IncHI1A plasmids potentially facilitate a horizontal flow of antibiotic resistance genes to pathogens in microbial communities of urban residential sewage

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Abstract

Horizontal gene transfer via plasmids is important for the dissemination of antibiotic resistance genes among medically relevant pathogens. Specifically, the transfer of IncHI1A plasmids is believed to facilitate the spread of antibiotic resistance genes, such as carbapenemases, within the clinically important family *Enterobacteriaceae*. The microbial community of urban wastewater treatment plants has been shown to be highly permissive towards conjugal transfer of IncP1 plasmids. Here, we tracked the transfer of the P1 plasmid pB10 and the clinically relevant HI1A plasmid R27 in the microbial communities present in urban residential sewage entering full-scale wastewater treatment plants. We found that both plasmids readily transferred to these communities and that strains in the sewage were able to further disseminate them. Furthermore, that R27 has a broad potential host range, but a low host divergence. Interestingly, although the majority of R27 transfer events were to members of *Enterobacteriaceae*, we found a subset of transfer to other families, even other phyla. Indicating, that HI1A plasmids facilitate horizontal gene transfer both within *Enterobacteriaceae*, but also across families of especially Gammaproteobacteria, such as *Moraxellaceae*, *Pseudomonadaceae* and *Shewanellaceae*. pB10 displayed a similar potential host range as R27. In contrast to R27, pB10 had a high host divergence. By cultivative enrichment of the transconjugant communities, we show that sewage strains of *Enterobacteriaceae* and *Aeromonadaceae* can stably maintain R27 and pB10, respectively. Our results suggest that dissemination in the urban residual water system of HI1A plasmids may result in an accelerated acquisition of antibiotic resistance genes among pathogens.

1 Introduction

The therapeutic success of antibiotics is challenged by the spread of antimicrobial resistance genes (ARGs) among bacteria that cause healthcare-associated and community-acquired infections, an escalating global health threat (Arias & Murray, 2009; Davies & Davies, 2010; WHO, 2014). In 2017, the World Health Organization released a list of pathogens against which healthcare systems urgently need new antimicrobial alternatives due to their commonly extensive antibiotic resistance profiles (WHO, 2017). These include*Klebsiella pneumoniae* , *Escherichia coli* ,*Enterobacter* spp., *Serratia* spp. and several other taxa commonly resistant to important beta-lactams, such as carbapenems and third-generation cephalosporins. Other critical pathogens on this list are fluoroquinolone-resistant *Salmonella* spp. and *Shigella*spp. These pathogens are closely related and belong to the family of*Enterobacteriaceae* . It is noteworthy that the medically-relevant ARGs within this family have been primarily found encoded by conjugative plasmids; mobile genetic elements that frequently mediate their dissemination among bacteria through horizontal gene transfer (HGT) (Carattoli, 2009).

The currently known variety of plasmids found within*Enterobacteriaceae* have been divided into 28 distinct

groups according to their incompatibility (Inc); i.e. their inability to coexist over time in the same cell-line (Couturier et al., 1988; Novick, 1987; Rozwandowicz et al., 2018). In order to understand the role of specific plasmid groups in the context of ARG dissemination, it is important to understand their ecology, including the diversity of their hosts (host range). Generally, plasmid host range denotes the phylogenetic or taxonomic breadth of organisms that can carry a plasmid; however, it can be further broken down into i) which recipients the plasmid can be transferred into; ii) which recipients the plasmid can replicate within; and iii) whether the plasmid is stably maintained over time in the cell-line. The two initial stages are known as the*transfer* - and *replication host range* s, respectively, and the latter is termed *evolutionary host range.* Since these stages occur sequentially, the host range narrows from one stage to the next (Suzuki et al., 2010). The evolutionary host range and the classification of plasmids are closely related (Redondo-Salvo et al., 2020), thus understanding the host range of archetypes representing a plasmid group reveals crucial knowledge about plasmid ecology. Recently, Redondo-Salvo and colleagues defined a host range scale for plasmids, which grades host range according to the highest taxonomic rank they distribute in; from I at the species level being very narrow, e.g. IncFIB, to VI at the phylum level being very broad, e.g. IncP1 (P1), which was shown to cross phylum level (Redondo-Salvo et al., 2020). Nonetheless, it is important to bear in mind that for specific strains or taxa within the plasmid host range, barriers may exist that prevent plasmid establishment. For example, host-encoded defense systems can form effective barriers against HGT by blocking the entry of foreign nucleic acids. These include restriction/modification (R/M) systems (Oliveira et al., 2014), CRISPR-Cas systems (Makarova et al., 2019), and Wadjet systems (Doron et al., 2018). In response, plasmids have developed various systems to evade host defense systems (anti-defense systems) e.g. Anti-R/M and Anti-CRISPR proteins, which directly interact with and inhibit R/M and CRISPR-Cas systems, respectively (Mahendra et al., 2020; Roy et al., 2020). Thus measurements of distribution breath, i.e. the taxonomic distance between the hosts in which a given plasmid is found (Redondo-Salvo et al., 2020), only describe the potential host range of a plasmid.

One of the *Enterobacteriaceae* plasmid groups, the IncHI1A group (HI1A), comprises conjugative plasmids typically in the size range of 75 to 400 kb (Rozwandowicz et al., 2018). HI1A plasmids are associated with the dissemination of ARGs such as the *bla* NDM-1 gene, which confers resistance towards carbapenems, a last resort drug used when treating extended spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* spp. (Carattoli, 2013; Dolejska et al., 2013). The transfer of some plasmids in the HI1A group, such as R27, is thermo-sensitive and primarily conjugate below 30° C, a feature that is speculated to promote the transmission of ARGs in the environment (Maher & Taylor, 1993; Sherburne et al., 2000). Additionally, plasmids in the HI1A group carry genes encoding thick flexible pili, which enable high conjugative transfer efficiency within both planktonic and surface-associated bacterial communities, thus emphasizing their potential to disseminate ARGs across a variety of environments (Bradley et al., 1980). Cultivation-based transfer experiments have found that HI1A plasmids can also be transferred to bacteria belonging to genera outside*Enterobacteriaceae,* such as *Vibrio* spp. and*Aeromonas* spp. (Maher & Taylor, 1993). Based on such experiments, and supported by bioinformatic predictions (Suzuki et al., 2010), HI1A plasmids are believed to have a wide or intermediate host range. However, an understanding of the initial host range stages, stage i) and ii), of HI1A is crucial for an improved understanding of the frequency and phylogenetic extent of plasmid-mediated HGT in the environment. Some transfer events may never extend beyond these initial host range stages, and therefore not result in stable plasmid-host association, however, still result in transfer of the plasmid from this host. Thus, a short-term host may function as a crucial stepping-stone for plasmids reaching into stable hosts.

Wastewater treatment plants (WWTPs) have been suggested to facilitate HGT of ARGs among bacteria (Guo et al., 2017; Li et al., 2018). Indeed, all known antibiotic resistance mechanisms have been found represented in WWTPs reservoirs (Rizzo et al., 2013), along with multiple mobile genetic elements, including plasmids (Rizzo et al., 2013; Zhang et al., 2011). Furthermore, studies have shown that the microbial communities of WWTPs are highly permissive towards broad host range plasmids (i.e. highly capable of taking up a given plasmid) (Jacquiod et al., 2017; Li et al., 2018). Moreover, it has been revealed that a core-permissive fraction of bacteria, capable of receiving several types of plasmids, are abundant across WWTPs (Li et al.,

2018). In addition, certain bacterial taxa have repeatedly been identified as a part of the core-permissive fraction of bacteria across diverse environments (Klümper et al., 2015; Li et al., 2018; Musovic et al., 2014; Pinilla-Redondo et al., n.d.).

