybean	+/-	*B. japoicum* USDA 123	*Bradyrhizobium* spp.		Kinkle et al., (1993)
1994	pQBR11	Soil Rhizosphere	Sugar beet	+	*P. marginalis* 376N	*P. aureofaciens* 381R		Lilley et al., (1994)
1995	R388::Tn1721 (IncW)	Soil Spermosphere Rhizosphere	Pea Pea	+/-	*P. cepacia* pCO12	*P. fluorescens* 2-79		Sudarshana and Knudsen, (1995)
1995	RP4 (IncP1)	Phyllosphere	Bush bean	+	*P.syringae* Cit7	*P. Syringae* Cit7	+	Björklöf et al., (1995)
1996	pTH16 (IncP) pIE1037 (IncN) pIE1056 (IncW) pIE639 (Inc Q)	Bulk soil (+nutrients) Rhizosphere	Grass	+	*E. coli* K12	*E. coli* CV601		Pukall et al., (1996)
1996	pQBR103	Rhizosplane Phyllosphere	Sugarbeet	+	*Indigenous bacteria*	*P. putida*		Lilley et al., (1996)

Table 4b. Plasmids transfer in plant environments

Year	Plasmid type and Inc-group	Environment	Plant	Non sterile Soil (+ yes; - no)	Donor	Recipient	Transfer to Indigenous population (+ yes; - no)	Reference
1998	RP4::*nap* (IncP1)	Sand Rhizosphere	Water-grass	-	*P. fluorescens* AS12	*Serratia sp. RF7*		Kroer et al., (1998)
1998	RP4 (IncP1)	Rhizosphere Spermosphere	Barley Barley	+	*P. putida* sp127 *P. fluorescens* As12	*P. putida* UWC1	+	Sørensen and Jensen., (1998)
1998	TOL::*gfp* (IncP9)	Phyllosphere	Bush Bean	+/-	*P. putida* KT2440::lacI^{q}	*P. putida* KT2440		Normander et al., (1998)
2000	TOL::*gfp* (IncP9)	Bulk soil Sand Residuesphere	Barley straw leaves	+/-	*P. putida* KT2440::*lacI*^{q}	*P. putida* KT2440		Sengeløv et al., (2000)
2000	TOL::Km (IncP9)	Rhizosphere	pine	+	*P. fluorescens* OS81		+	Sarand et al. (2000)
2001	RP4 (Incp1)	Rhizosphere	Pea Barley wheat		*P. fluorescens* AS12	*Serratia plymuthica* RF7		Schwaner and Kroer, (2001)
2003	pJp4	Rhizosphere	Arabidopsis thaliana	+/-	*P. chlororaphis* SPR044	Ralstonia eutropha		Schmidt-Eisenlohr and Baron (2003)

6. Epilog

For understanding the general evolutionary ecology of bacteria, it is important to study the mobility of plasmidic genes and MGE. The evolution of antibiotic resistance has shown that, under a strong selective pressure, evolutionary time can be short (Mazel and Davies, 1999). Studies of *Rhizobium etli* have shown that within a few generations, frequency of isolates with identifiable gene rearrangements reached a frequency of 3 % of the population (Flores et al., 1998). The sequencing of complete bacterial genomes has clearly shown that a large proportion of bacterial genes have been acquired by horizontal gene transfer (Smalla and Sobecky, 2002). In many species a high proportion of horizontally transferred genes can be attributed to plasmid, phage or transposons-related sequences because remnants of MGE are often found adjacent to genes identified as horizontally transferred (Ochman et al., 2002). Data from *H. pylori* has shown that considerable changes in genome architecture occurred naturally at the intra-strain level with the participation of repeated elements (Romero et al., 1999).

Host range studies will, in the future, aim at understanding the importance of the role that conjugative plasmids have as vehicle for MGE dissemination between the plasmid and the bacterial genome (and *vice versa*) and to understand under what conditions MGE become established (and/or maintained) in bacterial populations.

In Chapter 2 the potential phylogentic broadness of pKJK5::*gfp*, was shown and it was also highlighted that there can be considerable differences in conjugation efficiency at the intra-species level between natural isolates from the barley rhizosphere. In future work, it will be interesting to assess the factors (genomic or physiologically) that are responsible for this difference in plasmid transfer at the intra-specie or intra-clonal level.

There is only little information about structure / function relationships in relation to plasmid transfer in plant environments. Many studies have been done on the basis of CFU counts from the entire plant without the possibility to explain if a high transfer frequency was because of many cell-to-cell contacts or a higher or more efficient transfer rate. With the new technical approach to differentiate between donor, recipient and transconjugant bacteria at the single cell level on the root, developed and used in this study, it will be possible to start answering these questions.

The sequencing of whole bacterial genomes is revolutionizing the way to do modern biology. In Chapter 1 a plasmid genome database was made in cooperation between CEH in Oxford and CBS at DTU in Copenhagen. It is already the plan that this database can be integrated or combined with databases of transposons and whole genome sequences of bacterial species. This will give an enormous resource and it will be of high importance that a trinity is established between researcher in molecular biology, microbial ecology and bioinformatics to get the maximum output.

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Chapter 1.

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The Plasmid Genome Database

The Plasmid Genome Database (PGD) is a regularly updated collection of all fully sequenced plasmids (approaching 500 as of May 2003) with links to structural maps of each plasmid (http://www.genomics.ceh.ac.uk/plasmiddb/). The amount of whole genome and whole plasmid sequence data has been growing exponentially (see Fig. 1), generating enormous amounts of data that, if the information can be arranged in a comprehensive and structural way, represent a major resource for many researchers. To our knowledge, this is the first database that has collated all fully sequenced plasmids, including core features, their genetic composition and structural maps (Wackett, 2002).

By definition, plasmids are non-essential extra-chromosomal fragments of DNA that replicate with different degrees of autonomy from the hosts' replicative proteins. Plasmids are present in nearly all bacterial species (Amabile-Cuevas & Chicurel, 1992), range in size from a few to more than 1000 kbp and, as such, can represent a large proportion of the whole bacterial genome. In nature, plasmids appear to increase bacterial genetic diversity and to promote bacterial adaptation by horizontal gene spread (Bergstrom *et al.*, 2000; Gogarten *et al.*, 2002; Levin & Bergstrom, 2000).

The first plasmids were isolated and characterized in the 1950s and were associated with newly acquired antibiotic resistances. Plasmids have since been studied intensively for both their genetic and phenotypic properties, including antibiotic and toxic heavy metal resistance, degradation of xenobiotic compounds, symbiotic and virulence determinants, bacteriocin production, resistance to radiation and increased mutation frequency. These so-called 'accessory functions' (Levin & Bergstrom, 2000), which facilitate rapid adaptation to new or transient environmental selection pressures, are typically located on mobile genetic elements (MGEs) such as genomic islands, conjugative transposons, mobilizable transposons as well as plasmids. Evidence from bacterial sequencing projects clearly indicates that bacteria adapt and genomes evolve by rearranging existing DNA and by acquiring new sequences (Gogarten *et al.*, 2002; Levin & Bergstrom, 2000). Thus, MGEs have contributed to the evolution of bacteria.

Due to their physical separation from the chromosome, plasmids constitute a substantial and easily identifiable component of this accessory gene pool, but one that was not represented comprehensively in any database. The PGD contains all the plasmid genomes listed in the Entrez Genome pages of the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome) under Archaea/Plasmids ($n=18$), Bacteria/Plasmids ($n=305$), Eukaryotes/Plasmids ($n=13$) and Plasmids ($n=36$ on 2 May 2003). In addition, manual searches identified additional sequenced plasmids elsewhere in GenBank ($n=88$). At the time of writing, the database contains 460 plasmids, including ones of eukaryotic and mitochondrial origin, and meta-data from the informational categories included in NCBI submissions. These include plasmid name, host, NCBI genome number, accession number, genome size (bp), chromosome type (circular or linear) and date of submission/last update (when defined). Those genomes not included in the Refseq collection are listed by accession number only. Other features of the database include the ability to sort all data in the PGD by category and search locally held plasmid genomes using standard BLAST (Altschul *et al.*, 1990) tools.

One of the intriguing areas of biology that is being highlighted by sequencing large numbers of bacterial genomes is the blurred view of 'plasmids', mega-plasmids and secondary chromosomes. Comparative genomics is raising questions about how to differentiate between secondary chromosomes (apparently of plasmid origins in, for example, vibrio and rhizobium species) and mega-plasmids. Fig. 1 might suggest that there is a size cut-off between most bacterial plasmids and chromosomes at about 1×10^6 bp. This could be a consequence of a lack of basic knowledge; for example, whether a large replicon is truly a secondary chromosome or a mega-plasmid or *vice versa*, an artefact of sampling bias (of sequenced genomes) or simply a consequence of how we define what constitutes a plasmid or a chromosome. Certainly, the sequestration of plasmid

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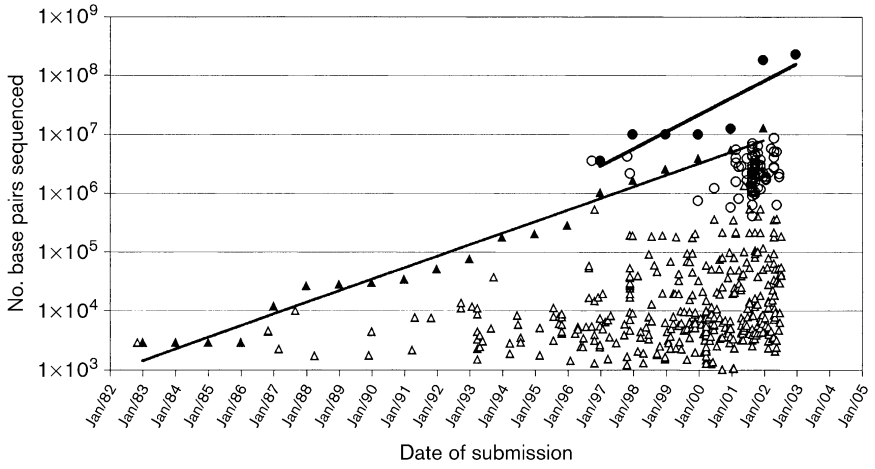

Fig. 1. Graph showing the size of all sequenced plasmids in the Plasmid Genome Database (△) and all sequenced chromosomes of bacteria and archaea from the NCBI web site (as of 6 January 2003) (○) according to date of submission. Additionally, the figure shows trend lines of the total number of base pairs for the sequenced plasmids (▲) and chromosomes (●).

genes to fulfil the role of secondary chromosomes has important general evolutionary implications.

Once all plasmid genomes have been collected into a single resource, analyses can be more easily applied across the entire data set. The PGD currently contains links to graphic structural maps for each plasmid in the database. These plots are constructed directly from the NCBI genome files by the Center for Biological Sequence Analysis (CBS) server in Denmark (http://www.cbs.dtu.dk/services/GenomeAtlas) (Pedersen *et al.*, 2000; Jensen *et al.*, 1999). The structural plasmid atlases provide an overview of plasmid structure, including features such as base composition, DNA flexibility, GC-skew, palindrome distributions, the presence of local and global repeats of various types, and gene content (when annotated). These plots highlight the mosaic structure of many plasmids, especially the larger ones. They clearly show that 'backbone' functions, responsible for self-maintenance, for example, genes encoding replication, copy number control, multimer resolution, partitioning, post-segregation killing and horizontal transfer, have similar physical characteristics. By contrast, adaptive genes, probably acquired relatively recently as a consequence of recent environmental selection, can be associated with blocks of DNA with distinct composition. These blocks often include gene cassettes including ones carried on smaller MGEs (transposons and IS elements) nested between the 'backbone' operons. The observation that recent horizontal gene acquisition gives rise to (or is associated with) atypical nucleotide signatures, relative to the rest of the genome, first proposed by Lawrence & Ochman (1997), has been highlighted in numerous genome sequencing projects since. That this phenomenon is observable among, at least, the larger plasmid replicons has clear implications for plasmid biology. In the context of bacterial adaptation, it perhaps indicates a hierarchy of horizontal gene spread. For example, self-mobilizing plasmids may act more as accidental mediators of intra- and inter-species spread of hitch-hiking adaptive traits associated with the smaller MGEs which are otherwise 'locked' within a host cell/clonal population. This contrasts with the concept of plasmids as drivers of adaptation *per se*, or with them existing as parasites within their host (for discussion, see Bergstrom *et al.*, 2000). Systematic interrogation of the PGD's comprehensive collection of plasmid genomes and structures should reveal patterns that improve our understanding of the roles that different types of plasmid contribute to the biology of their hosts in addition to plasmid biology.

A final point to make about plasmids is to emphasize their biological diversity and the resulting fact that plasmids currently lack a naming convention with real biological meaning. A number of sequenced plasmids lack any name. Plasmids do not share a single phylogenetic history and therefore can not be assigned a classic taxonomy, but they can move through bacterial populations in an independent manner acquiring and losing genes over time. The continued development of the PGD, including the collection of a large amount of meta-data describing each plasmid, should allow the selection and analysis of plasmids based on their phenotypic and genomic characteristics. Therefore, the PGD should improve the effective interrogation of these diverse but important genomic components.

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JWS Online Cellular Systems Modelling and *Microbiology*

As of July 2003, *Microbiology* has established a collaboration with JWS Online. Here, Jacky Snoep explains the significance of this

Introduction Rapid developments in the relatively new disciplines of 'bioinformatics', 'computational biology' and 'systems biology' have led to a marked increase in the use of kinetic models in the study of complex biological systems [for example, see 'Nature insight: Computational biology' (2002) *Nature* **420**, 205–251]. When reading publications in these 'new' fields, it is easy to overlook the fact that there exists a long-standing tradition of using kinetic models in biology. In the 1960s, pioneers such as Chance, Garfinkel, Higgins and Hess (e.g. Chance *et al.*, 1960) had already begun using kinetic models to explore biochemical systems. Since that time, running computer simulations has become easier. Faster personal computers and the development of dedicated simulation software have removed many of the numerical and computational obstacles to building and running kinetic models. Nevertheless, the construction of kinetic models, especially of detailed 'silicon cell' type models (http://www.siliconcell.net/) (e.g. Bakker *et al.*, 1997; Mulquiney & Kuchel, 1999; Teusink *et al.*, 2000; Hoefnagel *et al.*, 2002), can still be a tedious and time-consuming process. Considering the hard work involved in building such detailed kinetic models, it is rather surprising that so little attention is paid to presentation and conservation of existing kinetic models. Thus, no official repository of kinetic models currently exists and no standard method of presentation of kinetic models in scientific literature has been agreed upon. A number of initiatives have been started to collect kinetic models, such as the CellML (http://www.cellml.org) and SBML (http://www.sbw-sbml.org/) databases which have similar, but not identical, goals. Both projects use XML-based exchange formats. While CellML strives to describe the structure and underlying mathematics of cellular models in a very general way, SBML aims to be a generic platform for exchanging pathway and model reaction information between several existing applications. SBML compatibility is already integrated into several metabolic modelling packages, for example, SCAMP (Sauro, 1993), GEPASI (Mendes, 1997) and JARNAC (Sauro, 2000). However, neither of these databases is complete yet and the chances of finding 'the interesting model' that you have just read about in the literature are not necessarily good. Although a published model description should be sufficient for one to build the kinetic model, this could still be a daunting task since many model descriptions contain errors, are not complete or, due to a lack of a model description standard, are vague.

Aim of JWS Online In December 2000, we started building our JWS Online Cellular System Modelling (Snoep & Olivier, 2002) site with the aim of providing: (1) a user-friendly, internet-based, application for running kinetic models of biological systems; (2) a repository of such models; (3) a facility to make the reviewing of papers containing kinetic models easier.

How does it work? Currently, JWS Online has 22 models that can be interrogated via the internet using any browser that is capable of running JAVA2 applets (i.e. any modern web browsers that support the SUN Microsystems J2RE plug-in) such as INTERNET EXPLORER 5 under Windows 98, 2000, XP, SAFARI under Mac OS X, and MOZILLA under Linux. The application software is implemented in the JAVA™ programming language using a client server model. This set-up makes it possible to run relatively large models on clients with a minimal hardware specification. The JWS client is a JAVA™ applet and runs in any web browser that supports JAVA 1.4 (JAVA2) and above. The client provides a graphical interface for the user, establishing communication links with the server and displaying the results of the calculation. Users have control over various model parameters (typically kinetic parameters, time and integration steps) and may select either a steady-state calculation, time-course simulation, Metabolic Control Analysis or Structural Analysis of the model. The JWS server runs as a stand-alone JAVA™ program which uses J/LINK as an interface to facilitate all communication with MATHEMATICA by Wolfram Research (http://www.wolfram.com). All numerical calculations are performed using the server-side MATHEMATICA Kernel. The models are coded in J/LINK and dynamically linked into the server as modules allowing multiple, simultaneous modelling sessions.

Try it yourself The easiest way to get to know the system is to direct your browser to either of the JWS Online Cellular Systems Modelling mirror sites (http://jjj.biochem.sun.ac.za or http://www.jjj.bio.vu.nl). Make sure you have the J2RE plug-in installed, freely available for download from SUN Microsystems (http://java.sun.com/). After selecting 'database' on the home page, a selection of kinetic models is shown (Fig. 1), each of which can be selected by clicking on the model link. On doing so, an applet, functioning as a graphical interface, is downloaded (Fig. 2). The display is divided into two horizontal panels and one lower panel. The horizontal right-hand panel shows the pathway scheme of the system being modelled. If you place the cursor over any of the reaction steps (red dots) in this panel, the rate equation corresponding to that catalytic step is displayed in the bottom panel. The left-hand horizontal panel contains the applet. The applet has two tables where users can change any of the model's parameter values or select which variables they would like plotted in a time simulation. A tabbed pane allows the user to select between three main option tabs: **Sim**, a time simulation; **State**, a steady-state analysis; or **MCA**, a Metabolic

Chapter 2.

HOST RANGE OF A NATURAL BARLEY RHIZOSPHERE PLASMID AMONGST BACTERIA ISOLATED FROM A BARLEY FIELD

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ABSTRACT

Broad-host-range plasmids permit interspecies genetic exchange and may therefore be a major factor for the adaptation of microbial communities. In this study, the ability of a natural barley rhizosphere plasmid (pKJK5) to transfer to a collection of 1400 bacterial soil isolates was studied. The plasmid was tagged with *gfp* and a screening assay was developed based on the zygotic induction of Gfp in transconjugant cells in the contact zone of donor and recipient colonies. The number and diversity of isolates capable of receiving pKJK5::*gfp* as well as potential maximal transfer frequencies (transconjugants/(donors $\times$ recipients)$^{1/2}$) were determined. For comparison, the host range of plasmid RP4 to the 1400 isolates was also tested. The data showed that 3% of the 1400 strains were able to receive pKJK5 while 4% received RP4::*gfp*. Numbers of rhizosphere isolates acting as recipients of both plasmids were significantly higher for those isolated in August compared to May and July. Host ranges of pKJK5::*gfp* and RP4::gfp were broader than expected as α, β, γ-*Proteobacteria*, *Cytophaga-Flavobacterium-Bacteroides* (CFB), and Gram-positive bacteria were found to be recipients of both plasmids. Approximately 30% of the closest relatives to the strains that received pKJK5::*gfp* were hitherto uncultured clones. Transfer frequencies of pKJK5::*gfp* ranged between 2.7×10^{-5} and 1.7×10^{-1}. No clear relationship between frequency and phylogeny could be identified. The broad host range of pKJK5 suggests that it is a potent vector for the spreading of genes within soil microbial communities.

INTRODUCTION

Conjugal transfer of plasmids provides a means for genetic exchange between bacteria and may be a major factor for the adaptation of microbial communities to natural and man-made stresses (13). Plasmids that can be transferred and maintained in several host bacteria are of particular interests in this regard as they permit interspecies genetic exchange (12). Some of the most promiscuous plasmids transferring at high frequencies belong to the plasmid incompatibility group IncP1. The IncP1 plasmid RP4, for instance, is transmissible and stably maintained in almost all of the Gram-negative bacterial species tested (34).-

Environments reported as favoring conjugation include the intestinal tract of humans and animals, activated sludge and the plant rhizosphere (6,7). In the rhizosphere, bacteria benefit from the diffusion of a broad spectrum of soluble sugars, amino acids, mucilage, and sloughed off cell material from the plant roots (4,18,26). The enhanced availability of carbon and nutrients in the rhizosphere supports a high microbial density and activity (3,24,32), which are factors known to stimulate conjugal transfer (37).

Few studies have investigated the host range of natural soil plasmids amongst indigenous soil bacteria. Pukall et al. (23) reported that the majority of the bacteria receiving the rhizosphere IncP1 plasmid, pTH16, could be assigned to *Pseudomonas* spp., *Alcaligenes* spp., and *Rhizobium* spp. Geisenberger et al. (9) used green fluorescent protein (Gfp) in combination with fluorescent in situ hybridization to visualize transfer of RP4 to indigenous bacteria in activated sludge. They observed that more than 95% of all transconjugant cells hybridized to a probe targeting *γ-Proteobacteria*.

The objective of this study was to investigate the ability of a natural barley rhizosphere plasmid, pKJK5 (28), to transfer to 1400 soil isolates obtained from the barley rhizosphere and bulk soil in May, July and August (15). In order to accomplish the objective,

we constructed a gene transfer reporter system based on the green and red fluorescent proteins, and developed new techniques for the screening of plasmid host range and direct enumeration of donor and transconjugant cells. The number of isolates capable of receiving pKJK5::*gfp* was determined and compared to the number of isolates that could act as recipients of RP4::*gfp*. Furthermore, we determined the diversity of the recipients of pKJK5::*gfp* by partial 16S rDNA sequencing, and estimated potential maximal transfer frequencies in filter mating experiments. Finally, the Inc group of pKJK5 was determined. The data showed that approximately 3% of the 1400 strains were able to receive pKJK5 with a dominance of *α-Proteobacteria*. However, *β-Proteobacteria*, *γ-Proteobacteria*, *Cytophaga-Flavobacterium-Bacteroides* (CFB), and Gram-positive bacteria were also observed. Approximately 30 % of the closest relatives to the sequenced strains were found to be 16S rDNA sequences obtained only by cloning. Numbers of rhizosphere isolates acting as recipients of the two plasmids were significantly higher in August compared to May and July.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Characteristics of the used strains and plasmids are listed in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) medium (27) at 30°C, while *P. putida* stains were grown at 30°C in FAB minimal medium [1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.01 mM $FeCl_2$, 0.15 mM $(NH_4)SO_4$, 0.33 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 0.5 mM NaCl] (24). Minimal medium contained 10 mM sodium citrate as the sole carbon source. When needed, antibiotics were added at the following concentrations: kanamycin (Km) 25 μg/ml, nalidixic acid (Nal) 50 μg/ml, tetracycline (Tc) 10 μg/ml, and rifampicillin (Rif) 50 μg/ml).

Construction of donor strain. The *dsRed* gene fused to the *E. coli* ribosomal promoter, *rrnB*P1 (36) was inserted into the chromosome of *P. putida* KT2442::*lac*I^q by triparental mating with *E. coli* MV1090λ-*pir*/TTN151 and *E. coli* HB101/pRK600 according to Herrero et al. (14). A clone, which grew at the same rate as *Pseudomonas putida* KT2442 in minimal medium at 30°C, was named *P. putida* LM50 and served as donor strain in biparental experiments (see below).

