Extended-spectrum beta-lactamase antibiotic resistance plasmids have diverse transfer rates and can be spread in the absence of selection

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Abstract

Horizontal gene transfer, mediated by conjugative plasmids, is one of the main drivers of the global spread of antibiotic resistance. However, the relative contributions of different factors that underlie this plasmid spread are unclear, particularly for clinically relevant plasmids harboring antibiotic resistance genes. Here, we analyze nosocomial outbreak-associated plasmids that reflect the most relevant Extended Spectrum Beta-Lactamase (ESBL) mediated drug resistance plasmids to i) quantify conjugative transfer dynamics, and ii) investigate why some plasmid-strain associations are more successful than others, in terms of bacterial fitness and plasmid spread. We show that, in the absence of antibiotics, clinical *Escherichia coli* strains natively associated with ESBL-plasmids conjugate efficiently with three distinct *E. coli* strains and one *Salmonella* enterica Serovar Typhimurium strain. In more than 40% of the *in vitro* mating populations, ESBL-plasmids were transferred to recipients, reaching final transconjugant frequencies of up to 1% within 23 hours. Variation of final transconjugant frequencies was better explained by variation in conjugative transfer efficiency than by variable clonal expansion of transconjugants. We also identified plasmid-specific genetic factors, such as the presence/absence of particular transfer genes, that influenced final transconjugant frequencies. Finally, we validated the plasmid spread in a mouse model for gut colonization, demonstrating qualitative correlation between plasmid spread *in vitro* and *in vivo.* This suggests a potential for predictive modelling of plasmid spread in the gut of animals and humans, based on *in vitro* testing. Altogether, this may allow straightforward identification of resistance plasmids with high spreading potential and to implement quarantine or decolonization procedures to restrict their spread.

Author summary

Antibiotic resistance is a major obstacle to the treatment of bacterial infections in clinics. Plasmids encoding antibiotic resistance genes can spread between bacteria in a density-dependent manner and accelerate the rise of resistant bacterial strains. This is particularly important for densely inhabited ecological niches such as the guts of humans and animals, where many bacteria interact. Understanding the exact contribution plasmids make to the global spread of antibiotic resistance remains an obstacle, because we lack quantitative studies implementing large-scale experimental testing of conjugation rates between clinically relevant bacterial strains. To counteract this knowledge gap, we studied clinical *Escherichia coli* isolates from human patients that carry extended-spectrum beta-lactamase producing plasmids. We found that these plasmids spread extensively through different bacterial populations and that both bacterial- and plasmid-specific factors determined the extent of plasmid spread. Our study combines detailed bioinformatic analyses, high-throughput *in vitro* testing and validation in an animal model. It suggests a potential for laboratory testing to understand and predict the spread of clinically relevant plasmids, including in the human gut microbiota, and thereby generates insights into novel treatment strategies to manage antibiotic resistance spread mediated by plasmids.

Introduction

The prevalence of antibiotic resistant bacterial pathogens is rising and continues to challenge the clinical use of antibiotics [1]. Infections with bacteria resistant to antibiotics are increasingly common and can result in death [2,3]. Importantly, resistance determinants are often plasmid-encoded. Plasmids can be transferred horizontally between different bacterial cells by conjugation, allowing rapid spread through diverse bacterial communities. This includes transfer among and between clinically relevant bacterial pathogens and commensals found in the gut microbiota of humans, farm animals, or in the environment [4–6]. In some cases, the role of selection imposed by antibiotics may be overestimated, because certain resistance plasmids can increase in frequency even in the absence of antibiotics [7–9] .

Whether a plasmid persists within a population depends on the plasmid as well as its association with a bacterial host. Some of these associations, such as *Escherichia coli* sequence type ST131 and plasmids of the IncFII family, carrying the *bla CTX-M* gene (which encodes a cefotaximase, conferring resistance to extended-spectrum beta lactams; ESBLs), are very successful and have become global threats [10–12]. ESBLs provide their bacterial host with resistance to beta-lactam antibiotics, such as the widely used penicillins and cephalosporins, and are generally encoded on plasmids of the incompatibility groups IncF and IncI [13–15]. Other plasmids, such as those carrying the carbapenemase-producing gene blaOXA-48, remain a clinical problem despite no association with a specific strain. For instance, blaOXA-48 plasmids have been reported in *E. coli, Klebsiella spp., Citrobacter spp.,* and *Enterobacter spp*. [16]. The family of Enterobacteriaceae is a key public health concern, as it contains some of the most important nosocomial pathogens and is responsible for the pandemic spread of ESBLs [10,15,17,18].

To counteract resistant pathogens and their successful transmission in hospitals, communities, and the environment, we need to improve our understanding of the factors that determine successful plasmid-strain associations and to elucidate the drivers of resistance plasmid spread in the presence or absence of antibiotics. Recent *in vitro* studies revealed several key factors driving changes in resistance plasmid frequency over time. If a plasmid of the same incompatibility group is already established in a potential recipient, surface- or entry exclusion may prevent further plasmid acquisition [19]. Plasmids can also increase transfer rate of co-residing plasmids, or allow other (mobilizable) plasmids to co-transfer [20,21]. However, once a plasmid is taken up by a bacterium, the plasmid replication system and its interaction with host factors define whether it can be replicated and stably maintained in the lineage. For example, the efficiency of plasmid transfer decreases if a recipient strain encodes a restriction system, to which the incoming plasmid is susceptible [22–24]. In some cases, compensatory mutations have been shown to reduce plasmid cost in the presence and absence of selective pressure [25]. In others, it is the high rates of conjugation that allow a plasmid to persist despite a growth disadvantage [26]. With the recent discovery of several novel defense systems against foreign DNA in bacteria [24], interactions between bacteria, particularly during horizontal gene transfer, become increasingly interesting to study. This may allow us to find tools to prevent spread of plasmids carrying antibiotic resistance genes.

The relative contributions of the various factors that drive plasmid spread remain poorly understood, particularly for clinical plasmids [11]. Past studies have largely focused on types of plasmids not directly relevant for resistance in clinical settings, or on individual plasmids

after moving them into model strains [22,25–27] (notable exception [28]). Studies using mouse models have contributed to our understanding of the processes that drive conjugation within the gut, but these have mostly been limited to laboratory strains with conjugative plasmids that are not clinically relevant [8,9,29,30]. Human-based studies that demonstrate horizontal transfer of resistance plasmids in the human gut [5,31] are of strong clinical relevance, but often rely only on genomic evidence and thus do not allow to observe the factors driving plasmid spread. There is therefore a shortage of studies connecting mechanisms of plasmid spread found *in vitro* with corresponding patterns *in vivo* and with the effective spread of plasmids in the clinics [11,32,33]. To improve the predictability of clinically relevant plasmid spread, we need a quantitative understanding of resistance plasmid transfer from their clinical host strains to diverse recipient strains, the effect of plasmids on the growth phenotypes of these recipient strains, subsequent transfer of newly acquired plasmids to other recipients, and the impact of bacterial host factors on these properties.

Here, we use clinical *E. coli* strains with their natively associated plasmids carrying ESBL genes (ESBL-plasmids) to investigate the drivers of plasmid spread, and to study the correspondence between *in vitro* and *in vivo* spread. Based on *in vitro* liquid culture mating assays in the absence of antibiotic selection, we found that the final frequency of ESBL-plasmid carrying recipient strains (transconjugants) was determined by donor-, recipient-, and plasmid-specific factors. The plasmid itself had the largest contribution to final transconjugant frequency, as all plasmids that carry functional transfer (*tra*) genes were found to spread. Additionally, none of the tested plasmids show evidence of a growth cost in any recipient strain. Using a murine model for gut colonization, we validated that these plasmids can spread efficiently *in vivo* in the absence of antibiotic selection. As *in vitro*, plasmid carriage of the tested plasmids was

146 not associated with a major cost or benefit in the gut, confirming the critical role of
147 conjugation in driving the spread of antibiotic resistance plasmids. We showed that the range
148 of transfer efficiencies *in vivo* are qualitatively predictable based on our *in vitro* testing.

Results

Diverse clinical ESBL-plasmids spread extensively through recipient populations in the absence of antibiotics. We investigated the rate at which ESBL-plasmids from eight clinical donor *E. coli* strains spread through four non-resistant recipient populations. The donor strains stem from hospitalized patients and are representative of clinically relevant ESBL-producing *E. coli* [34]. Of the four recipients, two *E. coli* strains were isolated from healthy human subjects [35], one *E. coli* strain was previously isolated from mice housed in our facility [8], and one is a pathogenic wild type strain of *Salmonella enterica* Serovar Typhimurium. The chosen donor and recipient strains spanned the phylogenetic diversity of *E. coli* [36,37] (Fig 1) and contained many plasmids of various incompatibility groups (S1 Fig, S1 Table). Each donor carried one plasmid containing resistance genes, either of the plasmid family IncI1 or IncF (Table 1, S2 Fig). We found various virulence genes, including type 6 secretion systems (T6SSs, involved in bacterial killing), in donors and recipients (S3 Fig). The only strain with a pathogenic virulence profile is D4 (S3 Fig). All strains carry numerous intact prophage sequences (S1 File).

Table 1: Clinical ESBL-plasmids in their native host strains (donors).

