A culture-independent method for studying transfer of IncI1 plasmids from wild-type *Escherichia coli* in complex microbial communities

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Abstract

IncI1 plasmids play a central role in the transfer of antimicrobial resistance genes among *Enterobacteriaceae* in animals and humans. Knowledge on the dynamics of IncI1 plasmid transfer is limited, mainly due to lack of culture-independent methods that can quantify donor strain survival and plasmid transfer in complex microbial communities. The aim of this study was to develop a culture-independent method to study the dynamics of IncI1 plasmids transfer by fluorescence-activated cell sorting. We genetically modified three wild-type *Escherichia coli* of animal (n=2) and human (n=1) origin carrying $bla_{\text{CMY-2}}$ or $bla_{\text{CTX-M-1}}$ on two epidemic IncI1 plasmids (pST12 and pST7). Non-coding regions on the chromosome and on the IncI1 plasmid of each strain were tagged with mCherry (red) and GFPmut3 (green) fluorescent proteins, respectively, using lambda recombineering. A gene cassette expressing mCherry and $lacI^q$ was inserted into the chromosome, whereas the plasmid was marked with a GFPmut3 cassette with $LacI^q$ repressible promoter. Therefore, *gfpmut3* was repressed in donor strains but expressed in recipient strains acquiring the plasmids. We demonstrated that genetic engineering of the strains did not affect the growth rate and plasmid transfer-ability in filter and broth matings. A proof-of-concept experiment using the CoMiniGut, an *in vitro* model of the colon, proved the validity of our method for studying the survival of wild-type *E. coli* and horizontal transfer of IncI1 plasmids under different pH and oxygen conditions. The dual-labeling method by fluorescent proteins is useful to determine persistence of exogenous *E. coli* and transfer dynamics of IncI1 plasmids in microbial communities.

Keywords: horizontal gene transfer, antimicrobial resistance, *Enterobacteriaceae,* ESBL, $bla_{\text{CTX-M-1}}$, $bla_{\text{CMY-2}}$

1. Introduction

The spread of *Escherichia coli* producing extended-spectrum β-lactamases (ESBL) including CMY and CTX-M enzymes (Giske et al., 2009) is a threat to public health (Mathers et al., 2015). Bacteria that acquire these ESBLs become resistant to third generation cephalosporins, which are among the critically important antimicrobials in human medicine (WHO, 2017). Plasmids belonging to the Incompatibility group I1 (IncI1) have been associated with $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-1}}$ beta-lactamase genes in *E. coli* isolated from humans, animals and other sources worldwide (Accogli et al., 2013; Canton et al., 2012; EFSA. 2011). Nearly identical CMY-2 or CTX-M-1-encoding plasmids have been found in diverse *E. coli* isolated from humans and poultry, and various *in vitro* studies have shown that these plasmids can readily transfer between *E. coli* of human and animal origin (Touzain et al., 2018; Hansen, et al., 2016; De Been et al., 2014; Borjesson et al., 2013). Altogether, the data suggests that poultry meat is a likely source for zoonotic transmission of CMY-2 and CTX-M-1-encoding plasmids that may transfer via food to *E. coli* in the gut.

E. coli is part of the commensal microbiota in the intestine and causes opportunistic infections in both animals and humans, often as urinary tract infections in humans. Most *E. coli* infections are caused by strains that colonize the human intestinal tract (Nordstorm et al., 2013). The risk that exogenous *E. coli* of animal origin that transits the human intestinal tract can transfer ESBL-encoding plasmids to the indigenous microbiota remains poorly assessed. In order to determine the extent of this risk, there is need for an optimized tool to investigate this. The aim of this study was to develop a culture-independent method to track the dynamics of exogenous *E. coli* strains and their ESBL-encoding plasmids in complex microbiota. We have applied the dual-labeling technique with fluorescent reporter genes in wild-type *E. coli* strains with IncI1 plasmids encoding CMY-2 and CTX-M-1 beta-lactamases to construct model strains that can be used to study horizontal gene transfer *in situ*.

2. Materials and Methods

2.1. Strains and media

The strains used in the study were ESBL-producing *E. coli* isolated from poultry meat (strain code: 1061-1 and 6222) and human urinary tract infection (strain code: C20) (Hansen et al., 2016). Strains 1061-1 and C20 carried $bla_{\text{CMY-2}}$ on IncI1 plasmids belonging to sequence type (ST) 12 and displaying 99% nucleotide identity over 97% of the length (plasmid sequences deposited in the European Nucleotide Archives (ENA) under the study accession number PRJEB9625; Hansen et al., 2016), and p6222 carried $bla_{\text{CTX-M-1}}$ on IncI1 plasmid belonging to ST7 (deposited at ENA under sample accession number SAMEA4058419; unpublished).

Strains were cultured using Luria Bertani broth (LB) and Luria Bertani agar (LA) (Oxoid Ltd, Roskilde, Denmark). All reagents were purchased from Sigma-Aldrich, Copenhagen, Denmark unless stated otherwise. Complex colon (CC) media was prepared with the following recipe (g/L) in distilled water: starch, 5; peptone water, 5; tryptone, 5; yeast extract, 4.5; NaCl, 4.5; KCl, 4.5; mucin, 4; casein, 3; pectin, 2; xylan, 2; arabinogalactan, 2; $NaHCO_3$, 1.5; $MgSO_4.7H_2O$, 1.25; guar gum, 1; inulin, 1; cysteine.HCL, 0.8; KH_2PO_4, 0.5; K_2HPO, 0.5; bile salts, 0.4; $CaCl_2.6H_2O$, 0.15; $FeSO_4.7H_2O$, 0.005; haemin, 0.05; tween 80, 1 ml; vitamin K, 2 mg/L (Macfarlane et al., 1998).

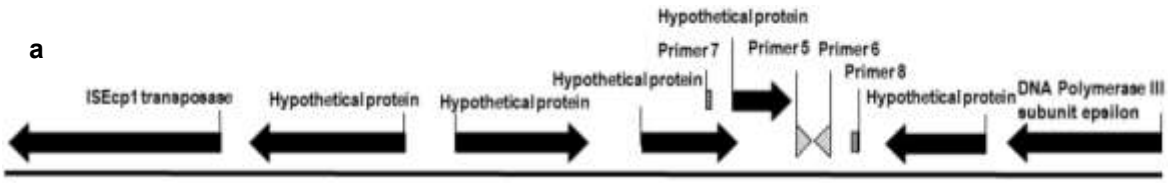
Antimicrobials were used in the following concentrations: cefotaxime (CTX) 1 mg/L, chloramphenicol (CHL) 15 mg/L, gentamicin (GEN) 10 mg/L, kanamycin (KAN) 50 mg/L, tetracycline (TET) 10 mg/L, trimethoprim (TMP) 10 mg/L.