Characterization of pKJK5::*gfp*. Plasmid DNA was isolated from one ml over night culture by use of a commercially available kit (Qiagen, Hilden, Germany). Lysis of bacterial cells and purification of plasmid DNA was carried out according to the protocol provided by the manufacturer of the kit. The size of pKJK5::*gfp* was determined by restriction analysis with *Not*I. To determine the incompability group of pKJK5, PCR primers for the amplification of regions specific for plasmids belonging to the incompatibility groups IncW (*oriT*), IncN (*kikA*), IncQ (*oriV*) and IncP1 (*korA*; *oriT*; *trfA*2; *traG*) (12) were employed. PCR reactions contained 250 µM of each dNTP (Roche, cat. no. 1277049), 2.5 µl 10×PCR Reaction Buffer (Roche cat. no. 1271318), 0.5 mM $MgCl_2$ (Roche cat. no. 1699113) and 0.5 U Taq DNA polymerase (Roche cat. no. 1435094). All PCR reactions were done in a HYBAID pcrEXPRESS HBPX-220 thermocycler. Amplification involved 25 cycles of 0.5 min at 94°C, 1 min at the annealing temperature of the respective primer system (12) and 2 min at 72°C. Finally, a primer extension reaction was performed for 6 min at 72°C. The PCR products were analyzed on 1.5% (w/v) NuSieve agarose gels (Medinova, Hellerup, Denmark) in 1×TBE buffer (Sharlau Chemie, Barcellona, Spain).

Filter mating experiments (see below) on two different substrates (10% TSB and M9) were performed between *P. putida* LM50 donors and *P. putida* SM1464 recipients to verify that insertion of the $P_{A1/04/03}$::*gfp*mut3b gene cassette into pKJK5::*gfp* and RP4::*gfp* had no

effect on the transfer frequency of the plasmids. No significant differences in transfer between the *gfp*-tagged and wildtype plasmids were observed (ANOVA, $P > 0.05$; data not shown).

Plasmid transfer to rhizosphere and soil isolates. A total of 1400 isolates from bulk soil and the rhizosphere of barley plants (*Hordeum vulgare* L.) were randomly selected from the collection of isolates described in Johansen and Binnerup (15) and screened for their ability to receive pKJK5::*gfp* and RP4::*gfp*. Approx. 1 µl glycerol suspensions (stored at –80°C) of the isolates were transferred by a 96-pin replicator (Life technology, Denmark) to the surface of 1/10 strength TSB square agar plates. Overnight cultures of donor strains *P. putida* LM50/pKJK5::*gfp* or *P. putida* LM50/RP4::*gfp* were similarly transferred to the agar surface by carefully placing them about 2 mm away from the soil isolates. After 2 days of incubation at 20°C, colonies of donor and isolate strains were in physical contact. Plasmid transfer was recorded during the following 8 days of incubation by observing the appearance of green fluorescent cells at the point of contact between the donor and recipient colonies. Isolates that were capable of receiving pKJK5::*gfp* were also tested for their ability to act as recipients of TOL::*gfp*.

Green fluorescing transconjugant cells were detected by use of an Axioplan epi-fluorescence microscope (Carl Zeiss) equipped with a 50-W mercury lamp, a plan-Neofluar x2.5/0.075 objective, and a no. 10 filter set (Carl Zeiss). Donor cells were detected using a XF40 filter set (Omega Optical Brattletror, Vt.) to visualize DsRed. Image acquisitions were performed with a model TCS SP1 3 channel scanning confocal laser microscope (Leica Microsystems Heidelberg GmbH, Germany) equipped with an argon laser (458nm, 476nm, 488 nm and 514 nm wavelength) and two HeNe lasers (543nm and 633nm wavelength) and a variable spectrophometric detection system that simultaneously monitored Gfp and DsRed fluorescence. Reflection scans were made to visualize the donor and recipient colonies.

Images were processed by use of Adobe Photoshop Version 6 (Adobe, Mountain View, CA.USA).

Transfer of the plasmids was confirmed by redoing the transfer experiments to the positive isolates three additional times (each with 3 replicates). Furthermore, to verify that putative transconjugant cells at the single cell level only fluoresced within the green light spectra, green fluorescing cells were picked from the meeting zone of the donor and recipient colonies, diluted in 0.8% NaCl and examined by epifluorescence microscopy using a ×63 objective.

Taxonomic identification of bacterial isolates. DNA was extracted by boiling cells from colonies in 200 µl Tris-EDTA buffer (1M Tris-HCl; 0.5M EDTA; pH 7.5) for 10 min. Cell debris was removed by centrifugation at 15,000×g for 5 min and the supernatant containing the DNA transferred to Eppendorf tubes and stored at 4°C. PCR amplification of the 16S rDNA gene (first 500 bp) was done in PCR tubes containing 1 µl of the DNA extract and 0.5 µM of each primer SDBact0008aS20 and S*UNIV518Aa18 (Alm et al. 1996), 250 µM of each dNTP, 1×PCR Reaction Buffer, 0.5 mM $MgCl_2$ and 0.5 U Taq DNA polymerase. Amplification involved 25 cycles of 30 s at 94°C, 30s at 61°C, 2 min at 72°C; 6 min at 72°C. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. MWG-BIOTECH (www.mwg-biotech.com) made partial sequencing of the PCR products on a NEN Global IR^2 DNA Sequencer Li-COR using the primer SDBact008aS20. The partial 16S rDNA sequences obtained were compared to sequences in nucleotide databases using NCBI/BLAST search program (http://www.ncbi.nlm.nih.gov). Last date for search was 3rd of January 2003. The closest match was accepted as the closest relative.

Transfer frequency of pKJK5::*gfp* to bacterial isolates. Transfer frequencies (transconjugants/(donors × recipients)$^{1/2}$) of PKJK5::*gfp* from the *P. putida* LM50 donor to the rhizosphere and soil isolates were determined in bi-parental filter mating experiments. This was done by mixing 100 µl of an o/n culture adjusted to OD_{450nm} = 1 of *P. putida* LM50/pKJK5::*gfp* with 100 µl of a colony suspension (OD_{450nm} = 1) of the soil isolates. An aliquot of 100 µl of the mixture was spotted onto a 0.22 µm pore-size cellulose nitrate filter (Sartorius, Germany) placed on a 10% TSB agar plate. As controls, 50 µl of donor culture and soil isolates suspension were spotted on separate membrane filters. Filters were inoculated for 8 days at 20˚C after which cells were extracted by vortexing in 1 ml filtered (0.22 µm pore size) 1.5 × PBS for 1 min.

Flow cytometry (FCM) was applied to determine the numbers of cells in the extracts by setting the flow cytometer so that discrimination between the red fluorescence of the donors and the green fluorescence of the transconjugants was possible. A Becton Dickinson FACS Calibur flow cytometer, equipped with a 15-mW, air-cooled argon ion laser excitation light source (488 mm) was used. Extracts were diluted to appropriate concentrations and an internal standard consisting of a known concentration of polystyrene fluorescent microspheres (Molecular Probes) added. FCM analysis was carried out thresholding on side scatter (SSC). The voltages were set to: FCS: E01; SSC: 343; FL1: 643; FL2: 628; and FL3: 319. A gate (Bacteria gate), defined around the bacterial cells in a SSC versus forward scatter (FSC) plot, was used to enumerate the total number of bacteria. This gate transposed to a SSC versus FL2 plot with 99.9% compensation for Fl1 was used to enumerate donor cells. Beads were counted in a separate gate in a SSC versus FL3 plot. Transconjugant cells were counted in a separate run using the settings described by Sørensen et al. (33).

A control filter mating experiment between *P. putida* LM50/pKJK::*gfp* and *P. putida* SM1464 was set up to allow comparison of cell counts based on flow cytometry with counts based on CFU.

RESULTS

Characterization of pKJK5. Restriction analysis by digestion with Not1 showed the size of pKJK5 to be approx. 58 000 bp (data not shown). pKJK5 was classified to plasmid incompatible group IncP1 on basis of the PCR-products obtained with the IncP1 primer systems *oriT* (110 bp), *trfA*2 (241 bp), *korA* (294 bp), and *traG* (762 bp) (Fig. 1). No PCR products were observed when the IncW, IncN, or IncQ primers were used. We sequenced a 190 bp fragment of the *trfA* gene and compared both the nucleotide and amino residue sequences to those of RP4 (IncP1-α) and R751 (IncP1-β) in an attempt to identify the IncP1 subgroup of pKJK5. Alignment of the amino residue sequences showed that *trfA* of RP4 and R751 were more similar to each other (87% similarity) than pKJK5 was similar to RP4 and R751 (84 % and 81 % similarity, repectively (Fig. 2). With a BLAST-X and BLAST-N search to the *trfA* sequence of pKJK5 in GenBank, a *trfA* sequence from an unidentified broad-host-range plasmid (GenBank accession no. AJ414161) with a 92 % amino residue similarity was found.

Host range of pKJK5 and RP4. The ability of pKJK5 to be transferred to the soil isolates was compared to that of plasmid RP4. An assay to screen for transfer was carried out by spotting the donor and the isolates close to each other on an agar plate. Generally, plasmid transfer was observed in a narrow meeting zone between the donor and recipient colonies (Fig. 3A-B), however, other patterns were also found (Fig. 3C-D).

Numbers of isolates receiving the two plasmids were not significantly different (χ^2-test; $P > 0.05$). A total of 38 isolates were found to be able to receive pKJK5::*gfp*, while 54 received RP4::*gfp* (Table 2), i.e. 2.7 % and 3.9 %, respectively, of the total number of isolates tested. The number of rhizosphere isolates acting as recipients of both plasmids were significantly higher ($P < 0.05$) for August than for May and July. Of the 38 isolates that received pKJK5::*gfp*, 15 could also act as recipients of RP4::*gfp* and 3 as recipients of TOL::*gfp* (Table 3).

Presence of IncP1 plasmids in the bacterial isolates could potentially have affected the number of isolates that were observed to receive pKJK5::*gfp* due to plasmid exclusion. By use of the IncP1 primer systems *oriT* and *korA*, we tested a 10% subsample of the 1400 isolates for content of IncP1 plasmids and found 7 isolates giving PCR products. This indicates that about 5% of the isolates contained an IncP1 plasmid. The 38 isolates that were able to receive pKJK5::*gfp* were also tested for content of IncP1 plasmids, however, in no cases were PCR products observed.

The 16S rDNA of the isolates that received pKJK5::*gfp* was sequenced and evaluated with BLASTN 2.2.1 to identify the closest relatives (Table 3). Of the 38 sequenced rDNA genes, 25 proved to be different. α-*Proteobacteria* dominated with 21 isolates, however, *β-Proteobacteria*, *γ-Proteobacteria*, *Cytophaga-Flavobacterium-Bacteroides* (CFB), and Gram-positive phyla were also represented (Table 3). Of the *α-Proteobacteria*, 38% belonged to *Rhizobium* or *Sinorhizobium*. Neither a clear taxonomic difference between bulk soil and rhizosphere isolates, nor a clear seasonal difference was observed. Approximately 30% of the closest relatives to the sequenced strains were hitherto uncultured clones (Table 3).

The fraction of isolates that acted as recipients of both pKJK5::*gfp* and RP4::*gfp* did not contain Gram-positive or CFB members. However, among the 16 isolates that only

received RP4*::gfp*, gram-positives (as tested by gram-staining (20) and lysis with 3% KOH (11)) and CFB (yellow/orange colonies, flexirubin positive (15,25)) were present. As expected, the host range of the TOL plasmid was narrow and the three strains tested positive were all *Pseudomonas* spp.

The isolates that appeared to contain natural IncP1 plasmids (i.e. tested positive with the *trfA*2 and *korA* primers) belonged to *β-Proteobacteria* (2 isolates), *γ-Proteobacteria* (1 isolate), CFB (3 isolates), and Gram-positive (1 isolate) phyla.

Transfer frequency of pKJK5::*gfp* to bacterial isolates. Numbers of cells determined by flow cytometry were 2-3 times higher compared to numbers determined by plating in the control mating experiment between *P. putida* LM50/ pKJK5::*gfp* and *P. putida* SM1464 (Fig. 4). The transfer frequency (transconjugants/(donors × recipients)$^{1/2}$) of pKJK5::*gfp*, however, was unaffected by the cell enumeration method ($3.48 \pm 1.51 \times 10^{-1}$ and $3.73 \pm 1.77 \times 10^{-1}$ for flow cytometry and plating, respectively).

Transfer frequencies of pKJK5::*gfp* to the indigenous bacterial isolates ranged between 2.7×10^{-5} and 1.7×10^{-1} (Table 3). No clear relationship between frequency and phylogeny was apparent.

DISCUSSION

Plasmid pKJK5 was isolated from the rhizosphere of barley (28). We PCR replicon typed pKJK5 to the IncP1 plasmid group. Based on DNA homology in the replication region, IncP-1 plasmids have been divided into two subgroups, IncP1-α and IncP1-β, representing two major evolutionary branches (30,38). Recently, complete sequencing of the RP4 (IncP1-α) and R751 (IncP1-β) plasmids (22,35) have confirmed that the basic organization of their replication and transfer functions is the same, and that the *trfA* ORF, coding for the activator

of the *oriV* region, shows a high degree of sequence homology (31). The *trfA* sequence has been used to assess the evolutionary branch of IncP1 plasmids (7). Due to the observed differences in the DNA and amino acid sequences of *trfA* between pKJK5 and the RP4 and R751 plasmids, pKJK5 could not be assigned to either the α- or the β branch. However, because a PCR product was obtained from the traG primer set, which is specific for the IncP-1α subgroup (12), this may indicate that pKJK5 may represent an out branch of the IncP1-α subgroup. Further investigations are needed to clarify the existence of new evolutionary branches.

In order to study the ability of pKJK5 to spread amongst a collection of 1400 natural isolates from a barley field, a screening assay for assessing the host range of plasmids was developed. The assay was based on the zygotic induction of Gfp in transconjugant cells (5) formed in the meeting zone of donor and recipient colonies. Although the assay was repeated three times for each isolate acting as recipient, and autofluorescence did not interfere with our ability to observe the transconjugants, we cannot rule out the possibility that transfer was overlooked in a few cases where lack of growth of the transconjugant cells resulted in an undetectable green fluorescent signal.

By use of the specific IncP1 primer systems, *trfA*2 and *korA,* IncP1 plasmids were shown to be present in the bacterial isolates at a frequency of about 5 %. This low occurrence of indigenous IncP1 plasmids suggests that the reported numbers of isolates capable of acting as recipients of pKJK5::*gfp* and RP4::*gfp* were not grossly underestimated due to plasmid exclusion.

The occurrence of rhizosphere isolates acting as recipients of pKJK5::*gfp* and RP4::*gfp* was highest in August (Table 2). Likewise, Lilley & Bailey (17) reported that an added *P. fluorescens* strain primarily took up plasmids from the indigenous bacterial

community of the sugar beat phytosphere during a defined period in the middle of the growth season. Changes in species composition of bacterial communities in the rhizosphere have frequently been reported. For instance, Johansen & Binnerup (15) reported that the relative importance of *Cytophaga*-like bacteria decreased from 25% in May to 10% in August in the rhizosphere of barley, and Mahaffee & Kloepper (19) observed a bacterial population shift in the rhizosphere of cucumber. Thus, it is likely that a temporal succession in the species composition of our collection of isolates may have affected the number of potential recipients and, hence, caused the observed change in numbers of rhizosphere isolates receiving pKJK5::*gfp* and RP4::*gfp*. Interestingly, however, no temporal species differences in the transconjugant populations of pKJK5::*gfp* were apparent.

The dominant recipients of pKJK5::*gfp* belonged to the α and γ subgroups of the *Proteobacteria*, however, β-*Proteobacteria*, *Cytophaga* sp. and Gram-positive bacteria (high and low G+C %) were also represented. Similarily, RP4::*gfp* was taken up α, β γ-*Proteobacteria*, Gram-positive bacteria and CFB, and the isolates that were tested positive for IncP1 plasmids belonged to *β-Proteobacteria*, *γ-Proteobacteria*, CFB, and Gram-positive phyla. To the best of our knowledge, conjugal transfer and replication of IncP1 plasmids into *Cytophaga* and Gram-positive bacteria has never been reported in the literature. Our data suggest, however, that the host range of IncP plasmids is much broader than previously believed, as both Gram-positive bacteria and CFB appear to be natural hosts of IncP plasmids.

Twelve of the 38 recipients of pKJK5::*gfp* had hitherto uncultured clones as their closest relatives. Unlike many previous plasmid host range studies, we worked with natural isolates that had been restricted to growth only once on 10% TSA (15) and this could have been the reason why many “unculturable” bacteria showed up in our study and why the broad host range was observed. As another explanation for the broad host range, we speculate that

pKJK5 may carry transposons that allow it to become integrated into the chromosme of *Cytophaga sp*. and Gram-positive bacteria. For instance, the IncP1 plasmid, R751, tagged with the *Bacteroides* transposon Tn*4351*, has been shown to stably integrate into the chromosome of *Bacteroides* (29). To test if a similar mechanism is involved in the transfer of pKJK5 into *Cytophaga sp*. and Gram-positive bacteria would require an investigation of whether the transconjugant bacteria contain pKJK5::*gfp* in the cytoplasm or integrated into the chromosome and/or a complete sequencing of pKJK5.

The potential maximal transfer frequency (transconjugants/(donors × recipients)$^{1/2}$) of pKJK5::*gfp* from *P. putida* LM50 to the isolates was determined in filter mating experiments in which cell numbers were counted by flow cytometry. Sengeløv et al. (28) studied transfer of pKJK5::*gfp* ("pKJK10" in Sengeløv et al.) between two *P. putida* strains and reported transfer frequencies of $6.08 \pm 1.76 \times 10^{-1}$, $8.20 \pm 11.6 \times 10^{-3}$ and $4.57 \pm 0.87 \times 10^{-2}$ for filter matings, and the rhizosphere and spermosphere of barley, respectively. Our transfer ratios ranged four order of magnitude between 2.7×10^{-5} and 1.7×10^{-1}. Thus, our filter mating frequencies were in many cases lower than those observed for the rhizosphere and spermosphere of barley (28). One reason for this apparent discrepancy could be that Sengeløv et al. looked at isogenic transfer between two *P. putida* strains whereas we looked at transfer to a broad spectrum of different species.

No clear correlation between transfer frequency and phyllogeny of the recipient strains was apparent, and the transfer frequency varied in many cases more than one order of magnitude between isolates having the same closest relative (e.g. LJ100 and LJ106) suggesting that the transfer process is isolate specific rather than species or genus specific. This agrees with Gordon (10) who studied transfer of plasmid R1 into ten strains of natural

isolates of *E. coli* and found that the rate of transfer varied between 5.2 x 10^{-11} to 1.1 $\times$ 10^{-18} ml per cell h^{-1}.

In conclusion, pKJK5 (and RP4) was shown to have a very broad host range that included several phyla. Both the propensity of pKJK5 to spread amongst different bacterial phyla, and the natural occurrence of IncP plasmids in *Cytophaga* and G-positive isolates, suggest that natural IncP rhizosphere plasmids may facilitate exchange of genes between distantly related bacteria in soil. Hence, presence of these plasmids may have a strong impact on the evolution of the microbial soil communities.

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FIGURE LEGENDS

Fig. 1. PCR amplification products using the IncP primers *korA* (lanes 2, 6, 10,14), *oriT* (lanes 3, 7, 11, 15), *traG* (lanes 4, 8, 12, 16) and *trfA*2 (lanes 5, 9, 13, 17) of RP4 (lanes 2-5), RP4::*gfp* (lanes 6-9), pKJK5 (lanes 10-13), pKJK5::*gfp* (lanes 14-17), and negative controls (lanes 18-21). The molecular marker was a 100 bp ladder (lanes 1, 22).

Fig. 2. Comparison of sequences upstream the N-terminal end of the *trfA* gene of pKJK5, RP4, R751 and an environmental plasmid (AJ414161). Grey shading indicates differences in the nucleotide sequence between pKJK5 and the other plasmids, while bold letters indicates differences in the amino residue sequences. The RP4, R751 and AJ414161 sequences were derived from NCBI (L27758, U67194, and AJ414161, respectively). The pKJK5 sequence is from this study.

Fig. 3. Reflection photographs of donor and recipient colonies (column A), location of green fluorescent transconjugant cells (column B) and red fluorescent *P. putida* LM50 /pKJK5::*gfp* donors (column C). The superimposed pictures in column D originate from column A, B and C and illustrate some of the different conjugation patterns observed. The recipient strains LJ51 (α-*Proteobacteria*), LJ68 (γ-*Proteobacteria*), LJ40C (*Cytophaga-Flavobacterium-Bacteriodes*) and LJ105 (α-*Proteobacteria*) are shown in rows 1-4, respectively.

Figure 4. Comparison of cell counts obtained using flow cytometry (light grey shading) and spread plating (dark grey shading) in filter matings between *P. putida* LM50/pKJK5::*gfp* and *P. putida* SM1464.

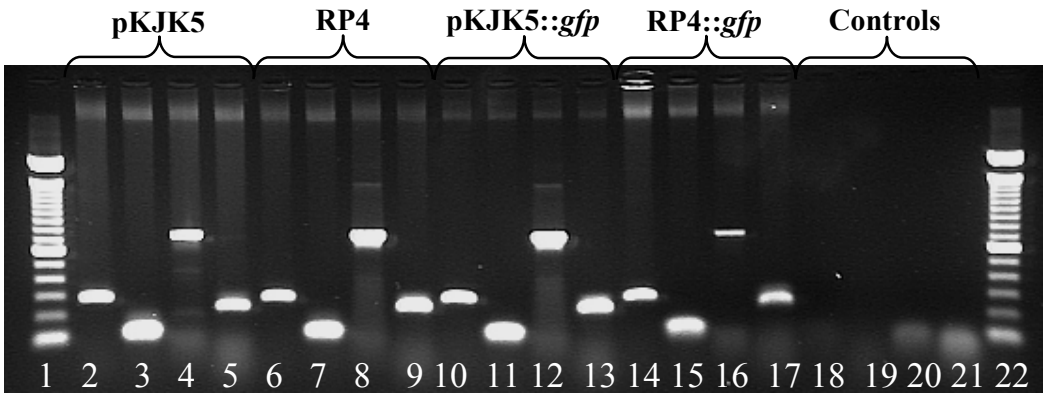
pKJK5
RP4
pKJK5::gfp
RP4::gfp
Controls
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Fig. 1


pKJK5 1
R M F D Y F A S H Q E P F P M K L E T F R L M
RP4
S R Y L
R751
T K Y L
AJ414161
Y
pKJK5 70
C G S Q S A R P K K W R E Q V G E A C E E L R
RP4
D T V
R751
D T D
AJ414161
Q
pKJK5 138
A S G L V E H A W V N N D L C H
RP4
G D V
R751
E N S D V
AJ414161
K S S

