

Permissiveness of soil microbial communities towards broad host range plasmids

Uli Klümper

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DTU Environment
Department of Environmental Engineering
Technical University of Denmark

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Preface

This thesis summarizes the results of the above-mentioned PhD project carried out at the Department of Environmental Engineering at the Technical University of Denmark from December 2011 to March 2015. Professor Barth F. Smets and Senior Researcher Arnaud Dechesne supervised the project, which was funded through the Villum Kann Rasmussen Centre of Excellence CREAM (Center for Environmental and Applied Microbiology). The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductory review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-V**.

- I Klümper U, Dechesne A, Smets BF. (2014).** Protocol for evaluating the permissiveness of bacterial communities toward conjugal plasmids by quantification and isolation of transconjugants. *In: Hydrocarbon and Lipid Microbiology Protocols (eds.: Timmis K), Springer Protocols Handbook, Humana Press.*
- II Klümper U, Riber L, Dechesne A, Sannazzarro A, Hansen LH, Sørensen SJ, Smets BF. (2015).** Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. *ISME J* 9:934–945.
- III Klümper U, Droumpali A, Dechesne A, Smets BF. (2014).** Novel assay to measure the plasmid mobilizing potential of mixed microbial communities. *Front Microbiol* 5. DOI: 10.3389/fmicb.2014.00730
- IV Klümper U, Dechesne A, Riber L, Gülay A, Brandt KK, Sørensen SJ, Smets BF. (2015).** Taxon specific modulation of soil community permissiveness towards broad host range plasmid pKJK5 under metal stress. *Submitted* May 2015
- V Musovic S, Klümper U, Dechesne A, Magid J, Smets BF. (2014).** Long-term manure exposure increases soil bacterial community potential for plasmid uptake. *Environ Microbiol Rep* 6:125–130.

TEXT FOR WWW-VERSION (without papers)

In this online version of the thesis, the papers are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljøvej, Building 113, 2800 Kgs. Lyngby, Denmark, reception@env.dtu.dk.

In addition, the following publications and presentations, not included in this thesis, were also concluded during this PhD study:

Presentations at international conferences:

Klümper U, Brandt KK, Dechesne A, Riber L, Sørensen SJ, Smets BF. (2015). Metal specific modulation of community permissiveness towards broad host range plasmids through stress. Abstract from: 6th annual congress of European Microbiologists (FEMS 2015), Maastricht, The Netherlands. (oral presentation)

Klümper U, Brandt KK, Dechesne A, Riber L, Sørensen SJ, Smets BF. (2015). Metal stress modulates the immediate plasmid uptake potential of soil microbes. Abstract from: 13th Symposium on Bacterial Genetics and Ecology (BAGECO13), Milan, Italy. (oral presentation)

Klümper U, Dechesne A, Smets BF. (2015). Magnitude and determinants of plasmid transfer from exogenous donor strains to complex microbial communities. Abstract from: 3rd International Symposium on the Environmental Dimension of Antibiotic Resistance (EDAR-3), Wernigerode, Germany. (oral presentation)

Klümper U, Brandt KK, Dechesne A, Riber L, Sørensen SJ, Smets BF. (2015). Modulation of microbial community permissiveness towards broad host range conjugal plasmid is metal specific. Abstract from: 3rd International Symposium on the Environmental Dimension of Antibiotic Resistance (EDAR-3), Wernigerode, Germany. (poster presentation)

Klümper U, Riber L, Dechesne A, Sannazzaro A, Hansen LH, Sørensen SJ, Smets BF. (2014). Deep sequencing of soil transconjugal pools reveals unexpected phylogenetic diversity of bacteria receiving broad host range plasmids. Abstract from: 15th International Symposium on Microbial Ecology (ISME15), Seoul, South Korea (oral presentation)

Klümper U, Brandt KK, Dechesne A, Riber L, Sørensen SJ, Smets BF. (2014). Metal stress alters a bacterial community's permissiveness towards plasmids. Abstract from: 15th International Symposium on Microbial Ecology (ISME15), Seoul, South Korea (poster presentation)

Klümper U, Brandt KK, Dechesne A, Riber L, Sørensen SJ, Smets BF. (2014). Metal stress response influences a soil bacterial community's permissiveness towards a broad-host-range plasmid. Abstract from: The Danish Microbiological Society Annual Congress 2014, Copenhagen, Denmark (poster presentation)

Klümper U, Riber L, Sannazzaro A, Dechesne A, Musovic S, Hansen LH, Sørensen SJ, Smets BF. (2013). Assessing the permissiveness of complex bacterial communities towards conjugal plasmids - A novel method. Abstract from: 12th Symposium on Bacterial Genetics and Ecology (BAGECO12), Ljubljana, Slovakia (poster presentation)

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Musovic S, **Klümper U**, Lundin L, Sørensen SJ, Smets BF. (2012). Permissiveness of soil microbial communities toward receipt of mobile genetic elements. Abstract from: 14th International Symposium on Microbial Ecology (ISME14), Copenhagen, Denmark (poster presentation)

Scientific reports to the Danish authorities:

Christensen SCB, Esbjørn A, Møllerup F, Musovic S, **Klümper U**, Albrechtsen, HJ. (2014) Mikrobiel vandkvalitet i rentvandsbeholdere efter inspektion og rensning.

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Summary

Horizontal transfer of mobile genetic elements facilitates adaptive and evolutionary processes in bacteria. Among the known mobile genetic elements, plasmids can confer their hosts with accessory adaptive traits, such as antibiotic or heavy metal resistances, or additional metabolic pathways. Plasmids are implicated in the rapid spread of antibiotic resistance and the emergence of multi-resistant pathogenic bacteria, making it crucial to be able to quantify, understand, and, ideally, control plasmid transfer in mixed microbial communities. The fate of plasmids in microbial communities and the extent of bacterial phyla permissive towards plasmid receipt are largely unknown. Historically, methods exploring the underlying genetic and environmental factors of plasmid transfer have been heavily reliant on cultivation and expression of plasmid encoded phenotypes. This has provided an incomplete and potentially cultivation biased image of the extent of plasmid transfer.

In this thesis, I investigated the extent of plasmid transfer in microbial communities at an unprecedented level of resolution and not reliant on cultivation. I focused on soil microbial communities. Their potential role as a reservoir for plasmids carrying antibiotic resistance genes is increasingly suspected to majorly contribute to the emergence of multi-resistant pathogens. More specifically, I examined what fraction of a soil microbial community is permissive to plasmids, identified the phylogenetic identity of this fraction and studied environmental factors that modulate plasmid transfer in soil microbial communities.

In order to attain these goals, I developed a high-throughput method that enabled me to evaluate the permissiveness of bacterial communities towards introduced plasmids. This new approach is based on the introduction of fluorescently tagged conjugative plasmids into a soil microbial community in solid-surface filter matings under maximized cell-to-cell contact, followed by quantification of transfer events through advanced fluorescent microscopy, isolation of transconjugants through triple-gated fluorescent activated cell sorting and finally 16S rRNA targeted pyrosequencing of the sorted transconjugal pools.

Employing this new method, I was able to map, for the first time, the diversity of all recipients in a soil microbial community for three broad host range model plasmids: RP4, pKJK5, and pIPO2tet. I found that a large fraction of soil the bacteria (up to 1 in 10,000) were able to take up any of these broad

host range conjugal plasmids. The transconjugal pools comprised 11 bacterial phyla. This finding indicates that the realized transfer range of broad host range plasmids in environmental microbial communities is much larger than previously assumed. I was able to show abundant plasmid transfer from the Gram negative donor strains to a wide diversity of Gram positive soil bacteria, formerly thought to constitute distinct clusters of gene transfer. Moreover, among the observed transconjugants, I identified a core super-permissive fraction of taxa prone to receive diverse BHR plasmids from diverse donors. This fraction comprised the proteobacterial genera *Pseudomonas*, *Enterobacterium* and *Burkholderia*. These taxa are known to be evolutionary interlinked through chromosomal gene exchange. Hence, I was able to show that the gene pool of microbial communities may be directly interconnected through transfer of BHR plasmids at a so far unrecognized level.

The developed method furthermore enabled me to explore how agronomic practices may affect gene transfer in soil microbial communities. I compared bacterial communities extracted from plots subjected to different treatments for their permissiveness towards the model BHR plasmids RP4, pRO101 and pIPO2tet. Periodic manure introduction increased the permissiveness of the community towards these plasmids by up to 100% compared to control treatments. However, the phylogenetic composition of the transconjugal pools remained similar. The underlying mechanisms remain unclear.

Subsequently, I focused on the effect of metal cations - Cu, Ni, Zn, and Cd – on community permissiveness. These cations are common environmental stressors associated with manure application to agricultural soils. I postulate an increased permissiveness of the community as a generic stress response to acquire foreign genes potentially conferring adaptive traits. I therefore evaluated to what extent short term metal stress modulated plasmid transfer. I analyzed both the transfer frequency and the phylogeny of the transconjugal pools using model BHR plasmid pKJK5 introduced through the γ -proteobacterial donor *E. coli*. I found that the permissiveness towards plasmids was modified through stress on a taxon specific basis and cannot be generally predicted for the whole community.

The response of the phylogenetic group was specific for the metal and level of stress imposed. The phylum *Bacteroidetes*, for example, displayed an increased permissiveness at moderate (20% growth inhibition) but not at severe (50% growth inhibition) applied Cu or Ni stress. I therefore showed that spe-

cific metal stress can increase or decrease gene transfer between phylogenetically distant groups.

Finally, I extended the high-throughput method to quantify the potential of a microbial community to actively mobilize and transfer exogenous mobilizable plasmids to its indigenous members. I evaluated the transfer frequency of model plasmid RSF1010 by comparing it to the community's permissiveness towards the mobilizing, conjugal plasmid RP4 and to the rate of transfer between isogenic strains. My results indicated that retromobilization takes place at frequencies only one order magnitude lower than permissiveness for conjugal RP4 transfer. Mobilizable plasmids transferred in the communities at frequencies of up to 30 times higher than the conjugal plasmid RP4 itself when co-resident with a conjugative plasmid.

In conclusion, in this thesis I developed a novel toolbox to study plasmid transfer of conjugal and mobilizable plasmids in mixed microbial communities. This method allows, for the first time, a detailed mapping of the realized transfer range of plasmids. I discovered that a previously far underestimated fraction of bacteria in natural communities is directly interconnected through BHR plasmid transfer. While a super-permissive fraction of bacteria were able to take up plasmids at high frequencies from diverse donors, I showed plasmid or donor dependence of plasmid transfer to other species. Additionally, environmental factors like stress also impact the permissiveness of phylogenetic groups towards plasmids. The developed method and results increase our ability to predict the fate and impact of plasmids in microbial communities.

Dansk sammenfatning

Horisontal overførsel af mobile genetiske elementer tilskynder de adaptive og evolutionære processer i bakterier. Blandt de kendte mobile genetiske elementer er plasmider i stand til at overdrage deres værter ikke-essentielle adaptive træk såsom resistens mod antibiotika eller tungmetaller, eller yderligere metaboliske reaktionsveje. Plasmider er indblandede i antibiotikaresistens' hurtige udbredelse og i fremkomsten af multiresistente patogene bakterier. Det er derfor afgørende at være i stand til at kvantificere, forstå og, ideelt, kontrollere overførselen af plasmider i diverse mikrobielle samfund. Plasmiders skæbne i mikrobielle samfund samt omfanget af bakterierækker, som er modtagelige overfor deres optagelse er stort set ukendt. Historiskset har metoderne hidtil benyttede for at undersøge de genetiske samt de miljømæssige faktorer, der ligger til grunde for plasmidoverførsel været stærkt afhængige af dyrkningen samt ekspresionen af plasmidkodede fænotyper. Dette har resulteret i et ukomplet og potentielt partisk billede af plasmidoverførsels udstrækning.

I denne afhandling undersøger jeg omfanget af plasmidoverførsel i mikrobielle samfund, på et hidtil uset niveau og uafhængigt af kultivering. Jeg fokuserede på mikrobielle samfund i jordmiljøet. Deres potentielle rolle som reservoir for plasmider bærende på antibiotikaresistente gener formodes nemlig at bidrage i høj grad til forekomsten af multiresistente patogener. Jeg undersøgte mere specifikt, hvor stor en andel af det mikrobielle samfund i jord er modtagelig overfor plasmider, identificerede denne brøkdels fylogenetiske identitet og vurderede de miljømæssige faktorer, der regulerer plasmidoverførsel i mikrobielle samfund, som stammer fra jord.

For at opnå disse mål udviklede jeg en high-throughput metode til at vurdere bakterielle samfunds modtagelighed overfor udefrakommende plasmider. Denne nye tilgang omfatter indførelse af fluorescensmærkede konjugative plasmider i et mikrobielt samfund fra jord ved parring under maksimeret celledkontakt på filtre med en fast overflade, efterfulgt af kvantificering af overførselshændelser via avanceret fluorescensmikroskopi, isolering af transkonjuganter ved tredobbeltportet fluorescensaktiveret celledsortering og til slut 16S rRNA målrettet pyrosekventering af de separerede transkonjugative puljer.

Ved at anvende denne nye metode var jeg i stand til at kortlægge diversiteten af alle modtagere i et mikrobielt samfund fra jorden for tre bredt værtsspektrum plasmider: RP4, pKJK5 og pIPO2tet. Det viste sig, at en stor del af jordbakterierne (op til 1 ud af 10.000) var i stand til at optage enhver af disse bredt værtsspektrum konjugative plasmider. De transkonjugative puljer beståede af 11 bakterierækker. Dette resultat indikerer, at den realiseret overførsel af bredt værtsspektrum plasmider i mikrobielle samfund fra jord er langt større end tidligere antaget. Jeg påviste omfattende overførsel fra den Gram-negative donorstamme til vidt forskellige Gram-positive jordbakterier, førhen ment at danne adskilte grupper for genoverførsel. Af de observerede transkonjuganter identificerede jeg en særlig modtagelig taxa, tilbøjelig til at optage diverse bredt værtsspektrum plasmider fra forskellige donorer. Denne andel beståede af Proteobakterielle slægter *Pseudomonas*, *Enterobakterie* og *Burkholderia*. Disse taxa er kendt for værende stærkt forbundet i evolutionsprocessens genudveksling af kromosomer. Derfor var jeg i stand til at påvise, at genpuljen i mikrobielle samfund kunne være direkte forbundet gennem overførselen af bredt værtsspektrum plasmider.

Den udviklede metode gjorde det endvidere muligt at udforske, hvorledes landbruget påvirker genoverførsel i mikrobielle samfund fra jorden. Jeg sammenlignede bakterielle samfund udtaget fra felter udsat for forskellige behandlinger for at undersøge deres modtagelighed overfor bredt værtsspektrum plasmiderne RP4, pRO101 og pIPO2tet. Periodisk husdyrgødning øgede samfundets modtagelighed overfor disse plasmider med op til 100 % i forhold til kontrol behandlinger. Dog forblev transkonjuganternes fylogenetiske sammensætning den samme og de underliggende mekanismer er fortsat uklare.

Efterfølgende fokuserede jeg på effekten af metal kationer – Cu, Ni, Zn, Cd – på mikrobielle samfunds modtagelighed overfor plasmider. Disse kationer repræsenterer en sædvanlig miljømæssig stressfaktor ofte associeret med gødning af landbrugsjord. Som generel stressrespons postulerede jeg en stigning i samfundets modtagelighed overfor fremmede gener til overdragelsen af potentielt adaptive træk. Derfor undersøgte jeg i hvilken grad kortvarigt metalstress regulerer plasmidoverførsel. Jeg analyserede både overførselsfrekvensen samt transkonjuganternes fylogeni ved brug af bredt værtsspektrum plasmidet pKJK5 introduceret i γ -proteobakterie donoren *E. coli*. Jeg fandt, at modtageligheden overfor plasmider ændredes ved stress på et taxaspecifikt plan og kan ikke forudses generelt for hele samfundet.

Den fylogenetisk gruppes respons afhæng af metallet samt graden af stress pålagt. For eksempel udviste rækken *Bacteroidetes* en øget modtagelighed ved moderat (20 % væksthæmning) men ikke ved svær (50 % væksthæmning) Cu eller Ni stress. Jeg påviste derfor, at specifik metalstress kan øge eller nedsætte genoverførsel blandt fylogenetiskdistancerede grupper.

Til slut udvidede jeg high-throughput metoden til at kunne kvantificere et mikrobielt samfunds evne for aktivt at mobilisere og overføre eksogene mobiliserbare plasmider til dets endogene medlemmer. Jeg evaluerede overførselsfrekvensen af model plasmidet RSF1010 ved sammenligning med både samfundets modtagelighed overfor det mobiliserende konjugativ plasmid RP4 samt overførselsraten mellem isogene stammer. Mine resultater indikerede, at frekvenser for retromobilisering finder sted blot i én størrelsesorden lavere end modtageligheden overfor det konjugative plasmid RP4. Overførselsfrekvenser af mobiliserbare plasmider til de mikrobielle samfund viste sig værende derimod op til 30 gange højere end det medfødte konjugative plasmid RP4.

Som konklusion udviklede jeg i denne afhandling en ny værktøjskasse til at undersøge overførsel af konjugative og mobiliserbare plasmider i diverse mikrobielle samfund. Dette muliggøre, for første gang, en detaljeret kortlægning af overførselsspektret for plasmider. Jeg opdagede, at en, førhen langt undervurderet, bakteriebrøkdæl af naturlige mikrobielle samfund, er direkte forbundet med bredt værtsspektrum plasmidoverførsel. Imens én andel bakterier udviste særlig modtagelighed ved at optage plasmider ved højere frekvenser og fra forskellige donorer, påviste jeg plasmid- eller donorafhængighed for plasmidoverførsel til andre arter. Derudover påvirker miljømæssige faktorer såsom stress ligeledes fylogenetiske gruppers modtagelighed overfor plasmider. Den udviklede metode og resultater øger vores evne til at forudse plasmiders skæbne samt indvirkning på mikrobielle samfund.

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Abbreviations

BHR	broad host range
cas	CRISPR-associated
CLSM	confocal laser scanning microscopy
CRISPR	clustered regularly interspaced short palindromic repeats
<i>ctl</i>	plasmid maintenance and partitioning control genes
DNA	deoxyribonucleic acid
FACS	fluorescence activated cell sorting
FSC	front scatter
GC-content	guanine-cytosine content
GFP	green fluorescent protein
<i>gfp</i>	green fluorescent protein encoding gene
HGT	horizontal gene transfer
ICE	integrative and conjugative elements
Inc	incompatibility
IS	insertion sequence
kbp	kilo base pairs
MGE	mobile genetic element
MOB	mobilization apparatus
MPF	mating pair formation
mRNA	messenger ribonucleic acid
NHR	narrow host range
NPK	nitrate-phosphate-potassium fertilized
oriT	origin of transfer
oriV	origin of replication

OTU	operational taxonomic units
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
rep	replication region
RM	restriction modification
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SM	stereo microscopy
SSC	side scatter
T/R	transconjugants per recipient
T4CP	type IV coupling protein
T4SS	type IV secretion system
TE	transposable elements
Tra	transfer region
<i>trfA</i>	replication initiation genes
<i>trfB</i>	mating bridge formation genes
VGT	vertical gene transfer

1 Aims and Objectives

Conjugal plasmid transfer is a key mechanism facilitating adaptive and evolutionary processes in bacteria (Gogarten *et al.*, 2002; Heuer & Smalla, 2012). Mobile genetic elements such as plasmids can confer accessory adaptive traits, such as antibiotic or heavy metal resistance, or additional metabolic pathways. Especially their involvement in the rapid spread of antibiotic resistance and the emergence of multi-resistant pathogenic bacteria make plasmid transfer and its underlying mechanisms a current research focus (WHO, 2014).