2.2. Strain construction

The chromosome of each strain was tagged with mCherry red fluorescent protein from the plasmid pGRG36-GEN using lambda red recombination system (Klümper et al., 2015; Datsenko and Wanner, 2000). Previous experiments showed that the *E. coli* strains were heteroresistant to aminoglycosides (data not shown). Hence the plasmid pGRG36-GEN was modified to include a CHL resistance marker using In-fusion cloning kit (Clontech Takara, Saint Germaine en-laye, France) following manufacturer's instructions. The new plasmid is called pGRG36-CHL. The gene cassette *GEN^R-CHL^R-pLpp-mCherry-lacIq* encoding mCherry was amplified from plasmid pGRG36-CHL using Primers 1 and 2 (Table 1) carrying overhangs homologous to the *ybeM* pseudogene (Kjeldsen et al., 2015). The wild-type *E. coli* were made electrocompetent using a standard protocol (Sambrook and Russell, 2001). Each strain was transformed with a temperature-sensitive recombineering plasmid pKD46 with TET resistance marker (courtesy of Bimal Jana, University of Copenhagen, Denmark) and transformants were selected on LA supplemented with TET following overnight incubation at 30 °C. The transformants were made electrocompetent again using the same method as above. The subculture was grown at 30 °C keeping the selection for pKD46-TET plasmid and 7 mM of L-arabinose was added to induce the recombinase. The strains were transformed with ~1 µg purified PCR product of mCherry gene cassette and cells were recovered at 37 °C. The cultures were spun down at 5000 x g, and the pellet was suspended in 100 µl LB and spread on LA plates supplemented with CHL to select mCherry inserted *E. coli*. After overnight incubation at 37 °C, colonies were screened for red fluorescence by epifluorescence stereomicroscopy and confocal microscopy, and subcultured on CHL-supplemented LA at 37 °C. The clones that fluoresced red under microscope were confirmed by PCR and Sanger sequencing using primers 3 and 4 (Table 1).

2.3. Plasmid construction

The IncI1 plasmids were marked with a conditionally expressible green fluorescent protein (GFP) amplified from plasmid pENT-pA10403-gfp (Klümper et al., 2015). The GFP cassette carried a *lacI^q* repressible promoter upstream of the *gfpmut3* gene, thus GFP expression is repressed in the host strain which encodes the LacI^q repressor constitutively in addition to mCherry. The GFP cassette containing a KAN resistance marker was amplified using primers 5 and 6 for insertion in plasmids pC20 and p1061-1, and primers 9 and 10 for insertion in p6222 (Table 1). The GFP cassette was inserted in a non-coding region of the three plasmids (Fig. 1) using lambda recombineering as described above. The colonies were selected on LA supplemented with KAN and confirmed by PCR amplification of the plasmid region where the cassette was inserted using primers 7 and 8 for pC20 and p1061-1, and primers 11 and 12 for p6222 (Table 1).



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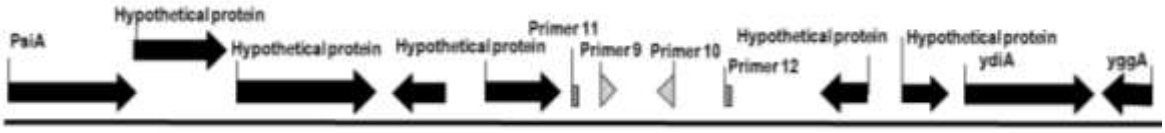

Fig.1 Region of plasmids (a) pC20, p1061-1 and (b) p6222 where the GFP cassette was inserted using lambda-red recombineering. (Figure not drawn to scale). Single column fitting image.

Table 1 Primers used for strain and plasmid construction. The bold sequence shows the overhang region of each primer that is homologous to the target site..

	Primer ID	Primer sequences (5′- 3′)
1.	Fwd YbeM/mCherry	**ACATCTGTGTGGGAAAAGAACGCTGAGATTTGTGCCTCGCA**GATCCCG GGTCAATAGCG
2.	Rev YbeM/mCherry	**GCGACGGTTGTTTAAGACGGGCAGTTGCGCGCGCACCTGGG**CTGAGC TTGGACTCCTG
3.	Fwd YbeM	ACATCTGTGTGGGAAAAG
4.	Rev YbeM	GCGACGGTTGTTTAAGAC
5.	Fwd pC20/1061-1	**GGTTTTACCTATTTT TGTGATTTTATTTCTGTATATTTG**CTGTGCAGGTCGTAAATCAC
6.	Rev pC20/1061-1	**GATAATTCTCATGTTTAGGGATGATAAATGTGACAAAAC**GTCAAGTCA GCGTAATGCTC
7.	Fwd pC20/1061-1 confirm	GAAGCACTTGATAACATC
8.	Rev pC20/1061-1 confirm	GAATGAGAACTTATCATGTC
9.	Fwd IncI1/6222 GFP	**GTGCCTGCGGGAGAATAAACCCCTGCACCGCCATACCCG**CTGTGCAGG TCGTAAATCAC
10	Rev IncI1/6222 GFP	**GCGTGGGCGTTGTGGTGTTTTTCTTCTTACTCACGGTTC**GTCAAGTCA GCGTAATGCTC
11	Fwd IncI1/6222 confirm	CTCTCTCCGCCGTTACTTC
12	Rev IncI1/6222 confirm	CAGATTTTCGGTGCTCTGG

