Estimating the Transfer Range of Plasmids Encoding Antimicrobial Resistance in a Wastewater Treatment Plant Microbial Community

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Title: Estimating the Transfer Range of Plasmids Encoding Antimicrobial Resistance in a Wastewater Treatment Plant Microbial Community

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Abstract

Wastewater treatment plants (WWTPs) have long been suggested as reservoirs and sources of antibiotic resistance genes (ARGs) in the environment. In a WWTP ecosystem, human enteric and environmental bacteria are mixed and exposed to pharmaceutical residues, potentially favoring genetic exchange and thus ARG transmission. However, the contribution of microbial communities in WWTP to ARG dissemination remains poorly understood. Here, we examined for the first time plasmid permissiveness of an activated sludge microbial community, by utilizing an established fluorescent bioreporter system. The activated sludge microbial community was challenged in standardized filter matings with one of the three multi-drug resistance plasmids (pKJK5, pB10 and RP4) harbored by *Escherichia coli* or *Pseudomonas putida*. Different donor-plasmid combinations had distinct transfer frequencies, ranging from 3 to 50 conjugation events per 100,000 cells of the WWTP microbial community. In addition, transfer was observed to a broad phylogenetic range of 13 bacterial phyla with several taxa containing potentially pathogenic species. Preferential transfer to taxa belonging to the predicted evolutionary host range of the plasmids was not observed. Overall, the ARG dissemination potential uncovered in WWTP communities calls for a thorough risk assessment of ARG transmission across the wastewater system, before identifying possible mitigation strategies.

Introduction

Wastewater treatment plants (WWTPs), at the interface between hospital/residential sewage and recipient surface water, have been proposed as overlooked reservoirs of antibiotic resistance genes (ARGs).[1–3] Indeed, there, the microbiomes indigenous to WWTP are intensely mixed with microbiomes of human enteric origin, in the presence of pharmaceutical residues and other selective agents, potentially stimulating the transfer of ARGs from pathogens and commensals to environmental bacteria. Among the gene transfer processes (e.g., transformation, transduction and conjugation), plasmid-mediated conjugation is characterized by its efficiency, even across distantly related taxa for broad host range plasmids. Therefore, the transfer of ARGs is facilitated by their frequent location on plasmids.[4–6] Several studies have provided evidence that WWTP microbiomes can contain significant amount of plasmids encoding multi-drug resistance.[7–9] Environmental bacteria receiving these plasmid-borne ARGs can persist in the receiving environments, facilitating their dissemination.[10,11] Considering the global public health threat posed by antimicrobial resistance and the obvious load from human waste collected and transported through sewage, it is crucial to evaluate the potential contribution of WWTP to plasmid mediated ARG dissemination.

In order to understand the fate of ARG-carrying plasmids in WWTP ecosystems, it is necessary to disentangle the roles of plasmid type, donor strain, and resident microbial community in shaping the plasmid transfer host ranges. The plasmid permissiveness assay, as originally introduced by Musovic et al,[12] provides a suitable platform to address this question. Combining a fluorescent reporter based plasmid detection assay with fluorescence-activated cell sorting (FACS) and 16S rRNA gene amplicon sequencing of transconjugant cells, it enables quantification and identification of the community fraction that receives the tested plasmid upon challenging this community with a plasmid donor strain.[13–15] Using this approach, extremely broad transfer host ranges of IncP-1

conjugative plasmid pKJK5 have been detected in microbial communities from agricultural soil,[13,14] as well as from the inlet and outlet of WWTPs.[15] Yet, the permissiveness of WWTP microbial communities for typical and relevant IncP-1 plasmids of different subgroups has not been examined. It has been argued - mainly based on metagenomic observations - that the high species diversity and cellular density of WWTP microbial communities creates a locale favoring horizontal gene transfer.[8,16] Predicting the range of plasmid-mediated genetic exchange at the community level has so far not been possible; host ranges inferred from bioinformatic analyses or traditional assays have been skewed toward only identifying evolutionary host taxa with preexisting genomic homogeneity or examining a limited number of well-studied model strains.[17–20] We believe that direct confirmation and quantification of this exchange is, however, necessary and possible via plasmid permissiveness assays.[13–15] By quantifying and identifying the permissive fraction, one can evaluate plasmid transfer potential as an essential community property and examine abiotic/biotic factors (e.g., environmental conditions, plasmid/donor type and recipient community) that shape permissiveness profiles, which together will help understand plasmid-mediated ARG spread.

