Permissiveness of soil microbial communities towards broad host range plasmids

Klümper, Uli; Smets, Barth F.; Dechesne, Arnaud

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Uli Klümper

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PhD Thesis
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The synopsis part of this thesis is available as a pdf-file for download from the DTU research database ORBIT: http://www.orbit.dtu.dk

Address: DTU Environment
Department of Environmental Engineering
Technical University of Denmark
Miljoevej, building 113
2800 Kgs. Lyngby
Denmark

Phone reception: +45 4525 1600
Fax: +45 4593 2850

Homepage: http://www.env.dtu.dk
E-mail: info@env.dtu.dk

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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 introductive 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.

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)

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: The Danish Microbiological Society Annual Congress 2013, Copenhagen, Denmark (oral & poster presentation)

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, Mollerup F, Musovic S, **Klümper U**, Albrechtsen, HJ. (2014) Mikrobiel vandkvalitet i rentvandsbeholdere efter inspektion og rensning.
http://www.vandcenter.dk/viden/forskningsprojekter/mikrobiel-vandkvalitet

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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 *Burkolderia*. 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

specific 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 permisssiveness 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 tilføre 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. Historisk set har metoderne benyttet til at undersøge de genetiske samt de miljømæssige faktorer, der ligger til grunde for plasmidoverførsel, været stærkt afhængige af dyrkningen samt ekspressionen 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 cellekontakt på filtre med en fast overflade, efterfulgt af kvantificering af overførselshændelser via avanceret fluorescensmikroskopi, isolering af transkonjuganter ved tredobbeltportet fluorescensaktiveret cellesortering 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 transkonjugantive puljer bestod 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 bestod af Proteobakterielle slægter Pseudomonas, Enterobakterie og Burkolderia. 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 transkonjuganters 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 afhang 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 derved, 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. Overførelsesfrekvenser 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, bakteriebrøkdel 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
ctl	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
gfp	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
trfA	replication initiation genes
trfB	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; Elass & 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 plasmid 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 of bactericidal properties. The first discovered antibiotic penicillin was isolated form 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.

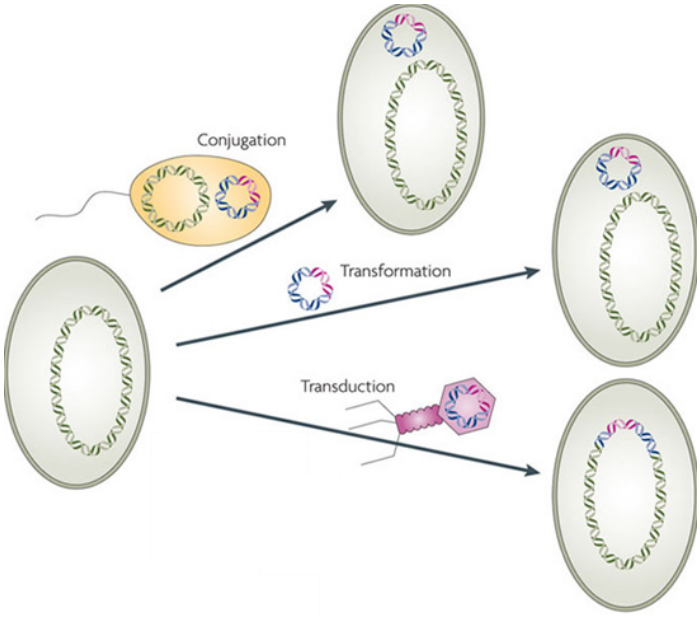

Figure 1 The three general modes of bacterial gene transfer: conjugation, transformation and transduction (modified from (Todar et al., 2008)).

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). Sill, 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 (Uhlig, 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

transduction 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).

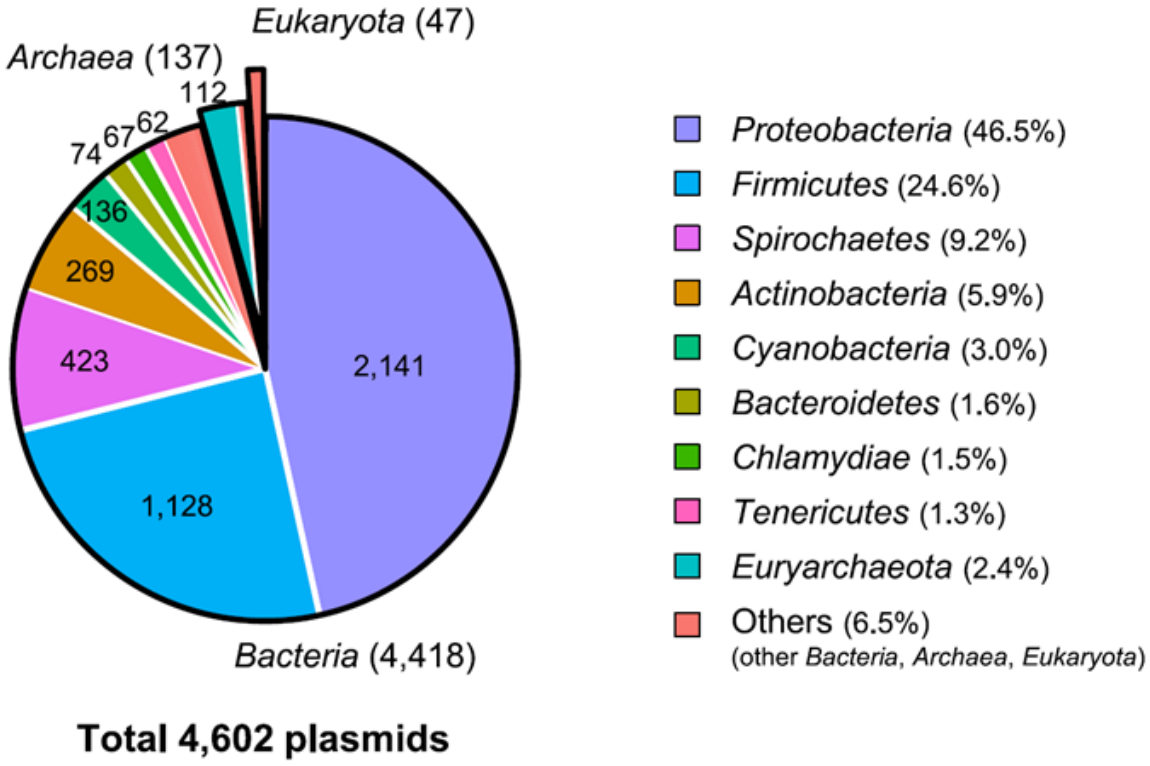

Figure 2 Phylogenetic distribution of the hosts of all currently fully sequenced plasmids (Shintani et al., 2015).

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.

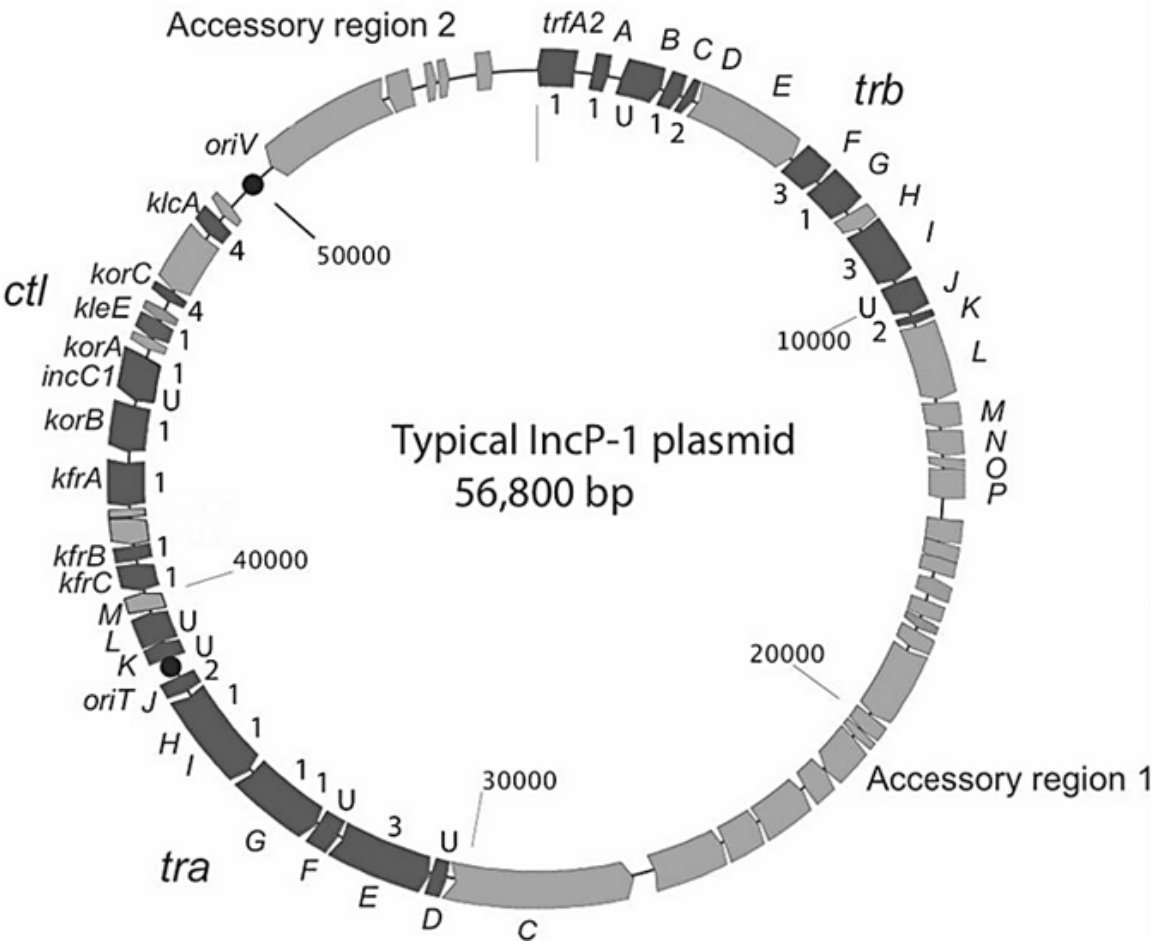

Figure 3 Genetic map of a typical broad host range conjugative IncP-1 plasmid. Two accessory gene regions with potentially adaptive traits for its host are found in addition to the plasmids backbone structure. The plasmid backbone consists of a replication and maintenance region (origin of replication (*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).

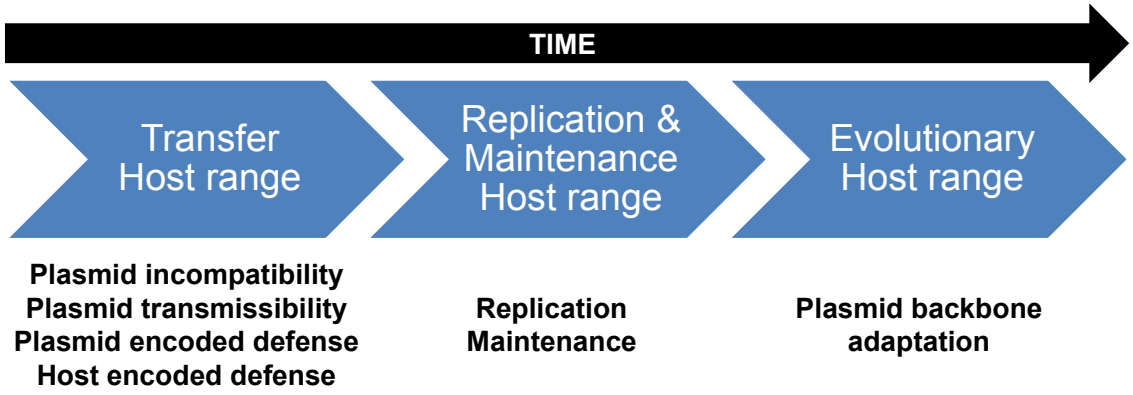

Figure 4 Plasmid host ranges based on residence time in the new host. The transfer host range includes all hosts that can initially receive a given plasmid. The replication & maintenance host range describes all those hosts in which a plasmid can be stably maintained over a short period of several vegetative 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; Elass & 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 transcongants, *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).

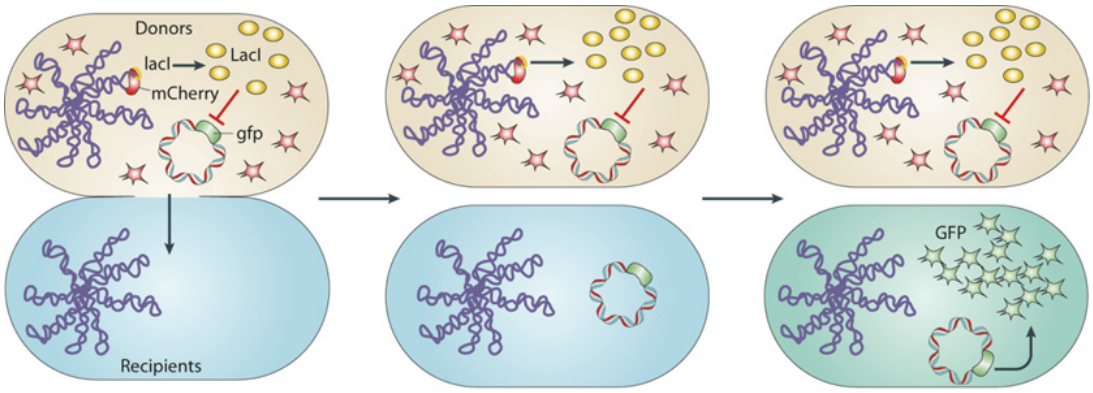

Figure 5 A schematic outline of the transfer reporter-gene approach. The donor cell contains a conjugative plasmid tagged with the green fluorescent protein (GFP) gene (*gfp*) downstream from a *LacI* repressible promoter. The donor chromosome encodes LacI, which represses the expression of GFP. During 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 works 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).

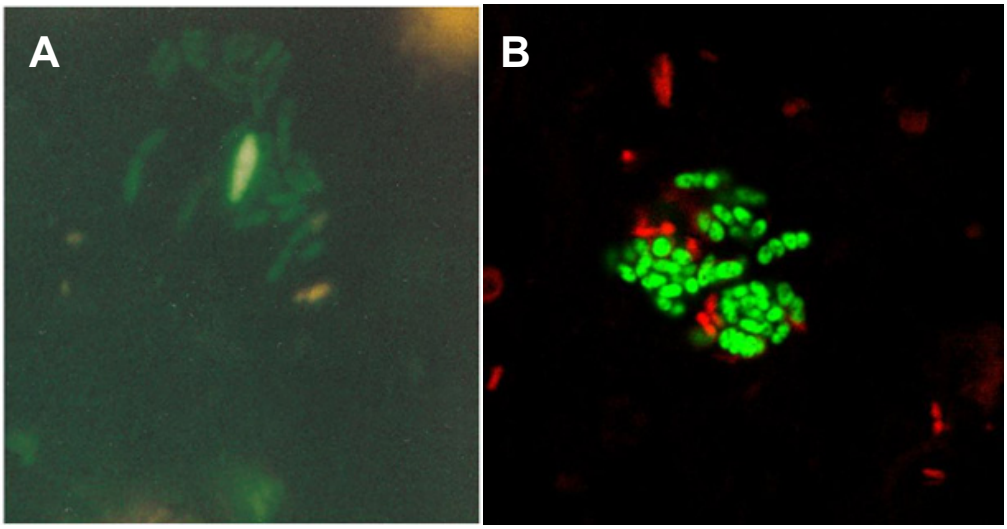

Figure 6 Improved fluorescent detection system. Original: GFP fluorescent transconjugant cells, formed after transfer of plasmid pBF1::*gfp* from *P. putida* KT2442 to marine bacteria in seawater (A) versus improved: *gfpmut3* fluorescent cells, formed after transfer of plasmid pKJK5::*gfpmut3* from 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:

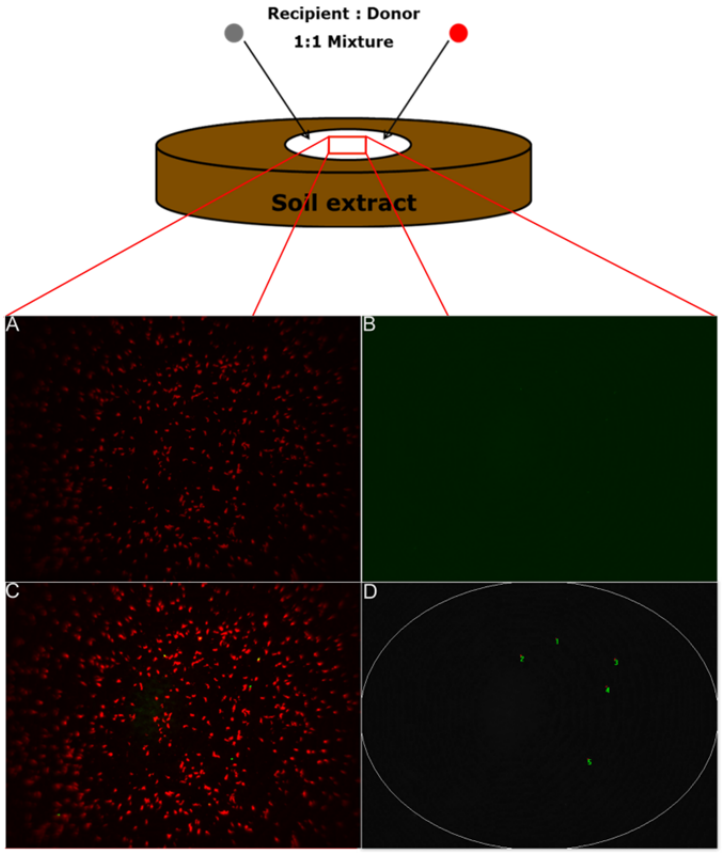

Figure 7 Fluorescence based stereomicroscopic images and image analysis of an example filter mating. Image A corresponds to the red fluorescent channel, displaying donor microcolonies. Image B shows the green fluorescent channel, corresponding to the transconjugal microcolonies that received the plasmid. 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 μm^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 transconjugant, 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.

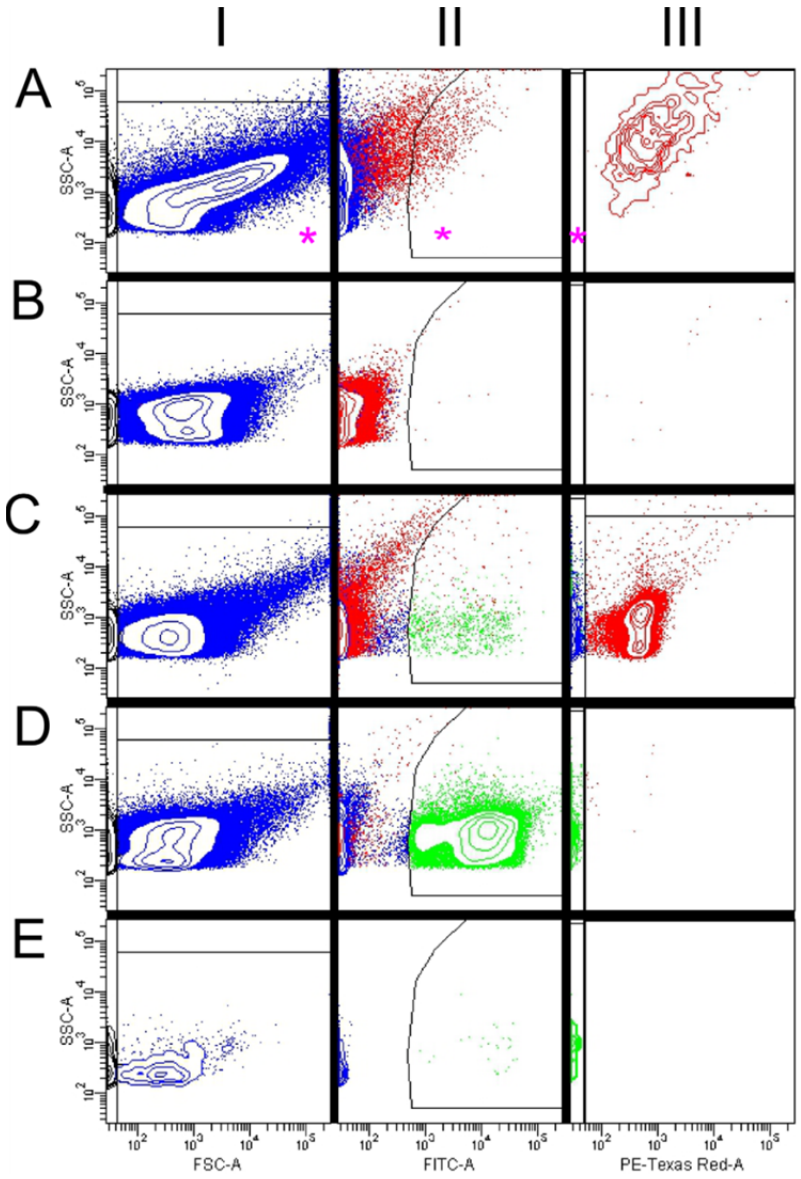

Figure 8 FACS sorting of transconjugal cells from a mating mixture initiated with soil bacteria and *E. coli* carrying pKJK5. The procedure consists in three successive gates (marked by pink stars in Panels A): Gate I sorts for bacterial size based on front and side scatter; Gate II sorts for green fluorescent 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**
Pseudomonas putida KT2440	*lacIq-pLpp-mCherry, Km^R*			This study
Escherichia coli MG1655	*lacIq-pLpp-mCherry, Km^R*			This study
Kluyvera sp.	*lacIq-pLpp-mCherry, Km^R*			This study
Plasmid	**Inc-group**	**Phenotype**	**Host range**	**Reference**
RP4::P*lac*::*gfp*	IncP-1α	Tet^R, Amp^R, Km^R	Broad	(Musovic *et al.*, 2010)
pIPO2tet::P*lac*::*gfp*	IncPromA	Tet^R	Broad	(Musovic *et al.*, 2014)
pKJK5::P*lac*::*gfp*	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).

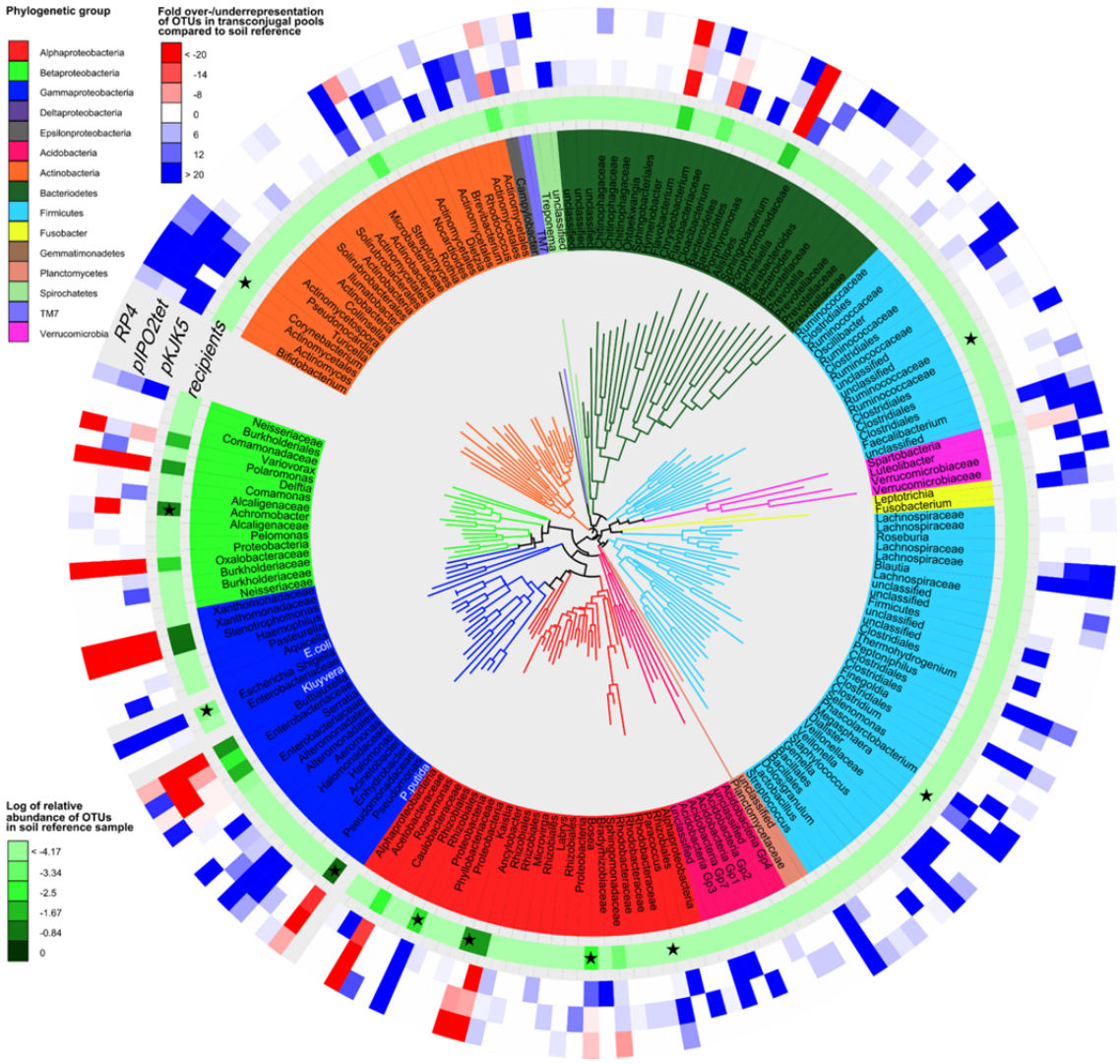

Figure 9 Phylogenetic tree showing all identified transconjugant OTUs for three different plasmids (pKJK5, RP4, pIPO2tet) from the same donor (*P.putida*). The colors of the branches mark different phylogenetic groups. The three donor strains are shown in white letters in the trees. Green heatmap-circle around the tree represents the log transformed relative OTU abundance in the soil reference recipient community. Three heatmap-circles 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 underdoing 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.

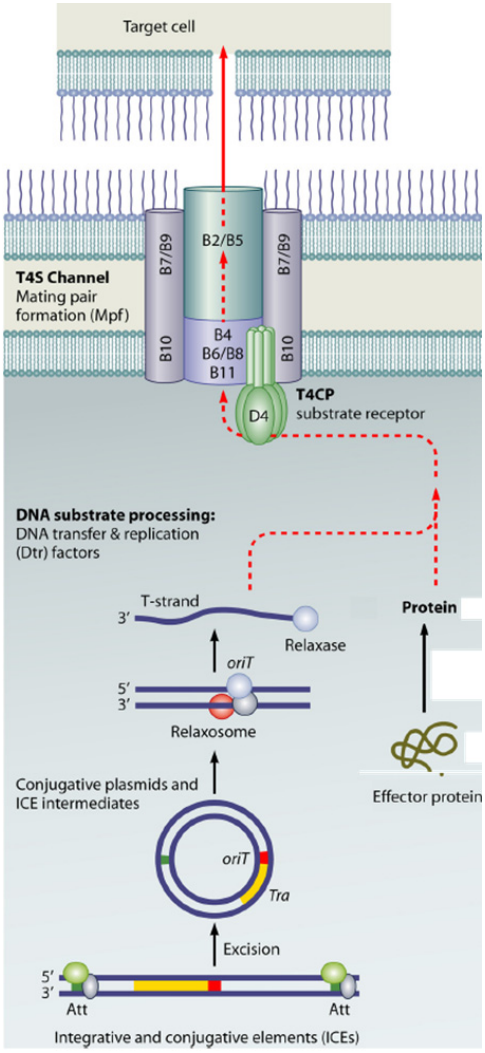

Figure 10 Mechanism of conjugative plasmid transfer through a type IV secretion system (T4SS). The steps include: 1. Processing of the plasmid through the MOB complex (*oriT*, relaxase), 2. binding of proteins to create the transferable relaxosome, 3. Nicking of the relaxosome to the type IV coupling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

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).

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). Conjugal 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).

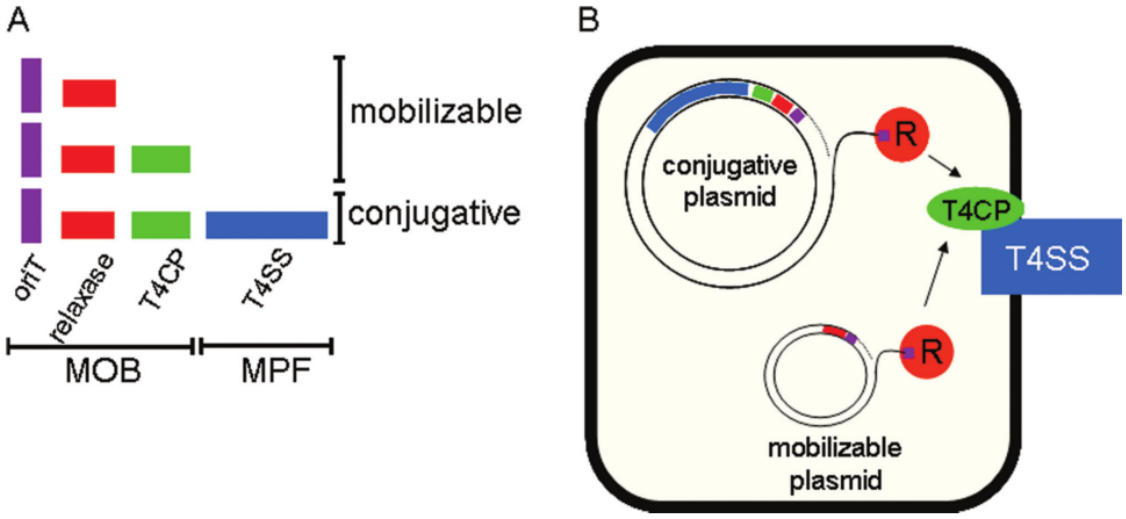

Figure 11 Classification of plasmids into mobilizable and conjugative ones based on the mobilization genes encoded (modified from (Smillie *et al.*, 2010)).

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).

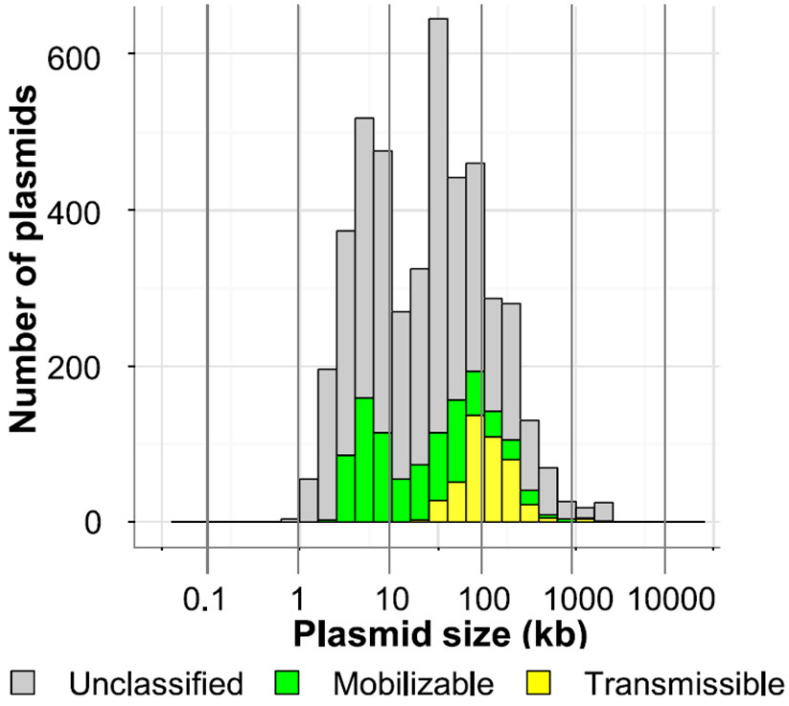

Figure 12 Histogram of plasmid size distribution and their classification into self-transmissible and mobilizable. Reprinted from (Shintani *et al.*, 2015).

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).