2.4. Strain modification for mating experiments

Conjugation frequencies of plasmids pC20, p1061-1 and p6222 were assessed using the respective wild-type hosts and their three genetically modified counterparts as donors, by *in vitro* conjugation experiments using laboratory *E. coli* MG1655-CHL^R as a recipient. As the recipient strain used was CHL^R, the CHL^R gene along with GEN^R gene was flipped out from the donor strains making them GEN and CHL sensitive. As the resistance markers are flanked by Flp recombinase target (FRT) sites in pGRG36-CHL. The plasmid pFLP2-FRT-GEN (Hoang et al., 1998) was used to flip out the genes. Since the cassette already contained a GEN resistance gene, pFLP2-FRT-GEN was modified by replacing the GEN resistance marker with a TMP resistance marker using In-fusion cloning kit following manufacturer's protocol. The TMP resistance marker was amplified using

dfrA14 cassette as template (*dfrA14* cassette was obtained courtesy of Prof. Paul Langford and Dr. Janine T. Bossé, Imperial College London). The engineered strains were made electrocompetent using a standard protocol and transformed with pFLP2-FRT-TMP. Following incubation at 37 °C for 2 hours, strains were selected on TMP-supplemented LA plates. Colonies were re-streaked keeping selection for pFLP2 plasmid and screened for loss of CHL resistance. CHL-sensitive colonies that fluoresced red under microscope were confirmed by PCR for loss of CHL resistance marker and were selected on LA plates supplemented with CTX and 5% sucrose. The presence of sucrose in agar plates ensured that cells without pFLP2-FRT-TMP were selected due to the presence of *sacB* gene in pFLP2-FRT-TMP, which is lethal for bacteria in the presence of sucrose.

2.5. Mating experiments

Filter-mating and broth-mating (with conjugation cultures under shaking at 200 rpm and non-shaking conditions) experiments were performed with six biological replicates each. Donor and recipient strains were subcultured from an overnight culture by diluting 100-fold separately in LB at 37°C. When OD_{600} reached 0.4, donor and recipient cultures (100 µl) were transferred in 1:1 ratio to 1 mL of pre-warmed LB or onto 0.45 µM nitrocellulose membrane filter placed on LA. The conjugation cultures were incubated at 37 °C for 30 minutes. The broth mating cultures were diluted 10 and 10^2 fold and 100 µL of each dilution were plated on CTX- and CAM-containing plates. For filter-mating culture filters were placed in 1 mL of 0.9% saline and vortexed, the suspension was diluted 10^2 and 10^3 fold, and 100 µL of each dilution was plated on CTX- and CHL-supplemented plates. Colony forming units (CFU) counts were also performed for each donor and recipient strain on LA plates supplemented with CTX and LA plates supplemented with CHL, respectively. Colonies from selection plates were counted after overnight incubation at 37 °C and conjugation frequencies were calculated as transconjugants/donors.

2.6. Growth studies

Growth studies were performed using a Bioscreen reader C (Labsystems Bioscreen C plate reader) to determine if genetic modifications in the strains affected fitness. Four independent overnight cultures of the wild type strains and the fluorescent tagged strains were made. The overnight cultures were diluted to 0.05 OD_{600}. Using 96 wells microtiter plates, 200 µL of each diluted culture was added in four wells (four technical replicates). OD_{600} was recorded every 4 min at temperature 37 °C under shaking at medium speed. Growth curves were made using Microsoft Excel and generation time was calculated using standard equation: Generation time (min) = LN(2)/m, where m = gradient of the slope at exponential growth.

2.7. Cell collection and multiple-gated FACS of transconjugants

Flow cytometric detection of cells was performed using a FACSAria IIIu (Becton Dickinson Biosciences, San Jose, CA, USA). The following settings and voltages were used: forward scatter=505 V, side scatter=308 V and detectors for green (bandpass filter 530/30 nm) and red fluorescence (bandpass filter 610/20 nm) were set at 500 and 500 V, respectively. A 70 µm nozzle was used at a sheath fluid pressure of 70 psi. The BD FACSDiva software v.6.1.3 was used for both operating and analyzing results. FACS analysis was performed using a 488 nm (20 mW) laser connected to the green fluorescence detector at 515–545 nm and a 561 nm (50 mW) laser connected to the red fluorescence detector at 600–620 nm. Three gates

were defined in bivariate plots to sort for transconjugants. On the side scatter-A vs forward scatter-A plot, a gate for only particles of bacterial size was used. On the FITC-A vs side scatter -A plot, a gate was set that covered all green fluorescent particles, while using an additional non-red gate on the PE-Texas Red-A vs side scatter-A plot excluded all small autofluorescent particles from CC media or leaking donors to sort out only transconjugants. All samples were diluted in 0.9% NaCl to ~2000 counting events s^{-1}.

2.8. The CoMiniGut model

The *in vitro* model known as CoMiniGut was used to determine horizontal plasmid transfer under conditions that mimic the human colon environment (Wiese et al., 2018). The CoMiniGut is an *in vitro* system simulating human colonic passage with 5 units running in parallel. The pH in the CoMiniGut varied from 5.7 to 6.9 over 24 hours (Wiese et al. 2018). In the first 8 hours, pH increases from 5.7 to 6.0 in order to simulate the proximal colon. Then it further increases to 6.5 to mimic the conditions in the transverse colon and finally reaches 6.9 to reproduce the environment of the distal colon (to mimic the transit through the adult human colon.

2.9. CoMiniGut experiment

The CoMiniGut was used to generate a proof-of-concept that our genetically engineered strains can be used for studying horizontal plasmid transfer in the human colon environment. Overnight cultures of the modified CHL and GEN-susceptible C20 strain (donor for mating experiment) and laboratory MG1655-CHL^R (recipient) were grown in LB. Two inocula were used, 10^7 and 10^2 CFU/mL in 1:1 ratio of donor and recipient. In vessels 1 and 2, 10^7 CFU/mL of each C20 donor and MG1655-CHL recipient was added, and in vessels 3-5, 10^2 CFU/mL each donor and recipient was added. The experiment was performed once under oxic conditions and once under anoxic conditions to assess whether plasmid transfer was affected by oxygen levels. Samples were collected 30, 300 and 1440 minutes after inoculation and analyzed by FACS. The samples from the anaerobic experiment were exposed to oxygen at 4 °C for 3 hours under shaking at 200 rpm before being analyzed in FACS to facilitate aerobic fluorescence recovery (AFR) (Pinilla et al., 2018). Samples were also plated on CTX- and KAN-supplemented LA, which selected for transconjugants and donors both in addition to CTX- and CHL-supplemented LA plates, which selected for only transconjugants. CFU counts of donors and transconjugants were performed after overnight incubation at 37 °C. The dilutions plated were 10^{-3}, 10^{-4} and 10^{-7} for the aerobic experiment and 10^{-2}, 10^{-3} and 10^{-6} for the anaerobic experiments. Plasmid transfer ratio was calculated to compare the numbers from FACS analysis and plating experiment. Plasmid transfer ratio for FACS analysis was calculated by dividing transconjugants/donors and for CFU from plates it was calculated by dividing transconjugants/(donors + transconjugants).

