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The transfer of chromosomal genes through bacterial conjugation in *Escherichia coli*

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Abstract

Evolution in bacteria occurs through the combined effects of spontaneous mutations and horizontal gene transfer (HGT). Several mechanisms can lead to HGT: (i) transformation, the uptake of DNA from the environment; (ii) transduction, the transfer of DNA carried by a bacteriophage into another bacterium during infection; and (iii) conjugation, bacterial mating mediated by a conjugative plasmid like the F-factor. HGT through conjugation can lead to the transfer of resistance and virulence genes, which often reside on conjugative plasmids. Conjugation can occur both within a species or between different species. The F-factor plasmid may sometimes integrate into the chromosome by recombination if there is homology between IS elements on the plasmid and the chromosome. Cells with an integrated F-factor can transfer chromosomal DNA with high efficiency and are called Hfr-cells. There are two clinical pathogens (*Klebsiella pneumoniae* ST258 and *Escherichia coli* ST1193) that are highly successful (pathogenicity-wise) and thought to originate through the transfer of chromosomal DNA via conjugation creating unique strains with hybrid chromosomes.

Our question was how frequently the transfer of chromosomal DNA occurs when we use clinical isolates of *E. coli* with a plasmid and mate them with an *E. coli* recipient. We hypothesized that any conjugative plasmid might integrate into the chromosome, thus creating Hfr-cells with the potential for transfer of chromosomal DNA to create hybrid strains. Our prediction was that some clinical isolates should be able to transfer chromosomal DNA to another bacterial strain. By plating conjugation mixtures on selective medium where neither donor nor recipient could grow, we were able to isolate 15 possible transconjugants with hybrid genotypes occurring at a frequency of $\sim 10^{-10}$.

Popular scientific summary

Bacterial hybrids - a looming threat

By Arijana Katana

Bacteria are living organisms that function as single cells and they contain their own DNA. The evolution of bacteria can occur in several different ways, either through spontaneous mutations, by transferring DNA to other bacteria through cell-cell contact, by picking up free DNA from the environment or by receiving DNA from a virus. The transfer of DNA between bacteria through cell-cell contact is called conjugation and it is a form of bacterial "mating". It requires one of the two bacteria to be able to attach to the other with a tube-like structure called a pilus. This will lead to the transfer of DNA through the pilus. Not all bacteria are able to conjugate, as it requires them to have a so-called conjugative plasmid. A conjugative plasmid is a piece of DNA that is not a part of the bacterial chromosome, and it enables bacteria to produce the pili required for conjugation. If the DNA that is transferred through conjugation contains genes that confer resistance to certain antibiotics or genes that make the bacteria more dangerous, the bacteria may become a serious threat to society. What is special about plasmids is that they can sometimes integrate into the chromosome and transfer parts of it during conjugation - creating what we call hybrids. Today, we have knowledge about two species of bacteria that have formed hybrids and that are very successful pathogens - *Klebsiella pneumoniae* ST258 and *Escherichia coli* ST1193. These hybrid bacteria are not only pathogenic, but also resistant to commonly used antibiotics.

There is currently little knowledge about how frequently hybrid bacteria occur, which is why our aim with this project was to create hybrids of *E. coli* and determine how frequently we can generate them. By using clinical isolates of *E. coli*, we believed that the plasmid they carry may integrate into their chromosome and that they would be able to transfer chromosomal DNA to another strain of *E. coli*. With this setup, we were able to select 15 possible hybrids at a very low frequency of ~10^{-10}. The next step is to analyze what has happened inside the chromosome of these possible hybrids. Although bacterial hybrids seem to occur infrequently, it is not impossible that there may be plenty of them across the world that we have not yet discovered.

By studying the potential of these hybrids as successful organisms, we can determine whether they pose a risk to the public health. The ability to transfer antibiotic-resistance genes or other genes that make the bacteria more dangerous is of great concern and may lead to the emergence of new bacterial species that can cause serious illness and that are very hard to treat. We will also gain new knowledge about bacterial evolution and we will have a better idea about why and how new bacterial species emerge. With this knowledge we can implement better surveillance methods and countermeasures to protect the health of both humans and animals.

1. Introduction

1.1 Evolution of bacteria

Bacterial evolution is largely driven by horizontal gene transfer (HGT) and by spontaneous mutations. HGT can occur through several mechanisms, such as transformation of foreign environmental nucleic acids, through transduction mediated by bacteriophages or through conjugation mediated by a conjugative plasmid (F-factor plasmid).[1] Cells with the F-factor plasmid can produce thread-like projections called pili, which can attach to other cells and help transfer DNA through their tube-like structure.[1] Conjugation can occur within a species (intraspecies conjugation) but also between species (interspecies conjugation) and may lead to acquisition of plasmids carrying virulence- and/or antibiotic resistance genes.[2] Examples of conjugative plasmids carrying antibiotic resistance genes in *Enterobacteriaceae* are IncF and IncA/C, of which the former often confer resistance to carbapenems and the latter often confer resistance to aminoglycosides.[3] Integrative conjugative elements (ICEs) can also be transferred through conjugation and may also lead to acquisition of resistance and/or virulence genes.[2] For example, several genes involved in virulence of oral streptococci are carried on ICEs.[4] Genes that confer resistance to the antibiotic trimethoprim, which is commonly used to treat urinary tract infections (UTIs), are often associated with integrons located on plasmids.[5] In *Escherichia coli*, clinical isolates with resistance to trimethoprim can be found at varying levels, ranging from ~15-60% depending on the country.[6–8]

1.2 Chromosomal transfer of DNA through conjugation by integration of the F-factor plasmid

The F-factor plasmid can recombine into the bacterial chromosome through homologous recombination if it carries insertion elements (IS elements) that have similarity with chromosomal IS elements.[9] Bacteria with an integrated conjugative plasmid are called Hfr-cells (high frequency recombination cells) which are typically generated at a low frequency of ~10^{-5} to 10^{-6}, and they may transfer a part of or even the entirety of their chromosome during conjugation.[9,10] As an example, it has been shown that transfer of chromosomal DNA through conjugation can recover metabolic functions in auxotrophic bacteria to wild-type levels.[11] A question that arises is whether Hfr-cells could potentially create hybrids or even novel species that are stable enough to make them relevant from a clinical point of view. Indeed, there are examples of successful pathogens that likely originated through intraspecies chromosomal conjugation, such as *Klebsiella pneumoniae* ST258 and *E. coli* ST1193. *K. pneumoniae* ST258 has a contiguous genome sequence (~1.1 Mbp, 20% of the genome length) that is homologous to DNA sequences in ST442-like *K. pneumoniae* strains, while the other 80% of the chromosome (~4.2 Mbp) is homologous to DNA sequences in ST11-like strains, making ST258 a hybrid clone.[12] ST258-strains produce a carbapenemase which makes them difficult to treat, and ever since their emergence they have continued to pose a threat to patients in hospitals and other healthcare facilities worldwide.[12] There is also evidence that these bacteria continue to evolve by acquiring advantageous mutations that increase their survivability, such as being able to produce effectors that mediate biofilm formation and iron acquisition.[13] *E. coli*

ST1193 is also a clinically relevant pathogen that originally gained a sequence through a single transfer of chromosomal DNA from another *E. coli* strain.[14] This sequence subsequently underwent 11 homologous recombination events (incorporated as discontinuous segments), conferring a high resistance to fluoroquinolones.[14] On a different note, interspecies conjugation has also been observed as another study has shown that conjugation between *E. coli* and *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) can generate high-fitness interspecies hybrids, where a contiguous segment of *E. coli* DNA (from ~100 to >4,000 kb in length in different transconjugants) replaces a homologous region of the *Salmonella* chromosome.[15]

In conclusion, transfer of chromosomal DNA occurs both within a species and between different species. Although resistance genes are not always involved in the transfer, there is a possibility that either resistance genes or virulence genes could be involved in the transfer. It is currently unknown if other pathogens similar to *E. coli* ST1193. or *K. pneumoniae* ST258 exist, or what kind of phenotypic potential new species and variants could have.