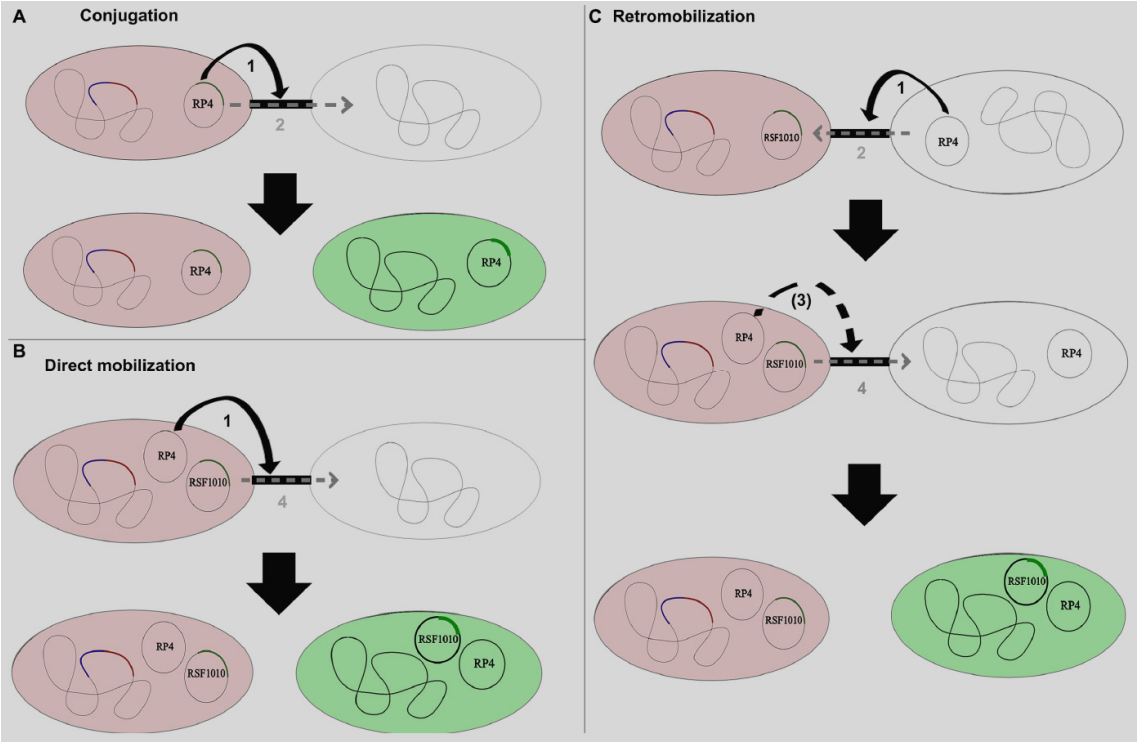

Figure 13 Conjugation, direct mobilization and retromobilization of a conjugative/mobilizable plasmid pair. Panel A: Conjugal transfer of a self-transmissible plasmid. Step 1 illustrates the establishment of a pilus between donor and recipient as part of the type IV secretion system (T4SS) encoded by 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 (Paper III)

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 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	Incompati-bility	Resistance (µg/mL)	Host range	Reference
RP4	Conjugal	60 kb	IncP-1α	*Amp*^{R}, *Km*^{R}, *Tet*^{R} (100, 50, 20)	Broad	(Barth & Grinter, 1977)
RSF1010	Mobilizable	8.7 kb	IncQ-1α	*Strep*^{R} (100)	Broad	(Honda *et al.*, 1991)

The community's permissiveness towards conjugative plasmid RP4 ($1.16x10^{-4}$ 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 $1.16x10^{-5}$ 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 $8.34x10^{-4}$ 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% ($8.34x10^{-4}$ T/R for *P. putida* (RSF1010::*gfp*) to *P. putida* (RP4) divided by $1.76x10^{-3}$ (T/R) for *P. putida* (RP4::*gfp*) to *P. putida*).

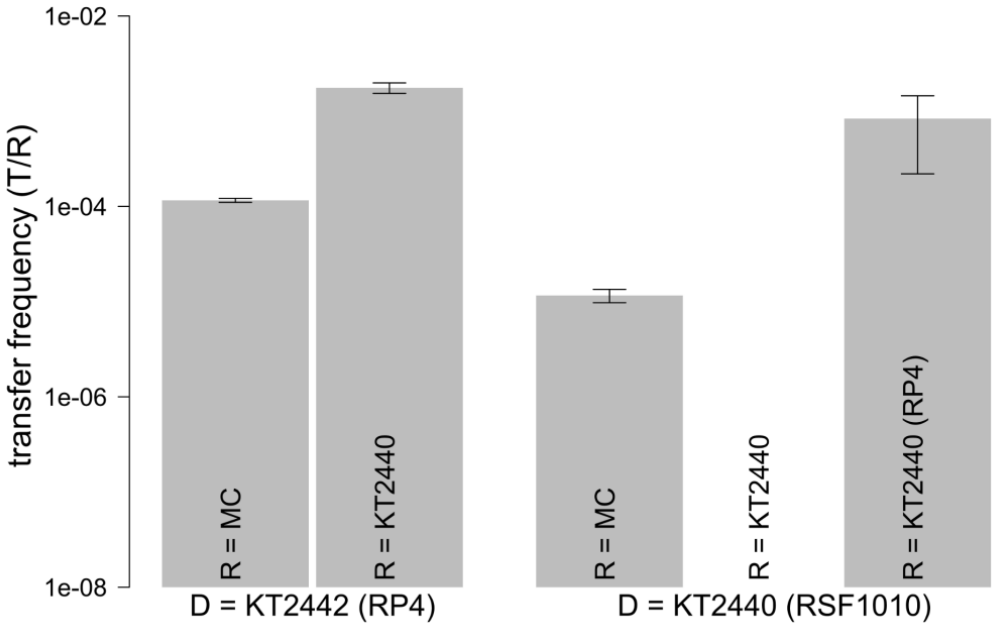

Figure 14 Transfer frequencies of RSF1010 and RP4. Transfer frequencies were defined as transconjugant microcolonies per initial recipient cells in solid surface filter matings with a mixed community (MC) or defined *P.putida* strains as recipients. Values are shown as mean of triplicates with stand 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 bar (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 conjugative 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 mechanism 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 temporary 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 of 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).

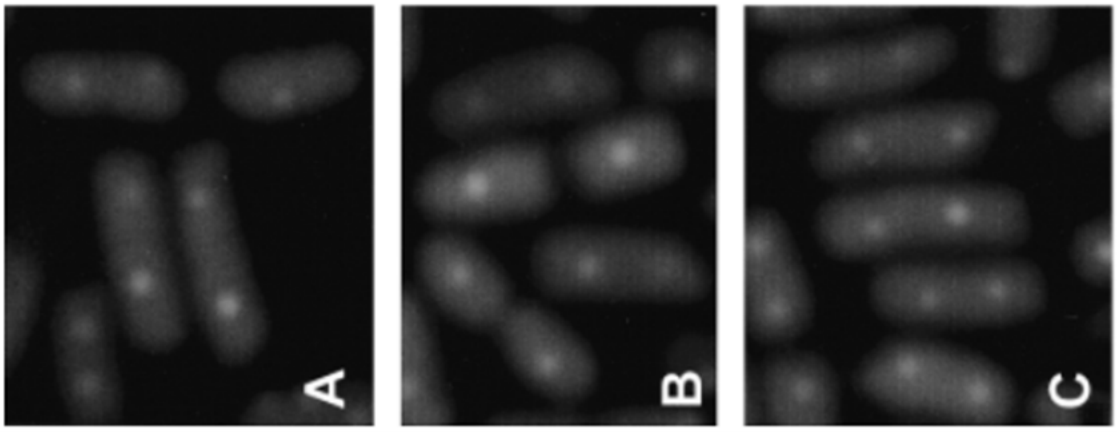

Figure 15: Partitioning cycle of a conjugative plasmid: Active partitioning of a conjugative plasmid at the quarter and three-quarter position of a cell (A). After segregation the plasmid is in the center of a cell, where it undergoes replication (B) before the active partitioning system leads it back 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. Rather 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 it 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 adaption 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 organisms. 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 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 wide realized transfer range and the narrower evolutionary range.

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 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.

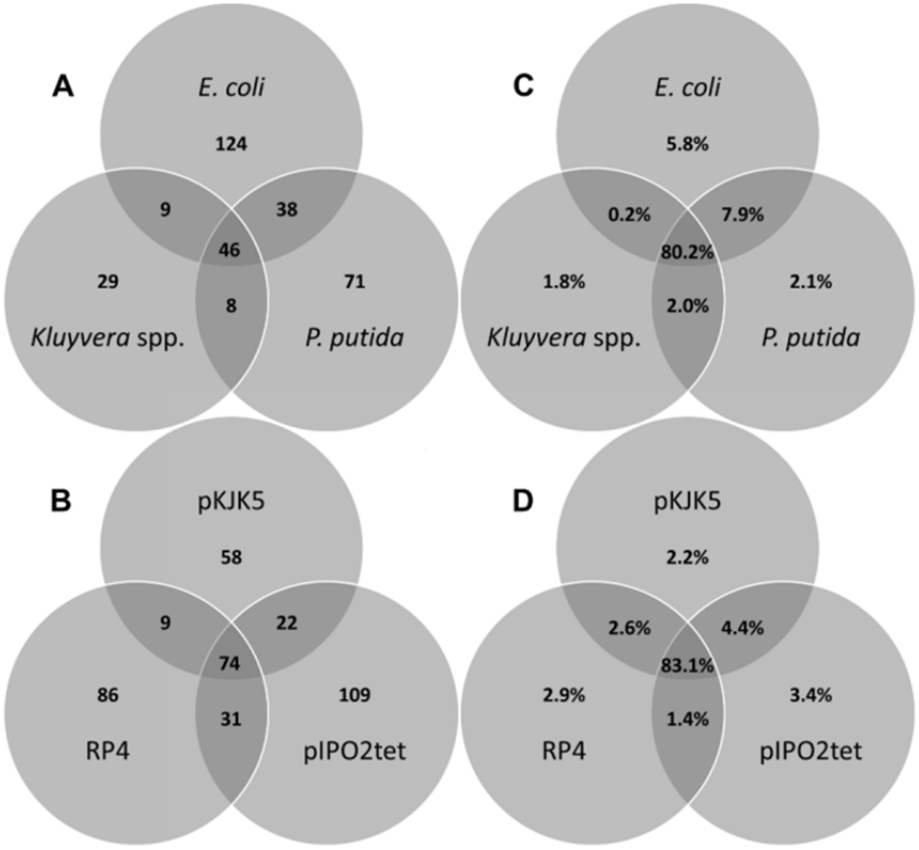

Figure 16 Venn diagram of transconjugal pools for plasmid pKJK5 transferred from three different donor strains (*E. coli*, *P. putida* & *Kluyvera* sp.) (A&B) and for three different plasmids (pKJK5, RP4, pIPO2tet) introduced through *P. putida* into the soil community. Venn diagrams are presented for 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)

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; Elass & 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).

Table 3 Inhibitory concentrations causing 20% and 50% bacterial growth inhibition (IC_{20} and IC_{50}, respectively) as extrapolated from [^{3}H]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.

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)

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 previously 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.

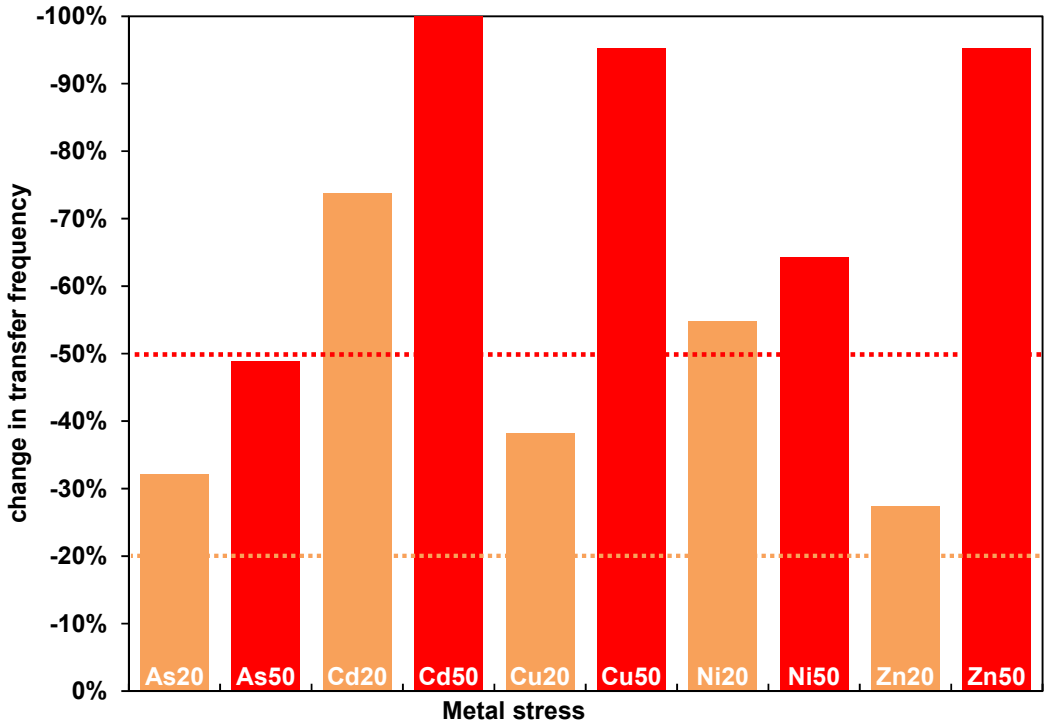

Figure 17 Plasmid transfer frequency reduced under stress conditions. Normalized plasmid transfer frequencies for each of the five tested metals at their respective IC_{20} (orange) and IC_{50} (red) and the non-stressed reference were calculated based on the analysis of 90-150 images each. Metal induced stress conditions were defined based on the ability of the different metals to inhibit [^{3}H]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 stochastics 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 ($T_{stress,obs}$) over its expected abundance in the transconjugal pool at the same metal stress ($T_{stress,exp}$). 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.

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

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).

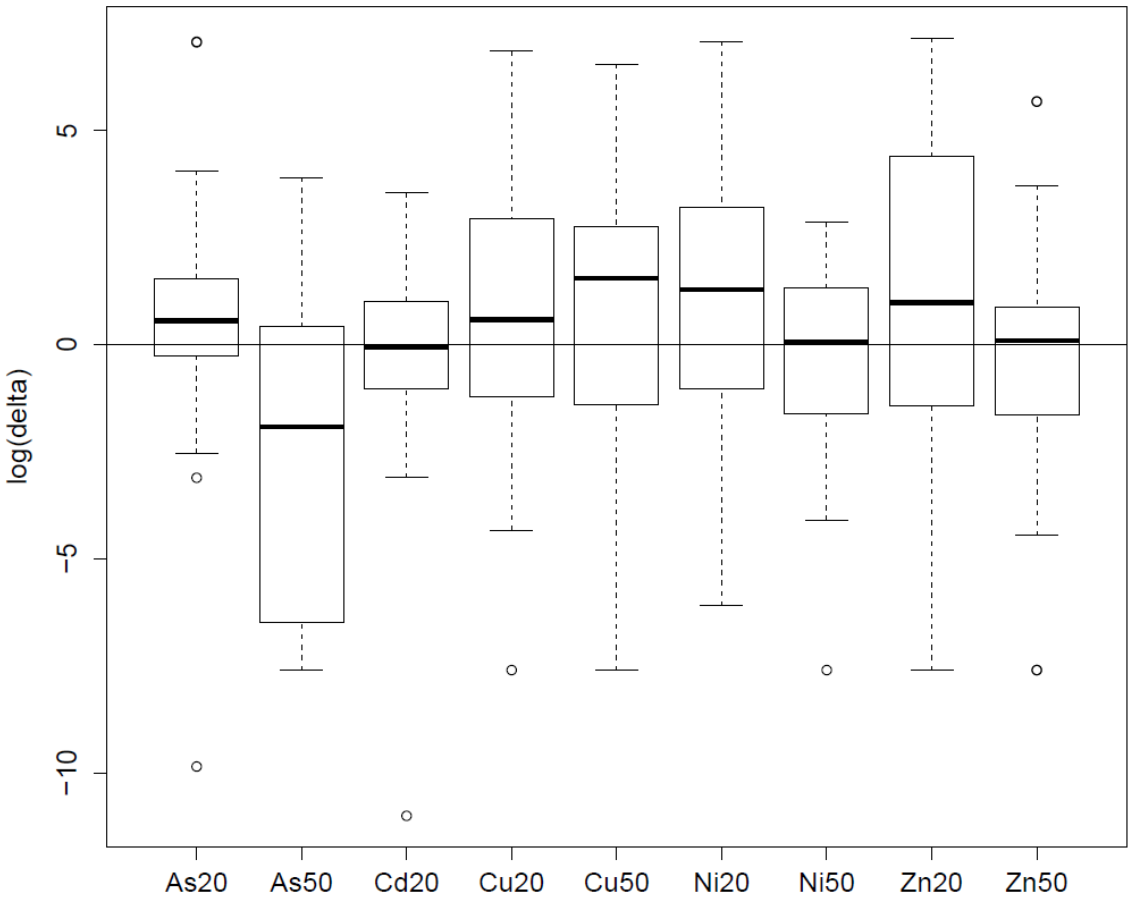

Figure 18 Boxplot diagram showing the distribution of δ values for the 39 most abundant OTUs in each transconjugal pool on the logarithmic scale. The line represents the complete absence of metal effect on permissiveness (all δ = 1).

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.

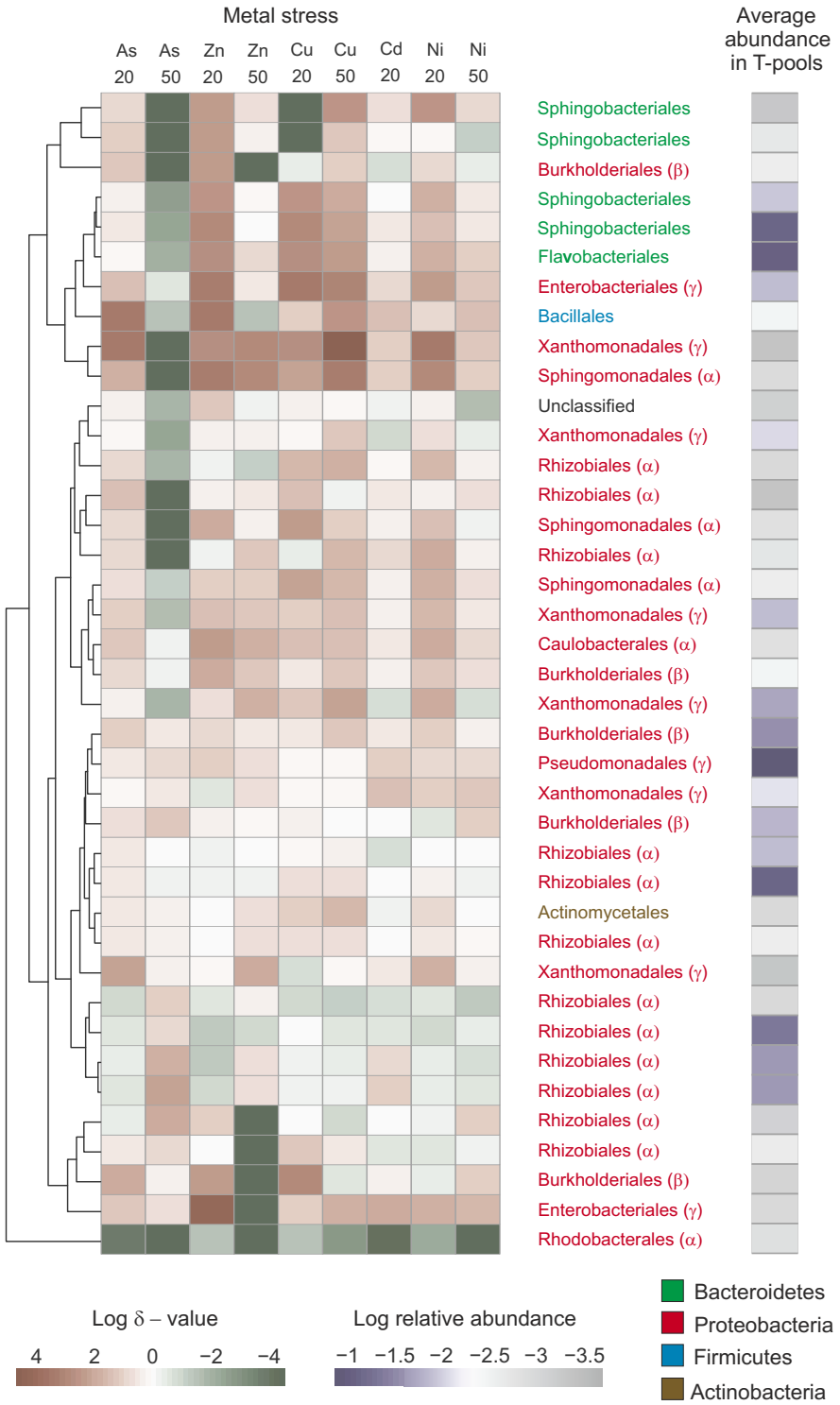

Figure 19 Heatmap showing the log scaled δ value of stress-imposed fold difference of an OTU's relative abundance due to stress. The 39 most abundant (>0.05%) OTUs, their relative abundance in log scale in violet as well as their phylogeny are shown (for Proteobacteria their class is shown in brackets) 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 when stressed. Most Proteobacteria, such as the Rhizobiales, do not respond to stress by modulating their permissiveness significantly. Rhodobacteriales, 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.

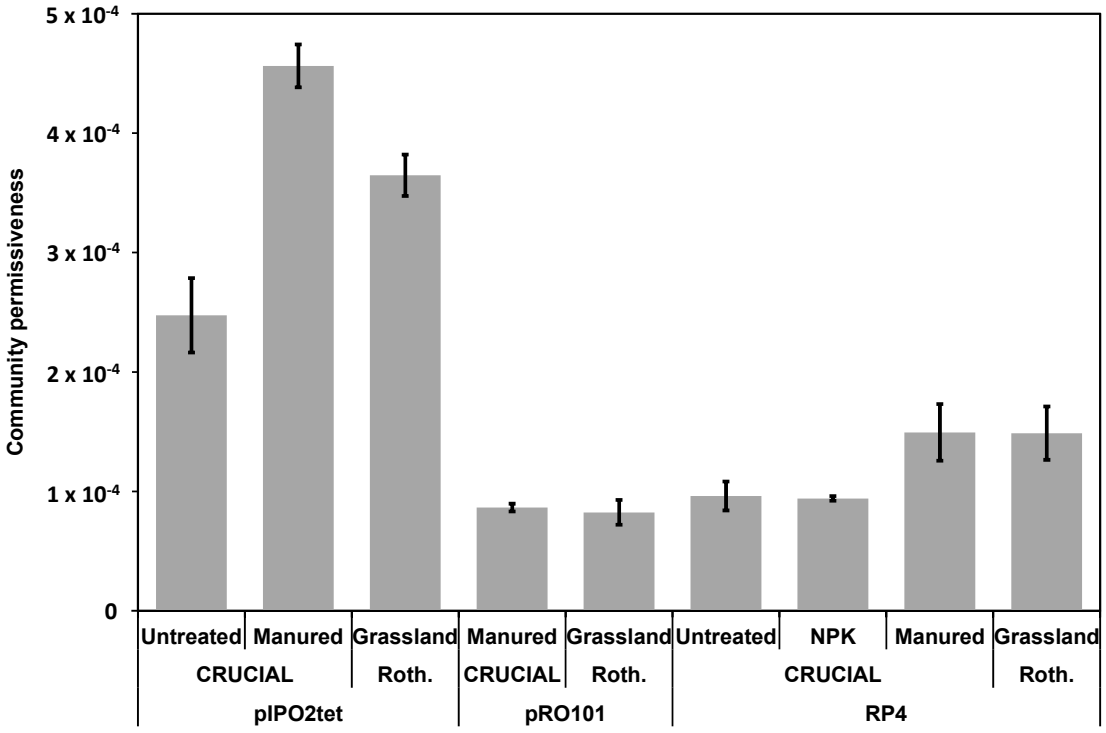

Figure 20 Transfer frequency of the introduced plasmid to the soil indigenous bacterial communities derived from the CRUCIAL and Rothamsted (Roth.) plots after 48 h of incubation in solid surface filter matings on soil extract medium. Values are displayed as the mean of triplicates with standard deviation.

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 intrastrain 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.*

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

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I

Protocol for evaluating the permissiveness of bacterial communities toward conjugal plasmids by quantification and isolation of transconjugants

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Protocol for Evaluating the Permissiveness of Bacterial Communities Toward Conjugal Plasmids by Quantification and Isolation of Transconjugants

Uli Klümper, Arnaud Dechesne, and Barth F. Smets

Abstract

The transfer of conjugal plasmids is the main bacterial process of horizontal gene transfer to potentially distantly related bacteria. These extrachromosomal, circular DNA molecules host genes that code for their own replication and transfer to other organisms. Because additional accessory genes may encode catabolic pathways, virulence factors, and antibiotic or metal resistances, it is of environmental, evolutionary, and medical relevance to track and monitor the fate of plasmids in mixed microbial community. When assessing the short-term and long-term implications of conjugal plasmid transfer, the ability of a plasmid to invade a mixed community is crucial. The main parameter that controls the possible extent of horizontal plasmid transfer in a bacterial community is the in situ community permissiveness towards the considered plasmid. Permissiveness describes the fraction of a microbial community able to receive an introduced plasmid at both quantitative and phylogenetic levels. In this chapter, we describe a protocol for simultaneous quantification of plasmid transfer frequency to and high-throughput isolation of transconjugants from a mixed bacterial community after introducing a *gfp*-tagged plasmid in a *mCherry* red fluorescently tagged donor strain repressing *gfp* expression. We take advantage of fluorescent marker genes to microscopically detect plasmid transfer events and use subsequent high-throughput fluorescence-activated cell sorting (FACS) to isolate these transconjugants from the complex community.

Keywords: Community permissiveness, Conjugation, Fluorescence-activated cell sorting (FACS), High-throughput isolation, Horizontal gene transfer (HGT), Plasmid transfer, Transfer frequency

1 Introduction

Conjugal plasmid transfer is the main bacterial process when horizontally transferring genes to other organisms. Conjugal plasmids are extrachromosomal, circular DNA molecules and can potentially transfer across bacterial phyla and even domains of life [1, 2]. Horizontal plasmid transfer events between different, potentially distantly related species are mediated by their intrinsic genes coding for autonomous replication and transfer and recognized as a common and major evolutionary process [3]. Additionally carried

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accessory genes encoding catabolic pathways, virulence factors, and antibiotic or metal resistances impose conjugal plasmids great environmental, evolutionary, and medical relevance as demonstrated in the historically heavily interconnected resistomes of human pathogens and soil bacterial communities [4]. Apart from long-term effects, also rapid adaptation within mixed bacterial communities to changing environmental conditions relies mainly on conjugal plasmid transfer, as an indispensable mechanism [5–7].

When studying the short-term and long-term implications of conjugal plasmid transfer with regard to environmental and medical aspects, the ability of a plasmid to newly invade a mixed community is crucial. Its host range, defined through the plasmids own genetic determinants [8], and the community's intrinsic ability to take up this plasmid [9] which might differ from strain to strain [10–12] are the key parameters controlling its ecology and fate. These two determinants are combined in the in situ community permissiveness, defined as the fraction of a microbial community able to receive a given plasmid [13].

Former common methods involving selection and cultivation steps for quantifying, isolating, and identifying transconjugants have nowadays been replaced through the introduction of fluorescent reporter genes for tracking the fate of plasmids [5] (*see* Fig. 1). Despite reporting a phylogenetically diverse fraction of a bacterial community being permissive toward broad-host-range plasmid receipt, studies have not been able to isolate more than a few hundred transconjugants [9, 14–16].

In this chapter, we describe the protocol that enabled for the first time simultaneous quantification of plasmid transfer frequency to and high-throughput isolation of transconjugants from a mixed bacterial community [17] which includes:

1. Setup of solid surface filter matings with plasmids tagged green fluorescently through Entranceposon [Km^R, PA10403-*gfpmut3*] (*see* **Note 1**). This one is carrying a *lacI*^{q}-repressible promoter upstream of the *gfpmut3* gene, encoding for the green fluorescent protein (*gfp*). Plasmids are introduced through donor strains tagged through a *lacI*^{q}-*pLpp-mCherry-*Km^R Tn*7* gene cassette (*see* **Note 2**) encoding for *mCherry*-induced red fluorescence and hosting a *lacI*^{q} repressor gene repressing *gfp* production in the donor strain into a mixed soil bacterial community. If the plasmid gets transferred to soil bacteria, they will subsequently express the encoded *gfp* and build green fluorescent microcolonies (*see* Fig. 1).
2. Acquisition and evaluation of fluorescence microscopic images to quantify the in situ community permissiveness toward the plasmid by calculating the transfer frequency. Rather than counting the number of transconjugant, we count the number of green fluorescent microcolonies and can thereby distinguish

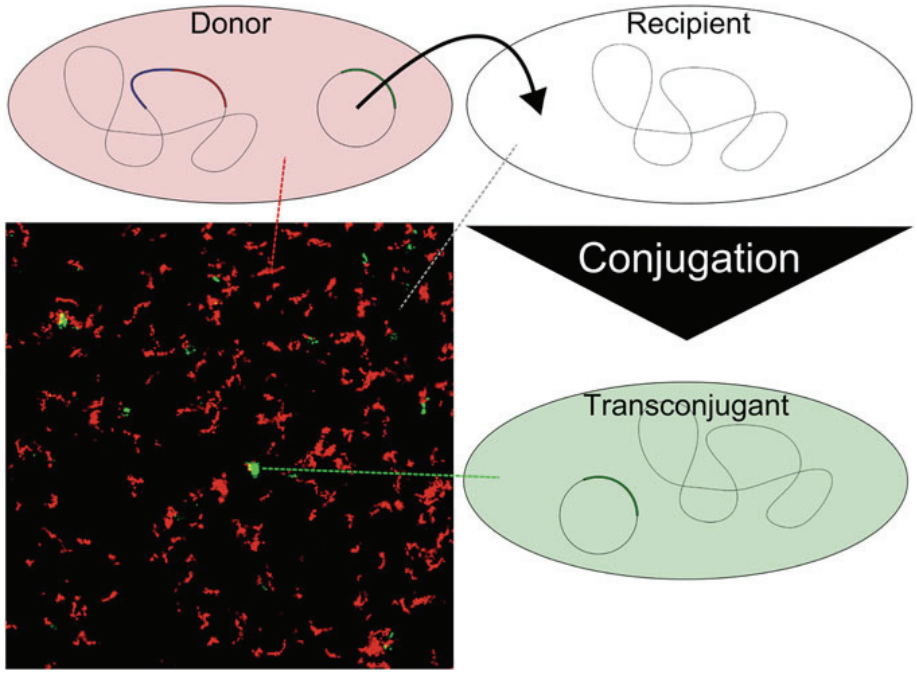

Fig. 1 Plasmids tagged with the green fluorescent protein gene (*gfp*) (*green region* on the plasmid) are introduced through a chromosomally tagged red fluorescent (*mCherry* gene as *red region* on chromosome) donor strain. These donor strains also encode a gene (shown on the chromosome as *blue region*) repressing the *gfp* production inside the donor strain. Therefore, the donor strain is red fluorescent and not green fluorescent. The donors are mated with non-fluorescent soil bacteria. These are responsible for the *black background* on the image. If the plasmid is transferred to soil bacterial strains through conjugation, they will subsequently express the encoded *gfp* as they will lack the *gfp* repressor and develop green fluorescent microcolonies, which are observable by microscopy

horizontal and vertical transfer of the plasmid and evaluate the number of transfer events that took place.

3. Isolation of transconjugants using high-throughput fluorescence-activated cell sorting, opening possibilities for complete sequencing of transconjugal pools through next-generation sequencing techniques, working with single transconjugal cells in single cell sorting or cultivation of sorted transconjugants.

We describe our method with the example of transfer of the broad-host-range plasmid RP4 from *Pseudomonas putida* to a complex soil community as recipient, but it can easily be adapted to other communities, plasmids, or donor strains.

2 Materials (*see* Note 3)

2.1 Production of Soil Extract Growth Medium

1. Fresh soil.
2. Mops buffer: 10 mM MOPS ($C_7H_{15}NO_4S$), adjusted to pH 7. MOPS was purchased through Sigma (http://www.sigmaaldrich.com).
3. Agar for solid growth media. Agar was purchased through Sigma (http://www.sigmaaldrich.com).
4. Nystatin (Sigma (http://www.sigmaaldrich.com)): 10 mg/mL in DMSO. Sterile filtered and stored in 1 mL aliquots at −20°C.
5. Petri dishes.

2.2 Donor Strain Preparation

1. Donor strain: *Pseudomonas putida* KT2440::*lacI*^{q}-*pLpp-mCherry*, *Km*^{R} (*see* **Note 1**) hosting plasmid RP4::*plac*::*gfp*, Tet^R (*see* **Note 2**).
2. Luria-Bertani (LB) growth medium: 10 g Bacto-Tryptone, 5 g yeast extract, 10 g NaCl per L water. LB was purchased through Sigma (http://www.sigmaaldrich.com).
3. Kanamycin (Sigma (http://www.sigmaaldrich.com)): 50 mg/mL in H_2O. Sterile filtered and stored in 1 mL aliquots at 4°C.
4. Tetracycline (Sigma (http://www.sigmaaldrich.com)): 25 mg/mL in H_2O. Sterile filtered and stored in 1 mL aliquots at −20°C.
5. Thoma counting chamber 0.1 mm depth.

2.3 Density Gradient Nycodenz Extraction of a Soil Bacterial Community

1. Fresh soil.
2. 2 mm hole size sieve.
3. Pyrophosphate buffer: 50 mM $Na_4O_7P_2$, 0.05% Tween 80; adjust pH to 8.0. Sterilize by autoclaving. The reagents were purchased through Sigma (http://www.sigmaaldrich.com).
4. Waring blender 8011ES Model HGB2WT53 (Waring Laboratory Science (http://www.waringlab.com/)).
5. Nycodenz solution: Add 800 mg Nycodenz® (Takeda Pharmaceuticals International GmbH (http://www.tpi.takeda.com/)) per 1 mL water. Dissolve Nycodenz® through sterilizing by autoclaving.
6. 0.9% NaCl solution. Sterilize by autoclaving. NaCl was purchased through Merck & Co., Inc. (http://www.merck.com).
7. 30 μm pore size filter (*see* **Note 4**).
8. Thoma counting chamber: 0.1 mm depth.