2.10. Statistical analysis

All statistical methods were performed using the base package in R (R core team, 2018). Data normality was analyzed by the Shapiro-Wilk test using the shapiro.test function in R. Conjugation frequency was compared between each wild-type strain and its genetically modified counterpart under three culture conditions: filter mating (FM), broth mating with shaking (SH) and without shaking (non-SH) using the t.test function in R. The generation time was compared between wild-types and their genetically modified strain using the t.test function in R. In case of negative or inconsistent normality results, t-test results were confirmed by the non-parametric

Wilcoxon test using the wilcox.test function in R. Statistical significance was set at 0.05. The number of donors and transconjugants present at each time point (30, 300 and 1440 minutes) in the FACS and the plating experiments were compared between oxic and anoxic conditions for two starting inocula (10^2 and 10^7 CFU/mL) using the wilcox.test function. All plots were produced using the ggplot2 package in R (Wickham, 2009).

3. Results

In our genetic constructions, *gfpmut3* expression was repressed in the plasmid host wild-type strain but expressed upon transfer of the GFP-tagged IncI1 plasmid to *E. coli* MG1655 CHL^R. This approach allowed easy quantification and sorting of red donor and green transconjugant fluorescent cells by FACS as well as rapid detection by fluorescence microscopy (Fig. 2).

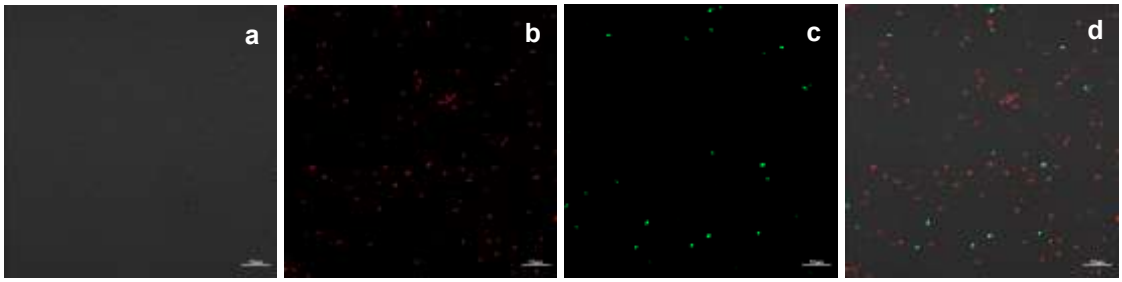

Fig.2 *E. coli* strain C20 (donor) and MG1655 CHL^R (recipient) under confocal laser scanning microscope. (a) Bacterial cells in the conjugation mix under bright field (b) donors fluoresce red because of mCherry tagging (c) transconjugants fluoresce green because of *gfp* expression (d) overlap image of (b) and (c) both donors and transconjugants are visible. Image was acquired using confocal microscope: CLSM (LSM800, Zeiss, Germany) and software Zen 2.3. Single-column fitting image.

Mating experiments were performed under different conditions to test if the genetic modifications had an effect on plasmid conjugal transfer frequency. Conjugation frequencies in liquid cultures with and without shaking were in the order of approximately 10^{-5} transconjugants per donor cell for pC20 and p1061-1 plasmids, and about 10-fold higher (10^{-4}) for p6222 without any significant differences between the wild-type and the genetically modified strains. The conjugation frequencies obtained by filter mating were approximately 10^{-4} transconjugants per donor for all wild-types and genetically modified strains. Conjugation frequencies are for each strain and culture conditions are shown in Fig. 3.