Fig. 2

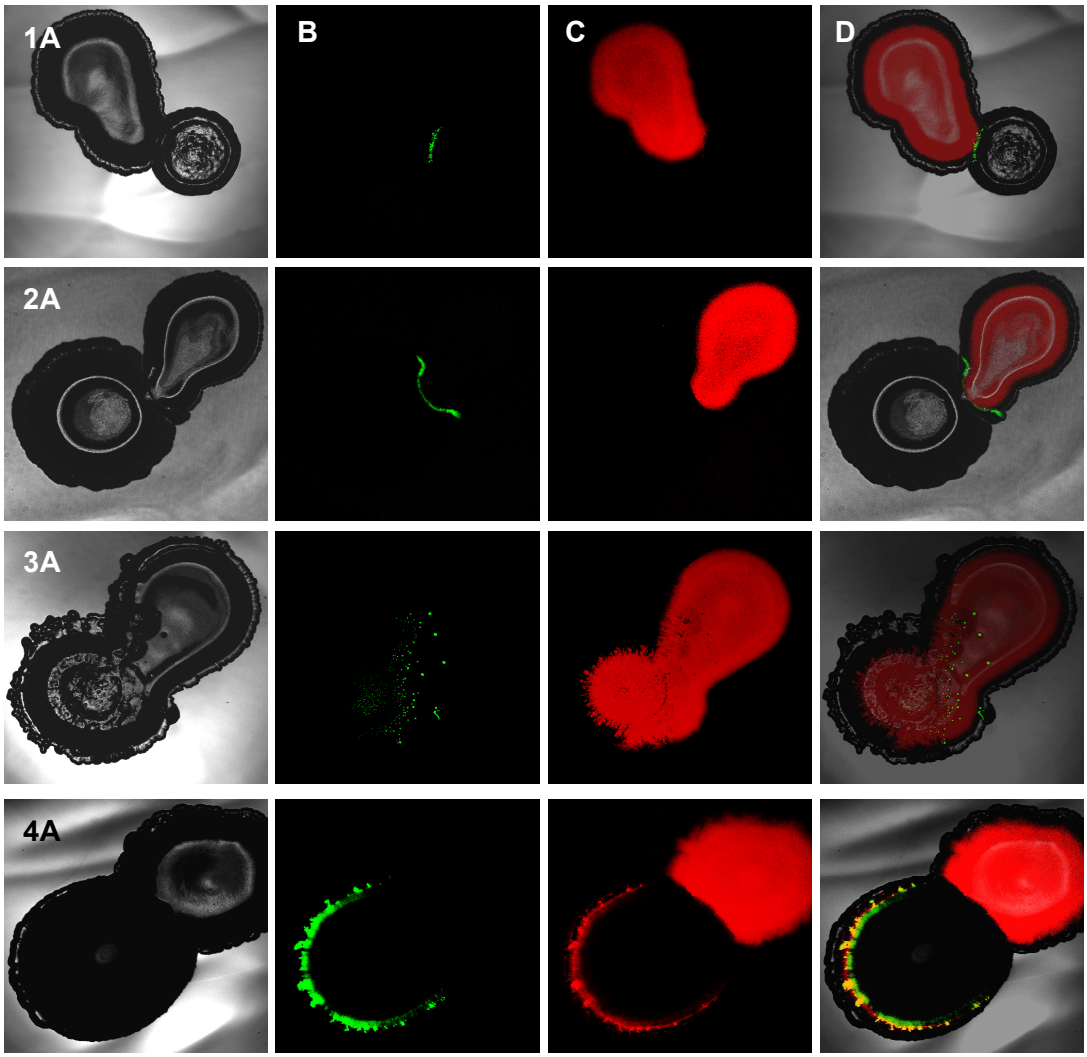

Fig. 3

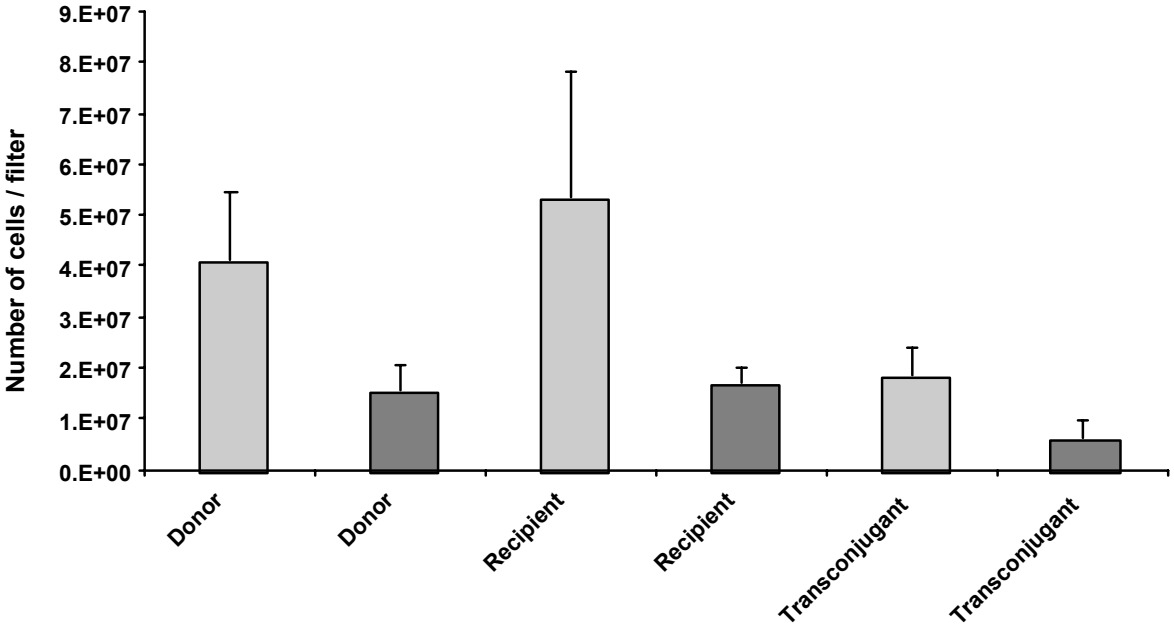
9.E+07
8.E+07
7.E+07
6.E+07
5.E+07
4.E+07
3.E+07
2.E+07
1.E+07
0.E+00
Number of cells / filter
Donor
Donor
Recipient
Recipient
Transconjugant
Transconjugant

Fig. 4

Table 1. Bacterial strains and plasmids used.

Strain or plasmid	Relevant genotype and/or characteristics	Reference
E. coli strains		
HB101	*Sm*^{r} *recA thi por leu hsdRM*$^+$	Kessler et al 1992
MV1190λ-*pir*	Δ(*lac proAB*) Δ(*srl-recA*)*306*::Tn*10* [F´*traD36 proAB lacI*^{q} Δ(*lacZ*)*M15*] t*hi supE*, lysogenized with *λ-pir* phage	Herrero et al, 1990
P. putida strains		
KT2440		Bagdasarian et al. 1981
KT2442	Rif^r mutant of KT2440	Bagdasarian et al. 1981
SM1464	KT2440 Nal^r	Søren Molin
KT2442::lacI^q	KT2442 with mini-Tn*5* insertion of lacI^q, Rif^r	Normander et al. 1998
LM50	KT2442 with mini-Tn*5* insertion of lacI^q, and a mini-Tn5 insertion of *dsRed*; Rif^r, Km^r	This study
Plasmids		
pRK600	Cmr ColE1oriV RP4oriT, helper plasmid in triparental matings	Kessler et al. 1992
TTN151	Ap^r Km^r, delivery plasmid for mini-Tn*5*-Km^r-rrnBP1::RBSII-dsRed-T_0-T_1 derived from pUT-mini-Tn*5*-Km^r	Tolker-Nielsen et al. 2000
RP4::*gfp*	RP4 with mini-Tn5 insertion of $P_{A1/04/03}$::*gfp*mut3b; Km^r.	Christensen, B.B. unpublished.
pKJK5	Conjugative plasmid recovered from barley rhizosphere by exogenous plasmid isolation, Tc^r	Sengeløv et al. 2001
pKJK5::*gfp*	pKJK5 with mini-Tn5 insertion of $P_{A1/04/03}$::*gfp*mut3b; Tc^r, Km^r, Sm^r	Sengeløv et al. 2001
TOL::*gfp*	TOL with mini-Tn5 insertion of $P_{A1/04/03}$::*gfp*mut3b; Km^r	Normander et al. 1998
AJ414161	Plasmid isolated from a polluted environment.	GenBank ass. AJ414161

Table 2. Ability of the 1400 isolates to receive and maintain pKJK5::*gfp* and RP4::*gfp*.

	Bulk soil			**Rhizosphere**			
	May	July	August	May	July	August	**Total**
Isolates tested	137	136	86	317	453	271	1400
Isolates receiving pKJK5::*gfp*	2 (1.5 %)	2 (1.5 %)	2 (2.3 %)	5 (1.6 %)	10 (2.0 %)	17 (6.3 %)*	38 (2.7 %)
Isolates receiving RP4::*gfp*	1 (0.7 %)	4 (2.9 %)	4 (4.7 %)	5 (1.6 %)	15 (3.3 %)	25 (9.1 %)*	54 (3.9 %)

* Significantly ($P \leq 0.05$) more rhizosphere isolates received pKJK5::*gfp* and RP4::*gfp* in August than in May or July. The statistical test was a two-way contingency table tested by a chi-square test.

Table 3. Identity of bacterial isolates capable of receiving pKJK5::*gfp* and transfer ratios of pKJK5::*gfp*. Isolates that also acted as recipients of RP4::*gfp* and TOL::*gfp* are indicated as well.

Isolate	Accession no.	Taxon	Closest relative (accession no.)	Similarity %	Recipient of RP4::*gfp*	Recipient of TOL::*gfp*	$T/(D \cdot R)^{1/2}$
LJ4	AJ605222	α	Grassland soil clone saf 3_112 (AF078301)	98			1.07×10^{-3}
LJ9	AJ605223	α	Uncultured α-proteobacterium clone SM1E02 (AF445680)	99			9.21×10^{-3}
LJ17	AJ605711	α	Uncultured α-proteobacterium clone SM1E02 (AF445680)	99			1.66×10^{-2}
LJ12	AJ605225	α	Grassland soil clone saf3_005 (AF078276)	96			3.83×10^{-3}
LJ51	AJ605235	α	Grassland soil clone saf3_005 (AF078276)	96			1.75×10^{-2}
LJ105	AJ605260	α	*Sinorhizobium* sp. S007 (AF285964)	99			7.76×10^{-2}
LJ26	AJ605227	α	Uncultured α-proteobacterium (AJ416679)	100	+		3.36×10^{-2}
LJ27	AJ605229	α	Uncultured α-proteobacterium (AJ416679)	99	+		1.78×10^{-2}
LJ28	AJ605230	α	*Rhizobium* sp. RM1-2001 (AF331662)	100	+		1.35×10^{-2}
LJ43	AJ605232	α	*Rhizobium* sp. OmegaB (AY064412)	99	+		2.33×10^{-2}
LJ52	AJ605236	α	*Rhizobium* sp. OmegaB (AY064412)	99			2.86×10^{-3}
LJ44	AJ605233	α	*Sphingomonas yanoikuyae* (AF182027)	99			2.29×10^{-4}
LJ45	AJ605234	α	*Sinorhizobium morelense* (AF452129)	100			5.38×10^{-2}
LJ76	AJ605246	α	*Sinorhizobium morelense* (AF452129)	100			7.65×10^{-3}
LJ104	AJ605259	α	*Sinorhizobium morelense* (AF452129)	100			1.29×10^{-2}
LJ66	AJ605240	α	α-Proteobacterium Kaza-27 (AF441726)	97			1.44×10^{-3}
LJ89	AJ605252	α	*Sphingomonas* sp. (U63962)	100	+		2.20×10^{-2}
LJ100	AJ605258	α	*Agrobacterium tumefaciens* strain HAMBI2405 (AF501343)	100	+		2.04×10^{-3}
LJ106	AJ605261	α	*Agrobacterium tumefaciens* strain HAMBI2405 (AF501343)	100	+		1.51×10^{-4}
LJ120	AJ605714	α	Uncultured bacterium TSA-14.3 (AF240153)	100			2.26×10^{-2}
LJ63	AJ605238	β	Uncultured bacterium BHB9 (AF090539)	99			1.19×10^{-4}
LJ97	AJ605712	β	*Variovorax paradoxus* strain dS (AF451851)	100	+		4.59×10^{-4}
LJ34	AJ605231	γ	Unidentified eubacterium (AF010020)	100	+		2.62×10^{-2}

LJ57	AJ605237	γ	Soil bacterium is110 (AF128760)	99			1.15×10^{-4}
LJ64	AJ605239	γ	*Pseudomonas* sp. DhA-91 (AF177916)	100	+	+	1.26×10^{-3}
LJ68	AJ605241	γ	*Pseudomonas* sp. PsI (AF105387)	100	+	+	1.21×10^{-2}
LJ70	AJ605242	γ	Uncultured γ-proteobacterium clone NMS8.133WL (AY043804)	100			7.19×10^{-3}
LJ72	AJ605244	γ	*Pseudomonas corrugata* (AF348508)	100	+	+	4.54×10^{-2}
LJ74	AJ605245	γ	*Pseudomonas putida*. ATCC 17527 (AF094743)	100			2.74×10^{-5}
LJ81	AJ605249	γ	γ-Proteobacterium PI_GH1.1.A2 (AY162032)	98	+		1.72×10^{-4}
LJ82	AJ605250	γ	γ-Proteobacterium PI_GH1.1.A2 (AY162032)	98			1.93×10^{-5}
LJ99	AJ605257	γ	γ-Proteobacterium PI_GH1.1.A2 (AY162032)	98	+		2.06×10^{-4}
LJ84	AJ605251	γ	Uncultured *Xanthomonas* sp. clone burs_22 (AF467297)	99	+		5.99×10^{-3}
CLB40	AJ605715	CLB	*Cytophaga* sp. (U63943)	95			1.65×10^{-1}
LJ11	AJ605224	GP-HGC	Rhizosphere soil bacterium isolate RSI-27 (AJ252594)	99			1.08×10^{-4}
LJ93	AJ605253	GP-HGC	Rhizosphere soil bacterium isolate RSI-27 (AJ252594)	98			1.05×10^{-4}
LJ77	AJ605247	GP-HGC	*Arthrobacter* sp. PF3 (AF500322)	100			2.75×10^{-3}
LJ118	AJ605713	GP-LGC	*Paenibacillus amylolyticus* strain B24 (AF406691)	99			3.91×10^{-3}

Legends: CFB, *Cytophaga-Flavobacterium-Bacteroides* phylum; α, *α-proteobacteria*; β, *β-proteobacteria*; γ, *γ-proteobacteria*; GP-HGC, Gram-positve bacteria with a high G+C content; GP-LGC, Gram-positve bacteria with a low G+C content.

Chapter 3.

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Plasmid Transfer from *Pseudomonas putida* to the Indigenous Bacteria on Alfalfa Sprouts: Characterization, Direct Quantification, and In Situ Location of Transconjugant Cells

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The transfer of the plasmids pJKJ5 and TOL (pWWO) from *Pseudomonas putida* to the indigenous bacterial community on alfalfa sprouts was studied. Tagging with fluorescent protein markers allowed direct quantification of the introduced donor bacteria and of indigenous bacteria that had received the plasmids. The sprouts were observed for 9 days; during this time alfalfa seeds, inoculated with donor bacteria, developed to edible and subsequently decaying sprouts. The first transconjugants were detected on day 6 after donor inoculation and occurred at frequencies of 3.4×10^{-4} and 2.0×10^{-6} transconjugant cells per donor cell for pKJK5::*gfp* and TOL::*gfp*, respectively. Confocal laser scanning microscopy revealed that the sprouts were heavily colonized with donors and that most transconjugants were located around the hypocotyl and root areas. Randomly selected members of the indigenous bacterial community from both inoculated and uninoculated sprouts, as well as a representative part of the community that had received the plasmids, were characterized by polymorphisms of PCR-amplified ribosomal DNA (rDNA) spacer regions between the 16S and 23S genes, followed by partial 16S rDNA sequencing. This showed that the initially dominating genera *Erwinia* and *Paenibacillus* were gradually replaced by *Pseudomonas* on the fully developed sprouts. Transconjugants carrying either of the investigated plasmids mainly belonged to the genera *Pseudomonas* and *Erwinia*. The numbers of transconjugant cells did not reach detectable levels until 6 days after the onset of germination, at which point these species constituted the majority of the indigenous bacteria. In conclusion, the alfalfa sprouts provided an environment that allowed noteworthy frequencies of plasmid transfer from *P. putida* in the absence of selective pressure that could favor the presence of the investigated plasmids.

Studies of horizontal gene transfer among bacteria colonizing natural or seminatural environments are important for the understanding of the mechanisms leading to bacterial adaptation to a given set of conditions (9, 23, 32), for assessing risks associated with spread of resistance genes to bacteria that infect humans and animals, and for the identification of bacterial populations in the environment that might play a role as reservoirs for resistance genes (for reviews, see references 4 and 35). Investigations contributing to the understanding of the putative transfer of mobile genetic elements through the human food chain have mainly been focused on gene transfer in the digestive tract, whereas only very few reports on gene transfer taking place directly on human food exist (19).

Conjugal gene transfer requires cell-to-cell contact and is thus most likely to occur in environments with a high bacterial density. Alfalfa sprouts sold for consumption constitute such an environment, since production of the sprouts takes place at temperatures and humidity conditions that are favorable for bacterial growth, and it is known that raw sprout products contain a high number of bacteria (10, 24). The same is true for sprouts that are cultivated at home by the consumer from seeds sold for this purpose (15). The bacteria present on sprout products originate from those colonizing the seeds and are thus predominantly rod-shaped soil microorganisms (10). Sprouts are usually consumed raw, and mobile genetic elements present on the sprouts might be transferred to bacteria in the human gut after consumption. To our knowledge, there have been no studies of gene transfer on sprouts, and we felt that such an investigation would be an important contribution to the understanding of the putative spread of mobile genetic elements through the human food chain. In addition, sprouts constitute a good model environment for investigations of plasmid spread on plant surfaces in general, since a single sprout represents an entire plant, including root, hypocotyl, stem, and cotyledons.

We studied the transfer and establishment of two different conjugative plasmids from *Pseudomonas putida* KT2442 (2) to the indigenous bacterial community of alfalfa sprouts grown from seeds sold for use in home sprouting kits. The first plasmid chosen was pKJK5, which is isolated from the barley rhizosphere and confers resistance to tetracycline (28). Recent results from our laboratories have shown that this plasmid is a broad-host-range plasmid belonging to the IncP1 group (L. Mølbak, unpublished data). The second plasmid was TOL (also designated pWW0), which has a narrower host range (26) and carries genes that allow the bacterial host to degrade certain organic compounds, including benzyl alcohol (33, 34).

Fluorescent protein markers have previously proved to be useful in studies addressing spatial distribution of plasmid-carrying bacteria in environmental or seminatural settings (7, 11, 12, 22, 31). In the present study, the plasmids were tagged with the green fluorescent protein (GFP), whereas the donor

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strain was labeled with the *Discosoma* sp. red fluorescent protein (DsRed), thereby allowing in situ visualization of donors and transconjugants directly on the sprouts, as well as quantification by direct counting of single donor and transconjugant cells in homogenized samples. After isolation, green fluorescent transconjugants were characterized by polymorphisms of PCR-amplified ribosomal DNA (rDNA) spacer regions between the 16S and 23S genes (17). Partial 16S rDNA sequencing was used to further identify selected strains representing the different rDNA spacer groups. The indigenous nontransconjugant population present on the sprouts was characterized in the same way. This approach, including direct counting of donor and transconjugant population and characterization of indigenous transconjugant bacteria, has not previously been used in studies of gene transfer on sprouting plants.

Our results suggested that alfalfa sprouts cultivated from commercially available seeds constitute an environment allowing efficient conjugal gene transfer between the colonizing bacteria and that the formation of transconjugants occurred later than reported in other gene transfer studies (18, 20, 22, 27).

MATERIALS AND METHODS

Bacterial strains and plasmids. In all studies, the strain serving as donor was a derivative of *P. putida* KT2442 (2), named LM50 (L. Mølbak, J. E. Johansen, S. J. Sørensen, N. Kroer, and S. J. Binnerup, unpublished data), carrying two independent chromosomal insertions. One is constructed by insertion of the *lacI*^{q} into the chromosome of *P. putida* KT2442 by triparental mating (22). The other is a mini-Tn*5* cassette containing the *dsRed* gene (21) fused to the *Escherichia coli* ribosomal promoter, *rrnB*P1 (31), and was transferred to the chromosome of *P. putida* KT2442::*lacI*^{q} as described by Herrero et al. (16). LM50 is resistant to rifampin (100 μg/ml) and kanamycin (50 μg/ml).

Plasmid pKJK10 is a *gfp*-tagged derivative of the barley rhizosphere conjugative plasmid pKJK5 encoding resistance to tetracycline. For clarity, pKJK10 is referred to as pKJK5::*gfp* here. This plasmid, as well as the *gfp*-labeled TOL plasmid, have been described by Sengeløv et al. (28) and Normander et al. (22), respectively. Both of the plasmids carry mini-Tn*5* insertions (16) of the *gfpmut3b* (8) genes under the control of the LacI-repressible $P_{A1/O4/O3}$ promoter, as well as resistance to kanamycin.

Growth media. Donors harboring pKJK5::*gfp* were grown overnight in brain heart infusion (BHI; Oxoid) supplemented with tetracycline (Sigma) prior to inoculation onto alfalfa seeds and were selectively isolated from sprout samples on 10 mM sodium benzoate supplemented FAB minimal medium (25) containing tetracycline. Indigenous transconjugants were recovered from the sprouts on BHI agar supplemented with tetracycline and identified by the green fluorescence of the colonies.

Donors harboring the *gfp*-labeled TOL plasmid were grown prior to inoculation in BHI supplemented with kanamycin (Sigma) and then selectively isolated from sprout samples on 6 mM benzyl alcohol-supplemented FAB minimal medium containing kanamycin. Indigenous transconjugants were recovered from sprouts on BHI agar supplemented with kanamycin and identified by the green fluorescence of the colonies. In all cases, the concentration of tetracycline was 10 μg/ml, while the concentration of kanamycin was 50 μg/ml.

Isolation and detection of coliform bacteria, pseudomonads, and enterococci from sprout samples was performed on MacConkey agar no. 3, *Pseudomonas* CFC agar, and Slanetz and Bartley agar, respectively. Total counts of bacteria were enumerated on BHI agar. All media were purchased from Oxoid.

Growth of alfalfa sprouts and inoculation of donor bacteria. Four grams of organic alfalfa seeds (*Medicago sativa* L.) imported from the United States (Urtekram, 9550 Mariager, Denmark) was soaked for 1 h at 37°C in 25 ml of (i) tap water, (ii) donor strain *P. putida* LM50/pKJK5::*gfp*, or (iii) donor strain *P. putida* LM50/TOL::*gfp*. Donor bacteria were grown overnight, washed twice, and subsequently diluted 100-fold in tap water before use. Seeds were placed in the sprouting kit (Bergs Biosalat; Dako), from which the liquid was drained off.

The sprouting process was designed to imitate household conditions (consumer-scale). Three perforated horizontal trays stacked on top of an unperforated bottom tray composed the household sprouting kit. The upper tray worked as a lid; the seeds were located in the second tray. During growth some of the sprouts produced long roots moving downwards, and the third tray ensured an adequate distance to the bottom tray, where the surplus of water was collected. For watering, ca. 50 ml of unchlorinated tap water was poured twice a day into the upper tray and recollected as wastewater in the bottom tray. This amount of water, which contained fewer than 10 bacteria per ml, as verified by plating on BHI agar, was sufficient to ensure that the sprouts were kept humid. Temperature was kept constant at 20°C. All experiments were performed at least twice.

Analysis of sprout samples on solid media. At days 0, 1, 3, 6, and 9 after the seeds were placed in the sprouting kit, three subsamples of 10 seeds or sprouts were collected (by random selection) with sterile tweezers and placed in a stomacher bag. The samples were weighed, diluted 10-fold (wt/vol) in sterile water, and homogenized (Stomacher model 80 laboratory blender; Seward) for 2 min at maximum speed. Dilutions of the homogenized sprouts were spread on solid media for determination of the total numbers of bacteria, pseudomonads, coliform bacteria, and enterococci, respectively. In addition, the dilutions of sprout samples that had been inoculated with the donor strains were spread on solid media (described above) for identification of donor and transconjugant bacteria.

Platemating controls. In order to estimate the amount of gene transfer potentially taking place on the selective plates, 500-μl volumes of overnight culture of the donor strain and 500 μl of homogenized sprouts (without donor strain) containing potential recipient bacteria were mixed in different selected proportions and immediately spotted onto transconjugant selective plates. This assay was performed on each sampling day.

Direct quantification of donor and transconjugant bacteria by microscopy. Samples of homogenized sprouts were diluted and filtered through 25-mm-diameter black polycarbonate membrane filters (pore size, 0.2 μm; Osmonics, Vista, Calif.). The volumes filtered depended on the desired detection level and were chosen so that counting by eye was facilitated.

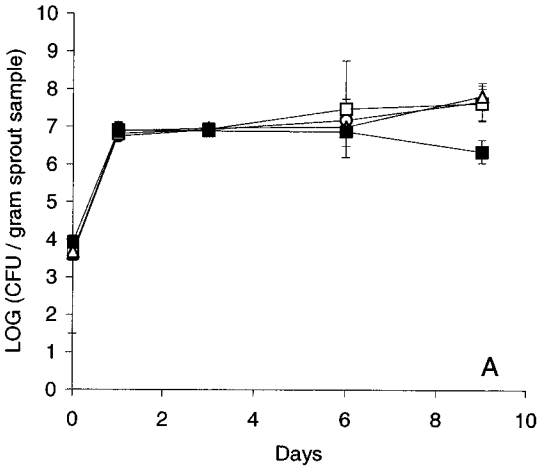
Bacteria were stained by placing the filters on a drop of 10 μg of DAPI (4′,6′-diamidino-2-phenylindole; Sigma)/ml and then washed twice by placing them on drops of distilled water.