Here, we report on the first permissiveness estimates of a WWTP microbial community towards several typical conjugative plasmids, and the first exploration of association between plasmid transfer and evolutionary host ranges. A WWTP community was challenged with three ARG-carrying plasmids from different subgroups in the incompatibility group IncP-1 (pKJK5, pB10 and RP4)[17] using either the prototypic member of Enterobacteriaceae - *Escherichia coli* or typical environmental bacterium - *Pseudomonas putida* as donor strains. Distinct transfer potentials were observed with the highest realized in *E. coli* (pKJK5) (50 conjugation events per 100,000 recipient cells). The transfer host ranges covered 13 phyla across the different donor-plasmid combinations; but no preferred transfer was observed to taxa predicted to belonging to the evolutionary host range

of the plasmids. It is noteworthy that plasmid acquisition was observed in several taxa with potentially pathogenic species. Overall, the wide transfer potential of plasmids experimentally revealed in this study confirms the importance of WWTP as a unique locale for plasmid mediated ARGs exchange between enteric and environmental bacteria.

Material and methods

Donor strain and WWTP recipient community

E. coli MG1655 and *P. putida* KT2440 (both chromosomally tagged by *lacI*^{q}-P*lpp-mCherry*) carrying one of the three plasmids pKJK5 (IncP-1ε), pB10 (IncP-1β) and RP4 (IncP-1α) (tagged with P*lac-gfp*), were used as donors (each combination group will be referred to as *donor* (plasmid), e.g., *E. coli* (pKJK5)) (Table S1). The donor strains were grown overnight in LB prepared as described previously.[13,14] Recipient community was phase-isolated activated sludge from a municipal WWTP (Mølleåværket, Lyngby-Taarbæk, DK). Briefly, bacteria were recovered by washing, sonication and settling. Cell numbers were adjusted to approx. 3.0 $\times 10^{7}$ cells per ml for filter mating assays.

Solid surface filter mating assay

Cell suspensions of donor strain and WWTP recipient community were mixed at 1:1 cell ratio and immediately filtered onto 0.2 μm Cyclopore membranes.[21] Filters were placed on a agar-solidified synthetic wastewater medium.[22] After incubation (48 hours at 25°C) and GFP maturation (48 hours at 4°C), transfer events were detected by epifluorescence microscopy and transfer frequency was quantified as the ratio of conjugation events (GFP-positive cells or microcolonies) to the original WWTP recipient cell number (CE/R), as per established procedures.[13,14,23]

Sorting and sequencing

For each mating condition, cells from triplicate filters were combined in 0.9% NaCl solution and detached by vortexing. Transconjugants and recipients were sorted using FACS by adjusting gating of bacterial size (forward scatter), green fluorescence, and red fluorescence as described earlier.[13,14] Sorted cells were subject to DNA extraction using GenePurgeDirect™ agent (NimaGen, NL). 16S rRNA gene fragments were amplified by the primer set 341F and 806R,[15] and subjected to paired-end sequencing on Illumina MiSeq platform.