Strain (*E. coli* sequence type)	**ESBL-plasmid**	**Plasmid size**	**Replicon** (incompatibility group)	**Resistance genes on the ESBL-plasmid†**	**Antibiotic resistance of strain***
D1 (ST 117)	p1B_IncI	111 kB	IncI1	aadA5; **blaCTX-M-1**; dfrA17; sul2	Amp, Ceftri, Cotri
D2 (ST 648)	p2A_IncF	165.7 kB	IncFIA; IncFIB; IncFII	mph(A); catB3; aadA5; aac(6')-Ib-cr; dfrA17; sul1; sul2; **blaOXA-1**; tet(A); **blaCTX-M-27**; aph(3'')-Ib; erm(B)	Amp, Amo/C; Ceftri; Tobra; Cotri; Cipro
D3 (ST 648)	p3B_IncI	59.8 kB	IncI1	**blaCMY-42**	Amp; Amo/C; Pip-T; Ceftaz; Ceftri;

					Tobra; Amika; Cotri; Cipro
D4 (ST 40)	p4A_IncI	88.9 kB	IncI1	**blaCTX-M-1**	Amp; Ceftri
D5 (ST 131)	p5A_IncF	160 kB	IncFIB; IncFIC(FII); IncFIA	mph(A); aadA5; aac(6')-Ib-cr; dfrA17; sul1; **blaCTX-M-15**; aac(3)-IIa; tet(A); catB3; **blaOXA-1**	Amp; Amo/C; Ceftaz; Ceftri; Cefe; Tobra; Cotri; Cipro
D6 (ST 131)	p6A_IncI/F	157 kB	IncI1; IncFIA; IncFIB; IncFII	**blaTEM-1B**, aac(3)-IId, **blaCTX-M-8**	Amp; Ceftri; Cefe; Tobra; Cipro
D7 (ST 131)	p7A_IncF/Col156	134.9 kB	IncFIA; IncFIB; IncFII; Col156	mph(A); aph(3'')-Ib; aadA5; dfrA17; sul1; sul2; tet(A); **blaCTX-M-27**	Amp; Ceftri; Cotri; Cipro
D8 (ST 69)	p8A_IncF	131 kB	IncFIA; IncFIB	dfrA14, mph(A), **blaCTX-M-14**, tet(B)	Amp; Ceftri; Cotri

†Genes encoding the ESBL phenotype are highlighted in bold.

*Antibiotic resistance is defined as being above the EUCAST defined minimum inhibitory concentration breakpoint (MIC; see S2 Table for all antibiotics tested and MIC information). Donor strains fulfilled criteria for ESBL production based on EUCAST recommendations. ESBL mechanism was phenotypically and genotypically confirmed. Abbreviations: Amp = Ampicillin; Amo/C = Amoxicillin/Clavulanic acid; Pip-T = Piperacillin-Tazobactam; Ceftaz = Ceftazidim; Ceftri = Ceftriaxone; Cefe = Cefepim; Tobra = Tobramycin; Amika = Amikacin; Cotri = Cotrimoxazol; Cipro = Ciprofloxacin.

First, we performed conjugation assays between all clinical donors and non-resistant recipients (approximately 1:1 ratio, referred to below as the "1st gen *in vitro* experiment") and measured conjugation as the final fraction of the recipient population that carried the plasmid after 23 hours of growth (hereafter termed "final transconjugant frequency"). Five of the eight ESBL-plasmids were transferred to more than one *E. coli* recipient (Fig 2) and transfer stemming from the donor-plasmid pair D4-p4A_IncI led to the highest final transconjugant frequencies of ~0.1%. Final transconjugant frequencies spanned 5 orders of magnitude (Fig 2A). For the donor-plasmid pairs where we observed plasmid spread to multiple recipients, average final transconjugant frequency varied depending on the donor-plasmid pair (effect of donor-plasmid pair in a two-way ANOVA, excluding D2, D3, D7: $F_{4,66} = 87.665$, $P < 0.01$) and among recipient strains (effect of recipient in the same two-way ANOVA: $F_{2,66} = 5.439$, $P < 0.01$). Furthermore, variation among donor-plasmid pairs depended on the recipient (donor-plasmid pair×recipient interaction: $F_{8,66} = 3.164$, $P < 0.01$). These plasmids, natively associated with *E. coli*, reached comparable maximal transconjugant frequencies in *S.* Typhimurium

recipient populations (RS, Fig 2B). In *S*. Typhimurium, variation across donor-plasmid pairs was similar to *E. coli* recipients, with the exception of p6A_IncI, which did not spread to recipient RS.

Qualitatively, phylogenetic relatedness (Fig 1) between donor strains did not explain similarities in their plasmid transfer dynamics in the 1st gen *in vitro* experiment. For example, D5, D6 and D7 have effectively the same host background (ST 131), yet transferred their plasmids with very different efficiencies (Fig 2). By contrast, D1 and D8 are relatively phylogenetically distant, yet showed very similar transfer dynamics across recipients (Fig 2). Phylogenetic relatedness between donor strain and recipient was not strongly associated with transfer efficiency either: D5, D6 and D7 are all equally closely related to recipients RE1 and RE2, yet we observed very different final transconjugants frequencies, spanning four orders of magnitude, among these 6 donor-recipient pairs (Fig 2). Finally, the *S.* Typhimurium (RS) recipient is roughly equally phylogenetically distant to all *E. coli* donors used, yet we observed widely varying transfer efficiencies, similar to those observed across *E. coli* recipients.

To investigate the effect of genomic factors on the observed plasmid spread, we related final transconjugant frequencies to the presence or absence of *tra* (transfer) genes and toxin-antitoxin systems (TA-systems) on plasmids and of restriction-modification systems and CRISPR-Cas in donor and recipient strains (S2 File). We found that the presence of relevant *tra* genes on a plasmid was the main genomic factor that affected whether plasmids were able to spread. *Tra* genes encode the sex pilus and the proteins required for the conjugative transfer, and thus, when missing, plasmids cannot conduct their own transfer [38–40]. All

three cases in which the plasmid showed no or almost no spread to any recipient (i.e. p2A_IncF, p7A_IncF/Col156, p3B_IncI) could be explained by their lack of functional *tra* genes (S2 File). All plasmids encode for numerous TA-systems, likely leading to plasmid addiction, and thus we could not find a relation between these and final transconjugant frequencies. Despite the large number of unique donor-recipient combinations, the analysis of CRISPR-Cas and restriction-modification systems did not allow a clear association between their presence and the observed plasmid spread (S2 File). *Tra* genes, were the only genomic factor for which presence or absence could be consistently associated with the final transconjugant frequency.

ESBL-plasmids from clinical donors carry no significant cost after transfer to new hosts. The final frequency of the ESBL-plasmids in different recipient populations spanned five orders of magnitude after 23 hours. This variation in plasmid spread could be driven by differences in horizontal transmission, or in clonal expansion of transconjugants. Thus, we investigated the effects of plasmids on bacterial growth in the absence of antibiotics for ten strain-plasmid combinations. We estimated growth rates of recipients and transconjugants over 24 hours and expressed plasmid cost as the growth rate of transconjugants relative to their respective plasmid-free strains (Fig 3A for *E. coli* recipients and 3B for *S.* Typhimurium). None of the novel host-plasmid combinations had a significantly different growth rate compared to the original host strain (Student's t-Test for *E. coli* hosts and Wilcoxon Rank Sum Test for *S.* Typhimurium, $P > 0.05$ in all cases, before and after Holm's correction for multiple testing). Although non-significant, the *S*. Typhimurium transconjugants showed a consistent trend towards higher relative growth rates when carrying an ESBL-plasmid, and were not normally distributed. Whether this trend can explain the final frequency of *S*. Typhimurium transconjugants (Fig 2B) depends on the magnitude of the growth advantage, and the duration of the conjugation

assay. We calculated whether, under the assumption of purely exponential growth, the mean difference in growth rate between recipients and transconjugants would be large enough to explain the observed final transconjugant frequencies (S3 File). This is the case for the combination of donors D1 and D8 with *S.* Typhimurium recipients, but for *E. coli* recipients, clonal expansion is unlikely to explain the observed variation in final transconjugant frequencies.

Plasmid transfer rate drives the final transconjugant frequency. Because for most cases clonal expansion alone did not explain the observed final transconjugant frequencies, we investigated whether variable rates of horizontal transfer (conjugation) could explain this. A widely used method to estimate plasmid transfer rates is the mass action model from Simonsen *et al.* [41]. When we applied it to the data obtained in the 1st gen *in vitro* experiment (S3 Table), we found that across all donor-recipient combinations, the estimated conjugative transfer rates correlate strongly with the observed final transconjugant frequencies (Fig 4, Pearson's test, $r = 0.99$, $P < 0.001$). This suggests that plasmid transfer rates dictate final transconjugant frequencies.