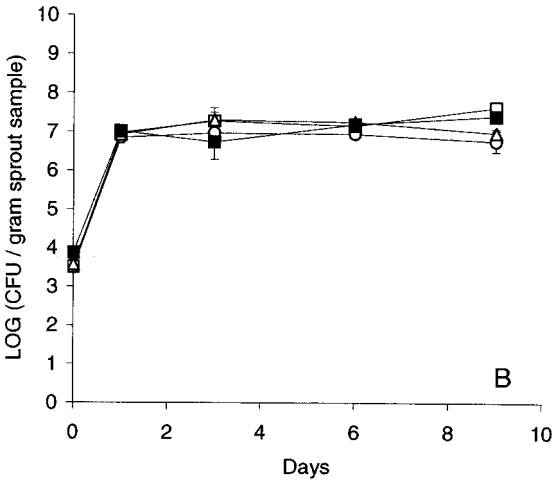
The numbers of DAPI-stained GFP- and DsRed-expressing cells were subsequently counted by using an Axioplan epifluorescence microscope (Carl Zeiss) equipped with a 50-W mercury lamp and the appropriate filter sets, allowing selective visualization of bacteria fluorescing at a given wavelength as desired (25).

Visualization of donor and transconjugant distribution on sprouts. On each day of sampling, three seeds or sprouts were picked from the home sprouting kits for microscopy. Each of the compartments—roots, seeds, stems, and leaves—was systematically investigated, and representative images of chosen areas were obtained. Visualization of GFP- and DsRed-tagged bacteria on sprouts was done by using a model TCS SP1 3 channel confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) equipped with an argon laser (458, 476, 488, and 514 nm) and two HeNe lasers (543 and 633 nm) and a variable spectrometric detection system that simultaneously monitored GFP and DsRed fluorescence. Images were processed for display by using Adobe Photoshop version 6.0.

Characterization of transconjugants and indigenous sprout bacteria. On days 1, 3, 5, and 9 of the experiment, ca. 30 colonies representing the indigenous sprout flora (including 10 from the uninoculated sprouts and 10 from the sprouts inoculated with each of the donor strains) were randomly picked, while the number of transconjugant colonies picked depended on the numbers of green colonies available. Several subcultivations (i.e., the transfer of green colonies to a new agar plate by using a sterile loop) were often required to obtain clean transconjugant colonies. A total of 394 colonies were isolated, including 120 representing the indigenous flora of the uninoculated sprouts, 118 representing the indigenous flora of spouts inoculated with a donor strain, 100 representing transconjugants carrying pKJK5::*gfp*, and 56 representing transconjugants carrying TOL::*gfp*. The 394 isolates were subjected to PCR amplification of the highly variable rDNA spacer regions between the 16S and 23S genes as described by Jensen et al. (17) by using the primers ITS-16S-1392-S-15 and ITS-23S-206-A-21 (P. Willumsen and B. M. Hansen, unpublished data). These isolates represented 20 different gel patterns and thus belonged to 20 different DNA spacer polymorphism groups. Of the 394 isolates, 59 were selected for partial 16S rDNA sequencing, ensuring that all DNA spacer groups were represented at least once.

PCR amplification of the 16S rDNA gene (first 500 bp) was performed with the primers SDBact0008aS20 and S*UNIV518Aa18 (1). PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Partial sequencing of the PCR products was carried out by MWG-Biotech AG by using an NEN Global IR^2 DNA Sequencer Li-COR and the primer SDBact0008aS20. The sequences obtained were compared to sequences in nucleotide databases by using the NCBI/BLASTN program (http://www.ncbi.nlm.nih.gov). The closest match was accepted as the closest relative.





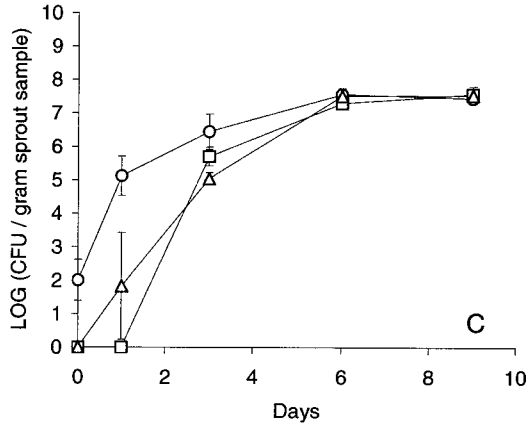


FIG. 1. Numbers of CFU of the total bacterial population (○), non-lactose-fermenting gram-negative bacteria (△), *Pseudomonas* including the donor strain (□), and the donor strain (■) measured on sprouts inoculated with donor bacteria containing either pKJK5::*gfp* (A), TOL::*gfp* (B), or without donor inoculation (C). The error bars represent the standard errors.

In vitro transfer of pKJK5::*gfp* and TOL::*gfp* to selected recipients. From the group of randomly isolated indigenous sprout bacteria, six representatives were chosen. The six isolates were collected on different sampling days and represented a number of different taxa on the uninoculated sprouts. Among the indigenous bacteria present at day 1, *Paenibacillus* sp. and *Erwinia* sp. were selected, while from day 3, another *Erwinia* sp. and a *Pseudomonas* sp. were selected. From days 5 and 9, strains of *Pseudomonas gessardii* and *Stenotrophomonas maltophilia*, respectively, were chosen. The six isolates were used as recipients of pKJK5::*gfp* and TOL::*gfp* in filter-mating experiments. From overnight cultures grown in BHI, 100 μl of donor and recipient strains (12 combinations) were mixed, placed on 25MM Whatman filters on nonselective agar plates, and incubated overnight at 30°C. Quantification of donor and transconjugant bacteria was done by fluorescence microscopy as described for sprout samples. The experiment was performed in triplicates.

RESULTS

Characterization of bacterial flora on alfalfa sprouts. Three days after introduction into home sprouting kits, most of the alfalfa seeds began to develop tiny sprouts. After 6 days, the sprouts appeared ready to eat, while advanced decay was observed after 9 days.

At the time of introduction into the home sprouting kit, the uninoculated seeds carried ca. 10^2 CFU/g, while the seeds inoculated with either of the two *P. putida* donor strains carried ca. 10^4 CFU/g. After 1 day of germination, the inoculated sprouts carried 10^7 CFU/g, and this number remained constant for the rest of the sampling period (Fig. 1A and B). On the uninoculated seeds, 10^5 CFU/g was detected after 1 day of germination. This number gradually increased to 10^7 CFU/g on day 6 and remained constant thereafter (Fig. 1C).

The donor bacteria constituted the major proportion of the bacteria present on the inoculated seeds (Fig. 1A and B), while bacteria belonging to the genus *Pseudomonas* were not detected on the uninoculated seeds until day 3 (Fig. 1C). A proportion of the bacteria present on the uninoculated seeds at day 1 represented non-lactose-fermenting gram-negative bacteria appearing as colorless colonies on MacConkey agar plates.

PCR amplification of the highly variable rDNA spacer regions between the 16S and 23S genes and subsequent partial 16S rDNA sequencing of randomly selected strains isolated from the uninoculated sprouts on BHI revealed that the dominant groups of bacteria at day 1 belonged to the genera *Erwinia* and *Paenibacillus.* With time, these genera were gradually replaced by *Pseudomonas* (Fig. 2). Low numbers of other bacteria (i.e., species of *Bacillus* and *Stenotrophomonas*) occurred occasionally (data not shown). Enterococci as defined by growth on Slanetz and Bartley agar or coliform bacteria as defined by red colonies on MacConkey agar no. 3 were not detected at any time (data not shown).

On alfalfa sprouts inoculated with donor bacteria, *Erwinia* and *Pseudomonas* were the only other genera found. Similar to what was observed for uninoculated sprouts, *Erwinia* spp. dominated the indigenous part of the microflora at day 1 but were gradually replaced by indigenous *Pseudomonas* spp. that dominated 9 days after the onset of the experiment (data not shown).

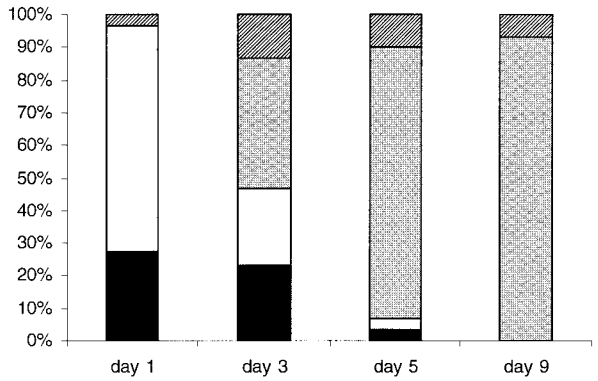


FIG. 2. Relative densities of major bacterial populations present on uninoculated sprouts at selected time points as identified by polymorphisms of PCR-amplified rDNA spacer regions and subsequent partial 16S rDNA sequencing. The results for *Pseudomonas* (▨), *Erwinia* (□), and *Paenibacillus* (■) spp. and for species either not identified or present in proportions of <5% (▨) are presented.

Transfer of pKJK5::*gfp* and TOL::*gfp* on sprouts. Direct counting of green (GFP), red (DsRed), and blue (DAPI) bacteria in sprout samples revealed the average numbers of transconjugants, donors, and total bacterial cells on the sprouts, respectively. In all cases the total numbers of bacteria per gram of sprout sample were ca. 10^8, gradually increasing to just less than 10^9 at the end of the experiments. The numbers of donor cells were generally between 5- and 10-fold lower (Fig. 3).

After 3 days of germination, the numbers of transconjugants were below or close to the detection limit of approximately two transconjugants per gram of sprout sample for both of the investigated plasmids (data not shown). After 6 days, however, ca. 10^4 transconjugants per g of sample were detected on the sprouts inoculated with *P. putida* LM50/pKJK5::*gfp*, and no noteworthy change in this number was seen after 9 days (Fig. 3A). The highest measured frequency of pKJK5::*gfp* transconjugant to donor cells occurred on day 6 and was ca. 3.4×10^{-4}.

The sprouts inoculated with *P. putida* LM50/TOL::*gfp* carried on the average 3.4×10^1 transconjugants per g of sample on day 6; this level increased to 2.7×10^2 per g of sample on day 9 (Fig. 3B). The highest measured frequency of TOL::*gfp* transconjugant cells per donor cell occurred on day 9 and was 2.0×10^{-6} per g of sample.

PCR amplification of the variable rDNA spacer regions between the 16S and 23S genes ($n = 394$), followed by partial 16S rDNA sequencing of a subset ($n = 59$) of strains together representing all of the 20 rDNA spacer groups identified, showed that most bacteria that had received plasmid pKJK5::*gfp* ($n = 100$) belonged to the genera *Erwinia* (64%) and *Pseudomonas* (28%), whereas 8% were not identified. Also, most indigenous sprout bacteria that had received TOL::*gfp* ($n = 52$) were identified as *Erwinia* (41%) and *Pseudomonas* (34%) spp., whereas 25% remained unidentified.

Control experiments performed to verify that the transconjugants picked for PCR amplification were not the result of conjugation events that took place after sampling showed that no transfer occurred on the selective plates used for transconjugant isolation.

Spatial distribution of donors and transconjugants on sprouts. Systematic confocal laser scanning microscopy of the sprouts revealed that the *P. putida* LM50 donor cells heavily colonized the alfalfa sprouts from day 1.

The rhizoplane was generally covered by a thick matrix of donor bacteria (Fig. 4A), many of which were motile. On the stem, the donor cells formed a monolayer, and the highest densities were observed in the curvatures of the epidermis cells. Transfer of pKJK5::*gfp* and TOL plasmids preferentially took place on the roots (Fig. 4A) and particularly at the hypocotyl (Fig. 4B) of the alfalfa seedlings. However, transconjugant cells were occasionally observed on the stem. Most of the bacterial cells that had received either of the plasmids were rod shaped and were observed as single cells, as well as clusters or chains of bacteria (Fig. 4B and C).

In vitro transfer of pKJK5::*gfp* and TOL::*gfp* to selected recipients. Filter-mating experiments showed that in vitro transfer of pKJK5::*gfp* occurred readily from the donor strain to three of the selected bacterial isolates naturally present on

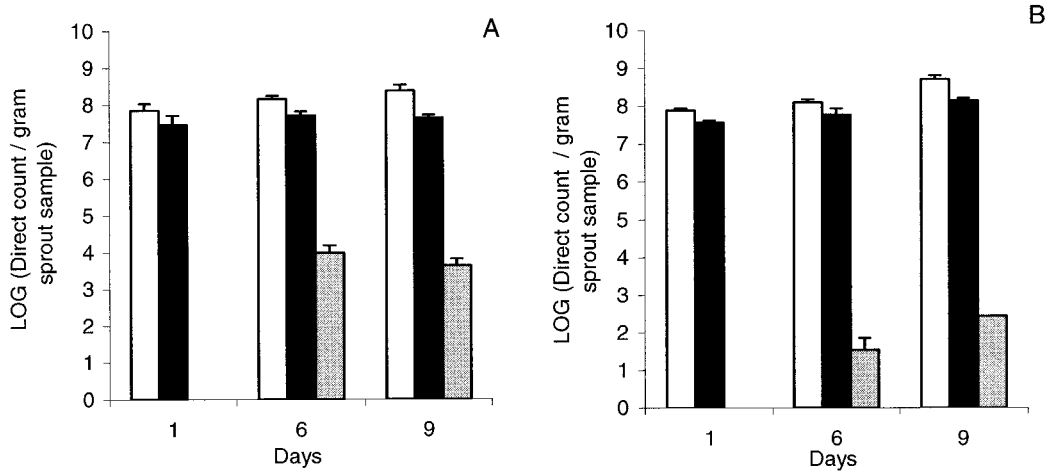


FIG. 3. Direct counts of total bacteria (□), donor cells (■), and transconjugant cells (▨) counted in sprout samples at days 1, 6, and 9 after inoculation with donor cells containing either pKJK5::*gfp* (A) or TOL::*gfp* (B). The error bars represent the standard errors.

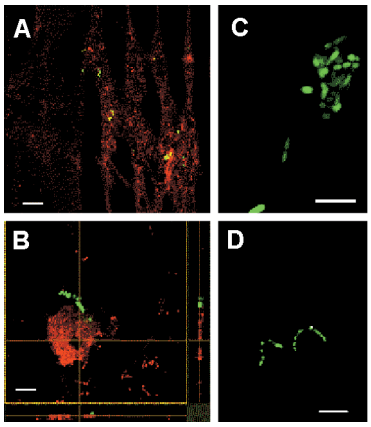


FIG. 4. Confocal laser scanning micrographs of alfalfa sprouts 6 days after the onset of germination and inoculation with red fluorescent donor bacteria *P. putida* LM50/pKJK5::*gfp*. Bacteria belonging to the indigenous microbial population on the sprouts, which have received the pKJK5::*gfp* plasmid, appear green. (A) Root area heavily colonized by donor cells. Several transconjugant bacteria are visible. Scale bar, 20 μm. (B) Xyz scan of hypocotyl area with microcolony of donor and transconjugant bacteria. Scale bar, 5 μm. (C and D) Micrographs of transconjugant bacteria with different cell morphologies. Scale bar, 5 μm. Observations of sprouts inoculated with *P. putida* LM50/TOL::*gfp* were similar to those of sprouts colonized with *P. putida* LM50/pKJK5::*gfp* (data not shown).

sprouts: one *Pseudomonas* sp. and two different isolates of *Erwinia* (isolates 1 and 2). However, filter-mating experiments with the donor strain containing TOL::*gfp* resulted in detectable transfer only to one of the six tested isolates: *Erwinia* isolate 2. Transfer of pKJK5::*gfp* to *Erwinia* isolate 1 and *Pseudomonas* sp. occurred at relatively high frequencies of ca. 5.6×10^{-2} (standard error of the mean [SEM] = 3.2×10^{-3}) and 1.6×10^{-2} (SEM = 5.5×10^{-3}) transconjugant per donor, respectively. Transfer to the third recipient, *Erwinia* isolate 2, occurred with significantly lower frequencies of ca. 1.5×10^{-4} (SEM = 1.0×10^{-4}) for pKJK5::*gfp*, and 7.2×10^{-4} (SEM = 1.7×10^{-4}) for TOL::gfp. Similar in vitro mating experiments with indigenous isolates of *Paenibacillus* sp., *S. maltophilia*, and *P. gessardii* as recipients did not result in detectable transfer of either of the investigated plasmids (data not shown). Consistently, these isolates represented rDNA spacer groups, which did not include transconjugants formed on sprouts.

DISCUSSION

Within the field of food microbiology, several studies addressing gene transfer in the intestinal tract of animals or humans have been performed. However, studies of gene transfer occurring directly on food products also contribute significantly to an understanding of the putative spread of genetic elements among bacteria throughout the human food chain.

Vegetables sold as ready-to-eat products are often colonized with a large amount of bacteria (10, 24). The conditions on the surface of such products are likely to be conducive to conjugational gene transfer, since it requires cell-to-cell contact, and for most plasmids present in gram-negative bacteria this takes place preferentially between surface-associated bacteria (5, 6). Alfalfa sprouts, cultivated in a home sprouting kit, provided a very useful nonsterile model system that allowed us to follow the development and decay of entire plants. The absence of soil in the system reduced the number of parameters to consider when interpreting the results, made the microscopy much easier, and reduced the necessary manipulations to a minimum. Also, a low content of chlorophyll, which is highly autofluorescent, facilitated the microscopy.

Our study included a rough mapping of the dominating groups of indigenous aerobic bacteria present on organically produced alfalfa seedlings during their development from seeds to edible and subsequently decaying sprouts. To the best of our knowledge, this has not previously been done. Bacteria belonging to the *Erwinia* and *Paenibacillus* genera dominated the microbial flora originally present on the seed but, as time progressed, these were outnumbered by pseudomonads (Fig. 1 and 2). These observations are consistent with another study showing that mainly rod-shaped bacteria are present on the surface of alfalfa sprouts (10).

Comparison of numbers from direct counts (Fig. 3) with data obtained by plating (Fig. 1) indicated that the donor cells were generally fully culturable, whereas a fraction of the total indigenous bacteria were not capable of growth on the media used in the present study. This should be kept in mind when the characterization of the indigenous sprout flora (Fig. 2) is evaluated, since only cultured bacteria were sequenced and identified. Also, it must be taken into account that the chosen agar medium (i.e., BHI) inevitably influenced which genera were found. The choice of this medium was based on the assumption that sprouts, which are known to be heavily colonized with bacteria (10, 24), represent an environment rich in nutrients, and that a rich medium would therefore support growth of the major fraction of sprout-associated indigenous bacteria.

Our data showed that the surface of alfalfa sprouts is an environment allowing plasmid transfer and that, in this environment, *Erwinia* and *Pseudomonas* spp. acted as recipients for both investigated plasmids. The DsRed-labeled *P. putida* donor strains readily colonized the surface of the sprouts (Fig. 1), and transfer of the GFP-labeled TOL and pKJK5 plasmids occurred (Fig. 3), resulting in transfer ratios comparable to (or lower than) the ratios obtained in other reports on *Pseudomonas* plasmid transfer in natural or seminatural systems (3, 22, 30).

Ratios of transconjugants per donor obtained in the filter-mating experiments by using selected indigenous isolates as recipients and designed to give a maximal yield of transconjugants were higher than the ratios observed on the sprouts. Isolates of *Paenibacillus* sp., *S. maltophilia*, and *P. gessardii*, which were present in significant numbers on the sprouts but not identified among the transconjugants, did not receive any of the two investigated plasmids in filter-mating experiments. *Erwinia* and *Pseudomonas* spp. were both identified among the transconjugants carrying either of the investigated plasmids, but the TOL::*gfp* plasmid was only transferred to one of the three selected representatives of these strains in filter-mating experiments. This indicates that the phenotype defined as the "putative TOL recipient" is not necessarily affiliated with a given species but more likely is affiliated with a given isolate. The isolates picked from the background microbial flora of uninoculated sprouts and subsequently used as recipients in filter-mating experiments may not have been identical to those identified as transconjugants from the sprout samples, and it is

known that conjugation ratios can vary considerably within different isolates of the same species (14).

On the sprouts, we observed a higher maximal frequency of transconjugants per donor cell for pKJK5::*gfp* (3.4×10^{-4}) than for the TOL plasmid (2.0×10^{-6}). We suggest that one explanation for this could be the difference in host range for these two plasmids. Although it has been shown that only intraspecies transfer of TOL takes place in soil (26), pKJK5 is an IncP1 plasmid and thus able to transfer to a number of different genera within the group of proteobacteria (Mølbak, unpublished). However, as mentioned above, characterization of transconjugant cells suggested that the indigenous bacteria acting as recipients for either of the plasmids belonged to the same genera (*Pseudomonas* and *Erwinia*). Still, as was the case for the characterization of indigenous sprout bacteria based on rDNA spacer regions, the choice of culture medium might have influenced the detection of these particular genera. An important difference between the two plasmids investigated was that filter-mating experiments to selected indigenous recipients showed generally higher transfer frequencies for pKJK5::*gfp* than for TOL:*gfp*. We therefore speculate that the lower mating efficiency of TOL donors than of pKJK5 donors observed on sprouts might be solely related to the maximal efficiency with which these plasmids are able to transfer.

Many reports on gene transfer in natural ecosystems show that transfer takes place, if at all, immediately after the introduction of a donor strain (13, 18, 20, 22). Still, in the present study, we detected no transconjugants until 6 days after introduction of the donor strain (Fig. 3) even though the concentration of donor bacteria reached its maximum already the first day after inoculation onto the seeds (Fig. 1). We suggest that the most important explanation for this is that the combined fraction of putative *Erwinia* and *Pseudomonas* recipients was initially low (Fig. 2). In some of the listed previous investigations (18, 20), a known recipient strain is added to the ecosystem and is thus present in relatively high numbers at the time of donor introduction. In the other studies (13, 22), which investigated plasmid transfer to indigenous microbial flora, the ecosystems were in a stable condition at the time of donor introduction, and it is therefore unlikely that large changes in the amount of putative recipients occurred as in the present study.

It is important to note that, unlike most other investigations of plasmid transfer, our study took place in an ecosystem that was undergoing a continuous development during germination of the seed and development of seedlings. In addition to the availability of recipients, other factors that were changing during the experiment and that might influence gene transfer could include the physical conditions on the surface of a seed versus that of a mature sprout. Transfer of each of the investigated plasmids took place preferentially on the root (Fig. 4), which was not fully developed until 6 days after the onset of germination, i.e., on the same day that the first transconjugants were detectable. Some studies indicate that plasmid transfer happens most efficiently when the availability of bacterial substrate is relatively high (29), and for the TOL plasmid it has been demonstrated that the physiological state of the recipient cells influence their ability to participate in transfer (7). We suggest that high concentrations of plant exudates around the root and the hypocotyl causes a high density of donors and putative recipients and perhaps also enhanced plasmid transfer and that these factors together explain why the large majority of transconjugants was observed in these zones of the seedlings (Fig. 4). An additional explanation could be that the roots of the sprouts growing in the home sprouting kit were exposed to higher humidity than other parts of the plants and that the presence of a liquid film on the surface of the roots would promote diffusion of nutrients, as well as bacterial motility. The latter would increase the number of encounters between donor and recipient bacteria and thus the frequency of transfer events. However, the decay of the sprouts that had taken place in the period between days 6 and 9 did not result in an increased amount of transfer (Fig. 3), even though it can be assumed that decay results in an increased release of nutrients as well as liquid from decomposed plant parts.