1.3 High-level resistance to fluoroquinolones in clinical isolates of *E. coli*

The evolution of high-level resistance to fluoroquinolones in clinical isolates of *E. coli* is typically a result of an accumulation of mutations in several genes, particularly in *gyrA* and *parC* (~1 Mb distance between each other), which are essential genes.[16] *gyrA* encodes DNA gyrase which is composed of two GyrA and two GyrB (encoded by *gyrB*) subunits.[17] DNA gyrase introduces negative supercoiling of dsDNA, which enables replication and transcription of the chromosomal DNA. *parC* encodes topoisomerase IV, which is composed of the subunits ParC and ParE (encoded by *parE*). Topoisomerase IV is required for segregation of chromosomes at the completion of a replication round. Ciprofloxacin is an extended-spectrum fluoroquinolone which inhibits both DNA gyrase and topoisomerase IV, leading to an extensive usage of the drug, particularly when treating UTIs.[17,18] As mentioned previously, high-level resistance to fluoroquinolones is often due to mutations in *gyrA* and *parC*, specifically in the *gyrA* residues S83 and D87, and in the *parC* residues S80 and E84.[16,19] Interestingly, the mutations occur in a certain order, starting with a mutation in *gyrA* (S83L), then a mutation in *parC* (S80I), and a second mutation in *gyrA* (D87N). Sometimes, a second mutation occurs in *parC* (E84K), but three mutations are enough to confer high-level resistance.[16,19] These mutational events are individually rare (each has a frequency of $<10^{-9}$) and high-level resistance is therefore extremely unlikely to occur in a single generation (predicted frequency $<10^{-27}$), however, it is currently unknown if the mutations can be transferred reliably through HGT-mechanisms.

1.4 Bottlenecks of chromosomal gene transfer

There are several possible bottlenecks to a successful transfer of chromosomal DNA. As mentioned earlier, Hfr-cells can transfer chromosomal DNA, which may recombine into the recipient's chromosome.[9] However, the generation of Hfr-cells has a low frequency and usually requires homology between some sequences in the plasmid and in the chromosome.[9,10] The conjugation itself can also be a barrier, since it requires cell-cell contact and gene expression

to make a functioning pilus.[1] The DNA also has to be processed and transferred properly via the pilus. The size of the DNA that is being transferred also has an impact as it is predicted to be easier to successfully transfer a smaller fragment than a larger one, because cell-cell contact must be maintained for a certain period of time.[1] Once the DNA has been transferred into the recipient, another set of issues may arise. Restriction enzymes, such as those encoded by the *mcr* or *mrr* genes, or other cellular exonucleases can cleave incoming DNA, while the fragment also needs to be sufficiently similar in sequence to be able to recombine into the recipient chromosome.[1,9,20] The transfer of chromosomal DNA between bacteria is accordingly complex and the question we are asking is how frequently such an event occurs in environments containing different bacterial variants.

1.5 Project aim

In this project, our aim is to generate chromosomal hybrids (transconjugants) of *E. coli* through conjugation. To do this, we will use specific donors and recipients that are suitable, and select transconjugants on the appropriate medium:

- Donors: clinical isolates related to human UTIs with a high-level resistance to ciprofloxacin. We predict that no spontaneous mutants with this phenotype will arise. Their Antibiotic Susceptibility Testing (AST) profile indicates the presence of a plasmid conferring resistance to trimethoprim.
- Recipients: resistant to apramycin due to insertion of resistance gene *aac(3)-IVa* in *galK* (*galK::pJ23100-aac(3)-IVa*), making them unable to degrade galactose. There are only two known genes that confer resistance to apramycin (*aac(3)-IV* and *npmA*) and the antibiotic is mostly used in veterinary medicine, which makes resistance less likely to be widespread in clinical isolates from humans.[21–24] In addition, several restriction enzyme genes are deleted (*Δ(mcrC-mrr)8*; *Δ(mcrA)*) in some recipients. Additionally, some recipients are also auxotrophic for histidine (*ΔhisC*) or tryptophan (*ΔtrpE*), providing other markers for the selection of transconjugant hybrid strains (Figure 1).
- The transconjugants will be selected on medium containing either ciprofloxacin + apramycin (selecting for transfer of both *gyrA* and *parC*), or on minimal medium containing apramycin (selecting for transfer of either *hisC* or *trpE*.

Our hypothesis is that a conjugative plasmid in a clinical isolate will be integrated into the chromosome (creating Hfr-strains) and that conjugative transfer of chromosomal DNA will create hybrid strains. We predict that some clinical isolates should be able to transfer chromosomal DNA to another strain.

By studying the potential of hybrid bacteria as successful organisms, we would gain a great deal of knowledge about bacterial evolution. Additionally, we would also gain new knowledge about the spread of resistance and virulence genes, which could help us predict the emergence of highly resistant and/or highly pathogenic bacteria.

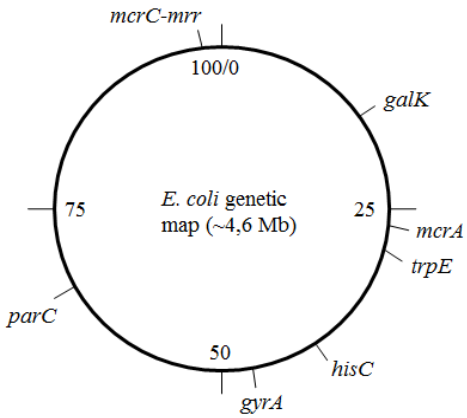

Figure 1. Genetic map of *Escherichia coli*. The genetic map indicates the approximate locations of *gyrA*, *parC*, *galK*, *hisC*, *trpE*, *mcrA* and *mcrC-mrr* on the *E. coli* chromosome. The numbers indicate the time (minutes) required for transfer of chromosomal segments.

2. Methods and Materials

2.1 Bacterial cultures and growth conditions

E. coli donors and recipients (Table 1 & 2) used in the conjugations were prepared from frozen stocks (-80°C) and grown on Luria Agar (LA) plates except for the donor CH10155, which was grown on M9 minimal medium for genetic stability. All strains were grown at 37°C.

2.2 Media

The M9 minimal medium contained 0,2% glucose but no additional supplements. MacConkey agar (Difco) plates were made using 1% galactose as a supplement.
Antibiotic concentrations for LA/M9 plates were used as follows:
Ciprofloxacin (CIP): 0,1/16 µg/mL
Apramycin (APR): 200 µg/mL
Trimethoprim (TMP): 16 µg/mL

2.3 Strains

All donors that were used (except TB56 and CH10155) were collected from patients in Pakistan with a confirmed or suspected UTI[25], and stocks are maintained at the Department of Medical Biochemistry and Microbiology (IMBIM) at Uppsala University. The TB56 strain was created by Talía Berruga-Fernández (IMBIM, Uppsala University). All other strains have been created by Douglas Huseby (IMBIM, Uppsala University) with the desirable traits for the conjugations. The donors were all tested and confirmed for growth on CIP-16 and TMP-16, while the recipients were tested and confirmed for growth on APR-200.

Table 1. Donor strains used

Strain	Source	Genotype
CH407	Clinical isolate	Cip^R, Tmp^R
CH409	Clinical isolate	Cip^R, Tmp^R
CH411	Clinical isolate	Cip^R, Tmp^R
CH412	Clinical isolate	Cip^R, Tmp^R
CH413	Clinical isolate	Cip^R, Tmp^R
CH418	Clinical isolate	Cip^R, Tmp^R
CH419	Clinical isolate	Cip^R, Tmp^R
CH420	Clinical isolate	Cip^R, Tmp^R
CH422	Clinical isolate	Cip^R, Tmp^R
CH427	Clinical isolate	Cip^R, Tmp^R
CH430	Clinical isolate	Cip^R, Tmp^R
CH435	Clinical isolate	Cip^R, Tmp^R
CH436	Clinical isolate	Cip^R, Tmp^R
CH438	Clinical isolate	Cip^R, Tmp^R
CH440	Clinical isolate	Cip^R, Tmp^R
CH441	Clinical isolate	Cip^R, Tmp^R
CH442	Clinical isolate	Cip^R, Tmp^R
CH443	Clinical isolate	Cip^R, Tmp^R
CH446	Clinical isolate	Cip^R, Tmp^R
CH451	Clinical isolate	Cip^R, Tmp^R
CH452	Clinical isolate	Cip^R, Tmp^R
CH454	Clinical isolate	Cip^R, Tmp^R
CH459	Clinical isolate	Cip^R, Tmp^R
CH460	Clinical isolate	Cip^R, Tmp^R
CH462	Clinical isolate	Cip^R, Tmp^R
CH464	Clinical isolate	Cip^R, Tmp^R
TB56	IMBIM	Hfr (PO66(ColV); *gyrA* S83L D87N; *parC* S80I E84K; *Δ(gpt-lac)5*; *glnX44*(AS); *λ-*; *srlD300::Tn10*; *relA1*; *spoT1?*; *thiE1*
CH10155	IMBIM	F'23; *gyrA* S83L D87N; *parC* S80I E84K; *pro-lac::kan*