A drawback of the Simonsen method is that it assumes that (i) the growth rates for donor, recipient and transconjugants are all equal, (ii) the conjugation rate does not change over time (i.e. that the rate of conjugation from the donors and newly formed transconjugants to the recipients is the same), and (iii) mating takes place in well-mixed liquid cultures. However, the transfer rate estimates of the experiments described here are not likely to be affected significantly by violation of these assumptions. Firstly, the observed differences in growth rate for donors, recipients, and transconjugants (Fig 3 and S3 File), are too small to drive shifts in

252 relative frequencies over the course of our conjugation assays. Secondly, transconjugants
253 reached maximally 0.1% of the donor population size, which is comparatively small (S4 Fig).
254 Therefore, the vast majority of transfer events results from donor-to-recipient conjugation
255 rather than transconjugant-to-recipient transfer. Thirdly, regarding assumption (iii), we
256 conducted a control experiment to estimate the number of transconjugants observed solely
257 due to surface mating during the plating step of our conjugation assay (S4 Table). For most
258 donor-recipient pairs we found that the number of transconjugants due to on-plate mating
259 was negligible. The pairs D1-RE1 and D1-RE3, however, reached high transconjugant CFU
260 counts due to surface-mating only (35% and 85% of the numbers observed in the 1st gen *in*
261 *vitro* experiment). Overall, we interpret estimates from the Simonsen method as an accurate
262 reflection of the conjugation rate in our experiments, but for D1-RE1 and D1-RE3, it may
263 reflect the combined effect of conjugation in liquid and on the agar surface after plating.
264
265 **Variation in plasmid spread stems from plasmid, donor and recipient factors.** In the 1st gen
266 *in vitro* experiment each plasmid was present in a single donor strain (Fig 2), preventing us
267 from disentangling the effects of donor strain and plasmid on plasmid spread. Thus, we next
268 performed an experiment under conditions identical to above, using 8 different plasmid
269 donors (2nd gen *in vitro,* Fig 5). Each donor is a unique combination of strain (one of three
270 different recipient strains from above) and plasmid (one of three different plasmids from
271 above), isolated from among the transconjugants of the 1st gen *in vitro* experiment. The final
272 transconjugant frequency varied among donor strains (effect of donor strain in a three-way
273 ANOVA with plasmid, donor strain and recipient as factors, excluding plasmid p1B_IncI, see
274 Materials and methods: $F_{2,90} = 150.133$, $P < 0.001$) and among plasmids ($F_{1,90} = 49.717$, $P <$
275 0.001). This observed variation among plasmids depended on both the recipient and the

donor strain. For instance, in cases where *S.* Typhimurium RS acted as the donor and recipient simultaneously, both IncI1 ESBL-plasmids yielded remarkably high final transconjugant frequencies of 40 % for p1B_IncI and 39 % for p4A_IncI (donor strain×plasmid interaction: $F_{2,90}$ = 96.352, P < 0.001; recipient×plasmid interaction: $F_{2,90}$ = 29.610, P < 0.001). A second analysis including plasmid p1B_IncI, but excluding donor RE2 (see Materials and methods) supported variation among donor strains ($F_{1,93}$ = 560.269, P < 0.001) and plasmids ($F_{2,93}$ = 156.075, P < 0.001), and that variation among plasmids depended on recipient and donor strain (recipient×plasmid interaction ($F_{4,93}$ = 26.104, P < 0.001; donor strain×plasmid interaction: $F_{2,93}$ = 3.999, P = 0.022). As in our 1st gen *in vitro* experiment, average final transconjugant frequencies also varied among recipients (P < 0.001 for effect of recipient in both three-way ANOVAs). Thus, the final frequency of transconjugants depended on all three factors of donor strain, plasmid, and recipient.

The final transconjugant frequencies in this 2nd gen *in vitro* experiment, where we replaced the native donor strains with transconjugants stemming from the 1st gen *in vitro* assay, differed strongly from those of the 1st gen *in vitro* experiment. They increased >10-fold in 11 and decreased >10-fold in 4 out of the 24 mating populations (Figs 2 and 5). Because the transferred plasmids and recipient strains were equal to, and the absolute frequencies of donor and recipient comparable to, those of the 1st gen in *vitro* experiment (S4-5 Figs), we tested whether these differing transconjugant frequencies stem from a difference in transfer from native host versus transfer from a secondary host (S7 Fig). As donors of plasmid p1B_IncI, we used its native host D1 and its transconjugants stemming from conjugation with recipients RE1 and RS, both carrying the marker plasmid pACYC184, and an additional D1 strain carrying pACYC184. First, we tested whether the presence of pACYC184 in donors could

affect the transfer of ESBL-plasmids. Indeed, pACYC184 in D1 had a significantly negative effect on transfer of p1B_IncI to RE1 (Wilcoxon Rank Sum Test, P = 0.035 after Holm's correction for multiple testing) and to RS (Student's t-Test, P = 0.042 after Holm's correction for multiple testing). Although significant, the effect of pACYC184 was small compared to the difference in transconjugant frequency that resulted from transfer from native versus 2nd gen donor strain. For instance, conjugation of the donor strains RE1 and RS with recipient RE1 resulted in a 45-fold and a 112-fold increase respectively, compared to conjugation of the native donor strain D1 with recipient RE1. When donor and recipient were RS, final transconjugant frequency even increased 2800-fold (S7 Fig). Overall, this experiment confirmed the significant difference in final transconjugant frequency resulting from transfer by native versus secondary donor strains and highlighted the great increase in final transconjugant frequency when donor and recipient are the same strain (self-self transfer).

A possible explanation for the altered transfer efficiency when transconjugants acted as plasmid donors, are mutational changes, in either the plasmid or the recipients, that might have accumulated during the 1st gen or the 2nd gen *in vitro* experiment. Thus, we analyzed transconjugants from the 1st and 2nd gen *in vitro* experiments by whole-genome sequencing (one clone for each transconjugant, except for RE3 carrying plasmid p4A_IncI and p8A_IncF, for which we sequenced three clones). We did not find any mutational changes in ESBL-plasmids but a range of chromosomal mutations in transconjugants (S5 Table). However, these mutations were not consistently present across transconjugants and could not be related to adaptation to plasmid carriage. Nevertheless, we expect that host factors play a major role in plasmid spread, and it is simply their interplay and our small sample size that prevent us from detecting one single crucial conjugation rate determinant.

ESBL-plasmids can spread rapidly *in vivo*, with efficiencies correlating with the *in vitro* trends. Sequence-based studies have shown that ESBL-plasmids can spread between bacteria in the human gut [5,31], but their actual conjugation dynamics have not yet been studied *in vivo* (exception using laboratory *S.* Typhimurium strains [9]). Therefore, we extended our study to a more realistic murine model. We performed conjugation assays in mice with a limited microbiota [42] with three of the clinical donor strains carrying ESBL-plasmids (D4, D8, and D7) and RE3 as a recipient (Fig 6). These mice allow colonization of approximately 10^8 *E. coli* per gram feces, densities of *E. coli* that can be found in the guts of humans and animals [37]. Donors and recipients were introduced at equal densities but at different timepoints, the recipient one day prior to the donor, and were allowed to grow and conjugate over 7 days (Fig 6A). In line with our *in vitro* conjugation assay, we observed transconjugants for p4A_IncI and p8A_IncF, but not for p7A_IncF/Col156 (Fig 6B). Similar to the *in vitro* results in Fig 2, transfer of p4A_IncI resulted in higher transconjugants frequencies than transfer of p8A_IncF (day 1-6, Fig 6B). Plasmid p4A_IncI reached maximal proportions of plasmid carrying recipients of ~1% within only 4-8 hours of colonization (Fig 6C). Overall, we found that the rank order of final transconjugant frequencies is conserved between *in vitro* and *in vivo* conjugation. Moreover, the speed at which these clinical plasmids can establish themselves in the gut in the absence of antibiotic selection is striking.

As was the case *in vitro*, the transconjugant population *in vivo* was relatively minor compared to the size of the donor population (S4-6 Figs). This suggests that most transfer events derived from donor-to-recipient transfer, rather than transconjugant-to-recipient transfer. Given this,

it is likely that the conjugation dynamics *in vivo* can be predicted by the 1st gen *in vitro* experiments (Fig 2).

To determine whether the increase in transconjugant population size in the gut over the seven-day experiment was driven by clonal expansion or by conjugation, we investigated the competitive advantage of transconjugants versus recipients *in vivo*. For two transconjugants from the 1st gen *in vitro* experiment, we deleted the ESBL-plasmid origin of transfer (*oriT*) and created "locked", non-conjugative plasmids. These were competed 1:1 with RE3, the recipient used in the *in vivo* conjugation assay. A change in fitness conferred by plasmid carriage would be reflected in the competitive index, which is calculated as the ratio between the population sizes of recipient and "locked" transconjugant. After seven days of competition, only transconjugants carrying p8A_IncF outcompeted RE3, with a competitive index of ~0.1 (Fig 6D). Over the course of the experiment, this plasmid-borne fitness advantage led the initial transconjugant frequency of 0.5 to increase to a final transconjugant frequency of 0.9. Because this two-fold increase in transconjugant frequency is small compared to the transconjugant frequencies observed in the conjugation experiment (increasing from the detection limit of 10^{-6} to a frequency of 0.01; Fig 6B), we conclude that final transconjugant frequencies in the conjugation assay were mainly driven by conjugative transfer, rather than by clonal expansion of transconjugants. This is seen to a larger extent for p4A_IncI, where the transconjugant frequency increased substantially faster than with p8A_IncF (Fig 6B), despite a total lack of a growth advantage over recipient RE3 (Fig 6D). Altogether, we show that in the murine gut, ESBL-plasmids can be transferred rapidly between *E. coli* in the absence of antibiotic selection.

Plasmid interactions: the effect of co-residence, co-transfer and plasmid exclusion on plasmid transfer. It has been proposed that the transfer rate of plasmids can be modified by either the presence of other plasmids in the same bacterial host, by the co-transfer of other plasmids or by the presence of other plasmids in recipients [19,43]. All donor and recipient strains used in this study carry at least one plasmid (S1 Table). We investigated whether interactions with these plasmids might affect transfer of the ESBL-plasmids, using sequenced transconjugants from the 1st and 2nd gen *in vitro* experiments, and 8 clones per plasmid-donor pair from the *in vivo* conjugation assays on day seven.