Sequence analysis

The forward reads of the 16S rRNA gene amplicon sequencing were analyzed using the DADA2 pipeline to infer exact sequence variants (ESV) (Table S2).[24,25] As estimating ESV-specific permissiveness is complicated by the (potential) growth of both transconjugants and recipients during mating incubation, we calculate apparent permissiveness (AP). It is defined as the ratio of the relative abundance of an ESV in the transconjugant pool and in the corresponding recipient community.[14] AP thus accounts for the fact that the abundance for an ESV in the transconjugant pool is partly dependent on their abundance in the recipient community. Phylogenetic relatedness between donor and transconjugant was calculated by DistanceMatrix in R package DECIPHER[26] and its correlation with AP values was calculated with Spearman correlation coefficient. Phylogenetic conservation of AP values was analyzed by calculating their phylogenetic signal in corresponding ESVs by multiPhylosignal in R package picante.[27] Plasmid transfer host range and evolutionary host range (i.e., hosts that have carried the plasmid during evolutionary time long enough to leave detectable sequence traits), were compared based on previous genomic analysis.[17] Occurrence of these evolutionary hosts in transconjugant pools was evaluated by t-test of both relative abundance and AP value. All sequences were deposited in NCBI under SRA accession

number SRP133153. Method details including experimental setups and statistical analyses are provided in Supporting Information.

Results and Discussion

Transfer frequencies across donor-plasmid combinations

Transfer frequencies in the WWTP microbial community ranged from 3.39×10^{-5} to 5.05×10^{-4} CE/R (i.e., from 3 to 50 conjugation events per 100,000 recipient cells) across donor-plasmid combinations (Figure 1); comparable transfer frequencies have been measured in soil microbial communities (6.8×10^{-5} CE/R of *E. coli* (pKJK5) and 1.0×10^{-4} CE/R of *P. putida* (RP4))[13,28] All three plasmids transferred at higher frequency from *E. coli* compared to *P. putida* with the highest transfer frequency observed with *E. coli* (pKJK5). Comparison among transfer frequencies of the three plasmids carried by the same host showed that pKJK5>pB10>RP4 in *E. coli* and pKJK5>pB10≈RP4 in *P. putida*. Despite belonging to the same incompatibility group (IncP-1), the three plasmids present genetic divergence in their transfer and regulatory regions,[17,29] which might explain the difference in observed conjugation behavior.

Transfer host ranges for different donor-plasmid combinations

Recipient communities were distinct from transconjugant pools (NMDS, ANOSIM P-value<0.01). While post filter-mating recipient pools were distinct from the raw AS communities, reasonable diversity was retained: the Shannon diversity index decreased slightly from 5.3 to 4.7. And notwithstanding the presence of a shared core (see below) the four transconjugant pools were distinct from each other (Figure 2). As expected, recipient pools were more diverse than transconjugant pools (Shannon diversity = 4.2-4.6; unique ESVs = 229-360 vs Shannon diversity = 1.3-3.2; unique ESVs = 73-126). Interestingly, distances within transconjugant pools and recipient

communities of *E. coli*/*P. putida* (pKJK5) were similar (Bray-Curtis distance within transconjugant pools vs within recipient pools = 0.55 vs 0.63). However, the three transconjugant pools of *E. coli* (pKJK5/pB10/RP4) were clearly distinct from each other, even though their recipient communities were close (Bray-Curtis distance = 0.43-0.57 vs 0.23-0.26). Hence, a plasmid type might shape transconjugant pool composition more than a plasmid donor.

The transconjugants across all donor-plasmid combinations comprised 308 distinct ESVs distributed over 13 phyla (Figure 2; Figure 3). While all transconjugant pools were dominated by genera from the Gammaproteobacteria class including *Escherichia*/*Shigella*, *Pseudomonas* and *Acinetobacter*, a few other Gram-negative (*Chloroflexi*, *Acidobacteria* and *Bacteroidetes*) and Gram-positive (*Actinobacteria* and *Firmicutes*) taxa were also noted. Overall, plasmid transfer was observed in 34-59% of the families present in the recipient community. Thirteen permissive genera were shared across all donor-plasmid combinations, representing >80% of each transconjugant pool. These core permissive taxa were mainly composed by *Enterobacteriaceae* and *Pseudomonadaceae*. These two lineages were also detected in transconjugant pools when permissiveness of inlet and outlet of the same WWTP was examined,[15] indicating their possible transmission from sewage to the environment. The frequent occurrence of *Acinetobacter*, *Aeromonas* and *Streptococcus* in the transconjugant pools highlights the possibility of ARG transmission to (opportunistic) pathogens. The high frequency and broad range of plasmid transfer to the examined WWTP community under the standardized experimental conditions, in the absence of selective pressure, suggests significant ability of ARG spread under actual WWTP conditions of intense microbial interaction[30] and the presence of residual antibiotics and other relevant co-selective stressors.[31]

