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ORIGINAL ARTICLE

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

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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

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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

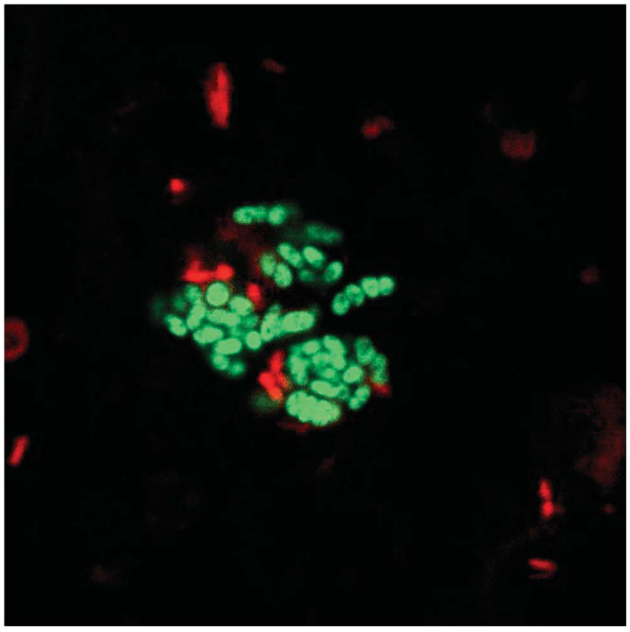

Figure 1 Typical transconjugal microcolonies for plasmid pKJK5::*gfp* introduced through *E. coli* MG1655::*lacI*^{q}-*pLpp-mCherry-Km*^{R}. Observation was carried out with a confocal laser scanning microscope (CLSM). Transconjugants are green fluorescent because of *gfp* expression, *gfp*-repressed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

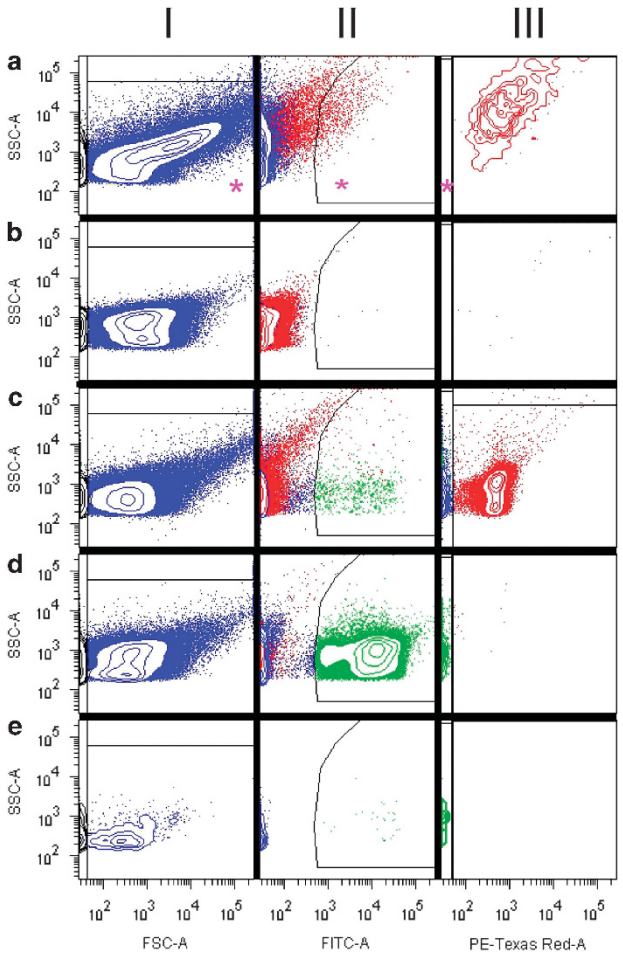

Figure 2 FACS sorting of transconjugal cells from a mating mixture initiated with soil bacteria and *E. coli* carrying pKJK5. The procedure consists in three successive gates (marked by pink stars in panel **a**). Gate I sorts for bacterial size based on forward and side scatter (SSC); Gate II sorts 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

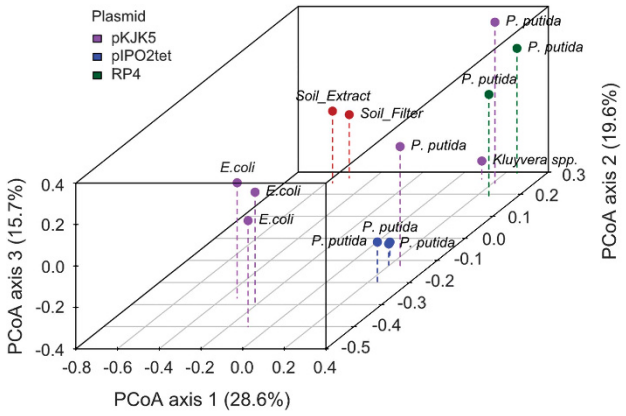

Figure 3 Principal coordinate analysis (PCoA) of individual transconjugal pools, as well as of the extracted soil community (Soil_Extract) and the reference soil community as grown on filters (Soil_Filter) based on the ThetaYC algorithm (Yue and Clayton, 2005). Each axis explains a certain fraction of 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;