We found multiple indications of plasmid interference in our conjugation experiments (Figs 2 and 5). Firstly, RE1, the only recipient without an IncFII-plasmid, received IncFII ESBL-plasmids (e.g. when mating with D5 and D6) at a higher rate than all other recipients. P6A_IncI/F of D6 did not transfer, and p5A_IncF of D5 only poorly, to recipient RS that carries a plasmid with IncFII/FIB replicons (Fig 2). On the other hand, plasmid p8A_IncF, carrying IncFIA/FIB-replicons, spread through RS recipient populations, despite RS having an IncFIB replicon (Figs 2 and 5). Because all but one of our conjugative plasmids carry multiple replicons [44], which is suggested to be a strategy to circumvent limitations to spread due to plasmid incompatibility, we argue that the plasmid incompatibility in our conjugation assays to might be permeable. Secondly, both *in vitro* and *in vivo,* transfer of ESBL-plasmid p8A_IncF to recipient RE3 resulted in the loss of the resident F-plasmid pRE3A_IncF (S8 Fig): in 6/11 re-sequenced transconjugants it was lost, retained in 2/11 and in 3 /11 it showed very low frequency in the sequencing reads. Similarly, pRE1A_IncI was lost by recipient RE1, irrespective of the donor strain and whether it acquired an IncI1 or IncF ESBL-plasmid (S8 Fig). The cause and effect we can not trace back: loss of resident plasmids could have allowed

subsequent acquisition of ESBL-plasmids, or the acquisition of ESBL-plasmids could have been followed by the loss of resident plasmids. Either way, despite this incompatibility of resident and newly-acquired ESBL-plasmids comparably high transconjugant frequencies could be reached (Figs 2 and 5).

In all the investigated conjugations, we only observed a few isolated cases of co-travelling plasmids, which we could not link to the observed variation in final transconjugant frequencies. We found plasmids co-transferred from donors D1 and D8 but not from D4 (S8 Fig). Although all three donors D1, D4 and D8 carry ColRNAI plasmids, we observed its transfer only from D1, alongside ESBL-plasmid p1A_IncF. *In vitro*, multiple plasmids were transferred from D8 alongside the ESBL-plasmid p8A_IncF, depending on the recipient: plasmid p8C_IncBOKZ was passed together with plasmid p8G_Col8282 to RE2, from which both plasmids were passed across the species boundary to *S.* Typhimurium RS in the 2nd generation *in vitro* experiment. Plasmid p8C_IncBOKZ also co-travelled to recipient RE1, but from there it was not passed to any 2nd generation recipient. No mutations were found on any of the co-transferred plasmids. The prophage p8B_p0111 (S8 Fig and S1 File) was passed to recipient RE3 *in vitro* but not *in vivo* and showed a deletion in the side tail fiber proteins stfE_2, and stfR in all three sequenced transconjugants. Despite the presence of their essential transfer genes (S2 File), we did not detect transfer of resident plasmids pRE2B_IncF of recipient RE2 or pRSA_IncF of recipient RS, to any recipient of the 2nd gen *in vitro* experiment.

Discussion

In this study, we demonstrated that transfer rates of ESBL-plasmids derived from clinical donors varied over five orders of magnitude and spread in the absence of antibiotics through various recipient populations. We confirm that, in the absence of external factors such as selection by antibiotics or competition against other bacterial species, conjugative transfer is the main determinant of the spread of ESBL-plasmids *in vitro* and *in vivo*. Like others, we found that ESBL-plasmids are not costly in our experiments [45,46]. When correcting for growth, we found that the increase in plasmid frequency was strongly correlated with plasmid transfer rate (Fig 4). This might hold true even for costly plasmids, as others have shown that transfer rates can surpass a fitness cost and allow plasmids to spread [26,47]. Foremost, we found that all plasmids carrying the required *tra* genes spread from various donors through a multitude of recipient populations. This suggests that conjugative plasmids have a strong drive to spread and that donor and recipient determinants play a lesser role. Since half of all plasmids can transfer via conjugation [20], it is tempting to speculate that the majority of these plasmids may spread in the absence of selection by antibiotics.

Although the estimates of our transfer rates are in line with results obtained with common laboratory strains [7,27], we have shown that it is crucial to use multiple and clinical strains to better understand the factors driving plasmid transfer. For instance, the pathogenic *S.* Typhimurium RS was able to receive 4 out of 5 conjugative plasmids. When acting as donor too, it could yield transconjugants at the highest efficiency compared to other recipients, particularly for IncI1 plasmids (Figs 2 and 5). This has important implications in the spread of antibiotic resistance plasmids, because the tissues of infected animals can serve as a reservoir for *S*. Typhimurium, which can lead to long-term potential for plasmid transfer into co-

colonizing Enterobacteriaceae [9]. An additionally important characteristic of clinical strains is the multitude of plasmids they harbor. It has been shown that their presence in donors and recipients may affect the transfer of the plasmid of interest. This has previously not been taken into account [27]. Here, we observed variation in plasmid transfer rate depending also on the recipient strain, and could recapitulate that some recipients are inherently more permissive to plasmids. The frequently used lab strain *E. coli* K12 seems to be an exceptionally good recipient, which may have led to a historical overestimation of transfer potential of plasmids [7,27] .

We found that *in vitro* transfer dynamics are a good qualitative predictor of *in vivo* plasmid transfer in a murine model, and discovered similar variation across donor and recipient combinations in both methods. Furthermore, we highlight the previously underappreciated speed with which ESBL-plasmids can spread through recipient populations *in vivo*. Plasmid donors colonized the gut within 4 hours and transferred ESBL-plasmids within the first 24 hours in the absence of antibiotic selection. This is particularly important, because our *in vivo* model recapitulates a natural situation in which *E. coli* is an abundant member of the microbiota of the gut lumen [37], and interacts with an invading ESBL-bearing *E. coli* [48,49]. However, contrary to our mouse experiments, not all natural Enterobacteriaceae populations reach densities of 10^8 CFU/g feces in farm animal or human guts [37]. This may lead to an overestimation of actual plasmid spread in our study. For such cases, the role of inflammation inducing pathogens such as *S.* Typhimurium may be especially important, because they can induce blooms of Enterobacteriaceae, leading to increased population densities and thus plasmid transfer [8]. The strains carrying clinical ESBL-plasmid rapidly colonized the mouse gut and thereby outperformed the resident strain (S6 Fig). All but one of our strains associated

464 with ESBL-plasmids encode a T6SS in their genome (S3 Fig), which may explain their
465 competitive success. Thus, especially *in vivo*, the strain background of the plasmid-host
466 association is crucial to long-term plasmid persistence.
467
468 To obtain exact quantitative predictions of *in vivo* plasmid spread based on *in vitro* data,
469 detailed mathematical modelling will be needed. Fischer *et al*. [50] showed that a very simple
470 model that does not take into account varying plasmid transfer rates to host microbiota,
471 underestimates observed *in vivo* transfer rates. Given the high transfer proficiency of RS
472 observed in this study, a realistic mathematical model will likely require detailed
473 understanding of inter-species aspects of plasmid transfer. Furthermore, one will need to
474 consider the recently discovered role of long-term reservoirs formed by plasmid-carrying
475 invasive enteropathogens, such as *S.* Typhimurium, in plasmid spread [9].
476
477 Although our broad study includes many of the factors known to determine plasmid transfer,
478 the biological basis of the variation in plasmid transfer we found remains unknown. For
479 instance, we found large differences in plasmid spread between transfer from native and from
480 secondary bacterial hosts and a consistent 1000-fold increase in final transconjugant
481 frequency when donor and recipient were the same strain (self-self transfer). Dimitriu *et al.*
482 suggested compatibility of restriction-modification systems as a likely mechanism for
483 increased transfer rates in such cases [27]. Here, neither the presence of restriction-
484 modification systems nor of CRISPR-Cas showed a consistent connection to the observed
485 plasmid spread. Because donors of the 1st gen and 2nd gen *in vitro* experiment had the same
486 handling before the conjugation assay, i.e. thawing and growing overnight, it is unlikely that
487 there is a role of plasmid de-repression. Moreover, other factors such as surface exclusion or

488 adaptive mutations seem, based on our genomic analysis, at most minor. Although evolution
489 experiments demonstrated extensive plasmid host co-adaptation upon plasmid acquisition
490 [51,52], neither for the 24 hours *in vitro* nor for the 7 days *in vivo* conjugation assay, could we
491 find any mutational changes that would suggest recipient-plasmid co-adaptation. Because
492 these ESBL-plasmids are non-costly in all tested recipients, we think further co-adaptation
493 might not be necessary for plasmid spread on a species level. An alternative explanation for
494 the lack of genomic modification related to plasmid-host adaptation could be transcriptional
495 changes in response to plasmid carriage [53]. To statistically test the relative significance of
496 donor, plasmid, and recipient factors, as those investigated here, in determining plasmid
497 transfer rates, even larger scale studies are needed. The feasibility of this has been
498 demonstrated by Alamam et al. [54], who studied horizontal resistance transfer for more
499 than 60.000 pairs of cell populations in parallel. We advocate for high-throughput screening
500 of clinically relevant donor and recipient strains *in vitro*.
501
502 Efforts must be made to minimize the transmission of strains containing antibiotic resistance
503 plasmids. Understanding the factors that make plasmid-host associations successful, can give
504 insight into novel measures to limit their spread. For instance, previous studies have
505 suggested to exploit systems like CRISPR-CAS, restriction modification or surface exclusion
506 [19,22,55,56]. We found multiple cases of plasmid interference due to plasmid
507 incompatibility, which may provide an important further avenue for future studies to block
508 the transfer and persistence of plasmids within a population of bacteria [57]. The results
509 presented here contribute to a growing scientific field aimed at determining fitness and
510 plasmid persistence factors that could ultimately inspire the development of anti-plasmid
511 spread strategies.