Heterogeneous apparent permissiveness profiles

The relative abundance profile of community members in transconjugant pools did not agree with their abundance in the recipient pools, indicating that capability in receiving plasmids varied among taxa (Figure S1): a few taxa with low abundance in the recipient communities were highly enriched in the transconjugant pools across all donor-plasmid combinations (e.g., *Escherichia*/*Shigella* <1% in recipient pools and >40% in all transconjugant pools; *Shimwellia* was <0.1% in recipient pools but >2% in transconjugant pools with both *E. coli* (pKJK5) and *P. putida* (pKJK5) groups). On the contrary, some highly abundant taxa were poorly represented in the transconjugant pools indicating their poor permissiveness (e.g., *Acinetobacteria* >40% in recipient but <0.1% in the transconjugant pool with *E. coli* (pB10) group). In several abundant taxa, no plasmid transfer was detected (e.g., *Flavobacterium* at 8-10% in recipient communities while absent in transconjugant pool of *E. coli* (pB10)).

Phylogenetic relatedness between recipient and donor did not explain the composition of the transconjugant pools for the three examined IncP-1 plasmids (Figure S1 and S2). Certainly, high intra-generic transfer was observed: from donor *E. coli* to *Escherichia*/*Shigella* (AP up to 704.1) and from donor *P. putida* to *Pseudomonas* (AP up to 294.2). However, transfer to distant phylogenetic groups, even across phylum borders, was equally observed, e.g., *E. coli* (pKJK5) transferred at high frequency to *Pseudobacteroides* (*Firmicutes*) (AP up to 448.1) and *Gardnerella* (*Actinobacteria*) (AP up to 429.6). Hence, the AP profile did not correlate with the phylogenetic distance between recipient and donor (Spearman correlation, P-value = 0.10~0.93). Within a single permissive genus, APs could be similar in magnitude or vary greatly: e.g., with *E. coli* (pB10) and *E. coli* (RP4), APs of *Staphylococcus* ESVs were within one order of magnitude; with *E. coli* (pKJK5), APs of *Acinetobacter* and *Pseudomonas* ESVs each ranged over three orders of magnitude. Such varying response at the ESV level indicates that AP is not significantly

phylogenetically conserved (phylogenetic signal, P-value = 0.61~0.98). Therefore, for the three IncP-1 plasmids, extrapolating permissiveness of a bacterial group to other phylogenetically similar groups in the WWTP community would not be valid. Future studies, including more plasmid groups, and especially plasmids with assumed narrow-host-range groups (e.g., IncF and IncI), will reveal the generality of this conclusion.

Comparing transfer host range to predicted evolutionary host range

While plasmid transfer host range can be inferred from experimental permissiveness assays, it is not clear how this range relates with a plasmid's long-term maintenance as plasmid acquisition is only the very first step of a possible long-term plasmid-host association. Since long-term adaptation between plasmid and host is achieved through genomic homogenization and subsequent cost amelioration, a plasmid's evolutionary host range can be inferred from genomic comparisons between bacterial chromosomes and plasmids (backbone).[17,18] For example, for the three examined plasmids, pKJK5 was predicted to have been evolutionarily present in the genera *Bordetella*, *Dechloromonas* and *Pseudomonas*, pB10 in *Ralstonia* and *Variovorax*, and RP4 in *Ralstonia*, *Slackia* and *Pseudomonas*.[17] These predicted evolutionary hosts might have more potential in taking up the plasmid and expressing its genes because of the preexisting genomic homogeneity. However, we did not detect such enrichment for members of the predicted evolutionary host range of the three plasmids in their corresponding transconjugant pools (Table S3). Among the six genera belonging to the predicted evolutionary host range of the three plasmids, only *Pseudomonas* (0.25%~17.60% with AP 0.5-294.2 across all groups) and *Dechloromonas* (0.14% with AP 2.3 in *E. coli* (pKJK5)) were detected in transconjugant pools. Even at higher taxonomical levels, there was little indication of enrichment of evolutionary host taxa in the transconjugant pools. For example, *Burkholderiaceae* (family) predicted as evolutionary host taxon of pB10, were not observed in the pB10