Some of the observed (green) bacterial cells on the sprout surface might originate from proliferation of the initially formed transconjugants, which would be expected to occur in areas with high substrate availability. Based on the observation that no increase in transconjugant numbers occurred from day 6 to day 9 (Fig. 3), we speculate that presence of the investigated plasmids did not result in increased bacterial fitness. On the contrary, transconjugants formed on the sprouts might have a lower potential for growth and colonization of the alfalfa plants than before reception of the plasmids due to the metabolic cost of expressing extra traits, which do not constitute a selective advantage in the given ecosystem. Since, to the best of our knowledge, the environment constituted by alfalfa sprouts did not contain benzyl alcohol, it was expected that the TOL plasmid would not provide the transconjugants with increased fitness. The rhizosphere plasmid pKJK5 has not yet been sequenced and might have provided the bacteria with an advantage by expression of uncharacterized phenotypes but, as explained here, this did not seem to be the case.

The present results can be relevant in the assessment of risks associated with the microbiological safety of ready-to-eat sprout products. We believe that our observations can be extrapolated to many other vegetables that are consumed raw, and we emphasize that mobile genetic elements can enter the human gastric system not only through food deriving from animals but also from ready-to-eat vegetable products. In addition, our observations contribute to the understanding of gene exchange between bacteria in dynamically developing ecosystems and underline the importance of describing the succession of bacterial populations indigenously present in such systems.

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Chapter 4.

Factors Affecting the Frequency of Conjugal Transfer in the Rhizosphere of Barley and Pea

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ABSTRACT

In this study the importance of bacterial density and distribution on the frequency of conjugal transfer in the rhizosphere of barley and pea was studied. *Pseudomonas putida* KT2442 was used as donor strain. It contained the natural broad host range IncP plasmid pKJK5 tagged with *gfp*. The donor strain was inoculated onto the seeds, while the recipient strain, *P. putida* LM24, was inoculated into the growth medium (soil or vermiculite). The recipient had a chromosomal insertion of the red fluorescent protein gene, *Dsred.* In this study the plasmid transfer ratios (transconjugant/donor) were as well higher for pea than for barley grown in ether vermiculite (pea_{max}= 2.5 x 10^{-2} and $barley_{max}$= 3.3 x 10^{-3}) or soil
(pea_{max}= 7.9 x 10^{-4} and $barley_{max}$= 7.9 x 10^{-5}) for all the sampling days. By using a combination of in situ visualization of single cells on the rhizoplane, macroscopic visualization of the colonization pattern of donor and transconjugant colonies in the phytosphere, and a quantification of the amount and quality of the root exudates, we were able to demonstrate that the higher transfer frequency in the pea rhizosphere was the consequence of approximately the double amount of exudates excreted from pea compared to barley. The higher exudate production resulted in a higher density of the donor cells, and hence, a higher probability of donor-to-recipient contact.

INTRODUCTION

Studies of conjugal transfer of broad host range plasmids in plant microcosms are important in terms of risk assessment of the spreading of genes and, for instance, in bioaugmentation strategies to spread plasmids having biodegradative capacity of specific xenobiotic compounds. Coating of seeds with bacteria may be an effective way of introducing bacteria for ether biocontrol or bioremediation purposes (Brazil et al. 1995).

The efficiency of bacterial conjugation depends on the conjugative element as well as the donor and recipient cells, and may be influenced by numerous biotic and abiotic factors (for a review, see Dröge et al. 1999). Several studies have shown that the phytosphere is conducive of conjugation compared to bulk soil (van Elsas et al. 1988; Lilley et al. 1994; Dröge et al. 1999). The elevated transfer frequency in the phytosphere has been suggested to be due to high cell density and/or that enhanced activity from exudates (van Elsas et al. 1988; Kroer et al. 1998). The causal relation-

ship between conjugation, root exudate production, metabolic activity and spatial distribution has, however, not been unequivocally resolved.

The aim of this study was to investigate the importance of bacterial density and distribution on the frequency of conjugal transfer in the rhizosphere of barley and pea. Previous studies by Schwaner and Kroer (2001) showed the pea rhizosphere to be ten times more conducive of transfer of RP4 than the rhizosphere of barley. As donor strain we used *Pseudomonas putida* KT2442 containing the natural broad host range IncP plasmid pKJK5 tagged with *gfp* (Sengeløv et al 2001; Mølbak et al. 2003). The donor strain was inoculated onto the seeds, while the recipient strain, *P. putida* LM24, was inoculated into the growth medium (soil or vermiculite). The recipient had a chromosomal insertion of the red fluorescent protein gene, *Dsred.* By using a combination of in situ visualization of single cells on the rhizoplane, macroscopic visualization of the colonization pattern of donor and transconjugant colonies in the phytosphere, and a quantification of the amount and quality of the root exudates, we were able to demonstrate a relationship between the higher transfer frequency in the pea rhizosphere and an elevated root exudate production. To the best of our knowledge, this is the first time that a causal relationship between production of root exudate and conjugal gene transfer has been established.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Characteristics of the used strains and plasmids are listed in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) medium at 30°C. *P. putida* strains were grown at 30°C in FAB minimal medium [1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.01 mM $FeCl_2$, 0.15 mM $(NH_4)SO_4$, 0.33 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 0.5 mM NaCl] (Ramos et al. 2000). Minimal medium contained 10 mM sodium citrate as the sole carbon source. When needed, antibiotics were added at the following concentrations: kanamycin (Km) 25 μg/ml, nalidixic acid (Nal) 50 μg/ml, tetracycline (Tc) 10 μg/ml, and rifampicillin (Rif) 50 μg/ml. A mini-Tn*5* gene cassette with the *Dsred* gene fused to the *E. coli* ribosomal promoter, *rrnB*P1 (Tolker-Nielsen et al. 2000) was inserted into the chromosome of *P. putida* SM1464 by triparental mating according to Herrero et al. (1990). *E. coli* Mv1190λ*pir*/TTN151 and *E. coli* HB101/pRK600 were used as donor and helper strains, respectively. A clone, which grew at the same rate as SM1464 in minimal

medium at 30°C, was selected and named *P. putida* LM24. The rhizosphere plasmid pKJK5::*gfp* (Sengeløv et al. 2001) was inserted into *Pseudomonas putida* KT2442 from *P. putida* KT2440/pKJK5::*gfp* by biparental mating as previously described (Mølbak et al. 2003).

Rhizosphere microcosms. Microcosms consisted of 50-ml plastic tubes filled with 45 ml autoclaved vermiculite or 55 g non-sterile soil. The soil was a sandy loam obtained from Taastrup near Copenhagen, Denmark (Kragelund and Nybroe 1996).

Bacterial inocula for seed coating or soil inoculation were prepared as follows. A colony of the *P. putida* strain was inoculated in 10 ml of FAB minimal medium containing 10 mM sodium citrate as the only carbon source, and the culture was incubated at 20°C until the stationary phase. Cells were washed twice in M8 buffer (22mM Na_2HPO_4, 22 mM $KHPO_4$, 100 mM $NaCl_2$) prior to use

Barley (*Hordeum vulgare* var. Alexis) and pea (*Pisum sativum* var. Ping Pong) seeds were coated with bacteria by soaking the seeds for 30 min at 20°C in a 10-ml bacterial inoculum of *P. putida* KT2442/pKJK5::*gfp* preparation adjusted turbidmetrically to the appropriate cell density. One donor-coated seed per microcosm was sown at about 1 cm depth. The recipient strain, *P. putida* LM24, was inoculated into the vermiculite and soil by diluting the appropriate amount of bacteria into germination buffer [22mM Na_2HPO_4, 22 mM $KHPO_4$, 100 mM $NaCl_2$, 1 mM $MgSO_4$, 6 mg/L Fe-cyt (28% Fe), 8.2 µM $ZnCl_2$, 0.6 µM H_3BO_4, 19.8 µM $MnCl_2$, 3.6 µM $CoCl_2$, 35.7 µM $NiCl_2$, 51.1 µm $CuCl_2$, 24.2 µM Na_2MoO_4] or sterile water used. A total volume of 30 ml germination buffer was added to the vermiculite while the soil moisture content was adjusted to 15% (wt/wt) with sterile water.

The tubes were placed in a glass beaker containing moist filter paper, and the system placed in transparent plastic bags in a growth chamber at 20°C to 22°C and a 12h:12h light-dark cycle.

Sampling of rhizosphere microcosms. At each sampling time, five replicate tubes containing vermiculite and five replicate tubes containing soil were sacrificed. The seedlings were carefully removed and the roots were separated from the seed and placed in 10-ml plastic tubes containing 5 ml cold (4°C) 0.9% NaCl. The bacteria were extracted from the rhizosphere by vortexing for 3 min and dilutions plated on FAB plates containing 10 mM sodium citrate and appropriate antibiotics for the

selection of donors, recipients and transconjugants. The plates were incubated at 30°C for 24 h before counting of numbers of CFU.

At each sampling time, the significance of mating on the transconjugant selective plates was assessed. This was done by combining extracts of tubes inoculated only with the donor with extracts of tubes inoculated only with the recipient and plating of the mixtures on transconjugant selective plates. No false positives were ever detected.

Bacterial distribution at the macroscale level. The distribution of the donor and transconjugant cells along the roots of the barley and pea plants was assessed at each sampling time. Two replicate roots of a barley/vermiculite and a pea/vermiculite microcosm were harvested by carefully removing the plants from plastic tubes. The plants were placed on separate FAB agar plates with 10 mM sodium citrate. Similarly, two other roots of each microcosm were placed on FAB agar plates containing 10 mM sodium citrate and 50 μg/ml Nal (Nal was added to prevent mating on the plates). Care was taken to insure that the tweezers were flamed before each touch on the agarplate and that the full length of the roots was in contact with the agar surface. The roots were photographed with white light using a CCD camera (Hamamatsu C5985). After one h of incubation, roots were removed and the plates incubated for another 18 h at 30°C. After incubation, bacterial colonies on plates without Nal were replica plated onto new FAB plates with Rif and Tc to allow growth of the donor cells. Colonies on FAB agar plates containing Nal were replica plated onto new FAB plates with Nal and Tc to allow growth of the transconjugant cells. Following incubation for 24 h at 30°C, plates were illuminated with UV-light and the auto fluorescent light from the colonies recorded by a CCD camera. Images of the roots were superimposed on the images of the bacterial colonies by using the Photoshop software (Adobe, Mountain view, Calif.).

Bacterial distribution at the macroscale level with growth inhibitors. Pea seedlings inoculated with donors were grown in vermiculite microcosms amended with different concentrations of nalidixic acid (0; 50, 100 mg/L) or carbenicillin (0, 50, 100, 300 mg/L). After 6 days of incubation , the colonization pattern of three replicate pea seedlings were harvest at day 6 and visualized as described above.

Bacterial distribution at the microscale level. Rhizosphere chambers were used for *in situ* visualization of donors, recipients, and transconjugants on the rhizos-

plane of the pea and barley seedlings. The rhizosphere chambers were constructed and used basically as described by Ramos et al. (2000). The chambers (length, 55 mm; width, 20 mm; depth, 10 mm) were constructed by gluing with silicone a plastic tube on top of a microscope slide whereupon a coverslip (24 mm by 60 mm) was sealed. The chambers were filled with vermiculite into which 1 ml recipient culture was inoculated. Pea and barley seeds, pre-germinated for one day and coated with donor bacteria, were sown just beneath the surface of the vermiculite. As controls, rhizosphere chambers consisting of donor-coated and pre-germinated seeds grown in uninoculated vermiculite, and recipient-coated seeds grown in uninoculated vermiculite were set up. Rhizosphere chambers were incubated at 20°C in the dark for 4-5 days. To ensure that the root system was localized in the vicinity of the coverslip, the growth chambers were placed on a rack at an angle of approximately 45°. At different time points, images of the rhizosphere bound cells were acquired with confocal microscopes (models TCS4D and TCS SP1; 3 channel Scanning; Leica Microsystems Heidelberg GmbH, Germany) equipped with detectors and filter sets that simultaneously monitored Gfp and Dsred. Simulated fluorescence projections and vertical cross-sections were generated using the IMARIS software package (Bitplane AG, Zürich, Switzerland). Images were processed for display using the Photoshop software 6.0.

Quantitative and qualitative measurements of root exudates. To estimate exudate production by the barley and pea seedlings, sterile seeds were prepared and grown aseptically. The barley seeds were sterilized by sequential treatment with 50% H_2SO_4 for one hour, 2 rinses in sterile water, and 6 min. in 0.1% $AgNO_3$. Finally, seeds were rinsed three times in sterile water. Bracts were removed manually after treatment with H_2SO_4. Pea seeds were sterilized as previously described for bean (Normander et al., 1998). After sterilization, seeds were pregerminated on moist filter paper for 48 h. To test for sterility, a subsample of 10 to 20 seeds was placed on LB agar plates for 48 h at 20°C.

Ten pregerminated seeds were sown in air tight glass jars (10 cm high and 10 cm wide) containing 200 ml sterilized vermiculite saturated with 130 ml germination buffer. The glass jars were incubated in a growth chamber at 20°C to 22°C and a 12:12 h light-dark cycle for 3 or 6 days.

Extraction of the exudates was done in a laminar flow bench. The glass jar was opened and 130 ml germination buffer added, followed by gentle stirring for 3 min. The solution was then decanted and filtered through a 0.2 µm Micron PES membrane (Frisenette; Ebeltoft; Denmark). To test for sterility, an aliquot of 100 µl was removed before the filtration and spread on a LB agar plate and incubated at 20°C for 5 days. The sterile exudate solutions were stored at 4°C. The procedure was repeated three times in order to obtain enough of the root exudates.

The content of total organic carbon was measured on a Shimadzu TOC 5000 Analyzer (oxidation at 680°C, Pt-covered alumina beads used as catalyst) (Kroer et al. 1998). To assess potential qualitative differences between the barley and pea root exudates with respect to support bacterial growth, an exponentially growing culture of *P. putida* KT2442/pKJK5::*gfp*, washed two times in 0.9% NaCl and incubated for 24 h at 30°C, was inoculated to an optical density of 0.005 (OD_{450} nm) in the different untreated exudate solutions and grown in batch experiments at 30°C. Growth of the strain in the root exudate solutions was followed by measuring the optical density at appropriate time points

Statistical tests. A multiple ANOVA analysis was done to test if there were significant differences between the means of the transconjugant populations in the barley and pea microcosms at the 95.0% confidence level.

Multiple Range Tests were used to determine significant difference among the different plant systems at the 95.0% confidence level.

RESULTS

Rhizosphere microcosms. The amount of donor bacteria (CFU/ g root) extracted from the four different microcosms types (Barley and Pea grown in ether vermiculite or soil) were not different at Day 1 ($P= 0.082$), but over the whole period there was a significant difference ($P<0.01$) between the different microcosms types (except within two of the combinations which were between pea grown in vermiculite and pea grown in soil and between barley grown in vermiculite and pea grown in soil). The donor was inoculated at densities of 4.8×10^6 CFU/seed on the pea seeds and 6.8×10^6 CFU/seed on the barley seeds (Fig. 1A).

The amounts of recipient bacteria extracted from the plant roots of barley and pea were not different through the experiment. In contrast, there was a significant

difference between recipient bacteria extracted from roots grown in vermiculite and soil ($P<0.01$). *P. putida* LM24 was inoculated to the soil at a density of 3.6×10^6 CFU/ g soil and to vermiculite at a density of 4.8×10^5 CFU/ ml germination buffer (Fig. 1B).

Numbers of transconjugant bacteria were different between the four microcosm types from Day 1, and stabilized at this level of CFU/g plant root for all 8 days (Fig. 1C). The amount of transconjugant extracted from pea and barley grown in vermiculite was approximately 1×10^5 CFU/g pea root and
1×10^4 CFU/g barley root, compared to pea and barley grown in soil that had values at approximately 1×10^3 CFU/g pea root and 1×10^2 CFU/g barley root. Transconjugant bacteria extracted from three of the barley samples grown in soil at day 6 and all five of the barley samples grown in soil at day 6 were below detection level.

The transfer efficiency (T/D) was significantly different ($p<0.01$) in T/D between the different plant systems both at day 1 and for the entire sampling period. The highest transfer ratio was observed in pea grown in vermiculite (T/D = 0.25).

Bacterial density and distribution at the macroscale level. This novel technique is an easy way to visualize colonization patterns of tagged bacteria at a macroscopic level on plant roots. *P. putida* KT2442 cells grown on FAB agar plates produce fluorescent siderophores that fluoresces when illuminated with UV light and simply by making an overlay of the fluorescence and the white light-picture of the roots it was possible to see where the bacteria had colonized the roots (Fig. 3).

On barley seedlings, donor colonies were only observed at the root base whereas pea roots were colonized along the whole root. Recipient bacteria were present all along the roots but for simplicity this information is not added to the pictures.

In vermiculite, the colonization patterns of the transconjugant bacteria were similar to what was observed for the donor bacteria. In soil microcosms, few transconjugant colonies were observed and only at the root base for all the tested plants.

In general, it was observed in the first 3 days that barley roots grew faster than pea roots, and the root diameter of barley was smaller than that of pea. The experiment, together with CFU measurements, was repeated three times and the results were reproducible.

Bacterial density and distribution at the microscale level. In general, pea roots were more heavily colonized by donor bacteria than the barley roots (Fig. 4). Also, it was possible to observe dense biofilm layers of donor bacteria at the root base. On pea, transconjugant cells were most often observed in the outer layer of the bacterial biofilm. On barley roots, cells were found in scattered microcolonies of single layers and particular at the root base of the seedlings, on roothairs and at the root tip. Motile bacteria were frequently seen on pea roots, whereas they were rare on barley roots. Recipient cells were the dominant colonizer of barley roots, but on pea roots donor and recipient microcolonies were more equally distributed. Transconjugant cells were observed on both plants as colonies mixed with donor and recipient cells.

Production and utilization of root exudates. The average amount of carbon were produced during 6 days of incubation were 2.71 (± 0.40) mg C/plant/day and 4.70 (±0.86) mg C/plant/day for barley and pea seedlings, respectively.

To test the quality of the exudates solutions as growth substrates batch experiments with *P. putida* KT2442(pKJK5::*gfp*) grown in the undiluted exudates samples were performed. No lag phase was observed and a max. doubling time of 40 min. was measured (Fig. 6). There was a linear correlation (R^2=0.99) between the maximum biomass (maximum optical density) of the different cell cultures and the exudates amount of total carbon monitored (Fig. 7).

Bacterial distribution at the macroscale level with growth inhibitors. It was not possible to visualize any difference in the colonization pattern of donor bacteria inoculated on pea seedlings in microcosm containing the two bacteriostatic antibiotics that inhibits cell divisions nalidixic acid (Fig .8) or carbenicillin. The growth of the pea seedlings was inhibited when grown in 100 mg/ L nalidixic acid (Fig. 8).

Discussion

In the context of biocontrol, colonization has been defined as the ability of bacteria, applied on the seedling, to reach the growing root tip (Lugtenberg and

Dekkers 2001). The aim of this study was to elucidate the importance of bacterial density and distribution on the frequency of conjugal plasmid transfer in the rhizosphere of barley and pea, when the donor was inoculated on the seeds. In a previous study, Schwaner and Kroer (2001) found that the pea rhizosphere was 10 times more conducive of plasmid transfer (between a *Pseudomonas fluorescens*/RP4 and a *Serratia plymuthica*) compared to the barley rhizosphere when the bacteria were inoculated together in the sand or the soil used. They concluded that the difference in transfer between the two rhizospheres could not be attributed to different metabolic activities of the bacteria.

In this study we compared plasmid transfer between *Pseudomonas putida* KT2442/pKJK5::*gfp* and *P. putida* LM24. pKJK5 is an IncP plasmid that original was isolated from the barley rhizosphere (Sengeløv et al. 2001 and Mølbak et al. 2003). In the present study we found a significant higher amount of donor bacteria (CFU/g root) in pea rhizosphere compared to barley, when grown in vermiculite or soil. *Pseudomonas putida* KT2440 has previously shown differences in the colonization of corn and broad bean (Molina et al., 2000).

There was a significant difference in the amount of T/D/ between the different plant systems for all 8 days. The pea rhizosphere
(max 2.5×10^{-2} and 7.9×10^{-4} in vermiculite and soil, respectively) had a higher transfer ratio than the barley rhizosphere (max 3.3×10^{-3} and 7.9×10^{-5} in vermiculite and barley, respectively). This is consistent with the factor 10 difference found by Schwaner and Kroer (2001) in spite of the differences in the experimental setups.

The transfer frequencies measured in the vermiculite plant systems were similar to those found by Sengeløv et al. (2001), who used the same plasmid, donor strain, and soil type. They found transfer frequencies of 4.4×10^{-3} in the barley rhizosphere and 8.1×10^{-2} in the barley spermosphere sampled after 48 hours. The higher transfer frequencies compared to ours could be due to the fact that Sengeløv et al. (2001) inoculated both the donor and the recipient cells into the soil. This means, donors and recipient were in contact throughout the experiment, whereas in our case, donor and recipient contacts were only established as the root grew.

The difference in transfer ratio between soil and vermiculite samples may be due to less frequent cell-to-cell contact between recipients and donor bacteria in soil possibly due to presence of indigenous microbes (Stotzky 1989; Top et al. 1990).

The replica plating method showed that the donor bacteria were not able to follow the growth of the barley root to same attends as for the pea root. Transconjugant cells were for 3 of the 4 plant systems located at the root base except for pea seedlings grown in vermiculite where transconjugant bacteria were located along the whole root.

The microscopic observations showed pea roots having a more dense colonization of donor bacteria along the entire pea roots compared to barley roots which were much more scattered colonized. Bloemberg et al. (2000) has previously used fluorescent proteins to simultaneously visualize different population of pseudomonads in the rhizosphere at the single cell level. In the present study it was possible to differentiate between donor, recipient and transconjugant cells at the single cell level in a non-invasive way. The hotspots of donor colonies on the rhizosplane of barley were typically monolayers. The general pattern was that the rootbase of pea grown in vermiculite was heavily colonized by donors, which often formed big microcolonies or dense biofilm layer.

Donor, transconjugant and recipient cells were always mixed and in close contact and transconjugant cells were most often observed in the outer layer of the bacterial biofilm on pea. These observations suggest that proliferation of donors happened before conjugation took places. We did not observe any difference in the ratio or pattern of donor, recipient and transconjugant cells in the single layers of microcolonies, suggesting no difference in transfer efficiency of the plasmid between *Pseudomonas putida* colonizing pea or barley. All these observation indicate that the higher transfer frequency in the pea rhizosphere was due to a higher density of the donor cells, and hence, a higher probability of donor-to-recipient contact.

The exudate excretion of barley and pea seedlings was investigated to find an explanation for the difference in bacterial density and spread of donor bacteria. The average amount of water soluble exudates, extracted from both seed and roots, and measured after 6 days, was approximately two fold higher for pea than barley (2.7 mg

C/ root/ day and 4.7 mg C/ root/ day). The amount of dry weight of roots was approximately the same for the two species, but the dry weight of the pea seed was several times higher than for barley seed (data not shown). The spermosphere was in a study by Sørensen and Jensen (1998) reported as a hot spot compared to rhizosphere and bulk soil, and Sørensen and Jensen (1998) suggested it might be due to higher metabolic activity, which is consistent with a model by Chin et al. 1997. The model suggested that upon inoculation, the cells proliferate on the seed coat, mainly using nutrients provided by the seed coat, and/ or seed exudates (Chin et al. 1997). During spermosphere colonization, chemotaxis towards seed exudates and active motility seems to play a major role (Benizri et al., 2001) and this may as well be important for the mixings of donor and recipient cells in the first days. The main difference in production of exudates by barley and pea seedlings may be the total amount of organic carbon because the amount of exudates carbon correlated surprisingly well with total biomass of *P. putida* KT2442 (pKJK5::*gfp*) that was recorded in a batch experiment with the extracted exudates and pea supported a higher bacterial density than barley.