2.4 Solid Surface Filter Matings

1. Filtration device 1225 sampling manifold (Merck Millipore (http://www.merckmillipore.com/)).
2. White GF/C glass microfiber filters, circles 25 mm (Whatman (http://www.whatman.com), Cat No 1822025).
3. Black polycarbonate filters, 0.2 μm pore size circle 25 mm (Maine Manufacturing, LLC (http://www.mainmanufacturingproducts.com/), Item Number 1215609).
4. Vacuum pump.
5. 0.9% NaCl solution. Sterilize by autoclaving. NaCl was purchased through Merck & Co., Inc. (http://www.merck.com).
6. Soil extract plates (*see* Subheading 3.1).
7. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Bio-Lab LTD. (http://www.biolab-chemicals.com)): 1 M in sterile double-distilled (MilliQ) water (SDDW) stored in 1 mL aliquots at −20°C.

2.5 Fluorescence Microscopic Observation of Transconjugants

1. Leica (www.leica-microsystems.com/) MZ16 FA fluorescence stereomicroscope (for settings, *see* Table 1).
2. Image-Pro Plus version 7.1 software (Media Cybernetics (http://www.mediacy.com/)).

2.6 Image Analysis and Calculation of Transfer Frequencies

1. Image-Pro Plus version 7.1 software (Media Cybernetics (http://www.mediacy.com/)).

Table 1
Stereomicroscope settings for quantification of transfer events

Objective	10× plan apochromatic
Magnification	40.0
gfp excitation wavelength	480/20 nm
gfp emission wavelength	525/40 nm
Red excitation wavelength	565/25 nm
Red emission detector	620/60 nm
Camera	Leica DFC300
Software	Image-Pro Plus V7.1
Image type	Monochrome
Image bit depth	8 bit
File format	.tif

Table 2
Flow cytometer settings for fluorescence-activated cell sorting

Forward scatter (FSC)	505 V
Side scatter (SSC)	308 V
Green excitation wavelength	488 nm/20 mW
Green fluorescent detector (BP filter 530/30 nm)	508 V
Red excitation wavelength	561 nm/50 mW
Red fluorescence detector (BP filter 610/20 nm)	500 V
Nozzle size	70 μm
Sheath fluid pressure	70 psi
Software	BD FACSDivaTM v6.1.3

2.7 Flow Cytometric Isolation of Transconjugants

1. Pyrophosphate buffer (*see* Subheading 2.3).
2. 30 μm pore size filter.
3. Flow cytometer FACSAria IIIu (Becton Dickinson Biosciences (https://www.bdbiosciences.com/)) (for settings, *see* Table 2).
4. 30% glycerol solution. Sterilize by autoclaving. Glycerol was purchased through Merck & Co., Inc. (http://www.merck.com).

3 Methods

The protocol below describes how to quantify transfer events and isolate transconjugants from a soil bacterial community which received the conjugal plasmid RP4 from an exogenously added donor strain. The main advantage of our method is that it allows isolating large numbers of transconjugants at high throughput through fluorescence-activated cell sorting (FACS), while microscopic quantification of conjugation frequencies is based on actual transfer events and not biased by growth of transconjugants.

3.1 Production of Soil Extract Growth Medium

A solid agar-based growth medium that represents soil nutrient conditions to support soil bacterial growth while prohibiting growth of fungi through nystatin is used:

1. Mix 500 g soil and 500 mL H_2O. Autoclave to sterilize.
2. Shake for 24 h at 300 rpm.
3. Let settle for 5 h.
4. Add 100 mL of the supernatant to 900 mL H2O and 15 g agar. Autoclave to sterilize (*see* **Note 5**).

5. Cool down to 50°C; add 5 mL nystatin solution.
6. Pour solid medium plates in to Petri dishes.

3.2 Donor Strain Preparation

1. Grow the donor strain in 50 mL LB medium supplemented with 50 μg/mL kanamycin and 15 μg/mL tetracycline overnight at 30°C.
2. Spin down the cells for 10 min at 10,000×*g* at 4°C and discard the supernatant.
3. Resuspend the cell pellet in sterile 0.9% NaCl solution.
4. Filter through 30 μm pore size filter (*see* **Note 4**).
5. Count cell density using the Thoma counting chamber under the microscope and adjust cell density to 3.38×10^7cells/mL (*see* **Note 6**) by diluting with NaCl solution.
6. Store on ice for an hour before use to give the slower growing soil microbial community a slight growth advantage on the filters.

3.3 Density Gradient Nycodenz Extraction of a Soil Bacterial Community

The extraction of the soil bacterial community is based on the density difference of bacteria and the solid soil particles they are residing on. Soil particles are usually denser than the Nycodenz solution and are therefore separated from the bacterial fraction that forms a layer on top of the Nycodenz fraction:

1. Sieve 25 g of fresh soil through 2 mm sieve.
2. Transfer sieved soil to Waring blender and add 100 mL pyrophosphate buffer.
3. Blend at maximum speed for 90 s (*see* **Note 7**).
4. Add 700 μL of Nycodenz solution to fifty 2 mL Eppendorf tubes.
5. Carefully add 1.2 mL of blended soil solution on top of the Nycodenz phase (*see* **Note 8**).
6. Centrifuge the tubes at 10,000×*g* at 4°C for 25 min (*see* **Note 9**).
7. Add 15 mL of pyrophosphate buffer to two 50 mL centrifuge tubes.
8. Transfer the phase on top of the Nycodenz layer containing the bacteria (Fig. 2) from 25 tubes each to the pyrophosphate buffer tubes and vortex for 30 s.
9. Spin down cells for 10 min at 10,000×*g* at 4°C and discard supernatant.
10. Resuspend cell pellet in 0.9% NaCl solution.
11. Filter through 30 μm pore size filter (*see* **Note 4**).
12. Count cell density using the Thoma counting chamber under the microscope (*see* **Note 10**) and adjust cell density to 3.38×10^7cells/mL (*see* **Note 7**) by diluting with NaCl solution.

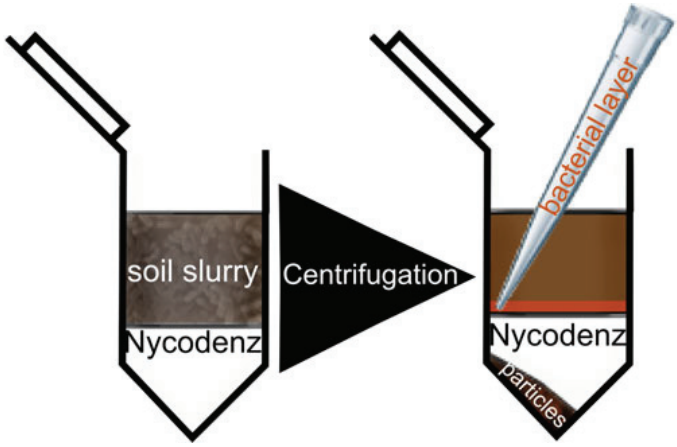

Fig. 2 Density-based Nycodenz extraction of a soil bacterial community. Soil slurry is carefully transferred on top of a dense Nycodenz layer. After centrifugation, the dense soil particles are on the bottom of the tube. The bacteria are slightly denser than water and are at the interface of the two liquid phases from where they can be extracted by pipetting

3.4 Solid Surface Filter Matings

1. Add black polycarbonate filter on top of a glass fiber filter onto the filtration device.
2. Pre-wet the filter by adding 2 mL of NaCl solution on top.
3. Load filter with 1 mL of extracted soil recipient community solution and 1 mL of ice-cooled donor solution. Mix with the pipette.
4. Vacuum filter at max. 400 mbar.
5. Transfer filter onto soil extract agar plate (*see* **Note 11**).
6. Prepare at least five replicates per mating combination.
7. Prepare additionally three filters of (a) donor strain only, (b) extracted soil recipient community only, and (c) only donor strain induced by 0.1 mM IPTG (*see* **Note 12**).
8. Incubate at 25°C for 48 h (*see* **Note 13**).

3.5 Fluorescence Microscopic Observation of Transconjugants

1. Check controls (a) and (b) (*see* step 7 in Subheading 3.4) to make sure there is no visible green fluorescence (*see* **Note 14**).
2. Place the Petri dish with the filter side up under the stereomicroscope (*see* **Note 15**).
3. Focus using the red fluorescent channel and capture an image. The image size will correspond to approx. 0.6 mm^2 per image.
4. Switch to the green fluorescent channel and capture an image.
5. Randomly switch position on the filter avoiding the outer rim; refocus and capture 49 additional images for a total of 30 mm^2 or approximately 10% of the total filter area (*see* **Note 15**).

3.6 Image Analysis and Calculation of Transfer Frequencies

1. Open all 50 images of the green fluorescent channel of one filter in Image-Pro Plus version 7.1 software.
2. Increase contrast of the images, subtract background, eliminate the poorly illuminated corners and threshold, and count any green fluorescent object larger than 7 μm^2 (*see* **Note 16**) (*see* Fig. 3).
3. Calculate the resulting transfer frequency according to:

$$\frac{\text{Transconjugants per picture} * \text{filter area } (\mu m^2)}{\text{picture area } (\mu m^2) * \text{recipients introduced originally}}$$

3.7 Flow Cytometric Isolation of Transconjugants

1. Add 3 mL pyrophosphate buffer into 15 mL centrifuge tubes.
2. Put one incubated filter per mating combination (after 48 h) into separate tubes.
3. Vortex for 60 s at highest speed to detach the cells from the filter.
4. Filter the suspension through 30 μm pore size filter (*see* **Note 17**).
5. Test the number of counting events/s and dilute with pyrophosphate buffer to approximately 2,000 counting events s^{-1} before fluorescence-activated cell sorting to assure for optimal sorting (*see* **Note 18**).
6. Set a gate for bacterial size on a bivariate SSC-A vs FSC-A plot for events of bacterial size by including the donor strain and excluding all events caused by a sterile pyrophosphate buffer control (*see* Fig. 4a).
7. Set a second gate for green fluorescence on a bivariate FITC-A vs SSC-A plot covering all events of bacterial size that includes green events from the IPTG-induced donor strain and excludes all events caused by the donor strain and a soil bacterial community control (*see* Fig. 4b).
8. Set a third gate for non-red fluorescence on a bivariate PE-Texas Red-A vs SSC-A plot covering all green events by excluding all events caused by IPTG-induced donor strain (*see* Fig. 4c).
9. Prepare a sterile sorting vial with 0.5 mL of pyrophosphate buffer.
10. Sort transconjugants into the sorting vial by using the FACS to sort for all three gates at highest speed by sorting for gain (*see* **Note 19**).
11. Prepare a sterile sorting vial with 30% glycerol solution.

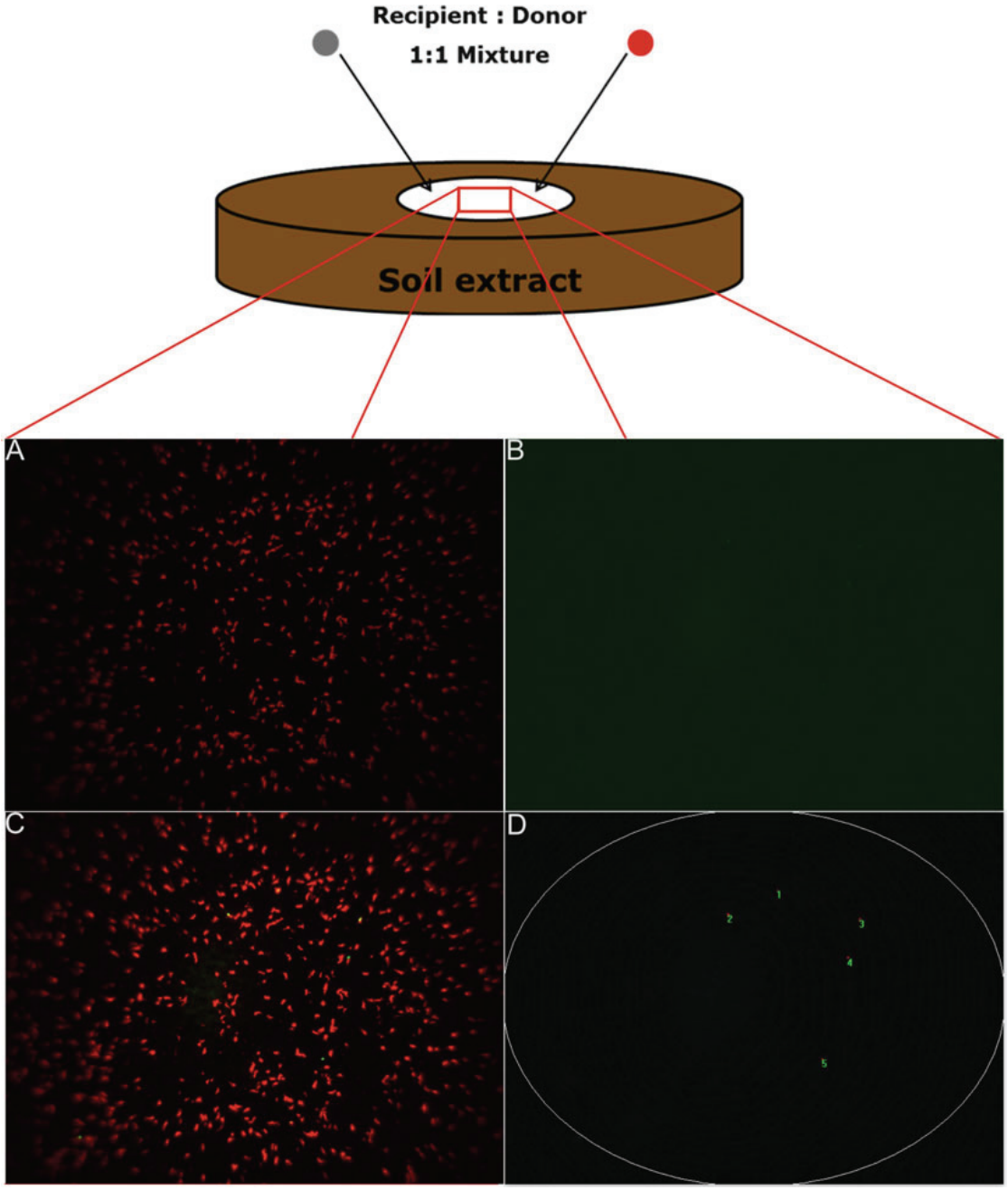

Fig. 3 Fluorescence-based stereomicroscopic images and image analysis of an example filter mating. *Image A* corresponds to the red fluorescent channel, displaying donor microcolonies. *Image B* shows the green fluorescent channel, corresponding to the transconjugal microcolonies that received the plasmid. *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 μm^2

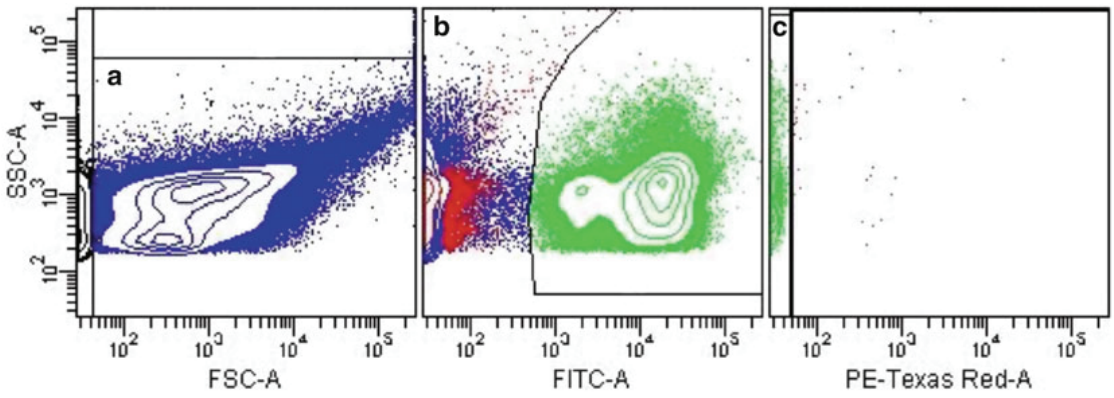

Fig. 4 Example gates set for flow cytometry for sorting transconjugants from the background microbial community. The field with the letter inside corresponds to the gate that sets the sorting. Gate A excludes all particles of smaller size than the smallest known bacteria (shown in *black*) originating either from sheath fluid or soil particles. Subsequent gate B sorts for green fluorescent events (bacteria and particles), excluding non-fluorescent soil bacteria (*blue*) and the donor strain repressing *gfp* expression (*red*). Gate C finally excludes all red fluorescent events (particles and bacteria) from the mixture, getting rid of autofluorescent soil particles or donor bacteria that were leaking in *gfp* repression

12. Use sorted transconjugants from the first vial for a second sort for all 3 gates at lowest speed possible and in purity mode into the glycerol vial.
13. Keep sorted transconjugants in 30% glycerol at 4°C. For prolonged storage (longer than 1–2 weeks), store at −80°C.

4 Notes

1. Entranceposon [Km^R, PA10403-*gfpmut3*], carrying a kanamycin resistance determinant and a LacIq-repressible promoter upstream the *gfpmut3* gene, encoding the green fluorescent protein (*gfp*), was derived from pEntranceposon [Km^R] (Finnzymes, F-766) and inserted into the plasmid as described in an earlier protocol [18].
2. Donor strains were tagged using plasmid pGRG36-$lacI^q$-Km^R-*pLpp-mCherry* carrying transposase genes and the Tn7 $lacI^q$-Km^R-*pLpp-mCherry* region for specific integration of the $lacI^q$-Km^R-*pLpp-mCherry* gene cassette into the chromosomal attTn7 site [19].
3. In the list of Materials (*see* Subheading 2), we provide the names and vendors from which we currently purchase reagents and equipment. We do by no means endorse these particular vendors. We provide the URLs of the vendor sites which are now more commonly used than their physical addresses.

4. Filtering is only required if cells have to be analyzed by flow cytometry afterward. Filter matings and quantification do not need filtered bacteria.
5. When using high-nutrient-content soils, a higher dilution of soil extract medium might be needed, to avoid excessive growth of the bacteria on the filters, hindering proper microscopic evaluation of transfer events.
6. Donor cells and recipient cells are used in a 1:1 ratio. The total filter area is 2.7×10^8 μm^2. The final recipient bacterial concentration is chosen so that assuming an average bacterial size of 1 μm^3 every bacterium will be in contact with the bacterium next to it after four doublings. A different filter area might therefore need a different final cell concentration.
7. Stop the blending process and cool the bowl on ice if the blender heats too much.
8. Achieving the density gradient becomes a lot easier if the soil suspension is added while tubes are at a 45° angle. It may be necessary to cut off the tip of the pipette tip to avoid clogging by the soil slurry. If the Nycodenz layer has been completely displaced after a non-careful addition of soil suspension, the tube should not be used.
9. The whole procedure works best in a swing-out rotor. If none is available, it is possible to use an angled rotor. The volume of Nycodenz might need to be increased for clear phase separation, so that the phase interface is still above the soil particle pellet.
10. For older soil samples potentially hosting a lot of dead cells, a life/dead staining is recommended before doing the counting. The cell density should then be adjusted according to the live cell count.
11. If not completely dry, dry filters on agar plate for 30 min before incubation to avoid cell motility and achieve clearly separated transconjugal microcolonies.
12. The additional filters work as controls both for the microscopy and for setting the gates in fluorescence-activated cell sorting. For setting the non-red fluorescent gate in FACS, it can be beneficial to use lower concentrations of IPTG since overexpression of *gfp* can reduce the expression of the red fluorescent *mCherry* gene.
13. An additional 2 days of storage at 4°C might increase the expression of *gfp*, if transconjugants show only a weak *gfp* signal.
14. If soil control (b) (*see* step 7 in Subheading 3.4) shows autofluorescent green particles, count those in the same manner as described for the samples and deduct the count from each of the sample counts.

15. For quality check, at least three images per filter should be captured with both channels. Green fluorescent transconjugal microcolonies should be detected touching directly red donor microcolonies. For all further images, the green channel is sufficient.
16. We use a custom-made macro in our software for all these operations. The code is available from the authors upon request, but different image analysis software or code can be used for the same operations.
17. Samples can be stored at 4°C for up to a week without loss of growth potential or community composition.
18. A density of at least 1,000 counting events/s is needed for time-efficient sorting. If density is not high enough for sorting, combine several filters in step 2.
19. To be able to perform an efficient second purification sort, at least 20,000 presumptive transconjugants should be sorted in the first sort to account for a sufficient concentration of cells for the second run.

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II

Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community

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ORIGINAL ARTICLE

Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community

Uli Klümper[1], Leise Riber[2], Arnaud Dechesne[1], Analia Sannazzarro[2], Lars H Hansen[2,3], Søren J Sørensen[2] and Barth F Smets[1]

[1]*Department of Environmental Engineering, Technical University of Denmark, Kgs. Lyngby, Denmark;* [2]*Section for Microbiology, Department of Biology, University of Copenhagen, Copenhagen, Denmark and* [3]*Department of Environmental Science, Aarhus University, Roskilde, Denmark*

Conjugal plasmids can provide microbes with full complements of new genes and constitute potent vehicles for horizontal gene transfer. Conjugal plasmid transfer is deemed responsible for the rapid spread of antibiotic resistance among microbes. While broad host range plasmids are known to transfer to diverse hosts in pure culture, the extent of their ability to transfer in the complex bacterial communities present in most habitats has not been comprehensively studied. Here, we isolated and characterized transconjugants with a degree of sensitivity not previously realized to investigate the transfer range of IncP- and IncPromA-type broad host range plasmids from three proteobacterial donors to a soil bacterial community. We identified transfer to many different recipients belonging to 11 different bacterial phyla. The prevalence of transconjugants belonging to diverse Gram-positive Firmicutes and Actinobacteria suggests that inter-Gram plasmid transfer of IncP-1 and IncPromA-type plasmids is a frequent phenomenon. While the plasmid receiving fractions of the community were both plasmid- and donor- dependent, we identified a core super-permissive fraction that could take up different plasmids from diverse donor strains. This fraction, comprising 80% of the identified transconjugants, thus has the potential to dominate IncP- and IncPromA-type plasmid transfer in soil. Our results demonstrate that these broad host range plasmids have a hitherto unrecognized potential to transfer readily to very diverse bacteria and can, therefore, directly connect large proportions of the soil bacterial gene pool. This finding reinforces the evolutionary and medical significances of these plasmids.
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Introduction

Conjugal plasmid transfer is a process by which bacteria horizontally transfer complete sets of genes to other, potentially distantly related, organisms. Conjugal plasmids frequently carry accessory genes, often encoding antibiotic or metal resistances, catabolic pathways or virulence factors. They are often implicated in the evolution of pathogenic bacteria and the rapid spread of antibiotic resistance, likely fostering the rise of multiple-resistant microbes in hospitals (Levy and Marshall, 2004) and animal husbandries (Zhu *et al.*, 2013). Although the relevance of plasmid transfer has become very acute in this age of massive antibiotic usage, plasmids have been exchanged for much longer, and many prokaryotic genomes present signs of intense past horizontal gene transfer (Ochman *et al.*, 2000).

Plasmids present different abilities to transfer into, and be maintained in, distantly related bacterial hosts and are loosely categorized as having a narrow or broad host range. The transfer of narrow host range plasmids is limited at one of the steps required for successful transfer, such as the formation of mating pairs, the avoidance of the recipient's restriction system or the correct expression of its replication and maintenance systems in the recipient (Thomas and Nielsen, 2005). Some broad host range plasmids can transfer across bacterial phyla and even across domains of life (Heinemann and Sprague, 1989; Waters, 2001), and several genetic determinants conferring broad host transfer capability have been identified (Jain and Srivastava, 2013).

The host range is thus a key parameter that controls the ecology and fate of plasmids. The evaluation of host range has traditionally been conducted using few individual pure strains as recipients, a situation that contrasts with the fact that most bacteria—and thus most plasmids—exist

Correspondence: BF Smets, Technical University of Denmark, Department of Environmental Engineering, Miljøvej 113, 2840 Kgs. Lyngby, Denmark.
E-mail bfsm@env.dtu.dk
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within complex communities of hundreds to thousands of species (Hong *et al.*, 2006; Brown Kav *et al.*, 2012). Among these diverse communities, all strains are obviously not equally permissive toward plasmid receipt, even for broad host range plasmids. This notion was supported when studying plasmid transfer to a range of strains isolated from marine water or wastewater treatment bioreactors (Sørensen, 1993; Inoue *et al.*, 2005). With the use of fluorescent reporter genes to track plasmids, which reduces the need for selection and cultivation steps to identify transconjugants, it has become apparent that, in complex communities, broad host range plasmids can indeed be received by bacteria distantly related to the donor, even in the absence of selective pressure for plasmid carriage (De Gelder *et al.*, 2005; Musovic *et al.*, 2006, 2014; Shintani *et al.*, 2014). However, these efforts, limited to inspection of a few hundred transconjugants at best, most likely underestimate the true diversity of transconjugal pools and do not accurately describe how plasmid permissiveness may vary across taxa in complex microbial communities.

Horizontal gene transfer between different species has been recognized as a common and major evolutionary process (Zhaxybayeva and Doolittle, 2011), most acutely demonstrated in the heavy interconnection between the resistome of soil dwelling bacteria and human pathogens (Forsberg *et al.*, 2012). The behavior of this environmental resistome may, thus, govern the spread of antibiotic resistance genes to pathogens (Finley *et al.*, 2013). Plasmids serve as main vessels of gene flow in microbial communities, linking distinct genetic pools (Norman *et al.*, 2009; Halary *et al.*, 2010). The *in situ* host range of plasmids may, then, well govern the taxonomic breadth across which gene flow occurs.

Here, taking advantage of high-throughput cell sorting and next-generation sequencing technologies, we map for the first time the intrinsic diversity of the bacterial recipients of broad host range plasmids in a microbial community extracted from soil, under conditions where cell-to-cell contacts are maximized. We analyzed matings initiated with combinations of three plasmid donors and three plasmids to identify how permissiveness toward broad host range plasmids is distributed across taxa among the recipient community.

Materials and methods

Donor strain construction

Soil bacterial communities were challenged with various plasmid–donor combinations through solid surface filter matings. The plasmids were marked with a genetic tag encoding conditionally expressible green fluorescent proteins (GFPs). The used entranceposon (Bahl *et al.*, 2009) carries a *lacI*^{q} repressible promoter upstream the *gfpmut3* gene, encoding for the GFP. Plasmid donor strains were all chromosomally tagged with a gene cassette encoding constitutive red fluorescence and constitutive *lacI*^{q} production. As a result, there is no *gfp* expression in the donor strains, but upon plasmid transfer to a soil bacterium, *gfp* expression is possible, resulting in green fluorescent cells or microcolonies, which can be detected and sorted by fluorescence microscopy or fluorescent activated cell sorting (FACS), respectively (Figure 1) (Sørensen *et al.*, 2005). *Pseudomonas putida* KT2440, *Escherichia coli* MG1655 and *Kluyvera* sp. served as donor strains, and were each electroporated with the plasmid pGRG36-*lacI*^{q}-*pLpp-mCherry-Km*^{R} carrying both the transposase genes and the Tn7 *lacI*^{q}-*pLpp-mCherry-Km*^{R} region for specific integration of the *lacI*^{q}-*pLpp-mCherry-Km*^{R} gene cassette into the chromosomal *att*Tn7 site. Colonies were selected for Km^R on Luria-Bertani (LB) agar plates at 30 °C. Colonies were restreaked on selective LB agar plates at 30 °C, incubated in liquid LB overnight culture without antibiotics at 30 °C and finally streaked on LB agar plates without selection at 37 °C for integration of the gene cassette and subsequent loss of the Tn7 helper plasmid. Colonies were tested for successful loss of helper plasmid and chromosomal integration of gene cassette by PCR (McKenzie and Craig, 2006). The same colonies were also phenotypically verified to be bright red fluorescent using stereomicroscopy.

Construction of gfpmut3-tagged plasmid pKJK5

Plasmids RP4 and pIPO2tet have been constructed earlier (Musovic *et al.*, 2010, 2014). The 54 kbp IncP-1

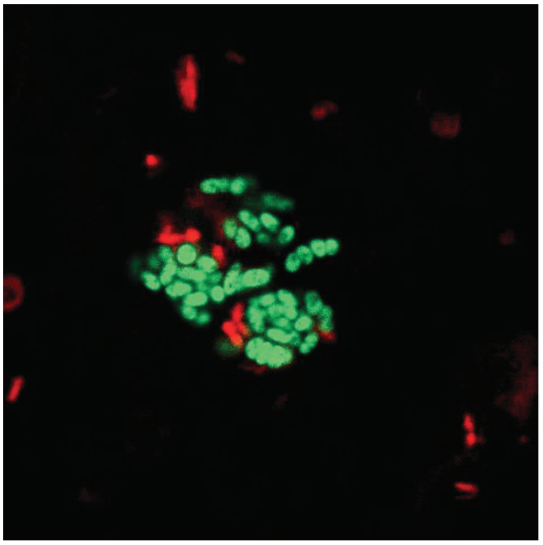

Figure 1 Typical transconjugal microcolonies for plasmid pKJK5::*gfp* introduced through *E. coli* MG1655::*lacI*^{q}-*pLpp-mCherry-Km*^{R}. Observation was carried out with a confocal laser scanning microscope (CLSM). Transconjugants are green fluorescent because of *gfp* expression, *gfp*-repressed donor cells are red fluorescent through chromosomal *mCherry* tagging, black background represents soil bacteria.

plasmid, pKJK5, originally isolated from a soil/manure environment, harbors a tetracycline and a trimethoprim resistance determinant, and a class 1 integron (Sengeløv *et al.*, 2001). The entranceposon [Km^R, PA10403-*gfpmut3*], carrying a kanamycin resistance determinant and a *lacI*^{q} repressible promoter upstream the *gfpmut3* gene, encoding *gfp*, was derived from pEntranceposon [Km^R] (Finnzymes, Thermo Scientific, Waltham, MA, USA; F-766) and randomly inserted into the plasmid pKJK5 using the artificial Mu transposon *in vitro* delivery system as described previously (Bahl *et al.*, 2009). Transformed *Escherichia coli* GeneHogs single colonies were selected for resistance toward trimethoprim and kanamycin and screened for sensitivity toward tetracycline to select for plasmid derivatives with an entranceposon insert location directed to an accessory element (the tetracycline resistance determinant), thereby excluding any potential impacts on conjugation transfer ability. The exact insert location of [Km^R, PA10403-*gfpmut3*] in the selected pKJK5 derivative of this study was determined by sequencing from the inserted fragment in one direction using primer Seq_Bw_Ent_gfp: 5′-GCCAGAACCGTTATGATGTC GG-3′. The insertion mapped to position 30.614 bp in the *tetA* gene (30.435–31.634 bp) of plasmid pKJK5 (accession no. AM261282). The selected *gfpmut3*-tagged pKJK5 plasmid was finally introduced into *E. coli* MG1655::*lacI*^{q}-*pLpp-mCherry-Km*^{R}, *P. putida* KT2440::*lacI*^{q}-*pLpp-mCherry-Km*^{R} and *Kluyvera* sp.::*lacI*^{q}-*pLpp-mCherry-Km*^{R} cells by transformation.

Soil sampling and community extraction

Soil samples were taken at the annually tilled CRUCIAL (Closing the Rural Urban Nutrient Cycle) agricultural field site (Taastrup, Denmark) from a plot subjected to no further agricultural treatment (Magid *et al.*, 2006). Soil samples were collected in late fall 2012. Samples were taken from three different plots of the treatment. Each plot was sampled for 1 kg of soil at five locations. The resulting soil volume was sieved and homogenized to obtain a representative sample. From a total of 30 g of the homogenized chosen soils, indigenous bacterial communities were isolated by Nycodenz extraction (Musovic *et al.*, 2010) and used as recipients in the mating assay. Donor strains were grown overnight in LB medium supplemented with the plasmid-specific antibiotics (Table 1) and were harvested by centrifugation.

Solid surface filter mating assay

The extracted recipient community was challenged with exogenous plasmids via solid surface filter matings (Musovic *et al.*, 2010) modified to an initial ratio of donor-to-recipient bacteria of 1:1 at a density of $\sim$30 000 bacteria mm^{-2} on the filter. As a growth medium, we used a 10% soil extract medium as described by Musovic *et al.* (2010) buffered at pH 7.2 with 5 mM 3-(N-morpholino)propanesulfonic acid and supplemented with 20 μg ml^{-1} nystatin to avoid fungal growth. Unlike in Musovic *et al.* (2010), we did not use additional nutrient additions, but only relied on soil-extracted nutrients to support activity during the mating incubations. Successful conjugation was checked after 48 h by epifluorescence stereomicroscopy and confocal laser scanning microscopy (Figure 1) (Musovic *et al.*, 2010).