transconjugant pool; *Burkholderiales* (order) were observed in the pKJK5 transonjugal pools with *E. coli* as donor but below 1% with AP ranging from 0.2-262.6; Gram-positive *Actinobacteria* (class) predicted evolutionary hosts of RP4, were minor fractions of the RP4 transconjugant pools (<4% with AP ranging 0.2-429.6). Hence, evolutionary host range predicted from genomic analysis does not seem to reflect extant plasmid transfer host rang in WWTP microbial communities.

In this study, the dissemination potential of ARGs in environmental communities was highlighted by the high transfer frequency (up to 50 conjugation events per 100,000 recipient cells) and the broad phylogenetic transfer range (covering 13 phyla) of the three ARG-carrying plasmids in a WWTP microbial community. Taxa belonging to a plasmid's predicted evolutionary host range do not necessarily exhibit high permissiveness. The plasmid permissiveness assay as adapted here for WWTP communities provides a quantitative assessment of a community property that is essential, but not sufficient, to describe, and ultimately predict the fate of plasmids in the environment. Indeed, the potential for plasmid uptake, as measured here, is not realized *in situ* in WWTP systems, and extrapolation to real environments will require additional experiments to identify the role of the environment, including conditions of (sub)inhibitory selective or co-selective pressure.

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Conflict of interest

The authors declare no competing financial interest.

Supporting Information

Supplementary methods of plasmid donor strain and recipient microbial community, solid surface filter mating assay, sorting and sequencing (sequence analysis); supplementary figures of relative abundance of genera across samples, AP profile of ESVs; supplementary tables of donor strains and plasmids, information of sequences, relative abundance of predicted evolutionary taxa.

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Figure legends

Figure 1. Transfer frequencies (CE/R: the ratio of conjugation events (CE) to the original WWTP recipient cell number (R)) from two donors (*E. coli* and *P. putida*) carrying one of three plasmids (pKJK5, RP4 and pB10) to an activated sludge microbial community. Error bar indicates 95% confidence interval of three replicates.

Figure 2. Diversity and phylogenetic composition of transconjugant and recipient communities. (A) and (B): Shannon index and NMDS (the same color scheme was applied in the two panels; for each donor-plasmid combination (circle dots), dark color indicates transconjugant pools and light color (within ellipse) indicates recipient pools; triangle dots indicate WWTP microbial communities. (C) and (D): phylogenetic composition at phylum level and relative abundance of phyla except Gamma- and Alpha-proteobacteria in transconjugant pools. (E) and (F): top 20 abundant orders and genera in the transconjugant pools.

Figure 3. Composition of the transconjugant pools across four donor-plasmid combinations. (A) and (B): phylogenetic tree showing the relative abundance of ESVs detected with *E. coli* (pKJK5/RP4/PB10) and *E. coli*/*P. putida* (pKJK5) as plasmid donor. Background colors indicate the 6 most abundant classes, and branch colors indicate the 13 core genera across all transconjugant pools (refer to panel (E)). (C), (D) and (E): Venn diagrams at the genus level of the transconjugant pools of *E. coli* (pKJK5/RP4/PB10), *E. coli*/*P. putida* (pKJK5) and all groups.