Microcosm experiments containing bacteriostatic antibiotics (growth inhibittors) were done to elucidate, at the macroscale level, if the higher exudate production by pea also could be responsible for longitudinally spread of donor bacteria. The antibiotics had no effect on the donors colonization potential. The difference between pea and barley in the longitudinal spread of donor bacteria might be that barley roots had a smaller diameter, grew faster and were longer than the pea roots, which may influence the movement of bacteria in percolation water and the range of motile bacteria. So the main difference between the spread of the donor bacteria on barley and pea roots may be in attachment or adsorption of bacteria to the plant roots. Plants secrete mucigel with agglutinins, more or less specific for microorganism, and bacterial synthesis of pili and O-antigenic chain of lipoplysaccharide (LPS) has been shown to have a positive effect of colonization (review see Benizri et al., 2001). Motility might as well influence the colonization ability of *Pseudomonas* spp. (Lugtenberg and Dekkers 1999).

In conclusion, the results and observation obtained in this study is in agreement with the colonization model by Howie et al. 1987 that proposed that

microbial colonization of the rhizosphere proceeds in two phases. In phase 1, bacteria become distributed by passive carriage downward with root extension through soil, thereby accounting for the progressively lower populations on the roots at increasing distances from the seed. *Phase* 2 is the multiplication and survival phase whereby the population increases to the limits of the ecological niche. We were able to demonstrate in this study that the higher transfer frequency in the pea rhizosphere was mainly the consequence of an elevated production of root exudates, resulting in a higher density of the donor cells, and hence, a higher probability of donor-to-recipient contact.

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TABLES

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant genotype and/or characteristics	Reference
E. coli strains		
HB101	*Sm*^{r} *recA thi por leu hsdRM*$^+$	Kessler et al. 1992
MV1190λ-*pir*	Δ(*lac proAB*) Δ(*srl-recA*)*306*::Tn*10* [F′*traD36 proAB lacI*^{q} Δ(*lacZ*)*M15*] t*hi supE*, lysogenized with *λ-pir* phage	Herrero et al. 1990
P. putida strains		
KT2442	Rif^r mutant of KT2440	Bagdasarian et al. 1981
KT2440	Nal^r	Sengeløv et al. 2001
SM1464	KT2440 Nal^r	Søren Molin
LM24	SM1464 with a mini-Tn*5* insertion of *dsRed*; Nal^r, Km^r	This study
Plasmids		
pRK600	Cm^r ColE1oriV RP4oriT, helper plasmid in triparental matings	Kessler et al. 1992
TTN151	Ap^r Km^r, delivery plasmid for mini-Tn*5*-Km^r-rrnBP1::RBSII-*gfp*mut3-T_0-T_1 derived from pUT-mini-Tn*5*-Km^r	Tolker-Nielsen et al. 2000
pKJK5::*gfp*	pKJK5 with mini-Tn5 insertion of $P_{A1/04/03}$::*gfp*mut3b; Tc^r, Km^r, Sm^r	Sengeløv et al. 2001

FIGURES

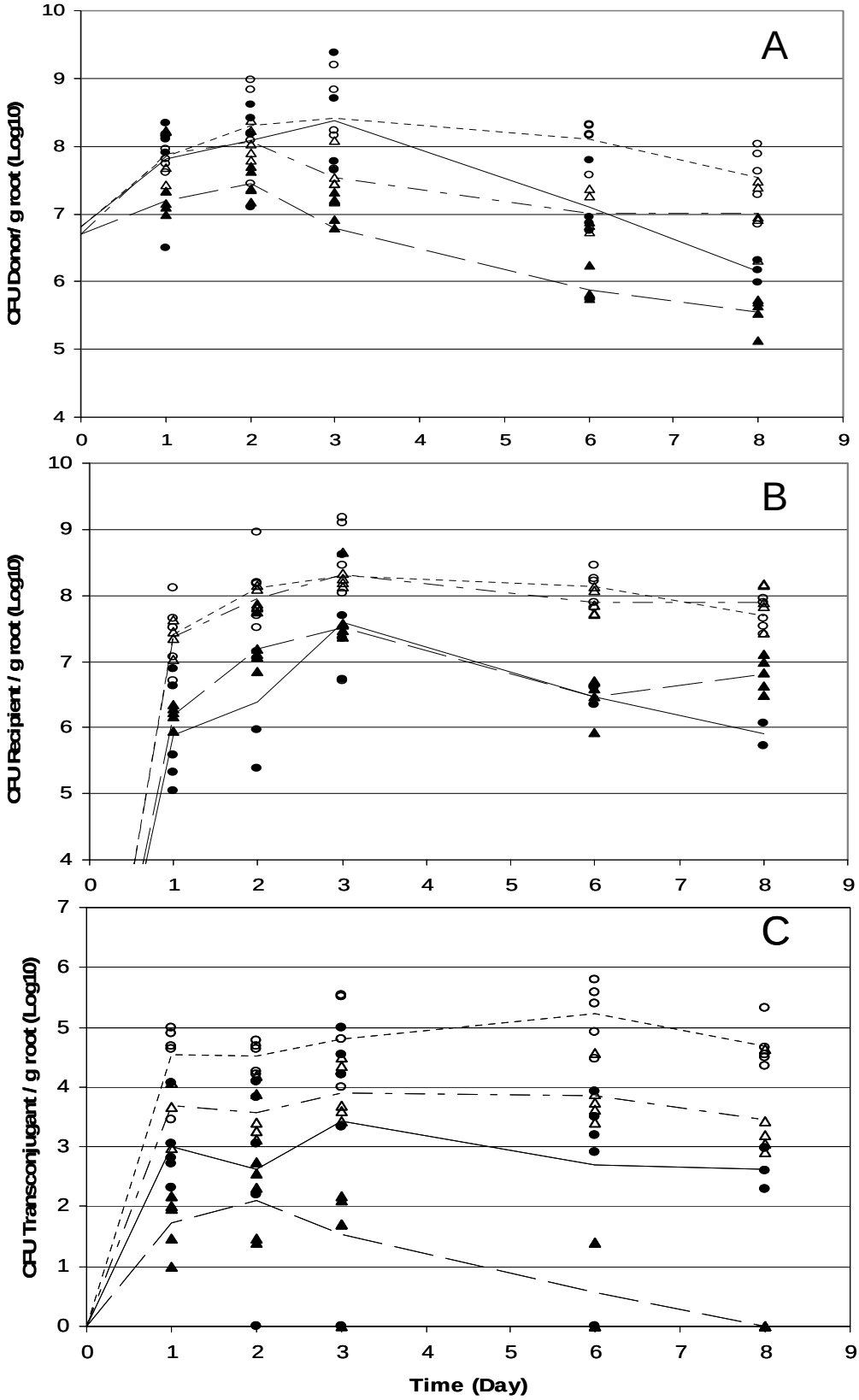

Figure 1. Densities of donors (A), recipients (B), and transconjugants (C) in the rhizosphere of barley grown in vermiculit (△ - - -), barley grown in soil (▲ — —), pea grown in vermiculite (○ ·····), and pea grown in soil (● ——). The lines are means of 5 replicate microcosms superimposed over individual data points.

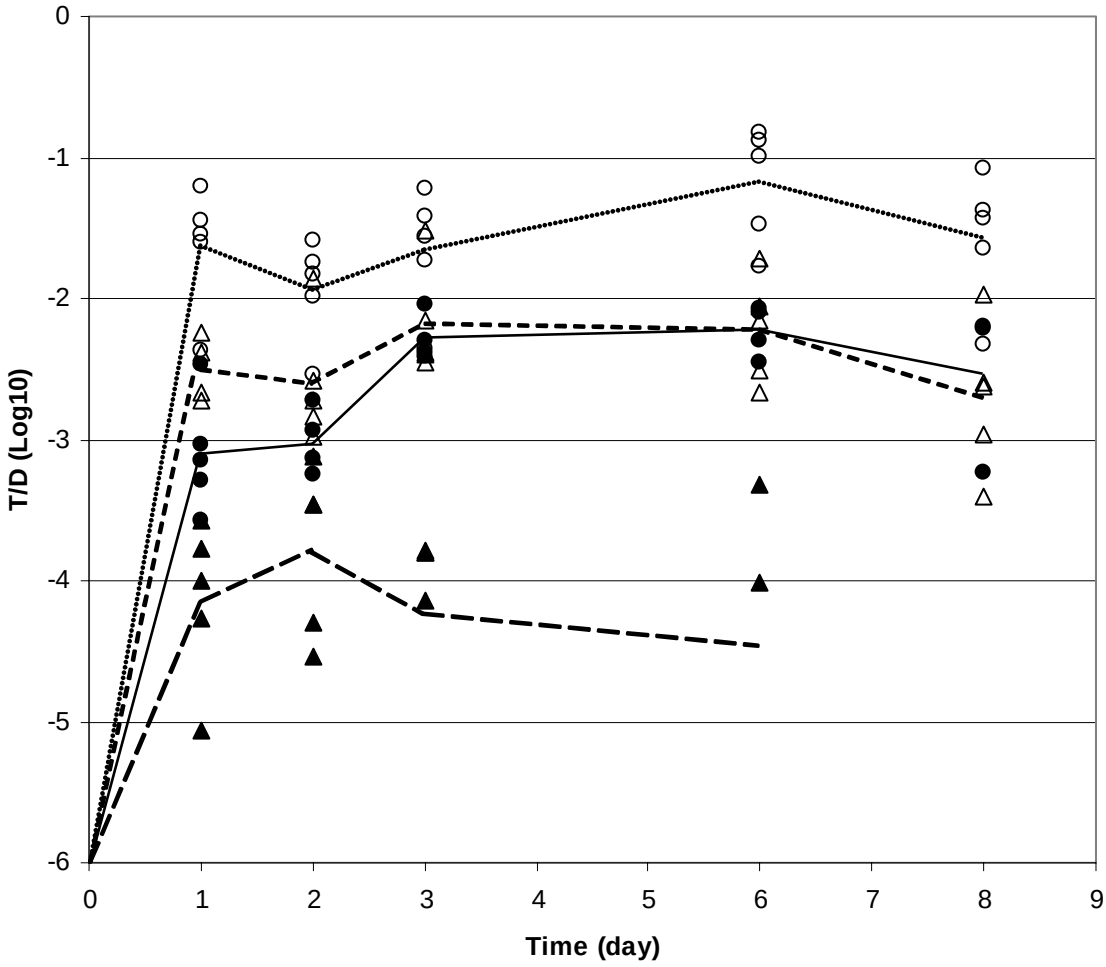

Figure 2. Transfer ratios (T/D) in in the rhizosphere of barley grown in vermiculite (△- - -), barley grown in soil (▲— —), pea grown in vermiculite (○ ·····), and pea grown in soil (● ——). The lines are means of five replicate microcosms superimposed over individual data points.

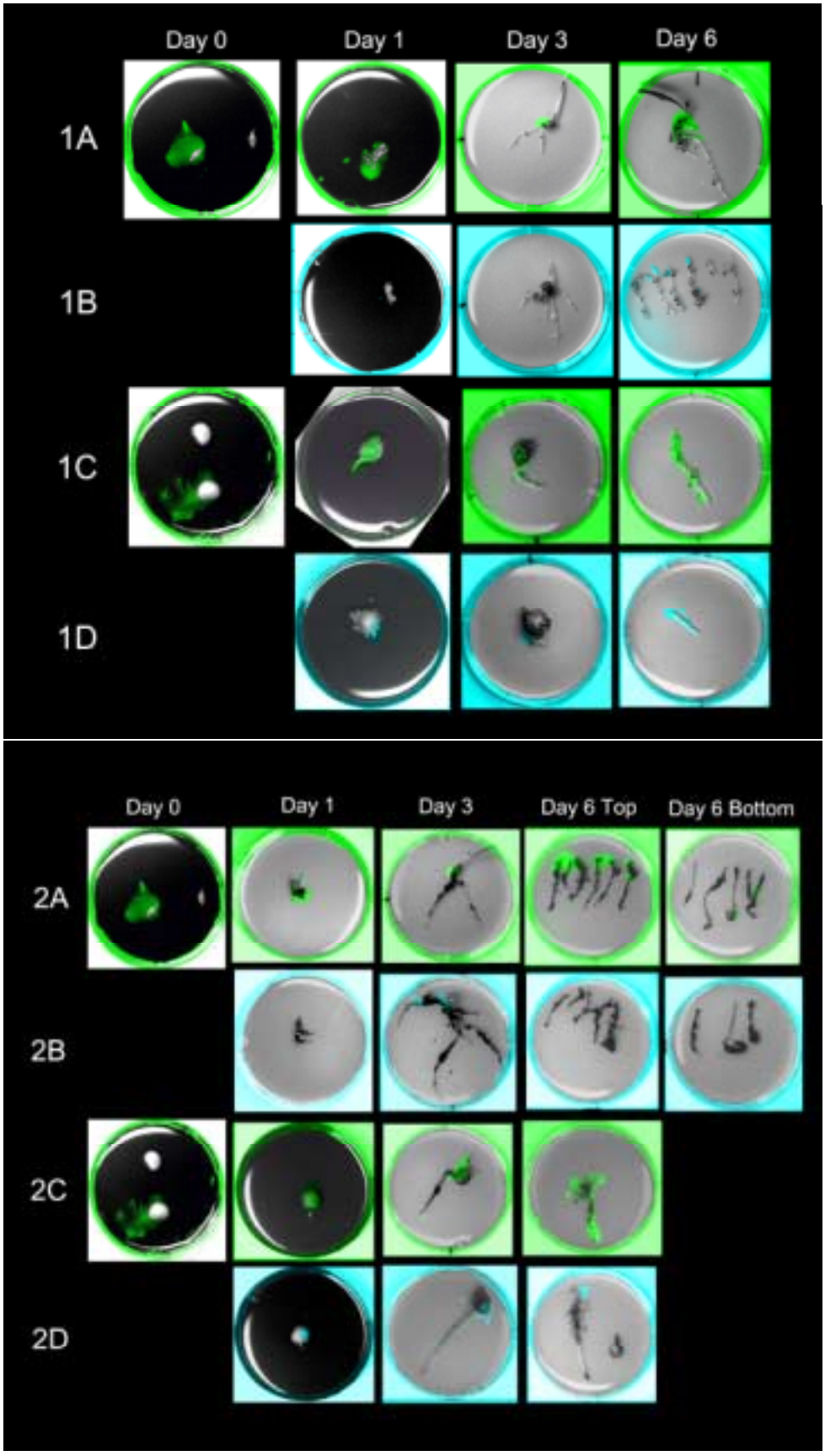

Figure 3. *Distribution of donors and transconjugants in the rhizospheres of barley (A and B) and pea (C and D) grown in vermiculite (1) or soil (2). Green colonies are donors, and blue colonies are transconjugants. At Day 0 both an inoculated (green colonies) and a non inoculated seed were placed on the agar dish as a control.*

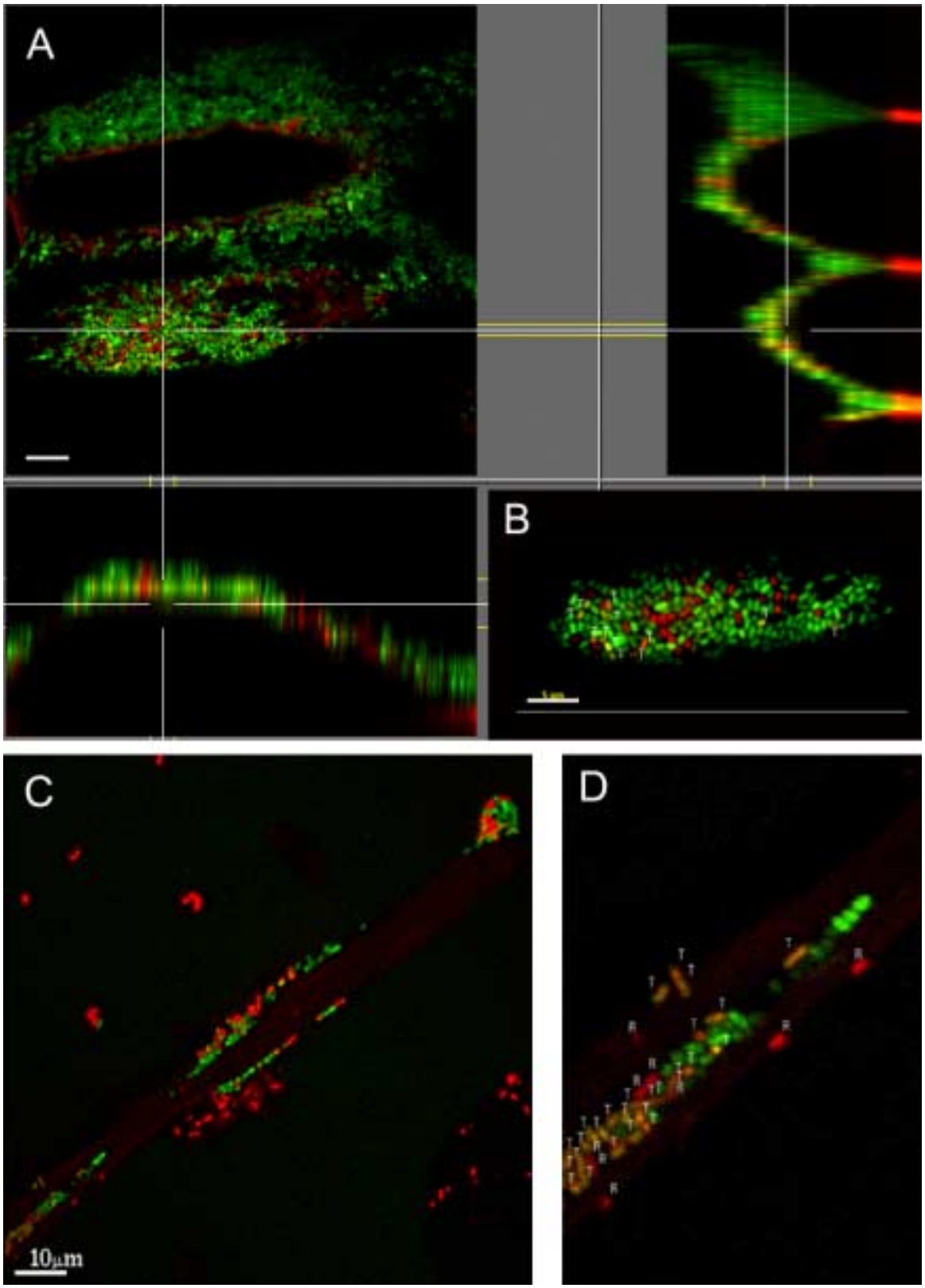

Figure 4. In situ visualization by CSLM of 2 hotspots colonized with green fluorescent donor cells, red fluorescenst recipient cells (R) and orange/yellow fluorescent transconjugant cells (T) on the root surface of a 3 day old pea seedling (A and B), and a root of a 3 day old barley seedling (C and D). Micrograph A is a xyz scan where vertical sections of the plant surface with microcolonies/ biofilm formation are shown to the right and below. Micrograph B is a magnified layer from the same scan as A. Micrograph D is a magnification of one of the microcolonies in C. The bar in A and B represents 5 µm, while and the bar in C represents 10 µm.

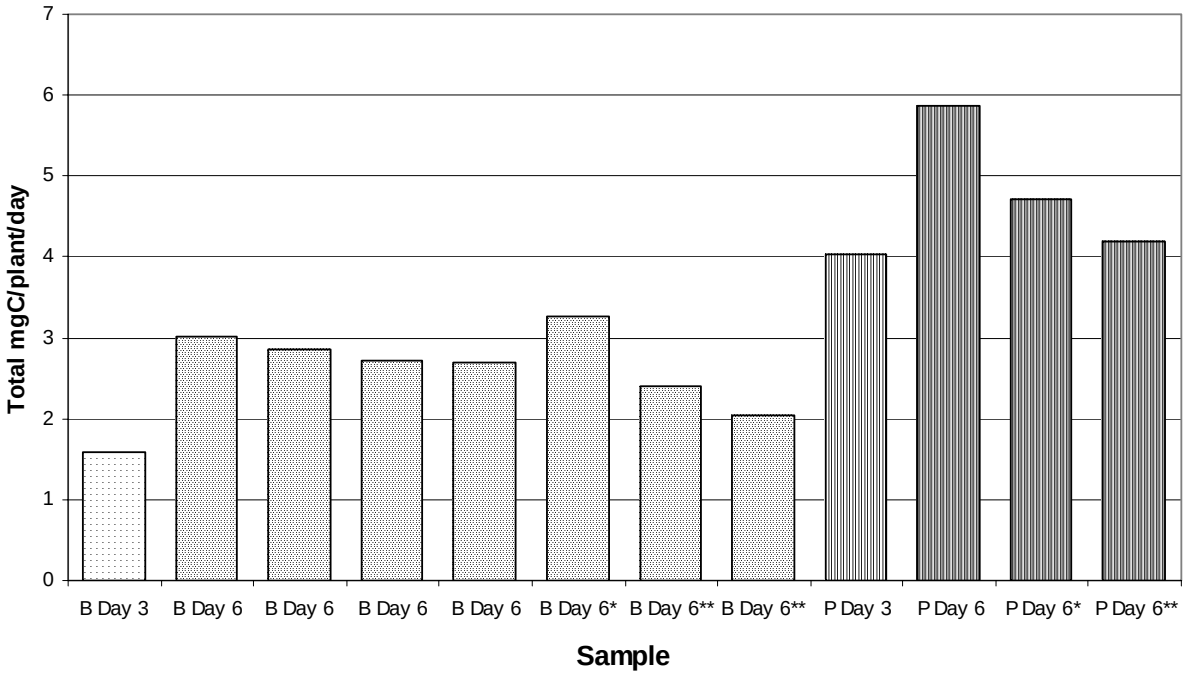

Figure 5. Root exudate production measured as mg organic C exuded per plant per day. Exudates were measured after 3 and 6 days of growth. P: pea; B: barley. Asterisks in the legend indicate from which of the three independent experiments the data correspond to.

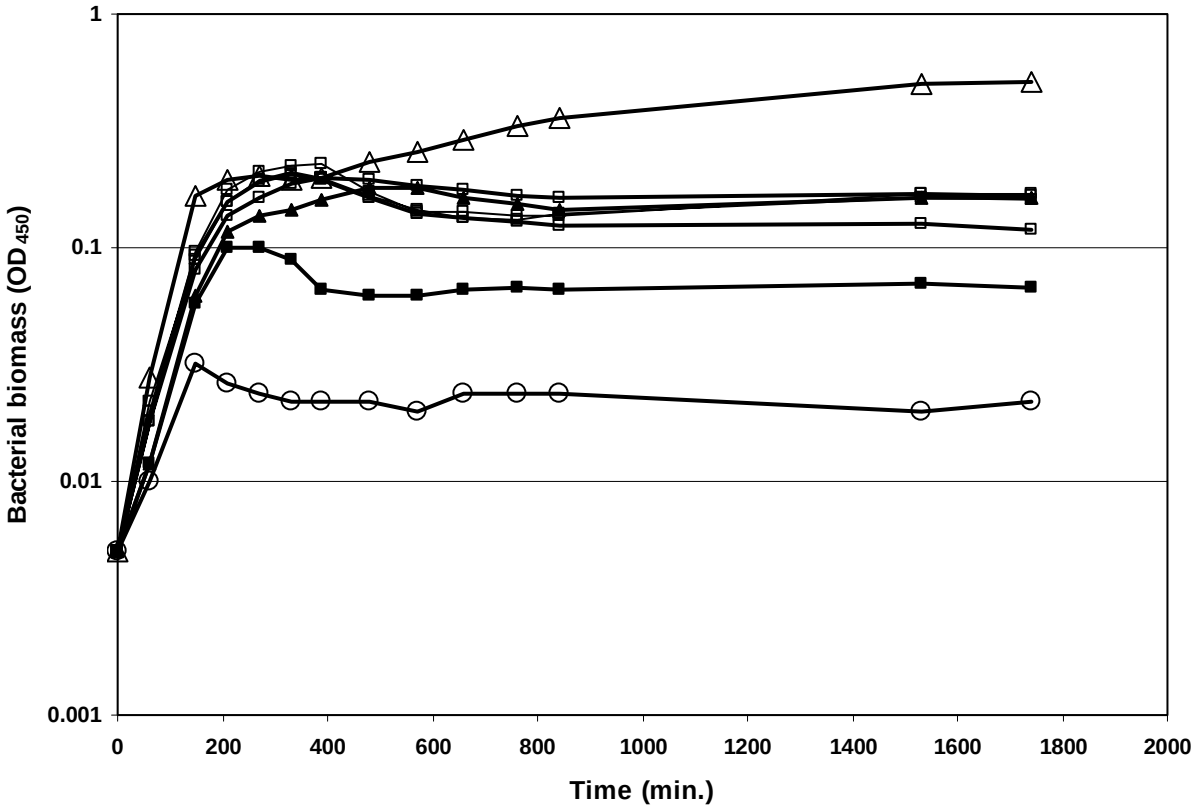

Figure 6. Growth of P. putida LM24/pKJK5::*gfp* at 30°C on barley exudates extracted after 3 days (■), barley exudates extracted after 6 days (□), pea exudates extracted after 3 days (▲), and pea exudates extracted after 6 days (△). Germination buffer without plants after 6 days (○) served as control.