In this study, we investigated the host range, including the initial host range stages, and transfer efficiency of the HI1A plasmid R27 in the microbial community of urban residential sewage collected at three WWTPs located in urban areas of southern Sweden. We utilized a dual fluorescent reporter gene platform, which previously has been used to examine transfer of various plasmid groups, including IncI1 (Anjum et al., 2018, 2019) and P1 (Jacquiod et al., 2017; Klümper et al., 2015; Li et al., 2018; Musovic et al., 2014; Pinilla-Redondo et al., n.d.). This platform is based on a plasmid-encoded green fluorescent protein (*gfp*) gene under the control of a $lacI^q$ repressible promoter. Donor cells, which introduce the plasmid to the community, carry a chromosomal*mCherry* along with $lacI^q$ and hence, although carrying the plasmid, do not express GFP. However, since indigenous bacteria in the sewage community likely do not encode$lacI^q$, which is a modified version of*lacI* , transconjugants express GFP constitutively. We used fluorescence-activated cell sorting (FACS) for high throughput identification of transconjugants in order to reveal the plasmids transfer host ranges (henceforth referred to as host range). Specifically, host range was investigated through post-mating sorting and 16S rRNA gene amplicon sequencing analysis of taxonomic and phylogenetic relationships of the transconjugant communities. Although the permissiveness towards broad host range plasmids of the microbial community in WWTPs have been assessed utilizing a similar strategy (Jacquiod et al., 2017; Li et al., 2018), the capability of the sewage transconjugant community to further disseminate plasmids to potential pathogens have not been investigated. Thus, subsequently, we assessed the donor potential of sorted transconjugants by further enriching this fraction in plasmid selective media to further track plasmid transfer to a model*Enterobacteriaceae* pathogen. In parallel, we performed the same experiments with pB10, a representative of the well-studied P1 group (Jacquiod et al., 2017; Klümper et al., 2015; Li et al., 2018; Musovic et al., 2014; Pinilla-Redondo et al., n.d.), which we hypothesized to represent the maximal host range grade VI (Redondo-Salvo et al., 2020).

2 Materials and Methods

For an overview of strains and plasmids used in this study, see table 1. and 2.

2.1 Construction of a dual fluorescent reporter gene system

For vector and primer information see Supplementary table 1 and 2, respectively. The insertion of the P_{lpp}*mcherry-*$lacI^q$*-*gen^Rsequence into the chromosomal attTn7 site of the *E. coli donor*was achieved by Tn7 transposition, as described previously (McKenzie & Craig, 2006), utilizing the vector pGRG36::P_{lpp}*mcherry-*$lacI^q$*-*gen^R(Klümper et al., 2015). The construction of the *E. coli* recipient strain was performed in a similar fashion, with the vector pGRG36::P_{lpp}*mCherry-*kan^R . PCR amplification and FACS were used to verify chromosomal integration and loss of vectors. The tagging of R27 with $P_{A1/O4/O3}$*gfpmut3-*kan^R was performed using the λ-red system as described previously (Anjum et al., 2018; Datsenko & Wanner, 2000). The insert sequence was amplified by PCR from the vector pENT::$P_{A1/O4/O3}$*gfpmut3-*kan^R*(Klümper et al., 2015)* with 40 nucleotides 5' overhangs homologous to the *tetR-D* region of the *Tn* 10 tetracycline resistance region of R27. Allelic exchange of this region generated a tetracycline sensitive phenotype. FACS was used to verify expression of the inserted fluorescence reporter gene in the resulting plasmid R27::*gfp* (referred to as R27). The construct was verified by Sanger and shotgun sequencing (Supplementary figure 1 and 2). For shotgun sequencing, R27 was extracted by Plasmid Mini AX kit (A&A Biotechnology) and sequencing libraries built using the Nextera XT DNA Library Preparation Kit (Illumina Inc.), and sequenced on the Illumina MiSeq Desktop Sequencer (Illumina Inc.) following the manufacturer's protocol. R27 and pB10::*gfp*(referred to as pB10) were conjugated into the constructed *E. coli* donor, respectively.

2.2 Flow cytometry and fluorescence-activated cell sorting (FACS)

Flow cytometry analysis and cell sorting (FACS) was done on a BD FACSAria IIIu (BD Biosciences) equipped with BD FACSDiva software v.6.1.3 (BD Biosciences) for general operation. GFP and SYBR Green were excited by a 488 nm laser (20 mW) and detected on a 530/30 nm bandpass filter. The mCherry signal

was excited by a 561 nm laser (50 mW) and detected on a 610/20 nm bandpass filter. Fluorescent minus one (FMO) controls of mCherry, GFP and non-fluorescent samples were used to set PMT voltages and appropriate gating. Samples were diluted in a 0.9% w/v NaCl solution until reaching ˜3000 evt/s, except for counting, where they were diluted 200X. Events were recorded for 60 sec at a flow rate of ˜15 µl/min. Thresholds on the forward scatter (FSC) were kept at 1200 with a "AND" threshold on the side scatter (SSC) kept at the minimum 200. The gating strategy can be seen in supplementary figure 3. Transconjugant cells were identified by sorting 10500 *gfp* expressing cells with purity precision and sample purity was subsequently analyzed by recording the sorted sample for 60 seconds.

2.3 sewage water collection, storage, and microbial community extraction

Sewage from urban areas was collected in late May 2018 from Ellinge (55°49'18.1"N, 13°18'10.3"E), Klagshamn (55°31'31.2"N, 12°55'55.4"E) and Sjölunda (55°37'59.4"N, 13°02'32.2"E) WWTPs in Southern Sweden. Sewage was kept at 4°C post sampling in plastic jerry cans. Sample preparation was as follows; for disruption of particles, the sewage was distributed into sterile 250 ml centrifuge bottles (Nalgene®, Sigma-Aldrich) containing metal beads and were horizontally attached to a shaker (IKA®KS 260 basic, Sigma-Aldrich), shaking at 500 RPM for 15 min and then followed by 1 min of sonication (1510, Branson). Bottles were then incubated on ice for 30 min for sedimentation of larger particles and the supernatant was filtered through a 10 µm coated cellulose acetate (CMF) filter (Advantec®), using a vacuum pump. Filtrates were centrifuged at 8000xG for 7 min at 4°C. Pellets were resuspended in 1 ml ice-cold 0.9 % NaCl solution and pooled together. The up-concentrated suspension was filtered through a 10 µm syringe filter (Frisenette) into a new 50 ml screw-cap tube (Sarstedt) and kept at 4°C until use. In order to enumerate bacteria in the sewage, cells were stained with SYBR green I 10000 X (Invitrogen, ThermoFisher Scientific) as described previously, with minor changes (Paerl et al. 2018). Briefly, 10 ul cell suspension were stained in a 990 ul 2 x SYBR green I solution with 1x TE buffer and 0.9% NaCl. The stained cells were incubated in the dark for 20 min and subsequently quantified by FACS.

2.4 Conjugation assay

Prior to the conjugation assay, the *E. coli* strains were incubated overnight at 30°C on solid lysogeny broth (Lennox) (LB) agar (VWR) supplemented with antibiotics, 30 ug kanamycin/ml for donor/R27 and 10 ug tetracycline/ml for donor/pB10. A single colony per replicate used, was grown in 5 ml LB for 3 hours and enumerated by flow cytometry. Donors and recipients were resuspended in 0.9 % (9 g/l) NaCl solution and mixed in a 1:1 ratio. The 0.2 µm mixed cellulose ester (MCE) filter (Advantec®) was placed on solidified LB agar plates and the mixture was pipetted on to 54 mm^2 corresponding to an initial cell density of 3.6 x 10^5 cells/mm^2. The plates were then incubated for 20 hours at 30°C, at which state cells were harvested; the filter was transferred to a 0.9 % NaCl solution where cells were washed off and the suspension was stored at 4°C.

2.5 Tracking transfer in sewage communities

The sewage community originating from the three WWTPs were supplemented with the *E. coli* donor harboring R27 or pB10 in a conjugation assay. To each donor/plasmid combination, six biological replicates were made and three controls of each donor and sewage community incubated individually. The dissemination of the plasmid was quantified by FACS, counting transconjugants (GFP positive), donors (mCherry positive) and recipients (colorless). To evaluate the host ranges, and the transconjugants donor potential of R27::gfp and pB10::gfp in influent sewage of Ellinge WWTP, 2 x 10500 transconjugants in 4 replicates, of each plasmid, were sorted out by FACS: one replicate for direct 16S rRNA gene sequencing, a second replicate for enrichment of transconjugants. The sorted sewage community/R27 and sewage community/pB10 were enriched in test tubes with 10% (10x diluted) LB supplemented with antibiotics, 30 ug kanamycin/ml for sewage community/R27 and 10 ug tetracycline/ml for sewage community/pB10, for 24 hours at 30°C with 250 RPM. Prior to conjugation assays, cell counts and purity were assessed by FACS ensuring GFP only from the donors. Three out of four biological replicates for each enriched transconjugant sewage community was found to be applicable. Next, enriched transconjugant sewage community samples underwent conjugation assays

with the *E. coli* recipient in three technical replicates per biological replicate, in order to investigate transfer using FACS. In this scenario transconjugants are characterized by bi-fluorescence events of mCherry and GFP, while donors are exclusively GFP positive and recipients are mCherry positive. In one biological replicate of each mating per plasmid the enriched transconjugant sewage community had outcompeted the recipient and the results were not used. Additionally, control experiments with the*E. coli* donor and an untagged strain of the *E. coli*recipient were conducted: Six replicates were made per donor/plasmid combination. See figure 1 a) and b) for an overview of how plasmid transfer was determined.