Table 2. Recipient strains used

Strain	Source	Genotype
CH10127	IMBIM	*galK::pJ23100-aac(3)-IVa* in MG1655
CH10138	IMBIM	*galK::pJ23100-aac(3)-IVa* in ATCC 25922 (EN0001)
CH10139	IMBIM	*galK::pJ23100-aac(3)-IVa*; *Δ(hsdR)*; *rpsL*(Str^R) in ATCC 25922 (EN0001)
CH10140	IMBIM	*Δ(mcrC-mrr)8*; *Δ(mcrA)*; *galK::pJ23100-aac(3)-IVa* in MG1655
CH10162	IMBIM	*Δ(hisC)*; *Δ(mcrC-mrr)8*; *Δ(mcrA)*; *galK::J23100-aac(3)-IVa* in MG1655
CH10163	IMBIM	*Δ(trpE)*; *Δ(mcrC-mrr)8*; *Δ(mcrA)*; *galK::J23100-aac(3)-IVa* in MG1655

2.4 Conjugation methods and conjugation frequency calculations

1. **Filter conjugations.** Overnight cultures were prepared by inoculating a colony in fresh LB medium and the cultures were incubated at 37℃ with shaking. 350 µL of the donor overnight culture was mixed with 350 µL of the recipient overnight culture. A nitrocellulose membrane filter (0,2/0,4 µM pore size; 82mM diameter, Protran®) was placed on an LA plate by using sterile tweezers and 600 µL of the bacterial mix was spread on the filter. The conjugation plate was incubated at 37℃ for ~18 hours (up to 20 hours). The filter was taken and folded using sterile tweezers and placed in a falcon tube with 2 mL phosphate-buffered saline (PBS). The tube was vortexed until all cells were suspended in the PBS which was used for plating. Serial dilutions were also prepared in PBS. All selection plates were incubated at 37℃ and monitored once a day for growth. The plates were kept for ~5-8 days before being thrown away.
    a. **Adding zeocin.** Overnight cultures of the donor CH10155 were prepared in M9 minimal medium and zeocin was added at concentrations of 0 µg/mL (negative control), 1µg/mL, 2 µg/mL and 4 µg/mL. The following procedures were the same as above for filter conjugations.
2. **Liquid conjugations.** Overnight cultures were prepared the same as for filter conjugations. 1 mL of the donor overnight culture was mixed with 1 mL of the recipient overnight culture. The whole bacterial mixture was added to a flask with 20 mL fresh LB and the flask was incubated (still) at 37℃ for ~18 hours (up to 20 hours). The liquid was used for plating and serial dilutions were prepared in PBS. 100 mL fresh LB was then added to the flask and ciprofloxacin was added at 16 µg/mL and apramycin was added at 200 µg/mL. The flask was incubated at 37℃ for ~18 hours (up to 20 hours) with shaking. The liquid was used for plating and serial dilutions were prepared in PBS. All selection plates were incubated at 37℃ and monitored once a day for growth. The plates were kept for ~5-8 days before being thrown away.

The conjugation frequencies were calculated using the following formula:

$$\frac{\text{cfu/mL of transconjugant}}{\text{cfu/mL of donor/recipient}} = \text{Conjugation frequency}$$

For filter conjugations, each cfu/mL was first multiplied by 2 and then applied in the formula. When no possible transconjugants could be found on the selection plates, the cfu/mL for these was set as 1.

2.5 Screening and PCR

Possible transconjugants, control transconjugants and recipients with a plasmid were screened by streaking colonies from the selection plate onto a fresh plate of the same selection to look for growth. The colonies were also streaked on MacConkey agar with galactose which indicated if they were able to degrade galactose (purple colonies) or not (white colonies).

PCR was used as a crude method to screen for the presence of the apramycin-resistance junction (*galK::J23100-aac(3)-IVa*) in transconjugants. *gyrA* and *parC* were amplified to confirm that the transconjugants were *E. coli* and to confirm that the control transconjugants

received the Cip^R quadruple mutation. The PCR products of *gyrA* and *parC* were purified using the QIAquick PCR purification kit (QIAGEN) and sent to Eurofins for local sequencing. The sequences were analyzed using the software CLC Main Workbench 8 (QIAGEN). Deletion of the restriction enzymes (*Δ(mcrC-mrr)8*; *Δ(mcrA)*) was also screened using PCR, along with the deletion of *hisC* and *trpE*. The primer sequences can be found in Supplementary Table 1 and the PCR programmes can be found in Supplementary Table 2. The PCRs were run in 25 µL reactions (2 µL template) and the Thermo Fisher Scientific Master Mix (TaqPol, 2X) was used at 1X concentration. Each primer was added at 0,4 µM and ddH_2O was added.

2.6 MIC determination for zeocin

The MIC of the donor strain CH10155 was determined through the following procedures:
1 mL of 128 µg/mL solution of zeocin was first prepared by dilution in MH-II broth. 100 µL of the solution was pipetted into each well of column 1 on a microtiter plate. 50 µL of MH-II media was then added into columns 2-12. 50 µL of the solution was transferred from column 1 to column 2, mixed, and the same dilution was repeated all the way across to column 10. Several colonies of the strains of choice (Supplementary Table 3) were picked from the plate and dissolved in 0,9% NaCl, and then measured with a nephelometer to match 0,5 MacFarland standard. 100 µL of the dissolved cells were added to 10 mL MH-II and mixed, and 50 µL was then used to inoculate columns 1-11. The microtiter plate was then covered with a polyester adhesive film and incubated at 37℃ and the plates were read after 18 hours.

3. Results

3.1 Filter conjugations selecting for high-level ciprofloxacin and apramycin resistance do not generate transconjugants when using clinical isolates as donors

The aim of our project was to generate chromosomal hybrids (transconjugants) of *E. coli* through conjugation. To do this, we selected three clinical isolates (CH411, CH422 and CH436) that were expected to carry a plasmid (indicated by resistance to trimethoprim, Tmp^R) which could integrate into the chromosome and transfer chromosomal genes. These clinical isolates were also resistant to ciprofloxacin (Cip^R – *gyrA* and *parC* mutants). TB56 (Cip^R Hfr-strain) was used as a control donor. As for the recipient, we selected an *E. coli* strain (CH10127) with resistance to apramycin (Apr^R – *galK::pJ23100-aac(3)-IVa*). With this, we wanted to select transconjugants who were Cip^R (received both *gyrA* and *parC* mutations) and Apr^R (carrying *galK::pJ23100-aac(3)-IVa*). The donors and the recipient were mixed on a filter and by selecting on plates containing CIP-16 + APR-200, we were able to generate transconjugants at a high frequency (10^{-3} and 10^{-4}) when using the Hfr-strain as a control (Table 3). The control transconjugants were confirmed as mentioned in the Methods and Materials section. No transconjugants were generated when using Cip^R clinical isolates as donors and their conjugation frequencies were very low ($<10^{-11}$). The formula we used for calculating all conjugation frequencies (for subsequent experiments as well) can be found in Methods and Materials.

Table 3. Filter conjugation frequency on CIP-16 + APR-200 plates

	Conjugation frequency	
Cross (Donor x Recipient)	**Per donor**	**Per recipient**
TB56 (Hfr) x CH10127	$5{,}53*10^{-3}$	$9{,}51*10^{-4}$
CH411 x CH10127	$<3{,}75*10^{-11}$	$<3{,}90*10^{-11}$
CH422 x CH10127	$<5{,}31*10^{-11}$	$<3{,}70*10^{-11}$
CH436 x CH10127	$<2{,}40*10^{-11}$	$<1{,}38*10^{-10}$

Hfr indicates the Hfr-strain control donor

3.2 The plasmid transfer frequency on a filter varies slightly when using recipients with different genotypes

Since we were unable to generate transconjugants when crossing Cip^R and Tmp^R donors (CH411, CH422 and CH436) with an Apr^R-recipient (CH10127), we considered the possibility that the plasmid transfer frequency of the donor and that the genotype of the recipient could affect the frequency of chromosomal DNA transfer. We believed that if we could find a donor with a high plasmid transfer frequency, it should be able to transfer chromosomal DNA at a high frequency (if it becomes an Hfr-strain). If the donor with a high plasmid transfer frequency

is crossed with a genetically compatible recipient, it should increase the chances of generating a transconjugant. With this in mind, we selected one Cip^R and Tmp^R clinical isolate (CH411) as a donor to determine its plasmid transfer frequency when crossed with different types of recipients that we constructed (CH10138, CH10139 and CH10140), while also using the same recipient (CH10127) as in the previous experiment. All recipients were highly resistant to apramycin (Apr^R) and either retained their genes encoding for restriction enzymes (CH10127 and CH10138) or had them deleted (R^- – CH10139 and CH10140). The recipients were constructed from lineages that have their chromosomes organized differently (MG1655 or ATCC), to see if this could affect the plasmid transfer frequency. The donor was mixed with the recipients on a filter and the selections were done on TMP-16 + APR-200 plates, with the expectation that the resulting bacteria would be Tmp^R and Apr^R.