The diverse soil environment serves as a reservoir of antibiotic resistance genes. The multitude of these diverse antibiotic resistance genes within a microbial community is known as its resistome. Recent genomic analysis indicated that the resistome of soil is highly interconnected through identical resistance genes with that in multi-resistant human pathogenic strains found in hospitals (Forsberg *et al.*, 2012). Plasmid transfer was found to be the main vector connecting the distinct genetic pools of soil and human pathogens (Finley *et al.*, 2013). Elevated levels of plasmid encoded antibiotic resistance have especially been found in agriculturally treated soils (Agersø *et al.*, 2006; You *et al.*, 2012). As an increased soil resistome might result in subsequent transfer of resistance to pathogens, it becomes crucial to understand the fate of plasmids, the extent of plasmid transfer, as well as the agronomic impact on the exchange of plasmids in soil.

Earlier studies identified several environmental factors affecting plasmid transfer to the complex soil communities. Several of those might be impacted by agronomic practice. These factors include, biological (Sengeløv *et al.*, 2000; Sørensen & Jensen, 1998) and abiotic ones such as nutrient availability (Sørensen & Jensen, 1998), stress exposure (Top *et al.*, 1995) or physicochemical soil parameters such as pH (Richaume *et al.*, 1989; Ellass & Trevors, 1990; Rochelle *et al.*, 1989). However, these former studies were purely based on transfer frequency and not initial horizontal transfer was not distinguished from subsequent vertical transfer of the plasmids during growth. Additionally, transfer frequencies were measured on the community average level, without taking into account that in mixed communities different strains might be not equally permissive towards plasmids.

Observing how different strains differ in their permissiveness became possible with the emergence of *in situ* monitoring tools relying on detecting plas-

mid transfer through plasmid encoded fluorescent reporter genes (Tolker-Nielsen *et al.*, 2000). Using fluorescent tools allowed also the isolation of transconjugants from mixed microbial communities (Gelder *et al.*, 2005; Musovic *et al.*, 2006; Shintani *et al.*, 2014). However, those studies were limited to few hundred transconjugants at most, therefore identifying only the main recipients of the studied plasmids. In the diverse soil environment this might provide an incomplete picture of the widespread range of plasmid transfer. For understanding the extent and factors influencing plasmid transfer in the highly diverse soil environment a new high-throughput method to isolate and identify the fraction of a soil microbial community able to take up plasmids is needed. In this PhD thesis I therefore aimed to:

- i. Develop a high-throughput method to quantify, isolate and identify that fraction of a bacterial community which is able to receive or actively mobilize introduced broad host range plasmids. (Paper I-III)
- ii. Identify how the type of broad host range plasmid and the plasmid donor affect the richness and the diversity of the permissive fraction of a mixed microbial community (Paper II)
- iii. Determine how the exposure to metal stress modulates horizontal gene transfer in soil bacterial communities (Paper IV) and how long-term agronomic practices alter the permissiveness of communities (Paper V).

2 Soil borne antibiotic resistance

Soil represents the thin layer of inorganic particles located at the interface of the atmosphere and the earth landmasses. This layer is comprised of different particles like sands, silts, and clays with various sizes and morphologies. These particles create an extremely rich and heterogeneous environment of aggregates and pores. Between the pores and surfaces of diverse architecture chemical parameters such as redox potential, exchange of gas and nutrients and water movement and retention can vary majorly (Pepper, 2013). The heterogeneity enables a huge variety of microorganisms to co-exist on a relatively small scale. This arguably makes soil the most complex biological system with an outstanding ability to retain, transform and release chemicals. It's very diverse micro-niches make soil one of the most diverse biohabitats with estimates ranging up to 8,300,000 bacterial species per gram of soil (Roesch *et al.*, 2007).

Soil microorganisms have been recognized as an important resource. Through their involvement in a huge variety of biotransformation processes they contribute significantly to soil fertility (Mäder *et al.*, 2002). For example the phosphorus uptake by plants and thus their growth and harvest can be increased through soil microorganisms (Richardson, 2001). Soil bacterial communities are also involved in the removal of organic pollutants through biodegradation (Ahmad *et al.*, 2011).

Apart from their biotransformation ability soil microbes are a major source of complex organic compounds. Most of today's antibiotics originate from the diverse soil microbial communities. Antibiotics are compounds produced by bacteria that have either bacteriostatic or bactericidal properties. The first discovered antibiotic penicillin was isolated from a soil fungus (Fleming, 1942). Soil bacteria, particularly Actinomycetes (Demain & Fang, 2000), are the source of a variety of currently used antibiotic compounds. The human health care sector nowadays majorly relies upon these soil born antibiotics in the treatment of bacterial infections.

Recently the rise of antibiotic resistance and especially the increasing number of multidrug-resistant pathogens, was considered as a major challenge for future human health (WHO, 2014). With its high content of antibiotic producing bacteria genes encoding for resistance to these compounds are ubiquitous in soil indigenous organisms (D'Costa *et al.*, 2006; Brooks *et al.*, 2007).

A major concern with many agricultural soils is the application of antibiotic residues through manure (Chee-Sanford *et al.*, 2009) which might enrich antibiotic resistant bacteria in the communities. Furthermore, a huge variety of non-indigenous, manure-borne microorganisms are introduced into the soil community. These come with increased levels of antibiotic resistance genes (Smalla *et al.*, 2000). While most of these enteric bacteria do not survive in soil (Pepper, 2013) their genes might survive after being horizontally transferred to soil indigenous bacteria and prevail in their new hosts.

To understand and tackle the spread of antibiotic resistance from and to soil the underlying mechanisms of horizontal gene transfer as well as the extent and range of the spread of resistance genes encoded on mobile elements needs further elucidation.

3 Significance and Modes of Horizontal Gene Transfer

Gene transfer refers to the movement of genetic material between microorganisms. As opposed to vertical gene transfer (VGT), defined as the genetic inheritance from evolutionary ancestors, HGT refers to the movement of genetic material between individual prokaryotes within a population or between different prokaryotes within a community (Francino, 2012). This uptake of foreign DNA may involve complete genes or operons, resulting, once established, in the acquisition of new phenotypic characteristics in the receiving bacteria.

Horizontal gene transfer (HGT) between different species has been recognized as a common and major evolutionary process among prokaryotes (Zhaxybayeva & Doolittle, 2011). HGT is the major force impacting the adaptive evolution and rapid adaptation (Daubin *et al.*, 2003; Gogarten *et al.*, 2002; Heuer & Smalla, 2012). Based on metabolic network analysis in *E. coli*, HGT is the main driver of adaptation to new environments, while mutations are the main evolutionary force when it comes to optimizations of the strains under fixed environmental conditions (Pál *et al.*, 2005).

HGT was first considered a relevant contributor to bacterial evolution to explain the rapid emergence of multidrug-resistant bacteria in the 1940's (Roberts, 1996; Davies & Davies, 2010). Still, the first appropriate quantification of the long-term impact of horizontal gene acquisition was not possible until approximately 20 years ago, when the emergence of whole genome sequencing allowed screening bacterial sequences for foreign genes (Lawrence & Ochman, 1998). Based on atypical nucleotide composition or restricted phylogenetic distribution of specific genes between related strains up to 16.6% of the *Escherichia coli* bacterial genome were identified as horizontally acquired DNA (Ochman *et al.*, 2000).

When analyzing the acquired genes, significant similarities with sequences of other bacterial species became apparent, allowing deducing the route of gene acquisition (Ochman *et al.*, 2000). All these identified, directed HGT events from donors to recipients of the acquired genes can then be combined to create networks of lateral gene acquisitions (Popa *et al.*, 2011). Most gene acquisitions were shown to occur between donors and recipients residing in the same habitat (Popa & Dagan, 2011). In most cases only a few closely related strains exchanged various genes and are the core nodes in an interconnected

cluster of lateral gene acquisition (Popa *et al.*, 2011). While gene acquisition in nature mainly occurs within taxonomically homogenous groups, some heterogeneous communities like the ones found in soil can provide hot-spots for gene acquisition from distant phylogenetic groups (Popa *et al.*, 2011).

Gene acquisition might not only appear among prokaryotes, as most recent genomic analysis of eukaryotes suggested that genes acquired through HGT are commonly integrated and expressed in most eukaryotic genomes including the human one at a unsuspected scale (Crisp *et al.*, 2015).

The long-term phylogenetic approach though constrains analysis to HGT events that were evolutionary conserved in the bacterial chromosome through recombination and integration of the formerly mobile DNA. Thus complementary approaches to study HGT events in situ (Sørensen *et al.*, 2005), trying to identify the forces that drive horizontal gene acquisition in diverse environments (Van Elsas *et al.*, 2003; Newby & Pepper, 2002; Arango Pinedo & Smets, 2005; Heuer *et al.*, 2011) were applied..

HGT among prokaryotes can occur through three main mechanisms: transformation, transduction and conjugation (Figure 1). While all these processes share the transfer of genetic material from one cell to another as a common characteristic, the transferable DNA fragments, known as mobile genetic elements (MGE), may be very diverse.

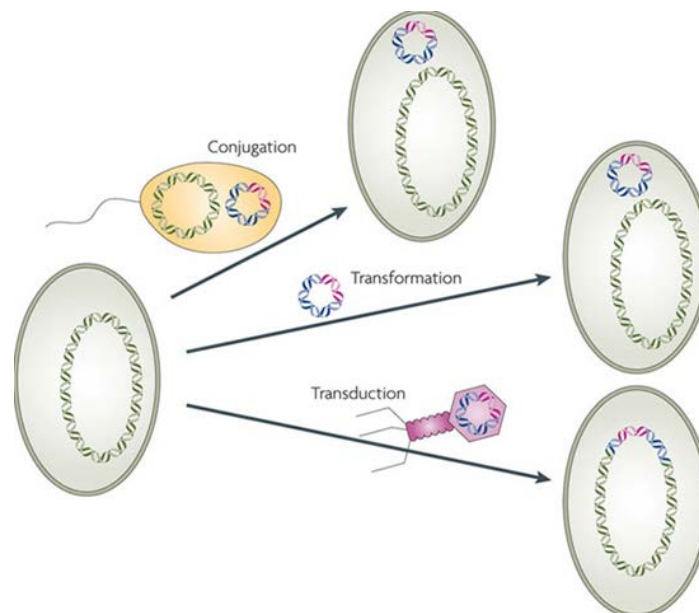


Figure 1 The three general modes of bacterial gene transfer: conjugation, transformation and transduction (modified from (Todar *et al.*, 2008)).

3.1 Transformation:

Transformation refers to the bacterial uptake of free exogenous environmental DNA. Only a minor fraction of bacterial cells in mixed or pure bacterial populations is able to take up this exogenous DNA and is, therefore, referred to as competent. Competence is a physiological state of bacteria, which can either occur constitutionally or induced by environmental factors (Hanahan, 1983; Nielsen & Van Elsas, 2001).

Transformation is the only HGT mechanism that is purely host dependent, since it only requires that the host, in a competent physiological state, is exposed to the free exogenous DNA. This host dependence makes transformation a process commonly used to introduce foreign DNA in gene technology, as the manipulation of environmental factors such as the availability and the type of nutrients or cell density allows the artificial creation of competent recipient cells.

Successful transformation events have been demonstrated in a wide variety of bacterial species and environments (Nielsen & Van Elsas, 2001; Averhoff & Friedrich, 2003; Matsui *et al.*, 2003; Sørensen *et al.*, 2005; Bräutigam *et al.*, 1997). It was also the first HGT mechanism that was established as able to transform an environmental bacterium into a virulent phenotype (Griffith, 1966). Still, the impact and extent of transformation in nature as well as its contribution in bacterial evolution is still relatively unexplored (Johnsborg *et al.*, 2007).

3.2 Transduction

Transduction is an indirect DNA transfer mechanism that is mediated by bacteriophages. It relies on mistakes in the viral packaging or prophage excision, during which genes from the current host are integrated in the phage DNA. When infecting a new host the phage DNA is integrated into the host's chromosome, including the former host's genes.

With numbers of up to 10^7 bacteriophages per mL seawater (Danovaro *et al.*, 2008) and thereby far outnumbering the 10^6 bacteria found per mL (Brüssow & Hendrix, 2002) transduction is a major contributor to DNA transfer in marine environments (Danovaro *et al.*, 2008; Zhao *et al.*, 2013). With an assumed number of 20,000,000 transduction events per second in the global marine waters (Uhlir, 2012), marine viruses are considered major players in the global ecosystem (Suttle, 2007). But, due to its reliance on mistakes most transduction events will not transfer functional DNA. The impact of transduc-

tion to adaptation and evolution of bacteria might therefore be relatively low. Still, unlike originally assumed (Bergh *et al.*, 1989) phages might not be limited to a small host range but can interconnect gene transfer networks in a wide range of bacterial genera (Chen & Novick, 2009).

3.3 Conjugation

Conjugation refers to the direct exchange of DNA between two bacterial cells that are connected through a mating pore in direct cell-to-contact. Conjugative transfer is one of the most efficient mechanisms for the exchange of mobile genetic elements (Halary *et al.*, 2010; Guglielmini *et al.*, 2011)

In theory, whole chromosomes could be transferred if they possess an origin of transfer location (*oriT*). But with a speed of around 45 kilo base pairs (kbp) per minute (Lawley *et al.*, 2004) the mating pair would need to be stable for longer than an hour to successfully transfer the whole chromosome of *E. coli* (Thomas & Nielsen, 2005). Hence, the transfer of complete bacterial chromosomes becomes rather unlikely during conjugation.

In general, small sized (up to 100 kb) mobile genetic elements are transferred in conjugative events. Plasmids are therefore the main vectors of genetic information transferred in conjugation events.

The type IV secretion systems involved in creating the mating connection are able to connect a huge variety of organisms across phyla and even domains of life (Thomas & Nielsen, 2005; Grahn *et al.*, 2000). Conjugative plasmid transfer through type IV mating systems thus becomes one of the most important mechanisms facilitating adaptive and evolutionary processes in bacteria (Aminov, 2011). Conjugative plasmids are also involved in the rapid spread of antibiotic resistance to pathogens, and remain key contributors in the rise of multi-resistant microbes in hospitals (Levy & Marshall, 2004) and animal husbandries (Zhu *et al.*, 2013).

4 Fate of plasmids in microbial communities

4.1 Plasmids

Joshua Lederberg proposed the generic term plasmid in 1952 as any extrachromosomal hereditary determinant independent of its genetic complexity (Lederberg, 1952). It was first used in bacteria for describing the fertility factor F in *E. coli*, which was known to promote mating contacts (Lederberg *et al.*, 1952). Nowadays, plasmids are defined as self-replicating genetic elements of linear or circular double-stranded DNA. Their size might range from as small as 1 to over 1000 kbp.

The ecology of plasmids in mixed communities relies mainly on three mechanisms: plasmid gain, maintenance and plasmid loss. A vital feature in the gain of plasmids is their ability to be transferred horizontally and consequently be maintained by autonomous replication in their host organism. The modules coding for these functions are referred to as plasmid-selfish modules only involved in their own propagation and proliferation, preventing plasmids from extinction from a microbial community, when environmental conditions suddenly change (Norman *et al.*, 2009). The modules and mechanisms of plasmid gain through transfer and their subsequent maintenance will be discussed in detail in the following section.

Due to the metabolic burden of their selfish modules plasmids have also been described as molecular parasites (Norman *et al.*, 2009) to their host organism. Their selfish nature allows plasmids to have alternative hosts within heterogeneous populations. Plasmids have been found in a huge variety of bacterial phyla, including Gram-negative as well as Gram-positive ones, cyanobacteria, archaea, fungi and even higher order eukaryotic organisms like plants (Figure 2). Transfer from bacteria to a huge variety of these organisms has been confirmed in lab experiments (Shintani *et al.*, 2014; Yano *et al.*, 2013; Musovic *et al.*, 2006).

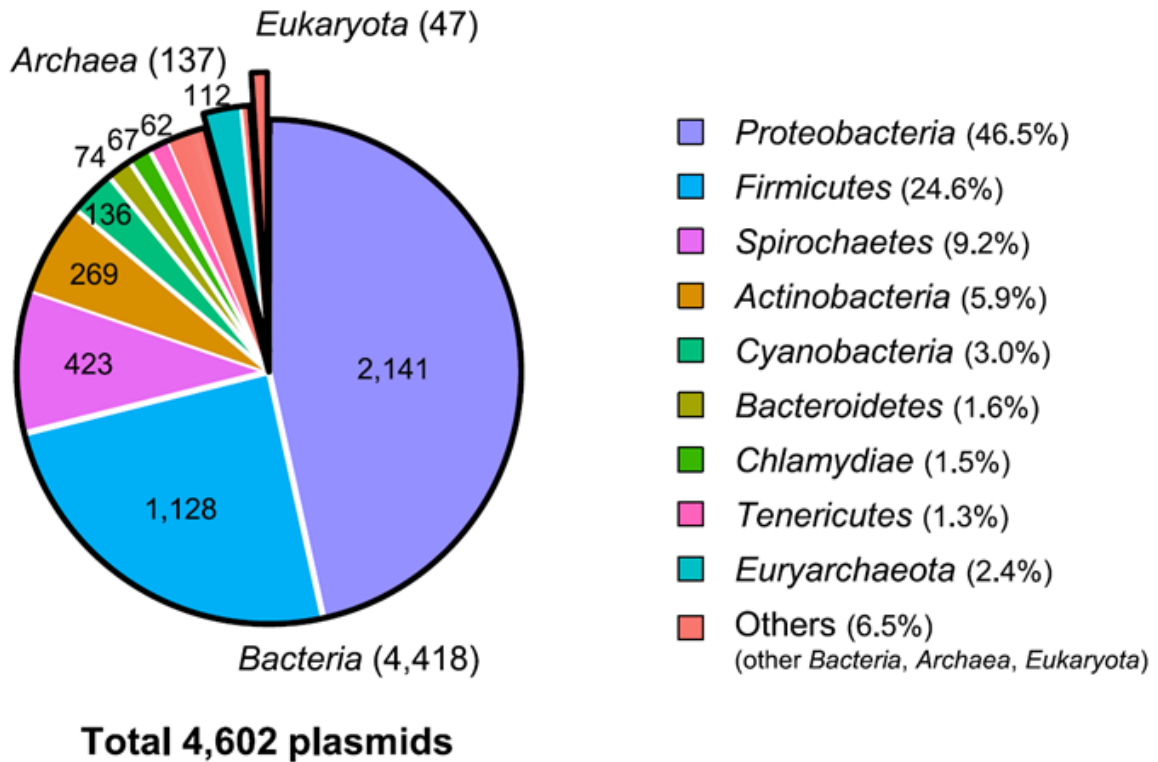


Figure 2 Phylogenetic distribution of the hosts of all currently fully sequenced plasmids (Shintani et al., 2015).

Apart from their selfish modules many plasmids encode accessory genes (Figure 3). Some plasmids, though, do not confer any beneficial traits to their hosts and are therefore called cryptic plasmid (Van Elsas *et al.*, 1998). Since cryptic plasmids do not have any selective advantages, their maintenance in microbial communities solely relies on a highly efficient plasmid encoded maintenance and transfer machinery.