Plasmid transfer was measured as transconjugants per recipient (T/R), calculated as the number of transconjugant (T) cells divided by the number of recipient cells $\frac{T}{R}$

2.6 DNA extractions and sequencing groups

DNA was extracted from 0.25 ml of high biomass samples by the Nucleospin® soil kit (Macherey-Nagel): three samples of the recipient sewage community, filter mated alone; three samples of enriched sewage community/R27; four samples of enriched sewage community/R27; 1 sterile control sample of 0.9 % NaCl solution and 1 sterile control sample of nuclease-free water (Sigma Aldrich). For sample groups with low biomass, DNA was extracted using the GenePurgeDirect(Nimagen) kit: 4 samples of sewage community/R27 with ˜ 10000 transconjugant cells per sample; 4 samples of sewage community/pB10 with ˜ 10000 transconjugant cells per sample; 1 sterile control sample of 0.9 % NaCl solution and 1 sterile control sample of nuclease-free water (Sigma Aldrich). For information on sequenced samples see supplementary table 3.

2.7 16S rRNA gene amplicon sequencing of the microbiome from Ellinge WWTP

For detailed information see supplementary method 1 and for information on sequenced samples see supplementary table 3. Briefly, sequencing libraries were made in a dual-PCR setup. In the first PCR, amplifying the 16s rRNA gene, primers Uni341F and Uni806R (Yu et al., 2005) were used, which amplifies the V3-V4 region of this gene. In the second PCR primers introducing sequencing adaptors and barcode tags were used (Nunes et al., 2016). 16s rRNA gene amplicon sequencing was done using an Illumina MiSeq Desktop Sequencer (Illumina Inc.). Raw sequence reads were trimmed using cutadapt version 2.3 (Martin, 2011). Primer-trimmed sequence reads were error-corrected, merged and amplicon sequence variants (ASVs) identified using DADA2 version 1.10.0 (Callahan et al., 2016) plugin for QIIME2 (Bolyen et al., 2019). For rarefaction curves see supplementary figure 6. A multiple sequence alignment of the ASVs was performed with mafft v7.407 (Katoh & Standley, 2013) and used to build an approximate ML tree with FastTree v2.1.10 (Price et al., 2010). R (R Core Team, 2020) was used for sequence and data analysis for the 16S rRNA gene community profiling. Furthermore were the tidyverse (Wickham et al., 2019) and phyloseq (McMurdie & Holmes, 2013) packages used for visualization and general data handling. Taxonomy was assigned with the dada2 package (Callahan et al., 2016) using the Genome Taxonomy Database (GTDB; https://doi.org/10.5281/zenodo.2541239) (Parks et al., 2018). The Alpha diversity metrics Faith's phylogenetic diversity (Faith, 1992), Mean pairwise distance (Webb et al., 2002) was calculated with the PhyloMeasures package (Tsirogiannis & Sandel, 2016). For the beta diversity, weighted Unifrac distances were calculated (Lozupone & Knight, 2005). The phylogenetic tree (figure 3.a) was made using the iTOL webtool (Letunic & Bork, 2019). For investigations of the low biomass samples sewage community/pB10 and sewage community/R27 such as alpha diversity measures, phylogeny, and abundances, a cleaned data object was used (see supplementary method 1), to avoid the influence of the kitome (i.e. the background signal of kits used) and other potential contaminants to which low biomass samples are more vulnerable than high biomass samples (Davis et al., 2018). Data cleaning for the eight low biomass samples resulted in the removal of 24165 reads, from 162040 to 137875 reads, thus removal of 14.9 % of the reads. The mean number of reads for the cleaned samples were 17234, and the minimum/maximum was 15038/20999 reads. The number of taxa for these samples was reduced from 299 to 65, thus removal of 78.26% of the taxa.

2.8 Data analysis

All pairwise comparisons were made using the Wilcoxon Rank-sum test of the stats package (R Core Team, 2020). Pearson correlation tests were added to plots with the ggpupr package (Kassambara, 2020). Per-

mutational multivariate analysis of variance (PERMANOVA) tests was performed using the vegan package ({Jari Oksanen et al., 2020), with a default 999 permutations.

3 Results

3.1 HI1A and P1 plasmids transfer to the sewage community

To investigate the transfer dynamics of the HI1A plasmid R27 across the selected sewage communities, we inserted the *gfp* tag into the Tn10 tetracycline resistance region (supplementary figure 4), resulting in its deletion. Performing conjugation assays, we introduced the donor strain, *E. coli* MG1655, carrying R27 into sewage communities from three Swedish urban area WWTPs (Ellinge-, Klagshamn-, and Sjolunda-WWTP). In parallel, we performed analogous matings with the IncP1 plasmid, pB10, as a benchmark for broad host range transfer (figure 2a).

We consistently observed plasmid transfer ranging from 2.6 x 10^{-5} to 2.8 x 10^{-4} T/R for R27 while the transfer of pB10 ranged from 2.6 x 10^{-2} to 3.2 x 10^{-1} T/R in all three sewage communities. This corresponds to an average ˜1150-fold lower transfer of R27 than pB10 ($p<0.001$) (figure 2a).

3.2 The sewage community is rich in potent plasmid donors

In order to assess the potential of HI1A plasmid dissemination within sewage communities, we sorted and enriched R27 and Pb10 transconjugants from conjugation assays from Ellinge sewage community. Briefly, the enriched transconjugant sewage communities were grown in broth with plasmid selective antibiotics, and GFP expression was verified, to ensure plasmid maintenance and trackability. We subsequently quantified the plasmid transfer to a *E. coli* MG1655 recipient not encoding $LacI^q$. This was furthermore compared to direct plasmid transfer from the *E. coli* donor to the *E. coli* recipient (figure 2b). The enriched transconjugant sewage community members harboring either R27 or pB10 and the *E. coli* donor were found to transfer their plasmids equally efficiently to the *E. coli*recipient ($p > 0.05$) (figure 2b). However, both the conjugation assays from *E. coli* to *E. coli* and enriched transconjugant sewage community to *E. coli* , revealed that R27 was transferred significantly less than pB10 ($p<0.05$) (figure 2b).

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3.3 R27 exhibits a broad potential host range, but a low host divergence and is less similar to the sewage community than pB10.

In order to investigate the phylogenetic host range of the two plasmids, we conducted 16S rRNA gene profiling of the transconjugants originating from conjugation assay challenging Ellinge sewage community with plasmids R27 (sewage community/R27) and pB10 (sewage community/pB10). Subsequently, we profiled the catalogue of recipients in the sewage community to examine how widely the plasmids transfer within this pool of recipients.

The plasmid transfer was initially evaluated in the two transconjugant sewage communities by alpha diversity analyses: Faith's phylogenetic diversity (PD) (Faith, 1992) measures the total phylogenetic distance among the hosts, which we interpret as a metric of the distribution breath describing a plasmids potential host range. Mean pairwise distance (Webb et al., 2002) describes the mean phylogenetic distance between hosts, which we interpret as average pairwise host divergence (Tucker et al., 2017). PD was similar for R27 and pB10 in this sewage community (figure 3a), revealing a broad potential host range for both plasmids. We observed a significantly higher mean pairwise distance of transconjugants for sewage community/pB10 compared to sewage community/R27 ($P<0.05$) (figure 3b). Thus, R27 has a broad potential host range but a low host divergence, whereas pB10 has a broad potential host range and a high host divergence.