As can be seen in Table 4, the plasmid transfer frequencies were low and varied between 10^{-7} and 10^{-8} per recipient, while they varied between 10^{-8} and 10^{-9} per donor. The MG1655 R^--strain (CH10140) yielded the highest plasmid transfer frequency (per donor and recipient) and was chosen as the recipient for subsequent experiments. The plasmid transfers were confirmed as mentioned in Methods and Materials.

Table 4. Plasmid transfer frequency on TMP-16 + APR-200 plates using different recipients (filter conjugation)

	Plasmid transfer frequency	
Cross (Donor x Recipient)	**Per donor**	**Per recipient**
CH411 x CH10127 (MG1655)	$9{,}16*10^{-9}$	$1{,}27*10^{-8}$
CH411 x CH10138 (ATCC)	$4{,}5*10^{-9}$	$1{,}12*10^{-8}$
CH411 x CH10139 (ATCC, R^-)	$5{,}55*10^{-9}$	$1{,}47*10^{-8}$
CH411 x CH10140 (MG1655, R^-)	$3{,}93*10^{-8}$	$1{,}01*10^{-7}$

R^- indicates that the recipient has its restriction enzymes deleted, while MG1655/ATCC indicates the recipient's lineage.

3.3 The plasmid transfer frequency on a filter varies when using different clinical isolates as donors

As a continuation of the previous experiment in section 3.2, we wanted to investigate if different Cip^R and Tmp^R clinical isolates (CH451, CH452, CH454 and CH459) yield varying plasmid transfer frequencies when using the same Apr^R R^--strain (CH10140) as recipient. The donors and the recipients were mixed on a filter, and by selecting on TMP-16 + APR-200 plates we were able to determine that different clinical isolates did indeed yield varying plasmid transfer frequencies (Table 5). Some clinical isolates (CH454) transferred their plasmid at a high frequency (10^{-3}), while other isolates (CH459) transferred their plasmid at a low frequency

(10^{-6}), varying between several orders of magnitude. The plasmid transfers were confirmed as mentioned in Methods and Materials.

Table 5. Plasmid transfer frequency on TMP-16 + APR-200 plates using different clinical isolates (filter conjugation)

	Plasmid transfer frequency	
Cross (Donor x Recipient)	**Per donor**	**Per recipient**
CH451 x CH10140 (10^{-3})	$5{,}42*10^{-4}$	$5{,}42*10^{-4}$
CH452 x CH10140 (10^{-3})	$6{,}22*10^{-5}$	$6{,}22*10^{-5}$
CH454 x CH10140 (10^{-3})	$2{,}65*10^{-3}$	$2{,}65*10^{-3}$
CH459 x CH10140 (undiluted)	$1{,}44*10^{-6}$	$1{,}44*10^{-6}$

10^{-3} or undiluted next to the cross indicates at what dilution the cross was plated.

3.4 Liquid conjugations using an F' donor can be used to enrich for transconjugants

For the next few experiments, we changed our approach and performed the conjugations in liquid instead of using filters to see if we could enrich the liquid for the desired transconjugants (Cip^R and Apr^R). We used the same Apr^R R^--recipient (CH10140) and constructed a Cip^R F'-donor (CH10155) as a control, and mixed them in LB. We also wanted to see if we would be able to generate transconjugants when diluting the control donor from 10^{-1} to 10^{-5}. By plating on both CIP-16 + APR-200 and CIP-0,1 + APR-200 we wanted to select for either high- or low-level ciprofloxacin resistance to determine if there was a difference in conjugation frequency or ability to generate transconjugants between the two selections. We plated the conjugations both before and after addition of antibiotics to the liquid (Table 6 & 7). When plating on CIP-16 + APR-200 before adding antibiotics to the flasks, we were unable to generate any transconjugants when diluting the donor (conjugation frequencies per donor: $<10^{-4}$ to $<10^{-8}$, $<10^{-10}$ per recipient), which can be seen in Table 6. Only the undiluted donor cross was able to generate transconjugants, at a low frequency (10^{-6} and 10^{-7}). When plating on CIP-0,1 + APR-200, we were able to generate transconjugants at low frequencies when diluting the donor to 10^{-3} (10^{-5} and 10^{-9} frequency) and 10^{-4} (10^{-5} and 10^{-8} frequency), and also when using the undiluted donor (10^{-7} frequency). This indicated that selections for low-level ciprofloxacin could generate transconjugants even when diluting the donor, and before enriching for transconjugants by adding antibiotics to the liquid.

When plating on CIP-16 + APR-200 after adding antibiotics to the flasks, we were able to generate transconjugants at very high frequencies for the undiluted donor and when diluting the donor to 10^{-1} and 10^{-2} (~2 to ~21 frequency per donor, 10^{-1} per recipient), confirming our approach (Table 7). These frequencies indicate that the liquid contained an enormous amount of transconjugants. We were unable to generate any transconjugants when diluting the donor

to 10^{-3}, 10^{-4} and 10^{-5} ($<10^{-4}$ to $<10^{-6}$ frequency per donor, $<10^{-9}$ to $<10^{-10}$ per recipient). This confirmed that we were able to enrich the liquid for transconjugants after adding the antibiotics. The transconjugants were confirmed as mentioned in Methods and Materials.

Table 6. Liquid conjugation frequency of F' donor before adding antibiotics to flasks

	Conjugation frequency (before adding antibiotics)			
	Selection: CIP-16 + APR-200		**Selection: CIP-0,1 + APR-200**	
Cross (Donor x Recipient)	**Per donor**	**Per recipient**	**Per donor**	**Per recipient**
CH10155 (undiluted) x CH10140	$1,6*10^{-6}$	$1,45*10^{-7}$	$6,6*10^{-7}$	$3*10^{-7}$
CH10155 (10^{-1}) x CH10140	$<2,5*10^{-8}$	$<8,33*10^{-10}$	$<5*10^{-8}$	$<8,33*10^{-10}$
CH10155 (10^{-2}) x CH10140	$<1,66*10^{-7}$	$<7,69*10^{-10}$	$<1,66*10^{-7}$	$<7,69*10^{-10}$
CH10155 (10^{-3}) x CH10140	$<1,66*10^{-6}$	$<8,33*10^{-10}$	$3,33*10^{-5}$	$8,33*10^{-9}$
CH10155 (10^{-4}) x CH10140	$<2,5*10^{-5}$	$<4*10^{-10}$	$4,29*10^{-4}$	$1,2*10^{-8}$
CH10155 (10^{-5}) x CH10140	$<3,33*10^{-4}$	$<8,33*10^{-10}$	$<5*10^{-4}$	$<8,33*10^{-10}$

Undiluted and 10^{-1} to 10^{-5} next to the donor in the cross indicates if it was diluted or not and at what level.

Table 7. Liquid conjugation frequency on CIP-16 + APR-200 plates using an F' donor (after adding antibiotics to flasks)

	Conjugation frequency (after adding antibiotics)	
Cross (Donor x Recipient)	**Per donor**	**Per recipient**
CH10155 (undiluted) x CH10140	2,144	$1,94*10^{-1}$
CH10155 (10^{-1}) x CH10140	5,36	$1,78*10^{-1}$
CH10155 (10^{-2}) x CH10140	21,44	$3,06*10^{-1}$
CH10155 (10^{-3}) x CH10140	$<5*10^{-6}$	$<1,11*10^{-9}$
CH10155 (10^{-4}) x CH10140	$<5*10^{-5}$	$<6,25*10^{-10}$
CH10155 (10^{-5}) x CH10140	$<3,33*10^{-4}$	$<8,33*10^{-10}$

Undiluted and 10^{-1} to 10^{-5} next to the donor in the cross indicates if it was diluted or not and at what level.

3.5 Liquid conjugations selecting for high-level ciprofloxacin and apramycin resistance do not generate transconjugants when using clinical isolates as donors

After determining that conjugations in liquid can generate Cip^R and Apr^R transconjugants at a high frequency when using a control donor (section 3.4), we tried using the same method with Cip^R and Tmp^R clinical isolates as donors (CH411, CH441, CH442, CH443, CH451, CH454 and CH459) and the Apr^R R^--recipient (CH10140). The clinical isolates were chosen based on the strains we used previously when determining the plasmid transfer frequency (section 3.2 and 3.3), while introducing three new isolates (CH441, CH442 and CH443). Selection on CIP-16 + APR-200 plates did not generate any transconjugants either before or after addition of antibiotics to the conjugation flasks, and the conjugation frequencies were low and varied between $<10^{-8}$ and $<10^{-9}$ (Table 8).