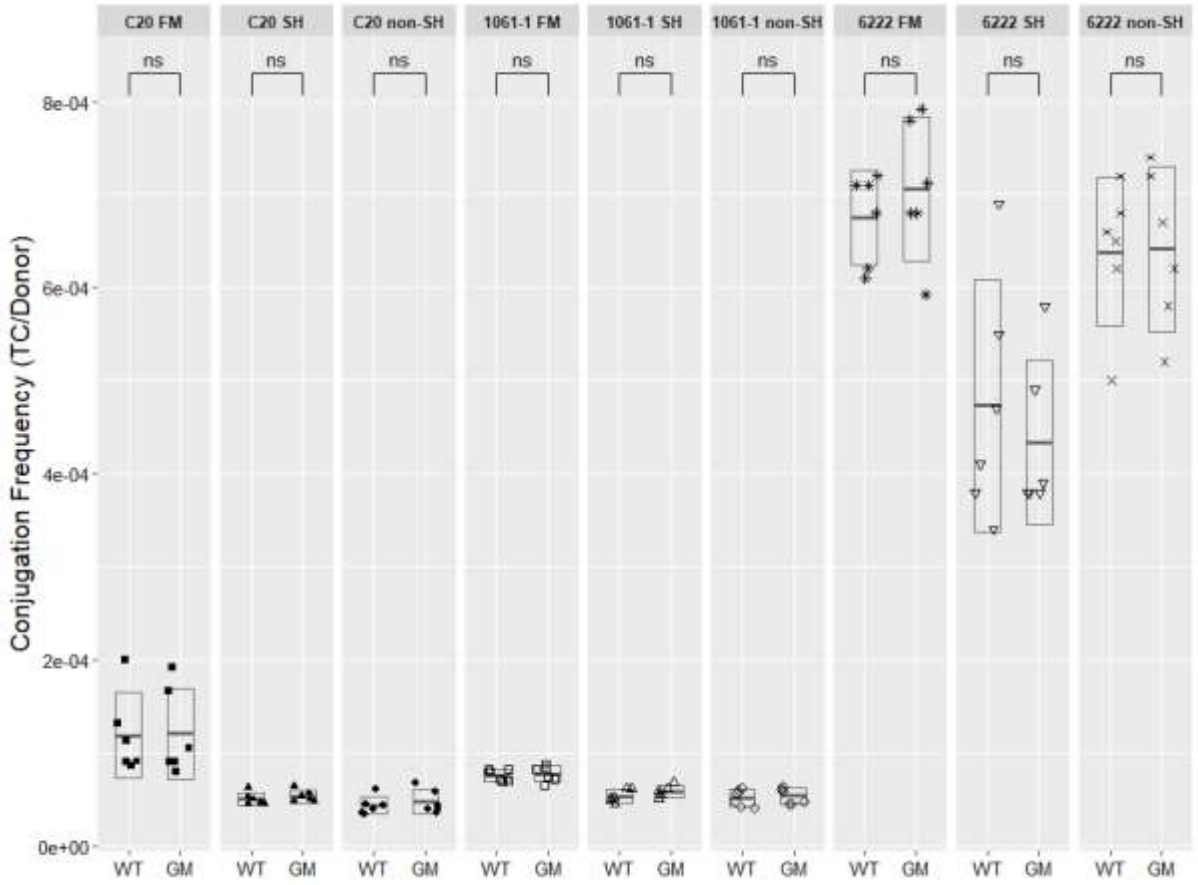

Fig.3 Conjugation frequency data of wild-type (WT) and genetically-modified (GM) populations of three *E. coli* strains (C20, 1061-1, 6222) under three culture conditions: filter mating (FM), broth mating w. shaking (SH), and broth mating w/o shaking (non-SH) with laboratory *E. coli* MG1655-CHL^{R} as recipient. The boxplots show the mean and 95% confidence interval. Top horizontal bars represent the lack of significant difference (ns) between the WT and GM groups based on Wilkinson test (significance set at 0.05). Conjugation frequencies were calculated by dividing transconjugants (TC) by donors. 2- column fitting image.

All data (grouped by strain and culture conditions) distributed normally based on the Shapiro-Wilk normality test, except for data corresponding to the genetically modified 6222 strain grown in shaking conditions. There were no significant differences in the frequency of conjugation of wild type and genetically modified populations in any of the strains under any of the culture conditions based on t-test (p-values 0.24-0.94). Due to the lack of normality in one of the groups, these results were re-tested and confirmed by the Wilcoxon test (p-values 0.26-0.93).

Growth studies were performed to assess possible effects of genetic manipulation on strain fitness. The generation times were 20, 21.5 and 20 minutes for wild-type strains C20, 1061-1and 6222 respectively and 19.8, 21 and 20 minutes for genetically modified strains C20, 1061-1and 6222 respectively (Fig. 4).

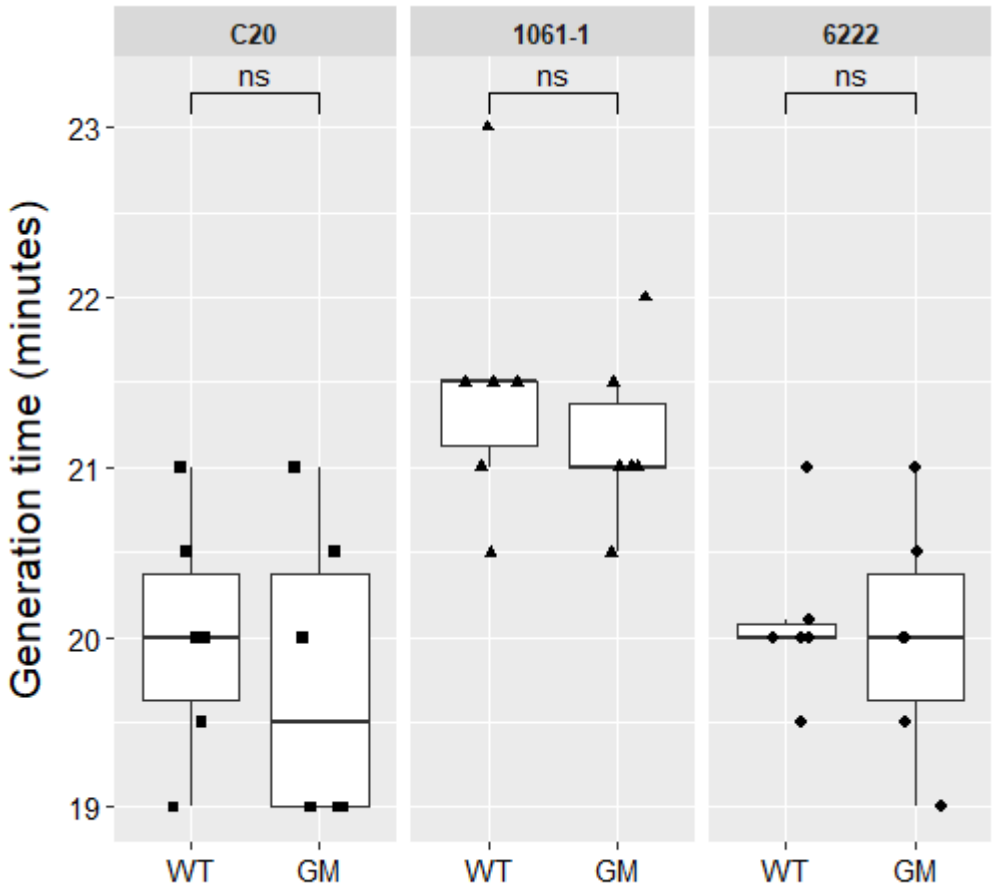

Fig.4 Generation time (in minutes) of wild type (WT) and genetically-modified (GM) populations of three *E. coli* strains (C20, 1061-1, 6222). The boxplots represent the mean and first and third quantiles. Top horizontal bars represent the lack of significant difference (ns) between the WT and GM groups based on t-test (significance set at 0.05). Single-column fitting image.

All data (grouped by strain) distributed normally based on the Shapiro-Wilk normality test. There were no significant differences in the generation time of wild-types and genetically modified populations of any of the strains based on t-test (strains C20, 1061 and 6222, p=0.6, p=0.42 and p=0.78 respectively).

In the CoMiniGut experiment, plasmid transfer occurred under both oxic and anoxic conditions (Fig. 5). At each time point (30, 300 and 1440 minutes), the numbers of donor cells detected by FACS were higher using the higher inoculum (10^7 CFU/mL) as compared to the lower inoculum (10^2 CFU/mL) regardless of oxygen levels. Fig. 5 shows the number of donors and transconjugants measured by FACS, during experiments (time points: 30, 300 and 1440 minutes) in oxic and anoxic conditions using two starting inocula (10^7 and 10^2 CFU/mL).