Next, we compared the composition of the two transconjugant communities to the entire recipient sewage community to investigate beta diversity. The diversities between the sample groups: recipient sewage community, sewage community/pB10, and sewage community/R27, were calculated using Weighted Unifrac. Ordination by Principal Coordinates Analysis (PCoA) with Weighted Unifrac distances between samples

revealed that samples cluster neatly according to the sample group, highlighting a strong biological underpinning for the observed trends. Furthermore, axis 1 and 2 comprise/explain a total of 93.8 % of the variance (figure 3c). This decisively shows less distance in-between replicates than between sample groups and suggests that plasmid-specific host range drives the divergence of transconjugant communities. Pairwise weighted Unifrac distances between sample groups revealed a higher distance between the recipient sewage community and sewage community/R27 than between the recipient sewage community and sewage community/pB10 (figure 3d). Additional PERMANOVA analyses of the weighted Unifrac distances found higher R^2 for sewage community/R27 (R^2=0.71, $p < 0.001$) than for sewage community/pB10 (R^2= 0.49, $p < 0.001$) (figure 3d). These results imply that the transfer of R27 is a stronger driver of phylogenetic distance between the resulting transconjugant sewage community and the recipient sewage community than the transfer of pB10.

3.4 R27 transfers primarily to *Enterobacteriaceae, and secondarily* across order and phylum

Next, we analyzed the taxonomic composition of the transconjugant communities sewage community/R27 and sewage community/pB10. Every ASV was associated with the gram-negative phylogroup. In total we identified 65 ASVs, of which 63 were distributed within six families of Proteobacteria, while the remaining two were members of the Bacteroides family *Flavobacteriaceae* (figure 4a). Transconjugants of R27 and pB10 were found within all these seven families (figure 4a). Every ASV was, however, not shared by pB10 and R27; six ASVs were unique to pB10, while 19 were unique to R27 (supplementary figure 5). R27 was identified in a relatively high number of closely related *Enterobacteriaceae*(figure 4a). Quantification of the relative abundance emphasized that sewage community/R27 predominantly consist of*Enterobacteriaceae* (= 77 %) (figure 3b). Other noticeable families of the sewage community/R27 were represented in considerable numbers (= >3 %), including *Aeromonadaceae* ,*Moraxellaceae* , *Shewanellaceae* and *Pseudomonadaceae*(figure 4b, supplementary table 4). In contrast, the relative abundance of *Enterobacteriaceae* was rather low *(* = 1.7 %) in sewage community/pB10, where the majority of ASVs belonged to the two families *Aeromonadaceae (* = 49 %) and *Pseudomonadaceae (* = 36 %) (figure 4b). Other families that represented = >3 % of the pB10 transconjugants were*Shewanellaceae* and *Flavobacteriaceae* (figure 4b & supplementary table 4). Lastly, we found a high degree of strain specificity in terms of plasmid uptake within similar genera for both transconjugant communities (figure 4a).

3.5 R27 transfer does not correlate with recipient abundance, unlike pB10

The most abundant transconjugant ASVs carrying pB10 were also the most abundant ASVs in the recipient sewage community. In contrast, the most abundant transconjugant ASVs in sewage community/R27 were some of the low abundant ASVs in the recipient sewage community (figure 4a). Accordingly, a Pearson correlation test indeed indicated a positive correlation between the log10-transformed relative abundance of an ASV in the recipient sewage community and its corresponding log10-transformed relative abundance in sewage community/pB10 (R = 0.67, p <0.001), while showing no positive correlation between the log10-transformed relative abundance in the recipient sewage community and transconjugants ASVs in sewage community/R27 (R = -0.055, p = 0.66) (figure 5a).

3.6 Culture enrichment of transconjugant sewage community resulted in single genera domination

Lastly, we assessed the taxonomy of the sorted and enriched transconjugant sewage community, in order to understand which hosts enable stable replication and maintenance of the two plasmids, and hence are able to support all three stages of plasmid host range. For all samples, enrichment promoted single-genus dominance, yet marked differences were observed between the two plasmid-associated sewage community transconjugant pools. While *Serratia* was primarily enriched in the transconjugant sewage community/R27 (= 99.2%),*Aeromonas* dominated the transconjugant sewage community/pB10 pool (= 99%) (figure 5b).

4 Discussion

4.1. The plasmids transfer in the sewage community

In this study, we investigated the transfer dynamics of the HI1A plasmid R27 in the sewage communities of the influent water of three WWTPs in Sweden (Ellinge-, Klagshamn-, and Sjolunda-WWTP). We recorded transfer of R27 to the sewage community from all three WWTP, indicating the potential of HI1A plasmids as effective vectors for the spread of ARGs to the microbial communities of WWTPs (Figure 2a). Yet, we found that the transfer of the P1 plasmid pB10 to the recipient sewage community was significantly higher than that of R27 (Figure 2a). This could be a result of the broader host range of pB10 compared to R27 in the sewage community, enabling more transfer events. However, we also show that pB10 has a higher transfer than R27, in *E. coli* to *E. coli*conjugation (Figure 2b), but the relative difference between the transfer of the two plasmids is remarkably higher in the sewage community. Thus the higher transfer in the sewage community of pB10 is likely a result of both a broader host range and higher transfer efficiency. Broad host range plasmids, such as P1 plasmids are known to be common in WWTPs (Bahl et al., 2009). Furthermore, P1 plasmids have been shown several times to transfer well to WWTPs communities (Jacquiod et al., 2017; Li et al., 2018) and even increase the fitness of strains originating from WWTPs without plasmid specific selection (Li et al., 2020). Thus, we expected that pB10 would transfer well in the sewage community. However, the higher transfer of pB10 to the recipient sewage community, compared to R27, is not necessarily of high medical relevance. Since the medical relevance of a plasmid is likely dependent both on its host and accessory genetic cargo (e.g. virulence and resistance determinants). Additionally, the medical relevance also depends on the capability of the recipient sewage community to further disseminate the plasmid, potentially reaching infectious human pathogens.

The conjugative assay described in this work, mixing the enriched transconjugant sewage community harboring either R27 or pB10 and a recipient *E. coli* , simulated the transfer of ARGs from sewage communities to a potential pathogenic *Enterobacteriaceae* . The enriched transconjugant sewage communities were, for both plasmids, found to be potent plasmid donors with high transfer to the *E. coli* recipient, corresponding to the transfer of the respective plasmid from *E. coli* to *E. coli* (Figure 2b). Thus, the sewage community were not only recipients of R27 and pB10 but could also efficiently disseminate them further. Furthermore we found that, regardless of the donor-recipient combinations tested, R27 transferred significantly less than pB10, indicating that the difference in plasmid transfer ratios is plasmid dependent. Our results support a growing body of work exposing the sewage microbiome as a facilitator of HGT (Guo et al., 2017; Jacquiod et al., 2017; Li et al., 2018). To our knowledge, this is the first study showing that the sewage community additionally could facilitate conjugative transfer of HI1A plasmids resulting in an environmental spread of medical relevant ARGs.

4.2 Host range and gene flow

The 16S rRNA gene profile of the transconjugant communities allowed us to analyze the host ranges of R27 and pB10 in the recipient sewage community extracted from Ellinge WWTP influent. By alpha diversity measures of phylogenetic distances in the transconjugant communities, we found that both R27 and pB10 had a relatively broad potential host range (figure 3a). However it was clear that the host divergence of R27 was low, as indicated by the mean pairwise distance in contrast to pB10 (figure 3b). We found that the ASVs to which R27 and pB10 were transferred to in our conjugation assay with sewage communities, were constrained to seven families. Namely, *Aeromonadaceae* ,*Burkholderiaceae* , *Enterobacteriaceae* ,*Flavobacteriaceae* , *Moraxellaceae* , *Pseudomonadaceae*and *Shewanellaceae* (figure 4a). R27 was predominantly transferred to members of the *Enterobacteriaceae* family (figure 4b). This observation justifies the low host divergence for R27 and emphasizes that this plasmid mainly is distributed within the*Enterobacteriaceae* family. In contrast, the transfer of pB10 is distributed more evenly among families and primarily occurring in two families i.e. *Aeromonadaceae* and *Pseudomonadaceae* (figure 4b). Other studies investigating transfer and following a similar approach have reported a far more diverse transconjugant community for the P1 plasmids. Indeed, the pool of transconjugants has been shown to include members from many phyla other than Proteobacteria, and even certain Gram positives (Klumper et al., 2015; Li et al., 2018; Musovic et al., 2014; Pinilla-Redondo et al., n.d.). This indicates that the conjugation assay used in this study is constraining the diversity of the recipient community (e.g., due to cultivation-related bias), thus limiting the maximum broadness of transfer.