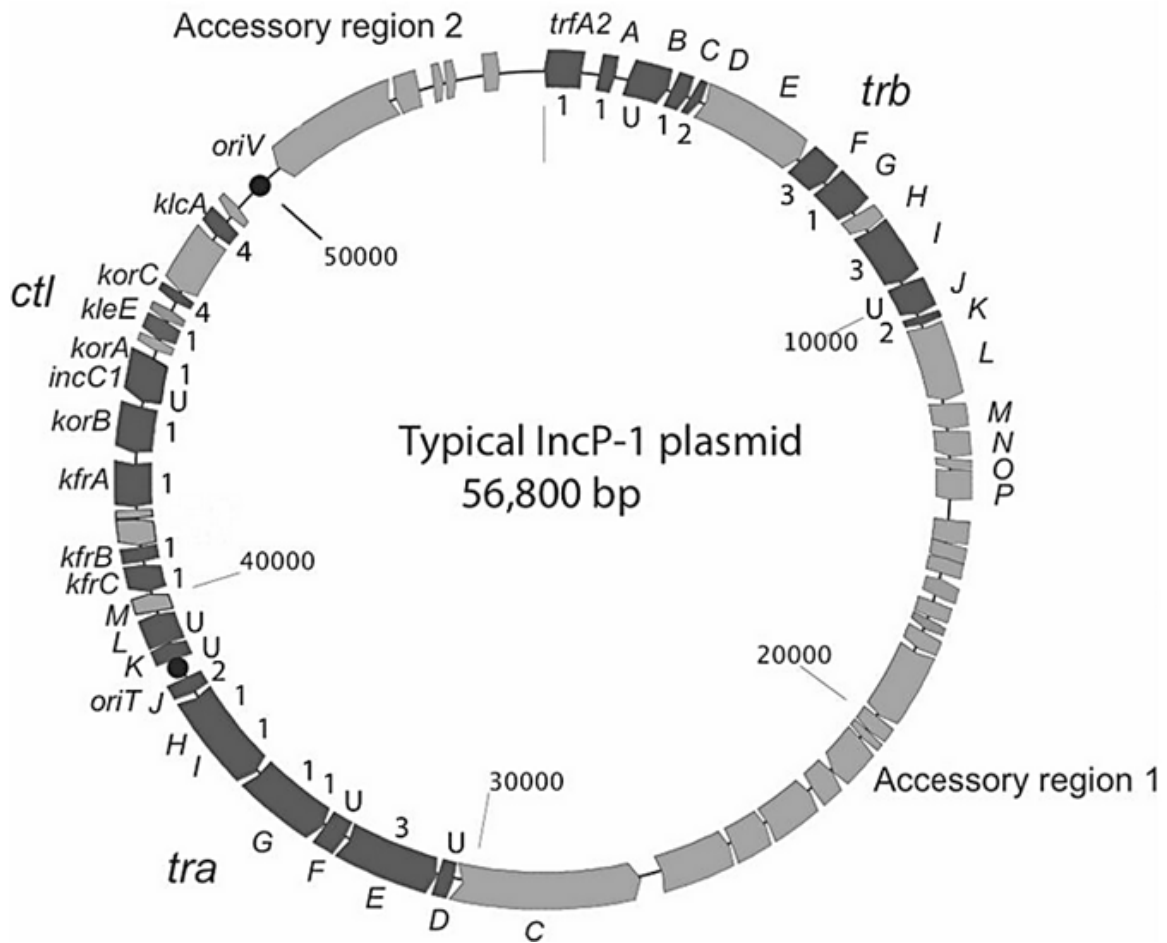


Figure 3 Genetic map of a typical broad host range conjugative IncP-1 plasmid. Two accessory gene regions with potentially adaptive traits for its host are found in addition to the plasmids backbone structure. The plasmid backbone consists of a replication and maintenance region (origin of replication (*oriV*), replication initiation genes (*trfA*) and plasmid maintenance and partitioning control (*ctl*)) and a mobility region (mating bridge formation (*trfB*), transfer region (*tra*)). While the selfish modules involved in transfer, maintenance and replication are shown in dark grey, the accessory regions are displayed in light grey (Sen *et al.*, 2013).

Genes found in the accessory regions can increase their host's fitness by supplying them with metal or antibiotic resistance, additional metabolic pathways or the ability to form stronger biofilms (Ghigo, 2001). Accessory traits of plasmids are regularly encoded within transposable elements (TE) which permit integration of these genes into the host chromosome (Schlüter *et al.*, 2007). The transfer and transposable nature of plasmid encoded genes make plasmids important tools in molecular microbiology allowing the manipulation of bacteria through introduction or removal of certain genes.

4.2 Plasmid host ranges

Plasmids are categorized into having a narrow or broad host range by their abilities to transfer and be maintained in a variety of phylogenetically distant bacterial hosts. While broad host range (BHR) plasmids are able to transfer across diverse bacterial phyla and sometimes even across domains of life (Waters, 2001; Heinemann & Sprague, 1989), narrow host range (NHR) plasmids are limited at one of the steps required for successful transfer or maintenance (Thomas & Nielsen, 2005).

Three different host ranges of plasmids are defined through the duration and intimacy of the considered plasmid-host relationship (Suzuki *et al.*, 2010). The transfer range is defined as the range of microbial organisms that are able to take up a certain plasmid. The replication and maintenance host range, includes all organisms in which the plasmid can be stably maintained and replicate independent of the host. The evolutionary host range describes the variety of organisms in which a given plasmid was maintained long enough to undergo adaptation of its backbone to the genetic code of its host organism (Suzuki *et al.*, 2010) (Figure 4).

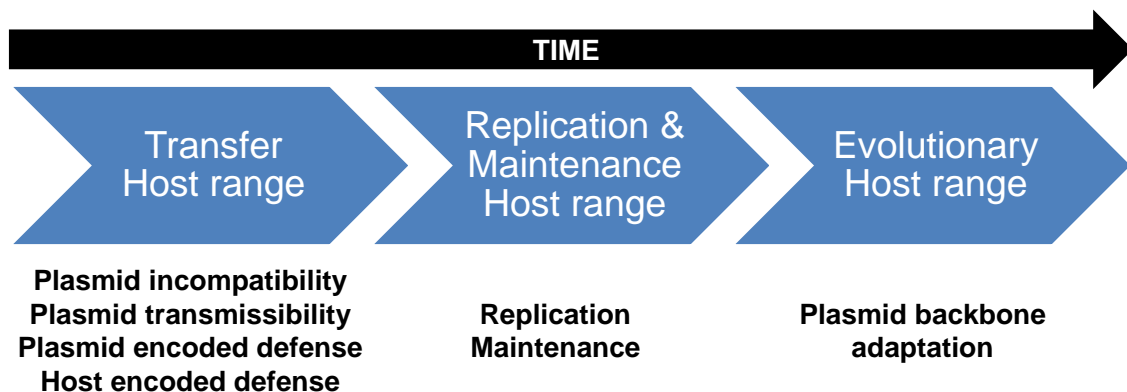


Figure 4 Plasmid host ranges based on residence time in the new host. The transfer host range includes all hosts that can initially receive a given plasmid. The replication & maintenance host range describes all those hosts in which a plasmid can be stably maintained over a short period of several vegetative growth cycles. The evolutionary host range includes all hosts in which the plasmid is maintained over an extended period of time, during which it can adapt to the genetic code of its host.

4.3 Plasmid gain

The diversity of microorganisms able to gain a given plasmid through conjugative transfer or retromobilization constitutes the plasmids transfer host range. Plasmid transmissibility and transfer system are the main distinctive

characteristics in deciding if a bacterium is able to gain a certain plasmid and thus included in its transfer range. Replication and maintenance, as well as defensive mechanisms of either hosts or co-resident mobile genetic elements are deciding factors in elevating from the transfer to the maintenance and replication host range and will be discussed later in this thesis.

Textbox 1 Important terms for studying plasmid transfer in microbial communities

Donor

A bacterium hosting a transferable plasmid

Recipient

A bacterium that has the theoretical ability to take up a transferable plasmid when encounters with the donor occur

Transconjugant

A recipient bacterium that received the plasmid from the plasmid donor strain after successful conjugation

Strain permissiveness

The fraction of bacterial cells within a single strain population that will successfully take up the plasmid after an encounter with a donor bacterium

Community permissiveness

The fraction of a bacterial community that is able to take up a newly introduced plasmid from a donor strain on the quantitative (transfer frequency) as well as the phylogenetic (transfer host range) level

Even if not stably maintained, the transient presence of a plasmid might provide a short-term, but highly significant, fitness gain through plasmid encoded accessory genes. But, purely transient hosts in which the plasmids are not maintained might not benefit from long-term adaptation through plasmid encoded features. However, the accessory gene pool of plasmids is often embedded within transposable regions flanked by insertion sequences (IS) (Heuer *et al.*, 2012). These are able to recombine with the new host's chromosome and remain even if the plasmid is subsequently lost.

Additionally, transient hosts can increase the transfer range further by allowing transfer to organisms that had a lower potential to gain the plasmid from the original donor strain (Yano *et al.*, 2013).

Therefore, determining the transfer potential of a plasmid, its transfer frequency to a community, as well as its transfer host range can resolve part of the ecology and fate of plasmids in microbial communities. The transfer potential becomes especially interesting when looking at plasmid encoded accessory antibiotic resistance genes.

4.3.1 Studying the extent of plasmid transfer in environmental systems

The evaluation of the transfer range has traditionally been conducted using individual strains as recipients (Lederberg *et al.*, 1952), a situation that contrasts with the fact that most bacteria - and thus most plasmids - exist within complex communities of hundreds to thousands of species (Hong *et al.*, 2006; Kav *et al.*, 2012).

The first methods to explore the extent of plasmid transfer at the community level were based on selective plating relying on plasmid-encoded traits. Plasmids used in these assays conferred heavy metal or antibiotic resistance or specific accessory metabolic pathways that allow the transconjugants to grow on selective media. Several environmental factors affecting plasmid transfer frequencies to microbial communities have been identified by selective plating experiments. These include biological ones like the co-occurrence of eukaryotes like fungi or protozoa (Sengeløv *et al.*, 2000; Sørensen & Jensen, 1998) and abiotic factors such as nutrient availability (Sørensen & Jensen, 1998), stress exposure (Top *et al.*, 1995) or physicochemical ones like temperature (Richaume *et al.*, 1989), water availability (Richaume *et al.*, 1989; Ellass & Trevors, 1990) or pH (Rochelle *et al.*, 1989).

While these methods can resolve plasmid transfer occurring at low frequency, they are limited to the culturable fraction of a community that is able to grow on the specific growth medium. This fraction can easily lie below 1% of the total cell counts (Amann *et al.*, 1995), and its phylogenetic composition might shift due to enrichment on plates compared to the original community (Wagner *et al.*, 1993). Quantitative methods relying on quantitative PCR (qPCR) (Götz *et al.*, 1996) of plasmid DNA can overcome the need for cultivation.

Among complex communities, strains might not be equally permissive towards plasmid receipt (Sørensen, 1993; Inoue *et al.*, 2005). Still, selective mating as well as qPCR approaches only deliver an community-averaged plasmid transfer frequency (Sørensen *et al.*, 2005).

Additionally, transfer in these studies is defined as the number of bacteria that hosted the introduced plasmid after a given amount of time. Plasmid acquisition through horizontal transfer to recipients can therefore not be distinguished from subsequent maintenance and vertical transfer to daughter cells. It can also not deliver insights in the spatial distribution or if different strains within a community might have a varying potential of plasmid receipt.

These weaknesses in detecting and quantifying plasmid transfer can be overcome by introducing *in-situ* reporter genes to plasmids, which confer no selective advantage. Transfer detection with those reporter genes relies on the detection of expression of these genes in the transconjugant. Therefore, reporter gene approaches do not rely on culturing transconjugants, avoiding the commonplace cultivation bias of selective plating. Diverse reporter gene systems have been used for monitoring plasmid transfer, including the β -galactosidase gene *lacZ* (Jaenecke *et al.*, 1996), the luciferase genes *luxAB* (Hoffmann *et al.*, 1998) and *luc* (Palomares *et al.*, 2001) and, most commonly employed, the fluorescent marker genes such as the one encoding for the green fluorescent protein (GFP) (Christensen *et al.*, 1996; Normander *et al.*, 1998; Dahlberg *et al.*, 1998).

The fluorescent marker gene approach is of specific importance in this thesis. Compared to the other reporter gene approaches it allows for quasi-immediate detection of plasmid transfer, even in individual cells, without the need of substrate addition or taking the strains out of their natural environment.

Early approaches to monitor plasmid transfer using reporter genes introduced an inducible *gfp* marker gene to the conjugative plasmid. To avoid *gfp* expression in the donor strain and enable selective quantification of transconjugants, *gfp* was introduced behind a *lacZ* promoter on the plasmid (Dahlberg *et al.*, 1998). This promoter was subject to inhibition by suppression. A constitutively expressed *lacI* repressor gene was additionally inserted in the donor's chromosome, thus avoiding *gfp* expression before transfer to the recipient was successful (Figure 5).

This reporter gene system was extensively used to make spatial observations through epifluorescence, stereo (SM) or confocal laser scanning microscopy (CLSM), as well as to quantify transfer frequencies using the fluorescent detectors of flow cytometers (Christensen *et al.*, 1996; Dahlberg *et al.*, 1998; Sørensen *et al.*, 2003). Transfer frequencies increased up to 1000 fold in microbial communities based on studies using flow cytometric quantification

compared to cultivation dependent methods (Musovic *et al.*, 2006). In-situ observation of fluorescent transconjugal microcolonies allowed additionally for the first time to distinguish between horizontal and vertical acquisition of plasmids (Arango Pinedo & Smets, 2005).

Tolker-Nielsen *et al.* (2000) improved the plasmid transfer detection system through introducing a zygotically expressed red-fluorescent marker gene (*DsRed*) into the chromosome of the donor strain, allowing simultaneous quantification and observation of donors, recipients and transconjugants (Figure 5).

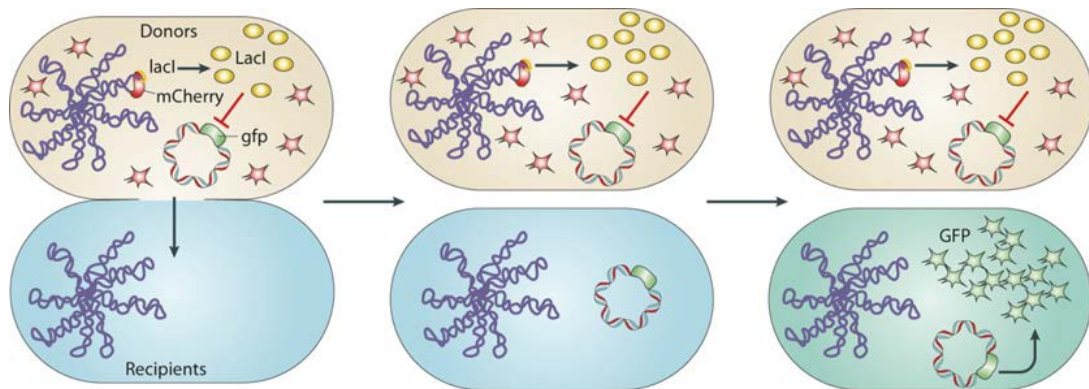


Figure 5 A schematic outline of the transfer reporter-gene approach. The donor cell contains a conjugative plasmid tagged with the green fluorescent protein (GFP) gene (*gfp*) downstream from a *LacI* repressible promoter. The donor chromosome encodes *LacI*, which represses the expression of GFP. During conjugation, the plasmid is transferred from the donor cells to the recipients, which become transconjugants. Expression of *gfp* is not repressed in the transconjugant cells, and these cells consequently fluoresce green. (reprinted from (Sørensen *et al.*, 2005)).

This system still prevails today and is extensively used in this thesis, even if the fluorescent system relied on in this work takes advantage of the advanced fluorescent properties of the *gfpmut3* variant of *gfp* and *mCherry* as the red fluorescent marker gene (Figure 6).

Earlier studies indicated that within complex communities, strains might be not equally permissive towards plasmid receipt (Sørensen, 1993; Inoue *et al.*, 2005). Emerging sequencing technologies allowed the determination of the permissiveness of a community towards an introduced plasmid at high resolution. Subsequently, the estimation of the plasmids transfer range by isolation and identification of transconjugants by 16S rRNA sequencing became an increased focus. Identification of transconjugants of the most common broad

host range IncP plasmids early on revealed a dominance of γ -Proteobacteria, as the main recipients (Sørensen & Jensen, 1998; Götz *et al.*, 1996). Furthermore, the identical plasmid introduced through different donor strains into the same soil community can transfer to a different subset of the community. (De Gelder *et al.*, 2005).

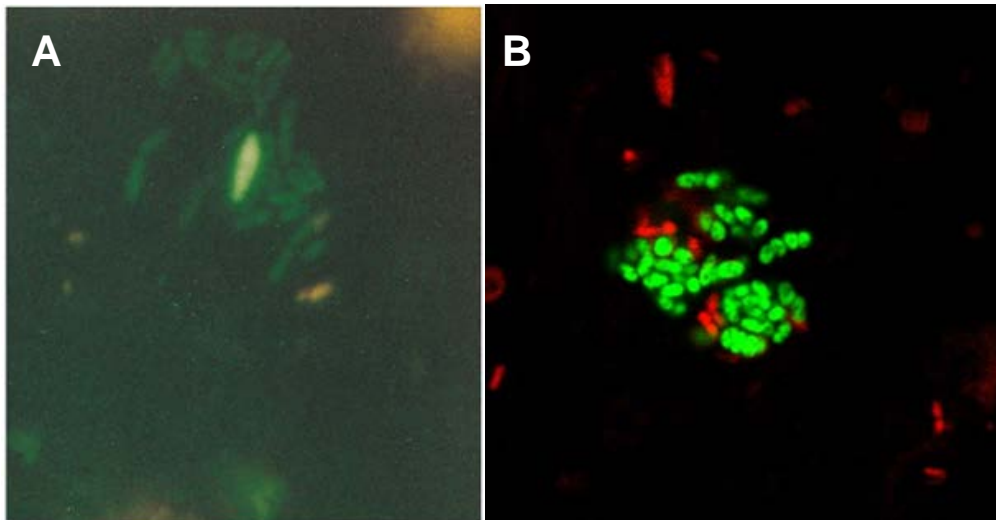


Figure 6 Improved fluorescent detection system. Original: GFP fluorescent transconjugant cells, formed after transfer of plasmid pBF1::*gfp* from *P. putida* KT2442 to marine bacteria in seawater (A) versus improved: *gfpmut3* fluorescent cells, formed after transfer of plasmid pKJK5::*gfpmut3* from *P. putida* KT2440::*mCherry-lacI^q* to soil bacteria on soil extract (B) (Images modified from (Dahlberg *et al.*, 1998; Klümper *et al.*, 2015)).

Plasmid transfer between phylogenetically more distant species was first observed, when using culture independent fluorescent activated cell sorting (FACS) to isolate transconjugants from in situ matings (Musovic *et al.*, 2006). A large fraction of the recipients of IncP-1 plasmid pKJK10 were identified as *Arthrobacter spp.*, a Gram-positive soil bacterium. Single-cell FACS sorting in combination with whole genome amplification of transconjugants confirmed that IncP plasmid transfer can cross the Gram border by identifying Firmicutes as plasmid recipients (Shintani *et al.*, 2014). However these efforts, limited to inspection of a few hundred transconjugants at best, most likely underestimated the true diversity of emerging transconjugants and did not accurately describe how plasmid permissiveness may vary across taxa in complex microbial communities. Therefore the true transfer potential as well as the realized host range in environmental systems could still not be determined.