Table 8. Liquid conjugation frequency on CIP-16 + APR-200 plates using different clinical isolates (before and after adding antibiotics to flask)

	Conjugation frequency (before and after adding antibiotics)	
Cross (Donor x Recipient)	**Per donor**	**Per recipient**
CH411 x CH10140	$<5*10^{-9}$	$<5*10^{-9}$
CH441 x CH10140	$<1*10^{-8}$	$<1*10^{-8}$
CH442 x CH10140	$<5*10^{-9}$	$<5*10^{-9}$
CH443 x CH10140	$<5*10^{-9}$	$<1{,}11*10^{-9}$
CH451 x CH10140	$<1*10^{-8}$	$<2*10^{-9}$
CH454 x CH10140	$<1*10^{-8}$	$<2*10^{-9}$
CH459 x CH10140	$<3{,}33*10^{-9}$	$<3{,}33*10^{-9}$

3.6 The plasmid transfer frequency in liquid varies when using clinical isolates as donors

While selecting for Cip^R and Apr^R transconjugants in liquid (section 3.5), we also wanted to determine the plasmid transfer frequency for the same crosses. This was done to investigate if the plasmid transfer frequency differs when doing conjugations in liquid and on a filter, and to investigate if we could find any donors with a high plasmid transfer frequency. The crosses were plated on TMP-16 + APR-200 (selecting for Tmp^R and Apr^R) and similarly to the plasmid transfer frequencies on a filter (Table 5), the frequency of transfer varied between several orders of magnitude for different crosses, as can be seen in Table 9. This time we were able to determine plasmid transfer frequencies as high as 10^{-1} (CH441) and as low as 10^{-5} (CH459), while two of the crosses (CH442/CH443 x CH10140) did not generate any colonies ($<10^{-9}$ plasmid transfer frequency). Some of the crosses had been done previously on a filter (Table 4

& 5), and we noticed that the plasmid transfer frequency for some of them was higher (at least one order of magnitude difference) when the conjugation was done in liquid (CH454 and CH459). The largest difference was observed for CH411 x CH10140, where the plasmid transfer frequency was 10^{-8} per donor and 10^{-7} per recipient on a filter (Table 4), while in liquid the frequency was as high as 10^{-4} (Table 9). The plasmid transfers were confirmed as mentioned in Methods and Materials.

Table 9. Plasmid transfer frequency on TMP-16 + APR-200 plates using different clinical isolates (liquid conjugation)

	Plasmid transfer frequency	
Cross (Donor x Recipient)	**Per donor**	**Per recipient**
CH411 x CH10140 (10^{-1})	$1{,}14*10^{-4}$	$1{,}14*10^{-4}$
CH441 x CH10140 (10^{-1})	$2{,}26*10^{-1}$	$2{,}26*10^{-1}$
CH442 x CH10140	$<5*10^{-9}$	$<5*10^{-9}$
CH443 x CH10140	$<5*10^{-9}$	$<1{,}11*10^{-9}$
CH451 x CH10140 (10^{-1})	$2{,}46*10^{-4}$	$4{,}92*10^{-5}$
CH454 x CH10140 (10^{-3})	$1{,}9*10^{-2}$	$3{,}8*10^{-3}$
CH459 x CH10140 (10^{-1})	$7{,}53*10^{-5}$	$7{,}53*10^{-5}$

10^{-1} or 10^{-3} next to the cross indicates at what dilution the cross was plated.

3.7 Adding zeocin at low levels to an F' donor does not affect the conjugation frequency

Since we were unable to generate any Cip^R and Apr^R transconjugants when using clinical isolates as donors with either filter or liquid conjugations, we tried another approach. Zeocin is a glycopeptide antibiotic that can bind and cleave DNA, and we considered the possibility that zeocin may induce DNA breaks resulting in recombination events that could increase the conjugation frequency. To test this, we wanted to generate Cip^R and Apr^R transconjugants by using the Cip^R F'-strain (CH10155) as a donor while inoculating it with zeocin, and we wanted to use the Apr^R R^--strain (CH10140) as a recipient. We determined the MIC to be 8 µg/mL for the donor by using a standard microtiter plate (Methods and Materials) and we inoculated the overnight cultures with 1, 2 or 4 µg/mL zeocin, with no zeocin as a control. We wanted to select for both high- and low-level ciprofloxacin resistance to see if there were any differences between the selections by plating the conjugations on CIP-16 + APR-200 and CIP-0,1 + APR-200, and the conjugations were performed on a filter. As can be seen in Table 10, the conjugation frequencies were low and there was no notable difference in conjugation frequency per donor (10^{-7}) whether the donor was inoculated with zeocin or not and neither when selecting for high- or low-level ciprofloxacin resistance. The conjugation frequency per recipient

decreased by one order of magnitude (10^{-8}) when inoculating the donor with 4 µg/mL zeocin. The transconjugants were confirmed as mentioned in Methods and Materials.

Table 10. Filter conjugation frequency when adding zeocin to control donor

	Conjugation frequency			
	Selection: CIP-16 + APR-200		**Selection: CIP-0,1 + APR-200**	
Cross (Donor x Recipient)	**Per donor**	**Per recipient**	**Per donor**	**Per recipient**
CH10155 (0 µg/mL) x CH10140	$1,47*10^{-7}$	$1,45*10^{-7}$	$2,07*10^{-7}$	$1,89*10^{-7}$
CH10155 (1 µg/mL) x CH10140	$1,92*10^{-7}$	$1,58*10^{-7}$	$2,42*10^{-7}$	$2,13*10^{-7}$
CH10155 (2 µg/mL) x CH10140	$1,86*10^{-7}$	$1,27*10^{-7}$	$2,09*10^{-7}$	$1,74*10^{-7}$
CH10155 (4 µg/mL) x CH10140	$1,37*10^{-7}$	$5,68*10^{-8}$	$3,33*10^{-7}$	$7,39*10^{-8}$

0, 1, 2 or 4 µg/mL indicates the concentration of zeocin that was added to the donor overnight culture.

3.8 Filter conjugations selecting for prototrophs with high-level apramycin resistance generate possible transconjugants when using clinical isolates as donors

For our final approach, we decided against selecting transconjugants with high-level ciprofloxacin resistance (Cip^R). We considered that it may be easier to select for a single gene instead of a larger fragment (*gyrA* and *parC*) and we constructed two different recipients; one that was auxotrophic for histidine – *his*$^-$ (CH10162), and one that was auxotrophic for tryptophan – *trp*$^-$ (CH10163), with the purpose of selecting transconjugants that were *trp*$^+$/*his*$^+$ and Apr^R. Both recipients were Apr^R and R^-. We used the Cip^R Hfr-strain (TB56) and the Cip^R F'-strain (CH10155) as control donors, and we picked 26 Cip^R and Tmp^R clinical isolates as donors. The conjugations were done on a filter and the selections were done on M9 minimal medium + APR-200 plates, and each donor was used twice (crossed with both recipients). When crossing the Hfr-strain with the *his*$^-$ recipient, we were able to generate transconjugants at a very high frequency (10^{-1} and 10^{-2}), while the frequency was much lower for the F'-strain (10^{-9}), which can be seen in Table 11. The control transconjugants were confirmed as mentioned in Methods and Materials. Two different clinical isolates were able to generate possible transconjugants (10^{-9} to 10^{-10} frequency), which are indicated in bold (CH411 x CH10162 and CH430 x CH10162). The cross between CH411 and CH10162 generated one colony while the cross between CH430 and CH10162 generated four colonies in total, which were all screened using PCR (Methods and Materials) and then saved (Table 13). The other clinical isolates did not generate any possible transconjugants, indicated by their low conjugation frequency of $<10^{-11}$ (Table 11).