Cell collection and triple-gated FACS of transconjugants

Cells from five filters per mating combination and replicate were harvested in 2 ml of 0.9% NaCl solution by vortexing for 3 min. Flow cytometric detection of cells was carried out using a FACSAria IIIu (Becton Dickinson Biosciences, San Jose, CA, USA). The following settings and voltages were used during analysis: forward scatter = 505 V, side scatter = 308 V and detectors for green (bandpass filter 530/30 nm) and red fluorescence (bandpass filter 610/20 nm) were set at 508 and 500 V, respectively. A 70 μm nozzle was used at a sheath fluid pressure of 70 psi. The BD FACSDiva software v.6.1.3 was used for both operating and analyzing results. Sorting was performed using a 488 nm (20 mW) laser connected to the green fluorescence detector at 515–545 nm and a 561 nm (50 mW) laser connected to the red fluorescence detector at 600–620 nm. Three gates were defined in bivariate plots to sort for transconjugants. On the side scatter-A vs forward scatter-A plot, a gate for only particles of bacterial size was used. On the FITC-A vs side scatter -A plot, a gate was set that covered all green fluorescent particles, while using an additional non-red gate on the PE-Texas Red-A vs side scatter-A plot excluded all small autofluorescent particles from soil or leaking donors (Figure 2) to sort out only transconjugants. All samples were diluted in 0.9% NaCl to $\sim$2000 counting events s^{-1} before FACS to assure for optimal sorting. Transconjugants that originally made up for $<$0.1% of the total community in the filter matings and were enriched to up to 82% in a first fast sorting step, before isolating over 10 000 transconjugants per sample in a second purification step, leading to 100% purity of green cells as observed by fluorescent counting in the flow cytometer. Plating of more than 200 isolated transconjugants on 10% soil extract medium (Musovic *et al.*, 2010) resulted in detection of green fluorescence in all colonies, additionally verifying purification of *gfp*-expressing transconjugants. Of the isolated transconjugants, 20 were subject to 16S rRNA gene sequences; the recovery of proteobacterial, sphingobacterial and actinobacterial phylotypes indicated diversity among transconjugants.

Table 1 Plasmids and donor strains used in this study

Donor	*Chromosomal marker*	*Reference*
Pseudomonas putida KT2440	*lacI^q-pLpp-mCherry-Km^R*	This study
Escherichia coli MG1655	*lacI^q-pLpp-mCherry-Km^R*	This study
Kluyvera sp.	*lacI^q-pLpp-mCherry-Km^R*	This study

Plasmid	*Inc group*	*Resistance conferred*	*Host range*	*Reference*
RP4::P*lac*::*gfp*	IncP-1α	Tet^R, Amp^R, Km^R	Broad	(Musovic *et al.*, 2010)
pIPO2tet::P*lac*::*gfp*	IncPromA	Tet^R	Broad	(Musovic *et al.*, 2014)
pKJK5::P*lac*::*gfp*	IncP-1ε	Tmp^R Tet^R	Broad	This study

Bacterial cell lysis, amplification and sequencing
Bacterial transconjugal cells from the second sort, initially collected in 5 ml sterile polystyrene round-bottom Falcon tubes (BD Biosciences, San Jose, CA, USA) with 0.5 ml of 0.9% NaCl solution, were transferred to 1.5 ml Eppendorf tubes and centrifuged at 10 000 *g* for 30 min to collect the cell pellets. The supernatant was carefully removed, the cell pellet suspended in 20 µl of Lyse and Go PCR Reagent (Thermo Scientific) and the lysis mixtures transferred to 0.2 ml amplification tubes. Cell lysis was subsequently performed in an Arktik Thermal Cycler (Thermo Scientific) using the program: one initial step at 57 °C for 30 s, a second step at 8 °C for 30 s, a third step at 65 °C for 90 s, a fourth step with heating to 97 °C for 3 min, a fifth step with cooling to 8 °C for 60 s, a sixth step with heating to 65 °C for 3 min followed by additional heating to 97 °C for 60 s and cooling to 65 °C for 60 s with a final end step at 80 °C. DNA-containing cell lysis products were immediately put on ice and used directly for subsequent PCR. Then, 5 µl of the cell lysis product from the previous step were used for sequencing library preparation. Tag-encoded 16S rRNA gene pyrosequencing was carried out after amplification of the V3 and V4 region (primers: 341F, 5′-CCTAYGGGRBGCASCAG-3 and 806R, 5′-GGACTACNNGGGTATCTAAT-3) using the PCR procedures and GS FLX Titanium chemistry as described previously (Hansen *et al.*, 2012).

Sequence analysis and tree construction
Sequence analysis was carried out using Mothur v.1.32.1 (Schloss *et al.*, 2009) and the 454 SOP (Schloss *et al.*, 2011) as accessed on 11 January 2013 on http://www.mothur.org/wiki/454_SOP. Sequences were classified based on the RDP (Ribosomal Database Project) classifier (Wang *et al.*, 2007). Phylogenetic trees were constructed using iToL (http://itol.embl.de/) (Letunic and Bork, 2007). All sequences have been submitted to the European Nucleotide Archive and can be accessed under study accession number PRJEB7443.

Results and discussion

High-throughput isolation and sequencing of transconjugants
We explored the ability of a bacterial community extracted from soil to engage in horizontal gene transfer and receive one of three *gfp*-tagged broad host range plasmids from three different red fluorescent-tagged donor strains in which plasmid-mediated *gfp* expression is repressed (Table 1). In soil, physical barriers limit contact between freshly introduced plasmid donors and potential recipients (Dechesne *et al.*, 2005); here we maximized cell-to-cell contact in a gene transfer assay (Musovic *et al.*, 2010) to study the intrinsic permissiveness of the recipient community. All three plasmids (RP4, pIPO2tet and pKJK5) were introduced to the soil community in matings with a *Pseudomonas putida* donor strain, whereas plasmid pKJK5 was also introduced via *E. coli* and *Kluyvera* sp. donors (Supplementary Table 1). After mating, the *gfp*-expressing transconjugant cells (Figure 1) were isolated from the mixed community by FACS. A novel triple-gated FACS approach based on size, green fluorescence and lack of red fluorescence allowed specific isolation of large numbers of transconjugant cells, in spite of their low relative abundance (<0.1%) in the mating mixture (Figure 2). At least 14 000 transconjugant cells were obtained for each mating replicate, corresponding to 28 000–116 500 transconjugants per donor–plasmid combination, depending on the number of replicate matings. The 11 pools of sorted transconjugants as well as the total soil recipient community were then subjected to deep amplicon sequencing of 16S rRNA genes, resulting in 29 894–50 398 sequences per sample after processing with the Mothur pipeline (Schloss *et al.*, 2009). This corresponds to more sequences than sorted transconjugants for most samples (Supplementary Table 1), providing an adequate picture of the observed plasmid transfer range.

Transconjugal pools are plasmid- and donor-specific
The phylogenetic structure of the transconjugal pools was compared after clustering the partial 16S

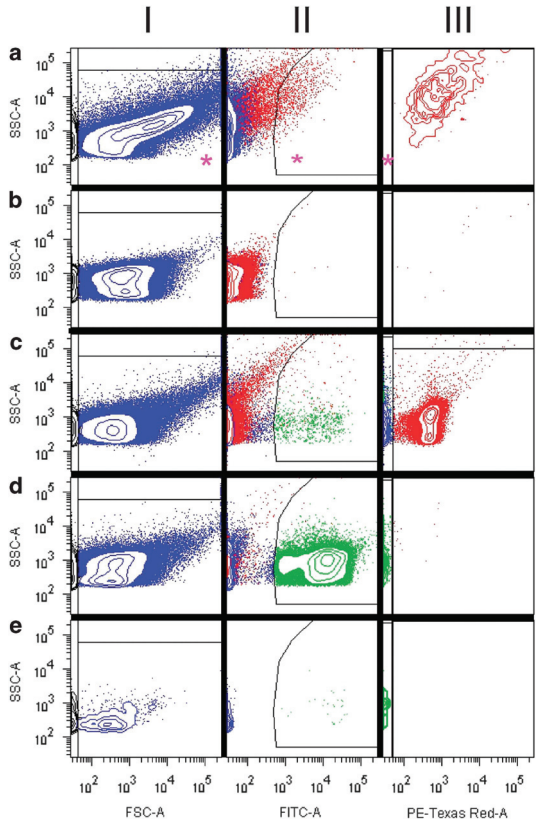

Figure 2 FACS sorting of transconjugal cells from a mating mixture initiated with soil bacteria and *E. coli* carrying pKJK5. The procedure consists in three successive gates (marked by pink stars in panel **a**). Gate I sorts for bacterial size based on forward and side scatter (SSC); Gate II sorts for green fluorescent cells; and Gate III selects only those green cells that display no red fluorescence. Line **a** shows the sorting of the initial soil bacterial recipient community in the absence of any donor strain and proves that the presence of green autofluorescent particles (**a**-II) does not yield false positives as they are excluded at the third gate, because of their red fluorescence (**a**-III). The sorting of a pure culture of the donor strain is shown in panel (**b**), where, again, no false-positive events are recorded at the final gate. Panel (**c**) represents the analysis of the mating mixture before sorting. Panel (**d**) shows the enrichment of transconjugants after the first fast enrichment sorting step to over 80% transconjugal cells, with minor contamination by donor or soil particles. Panel (**e**) shows how only pure transconjugants are obtained after the second purification sorting step.

rRNA gene sequences in operational taxonomic units (OTUs) at 97% similarity. The 11 transconjugal pools clustered clearly and significantly apart from the recipient community, as shown by principal coordinate analysis (Figure 3) and analysis of molecular variance (Excoffier *et al.*, 1992) ($P=0.028$). Mating plates contained soil extracts as nutrient sources and growth on filter did not significantly modify the soil community structure ($P=0.797$) based on UNIFRAC comparisons (Lozupone *et al.*, 2011), in spite of a diversity

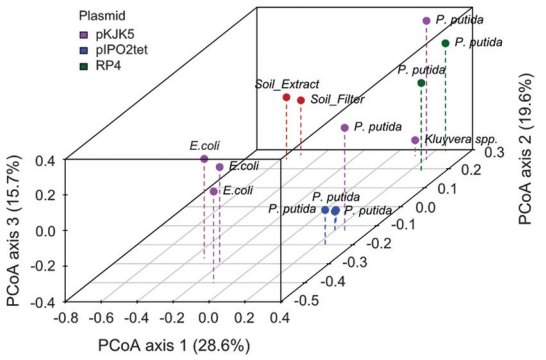

Figure 3 Principal coordinate analysis (PCoA) of individual transconjugal pools, as well as of the extracted soil community (Soil_Extract) and the reference soil community as grown on filters (Soil_Filter) based on the ThetaYC algorithm (Yue and Clayton, 2005). Each axis explains a certain fraction of dissimilarity according to the axis loading given within parentheses. The three different plasmids are represented by color. The three different donor strains are named next to the data points.

reduction by 72%. The transconjugal pools were clearly distinct from the recipient community, and also differed from each other based on plasmid or donor. Considering different plasmids in an identical donor strain (*P. putida*) and providing the same plasmid (pKJK5) in different donor strains revealed phylogenetically distinct transconjugal pools (analysis of molecular variance, $P<0.001$). Hence, plasmid acquisition is not a stochastic process, even for broad host range plasmids. Although replicates of the same donor–plasmid combinations differed based on weighted UNIFRAC comparisons ($P<0.05$), the average interreplicate dissimilarity ($W=0.36$) was clearly less than the dissimilarity between different plasmid–donor combinations ($W=0.49$) or between transconjugal pools and the soil community ($W=0.60$). Slight differences between the replicates can also be seen in the phylum level distribution of transconjugants (Supplementary Figure 1). This dissimilarity between replicates can most likely be decreased through sorting of higher numbers of transconjugants per replicate, as replicates from the same donor–plasmid combinations grouped significantly together in principal coordinate analysis ($P<0.01$) (Figure 3). Based on this principal coordinate analysis grouping and because the number of replicates per combination differed (Supplementary Table 1), replicates were pooled for subsequent phylogenetic analysis.

Transconjugal pools span most of the major bacterial phyla

More than 300 transconjugant OTUs were detected across all plasmid–donor combinations (Figures 4 and 5), a large expansion over the low number of distinct bacterial isolates identified previously from matings in complex environmental communities (De Gelder *et al.*, 2005; Musovic *et al.*, 2014, 2010;

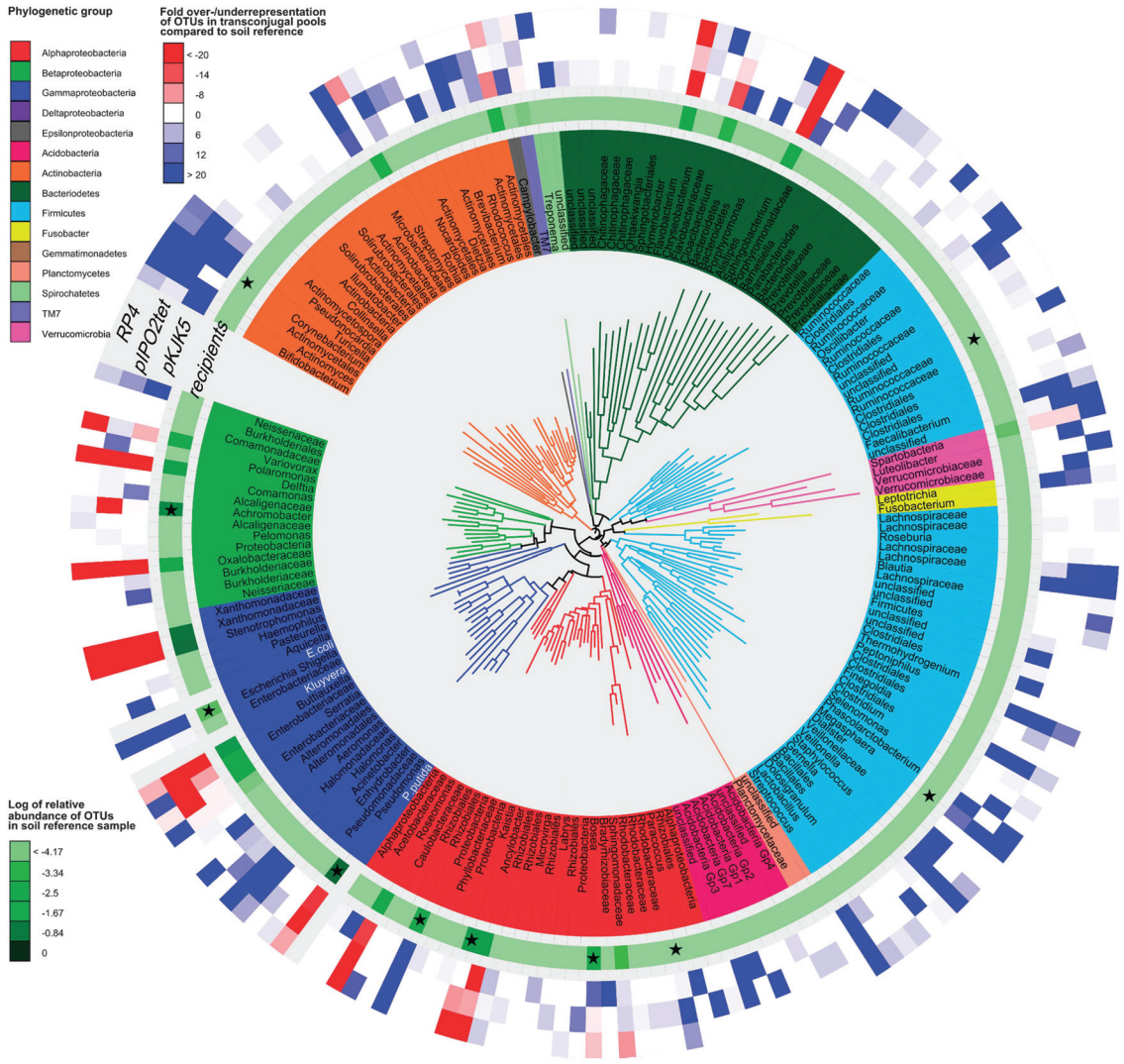

Figure 4 Phylogenetic tree showing all identified transconjugant OTUs for three different plasmids (pKJK5, RP4 and pIPO2tet) from the same donor (*P. putida*). The colors of the branches mark different phylogenetic groups. The three donor strains are shown in white letters in the trees. One green heatmap circle around the tree represents the log-transformed relative OTU abundance in the soil reference-recipient community. Three heatmap circles in blue and red display the *x*-fold over- and underrepresentation of the OTU in the respective transconjugal pool in comparison with the abundance in the reference soil sample. Stars mark the shared (present in all three 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 30 000 sequences per transconjugal pool.

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 represented. Unlike in previous studies (Musovic *et al*., 2010; Shintani *et al*., 2014), 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 10 additional phyla including Verrucomicrobia, Bacteroidetes and Actinobacteria, some of which are known as poorly cultivable (Joseph *et al*., 2003). The IncP transfer apparatus is known to build conjugative bridges between a huge variety of organisms (Grahn *et al*., 2000; Thomas and Nielsen, 2005). Shuttle vectors for gene transfer from Proteobacteria to distantly related recipients such as Cyanobacteria (Wolk *et al*., 1984) or Gram-positive bacteria and yeast (Heinemann and Sprague, 1989; Samuels *et al*., 2000) have, indeed, been built using the RP4 transfer system, an IncP-1α subgroup plasmid. Although the wide transfer

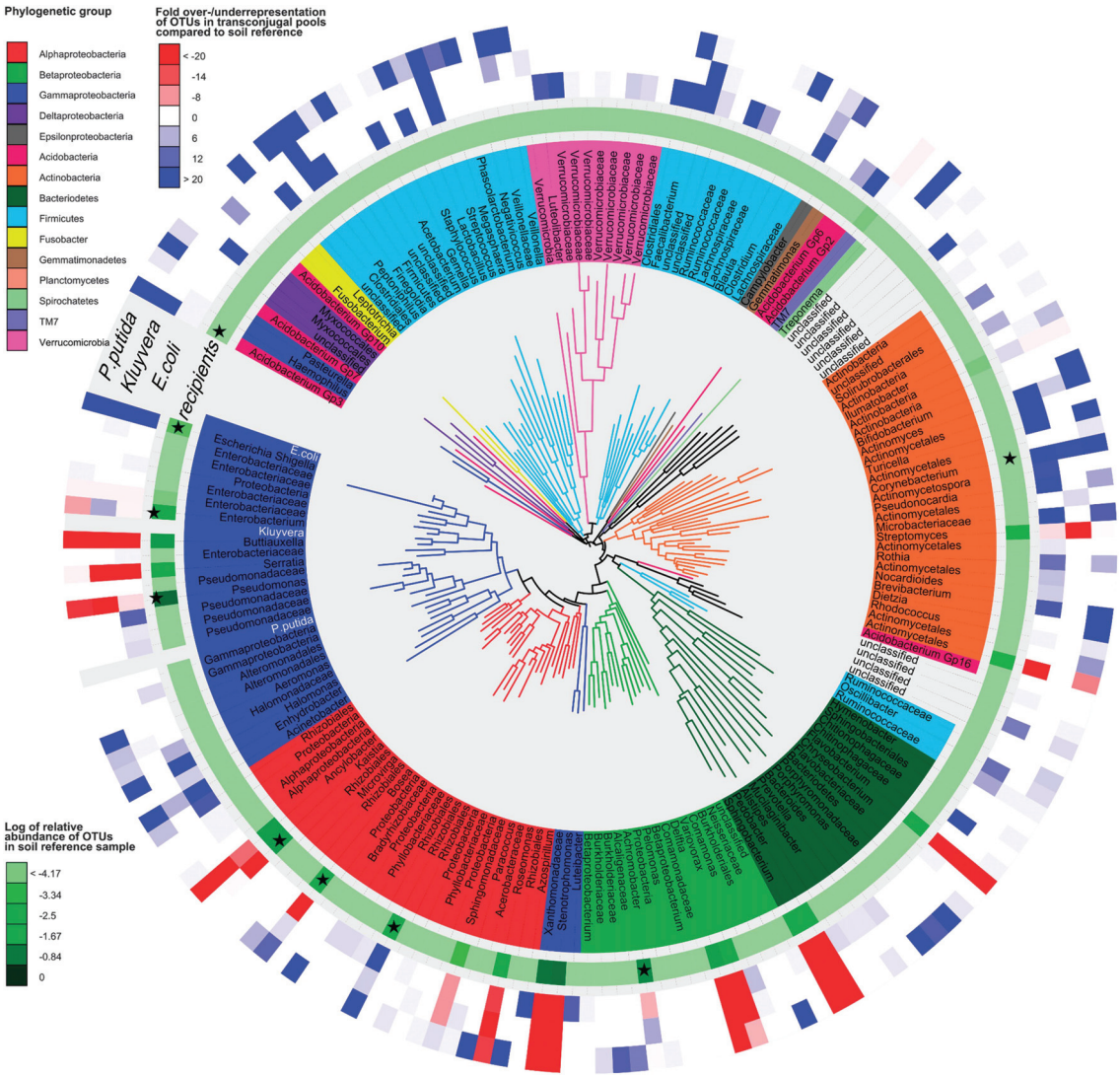

Figure 5 Phylogenetic tree showing all identified transconjugant OTUs for the same plasmid (pKJK5) introduced through three different donor strains (*P.putida*, *Kluyvera* sp. and *E. coli*). Colors of the branches mark different phylogenetic groups. The three donor strains are shown in white letters in the trees. One green heatmap circle around the tree represents the log-transformed relative OTU abundance in the soil reference recipient community. Three heatmap circles in blue and red display the *x*-fold over- and underrepresentation of the OTU in the respective transconjugal pool in comparison with the abundance in the reference soil sample. Stars mark the shared (present in all three 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 30 000 sequences per transconjugal pool.

potential of the RP4 conjugation system has therefore been known in artificial constructs under laboratory conditions, we are the first to prove that a large proportion of this transfer potential can be realized in nature. A similarly huge transfer potential is demonstrated for pKJK5, a plasmid closely related to RP4 belonging to the IncP-1ε subgroup and for pIPO2tet, an IncPromA-type phylogenetically more distant, cryptic plasmid that merely seems to provide plasmid mobilization capability to its host (Figure 4). We thus show that a variety of broad host range plasmids can effectively be transferred to, and encoded genes can be expressed in large proportions of the bacterial tree of life, much beyond the limited transfer range identified so far (Shintani *et al.*, 2014). The realized transfer range in the soil community under natural conditions might be even higher taking into account that Nycodenz

extraction might not be able to recover all bacterial phyla from the soil sample (Holmsgaard *et al.*, 2011). Of the total extractable soil microbial community, only the phyla Chloroflexi, Deinococcus-Thermus, Nitrospira and SR1 were not represented in the transconjugal pools in our experiments.

In particular, we identified transfer from the used Gram-negative donor strains to a wide variety of Gram-positive bacteria (Figures 4 and 5). Over 15 OTUs within the Actinobacteria phylum and more than 10 OTUs belonging to six different orders of Bacilli and Clostridia within the Firmicutes phylum were identified as transconjugants. Inter-Gram conjugal gene transfer has been shown with vectors consisting partly of the broad host range transfer machinery of RP4 recombined with the *sacB* gene from Gram-positive *Bacillus subtilis* (Schäfer *et al.*, 1994), but has only exceptionally been identified in natural habitats (Musovic *et al.*, 2006). Our observations suggest that it may be a more common process than previously considered.

Abundance in recipient community and phylogenetic distance to the donor do not explain the composition of transconjugal pools

In spite of the large diversity within the transconjugal pools, not all OTUs of the recipient community were represented in each pool and the relative abundance of OTUs in transconjugal pools was very heterogeneous. Our method cannot distinguish between original horizontal plasmid transfer events from subsequent vertical plasmid transfer through growth of transconjugants on the mating filter. Therefore, relative abundance in the transconjugal pools can be influenced by the relative growth rate of recipients. However, the fact that OTU abundance in the transconjugal pools is not explained by their abundance in the reference soil recipient community (Figures 4 and 5 and Supplementary Table 2) indicates that plasmid transfer occurs preferentially to some recipients and that transconjugal pools are not simply determined by the recipient's growth ability.

Next, we tested whether phylogenetic distance between donor and recipient, calculated based on the Sogin distance algorithm (Sogin *et al.*, 2006), influenced the abundance of individual OTUs among the transconjugal pools. We found no significant correlation between phylogenetic distance to the donor and recipient frequency in the transconjugal pools ($P = 0.09$–0.94) for any of the donor plasmid combinations (Supplementary Figure 2). For example, the most abundant OTUs in soil that do not appear in the transconjugal pools (Supplementary Table 2) are Gammaproteobacteria; they display more than 90% 16S rRNA gene sequence similarity to the donor strains, whereas other OTUs with $<70\%$ sequence similarity to donor cells, such as several members of the Flavobacterium phylum, did receive at least one of the plasmids. Transfer of an IncP-1 plasmid from *E. coli* to phylogenetically distant Flavobacteria was detected in soil microcosms (Pukall *et al.*, 1996), indicating that transfer to distant nodes of the phylogenetic tree is not only possible, but also realized in undisturbed soil environments. In pure culture, permissiveness toward broad host range plasmids of isolates that are indistinguishable by 16S rRNA gene analysis can differ by more than 100-fold (Heuer *et al.*, 2010). Here we confirm that inferring plasmid uptake and transfer frequency cannot be predicted based on the phylogenetic identity of an OTU.

However, we confirm the role of donors in defining the plasmid transfer host range (De Gelder *et al.*, 2005), and show that this effect is significant even for two donors belonging to the same family of Enterobacteriales (*E. coli* and *Kluyvera* sp.) and thus sharing a high genomic similarity. The reasons behind this are uncertain, but certain strains might have distinct abilities to achieve efficient cell-to-cell contact with a specific recipient, for example, through specific mating mediating pheromones (Hirt, 2002). Earlier studies have shown that plasmid exchange between two taxonomically different species can exceed intraspecies transfer frequencies (Bingle *et al.*, 2003), proving that the regulatory interactions of donor, recipient and plasmid can influence transfer efficiency.

Similarly, three broad host range plasmids, all carried by the same *P. putida* strain, were transferred to distinct pools of recipients. Yano *et al.* (2013) hypothesized that genetic differences appearing among closely related IncP-1 plasmids through plasmid backbone evolution can result in significant diversities in host range efficiency without affecting their broad host range nature. Such backbone alterations exist between the IncP-1α (RP4) and IncP-1ε (pKJK5) core regulatory proteins such as *KorB*, *TrfA*, *TrbA* and *Ssb* (Bahl *et al.*, 2007). Although these two plasmids are incompatible (both IncP-1), differences in gene silencing and expression of the different core proteins could explain the different transconjugal patterns. As already minor differences in regulation between two IncP-1 plasmids lead to distinct transconjugal pools, it is coherent that the unrelated transfer machinery of plasmid pIPO2tet caused significantly ($P<0.05$) dissimilar transconjugal pools when compared with the IncP ones.

A core superpermissive community fraction dominates gene transfer

Out of 281 OTUs identified in the transconjugal pools with the three different broad host range plasmids and *P. putida* as donor, 74 OTUs were common to all three pools (Figure 6a). A similar observation (46 out of 279 OTUs shared) held when comparing the transconjugal pools for plasmid pKJK5 introduced via three different donors

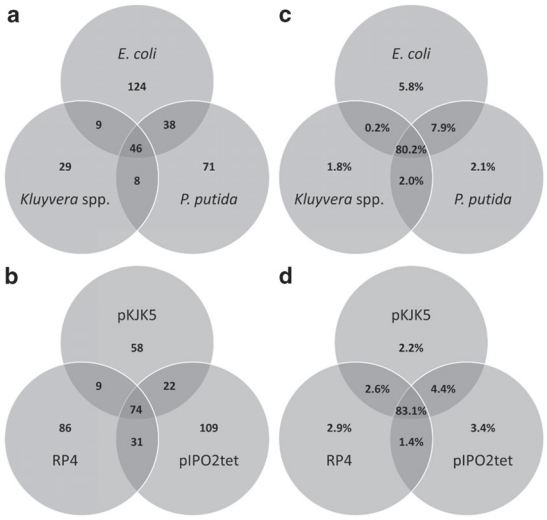

Figure 6 Venn diagram of transconjugal pools for plasmid pKJK5 transferred from three different donor strains (*E. coli*, *P. putida* and *Kluyvera* sp.) (**a** and **c**) and for three different plasmids (pKJK5, RP4 and pIPO2tet) (**b** and **d**) introduced through *P. putida* into the soil community. Venn diagrams are presented for OTU incidence (**a** and **b**) and for OTU relative abundance (**c** and **d**) 100% represents the total number of transconjugal sequences. OTUs were defined at 97% sequence similarity and sequence sample size was normalized to 30 000 per transconjugal pool.

(Figure 6b). Therefore, the majority of transconjugant OTUs were only identified in single donor–plasmid combinations. This might result from mating pair combinations that each favor or reduce gene transfer abilities (Bingle *et al.*, 2003; Thomas and Nielsen, 2005; Yano *et al.*, 2013).

Although only 74 and 46 OTUs are shared among the compared transconjugal pools, these OTUs represent over 80% of the transconjugal sequences (Figures 6c and d). This core super-permissive community fraction shared by all five transconjugal pools is able to take up diverse broad host range plasmids from diverse donor strains at high frequencies. The presence of this shared core in each analyzed transconjugal pool is the crucial discriminant that groups transconjugal pools apart from the original soil community (Figure 3). The core super-permissive community consists mainly of diverse Proteobacteria such as Enterobacteriales (γ), Burkholderiales (β), Pseudomonadales (γ) and Rhizobiales (α) (Figure 4). In addition, within this core super-permissive fraction, several OTUs that are rare in the recipient community (<0.001%) are more than 20-fold overrepresented in transconjugal pools (Figure 4). The participation of these rare community members in gene transfer might have a crucial role in increasing the communal gene pool through rapid recombination with plasmids, as the rare biosphere can harbor a great reservoir of genes (Sogin *et al.*, 2006).

Medical relevance

The large realized transfer potential of newly introduced plasmids in soil may be of medical importance. In recent EAHEC outbreaks in Germany, recombination of a pathogenic with the plasmid of a non-pathogenic *E. coli* strain increased the pathogenic potential to cause a deadly combination (Brzuszkiewicz *et al.*, 2011). Soil-borne antibiotic resistance has been found to be shared with human pathogens (Benveniste and Davies, 1973; Forsberg *et al.*, 2012). Several organisms among the identified transconjugants belong to groups known to contain opportunistic human pathogens, providing a direct link between the plasmid encoded mobile soil resistome and opportunistic pathogens. These groups include the proteobacterial *Enterobacteria*, *Pseudomonas* or *Campylobacter*, and also groups from other phyla such as *Fusobacterium*, *Streptococcus* and *Staphylococcus*, most of which are treated with antibiotic therapy. Especially the acquisition of new antibiotic resistance genes through plasmid-mediated gene transfer may push the pathogenic potential of *Staphylococcus*, originating from rapid evolution of virulence and drug resistance (Holden *et al.*, 2004), even further.

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, Actinobacteria, the origin of many soil-borne resistance genes (D'Costa *et al.*, 2006), which are sometimes identified in clinical isolates of Gram-negative antibiotic-resistant bacteria (Benveniste and Davies, 1973), are frequent among the transconjugants we identified. Broad host range plasmids of the IncP-1 and IncPromA group can thus provide a direct link between diverse bacterial groups. Especially, IncP-1ε plasmids such as pKJK5 have been identified as vectors of antibiotic resistance genes transfer among Proteobacteria by additionally hosting class 1 integron gene cassettes (Heuer *et al.*, 2012). These class 1 integrons may not only spread in their originally identified Gram-negative *Enterobacteriaceae* hosts but can also be found among many Gram-positive bacteria (Nandi *et al.*, 2004). Here, we demonstrated a possible direct way of accession of these class 1 integrons in Gram-positive bacteria through IncP-1ε plasmid transfer from Proteobacteria.

Ecological and evolutionary relevance

Plasmid host range can be defined in several ways depending on the duration and intimacy of the considered plasmid–host relationship, including the transfer host range, the replication and maintenance host range, or the evolutionary host range (Suzuki *et al.*, 2010). We show here that the immediate transfer range for IncP plasmids is much wider than previously reported, proving that in the 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.

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 these plasmids are not maintained long enough outside of this phylum to be significantly affected by non-Proteobacterial genomes. Long-term evolutionary adaptation of the plasmid backbone to the new host, as known for IncP plasmids (Norberg *et al.*, 2011), might therefore also not take place. 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. Mating pair formation and conjugation systems in these plasmids are evolutionarily adapted to connect and span Gram-negative membranes. The observed transfer to Gram-positive bacteria might therefore become a dead end in many cases for Gram-negative associated plasmids if the Type IV coupling and secretion system cannot efficiently spread the plasmid to other neighboring bacteria. However, an actinobacterial *Mycobacterium* strain has been shown to host and transfer an IncP-type plasmid, indicating that maintenance and transfer is possible across the Gram border (Leão *et al.*, 2013). Also, the transient presence of a plasmid can provide the new host with a punctual adaptive gene pool and result in a short-term, but highly significant, fitness gain. Accessory genes on plasmids are mostly arranged in transposons flanked by insertion sequence (IS) elements, which can recombine with the recipient bacterial chromosomes (e.g. class 1 integron of pKJK5) delivering packages of fitness altering DNA without the need for plasmid replication. Additionally, transient hosts can increase the transfer range further by allowing transfer to organisms that had a lower transfer potential from the original donor strain (Yano *et al.*, 2013).