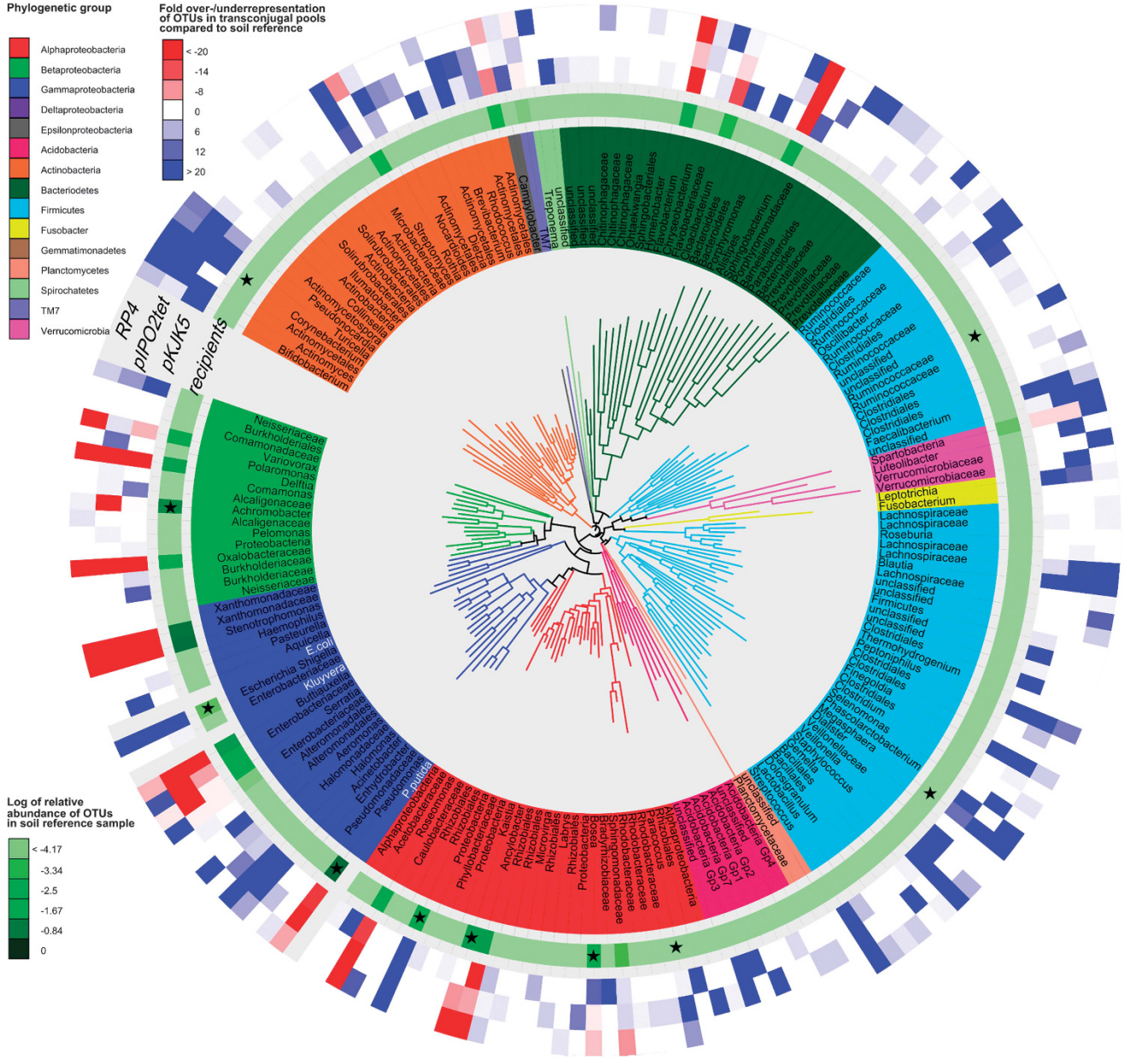

Figure 4 Phylogenetic tree showing all identified transconjugant OTUs for three different plasmids (pKJK5, RP4 and pIPO2tet) from the same donor (*P. putida*). The colors of the branches mark different phylogenetic groups. The three donor strains are shown in white letters in the trees. One green heatmap 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