Even though only a relatively low abundance of non-*Enterobacteriaceae* ASVs received R27, our observations expand the range of potential HI1A hosts considerably. We find transfer to genera within the families of *Flavobacteriaceae* ,*Burkholderiaceae* , *Moraxellaceae* , *Pseudomonadaceae*and *Shewanellaceae* (figure 4A). Remarkably, this results in a grade VI host range for both R27 and pB10, according to the host range grade scale proposed by Redondo-Salvo et al. (Redondo-Salvo et al., 2020). Previous studies have so far only identified HI1A plasmids in two genera outside *Enterobacteriaceae* : *Aeromonas* and *Vibrio,* although both belong to the Enterobacteriales order (Maher & Taylor, 1993; Suzuki et al., 2010). Likewise, the plasmid database PLSDB (Galata et al., 2019), V2020_06_29, only contains one HI1A plasmid found in a host outside*Enterobacteriaceae* , a *Pantoea* sp. (Conlan et al., 2014). According to the NCBI taxonomy (Schoch et al., 2020),*Pantoea* is assigned to the Enterobacteriales family*Erwiniaceae,* yet according to the GTDB taxonomy (Parks et al., 2018) used in this study, it is assigned to *Enterobacteriaceae* . Therefore, it is possible that the majority of these broad host range events of R27 only reflect transfer host range, and are not stably maintained over time. Importantly, such short-term events may reflect crucial intermediate stepping-stones that perhaps circumvent spatial segregation of donor and suitable hosts, increasing the likelihood of reaching evolutionary stable hosts. However, these events do still raise a concern in terms of spread of ARGs. Since plasmids can integrate, fully or partially, into their host chromosomes, they can potentially spread ARGs to bacteria in which they are not evolutionary stable. and hence to groups of bacteria that we did not previously expect. Integrational events of plasmid into the host chromosome are well-documented and have also been found to increase plasmid susceptibility of the bacterial host (Tardif & Grant, 1983). Furthermore, many ARGs carried on plasmids are located within transposons, possibly allowing the ARGs to jump into the host chromosome or hitchhike onto new plasmids colocated in the host cell (Razavi et al., 2020). Halary et al. found that plasmids have a central role as key vectors of gene flow between bacterial genomes in nature (Halary et al., 2010). Thus, the broad plasmid transfer host range of R27, may facilitate gene flow across a surprisingly broad phylogenetic span.

Furthermore, our results indicate that HI1A plasmids may be highly involved in genetic exchange between *Enterobacteriaceae* , since there is a high abundance of transfer events to a relatively wide range of *Enterobacteriaceae* , among which several genera are known to include human pathogens. Our results thus highlight a potential link for gene flow between these pathogenic types, which requires further investigations. Other studies have likewise found proof of plasmids ensuring a high genetic interconnectedness between*Enterobacteriaceae (Redondo-Salvo et al., 2020)* . We found that R27 was specifically transferred to 5 out of the 9 genera with pathogenic members classified as 'highest priority of critical pathogens' (WHO, 2017); namely, *Acinetobacter, Pseudomonas, Klebsiella, Escherichia and Serratia* . This suggests that HI1A plasmids could serve as a genetic link between these genera in which resistances to cephalosporins and especially carbapenems are a major clinical issue (WHO, 2017). Antibiotic resistances, which are known to be encoded by HI1A and the closely related IncHI1B plasmids (Carattoli, 2013; Dolejska et al., 2013; Zurfluh et al., 2014).

4.3 Barriers to plasmids transfer and the core permissive fraction

We observe a high degree of strain specificity for plasmid uptake within genera (figure 4a). Consistent with this, Maher & Taylor found that the uptake of different HI1A plasmids vary highly at the species level (Maher & Taylor, 1993). Likewise, another study also found that the capability of receiving plasmids is highly variable and strain specific, even within a single genus (Li et al., 2018). We speculate that this phenomenon can be attributed to a lack of host encoded defense systems e.g. R/M, CRISPR-Cas systems, etc. Moreover, other barriers to plasmid transfer that could cause strain-specific levels of permissiveness are plasmid incompatibility and plasmid entry exclusion mechanisms (Novick, 1987). Recently, it has been reported that a fundamental function of the diverse plasmid-encoded type IV CRISPR-Cas system is to target and eliminate competing plasmids (Crowley et al., 2019; Pinilla-Redondo et al., 2020).

We identify 40 shared ASVs (supplementary figure 5), which we define as the core permissive fraction. Within the core permissive fraction of our study, we do find ASVs representing the seven families identified in the overall pool of transconjugants: *Aeromonadaceae* ,*Burkholderiaceae* , *Enterobacteriaceae* ,*Flavobacteriaceae* ,

Moraxellaceae , *Pseudomonadaceae*and *Shewanellaceae* . The families *Aeromonadaceae* ,*Pseudomonadaceae* and *Shewanellaceae* are even found at relative abundances above 3% for both R27 and pB10 (figure 4b). Previous studies have identified *Aeromonadaceae* ,*Enterobacteriaceae* , *Moraxellaceae* and*Pseudomonadaceae* as part of the core permissive fraction, which extends across diverse environments (Klumper et al., 2015; Li et al., 2018; Musovic et al., 2014; Pinilla-Redondo et al., n.d.). Of special interest are *Enterobacteriaceae* and*Aeromonadaceae* , as these two families have been found to represent a large fraction of the core permissive fraction in WWTPs (Li et al., 2018). Suggesting further importance in the core-permissive fraction of these two families is the observation that a enrichment of R27 and pB10 transconjugants resulted in the domination of a single genus of*Serratia* (*Enterobacteriaceae*) or *Aeromonas*(*Aeromonadaceae*), respectively (Figure 5b). Thus we also show that members of the core-permissive fraction are fully capable of further carrying and disseminating plasmids. Given the positive impact a plasmid can have under selective conditions, and conversely, the parasitic burden it may represent when it does not grant advantages, it is not unimaginable that some bacterial families have evolved to embrace plasmids, while other families have evolved to categorically reject them. We speculate that a central feature of the core permissive fraction could be a lack of plasmid-targeting host defenses. Altogether, the families *Aeromonadaceae* , *Enterobacteriaceae* ,*Moraxellaceae* , *Pseudomonadaceae* and *Shewanellaceae*seem to play a central role in the dissemination of ARGs via plasmids and our work suggest that HI1A and P1 plasmids are included in this network of conjugation within the sewage microbiome.

4.4 Plasmid lifestyle

The concept of generalists (the capability of adapting to diverse habitats) and specialists (adapting to specific habitats) (Sriswasdi et al., 2017; Van Tienderen, 1991) have been extended to microbial ecology (Sriswasdi et al., 2017; Szekely & Langenheder, 2014). We propose this theory can be further extended to plasmids, the habitats of which are their host cell. Our analysis indicates that R27 is a specialist, transferring within*Enterobacteriaceae* . This is supported by both the low host divergence (figure 3b), lower similarity to the sewage community than pB10 transconjugants (figure 3c and d), and the lack of correlation between the ASV abundance in the recipient sewage community and the R27 transconjugant community (figure 5a). Contrarily, the transfer dynamics of pB10 resemble that of a generalist, with a high host divergence (figure 3b) higher similarity to the sewage community than R27 transconjugants (figure 3c and d) and a high correlation between ASV abundance in the recipient sewage community and the pB10 transconjugant community (figure 5a).

The specialization-disturbance hypothesis imposes that generalists are more resilient to disturbances altering niches than specialists (Vazquez & Simberloff, 2002). Thus a generalist plasmid lifestyle could ensure a higher resilience to change in the composition of available hosts. We argue that having a broad host range does not in theory have to be accompanied by being a generalist, yet being a generalist will likely be accompanied by also having a broad host range. The high transfer of the generalist pB10 could be considered as an adaptation to a low level of events that eventually are evolutionarily stable. Conversely, a specialist like R27, in which the majority of transfers are to very similar hosts, might have a high level of stable events due to low negative influence by host defence systems. Likewise this could result in a higher tendency of carrying more and/or diverse anti-defense systems for the generalist, than the specialist, as a co-evolutionary response to the encounter with diverse host defense systems. Further investigation on the long term persistence of plasmids in the host cell, derived from these two distinct competitive strategies in multispecies communities could be of interest to the field and our understanding of HGT mediated dissemination of ARGs.