We show within a bacterial community that there is a high variability in permissiveness to broad host range plasmids that cannot be explained by the phylogeny of the potential recipient. 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. We do not know if it is a strain-specific trait and how environmental conditions affect its manifestation. Also, we do not know to what extent the mating conditions used might have biased the observed pattern of super-permissive plasmid recipients. However, if strain-specific, these super-permissive strains would be expected to have a disproportionate role as central nodes in networks of lateral gene acquisitions (Popa *et al.*, 2011). Most gene acquisitions occur between donors and recipients residing in the same habitat (Popa and Dagan, 2011), and while gene acquisition in nature mainly occurs within taxonomically homogeneous groups, the heterogeneous soil community provides a hot-spot for gene acquisition from phylogenetically distant groups (Popa *et al.*, 2011). In soil, a few strains build the core nodes of a heavily connected network of lateral gene acquisition (Popa *et al.*, 2011), which could be a possible indication of being part of the super-permissive fraction. 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 there is indeed a link between increased plasmid uptake ability and long-term gene acquisition potential.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)

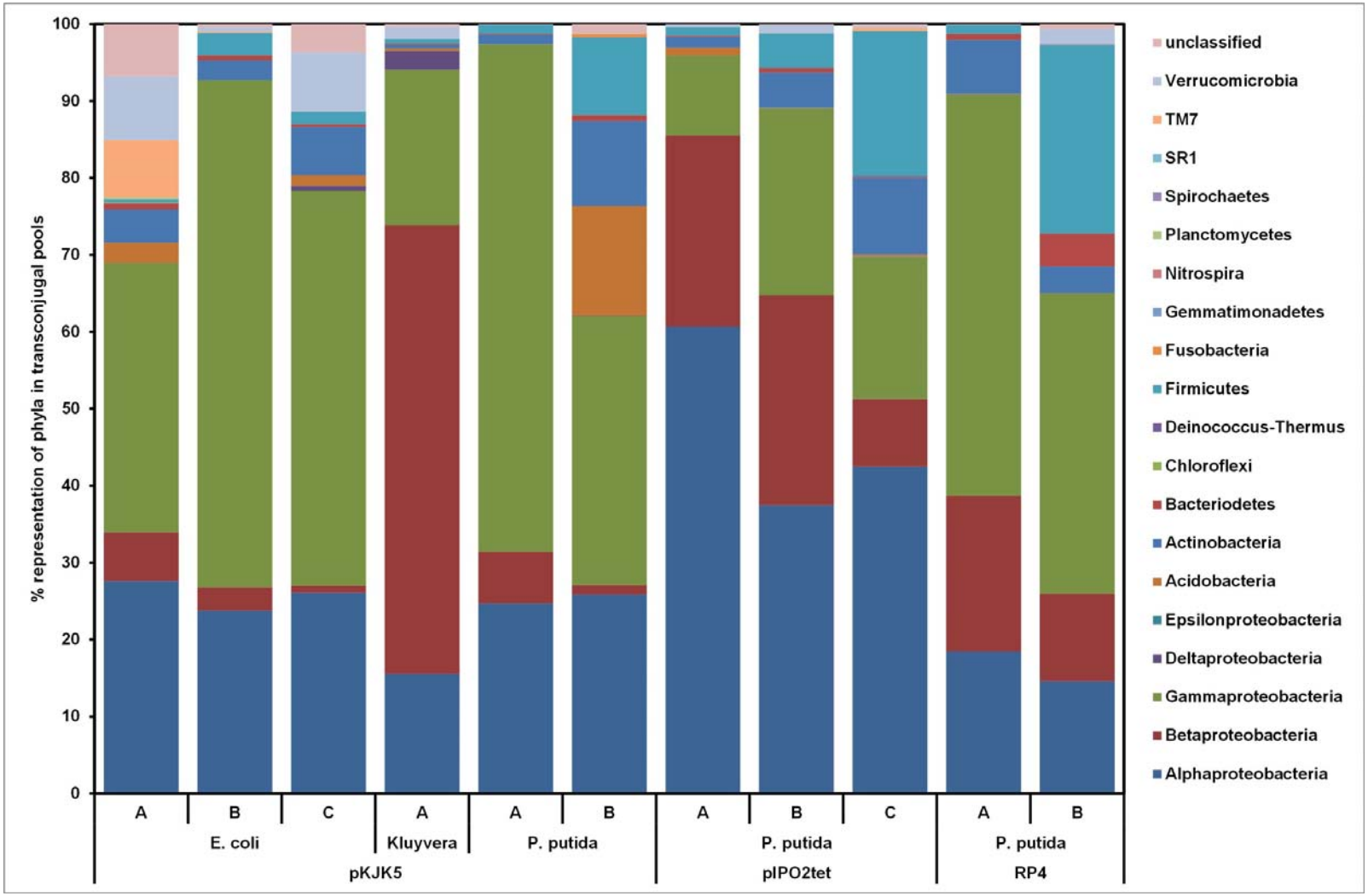
Supplementary Information

Supplementary Table 1: Number of FACS sorted transconjugant cells; raw and quality checked sequences for each mating combination and its replicates.

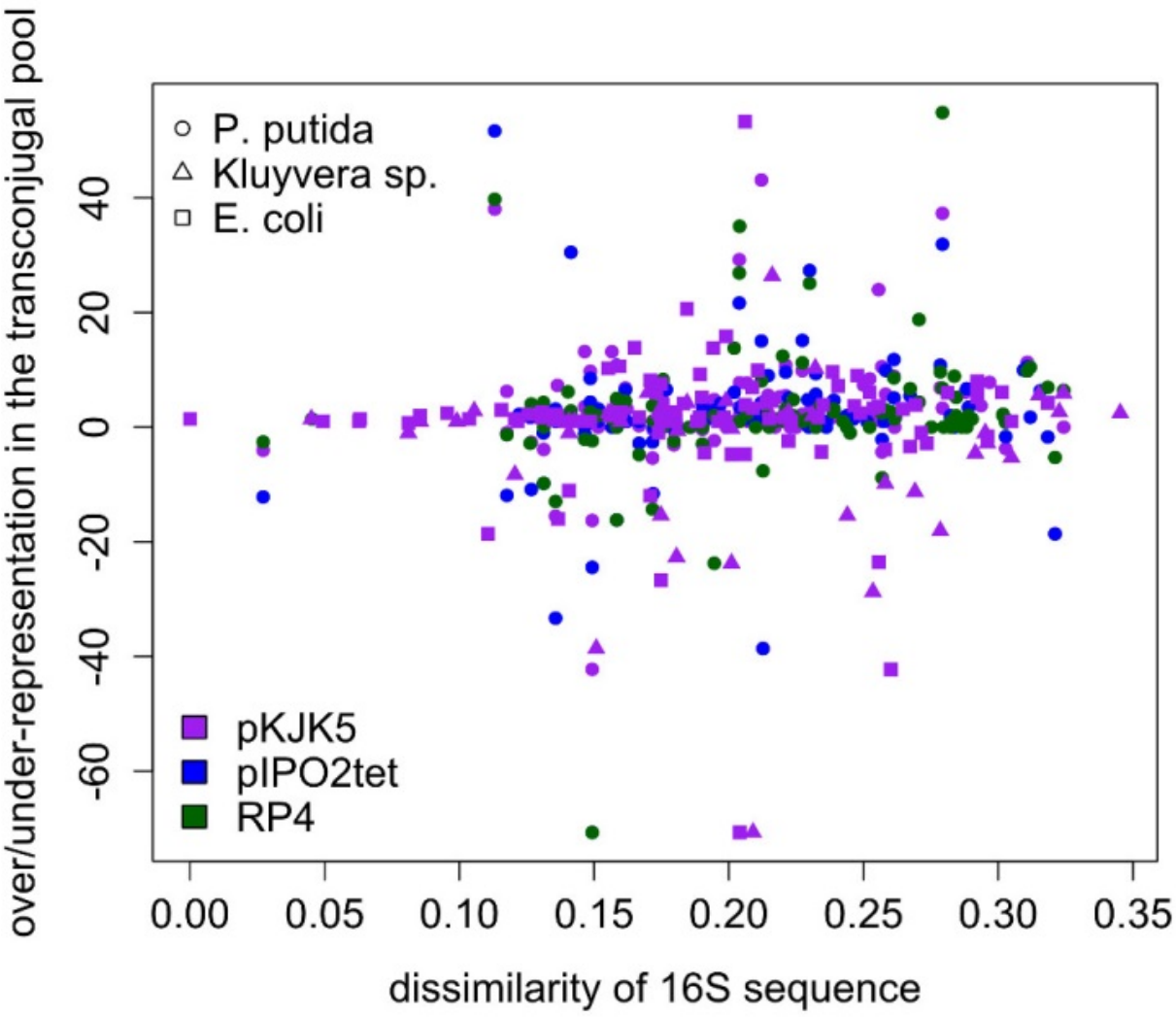
Donor	Plasmid	Replicate	Sorted Transconjugants	Raw reads	Sequences after mothur processing	Sequences/ Transconjugant sorted
E.coli	pKJK5	A	19000	65683	43227	2.28
E.coli	pKJK5	B	14500	57849	45870	3.16
E.coli	pKJK5	C	16500	60608	45292	2.74
Kluyvera	pKJK5	A	34500	52319	41510	1.20
P.putida	pKJK5	A	14000	47482	38931	2.78
P.putida	pKJK5	B	14000	63181	29894	2.14
P.putida	pIPO2tet	A	36500	55632	44156	1.21
P.putida	pIPO2tet	B	35500	65931	50398	1.42
P.putida	pIPO2tet	C	44500	57482	40027	0.90
P.putida	RP4	A	24000	52095	39972	1.67
P.putida	RP4	B	20000	61081	48986	2.45

Supplementary Table 2: OTUs with more than 0.01% sequence representation in the soil reference community that were not represented in the trans-conjugal pools. Sequences were classified using the RDP classifier. Numbers behind the name indicate % sequence similarity to the closest representative.

Phylum		Class		Order		Family		Genus	
Actinobacteria	100	Actinobacteria	100	Actinomycetales	100	unclassified	100	unclassified	100
Bacteroidetes	100	Sphingobacteria	100	Sphingobacteriales	100	Sphingobacteriaceae	100	Pedobacter	100
Bacteroidetes	100	Sphingobacteria	100	Sphingobacteriales	100	Sphingobacteriaceae	100	Sphingobacterium	100
Proteobacteria	100	Alphaproteobacteria	100	Rhizobiales	67	unclassified	67	unclassified	67
Proteobacteria	100	Alphaproteobacteria	100	unclassified	100	unclassified	100	unclassified	100
Proteobacteria	100	Betaproteobacteria	100	Burkholderiales	100	Burkholderiaceae	100	unclassified	100
Proteobacteria	100	Betaproteobacteria	100	Burkholderiales	100	Comamonadaceae	100	Delftia	100
Proteobacteria	100	Betaproteobacteria	100	Burkholderiales	100	Comamonadaceae	100	unclassified	100
Proteobacteria	100	Betaproteobacteria	100	Burkholderiales	100	Comamonadaceae	100	Variovorax	100
Proteobacteria	100	Deltaproteobacteria	100	Myxococcales	100	Myxococcaceae	100	Corallococcus	100
Proteobacteria	100	Deltaproteobacteria	100	Myxococcales	100	unclassified	100	unclassified	100
Proteobacteria	100	Gammaproteobacteria	100	Pseudomonadales	100	Pseudomonadaceae	100	Pseudomonas	93
Proteobacteria	100	Gammaproteobacteria	100	Pseudomonadales	100	Pseudomonadaceae	100	Pseudomonas	100
Proteobacteria	100	Gammaproteobacteria	100	Pseudomonadales	100	Pseudomonadaceae	100	Pseudomonas	93
Proteobacteria	100	Gammaproteobacteria	100	unclassified	100	unclassified	100	unclassified	100
Proteobacteria	100	Gammaproteobacteria	100	Xanthomonadales	100	Xanthomonadaceae	100	unclassified	61
Proteobacteria	100	unclassified	100	unclassified	100	unclassified	100	unclassified	100



Supplementary Figure 1: Phylum level distribution of the isolated and sequenced transconjugants in each of the 11 samples including all replicates per donor and plasmid combination.



Supplementary Figure 2: Relative over-/underrepresentation of OTUs in transconjugal pools compared with the soil reference recipient community as a function of the OTU's Sogin phylogenetic dissimilarity to the respective plasmid donor strain. Overrepresentation is calculated as relative abundance of the OTU in the transconjugal pool divided by relative abundance in soil reference community substracted by 1. Underrepresentation is calculated as the inverse of overrepresentation. Values given in the figure display the square root of the absolute over or underrepresentation.

III

Novel assay to measure the plasmid mobilizing potential of mixed microbial communities

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Novel assay to measure the plasmid mobilizing potential of mixed microbial communities

Uli Klümper*, Ariadni Droumpali, Arnaud Dechesne and Barth F. Smets*

Department of Environmental Engineering, Technical University of Denmark, Kongens Lyngby, Denmark

Edited by:
Kornelia Smalla, Julius Kühn-Institut, Germany

Reviewed by:
Sven Jechalke, Julius Kühn-Institut, Germany
Isabel Henriques, University of Aveiro, Portugal
Masaki Shintani, Shizuoka University, Japan

****Correspondence:***
Uli Klümper and Barth F. Smets, Department of Environmental Engineering, Technical University of Denmark, Miljøvej 113, 2800 Kongens Lyngby, Denmark
e-mail: ulik@env.dtu.dk; bfsm@env.dtu.dk

Mobilizable plasmids lack necessary genes for complete conjugation and are therefore non-self-transmissible. Instead, they rely on the conjugation system of conjugal plasmids to be horizontally transferred to new recipients. While community permissiveness, the fraction of a mixed microbial community that can receive self-transmissible conjugal plasmids, has been studied, the intrinsic ability of a community to mobilize plasmids that lack conjugation systems is unexplored. Here, we present a novel framework and experimental method to estimate the mobilization potential of mixed communities. We compare the transfer frequency of a mobilizable plasmid to that of a mobilizing and conjugal plasmid measured for a model strain and for the assayed community. With *Pseudomonas putida* carrying the *gfp*-tagged mobilizable IncQ plasmid RSF1010 as donor strain, we conducted solid surface mating experiments with either a *P. putida* strain carrying the mobilizing IncP-1α plasmid RP4 or a model bacterial community that was extracted from the inner walls of a domestic shower conduit. Additionally, we estimated the permissiveness of the same community for RP4 using *P. putida* as donor strain. The permissiveness of the model community for RP4 [at 1.16×10^{-4} transconjugants per recipient (T/R)] was similar to that previously measured for soil microbial communities. RSF1010 was mobilized by the model community at a frequency of 1.16×10^{-5} T/R, only one order of magnitude lower than its permissiveness to RP4. This mobilization frequency is unexpectedly high considering that (i) mobilization requires the presence of mobilizing conjugal plasmids within the permissive fraction of the recipients; (ii) in pure culture experiments with *P. putida* retromobilization of RSF1010 through RP4 only took place in approximately half of the donors receiving the conjugal plasmid in the first step. Further work is needed to establish how plasmid mobilization potential varies within and across microbial communities. This method has the potential to provide such insights; in addition it allows for the direct isolation of *in situ* mobilizing plasmids together with their endogenous hosts.

Keywords: plasmid mobilization, permissiveness, RSF1010, RP4, plasmid transfer, conjugation, horizontal gene transfer

INTRODUCTION

Plasmid transfer is believed to be a main mechanism in rapid bacterial adaption to environmental changes (Sørensen et al., 2005; Grohmann, 2011; Heuer and Smalla, 2012). Plasmids can be classified into two main groups based on the presence of genes associated with the transfer phenotype (Smillie et al., 2010). Conjugal plasmids encode a complete set of transfer genes needed to be self-transmissible. Mobilizable plasmids, on the other hand, lack some of the genes encoded in the transfer operon (*tra*), which encodes most of the functions involved in mating pair formation (MPF; Thomas and Nielsen, 2005).

Conjugal plasmids possess an origin of transfer (*oriT*), a relaxase, type IV coupling proteins (T4CP) and a type IV secretion system (T4SS). The relaxase is a key protein of the conjugal machinery, common to all conjugal and mobilizable plasmids. Conjugal transfer of self-transmissible plasmids like the IncP-1α plasmid RP4 is based on pilus establishment between donor and recipient cells coded by the T4SS. The plasmid then transfers through the pilus into the recipient (**Figure 1**). Mobilizable plasmids encode only a *MOB* module (with or without the T4CP) and need the MPF apparatus of a co-resident (i.e., located within the same cell) conjugal plasmid to be transmissible by conjugation (Smillie et al., 2010). To be transferred, they take advantage of a conjugal plasmid that initiates replication through expression of its *rep* genes. These genes are involved in pilus formation and connection of the relaxosome with proteins enabling passage of the DNA across the membranes (Yano et al., 2013). Direct mobilization involves a presently co-resident conjugal plasmid; in retromobilization the donor cells (harboring the mobilizable plasmid) must first receive a mobilizing conjugal plasmid from the recipient, which thereafter mobilizes the mobilizable plasmid toward the recipient (**Figure 1**).Therefore, microbial communities need a high intrinsic conjugal plasmid content to allow mobilization of mobilizable plasmids with potentially useful genetic content, when no co-resident conjugal plasmids are present in the newly introduced donor strain.

The most well-studied non-self-transmissible, mobilizable plasmids belong to the IncQ group. Compared to the broad

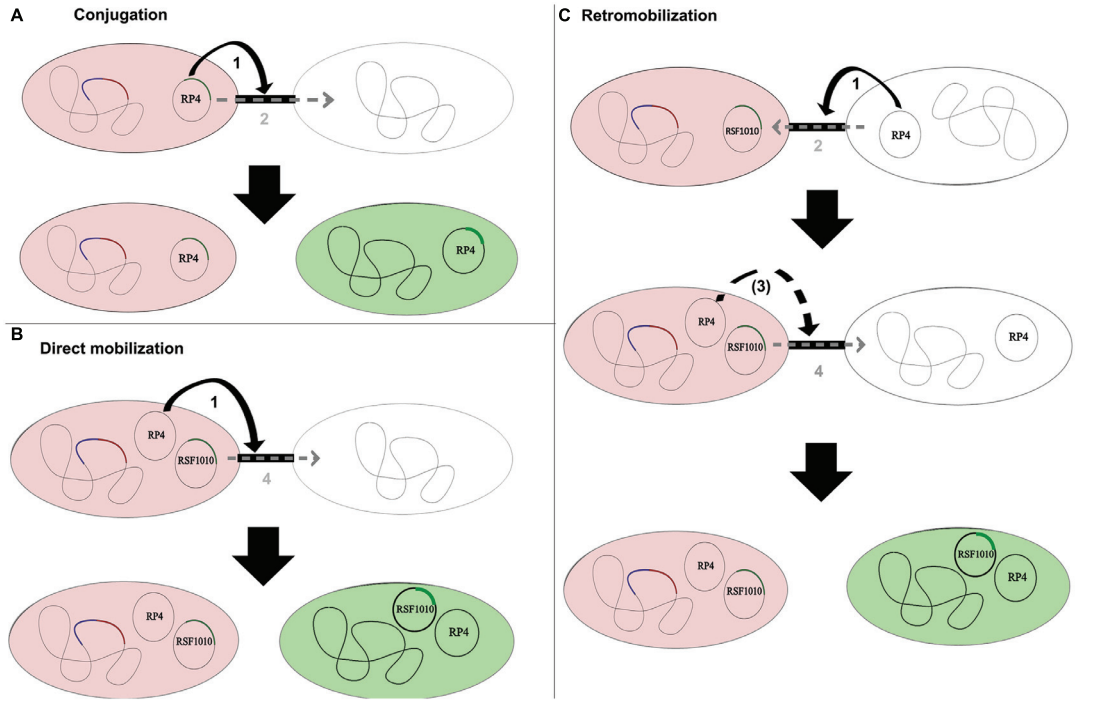

FIGURE 1 | Conjugation, direct mobilization, and retromobilization of the conjugative/mobilizable plasmid couple RP4/RSF1010. In all shown combinations, the donor strains are displayed in red as chromosomally tagged with the red fluorescent protein gene *mCherry* and a *gfp* repressor gene (blue). Recipients transition from being colorless to being green after the *gfp*-tagged plasmid is transferred and thus freed from the chromosomal repression of the donor. **(A)** Conjugal transfer of the self-transmissible IncP-1 plasmid RP4. Step 1 illustrates the establishment of the pilus between donor and recipient as part of the type IV secretion system (T4SS) encoded by the conjugal plasmid. Step 2 displays the transfer of the conjugal plasmid through its own secretion system into the recipient. **(B)** Direct mobilization of the mobilizable IncQ plasmid RSF1010 from the donor to the recipient by the co-resident conjugal plasmid RP4. The conjugal plasmid establishes the pilus as part of its T4SS and interconnects donor and recipient strain (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 might or might not transfer along with the mobilizable plasmid in the direct mobilization process. **(C)** Retromobilization process of plasmid RSF1010, mobilized by a conjugal plasmid from the recipient cell. In this process, the conjugal plasmid from the recipient establishes the conjugal connection between recipient and donor (Step 1) and transfers from the recipient to the donor (Step 2). The mobilizable plasmid can subsequently transfer through the established connection (Step 4) or through a potential new connection established by the now co-resident conjugal plasmid (Step 3).

host range IncP-1 conjugal plasmids, they are relatively small (5.1–14.2 kb; Loftie-Eaton and Rawlings, 2012). Thanks to their host independent replication system, these plasmids have a broader host range than any other known replicating components in bacteria (Meyer, 2009). They can be conjugally mobilized by a variety of different plasmid encoded type IV transporters (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).

Mobilization by the IncP-1 plasmids has contributed extensively to the dissemination of IncQ plasmids (Meyer, 2009) and the coupling of the transfer machinery of the IncP-1 RP4 plasmid to mobilize the IncQ RSF1010 plasmid has been well studied (Lessl et al., 1993; Haase et al., 1995).

In order to assess a conjugal plasmid's potential contribution to horizontal gene transfer in a microbial community, the permissiveness of the community toward the plasmid is a main parameter. We have defined permissiveness as the fraction of a community able to receive and maintain a target exogenous plasmid (Musovic et al., 2010; Klümper et al., 2014). Different factors such as phylogenetic diversity, cell density, and various environmental stress factors may affect community permissiveness (Musovic et al., 2010; Heuer et al., 2011). While some bacteria are known to exude signal molecules in order to obtain plasmids (Hirt, 2002), permissiveness toward a self-transmissible, conjugal plasmid is probably a passive trait of the bacterial community. The ability of a community to receive genes located on mobilizable non-self-transmissible plasmids, on the other hand, would rely on the community's own content of conjugal plasmids. While the spread and contribution of conjugal plasmids to gene exchange has been intensely studied (Heuer et al., 2012; Shintani et al., 2014; Zhang et al., 2014), the mobilization potential of microbial communities and the contribution of mobilizable plasmids to horizontal gene flow have been comparably poorly studied (Top et al., 1995). Exogenous isolation techniques to capture mobilizing and mobilizable plasmids from natural communities have been

developed (Top et al., 1994; van Elsas et al., 1998; Smalla et al., 2000). However, the characterization of communities based on their mobilization potential has mainly been carried out using indirect measures through triparental matings where both donor and terminal recipient were artificially introduced to the communities (Hill et al., 1992). For example, manure addition was shown to increase a soil microbial community's ability to support mobilization of a mobilizable plasmid between two introduced strains through an increased intrinsic plasmid content (Götz and Smalla, 1997). Direct mobilization of mobilizable plasmids into indigenous bacteria of a mixed community has been detected before (Hill et al., 1992; van Elsas et al., 1998), but was never directly quantified.

Here, we present a novel experimental method to estimate the plasmid mobilization potential of a mixed bacterial community, using IncQ RSF1010 as model plasmid. We quantify the mobilization potential of a model community extracted from a domestic shower conduit. We evaluated the transfer frequency by comparing it to the community's permissiveness toward the mobilizing, conjugal plasmid RP4. We finally related the observed transfer frequencies to those measured in transfer between isogenic strains. We additionally aimed to isolate transconjugants that mobilized the RSF1010 plasmid, assuming that retromobilization is the main mobilization process.

MATERIAL AND METHODS

PRINCIPLE OF PLASMID TRANSFER DETECTION

The recipient community was challenged with various plasmid combinations introduced through *Pseudomonas putida* in solid surface filter matings (**Figure 2**). All strains used or constructed for this study can be found in **Table 1**. The plasmids (**Table 2**) were marked with a genetic tag encoding a conditionally expressible fluorescent marker. The used entranceposon (Bahl et al., 2009) carries a *lacI*^{q} repressible promoter upstream of the *gfp-mut3* gene, coding for the green fluorescent protein (*gfp*). The plasmid donor strain was chromosomally tagged with a gene cassette encoding constitutive red fluorescence and constitutive *lacI*^{q} production. As a result, there is no *gfp* expression in the donor strain, but upon plasmid transfer to recipient bacteria, *gfp* expression is possible, resulting in green fluorescent cells or microcolonies, which can be detected and quantified by fluorescence microscopy or sorted by fluorescent activated cell sorting (FACS), respectively. *P. putida* KT2440 served as the donor strain in all the experiments, and was tagged through electroporation with plasmid pGRG36-*lacI*^{q}-*Km*^{R}-*Lpp-mCherry* carrying both the transposase genes and the Tn7 *lacI*^{q}-*Lpp-mCherry-Km*^{R} region for specific integration of the *lacI*^{q}-*Lpp-mCherry-Km*^{R} gene cassette into the chromosomal attTn7 site as described earlier (Bahl et al., 2009).

The 8.7 kbp IncQ plasmid, RSF1010, originally isolated from *Escherichia coli* (Scholz et al., 1989), harbors streptomycin and sulphonamide resistance determinants and genes for the degradation of arginine and ornithine. For *gfp*-tagging the P_{A10403}-*gfpmut3-Km*^{R} section of entranceposon [*Km*^{R}, P_{A10403}-*gfpmut3*] was amplified by PCR, subjected to subsequent enzyme digestion and ligated to the RSF1010 vector cut with the same enzyme. The correct insert location at the enzyme cut site of [*Km*^{R}, P_{A10403}-*gfpmut3*] in plasmid RSF1010 was confirmed by sequencing from the inserted fragment in one direction using primer Seq_Bw_Ent_gfp: 5′-GCCAGAACCGTTATGATGTCGG-3′. The selected *gfpmut3*-tagged RSF1010 (abbreviated as RSF1010::*gfp*) plasmid was finally introduced by transformation into the donor strain, *P. putida* KT2440::*Km*^{R}-*Lpp-mCherry*.

A donor *P. putida* KT2440::*Km*^{R}-*Lpp-mCherry* with both RSF1010::*gfp* and the wild type conjugal plasmid RP4 was also constructed. The previously created donor strain *P. putida* KT2440::*Km*^{R}-*Lpp-mCherry* carrying the RSF1010::*gfp* plasmid was mated with *E. coli* J5 harboring an untagged version of the RP4. Mating was carried out on microfiber filters (GF/C Whatman filter, 24 mm). Cells were detached from the mating filters and *P. putida* donor strains hosting both plasmids were selected for on 10 mM citrate medium supplemented with streptomycin and tetracycline and checked for red and green fluorescence after IPTG induction of *gfp*.

DONOR AND RECIPIENT STRAIN GROWTH AND PREPARATION

The *P. putida* recipient and donor strains were grown overnight on R2A medium supplemented with the plasmid specific antibiotics (**Table 2**) and harvested by centrifugation at 10,000 × *g* for 10 min. Harvested cells were resuspended and washed twice with sterile 0.9% NaCl solution to remove residual antibiotics and thereafter adjusted to a bacterial density of 3×10^6 bacteria/mL using Thoma chamber counts and sterile 0.9% NaCl solution for dilutions.

RECIPIENT COMMUNITY EXTRACTION AND PREPARATION

As model recipient microbial communities, we extracted biofilms that colonized the inner walls of a domestic shower PVC hose from a private residence. The shower hose was first drained in a sterile 50 mL Falcon tube. The emptied hose was then incised with a sterilized steel scalpel blade and the biofilm at its inner surface removed by scraping. The removed biofilm was transferred to the same 50 mL Falcon tube. The suspension was centrifuged for 8 min at 10.000 × *g*. The pellet was resuspended in 5 mL TTSP [tetrasodium pyrophosphate (50 mM), Tween 80® (0.05%)], vortexed at maximum speed for 5 min, and sonicated 60 s in a Branson Sonifier 250 (Branson, MO, USA) at 40% power at 200 W to disrupt cell aggregates. The bacterial suspension was then filtered through a sterile 20 μm pore-size filter. This filtrate was used as the recipient community in mating assays after adjusting the bacterial density to ∼3×10^6 bacteria/mL, as confirmed by Thoma chamber counts.

SOLID SURFACE FILTER MATING ASSAY

The recipient communities were challenged with the plasmids introduced through the constructed donor via solid surface filter matings (Musovic et al., 2010) at a 1:1 initial donor to recipient cell ratio and an initial density of approximately 30,000 bacteria/mm^2 filter surface area, with 10-fold diluted R2A as solid 1.5% agar mating medium. Conjugation was verified by epifluorescence stereomicroscopy after 48 h incubation at room temperature and the transfer events quantified (Musovic et al., 2010). R2A was chosen as filter mating medium as it is presumed optimal for water borne organisms (Reasoner et al., 1979). However, to simulate low nutrient conditions typical of drinking water distribution systems

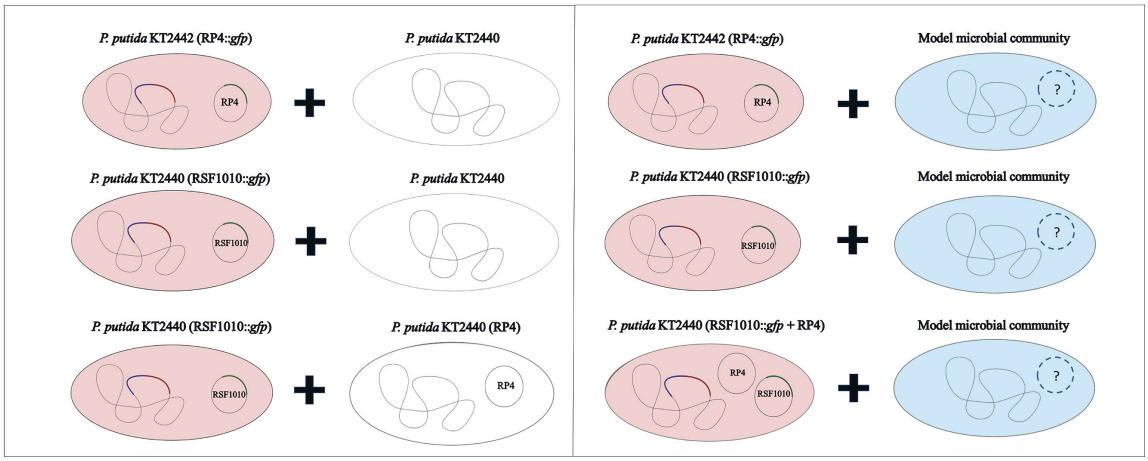

FIGURE 2 | Overview of executed filter mating combinations.

Table 1 | Donor and recipient strains used in this study.

Species/strain	Plasmid	Resistance (μg/mL)	Chromosomal markers	Reference
Pseudomonas putida KT2442	RP4::*gfp*	Km^R, Amp^R, Tet^R (50, 100, 10)	Rif^R	Musovic et al. (2010)
P. putida KT2440	RSF1010::*gfp*	$Strep^R$ (100)	$lacI^q$-*pLpp-mCherry*, Km^R	This study
P. putida KT2440	RSF1010::*gfp*, RP4	$Strep^R$, Amp^R, Tet^R, Km^R (100, 100, 40, 50)	$lacI^q$-*pLpp-mCherry*, Km^R	This study
P. putida KT2440	–	–	–	Nelson et al. (2002)
P. putida KT2440	RP4	Tet^R, Km^R, Amp^R (40, 50, 100)	–	This study

Table 2 | Plasmids used in this study.

Plasmid	Transfer	Size	Incompatibility	Resistance (μg/mL)	Host range	Degradation pathways	Reference
RP4	Conjugal	60 kb	IncP-1α	Amp^R, Km^R, Tet^R (100, 50, 20)	broad	BP, 4CBP	Barth and Grinter (1977)
RSF1010	Mobilizable	8.7 kb	IncQ	$Strep^R$ (100)	broad	Arginine, Ornithine	Honda et al. (1991)

(Boe-Hansen et al., 2002), the R2A medium was diluted to the maximum extent possible, while maintaining high enough bacterial activity for growth of microcolonies, to establish donor to recipient cell contact during the mating, and for expression of the plasmid encoded *gfp*-gene after plasmid transfer. Five different dilutions of R2A (1:5, 1:10, 1:50, 1:100, 1:1000) were tested and the 10-fold diluted R2A was finally chosen, as it was the highest dilution at which transconjugants were still observed for all tested plasmids.