Table 11. Filter conjugation frequency on M9 + APR-200 plates (*his*$^-$ recipient)

	Conjugation frequency	
Cross (Donor x Recipient)	**Per donor**	**Per recipient**
TB56 (Hfr) x CH10162	$4{,}16*10^{-1}$	$4{,}26*10^{-2}$
CH10155 (F') x CH10162	$6{,}38*10^{-9}$	$1{,}11*10^{-9}$
CH407 x CH10162	$<3{,}84*10^{-11}$	$<2{,}24*10^{-11}$
CH409 x CH10162	$<2{,}23*10^{-11}$	$<4{,}50*10^{-11}$
CH411 x CH10162	$\mathbf{2{,}69*10^{-10}}$	$\mathbf{2{,}38*10^{-10}}$
CH412 x CH10162	$<4{,}46*10^{-11}$	$<4{,}34*10^{-11}$
CH413 x CH10162	$<3{,}40*10^{-11}$	$<3{,}90*10^{-11}$
CH418 x CH10162	$<1{,}97*10^{-11}$	$<2{,}29*10^{-11}$
CH419 x CH10162	$<2{,}51*10^{-11}$	$<2{,}89*10^{-11}$
CH420 x CH10162	$<2{,}45*10^{-11}$	$<2{,}19*10^{-11}$
CH422 x CH10162	$<5{,}37*10^{-11}$	$<2{,}60*10^{-11}$
CH427 x CH10162	$<3{,}04*10^{-11}$	$<4{,}13*10^{-11}$
CH430 x CH10162*	$\mathbf{1{,}13*10^{-9}}$	$\mathbf{7{,}75*10^{-10}}$
CH435 x CH10162	$<7{,}46*10^{-11}$	$<4{,}23*10^{-11}$
CH436 x CH10162	$<7{,}81*10^{-11}$	$<5{,}74*10^{-11}$
CH438 x CH10162	$<4{,}71*10^{-11}$	$<3{,}54*10^{-11}$
CH440 x CH10162	$<3{,}62*10^{-11}$	$<2{,}5*10^{-11}$
CH441 x CH10162	$<1{,}40*10^{-11}$	$<2{,}03*10^{-11}$
CH442 x CH10162	$<4{,}16*10^{-11}$	$<3{,}52*10^{-11}$
CH443 x CH10162	$<8{,}92*10^{-11}$	$<3{,}90*10^{-11}$
CH446 x CH10162	$<5{,}26*10^{-11}$	$<3{,}44*10^{-11}$
CH451 x CH10162	$<2{,}76*10^{-11}$	$<3{,}40*10^{-11}$
CH452 x CH10162	$<4{,}09*10^{-11}$	$<4{,}42*10^{-11}$

	Conjugation frequency	
Cross (Donor x Recipient)	**Per donor**	**Per recipient**
CH454 x CH10162	$<3{,}40*10^{-11}$	$<3{,}37*10^{-11}$
CH459 x CH10162	$<3{,}60*10^{-11}$	$<6{,}17*10^{-11}$
CH460 x CH10162	$<3{,}25*10^{-11}$	$<4{,}17*10^{-11}$
CH462 x CH10162	$<7{,}25*10^{-11}$	$<6{,}94*10^{-11}$
CH464 x CH10162	$<2{,}90*10^{-11}$	$<5*10^{-11}$

Bold indicates the crosses using clinical isolates that generated a colony on the selection plate. The superscript * indicates that the cross generated more than one colony.

When crossing the Hfr-strain with the *trp*$^-$ recipient, we were able to generate transconjugants at a high to low frequency (10^{-3} per donor, 10^{-5} per recipient), which can be seen in Table 12. The frequency was lower when compared to the conjugation frequency with the *his*$^-$ recipient (Table 11). The conjugation frequency when crossing the F'-strain with the *trp*$^-$ recipient was a little higher (10^{-7} per donor, 10^{-8} per recipient) than when crossing the F'-strain with the *his*$^-$ recipient (Table 11 & 12). The control transconjugants were confirmed as mentioned in Methods and Materials. Several clinical isolates (CH412, CH413, CH418, CH419, CH427, CH430, CH435, CH442 and CH452) were able to generate possible transconjugants at a frequency of 10^{-10} per donor and 10^{-9} to 10^{-10} per recipient (Table 12). Only the cross between CH442 and CH10163 generated more than one colony (2 colonies total), and all colonies were screened using PCR (Methods and Materials) and then saved (Table 13). None of the other clinical isolates generated any possible transconjugants, indicated by their low conjugation frequency of $<10^{-10}$ to $<10^{-11}$ (Table 12).

Table 12. Filter conjugation frequency on M9 + APR-200 plates (*trp*$^-$ recipient)

	Conjugation frequency	
Cross (Donor x Recipient)	**Per donor**	**Per recipient**
TB56 (Hfr) x CH10163	$1{,}41*10^{-3}$	$7{,}76*10^{-5}$
CH10155 (F') x CH10163	$3{,}83*10^{-7}$	$3{,}25*10^{-8}$
CH407 x CH10163	$<3{,}64*10^{-11}$	$<1{,}60*10^{-11}$
CH409 x CH10163	$<1{,}81*10^{-11}$	$<3{,}08*10^{-11}$
CH411 x CH10163	$<1{,}66*10^{-11}$	$<2{,}48*10^{-11}$
CH412 x CH10163	**$3{,}24*10^{-10}$**	**$6{,}17*10^{-10}$**
CH413 x CH10163	**$1{,}49*10^{-10}$**	**$1{,}52*10^{-10}$**
CH418 x CH10163	**$2{,}61*10^{-10}$**	**$2{,}46*10^{-10}$**
CH419 x CH10163	**$4{,}27*10^{-10}$**	**$4{,}58*10^{-10}$**
CH420 x CH10163	$<3{,}40*10^{-11}$	$<4*10^{-11}$
CH422 x CH10163	$<5{,}55*10^{-11}$	$<3{,}03*10^{-11}$
CH427 x CH10163	**$4{,}23*10^{-10}$**	**$1*10^{-9}$**
CH430 x CH10163	**$6{,}49*10^{-10}$**	**$5{,}05*10^{-10}$**
CH435 x CH10163	**$7{,}14*10^{-10}$**	**$5{,}74*10^{-10}$**
CH436 x CH10163	$<6{,}94*10^{-11}$	$<6{,}49*10^{-11}$
CH438 x CH10163	$<4{,}50*10^{-11}$	$<4{,}50*10^{-11}$
CH440 x CH10163	$<3{,}81*10^{-11}$	$<4*10^{-11}$
CH441 x CH10163	$<1{,}76*10^{-11}$	$<3{,}26*10^{-11}$
CH442 x CH10163*	**$9{,}80*10^{-10}$**	**$9{,}25*10^{-10}$**
CH443 x CH10163	$<7{,}14*10^{-11}$	$<3{,}93*10^{-11}$
CH446 x CH10163	$<5*10^{-11}$	$<3{,}37*10^{-11}$
CH451 x CH10163	$<1{,}83*10^{-11}$	$<2{,}02*10^{-11}$
CH452 x CH10163	**$4{,}58*10^{-10}$**	**$1{,}08*10^{-9}$**

	Conjugation frequency	
Cross (Donor x Recipient)	**Per donor**	**Per recipient**
CH454 x CH10163	$<3{,}16*10^{-11}$	$<2{,}45*10^{-11}$
CH459 x CH10163	$<4{,}67*10^{-11}$	$<1{,}04*10^{-10}$
CH460 x CH10163	$<3{,}40*10^{-11}$	$<5{,}10*10^{-11}$
CH462 x CH10163	$<5{,}68*10^{-11}$	$<6{,}32*10^{-11}$
CH464 x CH10163	$<2{,}52*10^{-11}$	$<6{,}49*10^{-11}$

Bold indicates the crosses using clinical isolates that generated a colony on the selection plate. The superscript * indicates that the cross generated more than one colony.

Table 13 shows all possible transconjugants that were generated by crossing various Cip^R and Tmp^R clinical isolates with either the *his*$^-$ recipient (CH10162) or the *trp*$^-$ recipient (CH10163). The crosses in bold generated large and healthy colonies when re-streaked on the M9 minimal medium + APR-200. The crosses with a * superscript generated healthy colonies that were medium/small-sized. The crosses in bold and with the * superscript all took 1-2 days to show detectable growth after the restreak. The unmarked crosses grew weakly and generated very small colonies after the re-streak, taking ~5-8 days to show any detectable growth. We expected any possible transconjugants to contain the *galK::pJ23100-aac(3)-IVa* junction (indicating resistance to apramycin and inability to degrade galactose) as well as fixing either the *his*$^-$ or *trp*$^-$ auxotrophy (*hisC*$^+$ or *trpE*$^+$ in a PCR). The deletions of the restriction enzymes (*Δ(mcrC-mrr)8* and *Δ(mcrA)*) could either still be present or fixed . All crosses except the two between CH442 and CH10163 appeared to have the *galK::pJ23100-aac(3)-IVa* junction. The crosses CH413 x CH10163, CH430 x CH10162 and CH442 x CH10163 appeared to have fixed the *his*$^-$ or *trp*$^-$ auxotrophy. The clinical isolate CH430 was able to generate possible transconjugants both when crossed with the *his*$^-$ recipient or the *trp*$^-$ recipient, with the difference that the cross with the *trp*$^-$ recipient showed as *Δ(trpE)* in the PCR. CH430 x CH10163 was also the only cross that generated purple colonies on the MacConkey galactose plate, while all others generated white colonies. Some crosses appeared to have fixed *mcrC-mrr* and *mcrA* (CH419 x CH10163 and CH442 x CH10163) while some have only fixed *mcrA* (CH412 x CH10163, CH418 x CH10163 and CH427 x CH10163). None of the crosses had fixed only *mcrC-mrr* and no crosses had the *galK::pJ23100-aac(3)-IVa* junction along with having fixed the auxotrophy and the restriction enzymes.