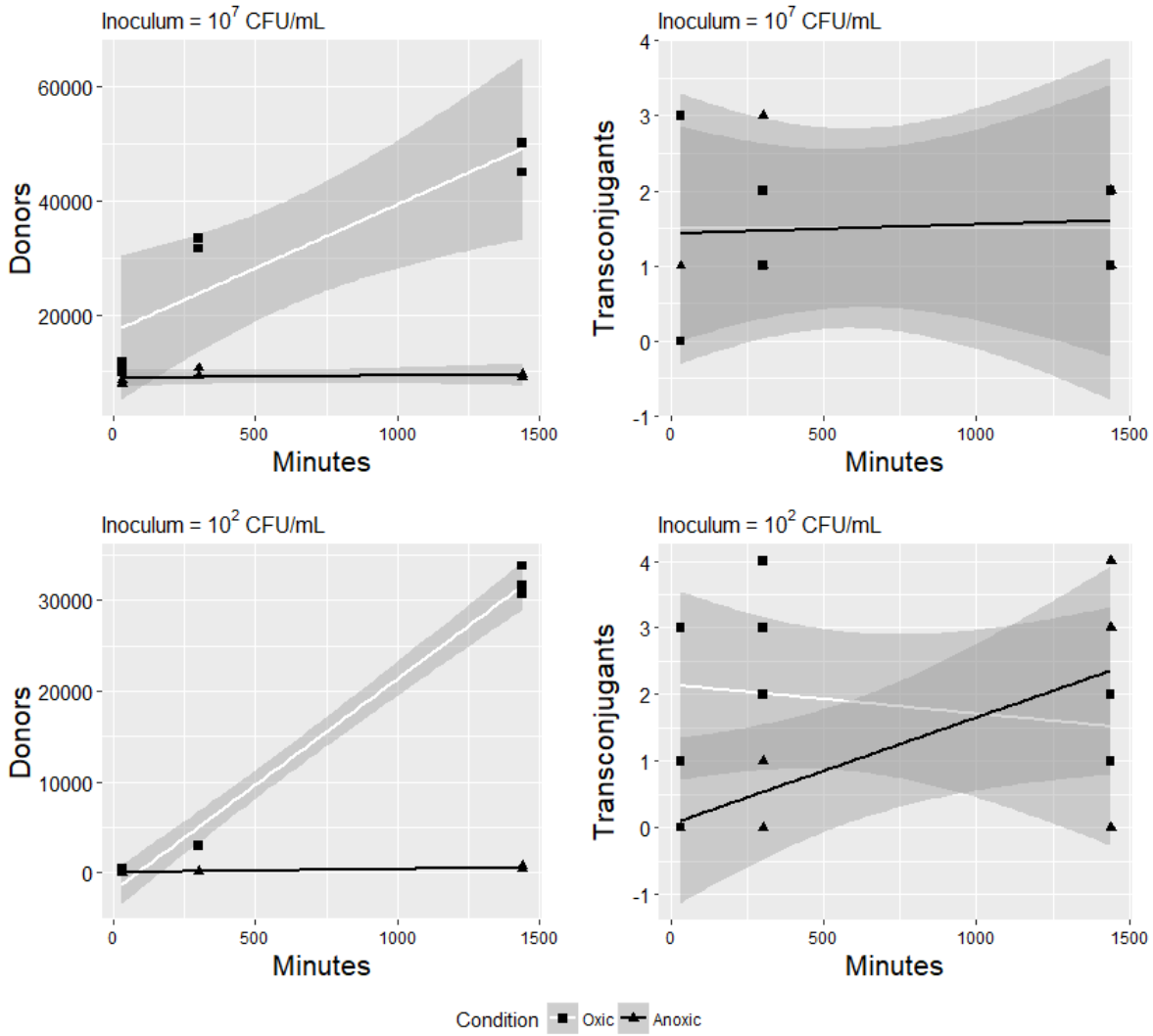

Fig.5 Number of donors and transconjugants measured by FACS (out of a total 10^5 cells) at three time points (30, 300 and 1440 minutes) under oxic and anoxic conditions, using two starting inoculum concentration (10^2 and 10^7 CFU/mL). Regression lines and confidence intervals (grey areas at 95%) have been added to the plots fitting a linear model. 2-column fitting image.

Shapiro-Wilk test was used to test normality of the grouped data (donors or transconjugants grouped by time point and starting inoculum). For 10^7 CFU/mL, out of 10^5 cells analyzed in FACS, the donors ranged between 10,000 cells at 30 minutes to 50,000 cells at 1440 minutes under oxic condition; for anoxic condition they ranged from 8000 cells at 30 minutes to 10,000 cells at 1440 minutes. The transconjugants were in the range of 0-3 out of 10^5 bacteria analyzed in FACS, for both oxic and anoxic condition. For 10^2 CFU/mL, out of 10^5 cells analyzed in FACS, the donors ranged between 400 cells at 30 minutes to 30,000 cells at 1440 minutes under oxic condition; for anoxic condition they ranged from 30 cells at 30 minutes to 700 cells at 1440 minutes. The transconjugants were in the range of 0-4 out of 10^5 bacteria analyzed in FACS, for both oxic and anoxic condition. Due to the presence of only two observations per group in the 10^7 inoculum, normality could not be testsed by the Shapiro-Wilk test. The difference observed in donors and transconjugants detected by FACS using the 10^7 inoculum , was not significant at each time point between the oxic and anoxic growth conditions

based on the Wilcoxon test. As for the 10^2 inoculum (with three observations per group), normality was confirmed in two out of six groups (number of donor cells at 30 min, and number of donor cells at 1440 min). In these two normally distributed cases, there was a significantly different number of donor cells under oxic conditions (at 30 and 1440 minutes, p=5.9x10-5 and p=0.00076 respectively).

The culture from CoMiniGut was also spread on agar plates to detect transconjugants and donors at the three time points of 30, 300, and 1440 minutes. We confirmed, by fluorescent microscopy, that the colonies yielded on CTX- and KAN-supplemented LA plates included both donor cells and transconjugant cells that had acquired the IncI1 plasmid, whereas colonies growing on CTX- and CHL- supplemented LA plates were only transconjugant cells. CFUs were counted for transconjugants and donors on selective plates. Donors and transconjugants per mL at each time point (30, 300 and 1440 minutes) were counted in the culture experiment from oxic and anoxic conditions using the two starting inocula (10^7 and 10^2 CFU/mL) (Fig. 6).