4.3.2 Determining the transfer potential and transfer host range of plasmids (Paper I & II)

With the ability to quantify transfer while simultaneously isolating and identifying the transconjugal fraction Musovic *et al.* (2010) defined the term community permissiveness as that fraction of a community that is able to take up a newly introduced plasmid on both the quantitative as well as the phylogenetic level. To assess the permissiveness of a soil community they developed a novel assay. It combined the improved quantification of horizontal transfer events through detection of fluorescent microcolonies (Arango Pinedo & Smets, 2005) in filter matings on soil extract medium with the isolation of transconjugal microcolonies by micromanipulation with a glass capillary and subsequent sequencing of the transconjugants (Musovic *et al.*, 2010). Every time a donor and a recipient get in close enough proximity conjugation becomes possible. To be comparable across different studies and environments the transfer frequency needs to be expressed as the subset of donor-recipient encounters at which successful transfer occurs (Sørensen *et al.*, 2005). In the filter mating assay as presented by Musovic (2010) and used throughout this thesis cell-to-cell contact is maximized. If each recipient is ensured to be in contact with at least one donor bacterium, the number of donor-recipient encounters is equal to number of originally introduced recipients. This allows a simple and comparable quantification of the transfer frequency based on transfer events per originally introduced recipient.

The micromanipulation based subsequent isolation method was used in this thesis to assess the permissiveness soils of different agricultural treatments towards different plasmids (see Paper V). Still the isolation of transconjugants through micromanipulation was time consuming, therefore only resulting in limited transconjugal pools (Musovic *et al.*, 2014) and depended on cultivation after isolation.

Therefore, a cultivation-independent high-throughput method to combine with the present microscopic quantification of plasmid transfer was needed.

4.3.2.1 A high-throughput method to quantify plasmid transfer and determine the host range in mixed microbial communities

The *in situ* host range of plasmids describes the taxonomic breadth across which gene flow occurs. The depth at which the host range is studied was so far limited to maximal few hundred transconjugants (Musovic *et al.*, 2006; De Gelder *et al.*, 2005; Shintani *et al.*, 2014) , therefore monitoring only the

main recipients of the studied plasmids. In the diverse soil environment this might provide an incomplete picture of the widespread range of plasmid transfer. For understanding the extent and factors influencing plasmid transfer in the highly diverse soil environment a new high-throughput method to isolate and identify the fraction of a soil microbial community able to take up plasmids is needed. I therefore developed a protocol for simultaneous quantification of plasmid transfer frequency to and high-throughput isolation of transconjugants from a soil bacterial community after introducing a *gfp*-tagged plasmid in a *mCherry* red fluorescently tagged donor strain repressing *gfp* expression (Figure 7). The high-throughput method consists of 3 main steps:

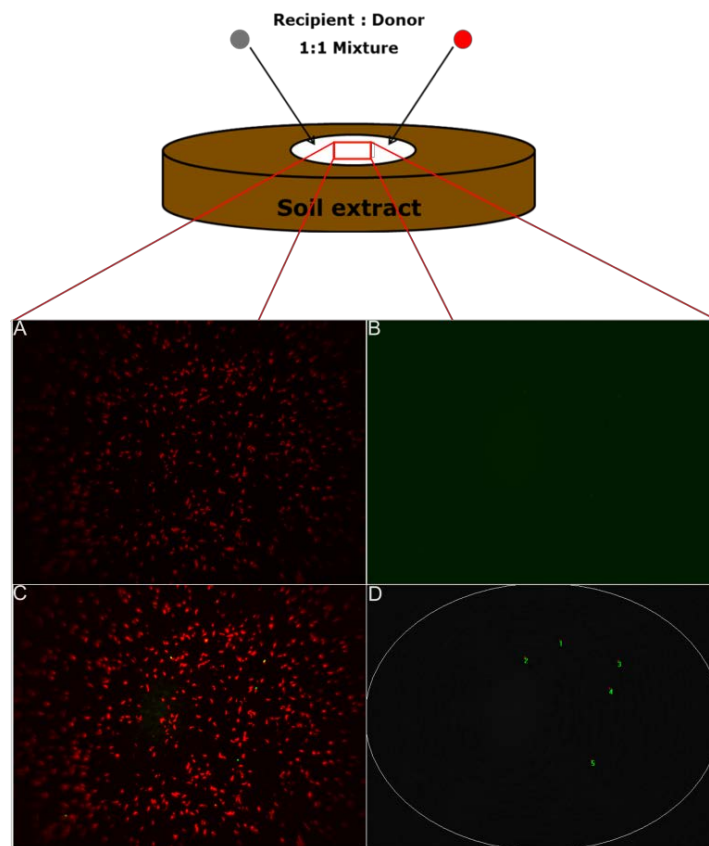


Figure 7 Fluorescence based stereomicroscopic images and image analysis of an example filter mating. Image A corresponds to the red fluorescent channel, displaying donor microcolonies. Image B shows the green fluorescent channel, corresponding to the transconjugal microcolonies that received the plasmid. Image C is a composite image of both channels with increased contrasts. Transconjugal microcolonies can be found in direct proximity to donor colonies. Image D illustrates counting of transconjugal colonies through a macro that increases contrast of the images, subtracts background, eliminates the poorly illuminated corners and threshold and counts green fluorescent object larger than $7 \mu\text{m}^2$ (Klümper *et al.*, 2014a).

First we set up a solid surface filter mating (Musovic *et al.*, 2010) wherein the plasmid donor strain is mixed with a Nycodenz[®]-extracted soil bacterial community under maximized cell-to-cell contact conditions to ensure that every single recipient is in contact with a donor bacterium.

The second step consists of the acquisition and evaluation of fluorescence microscopic images to quantify the community permissiveness towards the plasmid by calculating the transfer frequency. Rather than quantifying the number of transconjugants, I count the number of green fluorescent microcolonies (Figure 7) and can thus distinguish horizontal from vertical transfer of the plasmid (Arango Pinedo & Smets, 2005). Quantifying the number of successful conjugation events per recipient becomes thus possible.

In a final step, transconjugants are isolated using a new high throughput FACS method based on triple gating. The three gates are defined in bivariate plots (Figure 8). On the side scatter (SSC) vs front scatter (FSC) plot, a gate corresponding to particles of bacterial size was used. On the green fluorescence (FITC) vs SSC plot a gate was set that covered all green fluorescent particles, while using an additional non-red gate on the red fluorescence (PE-Texas Red) vs SSC plot excluded all small autofluorescent particles from soil to sort out only transconjugants.

Transconjugant cells that originally made up less than 0.1% of the total cell count in the filter matings were enriched to up to 82% in a first fast sorting step. Then over 10,000 transconjugants per sample were isolated a second purification step, at 100% purity of green cells as observed by fluorescent counting in the flow cytometer (Figure 8). Plating of more than 200 isolated transconjugants resulted in detection of green fluorescence in all colonies, verifying purification of *gfp*-expressing transconjugants. Thus, transconjugal pools obtained could subsequently be taxonomically analyzed by 16S rRNA based amplicon pyrosequencing.

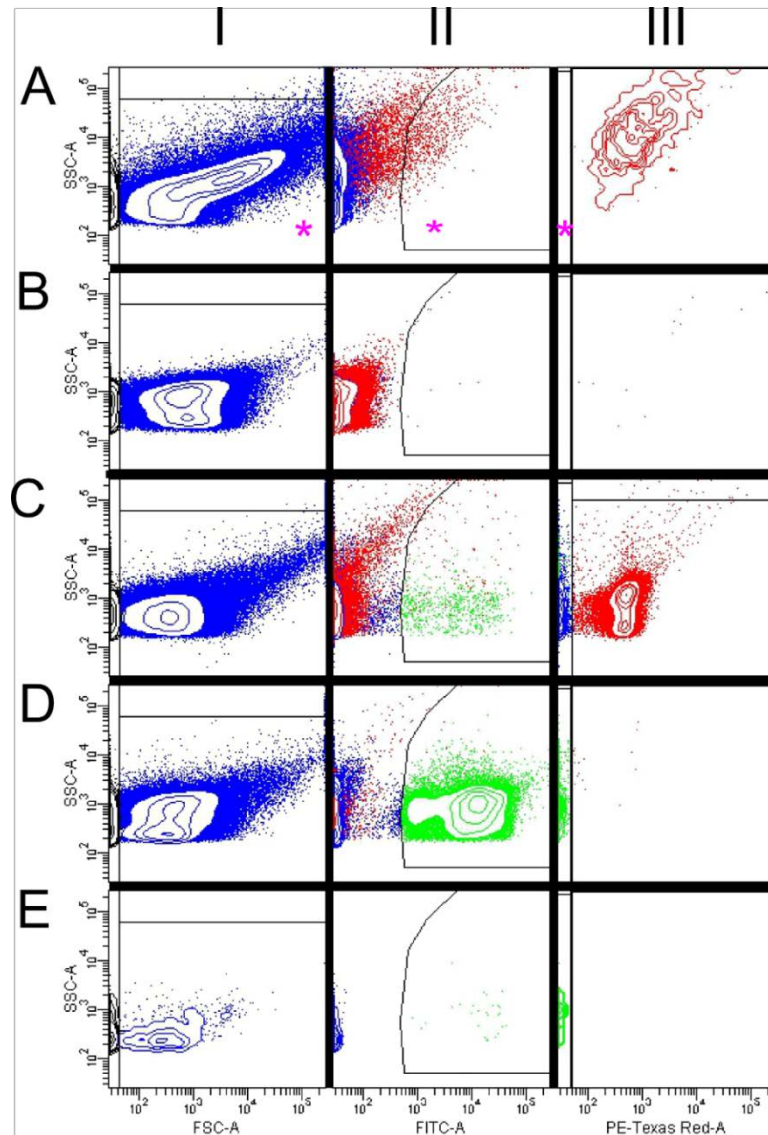


Figure 8 FACS sorting of transconjugal cells from a mating mixture initiated with soil bacteria and *E. coli* carrying pKJK5. The procedure consists in three successive gates (marked by pink stars in Panels A): Gate I sorts for bacterial size based on front and side scatter; Gate II sorts for green fluorescent cells; Gate III selects only those green cells that possess no red fluorescence. Panel A shows the sorting of the initial soil bacterial recipient community in absence of any donor strain and proves that the presence of green autofluorescent particles (A-II) does not yield false positive as they are excluded at the third gate, due to their red fluorescence (A-III). The sorting of a pure culture of the donor strain is shown in Panels B, where, again, no false positive events are recorded at the final gate. Panel C represents the analysis of the mating mixture before sorting. Panels D show the enrichment of transconjugants after the first fast enrichment sorting step to over 80% transconjugal cells, with minor contamination by donor or soil particles. Panels E show how only pure transconjugants are obtained after the second purification sorting step (reprinted from (Klümper *et al.*, 2015)).

4.3.2.2 Broad host range plasmids have an unexpectedly diverse transfer host range

Taking advantage of high throughput cell sorting and next-generation sequencing technologies, I mapped for the first time the diverse transfer host range of three broad host range IncP and IncPromA plasmids in a microbial community extracted from soil. All three plasmids (RP4, pIPO2tet, and pKJK5) were exposed to the soil community in matings with a *Pseudomonas putida* donor strain, while plasmid pKJK5 was also introduced via *Escherichia coli* and *Kluyvera* spp. donors (Table 1).

Table 1 Plasmids and donor strains used in this study

Donor	Chromosomal marker		Reference	
<i>Pseudomonas putida</i> KT2440	<i>lacIq-pLpp-mCherry</i> , Km ^R		This study	
<i>Escherichia coli</i> MG1655	<i>lacIq-pLpp-mCherry</i> , Km ^R		This study	
<i>Kluyvera</i> sp.	<i>lacIq-pLpp-mCherry</i> , Km ^R		This study	
Plasmid	Inc-group	Phenotype	Host range	Reference
RP4:: <i>Plac::gfp</i>	IncP-1α	Tet ^R , Amp ^R , Km ^R	Broad	(Musovic <i>et al.</i> , 2010)
pIPO2tet:: <i>Plac::gfp</i>	IncPromA	Tet ^R	Broad	(Musovic <i>et al.</i> , 2014)
pKJK5:: <i>Plac::gfp</i>	IncP-1ε	Tmp ^R Tet ^R ,	Broad	This study

More than 300 OTUs (defined at 97% sequence similarity) among the transconjugal pools across all plasmid/donor combinations, a large expansion over the low number of distinct bacterial isolates identified previously (De Gelder *et al.*, 2005; Musovic *et al.*, 2010, 2014; Shintani *et al.*, 2014).

As expected, Proteobacteria, known to be the main hosts for the studied broad-host-range plasmids (Suzuki *et al.*, 2010), were abundantly represented as more than 80% of the obtained sequences. Unlike in previous studies, all five classes (α-ε) of Proteobacteria were identified among the transconjugants. More strikingly, the diversity of transconjugants extended much beyond the proteobacterial phylum, and included diverse members of ten additional phyla including Verrucomicrobia, Bacteroidetes and Actinobacteria (Figure 9). Some of these taxa are known as poorly cultivable (Joseph *et al.*, 2003) and would not be detectable with traditional culture based methods.

I identified transfer from the Gram-negative donors to a wide variety of Gram positive bacteria. Over 15 OTUs within the Actinobacteria phylum and more than 10 OTUs belonging to 6 different orders of Bacilli and Clostridia in the Firmicutes phylum were identified as transconjugants (Figure 9).

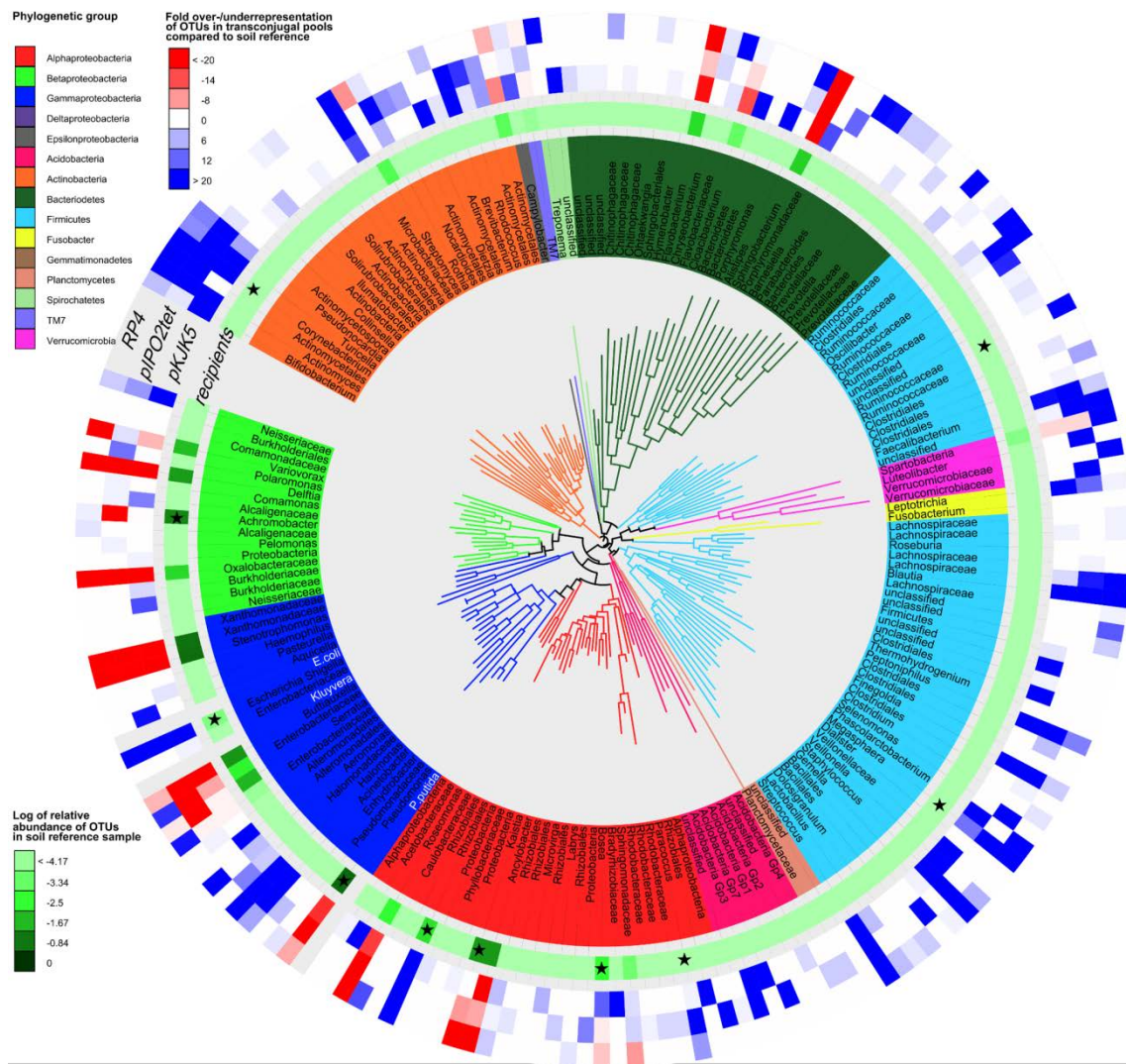


Figure 9 Phylogenetic tree showing all identified transconjugant OTUs for three different plasmids (pKJK5, RP4, pIPO2tet) from the same donor (*P.putida*). The colors of the branches mark different phylogenetic groups. The three donor strains are shown in white letters in the trees. Green heatmap-circle around the tree represents the log transformed relative OTU abundance in the soil reference recipient community. Three heatmaps in blue and red display the x-fold over- and underrepresentation of the OTU in the respective transconjugal pool in comparison to the abundance in the reference soil sample. Stars mark the shared (present in all 3 transconjugal pools) and abundant (present at more than 1% relative sequence abundance) transconjugant OTUs, which constitute the core super-permissive community fraction. Sample size was normalized to 30000 sequences per transconjugal pool. (reprinted from (Klümper *et al.*, 2015))

The large proportion of the transfer potential of plasmids proposed through artificial constructs (Wolk *et al.*, 1984; Heinemann & Sprague, 1989; Samuels *et al.*, 2000; Schäfer *et al.*, 1994), can thus actually be realized in nature. My observations suggest that conjugation among phylogenetically

distant organisms may be a more common process than previously considered.

The observed transfer of broad-host-range IncP-1 type plasmids between Gram negative and Gram positive bacteria might lead to a reassessment of the potential of soil bacterial communities to spread antibiotic resistance genes. Indeed, Gram positive Actinobacteria, the origin of many soil-borne resistance genes (D'Costa *et al.*, 2006) identified in clinical isolates of Gram negative antibiotic-resistant bacteria (Benveniste & Davies, 1973; Forsberg *et al.*, 2012), are frequent among the transconjugants identified. Broad host range plasmids of the IncP-1 and IncPromA group can thus provide a direct link between diverse bacterial groups.

I show here that the immediate transfer range for IncP plasmids is much wider than previously reported, proving that in absence of physical barriers to cell-to-cell contact, broad host range plasmids have a high likelihood to be, hosted by very diverse bacteria, at least transiently.

4.3.3 Plasmid transmissibility: Conjugative vs. Mobilizable plasmids

Independent of their host and origin all transmissible plasmids share two functionally identical subsets of genes for successful conjugation constituting the transfer operon (*tra*) (Willetts & Crowther, 1981).

The mobility subset (*MOB*), consists of the origin of transfer (*oriT*), the relaxase protein and the type IV coupling protein (T4CP). *MOB* is responsible for plasmid replication and converting the plasmid DNA into the relaxosome. When undergoing conjugation, the relaxase cleaves and binds to the originally double-stranded DNA of the plasmid at the *oriT* gene site. Thereafter it transforms the plasmid to a single strand which then becomes a protein-DNA complex (Alvarez-Martinez & Christie, 2009). This relaxosome then becomes transferable after the T4CP in combination with a VirB4-type ATPase couples it to the mating pore encoded by the second subset.

The second subset, responsible for mating pair formation (MPF) establishes the mating pore between donor and recipient. It consists of a type IV secretion system (T4SS) which produces exocellular pili that link the two cells via a mating channel enabling the relaxosome complex to path into the recipient cell (Alvarez-Martinez & Christie, 2009) (Figure 10).