Table 13. Possible transconjugants selected on M9 + APR-200 plates

Cross (Donor x Recipient)	Saved as strain	Genotype (PCR)	Phenotype on MacConkey galactose (Purple/White)
CH411 x CH10162	CH10234	*galK::pJ23100-aac(3)-IVa, Δ(hisC), Δ(mcrC-mrr)8, Δ(mcrA)*	White
CH412 x CH10163	CH10238	*galK::pJ23100-aac(3)-IVa, Δ(trpE), Δ(mcrC-mrr)8, mcrA*$^{+}$	White
CH413 x CH10163	CH10235	*galK::pJ23100-aac(3)-IVa, trpE*$^{+}$*, Δ(mcrC-mrr)8, Δ(mcrA)*	White
CH418 x CH10163	CH10239	*galK::pJ23100-aac(3)-IVa, Δ(trpE), Δ(mcrC-mrr)8, mcrA*$^{+}$	White
CH419 x CH10163	CH10242	*galK::pJ23100-aac(3)-IVa, Δ(trpE), mcrC-mrr*$^{+}$*, mcrA*$^{+}$	White
CH427 x CH10163	CH10240	*galK::pJ23100-aac(3)-IVa, Δ(trpE), Δ(mcrC-mrr)8, mcrA*$^{+}$	White
CH430 x CH10162	CH10241	*galK::pJ23100-aac(3)-IVa, hisC*$^{+}$*, Δ(mcrC-mrr)8, Δ(mcrA)*	White
CH430 x CH10162	CH10250	*galK::pJ23100-aac(3)-IVa, hisC*$^{+}$*, Δ(mcrC-mrr)8, Δ(mcrA)*	White
CH430 x CH10162	CH10251	*galK::pJ23100-aac(3)-IVa, hisC*$^{+}$*, Δ(mcrC-mrr)8, Δ(mcrA)*	White
CH430 x CH10162	CH10252	*galK::pJ23100-aac(3)-IVa, hisC*$^{+}$*, Δ(mcrC-mrr)8, Δ(mcrA)*	White
CH430 x CH10163*	CH10243	*galK::pJ23100-aac(3)-IVa, Δ(trpE), Δ(mcrC-mrr)8, Δ(mcrA)*	Purple
CH435 x CH10163*	CH10247	*galK::pJ23100-aac(3)-IVa, Δ(trpE), Δ(mcrC-mrr)8, Δ(mcrA)*	White

Cross (Donor x Recipient)	**Saved as strain**	**Genotype (PCR)**	**Phenotype on MacConkey galactose (Purple/White)**
CH442 x CH10163*	CH10244	*trpE*$^{+}$, *mcrC-mrr*$^{+}$, *mcrA*$^{+}$	White
CH442 x CH10163*	CH10249	*trpE*$^{+}$, *mcrC-mrr*$^{+}$, *mcrA*$^{+}$	White
CH452 x CH10163*	CH10248	*galK::pJ23100-aac(3)-IVa, Δ(trpE), Δ(mcrC-mrr)8, Δ(mcrA)*	White

Bold indicates conjugation crosses that generated healthy and large-sized colonies. The superscript * indicates conjugation crosses that generated healthy and medium/small-sized colonies.

4. Discussion

4.1 Filter and liquid conjugations selecting for high-level ciprofloxacin and apramycin resistance

In this project, we were aiming to generate transconjugants of *E. coli* with high-level resistance to ciprofloxacin and apramycin through conjugation. As mentioned previously, there are two examples of intraspecies transconjugants that are highly successful pathogens (*K. pneumoniae* ST258 and *E. coli* ST1193).[12,14] This indicates that we are either unaware (due to a lack of screening) of other similar transconjugants or that such transconjugants are rare to find. Indeed, we were unable to generate any transconjugants with high-level ciprofloxacin and apramycin resistance when crossing Cip^R and Tmp^R isolates with Apr^R recipients in either liquid or on a filter. As can be seen in Table 3 and 8, the conjugation frequencies of the crosses between the clinical isolates and the apramycin resistant recipients were very low ($<10^{-8}$). In comparison, the conjugation frequency when using an Hfr-strain as a donor on a filter was high (10^{-3} per donor and 10^{-4} per recipient) and we were able to generate many transconjugants (Table 3). When using an undiluted F'-strain as a donor in liquid, the conjugation frequency was low ($\sim10^{-7}$), regardless if transconjugants were selected to have low- or high-level resistance to ciprofloxacin (Table 6). Although, the liquid could still be enriched for transconjugants after adding antibiotics, where seemingly all cells that were remaining in the liquid were transconjugants (Table 7). However, if the donor was diluted enough, no transconjugants could be generated. We also tried using zeocin (a glycopeptide) when inoculating the F'-strain to see if the antibiotic could introduce DNA breaks leading to recombination events which could increase the conjugation frequency. As can be seen in Table 10, the conjugation frequency did not change considerably ($\sim10^{-7}$) and the conjugation frequency per recipient decreased (10^{-8}) at 4 µg/mL zeocin. These results could indicate that a higher concentration of zeocin may have to be used for any considerable effect, but that residual zeocin may also kill off bacteria.

These results are not surprising, considering the many barriers towards a successful transfer of chromosomal DNA. Hfr-cells are, as expected, efficient at transferring chromosomal DNA regardless of the size of the fragment that is being selected. The distance between *gyrA* and *parC* which confer the high-level resistance to ciprofloxacin is roughly ~1 Mb, which is quite a large part of the *E. coli* chromosome (Figure 1). The F'-strain was less efficient at transferring chromosomal DNA in non-enriched conditions, but it could still reliably generate the desired transconjugants. What could prevent an efficient transfer of chromosomal DNA when using clinical isolates as donors is firstly that they would need to be able to generate Hfr-cells. Not all conjugative plasmids may have sequences that are similar to chromosomal sequences, which would make an integration very unlikely, and even then, Hfr-cells are generated at a low frequency ($\sim10^{-5}$ to 10^{-6}).[9,10] The clinical isolates that we used as donors do not have extensive data about what type of plasmids they have, nor how large they are or what they may contain besides the indication of resistance to certain antibiotics. What also needs to be taken into consideration is that there needs to be cell-cell contact for a certain period of time, the pilus generated by the plasmid must be able to attach to other cells and that DNA must be processed and transferred.[1] Larger fragments of DNA, such as in the case of both *gyrA* and *parC*, are less

likely to be transferred unless cell-cell contact can be maintained long enough for the whole fragment to be transferred. Since we do not know much about the plasmids of the clinical isolates, we do not know their conjugational efficiency. Studying each individual plasmid of each donor in high detail would require extensive work, so it may be advantageous to conduct further experiments to determine plasmid transfer frequencies in different conditions (on filter, in liquid or at different temperatures). The next set of issues are present once DNA is being transferred. Restriction enzymes and exonucleases can prevent transfer of incoming DNA and the fragment may not even be able to integrate into the recipient chromosome.[1,9,20] We tried to avoid some of these issues by using R^- strains as recipients, but we do not know the full impact of the deletion of the restriction enzymes. Both the donor and the recipient need to be considered when performing these kinds of experiments, and although we were unable to generate the desired transconjugants, it does not mean that it is impossible, only extremely rare. Further experiments should be performed to evaluate the likelihood of generating such transconjugants.

4.2 Plasmid transfer frequencies

As mentioned, the clinical isolates we used as donors do not have extensive data about their plasmids. We were still able to determine that some clinical isolates transfer their plasmid with a higher frequency than others, and even that the same clinical isolate transfers its plasmid at different frequencies depending on the recipient. As can be seen in Table 4, when crossing one clinical isolate with four different types of recipients, different plasmid transfer frequencies were yielded. In this case the plasmid transfer frequencies were low and varied between 10^{-8} and 10^{-9} per donor, while they varied between 10^{-7} and 10^{-8} per recipient. The highest plasmid transfer frequency (10^{-8} per donor and 10^{-7} per recipient) was yielded when crossing the clinical isolate with an MG1655 R^--recipient. This could mean that there is a higher compatibility between this donor in particular and the MG1655 strain, and that being R^- helps to a small extent with the plasmid transfer. For the subsequent experiments we used the MG1655 R^--recipient and we crossed it with different clinical isolates, both on a filter and in liquid. In Table 5, the clinical isolates had varying frequencies of plasmid transfer (10^{-3} to 10^{-6}), indicating that some were more efficient at transferring their plasmid than others. In Table 9 we could see the same trend, the plasmid transfer frequencies between different crosses varied as high as 10^{-1} and as low as 10^{-5} and for two of the crosses we were unable to screen for any plasmid transfer. Interestingly, the plasmid transfer frequency for the cross between CH411 x CH10140 was much higher in liquid (10^{-4}), as seen in Table 9, than on a filter (10^{-8} per donor and 10^{-7} per recipient), as seen in Table 4. Some of the crosses that were done on a filter (Table 5) increased their plasmid transfer frequency by one order of magnitude in liquid (Table 9), but this difference is negligible. As for the cross between CH411 x CH10140 however, the results may indicate that conjugations in liquid (for this cross in particular) provide more favorable conditions than on a filter.