Materials and methods

Strains and growth conditions. As plasmid donor strains, we use 8 ESBL-plasmid positive clinical *E. coli* isolates, sampled from hospitalized patients at the University Hospital Basel, Switzerland [58]. Additionally, we worked with 4 ESBL-plasmid negative recipient strains: two clinical *E. coli* isolates from healthy patients, and mouse-derived *E.coli* and *Salmonella enterica* Typhimurium isolates. A comprehensive list of strains and associated plasmids can be found in S1 Table. Marker plasmids were introduced by electroporation, to mark recipients with either pACYC184 (New England Biolabs) encoding Chloramphenicol (Cm) resistance or pBGS18 [59] encoding Kanamycin (Kan) resistance. An exception was recipient RS, having chromosomal Cm resistance (*marT::cat*) [29]. Unless stated otherwise, we grew bacterial cultures at 37°C and under agitation (180 rpm) in lysogenic broth (LB) medium, supplemented with appropriates amounts of antibiotics (none, 100µg/mL Ampicillin (Amp) , 50µg/mL Cm and 50µg/mL Kan). We stored isolates in 25% glycerol at -80°C.

Antibiotic resistance profiling. We used microdilution assays with a VITEK2 system (bioMérieux, France) to determine the minimum inhibitory concentrations (MIC). MIC breakpoints for ESBL were interpreted according to EUCAST guidelines (v8.1). In addition, we confirmed the resistance mechanism of suspected isolates phenotypically using ROSCO disk assays (ROSCO diagnostica, Denmark) and/or genotypically with detection of CTX-M1 and -9 groups using the eazyplex Superbug assay (amplex, Germany).

Constriction of non-transferrable plasmids. We generated non transferrable plasmids, by deleting their origin of transfer (*oriT*) region, using the lambda red recombinase system with pKD4 as template for the Kan resistance marker[60]. The following primers were used (5' to

536 3'): For IncI1 plasmids (p4A_IncI) DIncI1_oriTnikA_f (GCA TAA GAC TAT GAT GCA CAA AAA
537 TAA CAG GCT ATA ATG GGT GTA GGC TGG AGC TGC TTC) and DIncI1_oriTnikA_r (CCT TCT CTT
538 TTT CGG AAT GAC TGC ATT CAC CGG AGA ATC CAT GGG AAT TAG CCA TGG TCC)[8] and for F
539 plasmids (p8A_IncF) D25_2_oriT-nikA-ko_vw (CCA TGA TAT CGC TCT CAG TAA ATC CGG GTC
540 TAT TTT GTA AGT GTA GGC TGG AGC TGC TTC) and D25_2_oriT-nikA-ko-rev (GTG CGG ACA
541 CAG ACT GGA TAT TTT GCG GAT AAA ATA ATT TAT GGG AAT TAG CCA TGG TCC). All mutants
542 were verified by PCR (IncI1_oriT _val_f: AGT TCC TCA TCG GTC ATG TC, IncI1_oriT _val_r: GAA
543 GCC ATT GGC ACT TTC TC, D25_oriT _val_fw: CAT ACA GGG ATC TGT TGT C and
544 D25_2_oriT_ver_rv: CAG AAT CAC TAT TCT GAC AC) and loss of transfer function.
545
546 **Sequencing, assembly, annotation**. We sequenced all donor and recipient strains on an
547 Illumina MiSeq machine (paired end, 2x250 bp), Oxford Nanopore MinION, and PacBio
548 Sequel. Unicycler [61] (v0.4.7) was used to produce hybrid assemblies (for D1,2,4,6,7,8 the
549 Oxford Nanopore – Illumina hybrid assembly proved most contiguous; D3,D5, RE1, RE2, RE3
550 were assembled using Pacbio Sequel – Illumina hybrid assembly). Manual curation involved
551 removing any contig smaller than 1kB, and sequences up to 5 kB that mapped to the own
552 chromosome. Quality control was performed by mapping the paired end Illumina reads to the
553 finished assemblies using samtools (v1.2) and bcftools (v1.7) [62,63]. The reference sequence
554 of RS was downloaded from NCBI Genbank under the accession numbers NZ_CP034230.1 and
555 NZ_CP034231.1.
556
557 We extracted an alignment of the concatenated core genome genes of all *E. coli* donor and
558 recipient strains, using chewBBACA for core genome Multi-Locus Sequence Typing (cgMLST)
559 against the Enterobase cgMLST scheme [64,65]. The phylogenetic tree was inferred using

BEAST2 [66], with an HKY substitution model and a strong prior on the mutation rate of *E. coli* (10^{-4} mutations per genome per generation, as estimated by Wielgoss et al. [67]).

We performed bacterial annotation using Prokka [68], and ST calling using mlst (Torsten Seemann, https://github.com/tseemann/mlst), which makes use of the PubMLST website (https://pubmlst.org/) developed by Keith Jolley [69]. Phylogroups were assigned using ClermonTyper [70].

We determined genomic features using a range of bioinformatic tools, and by BLAST comparison against various curated databases. Plasmid replicons and resistance genes were determined using abricate (Torsten Seemann, https://github.com/tseemann/abricate) with the PlasmidFinder [71] and ResFinder [72] databases respectively. Phages were located using PHASTER [73] (listing only those marked as "complete"), type 6 secretion systems using SecReT6 [74], virulence genes using the Virulence finder database [75], toxin-antitoxin systems using the database TADB 2.0 [76], and CRISPR-Cas loci using CRISPRCasFinder [77]. Restriction-modification systems were determined by using grep on the term 'restriction' in the prokka general feature format (GFF) files.

To determine the presence/absence of IncF and IncI1 transfer genes, we constructed our own database to compare against. IncF transfer genes were taken from the supplementary material of Fernandez-Lopez et al. [78], IncI1 transfer genes were copied from R64 using the annotations by Komano et al. [38], and R621a annotated by Takahashi et al. [39].

583 ***In vitro* conjugation assays**. We determined the final frequency of the recipient population
584 that obtained an ESBL-plasmid (T/(R+T)) in a high throughput, 96-well plate-based assay, with
585 two replica blocks per experiment. The experiments with *E. coli* recipients and the *S.*
586 Typhimurium recipient RS were performed independently. Donor and recipient grew over
587 night with or without ampicillin (100µg/mL), respectively. We washed the independent
588 overnight cultures (n=5-6 per strain) by spinning down and resuspending and added ~1µL of
589 6.5-fold diluted donor and recipient cultures into 150µL fresh LB with a pin replicator (total
590 ~1000-fold dilution, aiming to reach approximately a 1:1 ratio of donor and recipient). These
591 mating populations grew for 23 hours in the absence of antibiotics and were only shaken prior
592 to hourly optical density (OD) measurements (Tecan NanoQuant Infinite M200 Pro). To
593 determine the final cell densities, we plated the mating cultures at the end of the conjugation
594 assay on selective plates. In the 1st gen *in vitro* experiment, where the clinical strains
595 transferred their native plasmids to recipients, we selected for donors+transconjugants with
596 Amp, for recipients+transconjugants with Cm (*E. coli* recipients carried pACYC184-Cm and RS
597 chromosomal *marT::cat*) and for transconjugants with their combination. Four replicates had
598 to be excluded because the satellite colonies, a common phenomenon when plating on Amp,
599 could not be distinguished from true transconjugant colonies. For the 2nd gen *in vitro*
600 experiment, we chose a subset of transconjugants generated in the 1st gen *in vitro* experiment
601 that we used as plasmid donors. Transconjugants and recipients of the clone type EC3 were
602 omitted because of the size of the experiment, as well as transconjugant D1-EC2, carrying
603 p1B_IncI, because it could not be regrown from glycerol stock. We selected for donor with
604 Cm, for recipient with Kan (recipients carried pBGS18-Kan) and for transconjugants with
605 Kan+Amp. With this approach, we were able to detect transconjugant populations if they
606 were greater than 10^{-8} cells per mL.

607

608 Alongside the second replica block of the1st gen *in vitro* experiment, we performed a plate-
609 mating control experiment, to assess the extent of surface mating in our conjugation assay.
610 After washing and diluting the independent overnight cultures of the second replica block,
611 we grew clinical donors and *E. coli* recipients in isolation for the duration of the conjugation
612 experiment. Donors and recipients were mixed only before plating 20µL of the undiluted
613 mixed population (n_{total} = 1-3, S4 Table). In conjugations involving donors D4-D6 and D8, the
614 transconjugant CFU from plate-mating, was maximally ~15 % of the CFU observed in the 1st
615 gen *in vitro* experiment. For D1 in combination with RE1 and RE3, we found substantial
616 amounts of plate-mating, as high as fractions of 35 and 85 % of the transconjugants resulting
617 from the 1st gen *in vitro* experiment, respectively.

618

619 ***In vitro* plasmid cost and growth rate assays**. To investigate the effect of plasmid carriage on
620 bacterial growth, we measured the growth rate of transconjugants and corresponding
621 recipients. Per D and R combination, we used three clonal transconjugants (4 replicates each),
622 which were obtained from independent mating populations of the 1st gen *in vitro* experiment.
623 We grew bacterial cultures in absence of antibiotics overnight and diluted them 150-fold by
624 transfer with a pin replicator to a 96-well plate, containing 150µL fresh LB per well. The
625 cultures grew without shaking and we estimated growth rates of recipients and
626 transconjugants based on 10 OD measurements over 24 hours and expressed plasmid cost as
627 their relative growth rates. Because transconjugants have passed the 1st gen *in vitro*
628 experiment, we had to exclude that an effect of plasmid carriage could be obscured by
629 transconjugants having adapted to the LB growth conditions. Thus, we exposed recipients (n
630 = 4) to the same culture handling as a conjugation assay would and found their growth rates

not to be different from the naïve recipient strains used in the plasmid cost assay (S9 Fig). We had to exclude RE2 carrying p1B_IncI because it could not be regrown from glycerol stock and further excluded RE3 carrying p1B_IncI because we had not stored 3 independently arisen transconjugants.

For other growth measurements, we followed a similar approach of 150-fold dilution of overnight cultures by transfer with a pin replicator to a 96-well plate, containing 150µL fresh LB per well. Growth assays were performed in the plate reader with hourly OD measurements. We estimated growth rates using the R package Growthcurver [79].

