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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 $bla_{CMY\text{-}2}$ or $bla_{CTX\text{-}M\text{-}1}$ 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.

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 $bla_{CMY\text{-}2}$ and $bla_{CTX\text{-}M\text{-}1}$ beta-lactamase genes in *E. coli* isolated from humans, animals and other sources worldwide (Accogli et al., 2013; Cantón 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; Börjesson 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 (Nordstrom 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

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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_{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 $bla_{CTX-M-1}$ 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 pENTpA10403-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).

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

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′)
Fwd YbeM/mCherry	**ACATCTGTGTGGGAAAAGAACGCTGAGATTTGTGCCTCGCA**GATCCCGGGTCAATAGCG
Rev YbeM/mCherry	**GCGACGGTTGTTTAAGACGGGCAGTTGCGCGCGCACCTGGG**CTGAGCTTGGACTCCTG
Fwd YbeM	ACATCTGTGTGGGAAAAG
Rev YbeM	GCGACGGTTGTTTAAGAC
Fwd pC20/1061-1	**GGTTTTACCTATTTTTGTGATTTTATTTCTGTATATTTG**CTGTGCAGGTCGTAAATCAC
Rev pC20/1061-1	**GATAATTCTCATGTTTAGGGATGATAAATGTGACAAAAC**GTCAAGTCAGCGTAATGCTC
Fwd pC20/1061-1 confirm	GAAGCACTTGATAACATC
Rev pC20/1061-1 confirm	GAATGAGAACTTATCATGTC
Fwd IncI1/6222 GFP	**GTGCCTGCGGGAGAATAAACCCCTGCACCGCCATACCCG**CTGTGCAGGTCGTAAATCAC
Rev IncI1/6222 GFP	**GCGTGGGCGTTGTGGTGTTTTTCTTCTTACTCACGGTTC**GTCAAGTCAGCGTAATGCTC
Fwd IncI1/6222 confirm	CTCTCTCCGCCGTTACTTC
Rev IncI1/6222 confirm	CAGATTTTCGGTGCTCTGG