4.3 Filter conjugations selecting for prototrophy and apramycin resistance

Since we were unable to generate transconjugants with high-level resistance to ciprofloxacin and apramycin (Cip^R and Apr^R), we instead tried to select for prototrophic transconjugants with

resistance to apramycin by crossing Cip^R and Tmp^R isolates with auxotrophic Apr^R recipients. One recipient was auxotrophic for histidine (CH10162) while the other was auxotrophic for tryptophan (CH10163), and they were selected based on the distance between *hisC* and *trpE* to *gyrA* and *parC* (Figure 1). Our reasoning was that selecting for a single gene instead of a larger fragment of DNA would be a more efficient way to generate transconjugants. Other experiments where auxotrophs have been used in conjugations have been done previously, resulting in the successful transfer of chromosomal metabolic genes.[11] By selecting on minimal medium containing apramycin, we were able to select 15 possible transconjugants that are good candidates for a whole-genome sequencing analysis (Table 13). The conjugation frequencies of the different crosses were very low (~10^{-10}), but they were also low for the F-strain (10^{-9} with the *his*$^-$ recipient, 10^{-7} and 10^{-8} with the *trp*$^-$ recipient) (Table 11 & 12). The Hfr-strain had a conjugation frequency of 10^{-1} and 10^{-2} with the *his*$^-$ recipient and 10^{-3} and 10^{-5} with the *trp*$^-$ recipient.

Some of the crosses grew weakly on the selection medium (CH411 x CH10162, CH412 x CH10163, CH418 x CH10163, CH419 x CH10163 and CH427 x CH10163) and had a similar PCR profile to the recipient, meaning that they had the Apr^R-junction (*galK::pJ23100-aac(3)-IVa*) which makes them unable to degrade galactose and resistant to apramycin, but they did not fix the auxotrophy (*Δ(trpE)* or *Δ(hisC)*) (Table 13). One of the weak-growing crosses retained all deletions of its restriction genes (*Δ(mcrC-mrr)8, Δ(mcrA)*), while the other four all managed to fix the deletion of *mcrA*. One of these four managed to fix the deletion of *mcrC-mrr* and *mcrA*. These indications could mean that these bacteria are highly unstable and may have received a genetic fragment that cannot replicate, and which could be lost eventually if grown normally. Another possibility is that these bacteria have found a way to grow on minimal medium through another mechanism which is not related to the *hisC* or *trpE* genes.

All crosses that grew very well (CH413 x CH10163 and CH430 x CH10162) on the selection medium had the Apr^R-junction and fixed their auxotrophy, which is what we expected. None of these crosses fixed any of the restriction enzymes, which indicates that these crosses received only a small fragment of chromosomal DNA from the donor. One of the crosses, CH430 x CH10162 (*his*$^-$), managed to generate several possible transconjugants, which could indicate that the particular clinical isolate (CH430) that was used is efficient as a donor. The same donor (CH430) even managed to generate a possible transconjugant when crossed with the other recipient, CH10163 (*trp*$^-$). Interestingly, this cross grew well on the selection medium but produced smaller colonies. Its PCR profile indicated that it looked like the recipient, since it contained the Apr^R-junction and did not fix the auxotrophy or any restriction enzymes (*galK::pJ23100-aac(3)-IVa, Δ(trpE), Δ(mcrC-mrr)8, Δ(mcrA)*). However, it was also the only cross that appeared to be able to degrade galactose (purple on MacConkey galactose plates). These results could indicate that this cross has received a wild-type *galK* gene and possibly a fragment that has helped restore some of its metabolic ability (which is not picked up by the PCR amplification), and it is possible that the fragment it has received is larger than expected (duplication). Two of the other four crosses that grew well but produced smaller colonies (CH435 x CH10163 and CH452 x CH10163) had a similar PCR profile (*galK::pJ23100-aac(3)-IVa, Δ(trpE), Δ(mcrC-mrr)8, Δ(mcrA)*), but did not appear to be able to degrade

galactose. It is likely that these crosses have also received a larger fragment (duplication) of chromosomal DNA, which would explain why they produce small colonies that still grow well on the selection medium.

The donor CH442 crossed with CH10163 (*trp*$^{-}$) managed to generate two possible transconjugants. Surprisingly, these crosses did not contain any of the recipient markers (no detectable Apr^{R}-junction with a fixed auxotrophy and restriction enzymes) but they did grow well on the selection medium (produced small colonies) and showed as white on MacConkey galactose plates. These could have received a larger fragment of DNA which has integrated specifically across some of the areas on the chromosome where these genes are present and causing the primers to not detect any markers. However, this could also be due to a faulty PCR. Nevertheless, these crosses are still good candidates for WGS.

Another observation that we made was that most of the crosses (11 out of 15) came from the crosses with the *trp*$^{-}$ recipient. As can be seen in Figure 1, *trpE* appears before *hisC* on the chromosome, which could explain this observation. However, this does not mean that the origin of transfer is the same for all bacteria. On another note, there is also the chance that some of these possible transconjugants have not been generated through conjugation, but due to transduction via bacteriophages. Unless we know more about the clinical isolates, this possibility cannot be ruled out.

4.4 Conclusion

In conclusion, we have been able to generate 15 possible transconjugants by crossing Cip^{R} isolates with auxotrophic Apr^{R} recipients, at a very low frequency of ~10^{-10} per bacterial cell in a mixture of potential donors and recipients. These hybrid strains will have to be analyzed further using WGS to determine the actual genetic alterations that have occurred. Our current hypothesis to explain the rarity of transconjugant hybrids is that their creation depends on a sequential series of individually rare events. Our results imply that horizontal gene transfer through conjugation could lead to the creation of novel species and that resistance- and/or virulence genes may be transferred in this fashion. This poses a concern when it comes to the impact of bacteria on society, and what potential they have as highly successful pathogens.

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Supplementary Data

Supplementary Table 1. Primer sequences

Gene amplification	Sequence
gyrA	F: 5'-CTCCGTAATTGGCAAGAC-3' R: 5'-GGGATGTTGGTTGCCATA-3'
parC	F: 5'-TTGAAGGCTGGCGAATAAGT-3' R: 5'-GGTGCCGTTAAGCAAAATGT-3'
Apr^R-junction (*galK::J23100-aac(3)-IVa*)	F: 5'-CCTTTGGGCATGGAAACTGC-3' R: 5'-GGTGGCAGGGGCAATGGATC-3'
Δ(mcrC-mrr)8	F: 5'-CTGGGCAGGCCGGTAGAACTG-3' R: 5'-AGCGAAGTTTCCCGCGTGATGG-3'
Δ(mcrA)	F: 5'-CATGTGATGGGTGCCCTGGCTG-3' R: 5'-CAGCTTAGCGCCTTCCATCAG-3'
Δ(hisC)	F: 5'-GCCCGGAGCACCTGATCATTCAG-3' R: 5'-CTTACGGCAGTCGCACTCATCG-3'
Δ(trpE)	F: 5'-AAGCTGTGGTATGGCTGTGCAGG-3' R: 5'-AGAATTTCGCCCGCCTGACCG-3'

Supplementary Table 2. PCR programmes

Gene amplification	Programme
gyrA *parC* APR^R-junction (*galK::J23100-aac(3)-IVa*)	95℃ 5 min 95℃ 30s 55℃ 30s 72℃ 1 min 72℃ 7 min 4℃ ∞ x35 cycles
Δ(mcrC-mrr)8 *Δ(mcrA)* *Δ(hisC)* *Δ(trpE)*	95℃ 5 min 95℃ 30s 60℃ 30s 72℃ 1 min 72℃ 7 min 4℃ ∞ x35 cycles

Supplementary Table 3. Strains used in MIC determination

Strain (order of usage)	Genotype
CH1464	Wild-type MG1655
CH6017	Cip^R quadruple mutant
CH10154	Cip^R, *lac-pro::kan*
CH10155	(Cip^R, *lac-pro::kan*, F'23) - grown in both LB and M9 minimal medium
CH10129	Zeo^R

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