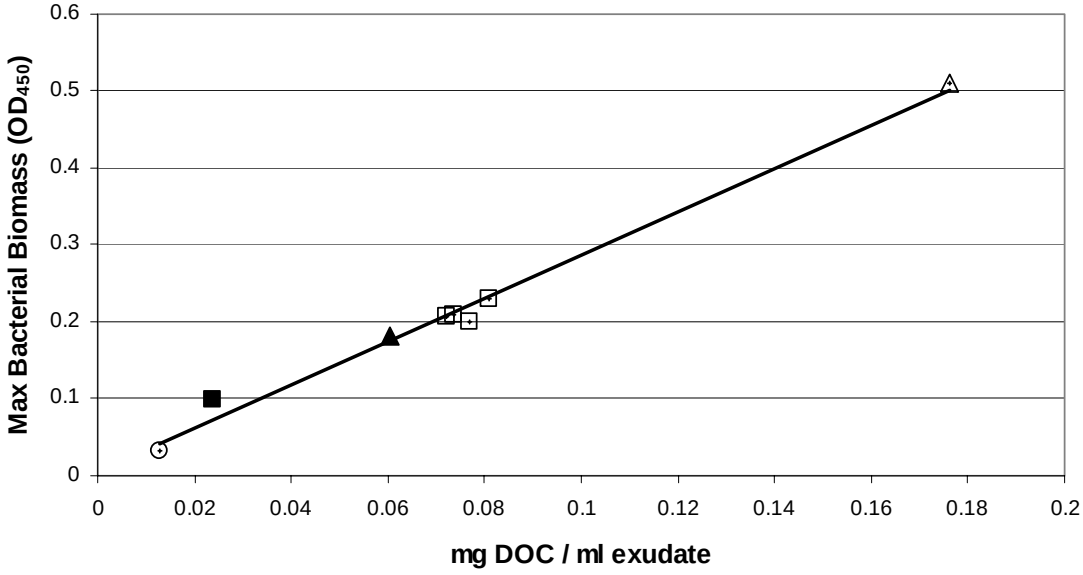

Figure 7. Correlation between concentration of root exudate (mg organic C/ml) and maximal biomass of *P. putida* LM24/pKJK5::*gfp* at 30°C.
Germination buffer without plants after 6 days (○); barley exudates extracted after 3 days (■); barley exudates extracted after 6 days (□); pea exudates extracted after 3 days (▲) and pea exudates extracted after 6 days (△)

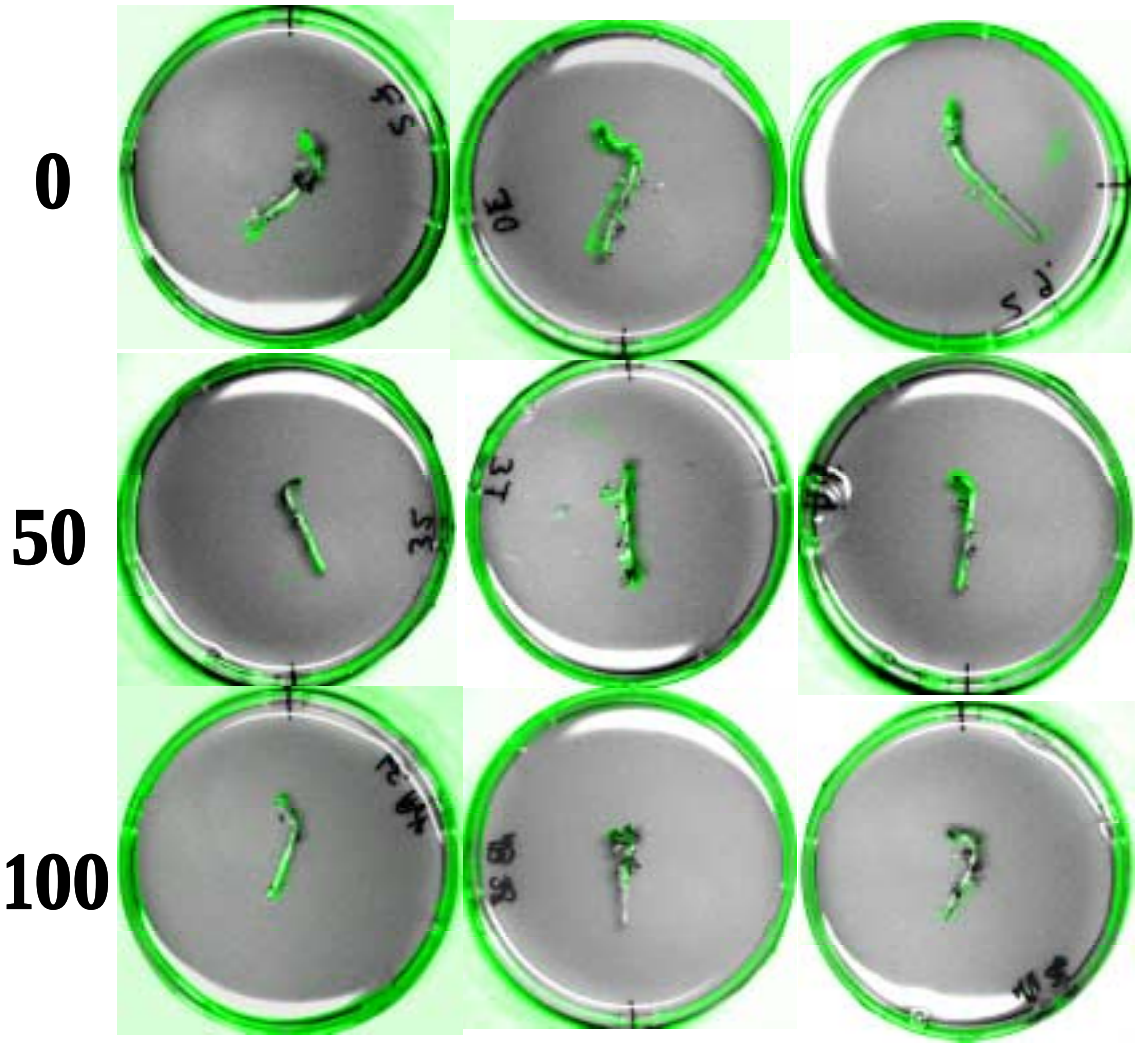

Figure 8. Distribution of donors (green colonies) in the rhizosphere of pea grown in vermiculite with 0 mg nalidixic acid, 50 mg nalidixic acid and 100 mg nalidixic acid. The results are in triplicates and there was no clear difference, between the 3 treatments, in the donor bacteria colonization ability. The pea roots were inhibited in growth at 100 mg nalidixic acid.

Appendix I.

APPENDIX I

This is a first draft, about the practical work I performed in Oxford in corporation with Mark Bailey's group at CEH and Poul Rainey's group at Plant physiology, Oxford University.

INTRODUCTION

Plasmids isolated from bacterial communities such as the rhizosphere tend to be large, and sequence analysis is increasingly showing that these plasmids apparently contain large amounts of non-coding, non-functional or uninformative DNA (see introduction to this thesis). It is therefore of high value to investigate if environmental plasmids actually contain novel environmentally specific genes or if large parts of the plasmid genomes are "junk" DNA, and use techniques which can link bacterial genotypes with bacterial phenotypes.

pQBR103 is a conjugative rhizosphere plasmid extracted from *Pseudomonas fluorescence S*BW25. Its size is 330 kb (4.76% of the total genome). The plasmid has a rather narrow host range and its known phenotype is mercury resistance (Lilley et al. 1996). The plasmid is one of a group of large genetically similar plasmids (group I) that have been regularly isolated from pseudomonad populations colonising both the leaves and roots of crops and wild plants of a mixed farmland and woodland site at Wytham, Oxfordshire (Lilley et al. 1996; Viegas et al. 1997). pQBR103 plasmid has an ecological exciting profile, because it may contain plasmid-encoded traits (as yet unidentified) conferring context-dependent benefits that facilitate host survival and colonization in the developing phytosphere (Lilley and Bailey 1997).

In vivo expression technology (IVET) is a genetic system that positively selects for bacterial genes that are specifically induced when bacteria infect their host (Mahan et al. 1993). IVET has been adapted by Dr. Rainey (Oxford University) to identify rhizosphere-specific genes of *P. fluorescens* SBW25 colonizing sugar beet (Rainey 1999). IVET has been employed to identify the genes of pQBR103 showing elevate level of expression in *P. fluorescens* SBW25 colonizing sugar beet seedlings (Bailey et al. 2001; Van elsas and Bailey 2002). The study by both Rainy (1999) and Bailey et al. (2001) revealed a very high percentage of selected IVET fusions having no homology with sequences from GenBank.

The aim of this study was to develop an assay to elucidate which factors in the phytosphere that induce promoters of selected IVET fusions. The IVET selection system used in this study is an extension of the promoter trapping system in which recombinants are screened for the complementation of the essential growth factor diamino pimelic acid (DAP) (Bailey et al. 2001). The chromosomal deletion of the *dapB* on the host chromosome ensure that thus organisms only will grow when the DAP is added or when the intact gene is introduced into the cell. Vector (pIVET) was constructed by Dr. Xeng and it carries a promoterless *dapB* (Bailey et al. 2001; van Elsas and Bailey 2002). Libraries of pQBR103 were constructed by insertion of small fragments of pQBR103 upstream of the promoterless *dapB* and the host (*Pseudomons fluorescens* SBW25Δ*dapB* (pQBR103)) was transformed. A pIVET vector-library insertion duplicates the promoter and does not interrupt the function of the gene in the plasmid. A second promoterless marker, *lacZ* was also included as an in-frame fusion downstream of the promoterless *dapB* on pIVET (see Fig. 1). In this study we used the *lacZ* reporter genes in a potentially high through put system to analyze under which specific conditions plant selected IVET fusions and pre-selected IVET fusions are induced.

MATERIALS AND METHODS

Bacteria strains and growth conditions.

The bacterial strains and plasmids used are listed in Table 1. *P. fluorescens* SBW25 was grown at 28°C in Luria-Bertani (LB) or M9 minimal medium. Extracted bacteria from alfalfa spouts were grown at 20°C on LB agar. When necessary antibiotic were used at the following concentrations: Tetracycline 10 μg ml^{-1}, Kanamycin 50 μg ml^{-1}. To supplement the growth of *P. fluorescens* SBW25*ΔdapB*, 80 μgml^{-1} of lysine and 800 μgml^{-1} DL-αε-diaminopimelic acid were added. To select for *P. fluorescens* SBW25*ΔdapB* (pQBR103), 0.1 mM $HgCl_2$ was used.

IVET libraries of SBW25 (pQBR103)

Previously described IVET libraries of *P. fluorescens* SBW25*ΔdapB* (pQBR103) were used (Bailey et al. 2001). Briefly described a pUC18 library containing 2000 clones of 1-3 kb DNA fragments from pQBR103 was divided into 5 groups (G1-G5)

and cloned into pIVETD. Additionally an extra pUC18 library was constructed in the same way but this was not subdivided. This library was called BB.

In this study a "White pQBR103 IVET library" was constructed from the 6 original pQBR103 IVET libraries by picking IVET colonies with no or minor β-galactosidase activity from M9 agar plates (Dap, Lys, Tc and X-gal) at Day 2 (Fig. 1). The IVET clones that were picked were in a replica-plating experiment tested for their ability to grow on M9 agar plates without Dap and Lys. Only the IVET clones that could not grow on M9 agar plates without Dap and Lys were stored in 20% glycerol in 96 wells micro plates. A constitutive expressed β-galactosidase IVET clone (pB) was added to 4 wells of one of the micro plates as a positive control and *P. fluorescens* SBW25*ΔdapB* was added to 4 wells of the same micro plate as a negative control.

IVET Sprout colonizing system. A sprout colonization system was developed to find and evaluate if clones from the "White IVET library" contained promoters induced on non-sterile alfalfa seedlings (Fig. 1). The sprout colonizing system was a 15 cm square plastic disk with 25 wells (Fig. 2). In each well was a folded filter paper saturated in tap water and on the top of the filterpaper was sown four alfalfa seeds (organic alfalfa seeds (*Medicago sativa* L.) imported from USA (Urtekram, 9550 Mariager, Denmark)). The system was placed in a plastic bag and the seeds were sprouted for 8 days at 20°C with a light cycle of 12 hours light and 12 hours dark.

Colonies from the white pQBR103 IVET library were pooled and inoculated onto 100 non-sterile alfalfa seeds. The seeds were then sown in the sprout colonizing system and grown for 8 days. At Day 8 the bacteria were extracted from the sprouts in 10 ml plastic tubes with 1 ml of PBS pr 4 sprouts. The tubes were wortexed for 1 min. The bacterial extracts were pooled from all the plastic tubes and plated on M9 agar plates (Dap Lys, Tc and Xgal). Three IVET isolates (W1; W5; W6) were selected after 2 days for further studies. (Fig 1.)

The 3 IVET isolates (W1; W5; W6) were reinoculated to alfalfa seeds to elucidate when and if they were induced in the sprout colonization system. The constitutive expressed IVET clone (pB) and *P.f* SBW25Δ*dapB* were also inoculated onto alfalfa seeds and grown in the sprout colonizing system as previous described. Uninoculated seeds were sown as controls. Sampling times were at Day 0; 1; 4 and 7. For each sampling day bacteria were extracted from the sprouts in triplicates and placed in 10 ml plastic tubes with 1 ml of PBS pr 4 sprouts. Each tube was wortexed for 1 min. The bacterial extracts were plated in appropriated concentration on LB agar plates and M9 agar plates (Dap Lys and Tc). At each sampling time plants were as well sacrificed for plant prints.

Plant-prints of the bacterial colonization were made by placing plants with flamed tweezers on a M9 plate with Tc and X-gal. The plates were incubated at 20°C for 24 hours after which the plants were removed and the plates were photographed. Two replicate plates with either M9 (Tc and X-gal) or M9 (Dap; Lys; Tc and X-gal) were made from the original plant plate, and after 24 hours at 28°C the plates were photographed.

Screening for plasmid promoters induced by bacterial interaction Approx. 1 µl of bacterial glycerol suspensions of the stored White IVET library at –80°C were transferred by a 96-pin replicator to the surface of M9 agar plates ((Dap; Lys; and X-gal). Overnight cultures of *P. putida* KT2440; *E. Coli* HB101; *P. fluorescens* SBW25, *P. fluorescens* SBW25 (pQBR103) were similarly transferred to the agar surface by placing them next to the IVET clones. After 2, 3 and 4 days of incubation at 20°C, colonies were analyzed for β-galactosidase activity in the interface between the colonies.

Alfalfa exudate production. Alfalfa exudates were produced as follows. Alfalfa seeds were sterilized by washing the seeds for 1 hours in 50 % H_2SO_4 followed by 3 times rinse in M8 media. The seeds were sown in a sterile sprouting system for 6 days at 20°C and a 12 hours light circle. On Day 6, a sterility test of the exudates was made by plating 100 µl from each system on LB agar plates. The LB agar plates were incubated for 2 days at 20°C and only the sprouting systems without

contamination were used. The sterile system was a 600 ml glass beaker containing an inverted 200 ml glass beaker. A double-layered filterpaper was placed on the bottom plateau of the 200 ml glass container. The system was filled with 300 ml M8 media and the filterpaper worked as a wick for the seeds placed on the bottom of the 200 ml glass container (Fig. 2). The system was autoclaved for 20 minutes, before the seeds were sown.

Chemiluminescent reporter gene assay. A Galacto-Light Plus kit (Applied Biosystems) was used to measure β-galactosidase activity with a micro plate luminometer (Lucy 2) in cell lysates of the White pQBR103 IVET library incubated under different growth conditions. The kit has a dynamic range of 2 fg to 20 ng of purified β-Galactosidase (Applied Biosystems). The Inoculation procedure of the micro plates was as follows: The -80°C frozen stock of the "White pQBR103 IVET library" was by a 96 wells replicator inoculated to 96 wells micro plates containing 100 µl M9 (Dap Lys and Tc) pr well. The micro plates were incubated at 28°C for 24 hours. 10µl from each well was transferred to new micro plates which contained 90 µl M9 (Dap Lys and Tc). After 24 hours of incubation turbidity (OD: 605 nm) was measured for all the micro plates (Lucy 2). The IVET cultures were now ready for different treatments.

In the first treatment, one alfalfa seedling was grown in each well in micro plates with IVET cultures. The procedure was as follows. The alfalfa seeds were pre-germinated for 2 days on filter paper saturated with M8 media. Hereafter all the seedlings were moved to micro plates containing 90 µl of M8. 10 µl of the IVET culture was then add to each well of the micro plates with the seedlings. Alfalfa sprouts without adding culture were as well grown in micro plates and followed the same procedure as the rest of the seedlings. Micro plates with the seedlings were incubated for 3 days at 20°C with a light circle of 12 hours. The seedlings were afterwards removed from each well and the Galacto-Light manual for detection with micro plate luminometers was followed. Briefly 10 µl of lysis solution was added to each well with cell culture and the plates were incubated for 10 minutes. 20 µl of bacteria lysate was mixed with 70 µl of reaction buffer in a new micro plate. After 50 min incubation at room temperature, 120 µl of Light Emission Accelerator was added

to the reaction mixture. Each sample was counted immediately OD(605 nm) and light luminescence was measured for 0.1 sec pr well (lucy 2). Enzyme activity is expressed in arbitrary light units (counts per second)

The second treatment was 90 µl of M9 (Dap Lys and Tc) where 10 µl of the White IVET library culture was added to each well and the plates were incubated for 24 hours or 3 days at 28°C. Hereafter was the Galacto-Light Plus kit protocol followed as previous explained. In the last treatment was 90 µl of alfalfa exudate-extraction added pr micro plate well followed by 10 µl of the white IVET library culture. The plates were incubated for 24 hours. The luminescence and turbidity measurements followed again the Galacto-Light Plus kit protocol.

RESULTS AND DISCUSSION

The IVET library of pQBR103, was in this study used in a novel way (See fig. 1). 127 of pQBR103 IVET clones not expressing β-galactosidase were picked at Day 2 from the 6 original IVET libraries. The pQBR103 fragments were approximately of 1-3 Kb DNA. Because of the relative little size of pQBR103 compared to the genome of *P. fluorescens* SBW25, this number of clones might represent a large part of the plasmid genome.

Ten IVET clones were selected that with a high probability have been induced and thereby been able to grow on the seedlings under a 7 d. long experiment. Most of the IVET clones will be represented because a pool of all the colonies from the "White IVET library" was inoculated to 100 non-sterile alfalfa seeds. The inoculum density was only approximately 1000 bacteria pr. seed, so that the IVET clones had to compete with the indigenous bacterial population.

To verify and elucidate when and where on the seedlings the isolated IVET clones from the previous experiment were induced, we re-inoculated individually three of the isolated IVET clones, a constitutive expressed IVET clone (pB) and SBW25Δ*dapB* to the alfalfa seeds. The amount of pB clone (CFU/g of seedling) increased exponential (R^2= 0.96) from Day 0 to Day 7 with a factor 0,45 pr day/g seedling (Fig. 3). The amount of SBW25Δ*dapB* increased as well exponential (R^2= 0,99) but with a factor 0,22 pr. day/g seedling. The W1 IVET clone was not different from the growth of SBW25Δ*dapB*. W5 increased from 2 x 10^5 CFU/g seedling at Day

one to 6,5 x 10^8 CFU/g seedling at Day 4 and decreased to 1,0 x 10^8 CFU/g seedling at Day 7. W6 increased in density from Day 4 to Day 7 from 6,3 x 10^6 CFU/g seedling to 1,5 x 10^9 CFU/g seedling.

W5 and W6 had at Day 4 induced β-galactosidase activity along the seedling placed on M9 agar plates without Dap visualized by plant prints (Fig. 4.2B and 4.2F). W4 and W6 made colonies on M9 plates (+Dap), but it was only W4 that had induced β-galactosidase activity (Fig. 4.3B and 4.3F). It was only W4 that was able to grow on M9 plates without Dap (after the replicaplating) (Fig. 4.4F). In conclusion it looks like W1 not contains any promoter that was induced in this experiment. W4 can be an IVET clone without a strictly regulated promoter because the clone could grow on M9 plates without Dap. The W6 IVET clone had an interesting profile because the density of the clone on the seedlings first increased at Day 6 and the clone could not grow on M9 plates without Dap. After the replica-plating most of the W6 colonies were located at the root of the seedling (see fig. 3.3b). In chapter 3 in this thesis it was found that pseudomonas first started to colonize alfalfa seedlings at Day 4, which makes it possible that the promoter is induced by interaction with the other pseudomonas or is a plant inducible promoter. Unfortunately the plasmid insertion has still not been sequenced, and before we have the result it is only possible to speculate about the gene and the promoter of W6.

An assay was developed to find plasmid promoters induced by bacterial interaction from the White IVET library (see fig. 1). The idea was to look after genes that are induced in the zone between colonies from the IVET library and colonies from different bacterial strains (i.e. *Pseudomonas* spp., *E. coli*). If there was an induction the zone should have been blue because of the β-galactosidase activity on the M9 plates with X-gal. We did unfortunately not find any such signal in the strains we tested against, there can be three explanations. First explanation might be that no clones had an inducible promoter for such an interaction. Second explanation may be that the conjugation frequencies of pQBR103 was too low (approximately 1 x 10^{-6} pr donor) to be detected. Finally the X-gal assay is not a very sensitive assay, and a luminescence or fluorescence detection system with a minimal background would have been preferable.

A commercial chemiluminescent reporter gene assay was used to screen one of the micro plates from the White library. There was a correlation between growth and luminescence when the white IVET clones were grown in M9 media (Dap + Lys + Tc) for 24 hours (see fig. 4); this is expected because the plasmid fragment is located upstreams for both the promoterless *dapB* and *lacZ*. There was no correlation between growth and luminescence when the IVET clones were incubated for 3 days. The turbidity decreased for most of the clones at Day 1 compared to Day 1. The luminescence increase for half of the clones at Day 3 compared to Day 1. An explanation for this increase in luminescent can be that there is some random promoter expression from the inserted plasmid fragment.

Both the luminescence and turbidity increased when a seedling was added to each well of the white IVET library. There was a high variance between the two independent experiments. Five clones changed expression pattern compared to M9 media (Dap + Lys), when the White IVET clones were grown in exudates from alfalfa. In conclusion 12 IVET clones were chosen for further studies because of their expression profile with all the different treatments. The next step will be to rescue the pIVET vector from pQBR103 and to sequence the plasmids fragments. Hopefully a promoter analysis and an ORF search well elucidate where the gene or at least the promoter is located. In conclusion, we found it easy with this assay to screen a high amount of IVET clones (both from the "traditionally" IVET procedure and from preselected IVET clones) for their growth and β-galactosidase expression under different conditions. This assay may have a future to find the stimuli that induce selected IVET clones with an unknown ORF.