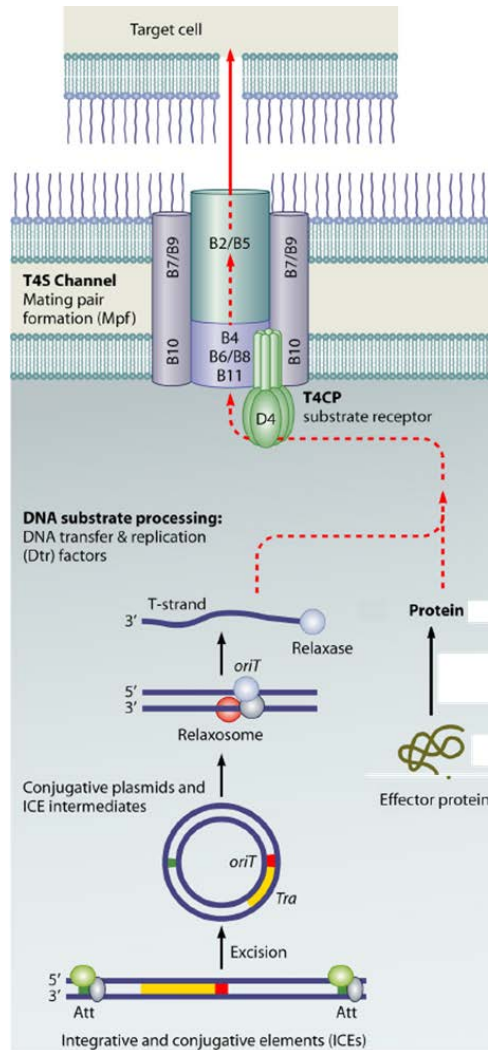


Figure 10 Mechanism of conjugative plasmid transfer through a type IV secretion system (T4SS). The steps include: 1. Processing of the plasmid through the MOB complex (*oriT*, relaxase), 2. binding of proteins to create the transferable relaxosome, 3. Nicking of the relaxosome to the type IV coupling protein (T4CP), 4. Translocation through the T4SS channel. (adapted from (Alvarez-Martinez & Christie, 2009)).

All transmissible plasmids can be classified into two contrasting main groups, conjugative and mobilizable plasmids. The classification is based on the presence of genes associated with their transfer (Smillie *et al.*, 2010). Conjugative plasmids encode a complete set of transfer genes which are essential for most of the functions involved in mating pair formation (Thomas & Nielsen, 2005). The genes needed to be self-transmissible, include *oriT*, the relaxase, T4CP, and T4SS. Mobilizable plasmids, on the other hand, lack some of the genes encoding the T4SS and sometimes also the T4CP (Figure 11) (Garcillán-Barcia *et al.*, 2009, 2011).

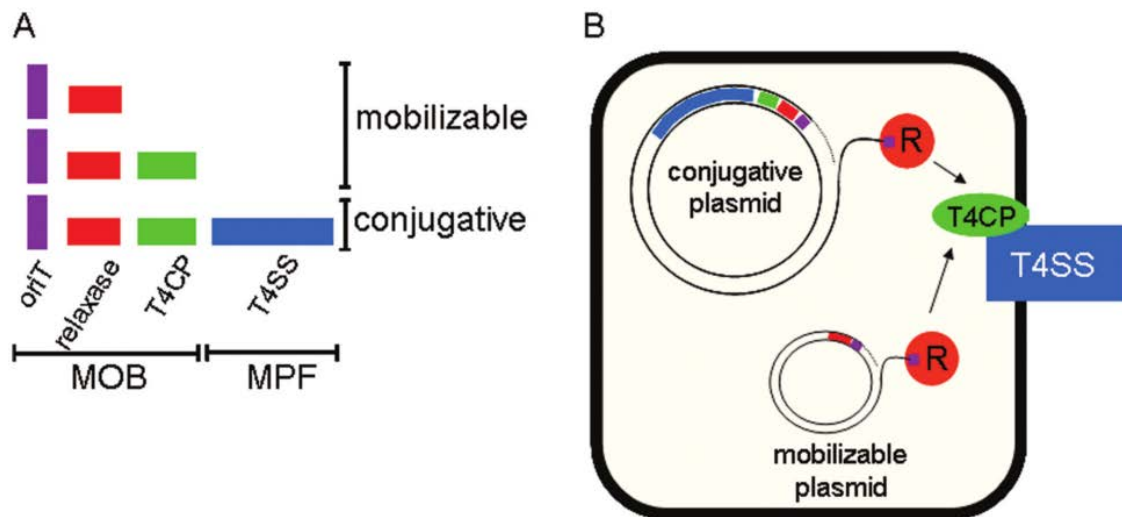


Figure 11 Classification of plasmids into mobilizable and conjugative ones based on the mobilization genes encoded (modified from (Smillie *et al.*, 2010)).

Classifying plasmids based on their mobilization apparatus (*MOB*) as proposed by Garcillán-Barcia *et al.* (2009) is more elegant than by incompatibility (see section 4.4.1). Plasmids usually carry only one relaxase gene and a *MOB* based classification can cover plasmids from all different phylogenetic hosts (Garcillán-Barcia *et al.*, 2009). Based on the amino acid sequence of their relaxase genes, encoded by both, conjugative and mobilizable plasmids, six different *MOB* classes (*MOBC*, *MOBF*, *MOBH*, *MOBP*, *MOBQ*, *MOBV*) have been defined. Furthermore, four different classes of T4SS involved in mating pair formation (*MPF*) during conjugation were identified (*MPFF*, *MPFG*, *MPFI*, *MPFT*) (Smillie *et al.*, 2010).

In 2010 14% of the 1,730 full-sequenced plasmids were classified as conjugative (Smillie *et al.*, 2010), a ratio that was confirmed in a more recent study working on all currently sequenced 4,602 plasmids (Shintani *et al.*, 2015). Similarly both studies reported that plasmids encoding the complete conjugative *tra* operon are generally bigger in size than those which are just encoding the *MOB* subset (Figure 12). While the *rep* region including initiation, elongation and termination of replication is usually only 1-3 kbp a complete set of *MOB* and *MPF* genes increases the size of the smallest known self-transmissible plasmids to around 10 kbp (Shintani *et al.*, 2015). These trends holding true over the last 5 years might indicate a good estimate of the occurrence and size of conjugative and mobilizable plasmids in nature, even if current sequencing technology will allow complete sequencing of more and

more environmental plasmids (Loftie-Eaton & Rawlings, 2012; Smillie *et al.*, 2010; Shintani *et al.*, 2015).

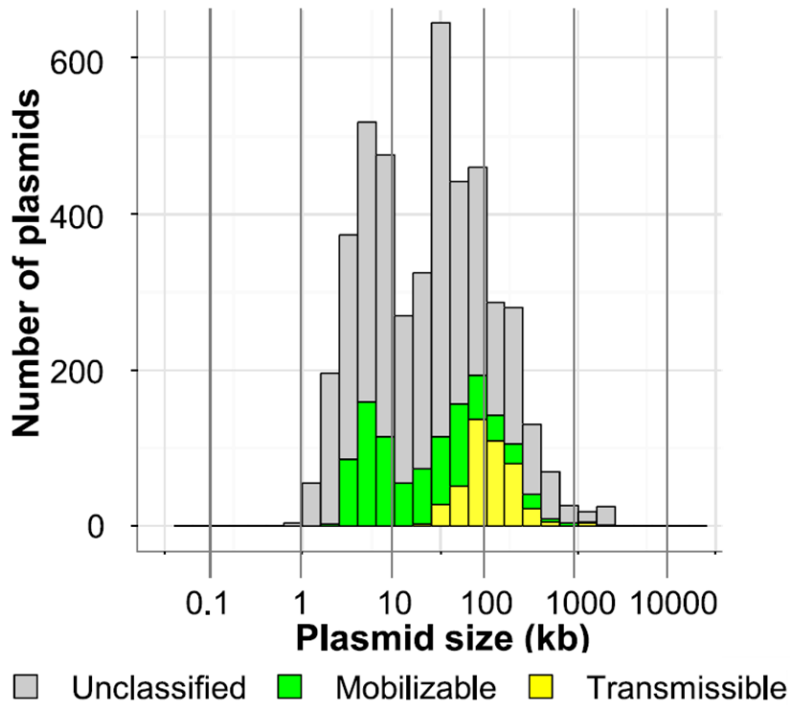


Figure 12 Histogram of plasmid size distribution and their classification into self-transmissible and mobilizable. Reprinted from (Shintani *et al.*, 2015).

Mobilizable plasmids can be mobilized by a variety of different plasmid encoded T4SSs (Meyer, 2009) as well as through integrative and conjugative elements (ICEs) (Lee *et al.*, 2012) both often at high frequencies (Gregory *et al.*, 2008; Meyer, 2009). In case of plasmid mobilization, co-resident conjugative plasmids have a beneficiary effect on transfer frequency and can increase the immediate transfer range. For transfer, mobilizable plasmids are activated and take advantage of the *tra* gene expression of the co-resident conjugal plasmid. The conjugative *tra* genes cause the formation of pili and modification of the mobilizable plasmid DNA into the transferable relaxosome (Yano *et al.*, 2013). If the mobilizing conjugative plasmid occurs in the same cell as the mobilizable one, the mobilization mechanism is called direct mobilization. In retromobilization the future recipient of the mobilizable plasmid first transfers a conjugative plasmid into the cell that harbors the mobilizable plasmid. After this conjugative transfer step the mobilizable plasmid gets mobilized into the host of the original conjugative plasmid (Figure 13).

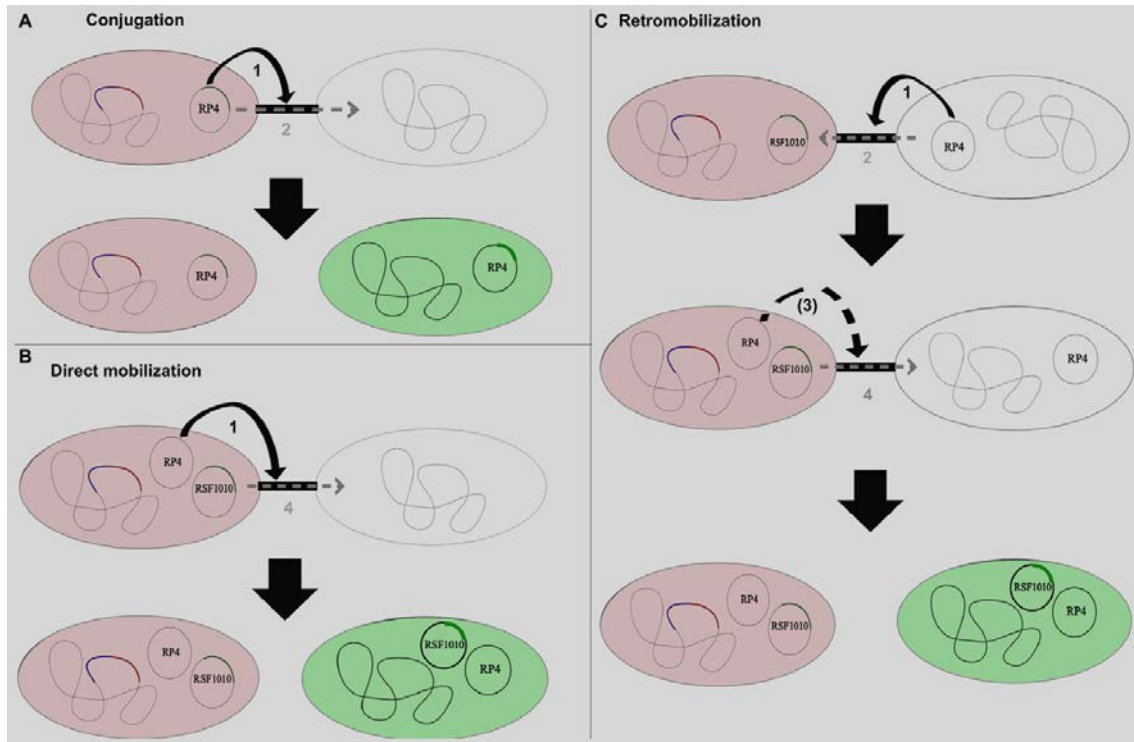


Figure 13 Conjugation, direct mobilization and retromobilization of a conjugative/mobilizable plasmid pair. Panel A: Conjugal transfer of a self-transmissible plasmid. Step 1 illustrates the establishment of a pilus between donor and recipient as part of the type IV secretion system (T4SS) encoded by the conjugative plasmid. Step 2 displays the transfer of a conjugative plasmid through its own secretion system into the recipient. Panel B: Direct mobilization of a mobilizable plasmid from donor to recipient by the co-resident conjugal plasmid. The conjugal plasmid establishes a pilus as part of its T4SS and interconnects donor and recipient cells (Step 1). The mobilizable plasmid does not encode for its own T4SS and transfers through the established pilus into the recipient cell (Step 4). The conjugal plasmid may or may not transfer along with the mobilizable plasmid in the direct mobilization process. Panel C: Retromobilization process of a mobilizable plasmid, mobilized by a conjugal plasmid from the recipient cell. In this process, the conjugal plasmid from the recipient establishes a conjugal connection between recipient and donor (Step 1) and transfers from recipient to donor cell (Step 2). The mobilizable plasmid can subsequently transfer through the established connection (Step 4) or through a new connection established by the now co-resident conjugal plasmid (Step 3). Reprinted from (Klümper, *et al.*, 2014b).

Since, mobilizable plasmids do not encode for their own replication system, but rely on that of the conjugative element, mobilizable plasmids reach a higher degree of host independence than those. Therefore, mobilizable plasmids have a broader replication host-range than any other known replicating mobile genetic element in bacteria (Meyer, 2009). Additionally, mobilizable plasmids are stably maintained by being characterized as high copy number

plasmids (Meyer, 2009) which increases their sustainability in a host until it can be mobilized from a co-resident conjugative plasmid. They were found to be stably maintained in Gram negative Proteobacteria, Gram positive Firmicutes, Actinomycetes and even Cyanobacteria (Meyer, 2009) or plants (Buchanan-Wollaston *et al.*, 1987).

Their extremely broad replication host range combined with an extremely efficient transfer mechanism (Gregory *et al.*, 2008; Meyer, 2009) and their small size result in faster transfer at frequencies far higher than those of most conjugative plasmids (Top *et al.*, 1995). A high mobilizing plasmid content within a community increases therefore the ecological and evolutionary importance of mobilizable plasmids.

4.3.3.1 Assessing a community's plasmid mobilization potential

Studies on conjugal gene flow in microbial communities have mainly focused on the community's ability to receive self-transmissible plasmids. This study is the first one to directly quantify the potential of a microbial community to actively mobilize non-self-transmissible, mobilizable plasmids to indigenous bacteria.

Exogenous isolation techniques to capture mobilizing and mobilizable plasmids from natural communities have been developed earlier (Top *et al.*, 1994; Smalla *et al.*, 2000; van Elsas *et al.*, 1998). Characterizing the mobilization potential of communities has been carried out with indirect triparental matings where both donor and the terminal recipient were artificially introduced to the communities and transfer from *E. coli* to *P. putida* was monitored (Hill *et al.*, 1992; Götz & Smalla, 1997). Direct mobilization of mobilizable plasmids into indigenous strains of mixed communities has been detected (Hill *et al.*, 1992; Van Elsas *et al.*, 1998), but never directly quantified.

We developed a novel framework and experimental method to estimate the plasmid mobilization potential of a mixed bacterial community. The well-studied mobilizable IncQ plasmid RSF1010 served here as the model plasmid introduced through *P. putida*. We quantify the mobilization potential of a model community. This community was extracted from a domestic shower conduit.

Taking advantage of the previously described fluorescent marker gene assay (see Paper I&II) and filter matings, we evaluated the transfer frequency of RSF1010 and compared it to the community's permissiveness towards the

mobilizing, conjugal plasmid RP4 (Table 2). We finally related the observed transfer frequencies to those measured between isogenic strains.

Table 2 Plasmids used in this study

Plasmid	Transfer	Size	Incompatibility	Resistance ($\mu\text{g/mL}$)	Host range	Reference
RP4	Conjugal	60 kb	IncP-1 α	<i>Amp</i> ^R , <i>Km</i> ^R , <i>Tet</i> ^R (100, 50, 20)	Broad	(Barth & Grinter, 1977)
RSF1010	Mobilizable	8.7 kb	IncQ-1 α	<i>Strep</i> ^R (100)	Broad	(Honda <i>et al.</i> , 1991)

The community's permissiveness towards conjugative plasmid RP4 (1.16×10^{-4} transconjugants per recipient (T/R)) was measured as 6.6% of that observed in *P. putida* intra strain matings, where all *P. putida* recipients can potentially take up RP4 (Figure 14). The higher transfer frequency observed using isogenic *P. putida* donor and recipient strains results from all recipients being part of the plasmid transfer range and absence of any incompatibility effect (see section 4.4.1) as recipients were plasmid-free. Hence, the observed transfer frequency in these intra-strain experiments was not limited by the recipient permissiveness, but only by the donor promiscuity, the fraction of donor cells expressing conjugal genes.

RSF1010 was mobilized by the model community at a frequency of 1.16×10^{-5} T/R, only one order of magnitude lower than the permissiveness towards RP4 (Figure 14). In these experiments RSF1010 must have been retromobilized into the recipient community by cells carrying IncQ mobilizing conjugal plasmids. In order to explore the retrotransfer frequency of RSF1010 further, isogenic *P. putida* strains were used to execute intrastrain matings. Here *P. putida* hosting the untagged wild-type of the conjugal, mobilizing RP4 plasmid served as recipient. Control experiments using a plasmid free version of *P. putida* as a recipient resulted expectedly in no observable plasmid transfer.

With *P. putida* (RP4) as recipient, retrotransfer was observed, with a measured frequency of 8.34×10^{-4} T/R (Figure 14). Successful RSF1010 retrotransfer requires establishment of a mating pair through a conjugal plasmid from recipients to RSF1010 donors, before RSF1010 is mobilized and retransferred to the recipients (Top *et al.*, 1992).

Here, the measured RSF1010 retrotransfer frequency by *P. putida* (RP4) results from a combination of the RP4 transfer process from the recipient to the

donor and the subsequent mobilization of RSF1010 through the now co-resident RP4 plasmid. It is possible to contrast the retrotransfer frequencies with the measured RP4 intrastrain transfer frequency which corresponds to the first two steps in RSF1010 retrotransfer (Figure 13). Hence, the probability of a cell that recently acquired RP4 via conjugal transfer to mobilize RSF1010 can be estimated at 47.4% (8.34×10^{-4} T/R for *P. putida* (RSF1010::*gfp*) to *P. putida* (RP4) divided by 1.76×10^{-3} (T/R) for *P. putida* (RP4::*gfp*) to *P. putida*).