Infection experiments. We have previously established a murine model for enterobacterial pathogen infection (Barthel et al, 2003) that has been used to measure conjugative plasmid transfer [8,9,29,30]. For conjugation experiments, 8-16 week old C57BL/6 mice containing an oligo microbiota [42], which allows colonization of approximately 10^8 *E. coli* per gram feces, densities of *E. coli* that can be found in the guts of humans and animals [37], were infected orogastrically with ~$5x10^7$ CFU of RE3, carrying marker plasmid pACYC184. 24 hours later, ~$5x10^7$ CFU of either D4, D7, or D8 were introduced orogastrically (Fig 6A). Feces were collected daily, homogenized in 1 ml of PBS with a steel ball by a Tissue Lyser (Qiagen) at 25 Hz for 1 min. Samples were enumerated for bacterial populations on MacConkey media containing the appropriate antibiotics (selection for donors+transconjugants with Amp, for recipients+transconjugants with Cm and for transconjugants with their combination) and final transconjugant frequencies T/(R+T) calculated.

For competition experiments, 8-16 week old C57BL/6 oligo microbiota mice were infected orogastrically with a 1:1 mixture of both competitor strains (~$5x10^7$ CFU total. Feces were collected and bacterial populations were enumerated daily as above. Bacteria were enumerated on MacConkey agar containing chloramphenicol and replica-plated on media containing chloramphenicol, kanamycin, and ampicillin to enumerate the locked transconjugants. Competitive index is calculated by dividing the recipient population by the locked transconjugant population.

Prior to all infections, overnight cultures in LB containing the appropriate antibiotics were subcultured for 4 hours at 37°C without antibiotics (1:20 dilution) to ensure equal densities of bacteria. Cells were washed in PBS and introduced into mice.

All infection experiments were approved by the responsible authority (Tierversuchskommission, Kantonales Veterinäramt Zürich, license 193/2016).

Resequencing. We re-sequenced isolates from the 2nd generation *in vitro* experiment as well as the *in vivo* transfer experiment, to study the genetic contribution to the observed transfer rates. Resequencing was performed on an Illumina MiSeq (paired end, 2x150 bp). Reads were mapped to the closed assemblies of respective donor and recipient strains using the breseq pipeline (v 0.32.0). Mutations or indels shared by all re-sequenced strains were treated as ancestral (S5 Table).

Statistical analyses. We performed statistical analyses using the software R (version 3.4.2). The effect of donor, recipient and plasmids on the final transconjugant frequency was

678 analyzed with either a two-way ANOVA (1st gen *in vitro* experiment, factors donor-plasmid
679 pair and recipient) or a three-way ANOVA (2nd gen *in vitro* experiment, factors donor, plasmid,
680 recipient). For the 1st gen *in vitro* experiment, we excluded from this analysis strain-plasmid
681 pairs which did not result in transconjugants in the 1st gen *in vitro* experiment (D2, D3, D7)
682 and *S.* Typhimurium recipient RS. When single replicates for a given donor-recipient
683 combination lacked transconjugants (D5 and D6), we assigned these replicates a final
684 transconjugant frequency at the detection limit of 10^{-8}. The same data was used to perform
685 the correlation of transconjugant frequency and transfer rate. Data of the 2nd gen *in vitro*
686 experiment was not fully factorial. To enable testing of higher-order interactions here, we
687 therefore performed two 3-way ANOVAs: one excluding plasmid p1B_IncI and one excluding
688 donor RE2, for which we had to take the two replica blocks into account: $P < 0.001$). For two
689 replica populations of donor RE2 transferring p4A_IncI to RE1, we were not able to detect
690 donors on plates and assigned them a donor population size at the detection limit of 1 colony
691 per plate (10^8 cells/mL). For two replica populations (RS self-self transfer with p1B_IncI), we
692 had higher counts on plates selecting for transconjugants than on plates selecting for
693 recipients+transconjugants and replaced the resulting negative CFU/mL for recipients with 1
694 CFU/mL.
695
696 To express the final fraction of transconjugants as a plasmid transfer rate, we used the cell
697 densities of donor, recipient, transconjugant and their sum (CFU/mL), and growth rates of
698 mating populations, both estimated in the 1st gen *in vitro* experiment. Introducing these
699 values to the mass action model as previously described by Simonsen *et al.* [41] yields the
700 transfer rate constant $\gamma\ (\mathrm{mL}(\mathrm{CFU} * \mathrm{t})^{-1})$. For statistical comparisons derived from *in vivo*

experiments, Kruskal-Wallis tests were performed with Dunn's multiple test correction using GraphPad Prism Version 8 for Windows.

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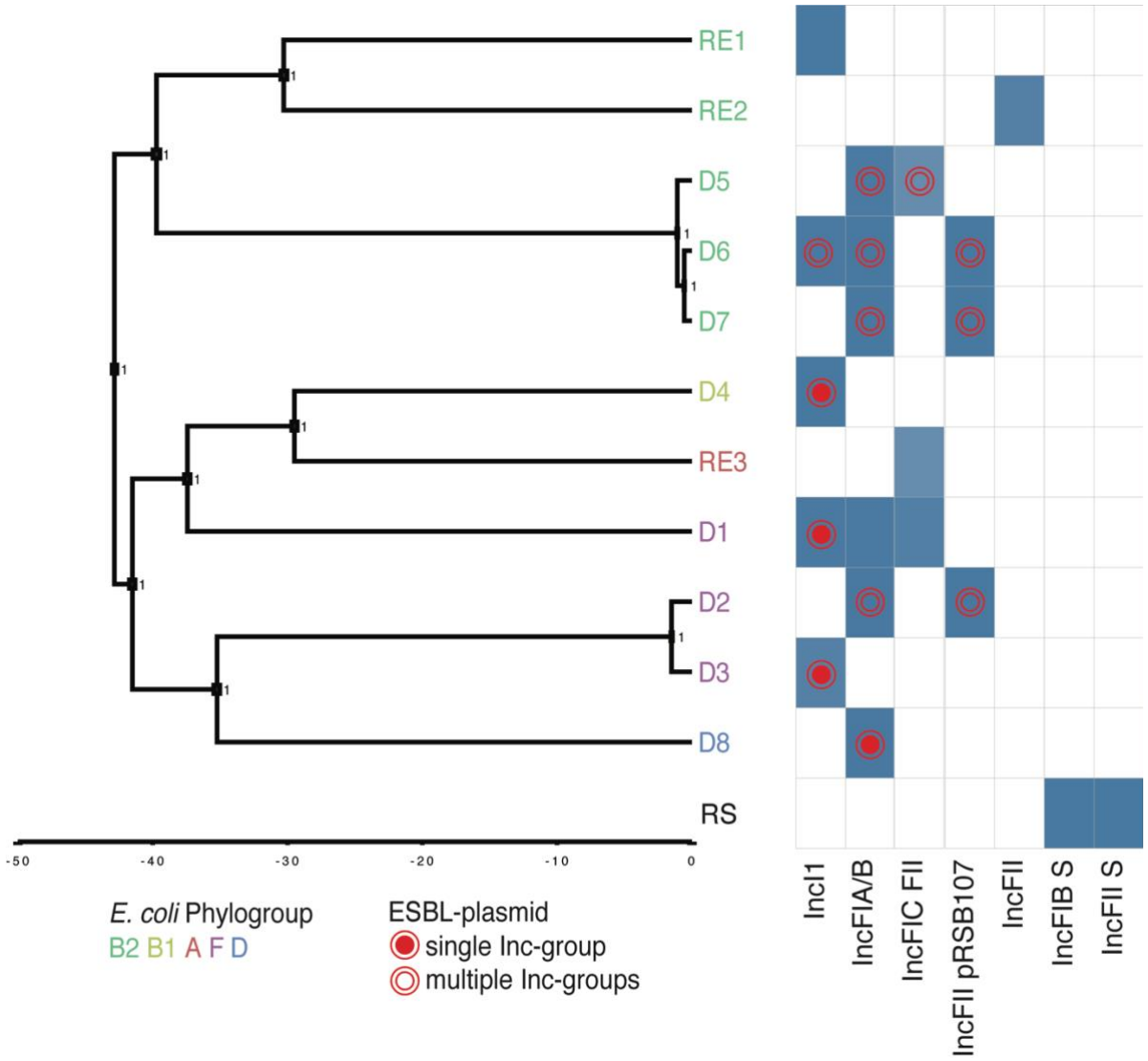
945

946 **Fig 1. Phylogenetic tree of the *E. coli* donor and recipient strains, inferred using Bayesian**
947 **inference on a core genome alignment.** The names at the tips are colored by *E. coli*
948 phylogroup. The *S.* Typhimurium recipient RS was not included in the phylogeny, but listed
949 here to allow comparison of the plasmid content. Blue rectangles indicate that a plasmid
950 carrying the given IncF or IncI incompatibility marker (replicon) was present in the strain. The
951 intensity of the shade of blue reflects the percent identity and coverage of the BLAST match
952 to the listed genes. A red dot indicates the ESBL-plasmid. If multiple replicons were found on
953 the same ESBL-plasmid this is indicated with open red circles.
954
955 **Fig 2. ESBL-plasmids spread at variable rates in the absence of antibiotics (1st gen *in vitro***
956 **experiment)**. Plasmid spread was measured as the final frequency of the recipient population
957 carrying the ESBL-plasmid (transconjugants), for three different *E. coli* (A) and one *S.*
958 Typhimurium (B) recipient populations. Circles represent independent replicates (n=4-6) and
959 the beams are mean values ± standard error of the mean (SEM). The detection limit was at ~10^{-}
960 ^{8}.
961
962 **Fig 3. No evidence for cost of plasmid carriage for transconjugants.** Plasmid cost was measured
963 for ten strain-plasmid combinations, with three independently isolated transconjugants each (n
964 = 4; beams are mean values ± SEM). The growth rate of transconjugants and their plasmid free
965 complements was determined from independent cultures. Relative growth rate was calculated
966 by dividing the transconjugant growth rates by the mean growth-rate of plasmid-free strains.
967