4.5 Conclusive remarks

In conclusion, we show that the intermediate host range plasmid R27, of the HI1A group, transfers horizontally among the microbial communities extracted from three Swedish WWTPs. However, the transfer frequency of R27 was always lower than that of plasmid pB10, of the P1 group. The enriched transconjugant communities were found to be efficient plasmid donors to a model of a pathogenic *Enterobacteriaceae* strain, revealing that strains in the recipient sewage community are able to acquire and disseminate both plasmids. Our results do furthermore suggest that the consistently higher transfer frequency of pB10 compared to

R27 is a plasmid intrinsic trait. We find that R27 has a broad potential host range, but a low host divergence. Whereas pB10 has both a broad potential host range and a high host divergence. We discovered that the vast majority of R27 transfer events were distributed within members of the *Enterobacteriaceae* family. Nonetheless we also find a subset of transfer events to members of *Aeromonadaceae,Burkholderiaceae, Flavobacteriaceae* , *Moraxellaceae* ,*Pseudomonadaceae* and *Shewanellaceae* , thus revealing a transfer host range up to grade VI, even across phyla. It is very rare to isolate HI1A plasmids from hosts outside the Enterobacteriales order, thus we speculate that these phylogenetically broad transfer events are short term, consisting of hosts that do not stably maintain the plasmid. However, these hosts may act as intermediate hosts utilized as "plasmid stepping stones" ensuring transfer to compatible hosts within Enterobacteriaceae. These events could additionally also indicate that HI1A plasmids facilitate a horizontal gene flow between*Enterobacteriaceae* across phyla. Whereas R27 seems to be specialized in its transfer, the transfer of pB10 reflects that of a generalist transfering to the most abundant bacteria in the recipient sewage community. A strategy that could ensure a higher resilience to change in the microbial composition. The enriched transconjugant sewage community, harboring R27 or pB10, were almost completely dominated by a single genus of *Enterobacteriaceae* or *Aeromonadaceae* , respectively. Nevertheless, as in our study these families are often found as the core permissive fraction, across diverse environments, suggesting a pivotal role in plasmid dissemination. In a broader context, these results provide new insight to the transfer of resistance in complex communities and emphasize the role of HI1A plasmids as vectors for environmental spread of ARGs. By demonstrating that HI1A plasmids conjugate in sewage communities and ultimately end in a family which comprises a large number highly medical relevant pathogens, our results support previous studies and highlight that WWTPs are facilitating the dissemination of resistance genes.

Table 1. Strains used in this study

Strain	Naming in this study	Chromosomal insert	Reference
Escherichia coli MG1655	*E. coli* donor	P_{lpp}*mcherry-lacI*^{q}*-Gen*^{R}	This study
Escherichia coli MG1655	*E. coli* recipient	P_{lpp}*mcherry-Kan*^{R}	This study

Table 2. Plasmids used in this study

Plasmid	*Inc*	*Resistance conferred*	*Insert site*	*Reference*
pB10::$P_{A1O4/O3}$*gfpmut3*	P1	Tet^{R}, Kan^{R}, Amp^{R}, Sul^{R}, Str^{R}	*kfrB*	(Van Meervenne et al., 2012)
R27::$P_{A1O4/O3}$*gfpmut3*	HI1A	Kan^{R}	*tetR-tetD*	This study

Gentamicin resistance (Gen^{R}), kanamycin resistance (Kan^{R}), streptomycin resistance (Str^{R}), ampicillin resistance (Amp^{R}), tetracycline resistance (Tet^{R}), sulfonamide resistance (Sul^{R}).

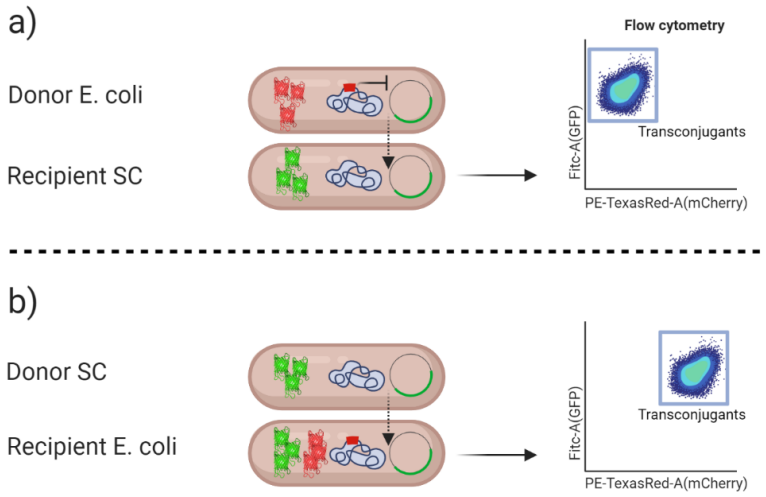

Figure 1. a) Shows the scenario of plasmid transfer from the red (mCherry) fluorescing E. coli donor strain to the sewage community (Recipient SC), where transconjugants will fluoresce green (GFP). b) Shows the scenario of plasmid transfer from the enriched transconjugant donor sewage community (Donor SC), fluorescing green (GFP) to the recipient E. coli strain fluorescing red (mCherry), where transconjugants will fluoresce both red (mCherry) and green (GFP). At the right hand side of both a) and b) is shown how the transconjugant will appear during flow cytometry. Created with BioRender.com

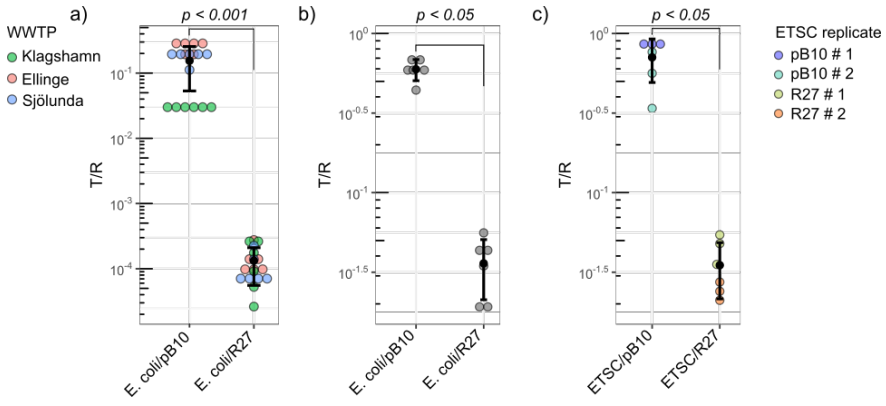

Figure 2. a) shows the transconjugants per recipient (T/R) of the plasmids R27 and pB10 from the E. coli plasmid donor (E. coli/pB10 and E. coli/R27) to the sewage community, in conjugation assays. The figure

summarizes transfer to the sewage community of Ellinge-, Klagshamn- and Sjölunda WWTP, WWTP are additionally indicated by color. Six biological replicates were made for each plant per donor/plasmid.**Dot plot b)** shows the T/R from the sorted and enriched sewage community, harboring either pB10 (Enriched T. sewage/pB10) or R27 (Enriched T. sewage/R27), to the recipient E. coli strain. Each enriched transconjugant sewage community sample number is indicated by color, and conjugation assays were performed as three technical replicates per enriched transconjugant sample. In comparison plasmid transfer from the E. coli plasmid donor strain (E. coli/pB10 and E. coli/R27) to the E. coli recipient strain is shown and color coded gray. **For both plots** are donors shown on the x-axis and the y-axis represents T/R, shown as a logarithmic scale. P values indicate significant differences (Wilcoxon rank-sum test, adjusted p values) in T/R. Error bars represent the standard deviation and the black dot the mean.