Figures

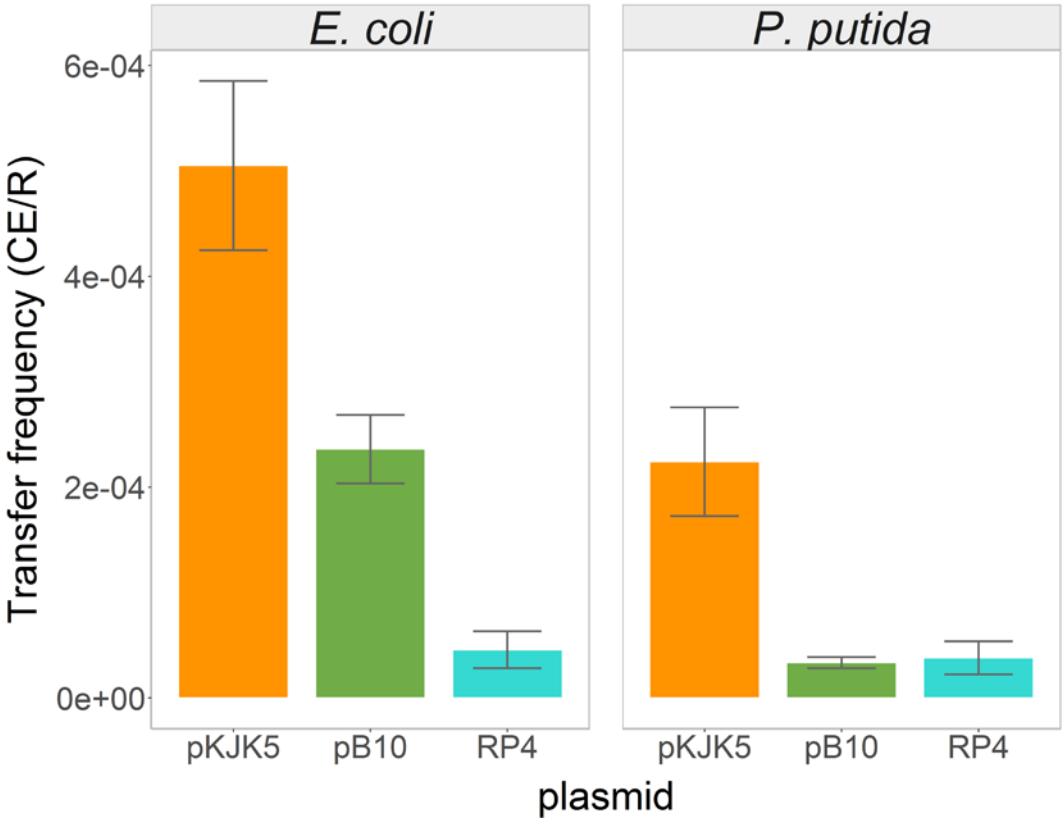

Figure 1. Transfer frequencies (CE/R: the ratio of conjugation events (CE) to the original WWTP recipient cell number (R)) from two donors (*E. coli* and *P. putida*) carrying one of three plasmids (pKJK5, RP4 and pB10) to an activated sludge microbial community. Error bar indicates 95% confidence interval of three replicates.