VISUALIZATION AND QUANTIFICATION OF TRANSFER EVENTS BY STEREOMICROSCOPY AND IMAGE ANALYSIS

Successful plasmid transfer was visualized *in situ* by stereomicroscopy and quantified by automated image analysis (Image Pro Plus 7.1; Media Cybernetics, Silver Spring, MD, USA) as previously described (Musovic et al., 2010), using a Leica MZ16 FA fluorescence stereomicroscope equipped with a 10x plan apochromatic objective, a 10× eyepiece (10×/21B), a 40× magnification zoom. Conditions for *gfp*- and *mCherry*-based fluorescence detection were 480/20 nm with emission at 525/40 nm and 580/25 with emission at 650/60 nm, respectively, and images were acquired with a Leica DFC300 fluorescence camera. A representative scanning zone of 7 × 7 fields of 980 × 732 μm each were analyzed per filter. With a total filter area of 270 mm^2, the scanned and quantified area corresponded to approximately 13% of the total filter area. Triplicate filters were analyzed for each donor/recipient combination.

Quantification of transfer events was performed with a custom-made macro written in Image Pro Plus 7.1. This macro successively extracts and subtracts the background from the original image, performs a best-fit equalization of the image intensity, before detecting bright objects larger than 4 μm^2 based on automatic segmentation. Analysis of images was limited to the brightly illuminated elliptic central area of the field of view (**Figure 3**). All

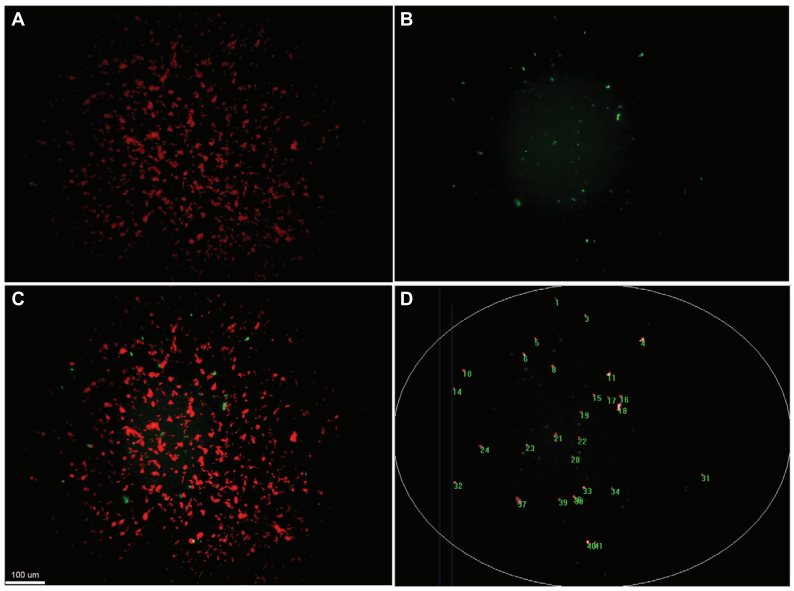

FIGURE 3 | Detection and quantification of transconjugant microcolonies by fluorescent microscopy. Fluorescence based stereomicroscopic images and image analysis of an example filter mating of *Pseudomonas putida* KT2440::*lacI*^{q}-*Lpp-mCherry-Km*^{R} (RSF1010::*gfp*) with the recipient community. **(A)** corresponds to the red fluorescent channel, displaying donor microcolonies. **(B)** shows the green fluorescent channel, corresponding to the transconjugal microcolonies that received the plasmid through retromobilization. **(C)** is a composite image of both channels with increased contrasts. Transconjugal microcolonies can be found in direct proximity to donor colonies. **(D)** illustrates counting of transconjugal colonies through a macro that increases contrast of the images, subtracts background, eliminates the poorly illuminated corners and counts green fluorescent object larger than 4 μm^2.

images were manually controlled for enumeration errors, and values corrected if deviations were noted. The number of *gfp*-positive colonies (transfer events) detected was scaled up to the total filter area and transfer frequency was calculated by dividing this number by the number of potential recipients originally placed on the filter.

CELL COLLECTION AND FLUORESCENCE ACTIVATED CELL SORTING OF TRANSCONJUGANTS

Cells from the filter mating between *P. putida* (RSF1010::*gfp*) and the model community were removed by vortexing in 2 mL of a 0.9% NaCl-solution for 3 min. Flow cytometric detection of cells and *gfp*-based isolation of transconjugants were carried out using a FACSAria IIIu Flowcytometer (Becton Dickinson Biosciences, San Jose, CA, USA), as previously described (Klümper et al., 2014).

RESULTS

PERMISSIVENESS OF THE RECIPIENT COMMUNITY FOR CONJUGAL IncP-1 PLASMID RP4

We explored the intrinsic ability of an extracted model microbial community to mobilize the broad host range mobilizable plasmid RSF1010 as well as its ability to receive the conjugal broad host range plasmid RP4. Both plasmids were introduced via a red fluorescent-tagged donor *P. putida* in which plasmid encoded *gfp* expression is repressed (**Table 1**). Microscopic examination and enumeration of the mating events (**Figure 3**) between the recipient microbial community and *P. putida* (RP4::*gfp*) revealed a transfer frequency of 1.16×10^{-4} transconjugants per potential recipient (T/R; **Figure 4**). A higher transfer frequency (1.76×10^{-3} T/R) was observed in the mating assay using isogenic *P. putida* donor and recipient strains (**Figure 4**). In this experiment, all recipients were obviously within the plasmid host range and any incompatibility effect with RP4 could be ruled out because they were all initially plasmid-free. Hence, the observed transfer frequency in these intrastrain experiments was not limited by the recipient permissiveness, but only by donor promiscuity (the fraction of donor cells expressing conjugal genes), successful completion of initiated plasmid transfer events to *P. putida* recipient cells, and the degree of donor–recipient contact saturation.

We can now express the community's permissiveness against the defined co-culture experiments: The community permissiveness for the conjugal RP4 (1.16×10^{-4} T/R) is divided by the conjugal transfer frequency of plasmid RP4 in intrastrain matings, where all *P. putida* recipients can potentially take up RP4 (1.76×10^{-3} T/R), as a standard. The resulting community permissiveness for RP4 is 0.066 RP4 intrastrain equivalents.

MOBILIZING POTENTIAL OF THE RECIPIENT COMMUNITY FOR PLASMID RSF1010

When the model community was challenged with *P. putida* (RSF1010::*gfp*), a transfer frequency of 1.16×10^{-5} T/R was measured. This value is one order of magnitude lower than the

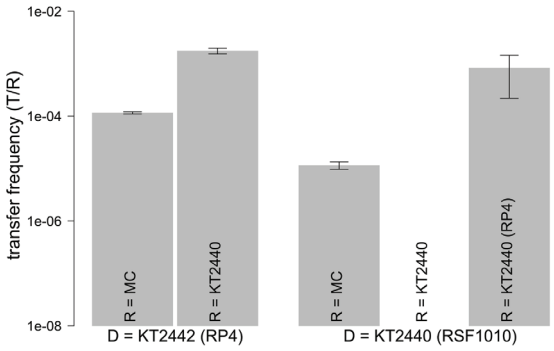

FIGURE 4 | Transfer frequencies of RSF1010 and RP4. Transfer frequencies defined as transconjugant microcolonies per recipient were obtained in solid surface filter matings with the recipient community and in *P. putida* intrastrain matings. Values are shown as mean of triplicates with standard error of mean. The *gfp*-tagged plasmid in the donor strain (D) is shown on the x-axis. RP4 or RSF1010 were each introduced through KT2440 or KT2442 (*P. putida* KT2440/KT2442::*lacI*^{q}-*Lpp-mCherry-Km*^{R}) into the recipients. Recipients (R) are shown within the bars (MC: model community; KT2440: *P. putida* KT2440). For the combination D= KT2440 (RSF1010) with R= KT2440 no transfer was observed.

community's measured permissiveness for the conjugal plasmid RP4 (**Figure 4**).

In these experiments RSF1010 must have been retromobilized into the recipient community by cells carrying IncQ compatible mobilizing conjugal plasmids (**Figure 1**). In order to explore the retrotransfer frequency of RSF1010 further, isogenic *P. putida* strains were used to execute two different intrastrain matings, taking advantage of all *P. putida* recipient cells being potential RSF1010 hosts. In the first experiment, a plasmid-free, non-*mCherry*-tagged *P. putida* strain served as recipient. In the second experiment, a non-*mCherry*-tagged *P. putida* strain hosting the untagged wild-type conjugal, mobilizing RP4 plasmid served as recipient. In the first experiment no RSF1010 transfer was observed, consistent with RSF1010's non-self-transmissible nature. In the second experiment with *P. putida* (RP4) as recipient, retrotransfer was observed, with a measured frequency of 8.34×10^{-4} T/R. Successful RSF1010 retrotransfer requires initial conjugal plasmid transfer from recipients to RSF1010 donors, before RSF1010 is mobilized and retransferred to the recipients (Top et al., 1992; **Figure 1C**).

RSF1010 retrotransfer frequency by *P. putida* (RP4) results from a combination of the RP4 transfer process from the recipient to the donor (**Figure 1C** Steps 1 and 2) and the subsequent mobilization of RSF1010 through the now co-resident RP4 plasmid (**Figure 1C** Steps 3 and 4). It can be contrasted with the measured RP4 intraspecies transfer frequency of 1.76×10^{-3} T/R. RP4 intrastrain transfer corresponds to the first two steps in RSF1010 retrotransfer (**Figure 1A**). Hence, the probability for 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*]. For this specific pair of mobilizing and mobilizable plasmid, retrotransfer is high (**Figure 4**).

The retrotransfer of RSF1010 to the recipient community occurs at a frequency of 10% compared to its permissiveness for the RP4 plasmid. Still, as shown above, mobilization of RSF1010 is realized only approximately every second time a conjugal plasmid is transferred from the recipient community into the donor strain, based on mobilization through RP4. If all these potential mobilization events were realized, the maximal mobilization potential of the recipient community is reached. The theoretical maximal mobilization potential toward RSF1010 can be quantitatively assessed as 2.45×10^{-5} T/R by dividing its transfer frequency toward the community (1.16×10^{-5} T/R) by the now established 47.4% probability of retrotransfer. When subsequently dividing 2.45×10^{-5} T/R through the community's permissiveness toward RP4 (1.16×10^{-4} T/R) as a standard, this results in 0.211 RP4 permissiveness equivalents as the maximal mobilization potential.

POTENTIAL COMMUNITY PERMISSIVENESS TOWARD MOBILIZABLE PLASMID RSF1010

In a final experiment, we quantified the intrinsic permissiveness of the model community for RSF1010. To do so, we augmented the community's own RSF1010 mobilizing potential by adding an exogenous RSF1010 mobilizing strain. Hence, the recipient community was challenged with *P. putida* hosting both the RSF1010::*gfp* and the wild-type RP4, which can directly mobilize RSF1010 (**Figure 1B**). The observed transfer frequency of RSF1010 in this mating was 3.14×10^{-3} T/R. This frequency is, surprisingly, higher (∼30-fold) than the community's permissiveness for RP4. As expected, this value is also substantially higher (∼2 orders of magnitude) than the RSF1010 mobilization frequency (**Figure 4**) relying on the community's inherent retromobilization potential only.

FACS BASED SORTING OF RSF1010 TRANSCONJUGANTS

Cell suspensions from matings between the recipient community and *P. putida* (RSF1010::*gfp*) were collected, resuspended and subjected to FACS to isolate green fluorescent transconjugants (Klümper et al., 2014). 200 transconjugants were successfully sorted, despite a sorting time exceeding 24 h, due to the low initial relative abundance of transconjugant cells at less than 1:1,000,000 events sorted.

DISCUSSION

Plasmids of the promiscuous, conjugal IncP-1 group illustrate the enormous potential of horizontal gene transfer among an extremely wide variety of gram-negative and gram-positive bacterial species (Gelder et al., 2005; Klümper et al., 2014; Musovic et al., 2014; Shintani et al., 2014). Studies on conjugal gene flow mainly focused on the passive characteristics of a mixed community to receive self-transmissible plasmids. Former approaches to assess the mobilization potential of mixed communities were using an indirect approach through triparental matings where both donor and terminal recipient were artificially introduced to the communities (Hill et al., 1992; Götz and Smalla, 1997) and even capture the mobilizing (van Elsas et al., 1998) or mobilizable (Smalla et al., 2000) genetic elements from natural communities. This study is the first one to directly quantify the potential of a

microbial community to actively mobilize non-self-transmissible, mobilizable plasmids to its indigenous bacteria. It also illustrates how the community's intrinsic plasmid content can contribute to an increased gene uptake potential. To estimate the maximum mobilization potential of a community, we utilized filter matings at maximized cell-to-cell contact of donor and potential recipients (Musovic et al., 2010). The spatial limitations for contact in water distribution systems might be small compared to other environments like the ones reported for soil (Dechesne et al., 2005). However, the initial invasion of the plasmid donor into the biofilm community might be limited to the surface of the biofilm and further reduced at high water flow conditions (Licht et al., 1999; Merkey et al., 2011; Król et al., 2013). Therefore, using our maximum cell-to-cell contact assay instead of natural conditions allows every single recipient cell to establish contact with donor cells and potentially engage in gene transfer. However, using this assay might limit the retransfer potential of the plasmid from new transconjugants to further recipients. Recipients that newly acquired the plasmid might only be surrounded by *P. putida* donor cells and not by other cells from the recipient community and can thus not retransfer the plasmid to other recipients. This retransfer process can especially be crucial for mobilizable plasmids. The first retromobilization transfer event leads to the co-occurrence of the mobilizable plasmid with the mobilizing conjugal plasmid(s) in the same cell. Through this co-occurrence the transconjugant cell significantly increases its transfer frequency of the mobilizable plasmid to the recipient community by switching the mechanism from retromobilization to direct mobilization, thereby omitting the steps involved in transferring the mobilizer to the donor cell. We measured a more than 300-fold increase in plasmid transfer for *P. putida* to the mixed community between retro- to direct mobilization. This large increase in transfer frequency was also reported earlier with a difference of over three orders of magnitude for direct mobilization versus retromobilization for a different mobilizable plasmid among pure strains (Top et al., 1995). Therefore, experiments that assess how this retransfer process influences the mitigation and invasion of a mobilizable plasmid from the initial donor through a mixed and spatially stratified biofilm community might be needed. To conclude, once mobilizable plasmids are in co-occurrence with a promiscuous mobilizing plasmid, they can significantly contribute to horizontal gene transfer in mixed communities.

We show here that the IncQ model plasmid RSF1010 can be easily mobilized by the bacterial community extracted from a household water distribution system. The permissiveness of this microbial community toward the conjugal plasmid RP4 is comparable in magnitude with that measured in diverse soil communities (Musovic et al., 2014). The lower permissiveness toward RP4 measured for mixed recipient communities compared to *P. putida* intraspecies transfer results primarily from the inability of a fraction of the bacterial community to either receive, transiently maintain, or express plasmid encoded genes.

The community's potential to retromobilize and subsequently receive RSF1010 is only one order of magnitude lower than its permissiveness toward 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). The numbers appear even higher taking into account that in pure culture experiments with *P. putida*, only half of the microcolonies that recently received RP4 retromobilized RSF1010. Earlier retrotransfer experiments between two *E. coli* strains (Top et al., 1992) showed T/R ratios within the same orders of magnitude (10^{-3}–10^{-4}) as our intrastrain matings. But, they suggested that retrotransfer of the mobilizable plasmid appears at rates lower than 1% once the first step of acquiring a conjugal plasmid is realized. In that work transfer was quantified based on single cells and after 2.33 h. Our far higher numbers (∼50%) might therefore result from quantifying transfer on a microcolony basis after 48 h. Only one retrotransfer event within a microcolony is needed for quantification as successful transfer event and due to increased incubation time retrotransfer can happen not only through the initial, but also through newly established conjugal pili. Nonetheless, the observed retromobilization requires the presence of mobilizing, conjugal plasmids within the permissive fraction of the recipients. Other mobilization possibilities involve conjugation-independent transfer of plasmids through the formation of nanotubes from members of the complex community toward the donor cells (Dubey and Ben-Yehuda, 2011), but are only realized if nanotubes from the recipient to the *P. putida* donor are established. 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.

IncP type IV secretion systems can conjugally connect a large variety of organisms (Grahn et al., 2000; Thomas and Nielsen, 2005; Klümper et al., 2014). But like the plasmids encoding them, they are evolutionary adapted to connect their mainly Gram-negative hosts. These self-transmissible plasmids might easily reach dead ends after being transferred, if the secretion system is not encoded efficiently for retransfer in the new host. Contrarily, mobilizable plasmids might less frequently reach dead ends once acquired, since they can utilize the conjugal connections build through adapted resident plasmids in their new host (Meyer, 2009) or through ICEs (Lee et al., 2012). Additionally, mobilizable plasmids are relatively stable, as their high copy number (Meyer, 2009) increases retention in a host until new transfer becomes possible. These two facts in connection with their strictly host-independent initiation of replication helps them to sustain in a very broad host range, including *Pseudomonas sp.*, related species in the Proteobacteria, as well as phylogenetically distant species within the Firmicutes, Actinomycetes and even Cyanobacteria (Meyer, 2009) or plants (Buchanan-Wollaston et al., 1987). Consequently, RSF1010, as a mobilizable plasmid, has a far higher replication host range than RP4. RSF1010 can even spread to a mixed community at a more than 30-fold higher transfer frequency when directly mobilized through co-occurring plasmid RP4 in the same donor cell compared to RP4 itself. Therefore, mobilizable plasmids might contribute to long term gene spread and acquisition to a so far underestimated extent, especially in environments with high intrinsic mobilizing plasmid content. In our current experiment, we use a simplified system and are able to deliver insights into the mobilization potential of a community at

the first acquisition event of a newly introduced mobilizable plasmid. The wide variety of mobilization systems possibly involved might not resemble the one encoded by RP4 in efficiency. Still, equivalents based on the community's permissiveness toward RP4 can be used here, since long term maintenance and retransfer are not taken into account. For more complex natural systems and experiments that allow extensive retransfer we recommend assessing the intrinsic mobilization potential of microbial communities based on absolute transfer frequencies, as the transfer and maintenance processes of RSF1010 and RP4 differ too much in the long term.

Apart from quantification of the mobilization potential, the method presented here provides several possibilities to study plasmid ecology and mobilization mechanisms. FACS based sorting of RSF1010 carrying transconjugants from the recipient community was possible. Studying the diversity of transconjugants might provide insights into the enormous host range of mobilizable plasmids, compared to those of broad host range conjugal plasmids (Klümper et al., 2014). But the high amount of sorting time prohibits intensive studies at this point. However, taking advantage of FACS sorting, even at low speed, new possibilities for plasmid isolation emerge. The mobilizing, conjugal plasmid can, now, after retromobilization, co-occurring with RSF1010 in the transconjugant, be subsequently isolated within its original environmental host. Compared to common exogenous plasmid isolation techniques our method has the potential to also capture plasmids that are only transiently hosted and therefore quickly lost in the introduced capturing strains. Since these plasmids remain stable in their original hosts, we gain the ability to isolate them with our method. Isolated plasmids need therefore only stable maintenance in their natural hosts rather than in an artificially introduced strain. This increases the range of obtainable plasmids and immediately supplies information on where they naturally occur. This method reverses the exogenous isolation technique for mobilizable plasmids (Top et al., 1994) and is cultivation independent. Additionally using the tools presented here in combination with FACS sorting, single cell observations to better understand the exact mechanisms proposed for retromobilization (Top et al., 1992, 1995) 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 transfer frequencies through fluorescent microscopy. Using the new method, we discovered that a mixed microbial community has the potential to easily mobilize a newly introduced mobilizable plasmid at high rates compared to a conjugal plasmid. We also showed that the mobilizable plasmid is spread at far increased frequencies once directly mobilized by a co-occurring conjugal plasmid from within the same cell.

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IV

Taxon specific modulation of soil community permissiveness towards broad host range plasmid pKJK5 under metal stress

Klümper, U., Dechesne, A., Riber, L., Brandt, K.K., Gülay, A., Sørensen, S.J. & Smets, B.F.

Submitted

Taxon specific modulation of soil community permissiveness towards broad host range plasmid pKJK5 under metal stress

Klümper, U[1]., Dechesne, A. [1], Riber, L. [2], Brandt, K.K. [2], Gülay, A. [1], Sørensen, S.J. [2] & Smets, B.F. [1]

[1] Technical University of Denmark, Department of Environmental Engineering, Kgs. Lyngby, Denmark
[2] University of Copenhagen, Copenhagen, Denmark

Abstract

Metal cations introduced to soils through manure can be the source of significant stress exposure to soil microbial communities. A major process in the adaptation of bacteria to stress is horizontal gene transfer through conjugal plasmids. However, it is unknown how the permissiveness of a community is altered under stress conditions. Here, we stressed a soil bacterial community with 5 metals (Cu, Cd, Ni, Zn, As) at 20% and 50% growth inhibition level in order to investigate how metal stress modulates the ability to take up a model broad host range IncP-type plasmid (pKJK5) from an exogenous *Escherichia coli* host.

Metal stress decreased the permissiveness of the community towards pKJK5 in transfer frequencies between 30% and 100%, while the richness of transconjugal pools remained stable at 190 OTUs. We demonstrated for the first time that metal stress modifies the permissiveness of each community member individually. Permissiveness can thus in- or decrease by more than 1000-fold. 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. Species from similar phylogenetic groups respond similarly to specific stresses with regard to their permissiveness. Plasmid transfer across the phylum border from Proteobacteria to Bacteroidetes was triggered under metal stress conditions.

We identified that no general response to stress exists within a community, or certain phylogenetic groups. The modified permissiveness could moreover neither be predicted through the metal stressor introduced, nor the inhibitory concentration it was applied at.

Our results indicate that the question on how the exposure to heavy metals determine the spread of mobile genetic elements needs to be answered at the individual strain rather than the community level.

1 Introduction

Horizontal gene transfer across bacterial species has been recognized as a common and major adaptive and evolutionary process (Zhaxybayeva & Doolittle, 2011). Mobile genetic elements can spread across diverse bacterial phyla and sometimes even across domains of life (Waters, 2001; Heinemann & Sprague, 1989) potentially linking distinct genetic pools (Halary *et al.*, 2010; Norman *et al.*, 2009). In this network of horizontal gene exchange, conjugal plasmids serve as main mediators, as which they are key elements of community resistance against stresses by carrying sets of genes coding for resistance mechanisms. (Sørensen *et al.*, 2005; Heuer & Smalla, 2012).

If a stress is imposed on a community, different bacterial species might have distinct responses based on the length and dose of exposure. Low exposure levels might serve as a stimulant or signal for the transcription of certain catabolic genes (Pérez-Martín *et al.*, 1996) . Elevated doses may cause expression of stress-response mechanisms. Results of stress at elevated 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). Direct stress response mechanisms include switching to slow growth, biofilm formation or the activation of efflux pumps (Mah & O'Toole, 2001), but an organism might also benefit from gaining a new set of stress resistance genes. Therefore, an indirect potential stress-response mechanism might be an increased permissiveness towards mobile genetic elements carrying useful genes.

Single strain experiments have revealed that horizontal gene uptake can be triggered as a stress response. In *Streptococcus pneumonia*, exposure to antibiotics leads to an increased promiscuity towards foreign DNA via increased competence (Slager *et al.*, 2014). In *Pseudomonas putida* pre-exposure to sodium dodecyl sulfate (SDS) also increased its plasmid receipt and maintenance, possibly by repressing restriction-modification systems (Arango Pinedo & Smets, 2005). Contrarily, in *Escherichia coli* stress imposed on the cell envelope induces the expression of CRISPR associated (CRISPR-cas) genes which would defend against foreign DNA (Perez-Rodriguez *et al.*, 2011), thus decreasing its permissiveness towards plasmids.

A typical environmental stress for soil microbes is caused by the accumulation of heavy metals (e.g. Cu, Zn) due to agronomic practices, industrial activities, or atmospheric deposition (Nicholson *et al.*, 2003; Zhao *et al.*, 2014).

Metal concentrations may reach toxic levels and can affect both composition and function of soil microbial communities (Giller *et al.*, 1998; Gans *et al.*, 2005; Berg *et al.*, 2012). Development of community tolerance to metals is a general consequence and may occur even in weakly contaminated soils when no effects are observed at the level of microbial activity or community composition (Brandt *et al.*, 2010).

At the community level, exposure to selective pressures like elevated metal concentrations was hypothesized to propagate transfer of plasmids encoding adaptive genes (Rensing *et al.*, 2002). Still, the underlying mechanisms and the direct impact of stress on the permissiveness of microbial communities remain poorly understood. Community permissiveness refers here to the fraction of a microbial community able to receive a given plasmid at the quantitative and phylogenetic level (Musovic *et al.*, 2010).

We, hypothesize that stress-response mechanisms affect the permissiveness of a soil bacterial community towards plasmids. We aimed to explore if this expected modulation is a general response to metal stress or if it is metal and/or dose dependent.

Hence, we introduced the model broad host range plasmid pKJK5 into a community extracted from soil. We challenged this community with five environmentally relevant metals (Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, AsO_3^{3-}) at moderate and elevated doses that correspond to 20% and 50% community level growth rate inhibition to compare transfer frequencies and the phylogeny of the transconjugants.

2 Material & Methods

2.1 Soil sampling

Soil was sampled from unfertilized control plots (*n*=3) belonging to the CRUCIAL agricultural field site (Dec 2013, Taastrup, DK) (Poulsen *et al.*, 2013; Lekfeldt *et al.*, 2014). Each plot was sampled for 500 g of soil at 3 locations. The resulting soil volume was sieved through a 1 mm^2 mesh filter and homogenized. The CRUCIAL field experiment was initiated in 2002 using a randomized block design with three replicate blocks of different agricultural treatments on a sandy loamy soil (clay content 12–19%, pH(H_2O) 6.6–7.5, %C 1.1–3.2) (Magid *et al.*, 2006).

2.2 Soil bacterial community extraction

Indigenous bacterial communities were recovered from 30 g of homogenized soil by Nycodenz®-extraction (Klümper *et al.*, 2014a). Extracted bacteria were resuspended in sterile 0.9% NaCl solution, washed, quantified using a Thoma counting chamber and adjusted to appropriate cell densities to quantify metal induced stress and to be used as recipients in the filter mating assay (see below).

2.3 [^{3}H]leucine incorporation inhibition assay

In order to select appropriate metal concentrations ensuring similar degrees of stress imposed by different metals to the total community in our filter mating experiments (see below), we used the [^{3}H]leucine incorporation technique to measure bacterial growth inhibition in buffered suspensions (Lekfeldt *et al.*, 2014). The leucine incorporation approach allows to measure the growth inhibition of the chosen stress dose on the community level by integrating both, responses at sub-toxic and toxic levels (Rousk & Bååth, 2011).

In brief, approximately 10^6 of the extracted soil bacterial cells were amended with 10% liquid soil extract medium (Klümper *et al.*, 2014a) buffered with 5 mM 3-morpholinopropane-1-sulfonic acid (MOPS) at pH 7.2 and supplemented with 20 µg/mL nystatin to avoid fungal growth. Cells were pre-incubated (30 min, 22 °C) in 2 mL micro-tubes with different concentrations of $CuSO_4$, $NiSO_4$, $ZnSO_4$, $CdCl_2$, and Na_2HAsO_3. [^{3}H]leucine incorporation incubations were then initiated by adding 50 µL of a mixture of [^{3}H]leucine (2.59 TBq $mmol^{-1}$, 37 MBq mL^{-1}, Amersham, Hillerød, Denmark) and unlabeled L-leucine to give 6 kBq per microtube and 200 nM L-leucine. Negative

controls were amended with 160 µL of 50% trichloroacetic acid (TCA) before adding [^{3}H]leucine. The incubations were stopped after 4 h by the addition of 160 µL ice cold 50% TCA. The incorporation of [^{3}H]leucine into precipitated proteins was separated from non-incorporated [^{3}H]leucine through a series of washing and centrifugation steps (Bååth *et al.*, 2001) and quantified by scintillation counting (Brandt *et al.*, 2004). The leucine incorporation data were fitted with the 4 parameter Log-logistic model using the drc (Analysis of Dose-Response Curves*)* package for R (Knezevic *et al.*, 2007). The fitted models were then used to estimate, for each metal, the concentrations that caused 20% and 50% bacterial growth inhibition based on [^{3}H]leucine incorporation data (IC_{20} and IC_{50}, respectively) (SI Figure 1) (Rousk & Bååth, 2011).

2.4 Plasmid and donor strain used for filter mating experiments

Cells of *Escherichia coli* MG1655::*Km^R-Lpp-mCherry* hosting the IncP-1ε broad host range plasmid pKJK5::*gfp* were used as donors. Plasmid pKJK5 was previously shown to have an extremely broad transfer range in the soil bacterial community including various Gram negative as well as Gram positive transconjugants (Klümper *et al.*, 2015) allowing to monitor metal stress effects towards a wide variety of bacterial groups. Additionally, pKJK5 does not encode for any known metal related resistance mechanisms (Bahl *et al.*, 2007). Therefore, selection or adaptation processes towards the imposed metal stress through plasmid encoded accessory genes can be ruled out.

The plasmid was marked with an entranceposon (Bahl *et al.*, 2009) derived genetic tag that carries a LacI^q repressible promoter upstream the conditionally expressive *gfpmut3* gene, encoding the green fluorescent protein (GFP). The plasmid donor strain was chromosomally tagged with a gene cassette encoding constitutive red fluorescence, expressed by the *mCherry* gene, and constitutive *lacI^q* production. As a result, *gfp* expression is repressed in the donor strain, but upon successful conjugative transfer to a soil bacterium, plasmid encoded *gfp* expression is possible, resulting in green fluorescent cells or microcolonies, which can be detected and quantified by fluorescence microscopy and retrieved by fluorescence activated cell sorting (FACS), respectively (Sørensen *et al.*, 2005; Klümper *et al.*, 2015). The donor strain was grown overnight in LB-medium supplemented with the plasmid specific antibiotic Trimethoprim (TMP) (30 µg mL^{-1}), harvested by centrifugation,

washed in 0.9% NaCl-solution, adjusted in OD_{600} and used in the filter mating assays as described previously (Klümper *et al.*, 2014a).

2.5 Filter matings

The extracted recipient bacterial community cells were challenged with the exogenous plasmid via solid-surface filter matings (Musovic *et al.*, 2010). Filter matings were used to ensure maximized cell-to-cell contact, as in the original soil environment physical barriers limit contact of freshly introduced plasmid donors and potential recipients (Dechesne *et al.*, 2005). Compared to the original mating protocol (Klümper *et al.*, 2014a) the initial ratio of donor to recipient bacteria was increased to 10:1 This modification was implemented to ensure that even if the donor cells were partially growth-inhibited, each recipient microcolony were in contact with at least one donor after 48h incubation. Filter matings were initiated with a bacterial density of 30,000 bacteria/mm^2.

The same medium used for the [^{3}H]leucine incorporation assay (10% soil extract medium) was supplemented with 15 g/L agar and used as solid medium in this assay. Neither donor strains nor the recipient community were pre-exposed to metal stress before the filter matings. For the stress exposure experiments, the medium was amended with $CuSO_4$, $NiSO_4$, $ZnSO_4$, $CdCl_2$ or Na_2HAsO_3 at the IC_{20} and IC_{50} concentrations previously determined. Filter matings lasted for 48 hours at 25 °C followed by storage at 4 °C for 72 hours for maturation of *gfp*. Successful conjugation was subsequently checked by epifluorescence stereo microscopy and confocal laser scanning microscopy (CLSM) (Musovic *et al.*, 2010).

2.6 Transfer frequency quantification

Successful plasmid transfer was visualized by stereomicroscopy and quantified by automated image analysis (Image Pro Plus 7.1; Media Cybernetics, Silver Spring, MD) as previously described (Musovic *et al.*, 2010), using a Leica MZ16 FA fluorescence stereomicroscope equipped with a 10 × plan apochromatic objective, a 10 × eyepiece (10x/21B), and a 40 × magnification zoom. Donor and transconjugant fluorescence detections were based on excitation at 480/20 nm with emission at 525/40 nm (*gfpmut3*) and excitation at 580/25 with emission at 650/60 nm (*mCherry*), respectively. Images were acquired with a Leica DFC300 fluorescence camera. A representative scanning area of 40 randomly chosen microscopic fields of 980 µm times 732 µm each, corresponding to approximately 11% of the total filter area, was ana-

lyzed per filter. Triplicate filters were analyzed for each metal concentration. Quantification of transfer events was performed with a custom-made macro written in Image Pro Plus 7.1. This macro successively extracts and subtracts the background from the original image, performs a best-fit equalization of the image intensity, before detecting bright objects larger than 4 μm^2 based on automatic segmentation (Klümper, *et al.*, 2014b). Analysis of images was limited to the brightly illuminated elliptic central area of the field of view (Klümper *et al.*, 2014b). The number of *gfp*-positive colonies (transfer events) detected was scaled up to the total filter area and transfer frequency was calculated by dividing this number by the number of originally introduced recipients.