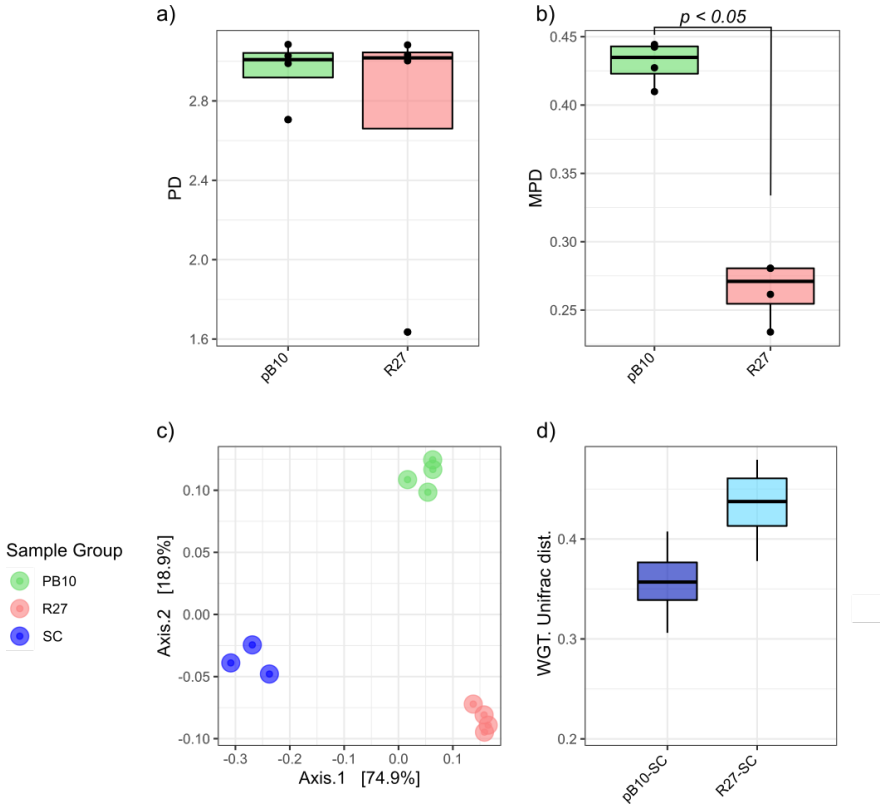

Figure 3. Diversity measure of the transconjugant sewage community**.** The top row of plots shows phylogenetic alpha diversity measures of the sewage community transconjugant pools of pB10 and R27, colored and plotted on the x-axis as "Plasmid". Boxplot a) shows on the y-axis Faith's Phylogenetic Diversity (PD). Boxplot b) shows on the y-axis mean pairwise distance (MPD). The bottom row of plots shows beta diversity, measured by weighted Unifrac distances, between the sewage community transconjugant pools and the recipient sewage community. Dot plot c) shows a Principal Coordinates Analysis (PCoA) of the weighted Unifrac distance, colored according to sample group. Boxplot d) shows on the y-axis the pairwise weighted Unifrac distance (WGT. Unifrac dist.), colored and plotted on the x-axis according to the group pair (Sample Group).

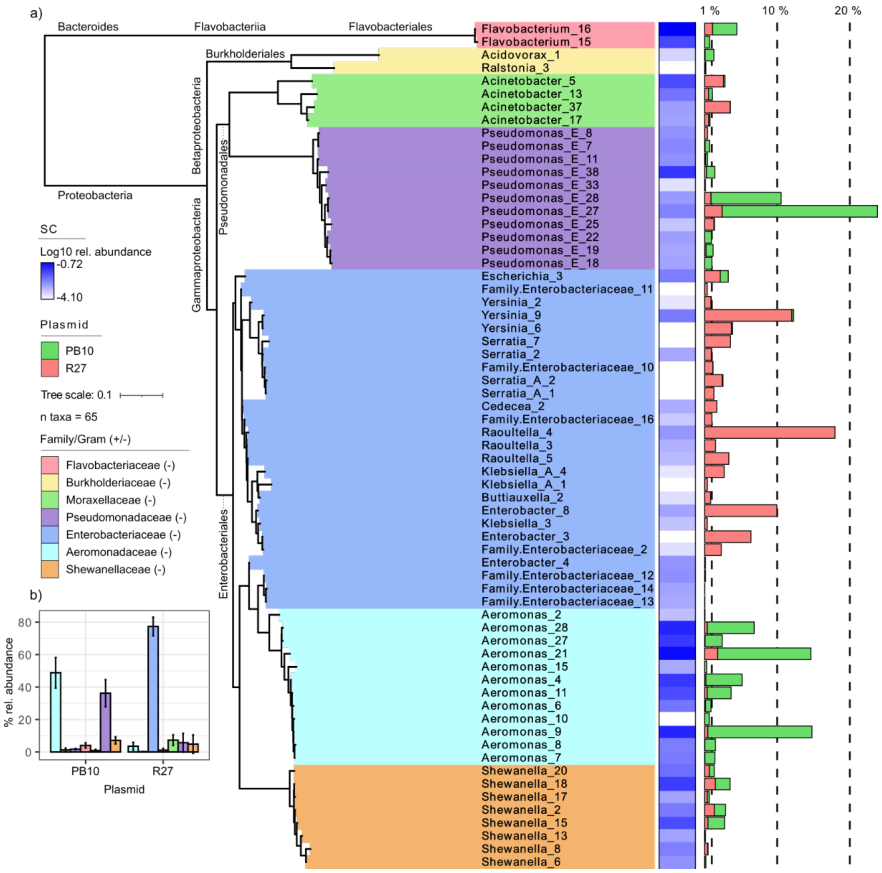

Figure 4. Phylogeny and relative abundance of the transconjugant sewage community**.** a) Phylogenetic tree, based on Maximum Likelihood, for the 65 ASVs present (n taxa) in the transconjugant sewage community after data cleaning. The ASVs are named according to genus annotation. As the legends at the left indicate, ASVs are colored according to family. ASVs were only assigned to Gram-negative phylogroups indicated by Gram (+/-). The log10 relative abundance of each ASV in the recipient sewage community (SC) is shown as the white to blue scaled heatmap. A pseudo count of 1 read was added to the OTU table before log transformation. The bar chart shows the distribution of mean % relative abundance for each transconjugant sewage community (pB10 and R27) between the ASVs, colored according to the plasmid. Taxonomy of clades is indicated by the text on branches, the phyla Proteobacteria, Bacteroides, the classes Flavobacteriia, Gammaproteobacteria, Betaproteobacteria and orders Enterobacteriales Pseudomonadales, Burkholderiales and Flavobacteriales. The bar plot b) shows the mean % relative abundance (% rel. abundance on the y-axis) between families in the two transconjugant sewage communities (Plasmid), plotted on the x-axis. Colored according to families, this legend is shared between figure a) and b). Error bars represent standard deviation.

Figure 5. Analysis of relative abundances of the transconjugant communities and enriched transconjugant communities. The scatterplot a) comparing the relative abundance of an ASV in the recipient sewage community, plotted on the x-axis to its relative abundance in the transconjugant sewage community, plotted on the y-axis. Both axes are shown as logarithmic, the blue line indicates a linear regression line and the gray area indicates a 95% confidence interval. The Pearson correlation coefficient (R) and corresponding p-value (p) are shown at the top of the plot. A pseudo count of 1 read was added to each count before transformations. Plots are divided according to the plasmid of the transconjugant sewage community. The bar plot b) shows the % relative abundance (% rel. abundance), plotted on the y-axis, of

families/genera in the enriched transconjugant sewage community (ETSC) plotted on the x-axis. Colored according to family/genus, genera below 1% relative abundance are shown as "Other". Error bars represent standard deviation.

Data accessibility

All raw paired-end sequence data, including negative controls and a mock community sample are accessible on EBI-ENA under study accession number PRJEB44804.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions:

All work was supervised by S.M. and S.J.S.; A.K.O., R.P.R., A.D., B.F.S., J.S.M. and S.J.S. planned the experiments; A.K.O. carried out plasmid and strain tagging under the guidance of J.S.M.; Vector construction for attTn7 chromosomal tagging where carried out by M.F.H.; A.K.O carried out sampling, planned by A.D and B.F.S.; A.K.O. carried out conjugation assays, flow cytometry and cell sorting under the guidance of R.P.R., J.S.M. and S.J.S.; DNA extraction and sequencing were carried out by A.K.O and J.N.; Sequence analysis and other data treatment were carried out by A.K.O under the guidance of J.N. and J.R.; A.K.O. wrote the article and all authors contributed to the manuscript with feedback and input.