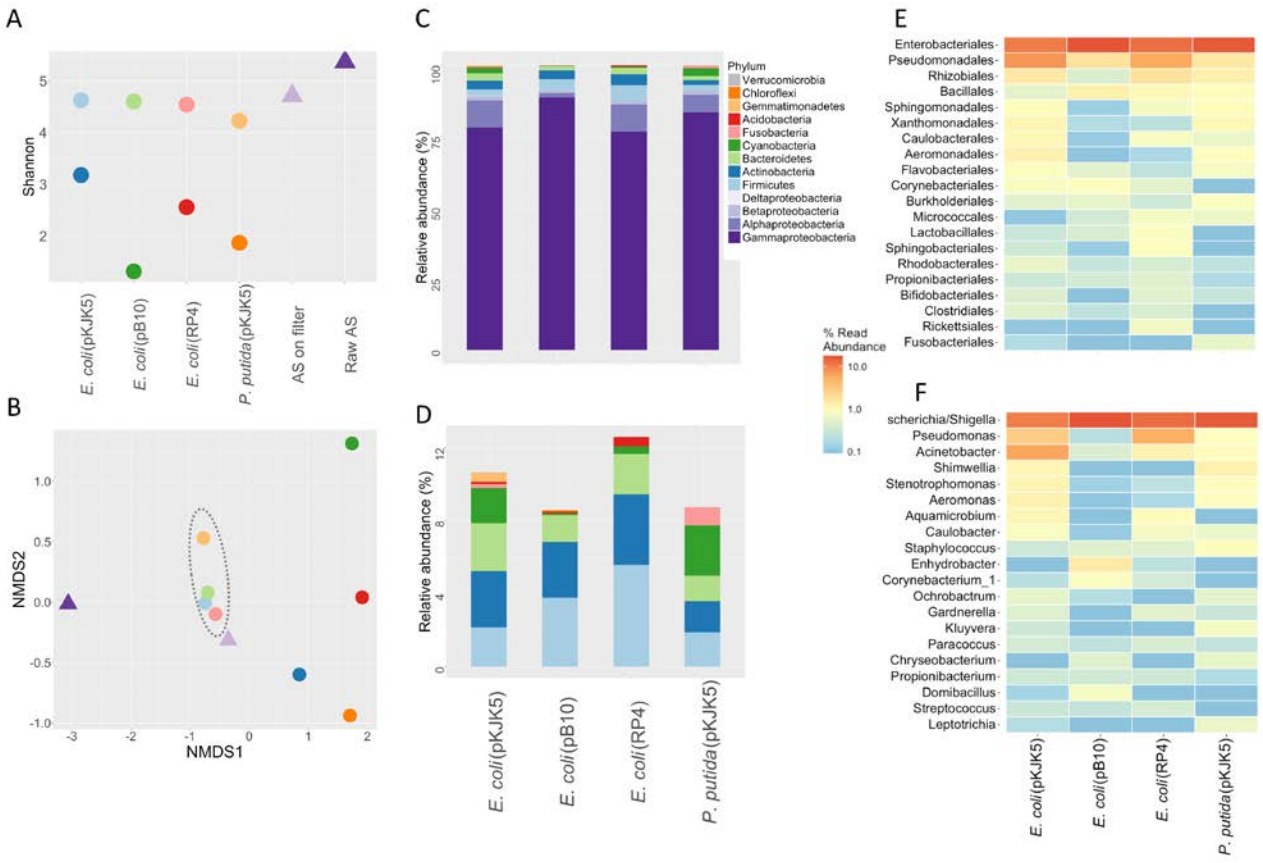

Figure 2. Diversity and phylogenetic composition of transconjugant and recipient communities. (A) and (B): Shannon index and NMDS (the same color scheme was applied in the two panels; for each donor-plasmid combination (circle dots), dark color indicates transconjugant pools and light color (within ellipse) indicates recipient pools; triangle dots indicate WWTP microbial communities. (C) and (D): phylogenetic composition at phylum level and relative abundance of phyla except Gamma- and Alpha-proteobacteria in transconjugant pools. (E) and (F): top 20 abundant orders and genera in the transconjugant pools.