968 **Fig 4. Correlation of plasmid transfer rate and final transconjugant frequency estimated**
969 **using the Simonsen formula** [41]. Each data point results from the same liquid mating culture
970 shown in Fig 2. Transconjugant frequencies and transfer rates can be found in the S3 Table.
971
972 **Fig 5. Final transconjugant frequency depends on donor, recipient, and plasmid (2nd gen *in***
973 ***vitro* experiment).** Eight transconjugants isolated from mating assays in the 1st gen *in vitro*
974 experiment (Fig 2), used here as plasmid donor strains, transferred their plasmid to three
975 different recipients. Circles represent independent replicates (n= 6), the beams are mean values
976 ± SEM and different plasmids are indicated in color. The detection limit was at ~10^{-8}.
977
978 **Fig 6. ESBL-plasmids can spread in the gut in the absence of selection.** (A) Mouse model used
979 for *in vivo* conjugation assays. These mice have a minimal microbiota that offers intermediate
980 colonization resistance. Recipient RE3 was added one day before the plasmid donor.
981 Populations were enumerated in faeces by selective plating. (B) Conjugation assay for three
982 clinical donors and recipient RE3. (C) A subset of mice used in panel B were monitored for
983 conjugation within the first 24 hours of infection. (B-C) Plasmid spread is reported as final
984 transconjugant frequency. Dotted lines indicate the detection limit for selective plating. (D)
985 Competition assay performed by infecting mice with a 1:1 mix of a "locked" transconjugant
986 (*oriT* knockout) and recipient RE3. The competitive index was calculated by dividing the
987 recipient population by the locked transconjugant population. (B, D) Kruskal-Wallis test
988 $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

Fig 1.



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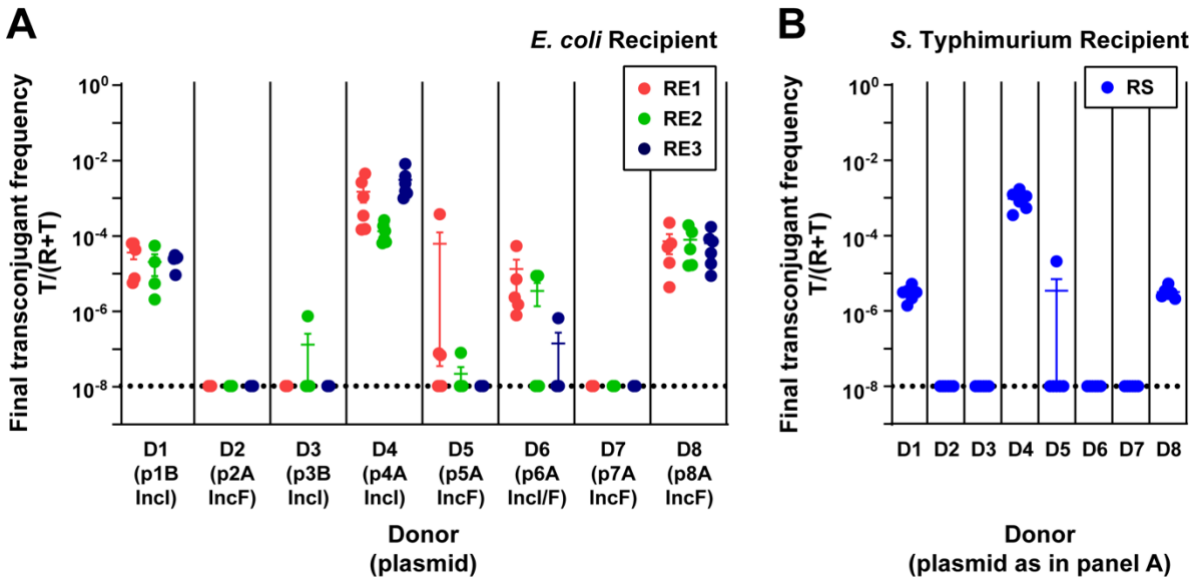

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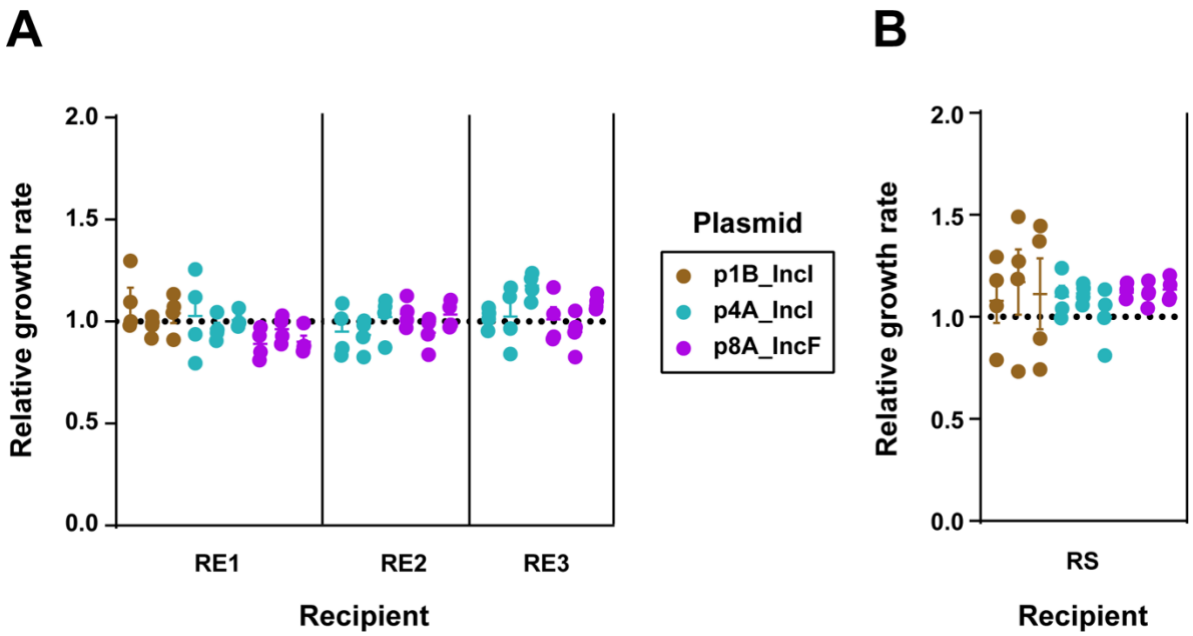

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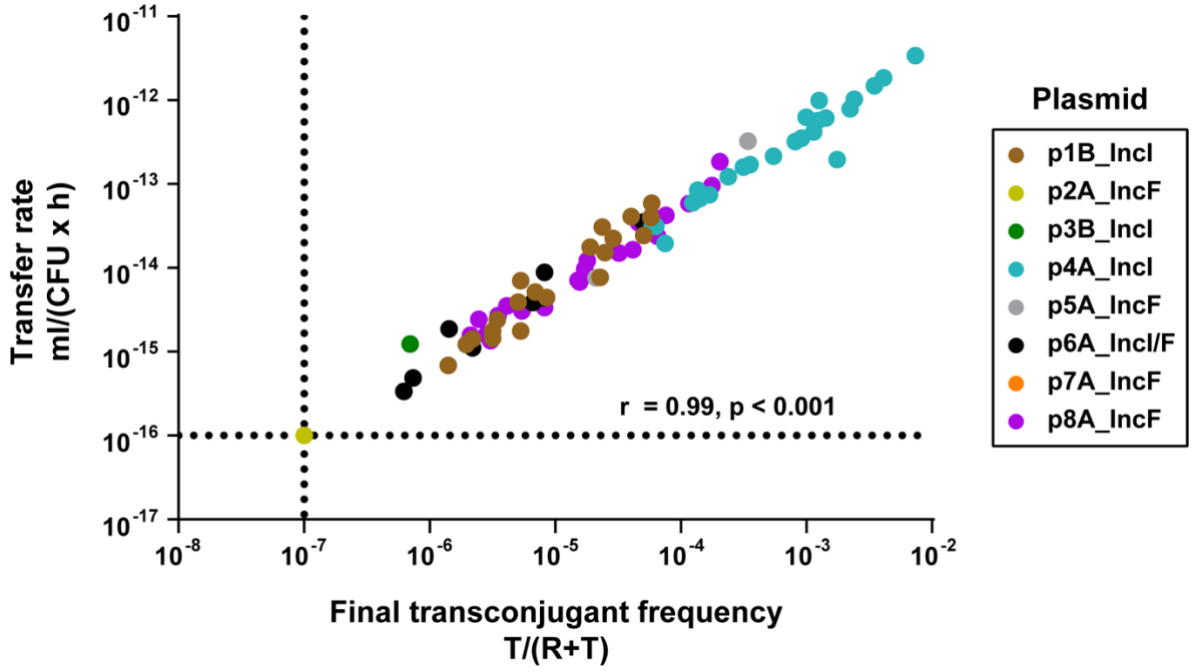