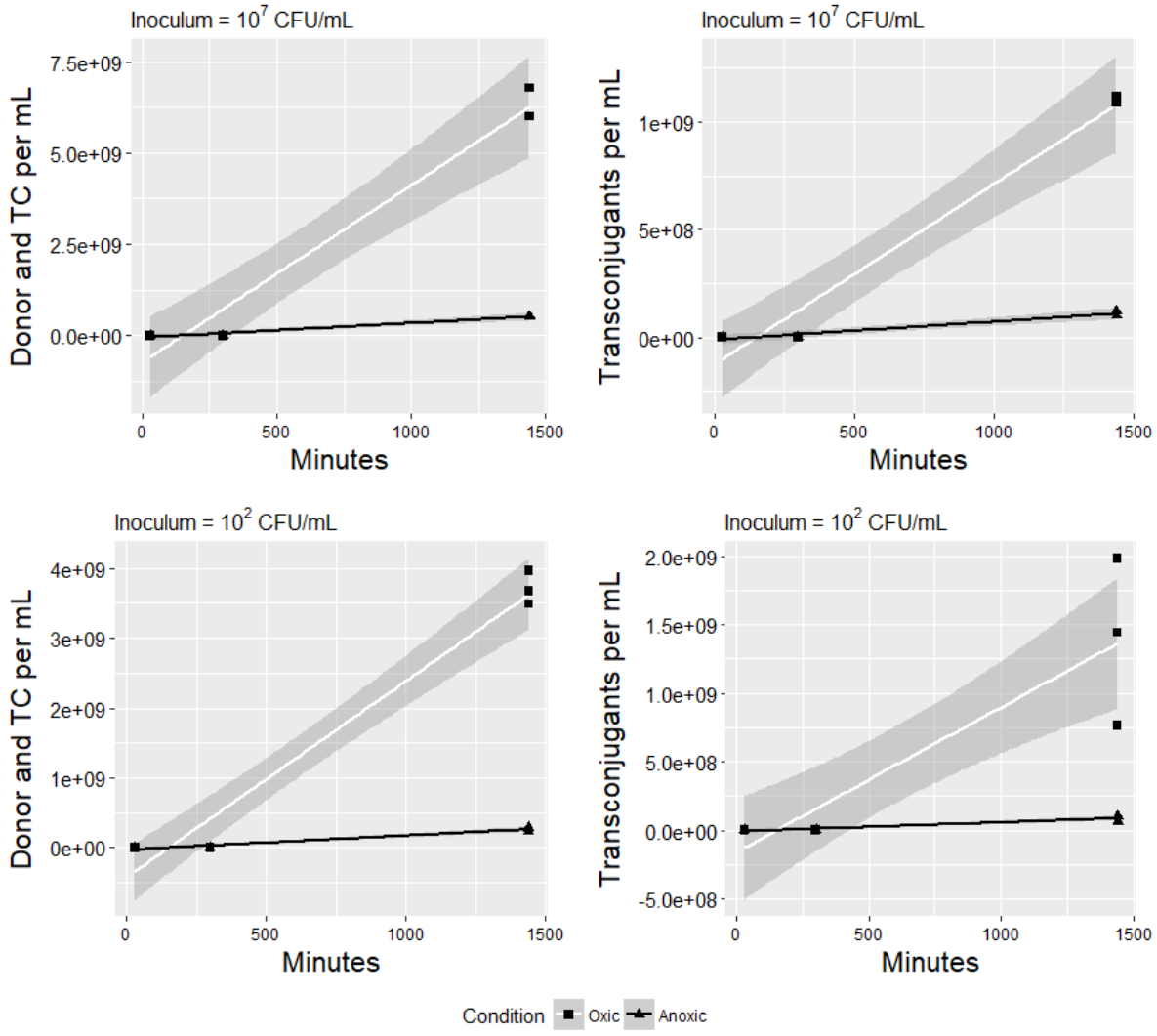

Fig.6 Number of donors and transconjugants (TC) per mL counted on selective plates from CoMiniGut experiments at three time points (30, 300 and 1440 minutes) under oxic and anoxic conditions, using two starting inoculum concentrations (10^2 and 10^7 CFU/mL). Regression lines and confidence intervals (grey areas at 95%) have been added to the plots fitting a linear model. 2-column fitting image.

For 10^7 CFU/mL, the donors ranged between 4 x 10^6 CFU/mL at 30 minutes to 7 x10^9 CFU/mL at 1440 minutes under oxic condition; for anoxic condition they ranged from 5 x 10^4 CFU/mL at 30 minutes to 5 x10^8 CFU/mL at 1440 minutes. The transconjugants were in the range of 1 x 10^4 - 10^9 CFU/mL for oxic and 1 x 10^3 - 10^8 CFU/mL under anoxic condition. For 10^2 CFU/mL, the donors ranged between 1x 10^5 CFU/mL at 30 minutes to 4 x 10^9 CFU/mL cells at 1440 minutes under oxic condition; for anoxic condition they ranged from 1 x 10^4 CFU/mL at 30 minutes to 3 x 10^8 CFU/mL at 1440 minutes. The transconjugants were in the range of 2 x 10^4 - 10^9 CFU/mL under oxic and 1 x 10^3 – 10^8 CFU/mL anoxic condition. Due to the presence of only two observations per group in the 10^7 inoculum, normality could not be tested by the Shapiro-Wilk test. There were no significant differences, using the 10^7 inoculum, in the number of donor or transconjugant cells at each time point between the oxic and anoxic growth conditions based on the Wilocxon test. As for the 10^2 inoculum (with three observations per group), normality was confirmed in all six groups (number of donors and TC/mL in all three time points, and number of TC/mL in all three time points). The number of donor and TC cells/mL were significantly different by t-test in all three time points (30, 300 and 1440 min, p=0.004, p=0.0005 and p=001 respectively). The number of TC/mL was significantly different at 30min (p=0.03), and borderline significant at 300 and 1440 min (p=0.06 for both).

The ratio of transconjugants over transconjugants plus donors obtained by bacterial counts was 1-4 logs higher than the transconjugant to donor ratio calculated by FACS (Table 2).

Table 2 Fold change comparison of plasmid transfer ratios from FACS analysis and plasmid transfer ratios obtained by CFU counts on selective plates. The fold change was calculated by plasmid transfer ratio from plates divided by plasmid transfer ratio from FACS. The plasmid transfer ratios on plates were higher than those detected by FACS at three time points (30, 300, 1440 minutes) with both inocula.