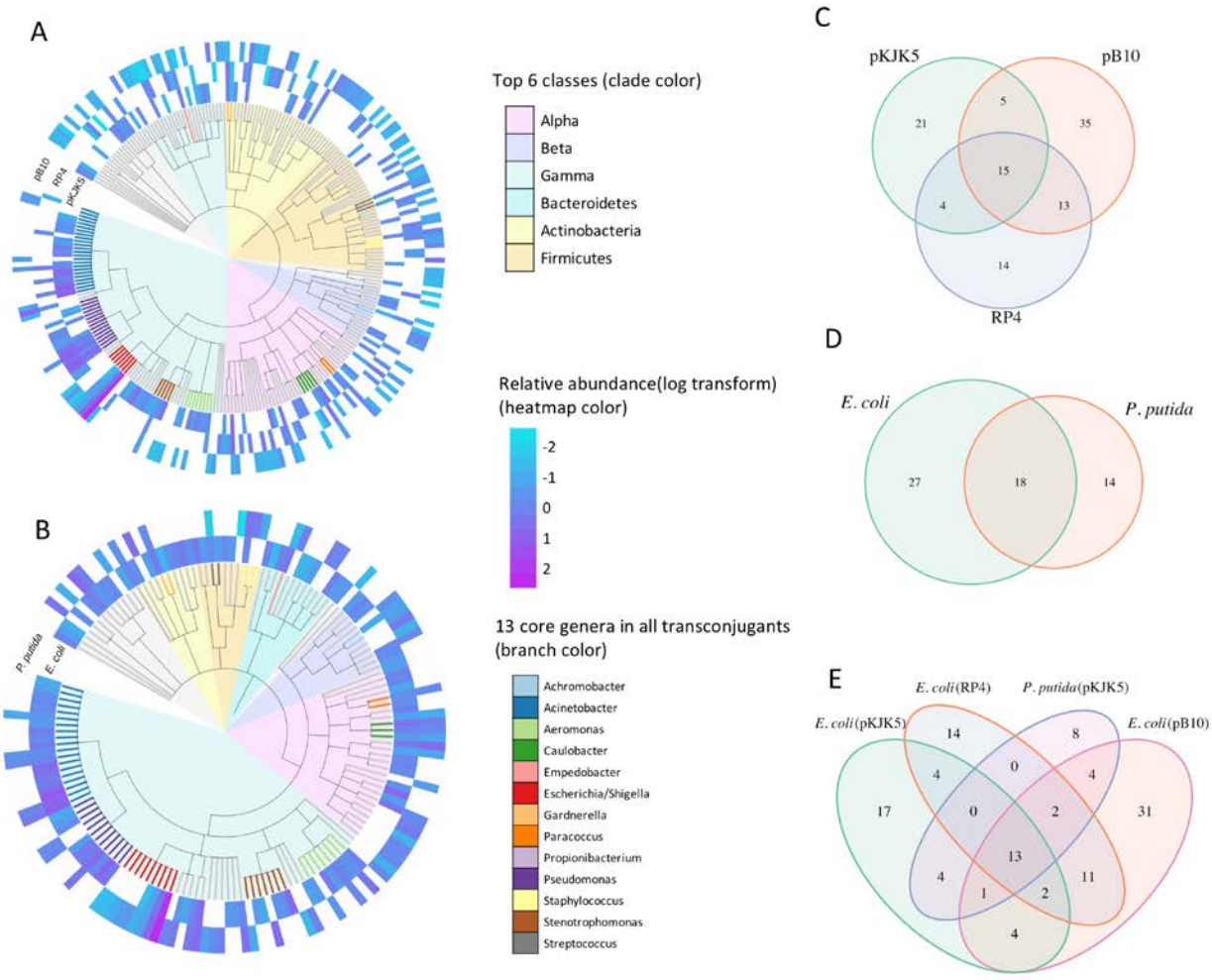

Figure 3. Composition of the transconjugant pools across four donor-plasmid combinations. (A) and (B): phylogenetic tree showing the relative abundance of ESVs detected with *E. coli* (pKJK5/RP4/PB10) and *E. coli*/*P. putida* (pKJK5) as plasmid donor. Background colors indicate the 6 most abundant classes, and branch colors indicate the 13 core genera across all transconjugant pools (refer to panel (E)). (C), (D) and (E): Venn diagrams at the genus level of the transconjugant pools of *E. coli* (pKJK5/RP4/PB10), *E. coli*/*P. putida* (pKJK5) and all groups.

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