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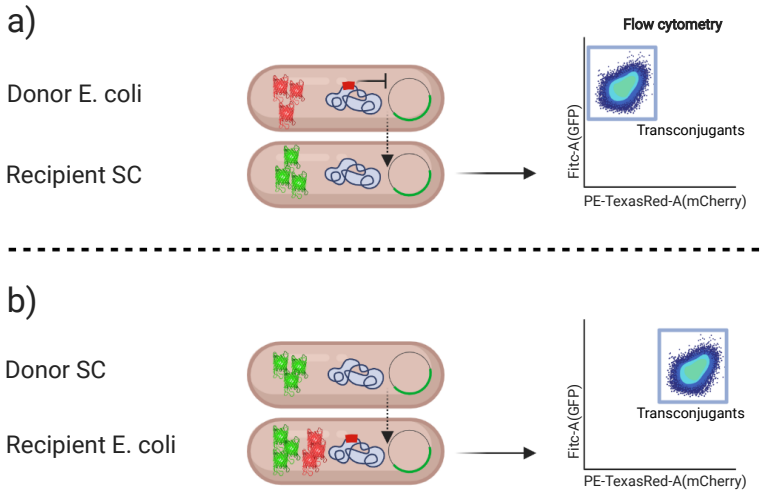
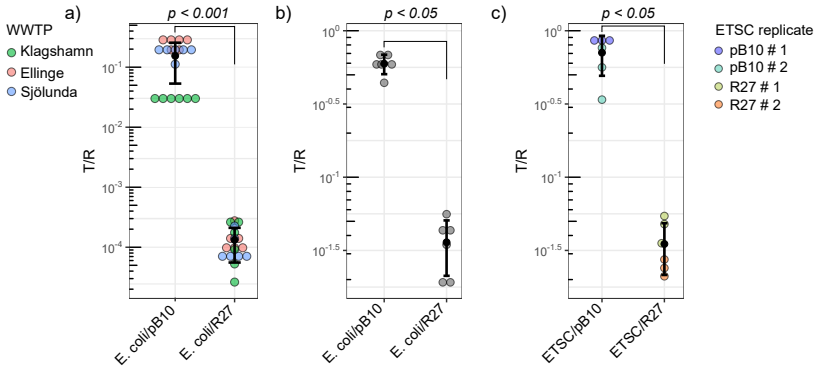
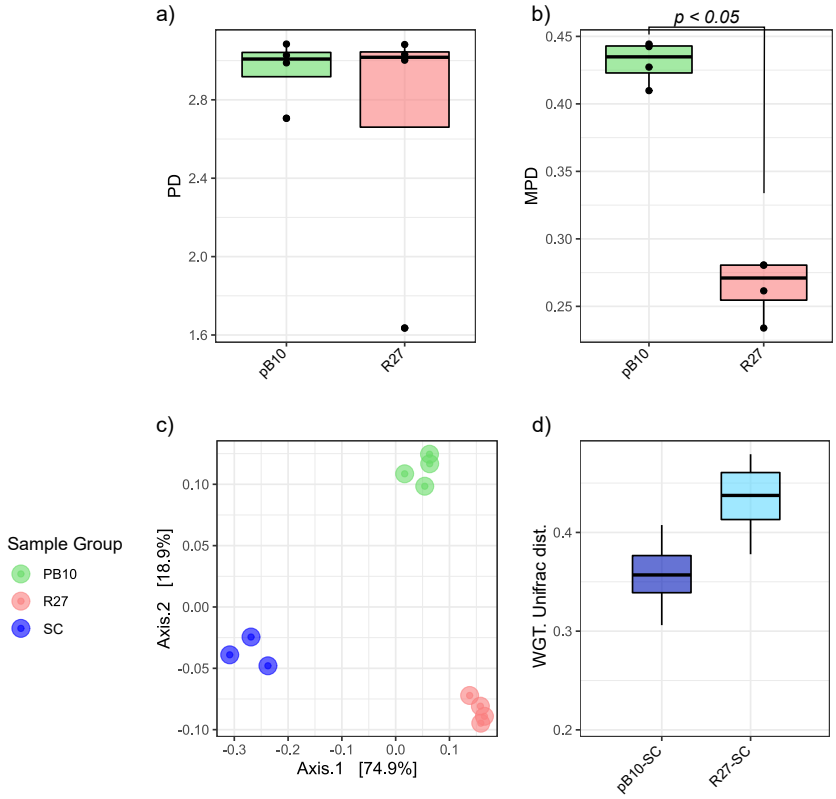
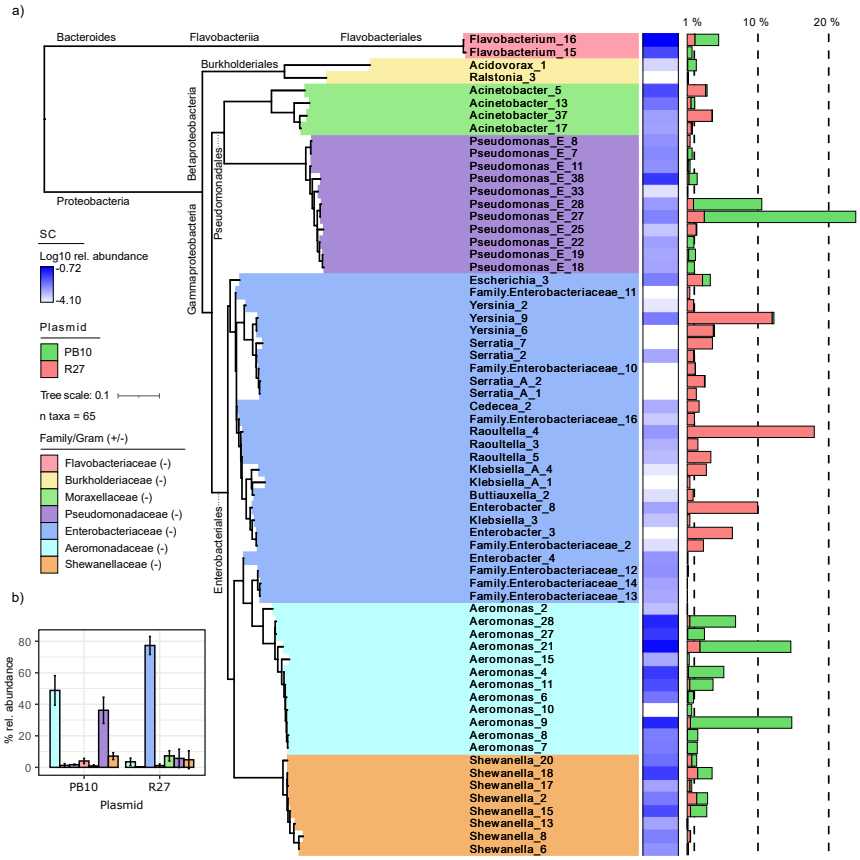
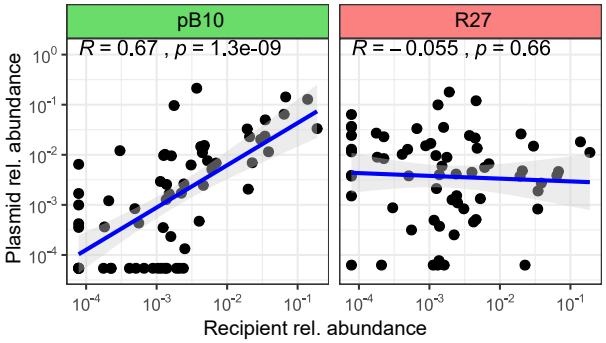








a)



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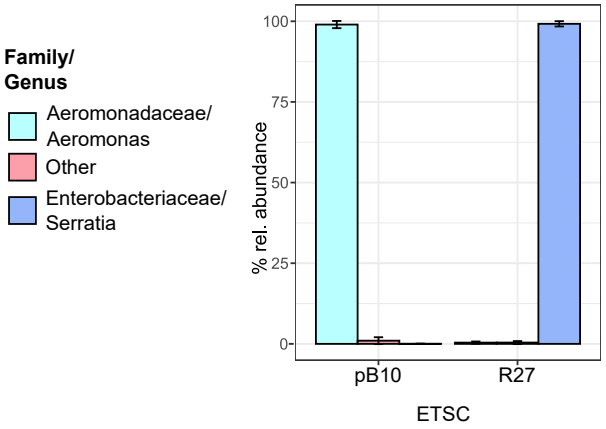

Table 1. Strains used in this study

Strain	Naming in this study	Chromosomal insert	Reference
Escherichia coli MG1655	*E. coli* donor	P_{lpp}*mcherry-lacI*^{q}*-Gen*^{R}	This study
Escherichia coli MG1655	*E. coli* recipient	P_{lpp}*mcherry-Kan*^{R}	This study

Table 2. Plasmids used in this study

Plasmid	*Inc*	*Resistance conferred*	*Insert site*	*Reference*
pB10::$P_{A1O4/O3}$*gfpmut3*	P1	Tet^R, Kan^R, Amp^R, Sul^R, Str^R	*kfrB*	(Van Meervenne et al., 2012)
R27::$P_{A1O4/O3}$*gfpmut3*	HI1A	Kan^R	*tetR-tetD*	This study

Gentamicin resistance (Gen^R), kanamycin resistance (Kan^R), streptomycin resistance (Str^R), ampicillin resistance (Amp^R), tetracycline resistance (Tet^R), sulfonamide resistance (Sul^R).