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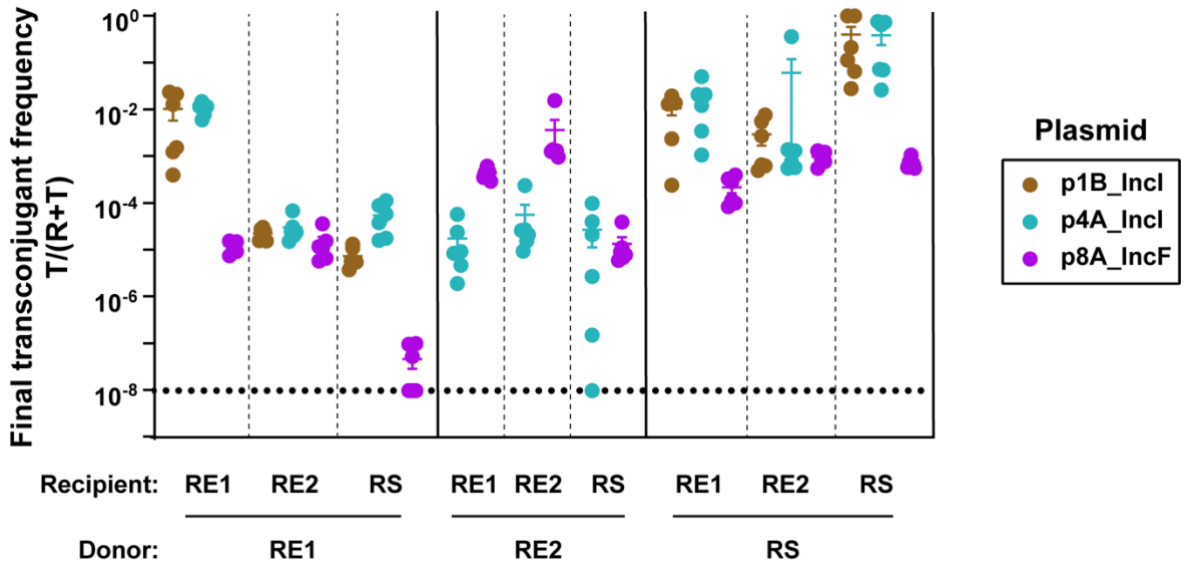

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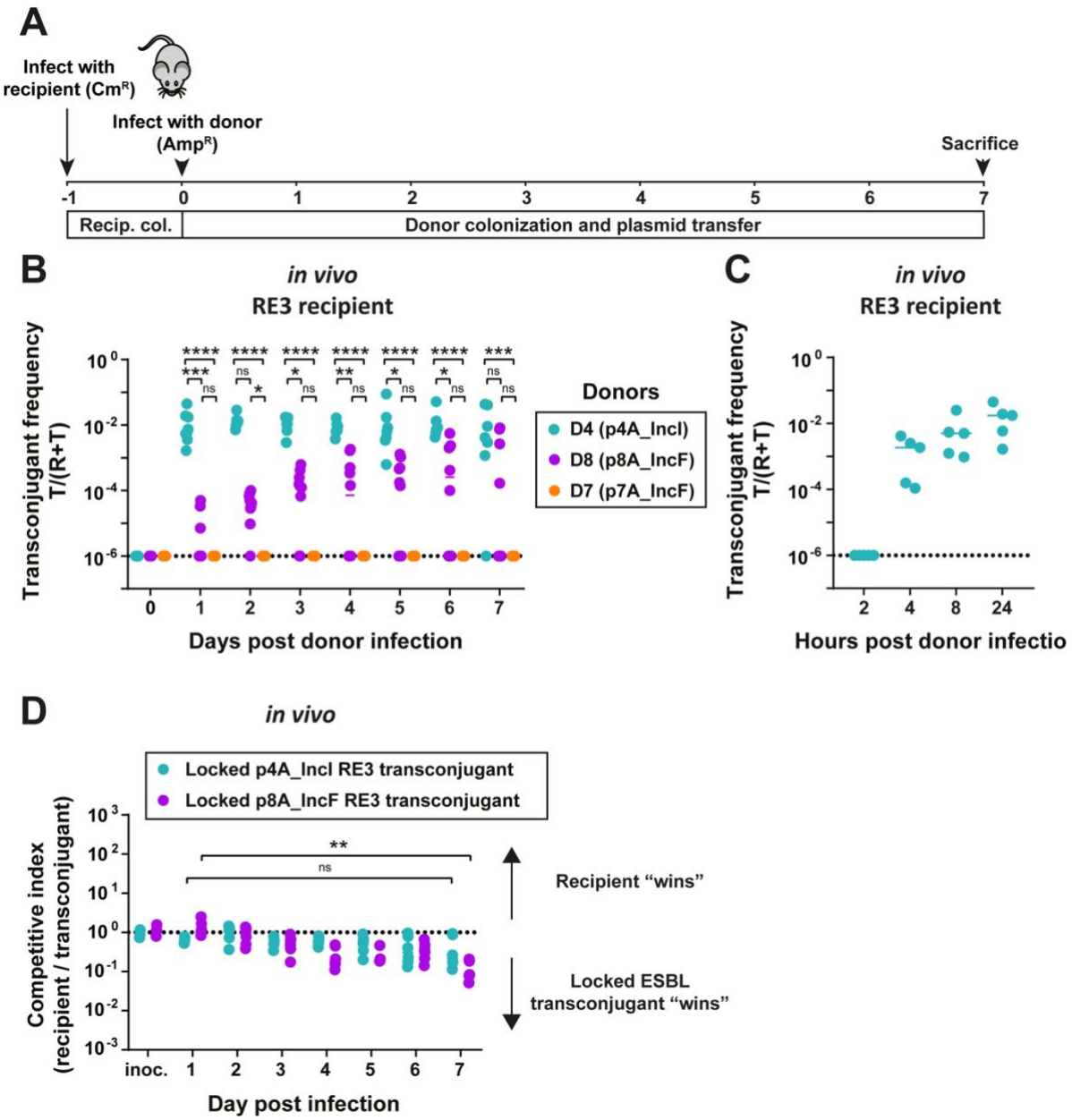
Fig 6.



994

Supporting information

S1 Fig. Plasmid replicons. Presence (blue) / absence (white) of plasmid replicons. The intensity of the shade of blue reflects the percent identity and coverage of the BLAST match to the listed genes.

S2 Fig. Resistance genes. Presence (blue) / absence (white) of resistance genes. The intensity of the shade of blue reflects the percent identity and coverage of the BLAST match to the listed genes.

S3 Fig. Type 6 secretion systems and virulence genes. Presence (blue) / absence (white) of T6SS (defined by the presence of more than one prodigal T6SS-encoding gene in an operon) and virulence genes (defined by BLAST match to the listed genes; the shades of blue indicate the percent identity and coverage). The only strain with a pathogenic virulence profile (Fig 1.3) is D4, which carries chromosomally encoded intimin genes (*eae*, *tir*) commonly associated with Enteropathogenic *E. coli*. All strains, except RE3 and D1, encode for a type 6 secretion system (T6SS).

S4 Fig. Absolute population sizes of donors, recipients, and transconjugants for 1st gen in vitro experiment. Conjugation experiments were performed with natural donor strains bearing the indicated plasmid, and recipients (A) RE1 (B) RE2 (C) RE3 and (D) RS. Dotted lines indicate the detection limit by selective plating. Beams are mean values ± SEM.

S5 Fig. Absolute population sizes of donors, recipients, and transconjugants for 2nd gen *in vitro* experiment. Conjugation experiments were performed with 2nd gen donor strains bearing the indicated plasmid, and recipients (A) RE1 (B) RE2 and (C) RS. Dotted line indicates detection limit by selective plating. Beams are mean values ± SEM.

S6 Fig. Absolute population sizes of donors, recipients, and transconjugants for *in vivo* experiments in Fig 6. Each population is indicated by a unique colored symbol (see figure

legend). (A, B, C) Fecal loads of donor, recipients, and transconjugants in Figure 6B determined by selective plating. Dotted line indicates the detection limit.

S7 Fig. Plasmid transfer from native versus secondary host. We marked D1 with the same Cm-resistance plasmid (pACYC184) as the *E. coli* recipients in the 1st gen conjugation experiment, to exclude pACYC184 having a major effect on transconjugants' ability to donate plasmids. Circles represent independent replicates (n= 5-6) and the beams are mean values ± SEM. The detection limit was at ~10^{-8}.

S8 Fig. Overview of co-transferring plasmids, showing the changing plasmid composition across both *in vitro* conjugation assays (1st gen is denoted as F_1, 2nd gen as F_2). Plasmids are indicated in italics. Because all the resident plasmids of recipients are listed in F_1, for simplicity in F_2 only changing resident plasmids are shown. Plasmids that transfer or get lost are highlighted (blue = ESBL-plasmids, green = changes originating from F_0, red = changes originating from F_1). Plasmids transferred across generations are only indicated with lines and not listed as resident plasmids. Skulls mark the loss of resident plasmids.

S9 Fig. Control for transconjugant growth rates. Recipient strains (n = 4) that were used in the 1st gen conjugation assay did not increase their growth rate compared to ancestral recipient strains. Thus, we can exclude that transconjugants have a growth advantage over ancestral recipients based on adaptation to laboratory conditions.

S1 Table. Strain overview. Overview of all strains used in this study, including their natural plasmid content and detected resistance genes. Replicon and resistance gene hits were recorded in this table if they had a coverage and percent identity of at least 70%.

S2 Table. Phenotypic resistance profile of donor strains. Minimum inhibitory concentration (µg/mL) measurements of ESBL donors used in this study.

1042 **S3 Table. Average transconjugant frequencies and average transfer rates in 1st gen *in vitro***

1043 **experiment.**

1044 **S4 Table. Conjugation on plate.**

1045 **S5 Table. Mutational changes that occurred during conjugation assays.**

1046 **S1 File. Prophages and plasmid cointegration.**

1047 **S2 File. Analyses to elucidate genomic effects on plasmid spread.**

1048 **S3 File. Growth rates of all strains in the 1st generation *in vitro* experiments.**