2.7 Fluorescent activated cell sorting (FACS)

For each mating condition, cells from 3 replicate filters were combined in 2 mL 0.9% NaCl-solution by vortexing for 3 minutes. Flow cytometric detection of cells was carried out using a FACSAria IIIu (Becton Dickinson Biosciences, San Jose, CA). The following settings and voltages were used during analysis: forward scatter (FSC) = 505 V, side scatter (SSC) = 308 V, and detectors for green (BP filter 530/30 nm) and red fluorescence (BP filter 610/20 nm) were set at 508 V and 500 V, respectively. A 70 µm nozzle was used at a sheath fluid pressure of 70 psi. The BD FACSDiva™ software v6.1.3 was used for both operating and analyzing results. Cell sorting was performed using a 488 nm (20 mW) laser connected to the green fluorescence detector at 515-545 nm and a 561 nm (50 mW) laser connected to the red fluorescence detector at 600-620 nm. Gating and sorting of transconjugants for each of the combinations was performed based on bacterial size, green fluorescence and exclusion of red fluorescent donor cells as described earlier (Klümper *et al.*, 2015). The recipient community members were also sorted for using the same conditions, but excluding green fluorescence in the gating, therefore only excluding all red fluorescent donor bacteria from the filter mating, while green transconjugants and colorless recipient bacteria which did not engage in conjugation, were sorted for. In all cases, a minimum of 20.000 *gfp* expressing transconjugal or soil recipient cells were sorted.

2.8 Bacterial cell lysis, amplification and sequencing

Sorted bacterial cells were collected by centrifugation at 7.000 rpm for 45 min. The supernatant was carefully removed, and the cell pellet was suspend-

ed in 20 µL of Lyse and Go PCR Reagent (Thermo Scientific, Waltham, MA, USA). Cell lysis was subsequently performed in an Arktik™ Thermal Cycler (Thermo Scientific, Waltham, MA, USA) using a program consisting of an initial step at 57 °C for 30 s, a second phase at 8 °C for 30 s, a third phase at 65 °C for 90 s, a fourth phase with heating to 97 °C for 3 min, a fifth phase with cooling to 8 °C for 60 sec, a sixth phase with heating to 65 °C for 3 min followed by additional heating to 97 °C for 60 s and cooling to 65 °C for 60 s with a final end-step at 80 °C. Cell lysis products were immediately put on ice and 2-8 µL of the lysis products from the previous step were used directly as template for the initial amplification step in sequencing library preparation (see below). Additionally, sequencing libraries from DNA directly extracted from the soil sample (PowerSoil® DNA Isolation kit, MoBIO, Denmark), from soil bacterial cells harvested after Nycodenz extraction.

Tag-encoded 16S rRNA gene amplicon sequencing was carried out using an initial amplification of the V3 and V4 regions of the 16S rRNA gene (Neefs *et al.*, 1990) with primers; 341F: 5′-CCTAYGGGRBGCASCAG-3 and 806R: 5′-GGACTACNNGGGTATCTAAT-3 (Yu *et al.*, 2004). A second round of amplification was performed on 2 µL of undiluted products from the initial PCR using primers; 515F: 5'-GTGCCAGCMGCCGCGGTAA-3 and 806R: 5′-GGACTACNNGGGTATCTAAT-3, both fused with adapters and commercially available index tags, which flank the V4 region of the 16S rRNA gene (Neefs *et al.*, 1990). Amplified amplicon fragments were purified by Agencourt AMPure XP beads (Beckman Coulter, CA, USA), and diluted to an equal molar concentration before they were pooled. The pooled sample was concentrated with the DNA clean and concentrator-5 kit (Zymo Research, CA, USA), adjusted to a final concentration of 4 nM and sequenced on an Illumina MiSeq instrument using a MiSeq reagent kit v2 for 2x250 bp Paired End sequencing, according to the manufacturer's instructions.

2.9 Sequence analysis and statistics

Sequence analysis was carried out using mothur v.1.32.1 (Schloss *et al.*, 2009) and the MiSeq SOP (Kozich *et al.*, 2013) as accessed on 01.12.2014 on http://www.mothur.org/wiki/MiSeq_SOP. Sequences were classified based on the RDP classifier (Wang *et al.*, 2007). Sequences are submitted under accession number XXX-XXX. Diversity was assessed based on observed OTUs at 97% sequence similarity and non-statistically significant linear regression was carried out. PCoA plots were designed based on the theta Yue Clayton diversity index (Yue & Clayton, 2005) using mothur software and

clustering of samples was tested for significance by analysis the molecular variance (AMOVA) (Excoffier *et al.*, 1992). Principle component analysis (PCA) was performed in R using the package ade4 (Dray & Dufour, 2007).

3 Results

3.1 Metal stress reduces transfer frequencies independent of activity reduction

We challenged a bacterial community extracted from soil with model broad host-range plasmid pKJK5 under metal stress. Stress was defined based on community level growth inhibition. Inhibitory concentrations of 20% and 50% growth inhibition (IC_{20} & IC_{50}) were determined (SI Figure 1) to define the doses of metal treatments for filter mating experiments (Table 1). Metal toxicity increased in the following order:

$$AsO_3^{3-} < Zn^{2+} < Cd^{2+} < Cu^{2+} < Ni^{2+}$$

Filter matings under metal stress conditions will subsequently referred to as a combination of the element symbol and the inhibitory concentration it was used at (e.g. As20 see Table 1) and compared to a non-stressed reference.

Table 1: Inhibitory concentrations causing 20% and 50% bacterial growth inhibition (IC_{20} and IC_{50}, respectively) as extrapolated from [^{3}H]leucine incorporation data (SI Figure 1). Abbreviations in brackets will be used throughout the paper to refer to results from filter matings under the diverse metal stress conditions.

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)

Conjugation events were detected through *gfp* expression of transconjugal microcolonies by epifluorescence microscopy. On all of representative images of filter matings at reference and metal stress conditions (Figure 1) the red fluorescent donor strains were detectable. The final amount of donors decreased slightly under stress conditions. Still, image analysis in combination with flow cytometric counts revealed that the high donor concentrations applied ensured that each recipient microcolony was in contact with multiple donor cells.

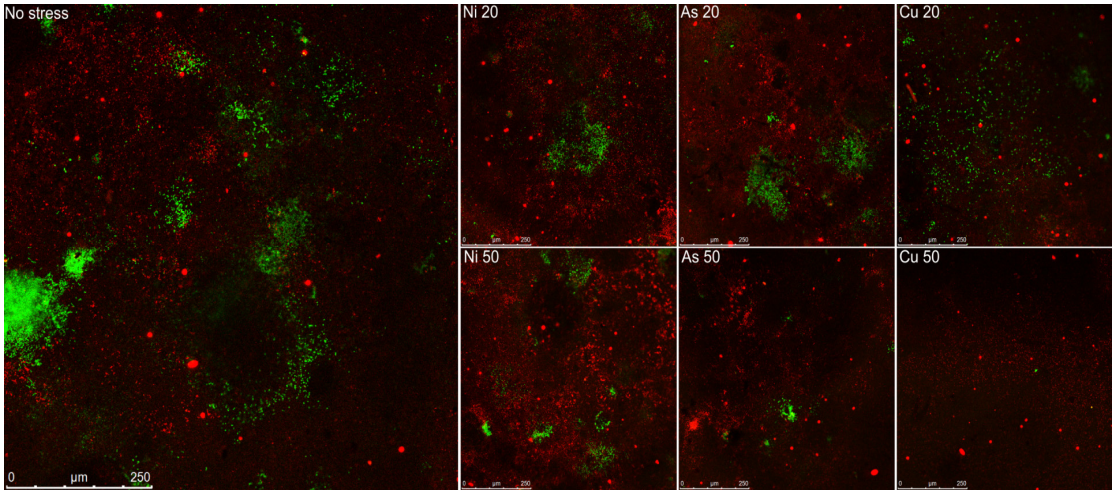

Figure 1 Confocal laser scanning microscopy images of plasmid transfer from an *E. coli* MG1655 donor strain to soil bacteria under reference conditions and under metal stress. Donor bacteria are shown in red based on a chromosomal *mCherry* tag, while plasmid encoded *gfp* expression was repressed in these cells. Recipient bacteria were colorless until successful plasmid receipt and subsequent *gfp* expression. Transconjugants therefore appear green. Metal induced stress conditions were defined based on the ability of the different metals to inhibit [^{3}H]leucine incorporation rates by 20% or 50% as indicated in the top left corner of each image.

gfp expressing transconjugants were detected at high frequencies under reference conditions, but decreased visibly in number under stress conditions for all applied metals (Figure 1). Image analyses revealed that 6.98 x 10^{-5} of the originally introduced bacteria were able to take up plasmid pKJK5 under reference conditions (Figure 2).

The decrease in permissiveness caused by metal stress was metal and dose specific. The number of green fluorescent transconjugant microcolonies significantly decreased ($p<0.05$) for all of the metals at both IC_{20} and IC_{50}. Furthermore, reduction in transfer frequency compared to the reference mating exceeded decrease in community growth of 20% or 50% respectively. Therefore, at the community level, plasmid receipt is quantitatively more sensitive to immediate metal stress exposure than growth. The normalized stress levels (20% or 50% growth inhibition) resulted in variable inhibition of plasmid transfer for different metals. The strongest reductions in transfer frequency were found for Cu50 and Zn50, when community permissiveness decreased by more than 90%. For Cd50 no transconjugants were detected.

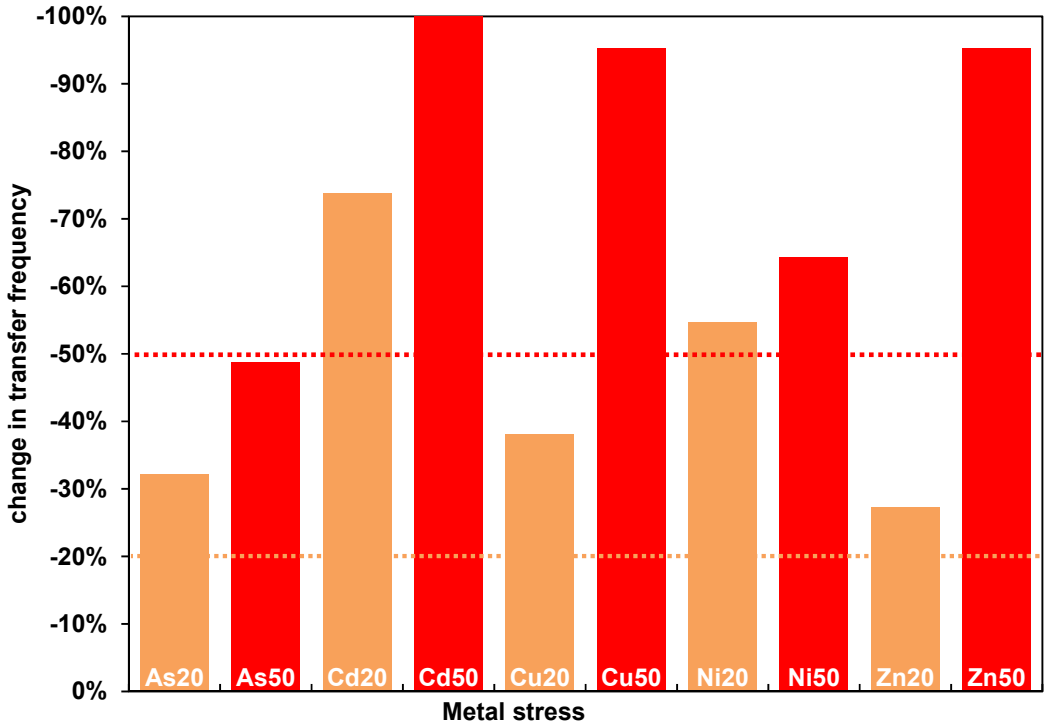

Figure 2 Reduction of transfer frequency under stress. Normalized plasmid transfer frequencies for each of the five tested metals at their respective IC_{20} (orange) and IC_{50} (red) and the non-stressed reference were calculated based on the analysis of 90-150 images each. Metal stress was defined based on the ability of the different metals to inhibit [^{3}H]leucine incorporation rates by 20% (orange) or 50% (red) as indicated by dotted lines.

3.2 Diversity of the permissive fraction is maintained in spite of transfer frequency reduction

The observed reduction in permissiveness towards plasmids could either be the result of a general decrease of transfer receipt across taxa or be caused by the disappearance of specific taxa from the transconjugal pool.

Transconjugant cells expressing *gfp* (Figure 1) were isolated from the mating mixture by FACS. Despite their low relative abundance, we were able to isolate at least 20,000 transconjugants for each condition for subsequent phylogenetic analysis. An exception was Cd50, since no transfer was detected at this stress level.

The phylogenetic richness of the transconjugal pools, subsampled to 50,000 sequences, was stably maintained at around 190 observed OTUs (97% sequence similarity) irrespective of how much transfer frequencies were re-

duced (p=0,798) (Figure 3). Consistently, 13 transconjugal phyla were identified in the diverse pools including the dominant Proteobacteria and Bacteroidetes, as well as other Gram-negative (Acidobacteria, Nitrospira, Fusobacteria, Planctomycetes, Gemmatimonadetes, Verrucomicrobia), diverse Gram-positive (Firmicutes, Actinobacteria, Chloroflexi) and two candidate phyla (BRC1, TM7) (SI figure 2). This adds two additional phyla (Nitrospira, BRC1) to plasmid pKJK5's transfer range compared to an earlier study (Klümper *et al.*, 2015). All transconjugal pools were sequenced at more than 98% coverage, indicating that the diversity of transconjugants is mapped completely.

Even though the permissiveness towards plasmid receipt was reduced by up to 95%, the diversity of transconjugal pools remained constant. Whereas under reference conditions only about 40% of the OTUs of the recipient community were detected as permissive to pKJK5, this ratio changed to 70-98% when the community was stressed (Figure 3). This increased ratio resulted from a significant decrease (p= 0.0053) in richness of the recipient community under stress conditions.

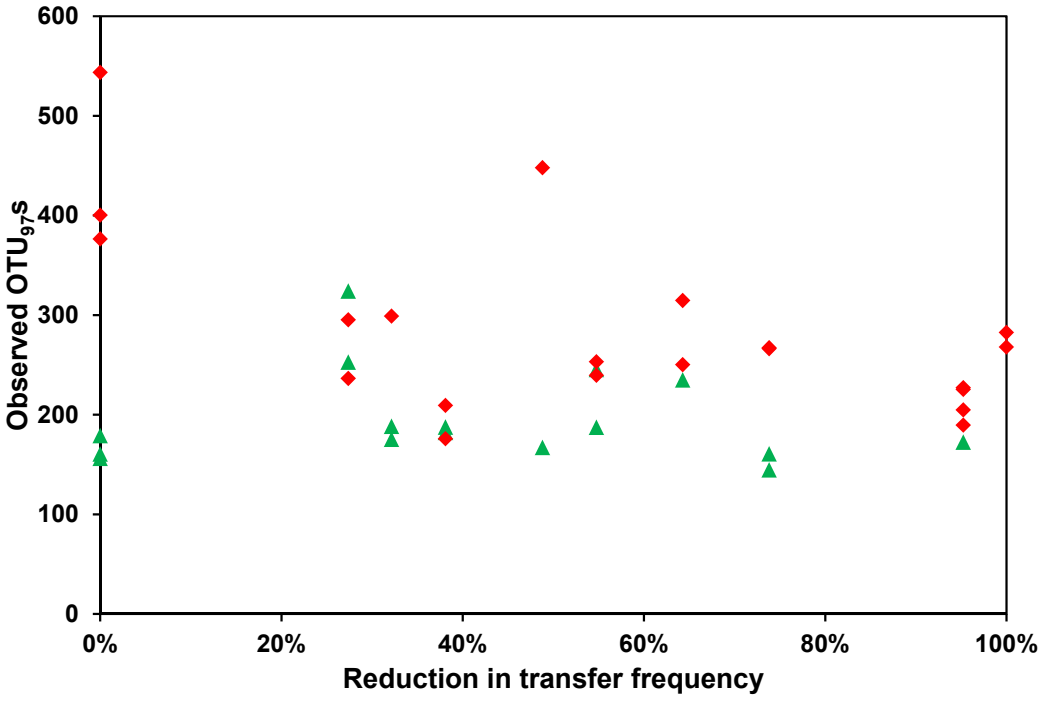

Figure 3 Number of observed OTUs (at 97% sequence similarity) as a function of metal stress induced reduction in transfer rate. The soil recipient communities (red) are shown versus the transconjugal pools (green).

Due to stress, either members of the recipient community unable to receive plasmids are lost, and/or the remaining OTUs became permissive towards plasmid receipt under stress conditions. More than 50% of the OTUs found in the soil reference community are lost under stress conditions. For the 9 stress conditions tested, between 94% and 97% of the transconjugal sequences belonged to OTUs which were found in the reference transconjugal pool. Together, these findings indicate that OTUs that are permissive to plasmids are positively biased in their ability to resist stress conditions.

3.3 Transconjugal communities cluster based on introduced stress

The phylogenetic richness of the transconjugal pools under stress conditions remained unchanged. We tested if the phylogenetic structure of the transconjugal pools was altered by stress.

Multidimensional analysis showed that all transconjugal pools clustered significantly apart from their respective recipient communities (Figure 4). This finding was supported by subsequent AMOVA (Excoffier *et al.*, 1992) ($p<0.001$).

Some OTUs present in the soil recipient communities were not permissive to plasmid pKJK5 under any of the tested conditions. These OTUs could cause the aforementioned clustering of the permissive transconjugal pools apart from their respective reference communities. Hence, they were removed from the soil recipient communities before PCoA analysis. Transconjugal pools still clustered apart from the reference communities, demonstrating that their phylogenetic composition is not a random subset of all recipient OTUs, but a function of the varying permissiveness of different OTUs.

Three clusters were observed among the stress-associated transconjugal pools ($p<0.001$) (Figure 4). A first cluster, comprised of the Cu50, Cd20, Ni20 and Ni50 replicate pools grouped significantly apart ($p<0.001$) from the reference pools. Replicate transconjugal pools obtained under Cu20 formed a separate cluster, with a phylogenetic composition significantly different ($p<0.05$) to pools obtained at any other stress. As20, As50 and Zn20 did not display any significant shifts in the transconjugal pool composition based on PCoA. Hence the latter plus the reference pool constituted a third cluster.

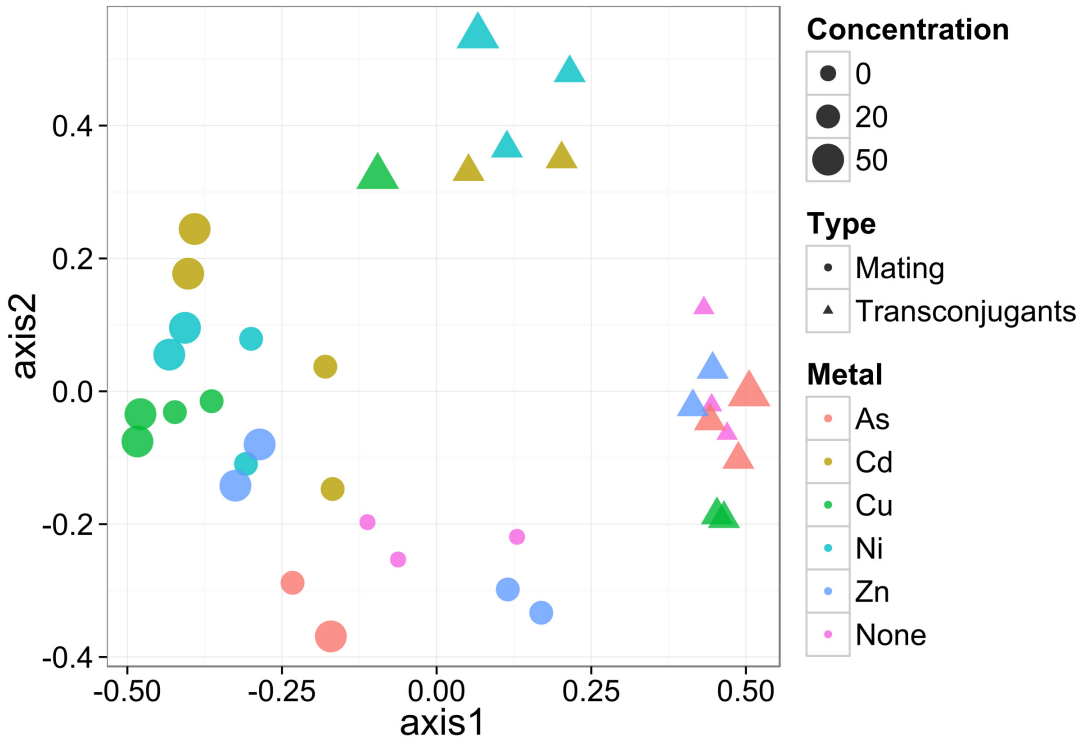

Figure 4 PCoA plot based on Theta Yue Clayton (Yue & Clayton, 2005) algorithm of weighted OTU abundance. Recipient communities (triangles) vs. transconjugal pools (circles) under different metal stress (color coded) at 20% and 50% inhibitory concentrations (size of the symbol). Sequencing pools were subsampled at 50.000 sequences. OTUs from the recipient pool that did not result in any transconjugants in any of the transconjugal pools were removed prior to ordination.

Replicates of different pools grouped with each other and were always found in the same cluster. Based on PCoA grouping and because the number of replicates per condition differed, replicates were pooled for subsequent phylogenetic analysis.

Furthermore, no subsampling effect was observed. For PCoA transconjugal pools were subsampled to 50,000 sequences. Different pools subsampled 10 times to a depth of 50,000 showed no significant dissimilarity ($p>0.99$) based on weighted UNIFRAC comparison.

3.4 Stress specific responses are resolved by transconjugal taxonomy

Almost all recipient OTUs are represented in the transconjugal pools under stress conditions (Figure 3). This fact, together with the observed differences in phylogenetic structure of transconjugal pools (Figure 4), can be explained

by two potential reasons. Metal stress induced shifts of the recipient community, or modulated permissiveness of specific bacterial OTUs as a stress response. Therefore, we separately analyzed if increased or decreased permissiveness of single OTUs occurred as part of the stress response and if stress specific patterns occur in their response.

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

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

Our quantification method does 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 5) 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 5).

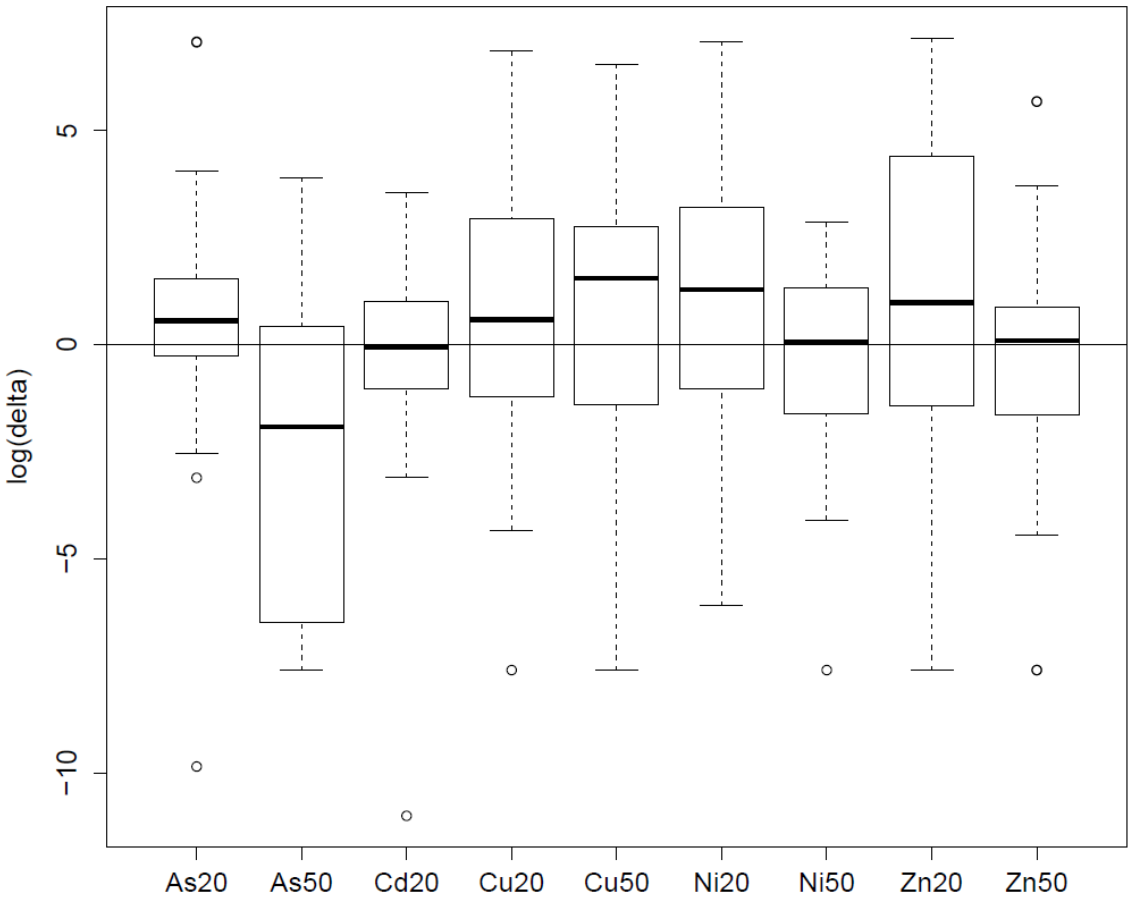

Figure 5 Boxplot diagram showing the distribution of δ values for the 39 most abundant OTUs in each transconjugal pool on the logarithmic scale. The line represents the complete absence of metal effect on permissiveness (all δ = 1).

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 6). All OTUs belonging to the phylum Bacteroidetes show a high degree of similarity in their response to different stress scenarios.

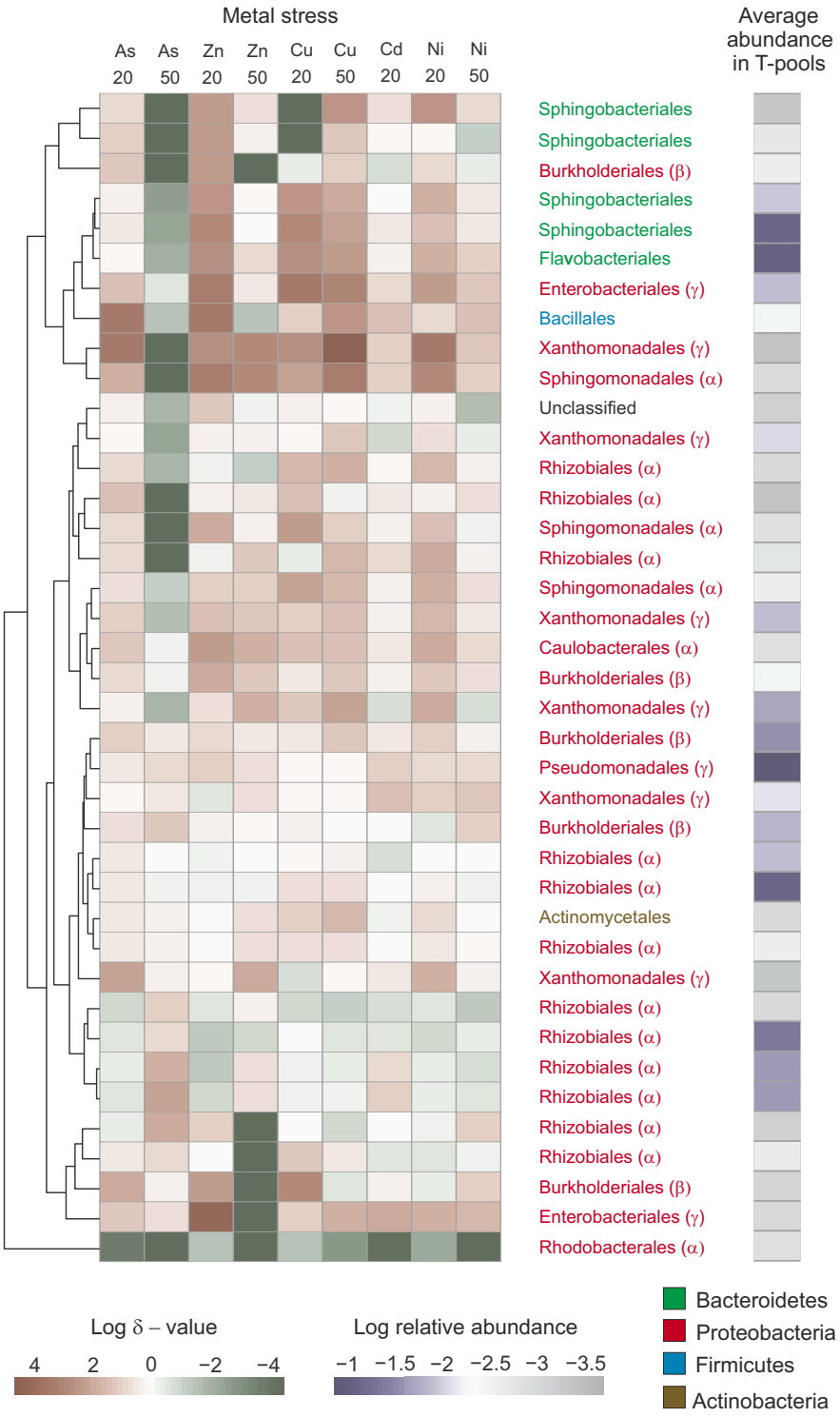

Figure 6 Heatmap showing the log scaled δ value of stress-imposed fold difference of an OTU's relative abundance due to stress. The 39 most abundant (>0.05%) OTUs, their relative abundance in log scale in violet as well as their phylogeny are shown (for Proteobacteria their class is shown in brackets) and sorted in a maximum likelihood tree based on their plasmid receipt dependent responses to stress at IC20 and IC50 through different metals. An increased plasmid receipt 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 1). 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. Rhodobacteriales, on the other hand, remarkably decreased their plasmid receipt under any applied stress conditions.

4 Discussion

Plasmids introduced to soil microbial communities can spread among an extremely wide variety of gram-negative and gram-positive bacterial species (De Gelder *et al.*, 2005; Shintani *et al.*, 2014; Musovic *et al.*, 2014; Klümper *et al.*, 2015). Former studies reported elevated levels of plasmids and plasmid encoded genes in soil microbial communities subject to selective pressure (Agersø *et al.*, 2006; You *et al.*, 2012; Heuer *et al.*, 2011). However, these elevated levels might result from increased permissiveness towards plasmids or from selective growth of plasmid bearing strains.

Here, for the first time, we resolve how metal induced stress directly modulates the permissiveness of a soil community towards a broad host range plasmid at the community, as well as the individual transconjugal species level.

Plasmid pKJK5 served as a model plasmid, since it does not encode for any known metal related resistance mechanisms (Bahl *et al.*, 2007). Therefore, selection or adaptation processes towards the imposed metal stress through plasmid encoded accessory genes can be ruled out, ensuring that our observations correspond to stress response affecting the general permissiveness of the recipient bacteria. pKJK5 has an extremely broad transfer range in soil bacterial community (Klümper *et al.*, 2015) allowing to monitor metal stress effects on the permissiveness of a wide variety of bacterial groups. Third, pKJK5 is a de-repressed plasmid with constitutive pilus synthesis (Bahl *et al.*, 2007). Therefore, plasmid transfer is independent of the donor's regulatory machinery, as long as sufficient donor cells are alive, which was the case in all our mating conditions.

Quantification of plasmid transfer revealed that more than 1 out of 20.000 indigenous soil bacteria can engage in plasmid transfer. These numbers are comparable to earlier studies on the permissiveness of diverse soil bacterial communities with different broad host range plasmids (Musovic *et al.*, 2014). Under all stress conditions, plasmid transfer frequency decreased significantly. A similar decrease of a metal resistance conferring plasmid's transfer frequency to a soil community were found under Zn stress (De Rore *et al.*, 1994). We showed here that this decrease at the community level seems to be general, independent of the type and dose of metal stress. Further, inhibition of permissiveness towards plasmids was even higher than the inhibition of bacterial growth at which the metal stresses were applied. It can therefore be

assumed that immediate metal stress, as applied here, has no general positive effect on the transfer frequency of exogenous plasmids in the soil environment at the community level. Microcosm experiments however indicated that heavy metal exposure increased the retromobilization frequencies of mobilizable plasmids (Top *et al.*, 1995). Moreover it allowed increased transfer as well as long-term retention of non-metal resistance coding plasmid RP4 (Smets *et al.*, 2003). The maintenance of conjugative plasmids might therefore very well be part of a stress-response as they allow for retromobilization and acquisition of a new gene pool.

Although studied for decades, we still have an incomplete understanding of the toxicity mechanisms underlying metal stress. However, several general and specific mechanisms are described. The cationic metals used in this study (Cd, Cu, Ni, Zn) are known to disrupt iron-sulfur clusters of metalloenzymes (Macomber & Hausinger, 2011; Macomber & Imlay, 2009; Xu & Imlay, 2012). Ni and especially copper have additionally been shown to indirectly cause oxidative stress, due to their ability to take part in single electron transfer reactions (Macomber & Hausinger, 2011). The uptake of metal ions such as Cu, Ni or Zn is essential as micronutrients for vital cell functions. Contrarily, Cd and As are not essential, but interfere with the uptake of essential metal ions (e.g. Cd for Zn and As for P). All bacteria have a certain tolerance level to metal stress. Thus, for a given exposure, a gradient of stress levels ranging from sub-toxic to toxic or even lethal can exist within the community (Rensing *et al.*, 2002). Due to the multitude of metal toxicity and resistance mechanisms, it is safe to assume that although introduced at the same community growth inhibition levels each element causes a diverse specific stress response in individual community members. Our work allows making observations and conclusions at both, the community level as well as the level of single members of the community.