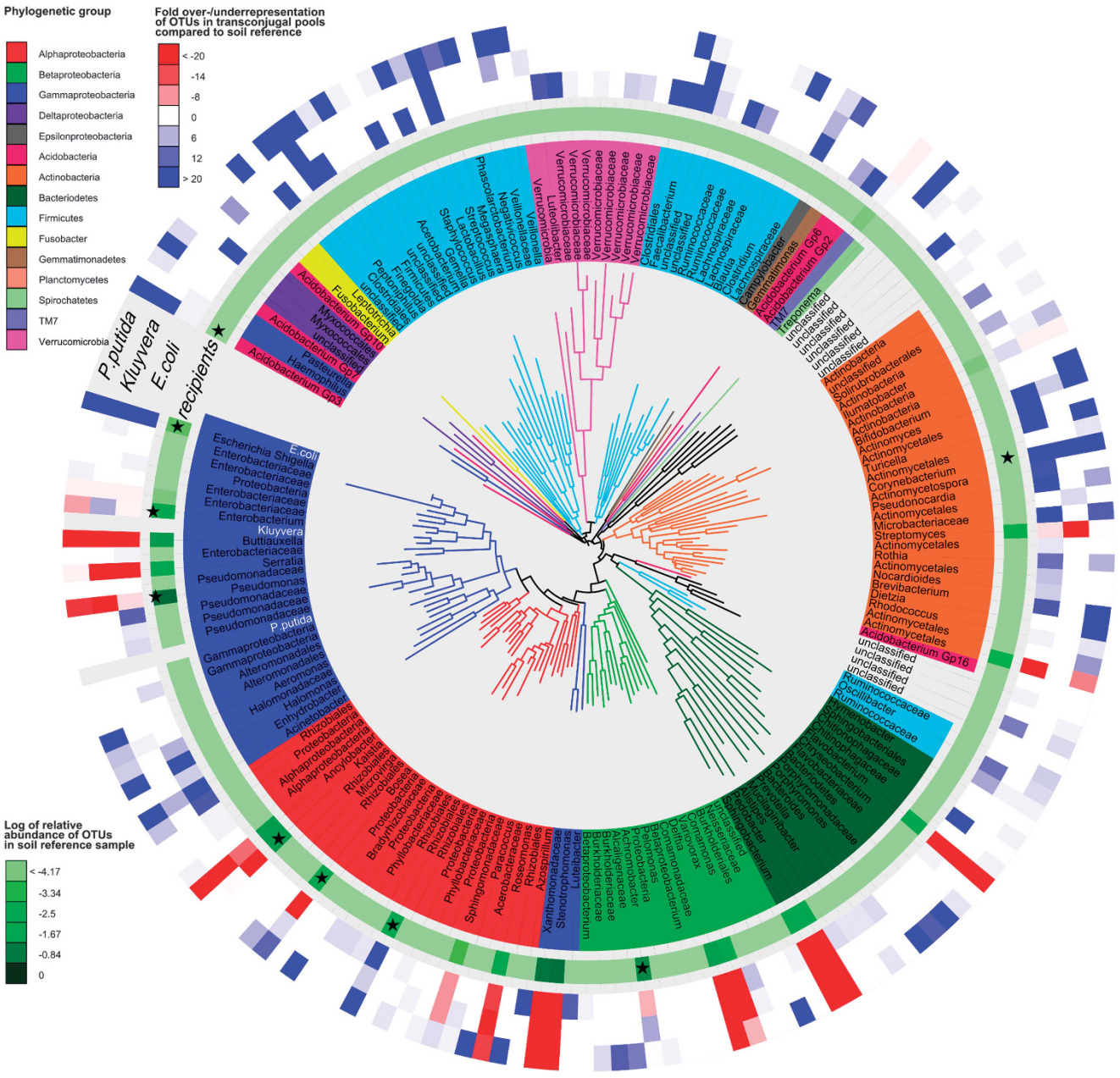

Figure 5 Phylogenetic tree showing all identified transconjugant OTUs for the same plasmid (pKJK5) introduced through three different donor strains (*P.putida*, *Kluyvera* sp. and *E. coli*). Colors of the branches mark different phylogenetic groups. The three donor strains are shown in white letters 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

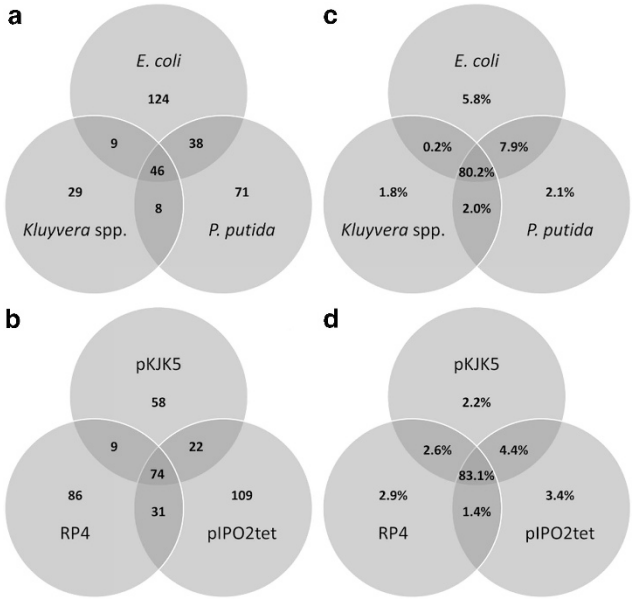

Figure 6 Venn diagram of transconjugal pools for plasmid pKJK5 transferred from three different donor strains (*E. coli*, *P. putida* and *Kluyvera* sp.) (**a** and **c**) and for three different plasmids (pKJK5, RP4 and pIPO2tet) (**b** and **d**) introduced through *P. putida* into the soil community. 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.

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