Inoculum	Plasmid transfer ratio (fold-change) at different time points		
	30’	300’	1440’
10^7 CFU/mL	0	4E+03	5E+03
	1E+02	2E+03	3E+03
10^2 CFU/mL	7E+01	9E+01	2E+04
	2E+01	5E+01	3E+03
	0	1E+02	3E+04
10^7 CFU/mL	2E+02	2E+03	1E+03
	3E+02	6E+02	2E+03
10^2 CFU/mL	0	3E+01	0
	0	0	4E+01
	0	3E+01	4E+01

4. Discussion

We have developed a model based on fluorescent reporter technology for studying transmission of IncI1 plasmids in complex microbial communities. The dual labeling technique used in this study was originally developed by Klümper et al. (2015) to study horizontal transfer of a GFP-tagged IncP plasmid harbored by a

laboratory strain in soil microbiota. We developed and validated this technique in wild type *E. coli* isolates from human and animal origin with different genetic background harboring IncI1 plasmids that carry ESBL genes of public health relevance. The principal advantage of this technique is that flow cytometry can be used not only to count cells and assess plasmid transfer frequencies, but also to sort transconjugants for subsequent 16S rRNA gene analysis, making it possible to identify the bacterial species that acquire the plasmids in a complex microbial community, including non-culturable species (Li et al., 2018; Sørensen et al., 2005). A multi-gated FACS approach based on size, green fluorescence and lack of red fluorescence allowed specific identification of transconjugant MG1655 cells in spite of their relative low abundance in the mating mixture. Moreover, the plasmid transfer occurred without the selection pressure of beta-lactam antibiotics, which has also been shown previously using a culture-based method with *in vitro* gut models (Smet et al., 2011; Card et al., 2017). We show that genetic modification of the wild-type ESBL-producing strains did not influence either plasmid transfer frequency or the growth rate as compared to their respective wild types. As such, the strains engineered in this study can be successfully used to mimic the fate of the wild type strains and their IncI1 plasmids in complex microbiota.

We performed a proof-of-concept experiment to demonstrate the validity of our method under *in vitro* conditions mimicking the human colon environment using media that has previously used to culture human colon bacteria in a chemostat model (Macfarlane et al., 1998). Plasmid transfer could be detected in both oxic and anoxic environment. However, the application of this dual-labeling technique in anoxic environments has its limitations since the fluorescent proteins used in this study do not exhibit their fluorescence properties in the absence of oxygen, because the final step in the maturation of their fluorophore forms strictly requires an oxygen environment. Nonetheless, even when anaerobically expressed, the possibility to recover their fluorescence has been described as AFR (Zhang et al., 2005). The fluorescence recovery used here has been explored in detail by Pinilla et al. who demonstrated that GFPmut3 showed completed AFR in less than 30 minutes while mCherry required 1-2 hours (Pinilla et al., 2018). We therefore included a 3-hour fluorescence recovery time before performing FACS analysis of the sample from the anaerobic experiment. Detection of both donors and transconjugants in oxygenated and oxygen depleted environments indicated that AFR was successful for both fluorescent proteins, even though it remains unknown whether fluorescence was recovered for 100% of donors and transconjugants.

The plasmid transfer frequencies determined by filter and broth mating experiments were in the same range as previously reported for other IncI1 plasmids (Carattoli, 2011). The transfer frequencies observed by standard mating experiments on LA media were 1×10^{-5} CFU/mL.

The culture samples collected during the CoMiniGut experiments were plated on antimicrobial selective agar to compare plasmid transfer frequency obtained by FACS and bacterial counts. The plasmid transfer ratio was 3 times higher on plates even though these were calculated as transconjugants divided by donors and transconjugants together, as opposed to plasmid transfer calculations in FACS where transconjugants are divided by donors. The results showed that transfer ratios were underestimated by FACS, possibly because the fluorescent proteins are not recovered after being exposed to low pH. The GFP fluorescent protein is sensitive to low pH and shows complete inhibition of fluorescence at pH 5 or lower. Previous studies have shown that low pH quenches the fluorescence of GFP (Pinilla et al., 2018; Hansen et al., 2011; Doherty et al., 2010). In contrast, mCherry is stable at low pH. In the CoMiniGut, during the first 8 hours, the pH is in the range of 5.7- 6.0 and

could be one of the reasons why fewer transconjugants were detected in FACS as compared to on plates. This highlights the importance of buffering the media in the slightly alkaline range when recovering cultures with these fluorescent markers. Alternatively a previous study by Smit and Van Elsas showed that plasmid transfer can be overestimated from plating experiments because transfer of plasmid also occurs on the selective plates (Smit and Van Elsas, 1990). It is also pertinent to mention that the plasmid transfer in FACS was determined without antibiotic selection whereas on plates there was selection of the plasmid due to LA supplemented with CTX- and KAN.

A disadvantage of this method is that whenever a new strain or plasmid has to be modified by fluorescent-labelling the validation steps have to be performed to ensure that the insertion of fluorescent markers on chromosome or plasmid does not cause any fitness disadvantage or affect the transfer ability of the plasmid. However the methods for validation are simple and have been well established as shown in our study.

The high-throughput, culture-independent method validated in this study can be used for studying persistence of exogenous *E. coli* and transfer dynamics of IncI1 plasmids encoding antimicrobial resistance in complex microbiota. The use of fluorescent markers to track plasmid and donor cells avoids the need for selection and cultivation steps to identify and isolate the bacterial species acquiring these plasmids. The strains and the plasmids used in our study belong to different STs associated with carriage of different ESBL genes, and can therefore be used to investigate the role played by strain and plasmid background in the spread of these clinically important resistance determinants. The chromosomal location has been used for genetic manipulation in at least four different *E. coli* (this study and Kjeldsen et al., 2015) thus the modifications in this area does not disturb the *E. coli* physiology as measured in standard growth and conjugation experiments.

We studied the plasmid maps to find non-coding regions and ensured that the genes flanking the inserted GFP cassette were not immediately adjacent to any known gene with function in the plasmid biology. We believe that, by following this approach, it is possible to find areas for labelling most of the well-described plasmids in *Enterobacteriaceae*.

Moreover, the genetic cassette for GFP has been placed in two different locations on the plasmids and validated to ensure that it has no influence compared to wild-type

strains. The use of the dual labeling technique in wild type strains opens up the possibility of studying horizontal gene transfer *in situ* by-passing culture-based methods that could limit the identification of potential plasmid recipients, thus providing data on the role of plasmid transfer in spread of antimicrobial resistance in complex microbiota.

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Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors

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