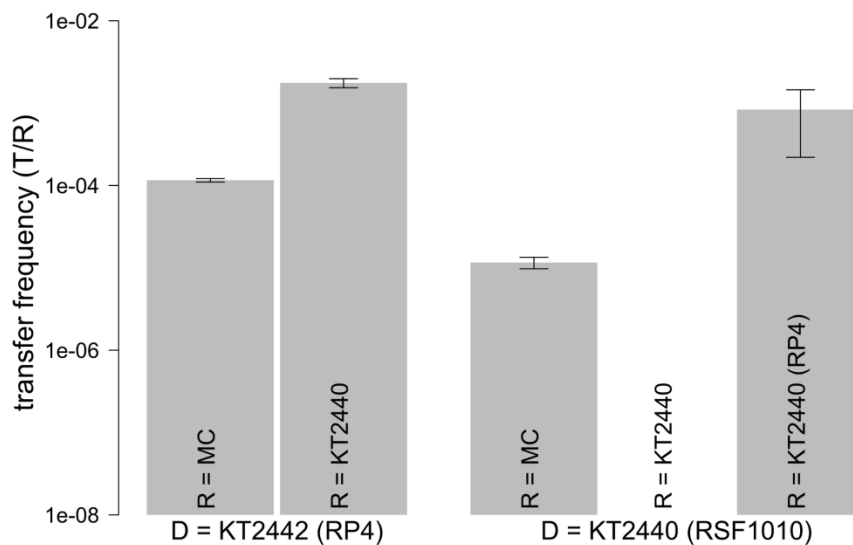


Figure 14 Transfer frequencies of RSF1010 and RP4. Transfer frequencies were defined as transconjugant microcolonies per initial recipient cells in solid surface filter matings with a mixed community (MC) or defined *P.putida* strains as recipients. Values are shown as mean of triplicates with stand error of mean. Donor strain (D) and plasmid are shown on x-axis. RP4 or RSF1010 were each introduced through KT2440 or KT2442 (*Pseudomonas putida* KT2440 /KT2442::*lacI^q-Lpp-mCherry-Km^R*) into the recipients. Recipients (R) are shown within the bars (MC = Model community; KT2440 = *Pseudomonas putida* KT2440). (Klümper, *et al.*, 2014b)

The retrotransfer of RSF1010 to the recipient community occurred at a frequency of 10% compared to its permissiveness for the RP4 plasmid. As estimated above for RP4 as mobilizing plasmid, RSF1010 is mobilized approximately every second time a conjugal plasmid is transferred from the recipient community into the donor strain. If all potential mobilization events were realized, the maximal mobilization potential of the recipient community is reached. This maximal mobilization potential describes the fraction of the

community able to mobilize an exogenously introduced plasmid and can be compared to its permissiveness towards a conjugal plasmid. The theoretical maximal mobilization potential towards RSF1010 can be quantitatively assessed as 2.45×10^{-5} T/R by dividing its transfer frequency towards the community (1.16×10^{-5} T/R) by the 50% probability of retrotransfer determined. When subsequently dividing 2.45×10^{-5} T/R through the community's permissiveness towards RP4 (1.16×10^{-4} T/R) the maximal mobilization potential of the community for RSF1010 can be assessed as approximately 20% of its permissiveness towards conjugal plasmid RP4.

The community's potential to retromobilize and subsequently receive RSF1010 is only one order of magnitude lower than its permissiveness towards RP4. This surprisingly high transfer frequency may result from the fact that IncQ plasmids have a broader host range than any other known replicating component in bacteria (Meyer, 2009) combined with an extremely efficient transfer mechanism (Gregory *et al.*, 2008; Meyer, 2009). Nonetheless, the observed retromobilization requires the presence of mobilizing, conjugal plasmids in the recipients. Therefore, a high intrinsic conjugal plasmid content of the model recipient community in combination with RSF1010's efficient transfer mechanism is the most likely reason for the observed high mobilization potential. However, we were not able to identify if the fraction taking up RSF1010 was identical with that permissive towards RP4. Isolation and identification of transconjugants might be needed. Studying the diversity of transconjugants might provide insights into the transfer range of mobilizable plasmids. Comparison with the transfer range of broad host range conjugal plasmids (Klümper *et al.*, 2015) might consequently become possible.

When directly mobilized through a co-resident RP4 plasmid the observed transfer frequency of RSF1010 into the mixed community was more than 30-fold higher than the community's permissiveness for RP4. As the first retromobilization transfer event leads to the co-occurrence of the mobilizable plasmid with the mobilizing conjugal plasmid(s) in the same cell, the main transfer mechanism switches to subsequent direct mobilization and can reach the up to 30 fold higher transfer frequencies observed.

Apart from quantification of the mobilization potential, the method presented here provides several possibilities to study plasmid ecology and mobilization mechanisms. Additionally isolation of mobilizing plasmids within the transconjugants might become possible.

In conclusion, this method is the first one to assess the plasmid mobilization potential of a microbial community on a quantitative level by estimating a mobilizable plasmids transfer frequency through fluorescent microscopy. Using this method, we discovered that some mixed microbial communities have the potential to mobilize a newly introduced mobilizable plasmid at high frequencies.

4.4 Defense mechanisms against plasmid establishment

Immediately after a plasmid is gained through horizontal gene transfer it has to overcome several barriers to establish itself in its new host. I earlier demonstrated the unexpectedly diverse transfer range of broad host range plasmids. I also established that co-residence of a plasmid can far increase the retromobilization potential of mobilizing plasmids. If a strain is among the potential hosts of a plasmid, strain specific or co-residential plasmid encoded defense mechanisms may determine the stability of the plasmid in the new host.

4.4.1 Plasmid incompatibility

If a potential host cell is already hosting another plasmid, it can affect acquisition of new plasmids (Fer & Francino, 2012). This includes positive effects for increased plasmid receipt through mechanisms such as mobilization (Buchanan-Wollaston *et al.*, 1987) as well as hindering their receipt potential through entry exclusion mechanisms (Garcillán-Barcia & de la Cruz, 2008). One of the earliest mechanisms of negative interactions discovered, called incompatibility (Novick, 1987), describes the inability of two plasmids to coexist in the same cell (Hedges & Datta, 1973). Incompatibility serves as a basis for classification of plasmids in incompatibility (Inc) groups. The main reasons for this phenomenon are either sharing the same replication mechanisms or actively partitioning towards the identical partitioning signals or locations involved in stable plasmid maintenance (Ebersbach *et al.*, 2005).

Two plasmids possessing similarly regulated replication mechanisms cannot be stably maintained, since their copy number control system, as well as their partitioning system cannot distinguish between the two plasmids and maintain them stably in the population during segregation. Therefore the maintenance of the plasmid becomes a purely probabilistic phenomenon (Novick, 1987), leading to the loss of the newly introduced plasmid that has a lower initial copy number in most cases.

While replication mediated incompatibility is purely plasmid dependent, partitioning dependent incompatibility is dependent on both the host strain and the plasmids involved (Grant *et al.*, 1980). Plasmids compatible by replication can still compete for the identical partitioning signal and location. This phenomenon is host specific. In one strain partitioning based competition and thus incompatibility might occur, in others they might partition to different locations and be compatible.

Classifying plasmids according to their replicon type has been a common practice for the last 40 years. However, classification based on the replicon signature can cause problems when plasmids host multiple replicon sequences and are therefore not unambiguously classifiable (Shintani *et al.*, 2015).

Historically plasmids were studied mainly in few proteobacterial families like Enterobacteriales or Pseudomonadales. Replicon types of those plasmids are well understood. Thus, classifying plasmids with different *rep* structures from less studied phyla like Firmicutes (Fukao *et al.*, 2013) or Actinobacteria (Ventura *et al.*, 2007) based on *rep* defined incompatibility groups becomes difficult.

4.4.2 Plasmid entry exclusion

Apart from incompatibility and increased retromobilization, a third plasmid encoded mechanism can affect a cell's uptake potential for MGEs. After receipt of any plasmid, the bacterial membrane is modified through plasmid encoded genes to prohibit its ability to take part in further conjugative events (Garcillán-Barcia & de la Cruz, 2008). This mechanism is known as plasmid entry or surface exclusion and gives the plasmid an evolutionary advantage by minimizing the chance of intra-cell competition with another conjugative plasmid (Thomas & Nielsen, 2005). This mechanism is unspecific and does not rely on the type of the second plasmid. It additionally avoids further reduction of the fitness of its host cell by preventing an additional metabolic burden by hosting a high amount of diverse plasmids (Garcillán-Barcia & de la Cruz, 2008).

The two main surface exclusion mechanisms mainly documented in Gram negative strains are either the modification of the outer or the inner membrane through entry exclusion proteins.

Entry exclusion proteins (Helmuth & Achtman, 1978) or sexual pheromone antibodies (Hirt *et al.*, 2002; Dunny *et al.*, 1995) on the outer membrane inhibit the binding of plasmid encoded pili, thereby preventing the formation of

a conjugative mating pore (Thomas & Nielsen, 2005). On the inner membrane the entry exclusion proteins interfere with the signaling pathway involved in DNA uptake and block the synthesis and transport of additional plasmid DNA between donor and recipient (Audette *et al.*, 2007).

At least one of these mechanisms was detected to be encoded on any conjugative plasmid (Garcillán-Barcia & de la Cruz, 2008) while most mobilizable plasmids are lacking any, thus enabling the acquisition of co-resident MGEs for further transfer.

However, entry exclusion mechanisms do not completely exclude the transfer of new plasmids to a host, but can decrease the plasmid uptake potential by more than 500-fold (Pérez-Mendoza & de la Cruz, 2009).

4.4.3 Host restriction-modification systems

To be expressed in a new host, the plasmid has to be established after transfer. The previously discussed defense mechanisms against establishment are all encoded on plasmids. New hosts might also feature some defense mechanisms. Directly after entering a new host cell, the then single stranded plasmid DNA has to overcome host encoded defensive barriers. One of the first barriers, universally encoded in bacteria are restriction-modification (RM) systems that enzymatically cleave foreign DNA. While the hosts own methyltransferases enzymes specifically methylate defined nucleotide positions in its own DNA, unmodified foreign DNA will be digested by the hosts endonucleases that can bind to unmethylated restriction sites of the introduced DNA (Blumenthal & Cheng, 2002; Wilkins, 2002).

Most endonucleases show far higher activity against double stranded DNA, proven by the fact that introduced double stranded DNA remained unrestricted at frequencies lower than 10^{-5} while single stranded DNA remained unrestricted in 50% of the cases in *Streptococcus pneumonia* (Lacks & Springhorn, 1984). Since plasmids are transferred in single-stranded form, they usually become susceptible to RM systems after synthesis of the second strand, needed for its subsequent establishment in the cell (Thomas & Nielsen, 2005; Fer & Francino, 2012). Still few organisms possess RM systems relying on endonucleases that can cleave single stranded DNA (Berndt *et al.*, 2003).

Plasmids that lose as many restriction sites as possible gain an advantage against evolutionary pressure imposed by RM systems (Wilkins, 2002). Being able to avoid restriction in diverse hosts can be an additional reason for

conferring a broad host range. The loss of specific recognition sites led consequently to the evolution of a multitude of diverse RM systems (Bayliss *et al.*, 2006) able to recognize various target structures.

Apart from losing the target sequences many plasmids developed mechanisms to inactivate the new host's RM systems through anti-restriction functions. A purely probabilistic approach is the transfer of multiple copies of the same plasmid, in an attempt to overload the endonucleases allowing one plasmid copy to survive and transcribe anti-restriction proteins (Matic *et al.*, 1995). Other plasmids possess promoters of genes encoding anti-restriction proteins temporarily transcribed already from the secondary structure of the single stranded DNA (Bates *et al.*, 1997) before the restrictable second strand is synthesized. The proteins involved in these processes inactivate the endonucleases by binding to their recognition sites through DNA mimicry (Atanasiu *et al.*, 2001; Dryden & Tock, 2006).

RM systems were shown to cause a dramatic reduction in plasmid transfer frequencies if the incoming plasmid was susceptible (Arango Pinedo & Smets, 2005; Tock & Dryden, 2005; Hoskisson & Smith, 2007). Comparison of a RM system knockout *E. coli* mutant caused 7-fold increased uptake of an unmethylated plasmid (Roer *et al.*, 2015). Still, only reduction, but not a complete exclusion of plasmid uptake was detected, since the wild type could also receive the plasmid (Roer *et al.*, 2015).

Additionally the expression of RM system genes seems not to be constant, but rely on environmental conditions (Bayliss *et al.*, 2006), and cells with a turned off RM system or RM mutants can become hypersusceptible for foreign DNA uptake (Corvaglia *et al.*, 2010). This observation needs further elucidation in complex communities, where modification of RM mechanisms through environmental conditions might shift phylogenetic composition of the permissive community fraction dramatically towards these hypersusceptible strains.

In conclusion, it appears RM systems can influence the transfer probability, but not eliminate a potential recipient from the transfer range of a plasmid.

4.4.4 Host CRISPR systems

Relatively recently a major defense mechanisms against the invasion of foreign DNA based on repetitive DNA sequences called CRISPR systems was discovered (Mojica *et al.*, 2005). CRISPRs are host chromosomally encoded clustered regularly interspaced short palindromic repeats, which consist of

highly variable spacer DNA sequences of phage or plasmid origin separated by repeated identical DNA sequences (Mojica *et al.*, 2005).

A huge variety of bacterial CRISPR defense mechanisms are known (Terns & Terns, 2011), and while their different regulatory control systems are not well understood (Mojica & Díez-Villaseñor, 2010), they share a general mechanism. The CRISPR encoded variable spacer segments are transcribed into small RNA fragments that can bind to complementary structures of a plasmid. The co-transcribed repetitive DNA sequences function then as a recognition site for CRISPR-associated proteins (Cas) which then degrade the mobile genetic element. As the variable spacer region consists of sequences from formerly encountered plasmid invasion, CRISPR-Cas systems have been described as a bacterial adaptive immune response system (Barrangou *et al.*, 2007; Marraffini & Sontheimer, 2008, 2010).

Bacteriophages with their mosaic genetic structure undergoing constant recombination, might be relatively resistant to CRISPR-Cas defense mechanisms (Andersson & Banfield, 2008). Contrarily, plasmids with low rates of evolutionary recombination are far more prone to Cas recognition and degradation. However, pre-exposure to plasmids of similar genetic content is needed to create the CRISPRs variable spacer regions in the host (Fricke *et al.*, 2011). Therefore, CRISPR-Cas systems might be very active while a bacterium remains in its original environment, while they allow plasmid acquisition once the host gets exposed to a new habitat and therefore a fresh adaptive genepool. Thus, like RM systems, CRISPR-cas systems might just govern a relative, but no absolute barrier to the transfer range of a plasmid.

4.5 Plasmid Maintenance

After transfer and avoidance of the early defense mechanisms a well regulated replication control and a diverse subset of maintenance strategies minimize the chance of plasmid loss after vegetative segregation. Thus, the plasmid can be successfully established in its new host. Plasmid maintenance mechanisms are exceptionally relevant for plasmids that appear at low-copy numbers, since daughter cells that might be cured of the metabolic burden might easily outgrow the plasmid carrying ones, leading to extinction from the community. The replication and maintenance host range of a plasmid includes all organisms in which the plasmid after successful transfer can replicate and be maintained over a short or long period of time.

4.5.1 Plasmid replication

One of the most vital features that cause the success of plasmids is the ability to self-replicate autonomously from the host organism.

Linear plasmids, mainly found in Actinobacteria (Ventura *et al.*, 2007) rely on a mechanism based on conserved telomeric replication proteins (Qin *et al.*, 1998). The far more abundant circular plasmids have two different types of basic mechanisms, the rolling circle mechanism and the theta-type and strand replacement mechanisms.

Rolling circle replication generally occurs in small (<10kbp), high copy number plasmids (Khan, 2005; Guglielmetti *et al.*, 2007). Proteins involved in the initiation, elongation and termination are all self-encoded in the plasmids replicon region (*rep*) (del Solar *et al.*, 1998). For successful segregation of multimers that might result from the rolling circle replication plasmids encode resolvases. These resolvases split the plasmid multimers at the plasmid resolution sites and ensure that the copy numbers per daughter cell stay stable. Plasmid loss rates as low as 10^{-4} despite having a copy number of 3-4 per cell can be reached with an efficient partitioning mechanism like that of exemplary plasmid P1 (Li *et al.*, 2004). Rather than positioning the plasmid actively in each daughter cell, many small-sized plasmids rely on a high copy number and random diffusion in dividing cells. For high copy number plasmids the probability of plasmid loss becomes thus a function of their copy number in binomial distribution (Summers, 1991).

In the theta-type and strand replacement mechanism, used in most conjugative, larger size plasmids, replication is initiated through the *rep* based synthesis of primer DNA that can bind at one or multiple DNA iterons of the denatured plasmid DNA (Krüger *et al.*, 2004). While some plasmids encode for their own DNA polymerase elongating the DNA on both denatured plasmid strands after initiation, some rely on the host's own DNA polymerase I (del Solar *et al.*, 1998). This reliance on the host can diminish their replication host range, if the DNA polymerase is not compatible with the initiation of replication of the plasmid.

After transfer and successful replication, all daughter cells are supposed to carry at least one copy of the transferred plasmid to establish the plasmid in the host population. This process is known as plasmid segregation.

The copy number, regulated by the replication machinery is herein decisive. Most replication control systems are ensuring one plasmid replication cycle

per segregation event of the host cell (Gerdes *et al.*, 2002). This keeps the number of plasmid copies per cell constant. Replication inhibiting proteins encoded by the plasmid additionally regulate the copy number if a too high number of plasmid copies are maintained after partitioning.

4.5.2 Active plasmid partitioning

For low copy number plasmids random distribution of plasmids in the partitioning cell would lead to a high probability of plasmid loss. Therefore, most low-copy number plasmids rely on an active partitioning process. Plasmids that are positioned in the center of the cell directly after segregation (Gordon *et al.*, 2004) will thus move to both the quarter and three-quarter position (Gordon *et al.*, 2004) or to the two poles of a cell (Jensen & Gerdes, 1999). This ensures that at least one plasmid copy will be present in each daughter cell (Figure 15).

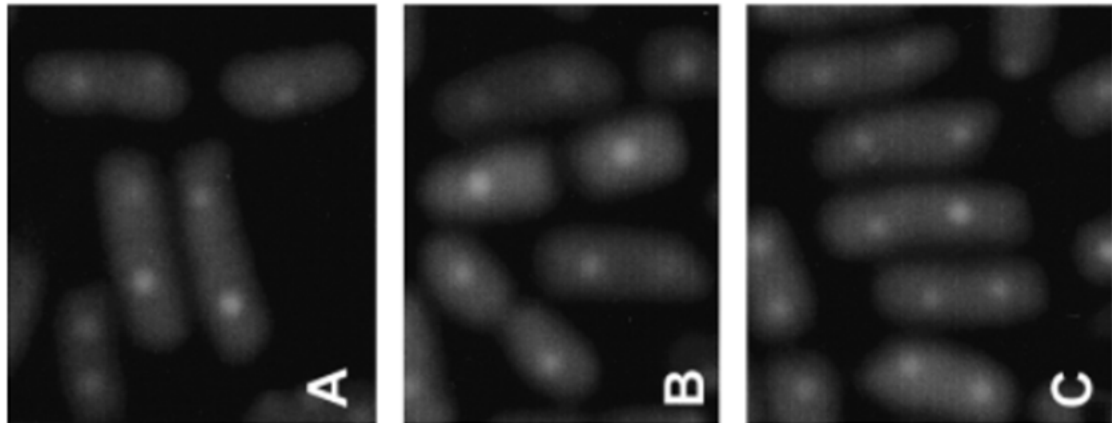


Figure 15: Partitioning cycle of a conjugative plasmid: Active partitioning of a conjugative plasmid at the quarter and three-quarter position of a cell (A). After segregation the plasmid is in the center of a cell, where it undergoes replication (B) before the active partitioning system leads it back to the quarter and three-quarter position of the cell (C) (modified from (Gordon *et al.*, 2004)).

Several different partitioning signals and locations within the same cell exist, which results in different plasmids within the same cell being either partitioned at two separate places (Ho *et al.*, 2002) or competing for the same partitioning location, a reason for the formerly discussed plasmid incompatibility.