We demonstrated here that a modified permissiveness is indeed unique for each bacterial OTU under stress conditions. Furthermore, 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. Community averaged analysis therefore draws an incomplete picture of the extent and modification of plasmid transfer through environmental stress.

The regulatory mechanisms 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.

Single strain studies reported that the ability of foreign DNA receipt can be increased or decreased as a response to stress and is very specific towards the kind of stress applied (Arango Pinedo & Smets, 2005; Pérez-Mendoza & de la Cruz, 2009). These responses might be closely connected to the regulation of mechanisms involved in the defense against foreign DNA, such as restriction-modification (RM) or CRISPR-cas systems. The expression of RM genes is not constant, but relies on environmental conditions (Bayliss *et al.*, 2006), therefore stress might very well play a role in their regulation. Cells with a turned off RM system were shown to become hypersusceptible to foreign DNA receipt (Corvaglia *et al.*, 2010) with more than 7-fold increased plasmid uptake (Roer *et al.*, 2015). While the different regulatory control systems for CRISPR-cas systems are not well understood (Mojica & Díez-Villaseñor, 2010), specific stress responses can lead to induced expression of CRISPR-cas genes, decreasing the plasmid receipt potential (Perez-Rodriguez *et al.*, 2011). We demonstrated that the stress-response mechanisms affecting the permissiveness of strains show a significant degree of similarity among some phylogenetically related groups. Therefore, extrapolation from the aforementioned single strain experiments to phylogenetically related groups of bacteria might be possible.

In spite of the observed decrease in permissiveness of the whole community, plasmid transfer across the phylum border from the gammaproteobacterial donor *E. coli* to Bacteroidetes was increased under specific metal stress conditions. This increased permissiveness becomes additionally interesting as model plasmid pKJK5 hosts a Class 1 integron gene cassette (Heuer *et al.*, 2012). These integrative elements are commonly found on plasmids and associated with antibiotic resistance gene integration into the chromosome of their originally identified Gram-negative hosts as well as many Gram-positive bacteria (Nandi *et al.*, 2004). The activation of transposons and integrating conjugative elements is well known as part of the SOS stress response machinery in multiple bacteria (Hastings *et al.*, 2004; Beaber *et al.*, 2004). Thus, increased recombinatory probabilities of plasmids acquired under stress conditions with the host genome can provide a direct connection of the genome and thus the resistome of diverse phyla in soil.

5 Conclusions

We demonstrated that a modified permissiveness is unique for each bacterium under stress conditions. 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. 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.

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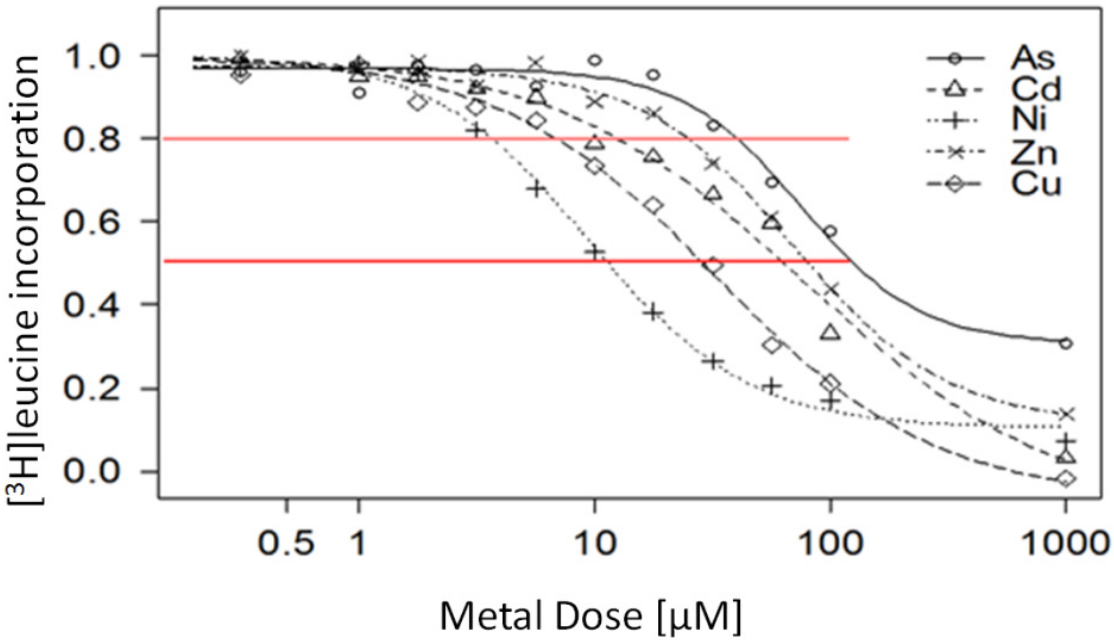
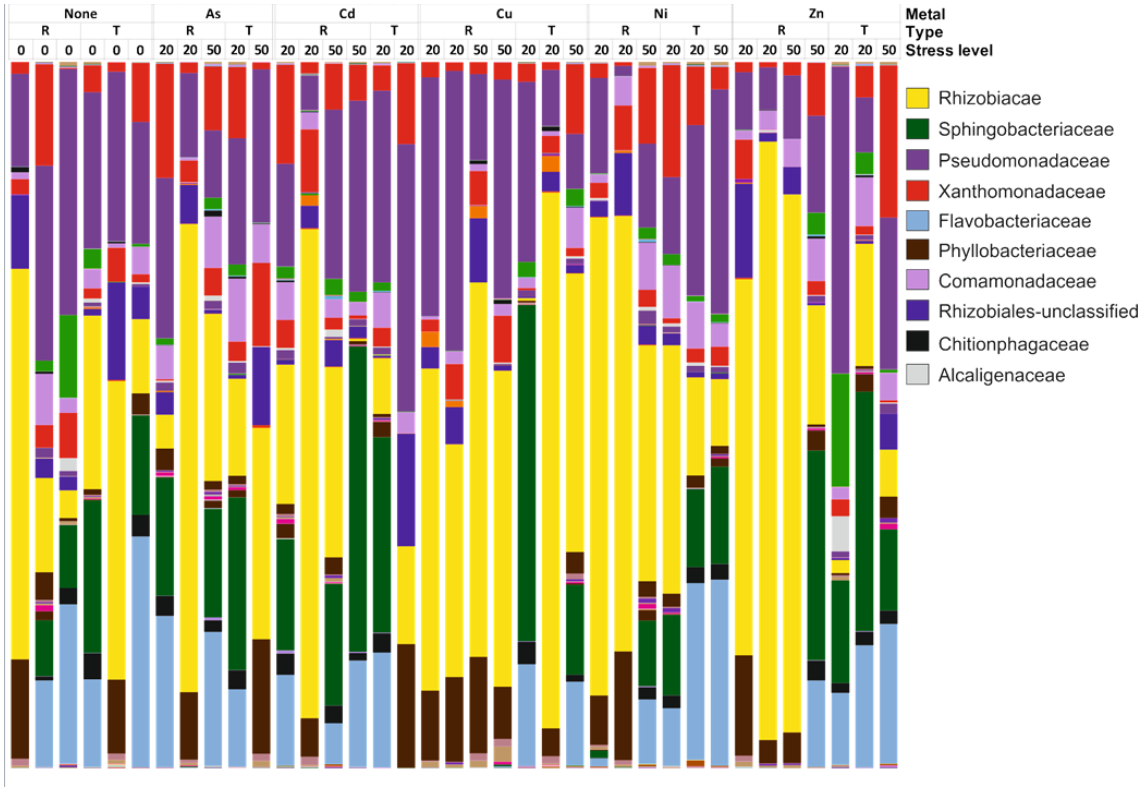
Supporting Information



SI Figure 1 Dose-Response curves for all 5 metals used in the study. Metal ion concentrations (Dose) are shown in µM. Reponse = 1 equals leucine uptake under no stress conditions. Response = 0 corresponds to the sorption of leucine to the dead negative controls. The leucine incorporation data were fitted with the 4 parameter Log-logistic model using the drc (Analysis of Dose-Response Curves) package for R (Knezevic et al., 2007). The fitted models were then used to estimate, for each metal, the concentrations that cause 20% and 50% growth inhibition (red lines).



SI Figure 2 Diversity observed in the different transconjugal pools

V

Long-term manure exposure increases soil bacterial community potential for plasmid uptake

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Long-term manure exposure increases soil bacterial community potential for plasmid uptake

Sanin Musovic,[1] Uli Klümper,[1] Arnaud Dechesne,[1] Jakob Magid[2] and Barth F. Smets[1]*

[1]*Department of Environmental Engineering, Technical University of Denmark, Kgs. Lyngby, Denmark.*

[2]*Department of Agriculture and Ecology, Plant and Soil Science, Faculty of Life Sciences, University of Copenhagen, Frederiksberg C, Denmark.*

Summary

Microbial communities derived from soils subject to different agronomic treatments were challenged with three broad host range plasmids, RP4, pIPO2tet and pRO101, via solid surface filter matings to assess their permissiveness. Approximately 1 in 10 000 soil bacterial cells could receive and maintain the plasmids. The community permissiveness increased up to 100% in communities derived from manured soil. While the plasmid transfer frequency was significantly influenced by both the type of plasmid and the agronomic treatment, the diversity of the transconjugal pools was purely plasmid dependent and was dominated by β- and γ-*Proteobacteria*.

Rapid adaption of bacterial communities to changing environmental conditions is believed to rely on lateral transfer of mobile genetic elements, such as plasmids, as an indispensable mechanism (Sørensen *et al.*, 2005; Thomas and Nielsen, 2005; Heuer and Smalla, 2012). One of the crucial parameters that determines the extent of conjugal plasmid transfer is community permissiveness, defined as the fraction of a microbial community able to receive a given plasmid (Musovic *et al.*, 2010). In agricultural soils, seasonal application of manure and fertilizers provokes intense and immediate changes to soil physical-chemical conditions that might modulate horizontal gene transfer (HGT). Indeed, both an increased nutrient availability (van Elsas *et al.*, 2003) and the introduction of selective stressors like metals or antibiotics (Newby and Pepper, 2002; Heuer *et al.*, 2011) may lead to increased rates of plasmid transfer. Apart from this immediate stimulation of HGT, it remains unknown whether agronomic soil treatment may have long-term effects on permissiveness. To test this, we investigated permissiveness towards three broad-host-range plasmids (Table 1) in four communities isolated from agricultural soils subjected to different long-term seasonal treatments (Table 2).

Microbial communities from three plots [untreated, manured and nitrate-phosphate-potassium-fertilized (NPK)], at the long-term Closing the Rural Urban Nutrient Cycle (CRUCIAL) experimental site (Taastrup, Denmark) (Magid *et al.*, 2006; Poulsen *et al.*, 2013b), were sampled (SI2) in order to assess permissiveness. Agricultural treatments at the field site were in place for 8 years at the time of sampling. Manure was derived from a conventional dairy cow farm. The estimated accumulative applications of C-N-P-K were estimated at 62800-3768-1184-2979 (manured), 0-840-120-400 (NPK) and 0-0-0-0 (untreated) kg ha^{-1} respectively (Magid *et al.*, 2006).

As reference, soil from an untreated plot of the well-known Rothamsted Park Grassland (Rothamsted, United Kingdom) (Silvertown *et al.*, 2006) site was included. From the chosen soils, indigenous bacterial communities were isolated by Nycodenz® extraction (Nycomed, Zürich, Switzerland) (Musovic *et al.*, 2010) and challenged with exogenous plasmids via solid-surface filter matings (Musovic *et al.*, 2010). *Pseudomonas putida* KT2442 (Bagdasarian *et al.*, 1981) served as the plasmid donor strain (Table 1). Conjugation events were detected by epifluorescence microscopy, allowing their quantification (Musovic *et al.*, 2010).

For all tested combinations, community permissiveness ranged from 8.24×10^{-5} to 4.56×10^{-4} conjugations per recipient (Fig. 1). Permissiveness was consistently higher (up to three times) for the soil-derived pIPO2tet plasmid than for the other two IncP plasmids, RP4 and pRO101. Therefore, for both sampling sites, the introduced plasmid significantly influenced the permissiveness ($p_{CRUCIAL} < 0.001$; $p_{Rothamsted} < 0.001$). Additionally, in CRUCIAL soils, a significantly higher permissiveness was measured for the manure-treated community compared with the untreated control ($p_{RP4} = 0.041$; $p_{pIPO2tet} = 0.001$). Previous studies have suggested that manure treatment may result in hot spots of gene transfer because of increased nutrient availability and cell density (van Elsas *et al.*, 2003), with

Received 27 August, 2013; revised 21 October, 2013; accepted 5 November, 2013. *For correspondence. E-mail bfsm@env.dtu.dk; Tel. +45 45252230; Fax +45 45932850.

Table 1. Plasmids[a] carried by the red fluorescent donor strain, *Pseudomonas putida* KT2442 *DsRed*::*lacIq*.

Plasmid	Inc-group	Type	Phenotype	Host range	References
RP4::P*lac*::*gfp*	IncP-1α	Resistance	Tet^R, Amp^R, Km^R	broad	Barth and Grinter (1977)
pIPO2tet::P*lac*::*gfp*	(IncQ-mobilizer)	Cryptic	Tet^R	broad	Tauch *et al.* (2002)
pRO101::P*lac*::*gfp*	IncP-1β	Catabolic	Tfd^+, Hg^R, Tet^R	broad	Harker *et al.* (1989)

a. Plasmids are tagged using a TN5 cassette with a constantly expressed *gfp* gene that is *lacI* repressed in the donor strains.

transfer frequencies increasing by up to one order of magnitude (Götz and Smalla, 1997). However, this does not explain the observed stimulation of permissiveness by manuring because, in our assay, heterogeneities in nutrient availability or cell density were excluded as matings were carried out under standard nutritional conditions on soil extract medium (Musovic *et al.*, 2010). While raised levels of plasmid shuttled tetracycline resistance genes were shown to occur in soils treated with piggery manure slurry (Agersø *et al.*, 2006) or chicken waste (You *et al.*, 2012) in selective and enriching environments, this study is the first to indicate that long-term manure treatment also changes the community permissiveness towards newly introduced plasmids under neutral conditions.

Nutrient addition has previously been shown to enhance gene transfer frequencies in soil (Smets *et al.*, 1995; Nielsen and van Elsas, 2001). To study the effect of fertilization, we investigated the permissiveness of RP4 in the NPK-fertilized soil bacterial community. Permissiveness in the NPK-treated soil was similar to the untreated control ($P = 0.79$) and significantly lower than in the manured ($P = 0.016$). Therefore, higher activity through previous nutrient addition is not the reason for increased permissiveness in manured soil. It thus seems that it appeared not due to immediate nutrient effects or increased cell density in hot spots. However, increased permissiveness might have been intrinsic to the community. Hence, we examined the diversity of the transconjugal pools to test whether they differ between soil treatments.

Random transconjugants were isolated from the matings using micromanipulation (Musovic *et al.*, 2010) to analyse, if apart from increased transfer frequencies, the phylogenetic composition of the transconjugal pool changed due to long-term manure application. Successfully isolated transconjugants were subjected to 16S ribosomal RNA (rRNA) sequencing (Musovic *et al.*, 2010). Sequences were analysed using MOTHUR v.1.30.0 (Schloss *et al.*, 2009) and the SILVA database (Quast *et al.*, 2013). The sequences have been submitted to GenBank and can be accessed under number KF590708 – KF591079.

Principal coordinate analysis (PCoA) revealed transconjugal pools separating primarily by introduced plasmid (Axis 1, Fig. 2). Independent of sampling site or treatment, pRO101, RP4 and pIPO2tet were associated with significantly different transconjugal pools as revealed by analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) ($P = 0.007$). Surprisingly, the number of operational taxonomic units (OTUs) was lowest for pIPO2tet (Supporting Information Table S1), the plasmid exhibiting the highest permissiveness. Although the effective phylogenetic host range in the tested soils appears smallest for pIPO2tet, it might exhibit higher transfer frequencies, resulting in a higher total permissiveness, possibly because of its nature as cryptic plasmid (van Elsas *et al.*, 1998). High transfer frequencies are especially important for the maintenance of those plasmids not conferring any beneficial traits to their hosts.

The second dimension of PCoA separated all transconjugal pools from the CRUCIAL site from those from Rothamsted ($p_{RP4} < 0.001$; $p_{pIPO2tet} < 0.001$). This separation likely derives from differences in their original bacterial community composition. Meanwhile, an earlier study – comparing deeply sequenced 16S rRNA community libraries – revealed no major difference in the total

Table 2. Origin of the recipient microbial communities.

Soil origin	Soil type	Soil treatment	Coordinates	References
CRUCIAL Taastrup Denmark	agricultural	Untreated	(55.681 N, 12.276 E)	Magid *et al.* (2006); Poulsen *et al.* (2013b)
		Manured	(55.681 N, 12.276 E)	Magid *et al.* (2006); Poulsen *et al.* (2013b)
		NPK-fertilized	(55.681 N, 12.276 E)	Magid *et al.* (2006); Poulsen *et al.* (2013b)
Park Grass Rothamsted, United Kingdom	grassland	Untreated	(51.811 N, –0.377 E)	Silvertown *et al.* (2006)

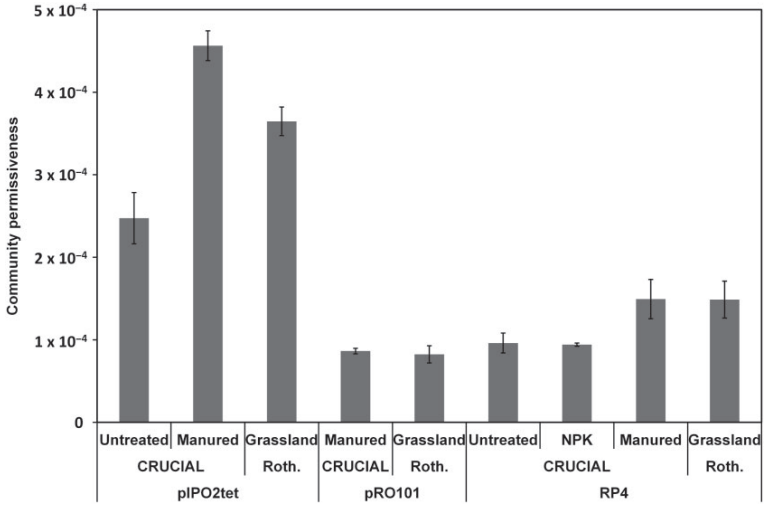

Fig. 1. Transfer frequency of the introduced plasmid to the soil indigenous bacterial communities derived from the CRUCIAL and Rothamsted (Roth.) plots after 48 h of incubation in solid surface filter matings on soil extract medium. Values are displayed as the mean of triplicates with standard deviation.

bacterial community composition for different treatments at the CRUCIAL site (Poulsen *et al*., 2013a), hinting towards the observed grouping of those transconjugal pools.

While plasmid and sampling site both affected transconjugal pools, no effect of agricultural treatment was detected ($P > 0.9$) for any of the plasmids in the CRUCIAL soil. A closer look at the six corresponding phylogenetic profiles (Fig. 3) confirms that transconjugal pools of the same plasmid within different soil communities are closely related. For instance, *Enterobacteriacae* sequences can only be found in both transconjugal pools associated with pRO101, although their relative fractions differ. All six pools were dominated by β- and γ-*Proteobacteria*. α-*Proteobacteria*, *Flavobacteria* and *Sphingobacteria* were detected in lower abundance, revealing a wide variety of transconjugants (Supporting Information Table S1), in spite of the low total number of isolates. High-throughput analysis of transconjugal pools could in the future lead to new insights in the extent of plasmid transfer in soils.

The similarity of transconjugal pools across soil treatments parallels that of the total bacterial community composition of CRUCIAL soils (Poulsen *et al*., 2013a). Therefore, the increased community permissiveness in manured soil cannot be explained by difference in community diversity. Increased seasonal nutrient availability can also be ruled out because high permissiveness was not observed for NPK-fertilized soil. Potentially, a higher indigenous plasmid content in the community associated with manure applications (Marti *et al*., 2013) can result in a higher permissiveness towards additional plasmids, by increasing the mating potential of the plasmid bearing cells. Additionally, the introduction of diverse plasmids through manure application may increase the community's permissiveness. Indeed previous hosting of a plasmid has been shown to significantly increase the permissiveness towards its renewed uptake in a *Dickeya* strain (Heuer *et al*., 2010).

On the other hand, the periodic introduction of stressors, such as metal ions (Nicholson *et al*., 1999) or antibiotics (Christian *et al*., 2003) present in manure, might lead to selection mechanisms favouring more robust populations adapted to environmental changes

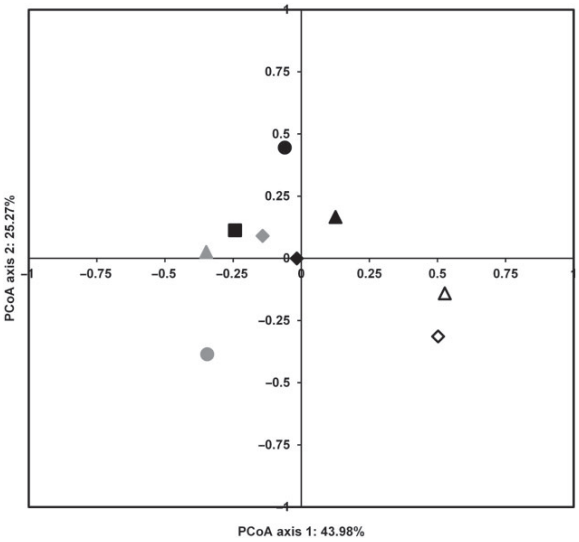

Fig. 2. Principal coordinate analysis (PCoA) of the sequenced transconjugal pools based on the Yue and Clayton measure of dissimilarity between the structures of different communities. Plasmids: pIPO2tet (grey), pRO101 (no fill) and RP4 (black); soil microbial communities: CRUCIAL manured (diamond), CRUCIAL untreated (triangle), CRUCIAL NPK-fertilized (square) and Rothamsted grassland (circle).

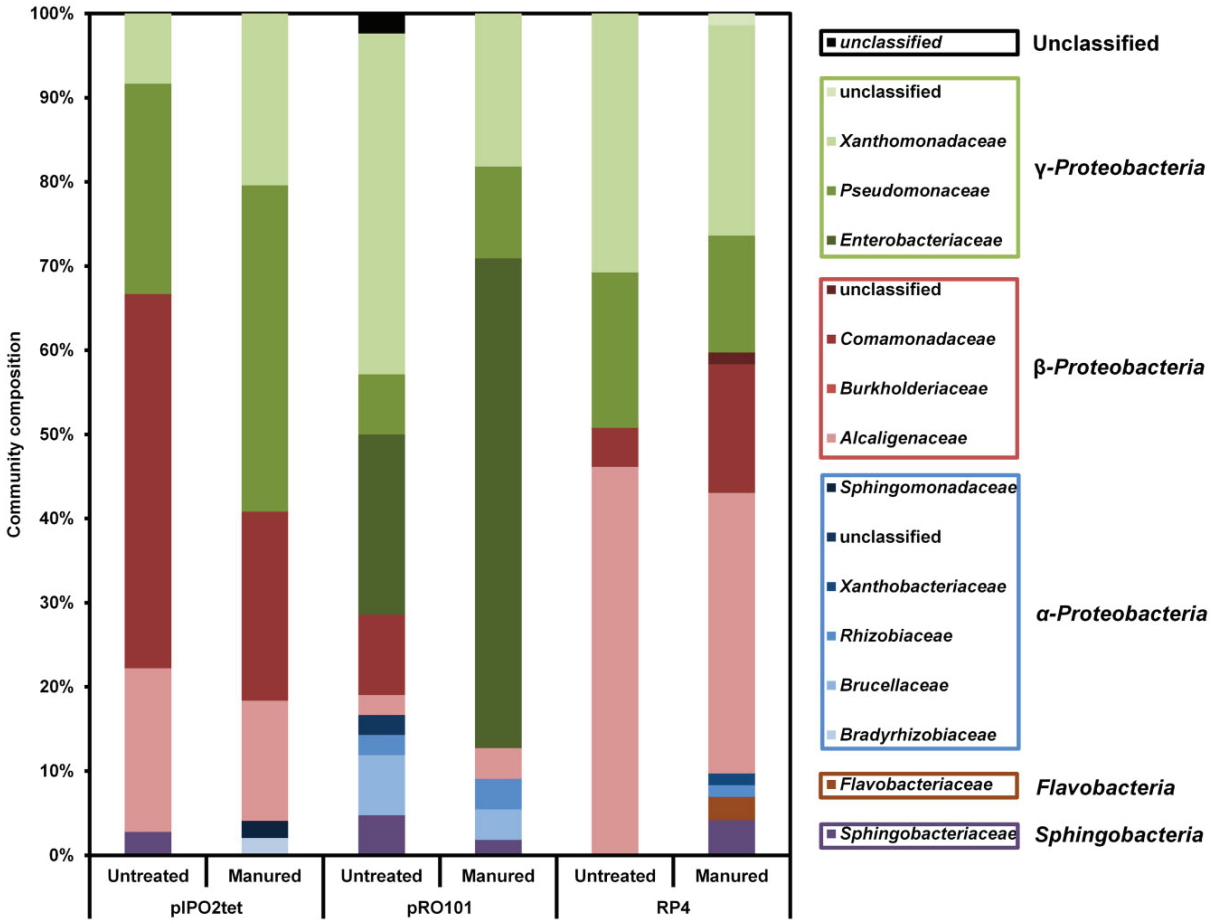

Fig. 3. Phylogenetic comparison of the transconjugal pools originating from the six combinations of CRUCIAL manured and CRUCIAL untreated soil microbial communities with all three tested plasmids.

through increased permissiveness towards foreign DNA uptake (Heuer *et al.*, 2008). These selection mechanisms for more permissive strains do not have to be associated with changes in the phylogenetic profile of the community since permissiveness towards broad host range plasmids of isolates from identical field plots that are genetically indistinguishable by 16S rRNA analysis can differ by more than two orders of magnitude (Heuer *et al.*, 2010). Still, the exact mechanisms of the observed enhanced permissiveness are yet to be elucidated. High-throughput analysis of the transconjugal pools could thus lead to insights into community dynamics of plasmid transfer in soil.

Here, we confirmed that soil communities can serve as significant reservoirs for exogenous conjugal plasmids. Approximately 1 in every 10 000 indigenous cells could receive and maintain the studied plasmids. This frequency is increased up to 100% in soils subject to manure treatment. Such an increase in the permissiveness of soil communities and therefore in their potential for contributing to the spread of antibiotic resistance genes is remarkable, making it crucial to elucidate the underlying mechanisms in further research with higher sampling depth. Additional work investigating dissemination and mobilization capacity of more diverse genetic elements among bacterial communities from differently treated soils is therefore necessary.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Diversity of transconjugants sorted by OTUs (97%) and occurrence per transconjugal pool. Sequences were analysed using MOTHUR v1.30.0 (Schloss *et al.*, 2009) and the SILVA database (Quast *et al.*, 2013). The sequences can be found in GenBank under accession numbers KF590708-KF591079.

Appendix S1. Sampling procedure and information.

Supporting Information

Appendix S1 Sampling procedure and information.

Soil samples of three different treatments were taken at the annually manured CRUCIAL (Closing the Rural Urban Nutrient Cycle) agricultural field site (Taastrup, Denmark) (Magid et al., 2006; Poulsen et al., 2013). Soil samples were collected in late fall 2010. Samples of each treatment were taken from three different plots of this treatment. Each plot was sampled for 1 kg of soil at 15 locations. The resulting soil volume was sieved and homogenized to obtain a representative sample. Twenty grams of this homogenized soil samples were used for Nycodenz®-extraction. The fourth sample was taken from the monitored untreated Park Grass field in Rothamsted (Silvertown et al., 2006) (Rothamsted, UK) in the exact same manner.

OTU	Kingdom	Phylum	Class	Order	Family	Genus	pIPO2tet				pRO101		RP4			total sequences
							CRUCIAL			Rothamstead	CRUCIAL		CRUCIAL		Rothamstead	
							Untreated	NPK	Manured	Park Grass	Untreated	Manured	Untreated	Manured	Park Grass	
1			Flavobacteria	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	0	0	0	0	0	0	0	1	0	**1**
2						unclassified	0	0	0	0	0	0	0	1	0	**1**
3						Pedobacter	0	0	0	0	0	0	0	2	1	**3**
4		Bacteroidetes					1	0	0	0	0	0	0	1	0	**2**
5			Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	0	0	0	0	1	0	0	0	0	**1**
6							0	0	0	0	0	1	0	0	0	**1**
7							0	0	0	0	1	0	0	0	0	**1**
8					Bradyrhizobiaceae	Bosea	0	0	1	0	0	0	0	0	0	**1**
9					Brucellaceae	Ochrobactrum	0	0	0	1	3	0	0	0	0	**4**
10							0	0	0	0	0	2	0	0	0	**2**
11			Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	0	0	0	1	1	2	0	1	0	**5**
12					unclassified	unclassified	0	0	0	0	1	0	0	0	0	**1**
13					Xanthobacteraceae	unclassified	0	0	0	0	0	0	0	1	0	**1**
14				Sphingomonadales	Sphingomonadaceae	Sphingomonas	0	0	1	0	0	0	0	0	0	**1**
15							7	9	7	2	1	2	27	21	4	**80**
16					Alcaligenaceae	Achromobacter	0	0	0	0	0	0	1	1	0	**2**
17							0	0	0	0	0	0	0	1	0	**1**
18			Betaproteobacteria	Burkholderiales			0	0	0	0	0	0	1	0	0	**1**
19						unknown	0	0	0	0	0	0	1	1	1	**3**
20					Burkholderiaceae	unknown	0	0	0	0	0	0	0	0	5	**5**
21					Comamonadaceae	Variovorax	16	0	11	0	4	0	3	11	0	**45**
22					unclassified	unclassified	0	0	0	0	0	0	0	1	0	**1**
23	Bacteria					Pantoea	0	0	0	0	1	0	0	0	0	**1**
24						unclassified	0	0	0	0	8	25	0	0	0	**33**
25				Enterobacteriales	Enterobacteriaceae	unclassified	0	0	0	0	0	2	0	0	0	**2**
26		Proteobacteria				unclassified	0	0	0	0	0	1	0	0	0	**1**
27						unclassified	0	0	0	0	0	1	0	0	0	**1**
28						unknown	0	0	0	0	0	3	0	0	0	**3**
29							8	4	18	1	2	5	11	10	2	**61**
30							0	0	0	0	1	0	0	0	0	**1**
31						Pseudomonas	0	0	0	0	0	1	0	0	0	**1**
32				Pseudomonadales	Pseudomonadaceae		0	0	0	0	0	0	1	0	0	**1**
33			Gammaproteobacteria				0	1	0	0	0	0	0	0	0	**1**
34							0	0	1	0	0	0	0	0	0	**1**
35						unclassified	0	0	0	1	0	0	0	0	0	**1**
36						unclassified	1	0	0	0	0	0	0	0	0	**1**
37							0	3	4	0	1	0	18	7	10	**43**
38							3	4	6	0	6	8	1	11	3	**42**
39						Stenotrophomonas	0	0	0	0	4	0	0	0	0	**4**
40				Xanthomonadales	Xanthomonadaceae		0	0	0	0	0	2	0	0	0	**2**
41							0	0	0	0	1	0	0	0	0	**1**
42							0	0	0	0	0	0	1	0	0	**1**
43						unclassified	0	0	0	0	4	0	0	0	0	**4**
44						unclassified	0	0	0	0	1	0	0	0	0	**1**
45			unclassified	unclassified	unclassified	unclassified	0	0	0	0	0	0	0	1	0	**1**
46		unclassified	unclassified	unclassified	unclassified	unclassified	0	0	0	0	1	0	0	0	0	**1**
						total OTUs	**6**	**5**	**8**	**5**	**18**	**13**	**10**	**16**	**7**	**46**
						total sequences	**36**	**21**	**49**	**6**	**42**	**55**	**65**	**72**	**26**	**372**

SI Table 1: Diversity of transconjugants sorted by OTUs and occurrence per transconjugal pool. Sequences were analyzed using mothur v1.30.0 (Schloss et al., 2009) and the SILVA database (Quast et al., 2013).
These sequence data have been submitted to the GenBank database under submission ID 1656830.

Table S1 Diversity of transconjugants sorted by OTUs (97%) and occurrence per transconjugal pool. Sequences were analysed using MOTHUR v1.30.0 (Schloss et al., 2009) and the SILVA database (Quast et al., 2013). The sequences can be found in GenBank under accession numbers KF590708-KF591079.

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DTU Environment
Department of Environmental Engineering
Technical University of Denmark

Miljoevej, building 113
2800 Kgs. Lyngby
Denmark

Phone: +45 4525 1600
Fax: +45 4593 2850
e-mail: info@env.dtu.dk
www.env.dtu.dk