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TABLE

Table 1. Bacterial strains used in this study.

Strain or plasmid	Relevant genotype and/or characteristics	Reference
E. coli strains		
HB101	*Sm^r recA thi por leu hsdRM$^+$*	Kessler et al 1992
P. putida strains		
KT2440		Bagdasarian et al. 1981
P. fluorescens strains		
SBW25		Lilley et al. 1996
SBW25*Δdap*		Bailey et al. 2001
pB	IVET clone with a pQBR103 fragment with a constitutive promoter.	X.X. Xeng, strain collection
W1	IVET clone	This study
W5	IVET clone	This study
W6	IVET clone	This study

FIGURES

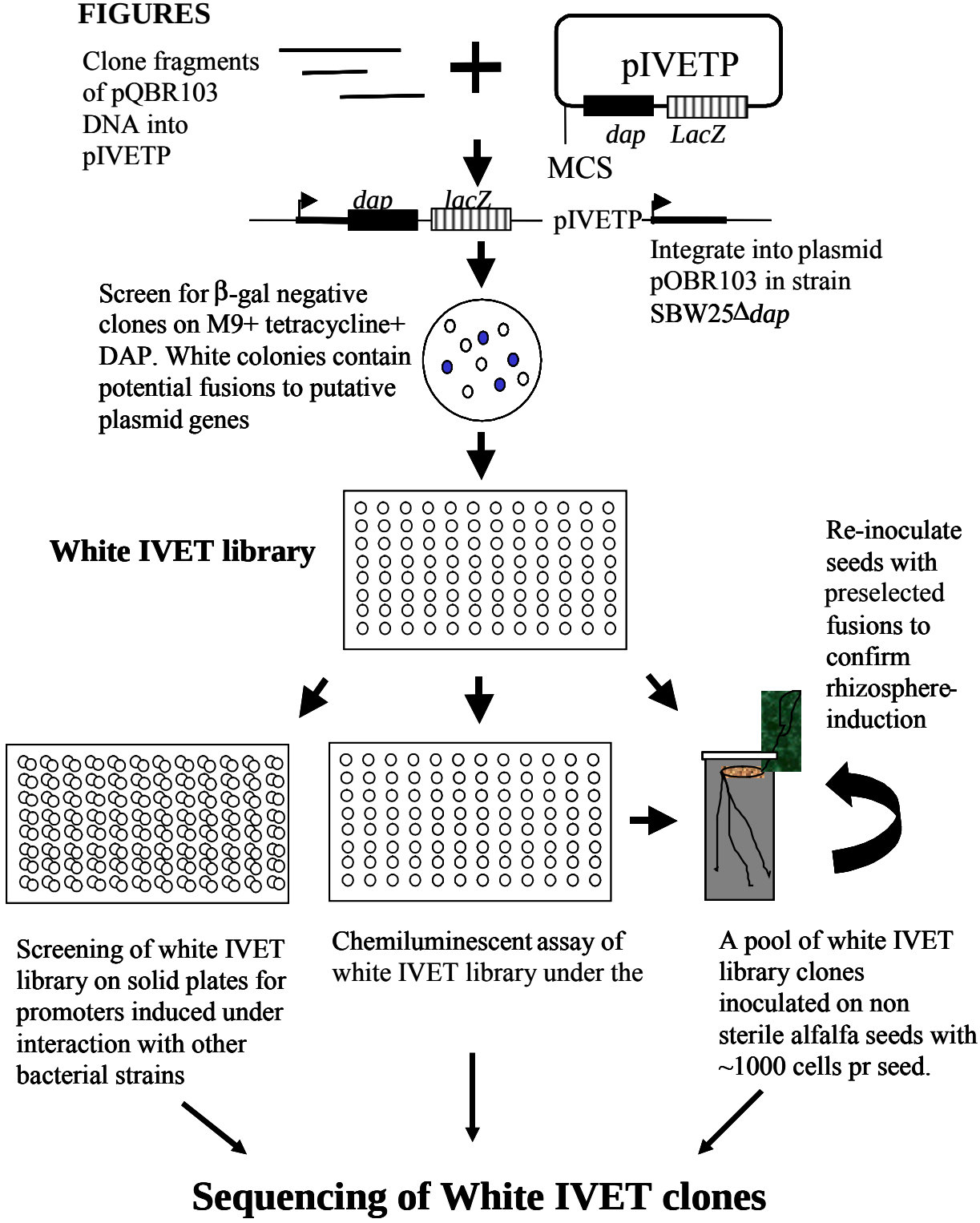

Figure 1. The"White IVET library was made in this study and it contained DNA fragments from pQBR103 with uninduced promoters (on M9 agarplates) or no promoters at all. We used the *lacZ* marker gene to distinguish between expression from the individually IVET clones. The *lacZ* genes are fused to the promoterless *dapB* gene. Only the white colonies that not was able to grow on M9 plates without DAP (tested in a replicaplating experiment) was picked and stored in micro-plates. We used three different assays, based on bacterial growth and β-galactosidase activity, to screen the White IVET library for regulated promoters.

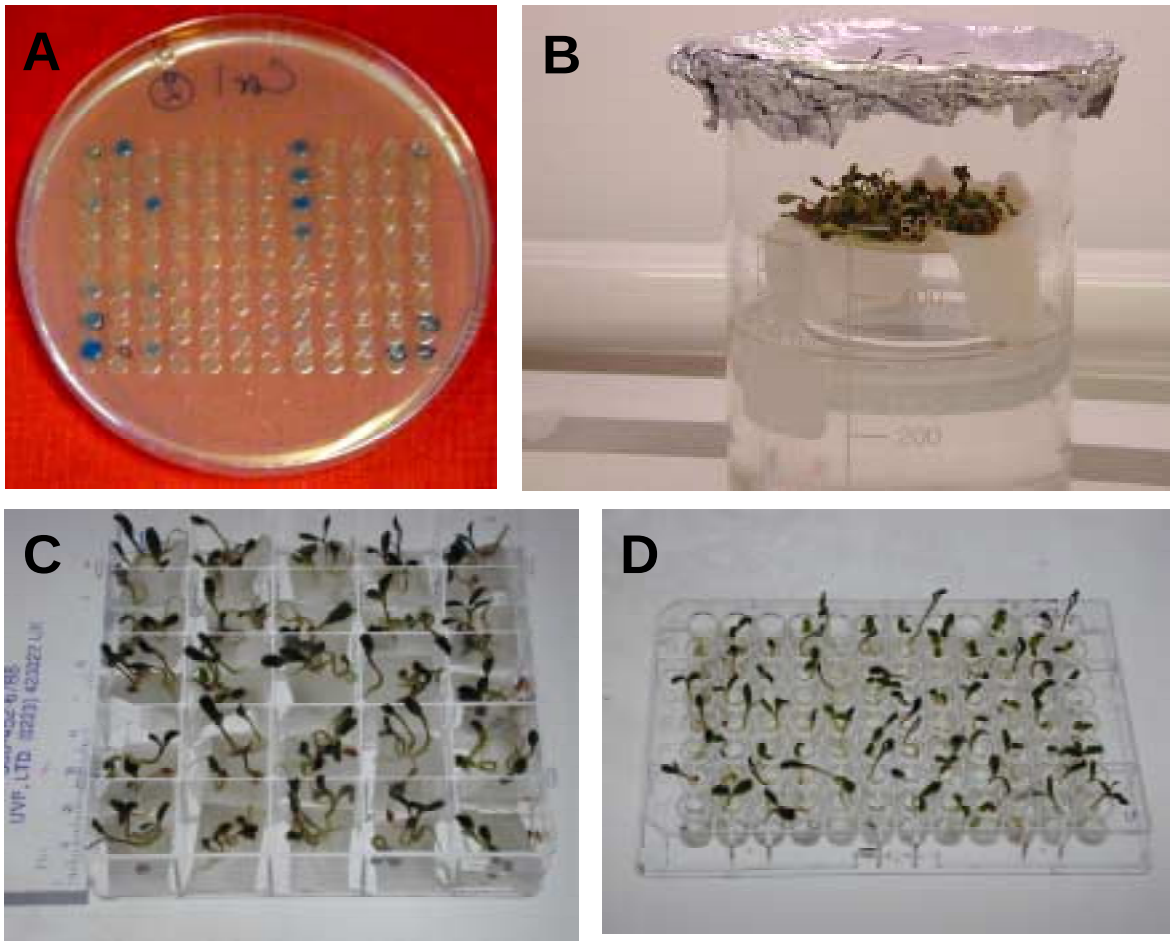

Figure 2. The different systems used in this study. **A)** Solid plate assay used to screen the White IVET library for clones induced by interaction with other bacteria. If a clone is positive there will be a blue zone between the IVET clone and the strain it is responding too. **B)** An alfalfa exudate system (see material and methods for further details). **C)** The IVET sprout colonization system used to screen for IVET clones induced by alfalfa seedlings. **D)** A microplates with alfalfa seedlings used in a chemiluminescent assay to screen the "White IVET library for plant induced promoters.

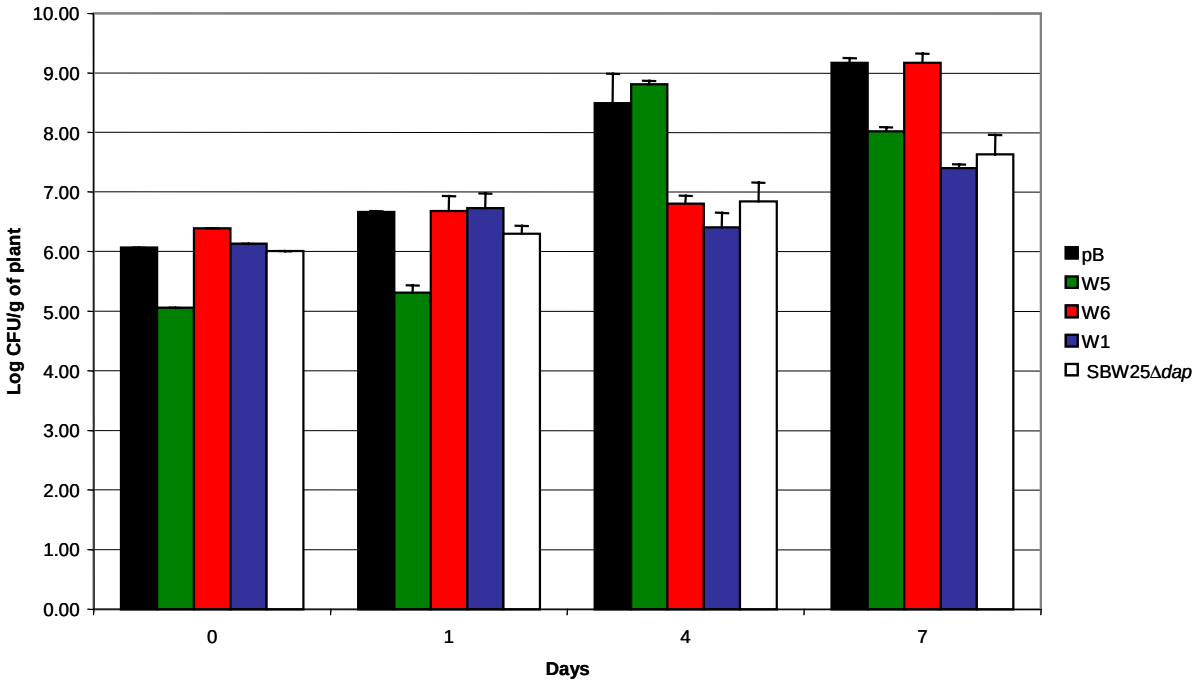

Figure 3. Re-inoculation of 3 IVET clones (W5; W6; W1) to the IVET sprout colonization system. pB is a positive control with a constitutive expressed IVET promoter and *P. fluorescens* SBW25*ΔdapB*. The three tested clones had different expression patterns; W1 did not grow differently from delta. W5 and W6 could grow on the alfalfa seedlings but W6 started first to increase in density from day 4.

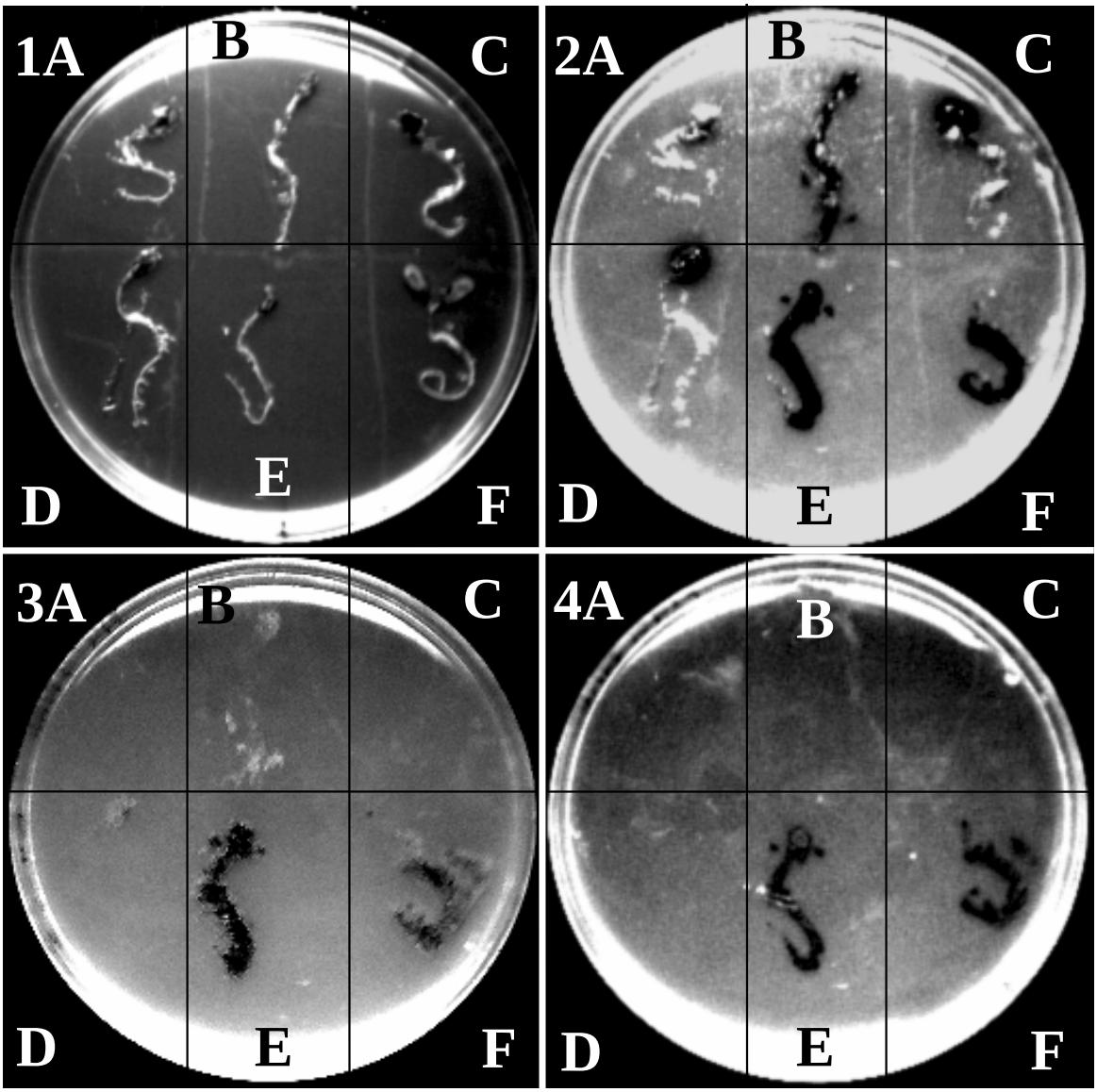

Figure 4. Plant prints of bacterial colonization of alfalfa seedlings made at day 4 from the reinoculation experiment. A) Seeds not inoculated; B)Seeds inoculated with clone W6; C) Seeds inoculated with clone W1; D)Seeds inoculated with SBW25*Δdap*; E)Seeds inoculated with clone pB; F)Seeds inoculated with clone W5.
Nr. 1) Photograph of plants placed directly on M9 agar plates (Tc + X-gal) from day 4. Nr. 2) The same plate as in 1 but after 24 hours of inoculation with the plants placed on the M9 agar plate (Tc + x-gal). Where the seed was placed there is a background of β-galactosidase activity from all the seedlings. pB, W5 and W6 inoculated seedlings had all β-galactosidase activity along the seedlings. Nr. 3 and Nr. 4 are plates replicated from plate Nr. 2. Nr. 3) Only pB, W5 and W6 did grow on the M9 agar plate (Dap + Lys + Tc + X-gal). pB and W5 had β-galactosidase activity and W6 did only grow at the original alfalfa root area. Nr. 4) pB and W5 were the only clones able to grow on M9 agar plates (Tc + X-gal). From this study it is possible to conclude that the IVET clone W5 both can grow and express β-galactosidase on M9 plates, W1 was not induced at all, and W6 had an exiting profile because it could only grow on M9 plates with Dap and the clone had only β-galactosidase activity towhen the seedling was present.

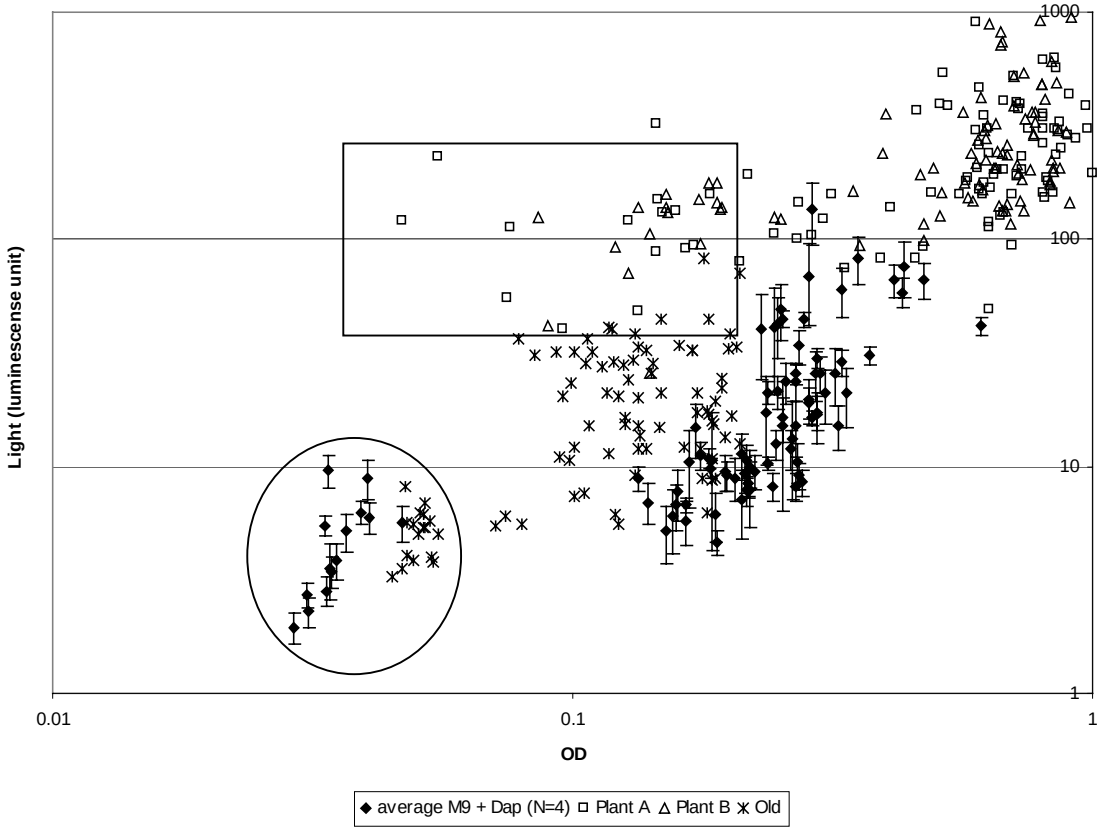

Figure 5. Luminescence screenings assay of 90 of the White IVET clones. The turbidity for each clone (well) is plotted against luminescence. The control treatment was incubated for 24 hours in M9 media (Dap+ Lys), and the points are plotted here as the average of 3 independent runs. If the incubation time was increased to 3 days there was a general increase in luminescence and a drop in OD for each individually IVET clone. The big circle is showing the background measurements for media not inoculated with bacteria. The background measurements were higher both in turbidity and luminescence in the screening where a plant was placed in each well (The box is surrounding the background values in the two seedlings experiments). The exudate experiment is not shown. From this experiment each single clone was evaluated if it was induced under specific condition compared to the background level of all the clones.

Appendix II.

Appendix II

Introduction to the Conju image analysis program.

The program Conju was developed together with Arne Heydorn who also created COMSTAT (Heydorn et al. 2000). Arne Heydorn wrote the program as a script in Matlab 5.1 (The Math Works), equipped with the Image Processing Tollbox.
The Conju program package can be found at the Internet site http://conju.dmu.dk.
The program is menu-controlled, user-friendly, and requires no prior knowledge in programming or image analysis (Heydorn et al. 2000).

Aim:
The aim of making the Conju image analysis program was an interest in analysing plasmid transfer at micro colony level between fluorescently marked Donor, Recipient and transconjugant cells. (See also Introduction).

Image requirement
It is necessary for image analysis that the plasmid is tagged with a fluorescent maker gene that gives the host cells a fluorescent color that can be separated from the fluorescent color of the recipient cells. Each fluorescent color needs to be recorded in a separate picture, and transconjugant cells will, therefore, be present in both pictures.

The bacterials have to be located in a single cell layer because Conju can only analyse images in two dimensions. To obtain high resolution pictures it is recommended to perform the image acquisitions with a confocal scanning laser microscopy equipped with a 100 x objective (see Fig. 13 in Introduction).

Image processing by Conju
Prior to quantification, each image is thresholded and median filtered. Additionally, the image may be filtered to get a minimum colony (or single cell) size. The program called looktifmore_new can be used to optimizs the different filter conditions. Thresholding of an image results in a two-dimensional matrix with a value of ONE in positions where the pixel values in the original image are above or equal to the threshold value, and ZERO where the pixel values are below the threshold value. The value ONE represents positions with bacteria, while ZERO represents the back-ground.

Conju makes an overlay of the two images, where the green color represent donor bacteria, the red color represent recipient bacteria and the yellow color (where there is an overlap between green and red) represent transconjugant bacteria. The image is divided out in three new images after the thresholding, where each picture represents one of the colors (green, red and yellow). All the image analysis is based on these three pictures. Optionally, the pre-processed image can be saved during execution of the program, and used later to check the input images for the quantification steps. Image information and quantification results are saved in two report files. One report file includes all the area quantification and the other report file contains the result calculated from the micro-colonies periphery.

Features calculated by Conju

Area occupied by donor, recipient and transconjugant bacteria. This is the fraction of the total area occupied by donor, recipient and transconjugant bacteria before and after thresholding.

Identification of area distribution of micro-colonies of donor, recipient and transconjugant bacteria. The function calculates the total number of identified micro-colonies, the area size of each micro-colony (pixel^2) and the mean micro-colony area (pixel^2).

Distance matrixes from the periphery of the donor micro-colonies to the recipient micro-colonies, and from the periphery of the transconjugant micro-colonies to the recipient micro-colonies. From each pixel in the periphery of donor and trans-conjugant micro-colonies is the shortest distance measured to the closest periphery of recipient micro-colonies. The circumference distance of all the donor and trans-conjugant micro-colonies can also be estimated from this parameter.

The idea of this parameter was to measure all the contact distances from plasmid hosts (donor and transconjugant) to the recipient cells. With this information it will be possible to see if there is a correlation in the distance between the donor and recipient bacteria and the transfer frequency. Additionally, it will be possible to investigate the plasmids re-transfer rate (transfer from a transconjugant cell to a recipient cell). This parameter, however, will only work in a system with minimal growth.

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