4.5.3 Toxin-antitoxin systems

Another mechanism that preserve plasmids in their hosts relies on toxin-antitoxin systems causes post-segregational killing of plasmid-free cells. Ra-

ther than preventing plasmid loss, these systems ensure the retention of the plasmid through reduction of the viability of plasmid-free cells (Jaffe *et al.*, 1985). Plasmids are therefore kept in the community with fidelity as their loss is punished. While there are five different types of post-segregational killing mechanisms (Wen *et al.*, 2014; Goeders & Van Melderen, 2014), all of them are based on the production of a plasmid encoded stable toxin and its likewise plasmid encoded but labile antitoxin counterpart (Hayes, 2003). The stable toxin proteins can either have bactericidal or bacteriostatic properties (Wright *et al.*, 2013). Antitoxins can be untranslated, antisense RNA species that bind to the toxin mRNA before translation and causes its degradation, or binds the toxin protein. Another option for antitoxins are proteins that degrade the toxin encoding mRNA, bind and inactivate the toxin protein or bind and modify the target structure of the toxin (Wen *et al.*, 2014).

4.6 Plasmid host evolution

If a plasmid is able to overcome all of the formerly established barriers it might be stably maintained in a host over a long time. There it might undergo adaptation of its backbone to the genetic code of its host organism (Suzuki *et al.*, 2010). While the exchange of accessory genes through recombinatory events, especially those on transposable elements, might happen in a rather short period of time, the evolutionary adaptation of the plasmid backbone to the genetic code of its host takes only place in long-term hosts.

The nucleotide composition of the plasmid might thus be altered on the evolutionary timescale, towards the GC content of its new host (Rocha & Danchin, 2002). Shintani *et al.* (Shintani *et al.*, 2015) suggested that the resident time of a plasmid in *Pseudomonas* hosts could be predicted based on a comparison of the GC contents of plasmid and host. However, the original GC content of plasmids before evolutionary adaptation remains unknown and thus disables an absolute measure. Additionally, when comparing all sequenced plasmids to their hosts, the general GC content of plasmids is slightly, but significantly, lower than the one of its host's genome (Nishida, 2012).

The adaptation of plasmid backbones to the hosts genetic content has been shown for the well-studied IncP-1 type plasmids (Norberg *et al.*, 2011). Based on genetic analysis completely sequenced plasmids Suzuki *et al.* (Suzuki *et al.*, 2010) proposed that for this group all candidate evolutionary hosts still belong to the identical phylum of Proteobacteria. Analysis was carried out based on GC content and similarity of genes to fully sequenced or-

ganisms. The results are consistent with the known long term host range for these kinds of BHR plasmids.

4.6.1 A core super-permissive fraction dominates plasmid transfer in soil (Paper II)

The transfer range of these plasmids is far more diverse than the evolutionary host range (Klümper *et al.*, 2015). Still, we found that long-term gene acquisition within the evolutionary long-term hosts can be resolved through the short-term transfer range of plasmids. We identified 281 diverse OTUs of 11 phyla as the transfer host range within a soil community with the three different broad host range plasmids and *P. putida* as donor. However, comparative analysis of plasmid sequences has indicated that the evolutionary host range of IncP plasmids seems to be mostly limited to Proteobacterial classes (Suzuki *et al.*, 2010). This suggests that plasmids are not maintained long enough outside of this phylum to be significantly affected by non-Proteobacterial genomes. Poor maintenance of these plasmids in non-Proteobacterial hosts is the likely bottleneck explaining the difference between the very wide realized transfer range and the narrower evolutionary range.

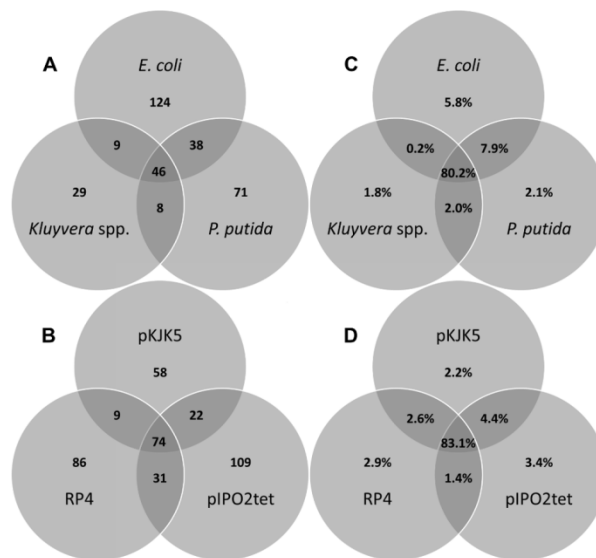


Figure 16 Venn diagram of transconjugal pools for plasmid pKJK5 transferred from three different donor strains (*E. coli*, *P. putida* & *Kluyvera* sp.) (A&B) and for three different plasmids (pKJK5, RP4, pIPO2tet) introduced through *P. putida* into the soil community. Venn diagrams are presented for OTU incidence (C&D) and for OTU relative abundance (right, 100% represents the total number of transconjugal sequences). OTUs were defined at 97% sequence similarity and sequence sample size was normalized to 30000 per transconjugal pool. (Klümper *et al.*, 2015)

However, I identified a core super-permissive community that consists mainly of diverse Proteobacteria like Enterobacteriales (γ), Burkholderiales (β), Pseudomonadales (γ) and Rhizobiales (α) (Figure 16). It consisted of 74 OTUs that were common to all three pools for plasmid pKJK5 (Figure 16). A similar observation (46 out of 279 OTUs shared) held when comparing the transconjugal pools for plasmid pKJK5 introduced via three different donors (Figure 16). These shared OTUs represent over 80% of the transconjugal sequences. This core super-permissive community fraction shared by all five transconjugal pools represents taxa that are able to take up diverse broad host range plasmids from diverse donor strains at high frequencies.

The ability to take up diverse broad host range plasmids from different hosts at high frequencies as represented by the super permissive fraction of the community has not previously been described.

In soil a few closely related strains exchanged various genes on the evolutionary scale. They are the core nodes in an interconnected cluster of lateral gene acquisition (Popa *et al.*, 2011). These species are mainly found within Enterobacteriales (Gammaproteobacteria), Burkholderiales (Betaproteobacteria), and Staphylococci (Bacilli), groups that contain most of our super-permissive OTUs. Finding the same group of bacteria as central nodes in lateral gene transfer networks (Popa *et al.*, 2011) and as main contributors to plasmid flow in soil suggests that we found a link between increased plasmid uptake ability and long-term gene acquisition and plasmid adaptation potential.

5 Agronomic practices modulate gene transfer in soil

Resistance genes originating from soil microbes can horizontally spread to pathogens if they are encoded on mobile genetic elements which can be transferred between distinct communities (Finley *et al.*, 2013). Recent genomic analysis indicated that many soil borne antibiotic resistance genes are identical to those in multi-resistant human pathogenic strains found in hospitals (Forsberg *et al.*, 2012). This indicates that resistance genes originating from soil have horizontally spread to pathogens. Through agronomic practice, especially the application of manure, a huge variety of non-indigenous, manure-borne microorganisms are periodically introduced into the soil community. These manure-borne microorganisms come with increased levels of antibiotic resistance genes (Smalla *et al.*, 2000). While most of these enteric bacteria do not survive in soil (Pepper, 2013) their genes might survive after being horizontally transferred to soil indigenous bacteria and become part of the soil resistome. Former studies have indicated that long-term agronomic practice might have a major influence on the mobile soil resistome. High levels of antibiotic resistance genes encoded on plasmids were shown in diverse soils treated with manure (Agersø *et al.*, 2006), biosolids (Brooks *et al.*, 2007) or fertilized with chicken waste (You *et al.*, 2012). Understanding the fate and ecology of plasmids in soil microbial communities might therefore be a crucial aspect when tackling the problem of multi-resistant strains.

5.1 Environmental conditions affect soil plasmid transfer

Several environmental factors affecting plasmid transfer in the complex soil community have been described. These effects can either increase or decrease plasmid transfer or plasmid maintenance in soil microbial communities. They can directly affect one or several of the processes described above in plasmids dynamics, or they might act indirectly by affecting growth rates, physiological state, creation of selective condition, or causing or alleviating spatial barriers between cell types (Dechesne *et al.*, 2005).

Natural effectors on plasmid transfer might involve the physicochemical parameters of soil, such as pH or water retention potential (Richaume *et al.*, 1989; Ellass & Trevors, 1990; Rochelle *et al.*, 1989). Also biological factors such as the abundance of earthworms or protozoa, increasing the transport of

bacteria within the community were shown to positively increase the abundance of transconjugants in soil (Sengeløv *et al.*, 2000; Sørensen & Jensen, 1998). Other parameters might be closely connected to agronomic practice, such as enhancing plasmid transfer by the introduction of metal stressors (Top *et al.*, 1995) or an increased nutrient availability (Sørensen & Jensen, 1998) through fertilization or manure treatment. Previous studies have suggested that manure treatment may result in hot-spots of gene transfer due to increased nutrient availability and cell density (Van Elsas *et al.*, 2003) with transfer frequencies increasing by up to one order of magnitude compared to the surrounding soil environment (Götz & Smalla, 1997).

However, these former studies were mainly based on community level transfer frequencies and could not distinguish between potentially related direct effects on the plasmid transfer and maintenance machinery or indirect effects.

Single strain experiments confirmed direct effects of stress exposure on horizontal gene uptake. The exposure to antibiotics in *Streptococcus pneumonia* lead to an increased promiscuity towards foreign DNA via increased competence (Slager *et al.*, 2014). Also pre-exposure to sodium dodecyl sulfate (SDS) in *Pseudomonas putida* increased its plasmid receipt and maintenance possibly by repressing restriction-modification mechanisms (Arango Pinedo & Smets, 2005). Contrarily, stress imposed on the cell envelope induces the expression of CRISPR associated (CRISPR-cas) genes involved in the defense against foreign invading DNA in *Escherichia coli* (Perez-Rodriguez *et al.*, 2011), thus decreasing its permissiveness towards plasmids.

These highly strain specific stress responses indicate that a community level evaluation of effectors might draw an incomplete picture of the processes agronomic practice might have on the soil permissiveness. An evaluation on the effect of stress on individual taxonomic groups might therefore further elucidate the direct impact of stress on the permissiveness of microbial communities towards plasmids transfer.

5.2 Short-term metal stress modulates soil permissiveness (Paper IV)

One of the most common environmental stresses is the frequent accumulation of metals (e.g. Cu, Zn) due to agricultural practices, industrial activities, or atmospheric deposition (Nicholson *et al.*, 2003; Zhao *et al.*, 2014). If a stress is imposed on an environment, different bacterial species have distinct responses based on the dose of exposition. Low exposure levels might serve as

a stimulant or signal for the transcription of certain catabolic genes (Pérez-Martín *et al.*, 1996) at sub-toxic levels. Increased doses above the toxic level will cause expression of stress-response mechanisms. Results of stress at toxic levels can be growth inhibition or even lethality, if the triggered stress-response machinery is not able to cope with the imposed stress (Cases & de Lorenzo, 2005). We speculate that an increased permissiveness towards mobile genetic elements, supplying potentially adaptive genes might be part of the yet not fully discovered stress-response system in bacteria. We aimed to explore if this expected modulation is based on a general response to metal stress or if a dependency on the type of metal stressor or the dose it is introduced at exists. Further, we investigated if the phylogenetic diversity of the transconjugal fraction changes as a result of metal stress.

Hence, we introduced the model broad host range plasmid pKJK5 into a soil microbial community in the previously described filter matings. We challenged the community with stress through five environmentally relevant metals (Cu^{2+} , Ni^{2+} , Zn^{2+} , Cd^{2+} , AsO_3^{3-}) at doses that correspond to 20% and 50% community level growth rate inhibition. Inhibitory concentrations of 20% and 50% community level growth inhibition (IC_{20} & IC_{50}) were obtained to define the doses of metal treatments for subsequent filter mating experiments (Table 3).

Metal	IC_{20}	IC_{50}
AsO_3^{3-}	40.5 μM (As20)	125.2 μM (As50)
Cd^{2+}	12.6 μM (Cd20)	63.6 μM (Cd50)
Ni^{2+}	3.7 μM (Ni20)	11.5 μM (Ni50)
Zn^{2+}	24.7 μM (Zn20)	80.7 μM (Zn50)
Cu^{2+}	6.9 μM (Cu20)	28.9 μM (Cu50)

Table 3 Inhibitory concentrations causing 20% and 50% bacterial growth inhibition (IC_{20} and IC_{50} , respectively) as extrapolated from [^3H]leucine incorporation data. Abbreviations in brackets will be used throughout the paper to refer to results from filter matings under the diverse metal stress conditions.

We detected that on the community level the transfer frequency of pKJK5 to the soil community was significantly reduced. This reduction compared to the reference mating even exceeded the decrease in growth of 20% or respectively 50%. Therefore, plasmid uptake activity appears to be more sensitive to immediate metal stress exposure than growth activity. However, the previous-

ly normalized and identical stress levels resulted in variable inhibition of plasmid transfer for different metals (Figure 17). While plasmid transfer was reduced by up to 90%, the diversity of the permissive community fraction community remained stable and included 13 different phyla.

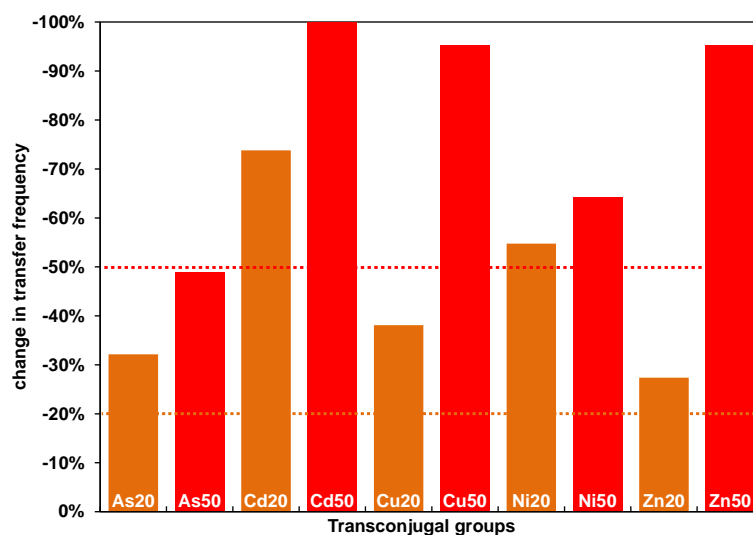


Figure 17 Plasmid transfer frequency reduced under stress conditions. Normalized plasmid transfer frequencies for each of the five tested metals at their respective IC₂₀ (orange) and IC₅₀ (red) and the non-stressed reference were calculated based on the analysis of 90-150 images each. Metal induced stress conditions were defined based on the ability of the different metals to inhibit [³H]leucine incorporation rates by 20% (orange) or 50% (red) as indicated by dotted lines.

Based on phylogenetic analysis transconjugal pools clustered significantly ($p < 0.001$) apart from their respective recipient communities in PCoA analysis. Bacterial OTUs that were not permissive to plasmid pKJK5 under any of the tested conditions exist in the soil recipient communities. These could have resulted in the aforementioned clustering the permissive transconjugal pools apart from their respective reference communities. Hence, those were removed from the soil recipient communities before PCoA analysis. Transconjugal pools still clustered apart from the reference communities thereby demonstrating that their phylogenetic composition is not based on stochastic selection process, but a function of the varying permissiveness of different OTUs. The phylogenetic composition of the transconjugal pools after the exposure to stress can significantly shift compared to the reference transconjugal pool.

5.2.1 Stress specific responses are resolved by transconjugal phylogeny

The observed differences in phylogenetic structure can be based on two potential reasons. Metal stress induced indirect effects causing shifts of the original recipient community, or a directly modulated permissiveness of specific bacterial OTUs as a stress response. We therefore aimed to separately analyze if increased or decreased permissiveness of single types of bacteria occurs as part of a direct effect in the stress response and if stress specific patterns occur.

Hence, we calculated for all OTUs the ratio δ of its observed relative abundance ($T_{\text{stress,obs}}$) over its expected abundance in the transconjugal pool at the same metal stress ($T_{\text{stress,exp}}$). The expected abundance was calculated as the relative abundance of the OTU in the reference transconjugal pool (T_{ref}) multiplied by the ratio of relative abundance of the same OTU in the metal stressed (R_{stress}) and reference (R_{ref}) recipient community. If stress does not affect the OTU abundance in the reference community, the latter ratio is 1. A δ value above 1 would indicate increased plasmid receipt in an OTU associated with metal stress. A δ value below 1 would indicate decreased plasmid receipt in that OTU associated with metal stress.

$$\delta = \frac{T_{\text{stress,obs}}}{T_{\text{stress,exp}}} = \frac{T_{\text{stress,obs}}}{T_{\text{ref}} * \frac{R_{\text{stress}}}{R_{\text{ref}}}}$$

Our isolation method could not distinguish original horizontal transfer of the plasmid from its subsequent vertical replication and maintenance through growth. The growth factor interferes in the original relative abundance of each OTU. It is corrected for in the δ value, by correcting for growth in the recipient community and its specific growth inhibition under stress conditions.

The 39 most abundant OTUs with a relative average abundance above 0.05% in transconjugal pools were analyzed for their δ value. The high variability of these OTUs δ values among the different transconjugal pools (Figure 18) demonstrates that indeed the relative permissiveness of an OTU is altered as part of the metal stress response.

Some metal stresses (As20, Cu20, Cu50, Ni20, and Zn20) might promote plasmid receipt in most OTUs observed, while As50 seems to considerably decrease the permissiveness of the majority of OTUs tested. The influence

stress can have on the permissiveness of an OTU is independent of its dose. The boxplot diagram shows that when exposed to Zn and Ni stress the permissiveness increases for most OTUs at lower dosed stresses (Zn20, Ni20) while at their corresponding heavier stresses (Zn50, Ni50) permissiveness varies around a median at the level of no effect (Figure 18).

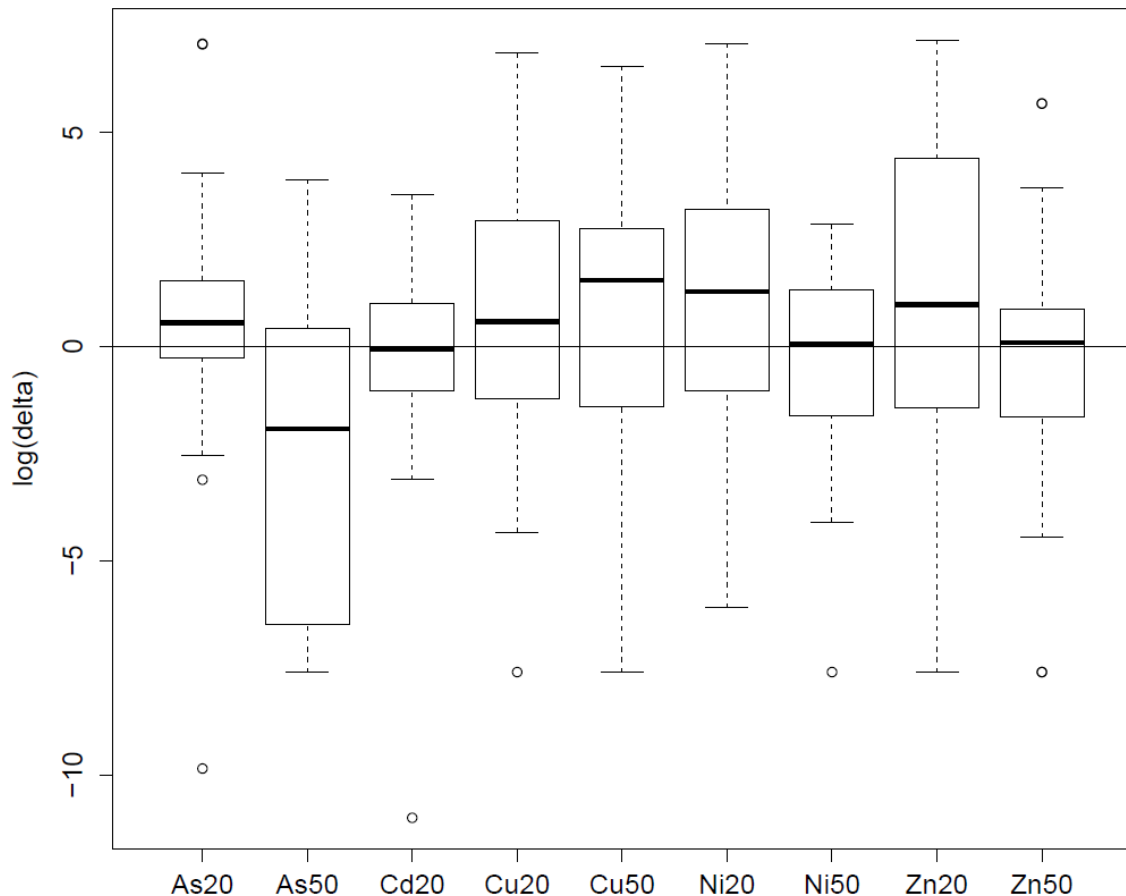


Figure 18 Boxplot diagram showing the distribution of δ values for the 39 most abundant OTUs in each transconjugal pool on the logarithmic scale. The line represents the complete absence of metal effect on permissiveness (all $\delta = 1$).

The phylogeny of a recipient OTU impacts its stress response with regard to plasmid receipt. This notion, already indicated in the PCA plot, is supported by a maximum likelihood tree constructed based on similarity of their delta value across stresses (Figure 19). All OTUs belonging to the phylum Bacteroidetes show a high degree of similarity in their response to different stress scenarios.

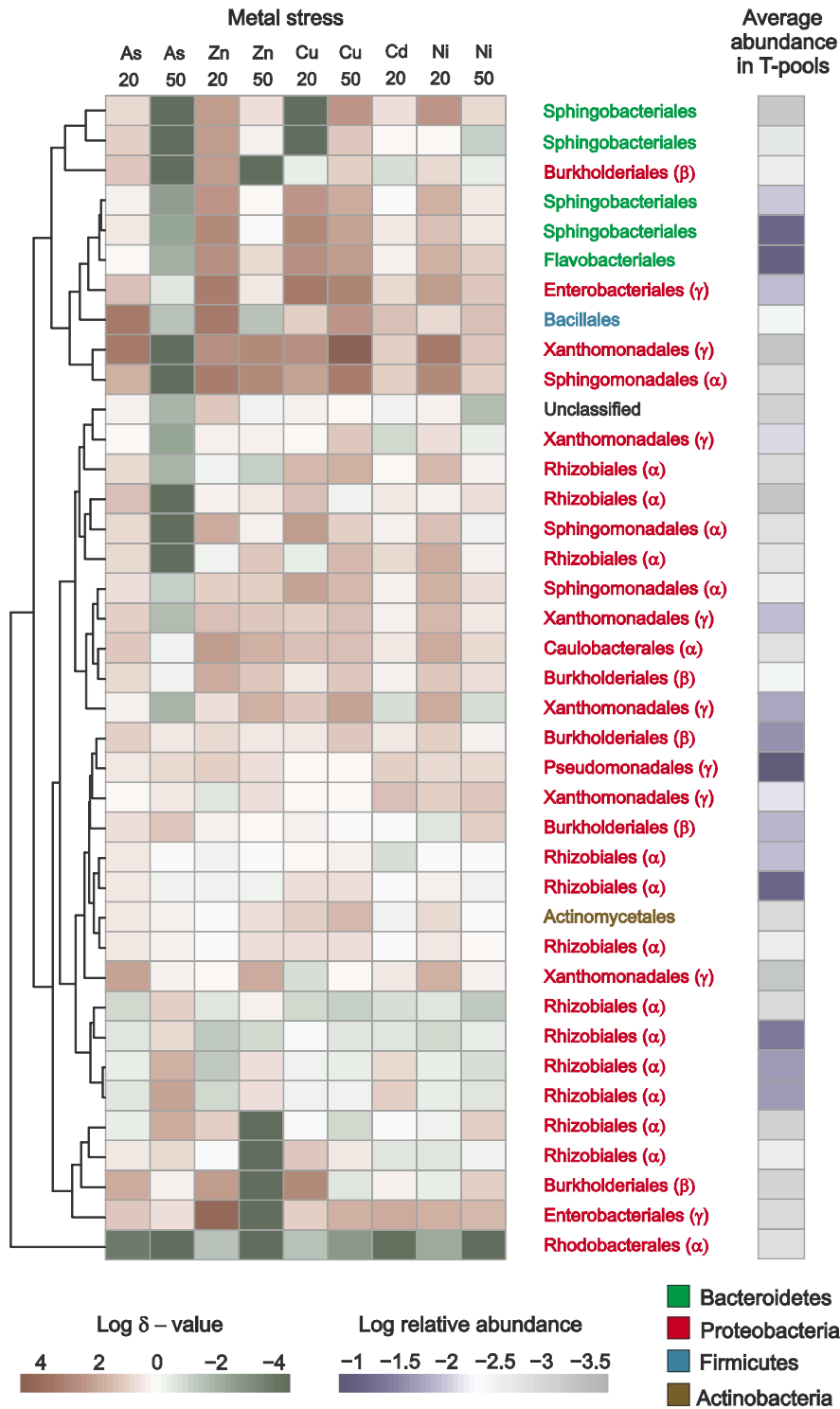


Figure 19 Heatmap showing the log scaled δ value of stress-imposed fold difference of an OTU's relative abundance due to stress. The 39 most abundant ($>0.05\%$) OTUs, their relative abundance in log scale in violet as well as their phylogeny are shown (for Proteobacteria their class is shown in brackets) and sorted in a maximum likelihood tree based on their plasmid uptake dependent responses to stress at IC20 and IC50 through different metals. An increased plasmid uptake response is shown in red, a decreased in green.

For all stresses except As50, plasmid transfer from the proteobacterial *E. coli* donor to Bacteroidetes is significantly increased. Metal stresses thus promoted increased plasmid transfer across phylum borders. A positive δ -value, indicating a relative increase of an OTU in the metal-associated transconjugant pool, could also correspond to another, dominant transconjugant OTU disappearing under stress conditions. Therefore, we calculated the absolute increase of those OTUs after correcting for the stress induced reduction in transfer frequency observed at community level (Figure 17). The total number of transconjugants belonging to the Bacteroidetes phylum would have the potential to more than double in soil communities under stress conditions. Thus, the observed propagation of plasmid transfer to other phyla is not only relative, but also absolute. The only gram-positive OTU among the 39 most abundant ones, part of the Firmicutes phylum, is also found in this cluster of increased plasmid receipt under stress conditions. This might indicate that plasmid transfer also to other phylogenetically distant phyla becomes increasingly relevant under metal stress conditions, but observing one single OTU might not be a high enough resolution to conclude.

For most of the transconjugal OTUs in the Proteobacteria, the stress response is more variable. Four of these OTUs show stress responses similar to Bacteroidetes and become increasingly permissive under stress conditions. Most Proteobacteria, such as the Rhizobiales, do not respond to stress by modulating their permissiveness significantly. Rhodobacterales, on the other hand, remarkably decreased their plasmid receipt under any applied stress conditions.

We demonstrated here that a modified permissiveness is indeed unique for each bacterial OTU under stress conditions. Stress can directly affect the uptake of foreign DNA by modulation of the previously described essential processes in plasmid gain or maintenance and defense. Assessing the individual permissiveness of each OTU within the community revealed that the response to a specific stress is dependent on the phylogeny of the OTU, since species from similar phylogenetic groups respond similar to specific stresses applied. However, prediction of a specific stress response might become difficult, since stress responses were neither dose nor metal dependent. The regulatory mechanisms involved in these stress responses of bacteria within the same family are highly evolutionary conserved. This could explain why the stress triggered regulation of plasmid receipt seems to be phylogenetically dependent. The question how the exposure to heavy metals determines the spread of

mobile genetic elements needs therefore to be answered at the individual strain rather than the community level.

5.3 Long-term agronomic effects on soil permissiveness (Paper V)

High levels of plasmid shuttled resistance genes were found to sustain in agronomic soils annually treated with piggery manure slurry (Agersø *et al.*, 2006). Previous studies on the seasonal introduction of manure have suggested that directly after application plasmid transfer frequencies may increase by up to one order of magnitude (Götz & Smalla, 1997). These might thereafter remain in the soil microbial community (Heuer *et al.*, 2011). Elevated levels of plasmid transfer after manure application can be explained by indirect effects such as increased nutrient availability, which promotes bacterial growth. With increased cell densities so called hot-spots of gene transfer develop in which plasmids transfer at high frequencies (Van Elsas *et al.*, 2003).

We hypothesized that an additional, so far unexplored indirect effect might be the long term selection for more permissive phenotypes that can adapt easier to the seasonal environmental changes going along with manure application through their ability to gain adaptive genes encoded on plasmids. In order to test this hypothesis we tested 4 different extracted soil microbial communities for their plasmid uptake potential in a filter mating assay (Musovic *et al.*, 2010). We chose 3 model broad host range plasmids (RP4, pIPO2tet and pRO101) introduced to the communities through a *Pseudomonas putida* donor strain.

we chose three agricultural plots (Untreated, Manured and Nitrate-Phosphate-Potassium-fertilized (NPK)), at the long-term CRUCIAL experimental site (Taastrup, Denmark) (Poulsen, Al-Soud, *et al.*, 2013; Magid *et al.*, 2006) to test if their plasmid uptake potential is altered through agronomic treatment. Earlier studies on the CRUCIAL soil showed that their phylogenetic composition is not altered by the different agronomic treatments (Poulsen, Magid, *et al.*, 2013). Further, samples were taken 3 month after the last manure application, to ensure that non-indigenous bacteria introduced to the soil community were outcompeted. As a reference, soil from an untreated plot of the well-known Rothamsted Park Grassland (Rothamsted, United Kingdom) (Silvertown *et al.*, 2006) site was included in the study.

In our assay, heterogeneities in nutrient or cell density were excluded as matings were carried out under standard nutritional conditions on soil extract medium (Musovic *et al.*, 2010). Any effects observed are therefore intrinsic to the soil microbial communities.

For all 4 different soil microbial communities the plasmid uptake potential was in the same order of magnitude. Approximately 1 in 10,000 soil bacterial cells could receive and maintain the different plasmids tested (Figure 20). The similarity between different treatments of the CRUCIAL soil and the Rothamsted reference suggests that a similar plasmid uptake potential for these broad host range plasmids under neutral conditions might be a general feature among diverse soil communities.

To study the effect of fertilization, we compared the permissiveness towards plasmid RP4 in the NPK-fertilized soil bacterial community with the untreated control. Permissiveness in the NPK-treated soil was similar to the untreated control ($p=0.79$). Therefore, a potentially higher activity through previous nutrient addition is not affecting the permissiveness in soil.

An up to 100% higher permissiveness towards RP4 and pIPO2tet was measured for the manure treated community compared to the untreated control ($p_{RP4}=0.041$; $p_{pIPO2tet}=0.001$) indicating that long-term manure treatment also changes the community permissiveness towards newly introduced plasmids under neutral nutrient conditions. This increased permissiveness appears intrinsic to the community, as the diversity of the recipient community was similar (Poulsen, Magid, *et al.*, 2013).

Although plasmid transfer frequency was in the same order of magnitude, both type of plasmid and applied agronomic treatment affected the absolute values. Contrarily, the diversity of the transconjugal pools, isolated by micromanipulation and subsequently sequenced was purely plasmid dependent based on PCoA analysis of the retrieved transconjugal pools. All pools were dominated by β - and γ -Proteobacteria. The increased community permissiveness in manured soil can thus not be explained by difference in community diversity. Increased seasonal nutrient availability can also be ruled out, since high permissiveness was not observed for NPK-fertilized soil.

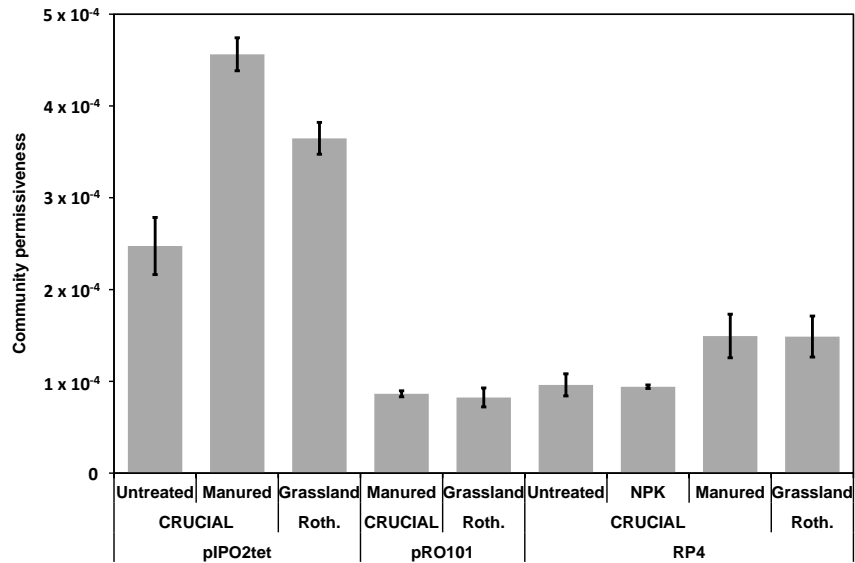


Figure 20 Transfer frequency of the introduced plasmid to the soil indigenous bacterial communities derived from the CRUCIAL and Rothamsted (Roth.) plots after 48 h of incubation in solid surface filter matings on soil extract medium. Values are displayed as the mean of triplicates with standard deviation.

Still, the exact mechanisms by which the permissiveness of manure treated soil microbial communities is increased are yet to be elucidated. The exposure to stress by metal cations, co-introduced through manure, might play a role in this phenomenon.

Earlier studies have shown that the permissiveness within a bacterial population can vary up to 100 fold (Heuer *et al.*, 2010). While short term stress may lead to decreased immediate plasmid transfer frequencies (see Paper IV), long-term adaptation to seasonal stress conditions might select for the more permissive subpopulations.

High-throughput analysis of the transconjugal pools in combination with analysis based on the δ value (see Paper IV) to evaluate an potentially increased permissiveness of specific OTUs as developed later during this PhD would probably have led to further insights how manure modulated the permissiveness of single bacterial taxa.

6 Conclusions

This thesis has focused on identifying the transfer ranges of plasmids and the extent of plasmid transfer in soil microbial communities. Additionally the effect of environmental conditions on plasmid transfer has been elucidated. I developed novel methods that allow high-resolution insights into plasmid transfer in soil microbial communities. I applied this novel toolbox to assess the quantitative and phylogenetic extent of plasmid transfer in soil. Furthermore, I assessed the impact of short –term metal exposure on plasmid transfer in soil communities and assessed to what extent long-term agronomic practices modulate their permissiveness. The main findings of each of these sections can be summarized as follows:

A new high-throughput method to analyze plasmid transfer in soil at high-resolution (Paper I-III):

- A triple-gated, double-sorting FACS approach allows high-throughput isolation of transconjugants at high purity.
- Our newly developed method is able to assess a bacterial community's permissiveness through simultaneous quantification of transfer and isolation of transconjugants.
- Sequencing of more than 10,000 sorted transconjugants for different donor-plasmid combinations far increases earlier approaches to map transconjugal diversity.
- A community's ability to actively mobilize plasmids can be quantified and assessed in comparison to a mobilizing plasmid and intrastain transfer experiments.

Mapping plasmid transfer host ranges in soil bacterial communities (Paper II, III & V)

- Broad host range plasmids can transfer to an unexpectedly diverse fraction of a soil bacterial community involving 13 different phyla.
- Plasmid transfer across the Gram border is a common phenomenon.
- The phylogenetic composition of transconjugal pools is plasmid and donor dependent.

- Mobilizable plasmids gain impact when co-resident with a mobilizing plasmid that is able to directly mobilize them.
- Stress can significantly shift the specific phylogenetic composition of the transconjugal pool of a plasmid-donor combination.
- A core super-permissive fraction that dominates plasmid transfer in soil is also found at the core nodes of a network of evolutionary gene acquisition.

Analyzing the agronomic impact on plasmid transfer in soil (Paper IV-V)

- The modulation of permissiveness by acute metal stress within a mixed microbial community is taxon dependent.
- Different metals introduced at identical stress levels do not modulate the permissiveness of the community in an identical way. The modulation is furthermore dependent on the metal introduced and the stress level at which it is applied.
- Long-term manure exposure increases a bacterial community's permissiveness towards broad host range plasmids, while its phylogenetic composition remains identical.

7 Future perspectives

The methods I developed in this thesis allow high-resolution insights into plasmid transfer in soil microbial communities. Exogenous donors introduced to the soil microbial community are often outcompeted by indigenous bacteria and subsequently lost in a relatively short after introduction. Contrary, plasmids and thus plasmid encoded antibiotic resistance genes can be maintained within the indigenous community. I so far identified the enormous short-term transfer range of diverse broad host range plasmids after being introduced to a soil microbial community.

To further understand and predict the fate of plasmids in soil the long-term replication and maintenance of the introduced plasmids within the permissive fraction of the soil microbial community needs to be elucidated additionally. Studying in which fraction of the transconjugants the plasmids remain established as a function of time after exogenous plasmid introduction should become the next focus.

The fraction in which the plasmid can be stably maintained becomes of acute relevance when testing which of the transconjugants are able to retransfer the plasmid to new recipients. Analyzing the retransfer ability from transconjugants to new soil recipients or human pathogens could reveal the potential of exogenous plasmids to not only remain within the soil community but also to subsequently spread to other environments.

I demonstrated that stress can play an important role in modulating plasmid transfer in soil bacterial communities. Extending my studies to selective stresses, which favor bacteria hosting a plasmid with adaptive features, might play an important role on which fraction of the soil community subsequently maintain, loses or retransfers a plasmid needs further elucidation. Moreover understanding the processes involved in maintenance and retransfer would allow extrapolating our studies from model exogenous plasmids used in my study to indigenous plasmids ubiquitous in soil bacterial communities.

A combination of my method with these future research perspectives could subsequently serve as an analytic and predictive tool for plasmid transfer dynamics in soil. This tool could help optimizing agronomic practices with a focus to avoid the spread of plasmids and thus plasmid encoded antibiotic resistance genes in and from the soil environment.

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9 Papers

- I Klümper U, Dechesne A, Smets BF. (2014).** Protocol for evaluating the permissiveness of bacterial communities toward conjugal plasmids by quantification and isolation of transconjugants. *In: Hydrocarbon and Lipid Microbiology Protocols (eds.: Timmis K), Springer Protocols Handbook, Humana Press.*
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- III Klümper U, Droumpali A, Dechesne A, Smets BF. (2014).** Novel assay to measure the plasmid mobilizing potential of mixed microbial communities. *Front Microbiol* 5. DOI: 10.3389/fmicb.2014.00730
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- V Musovic S, Klümper U, Dechesne A, Magid J, Smets BF. (2014).** Long-term manure exposure increases soil bacterial community potential for plasmid uptake. *Environ Microbiol Rep* 6:125–130.

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DTU Environment
Technical University of Denmark
Miljøvej, Building 113
2800 Kgs. Lyngby
Denmark

reception@env.dtu.dk.