RESEARCH ARTICLE

Monitoring horizontal antibiotic resistance gene transfer in a colonic fermentation model

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Abstract

The human microbiota is suggested to be a reservoir of antibiotic resistance (ABR) genes, which are exchangeable between transient colonizers and residing bacteria. In this study, the transfer of ABR genes from *Enterococcus faecalis* to *Listeria monocytogenes* and to commensal bacteria of the human gut microbiota was demonstrated in a colonic fermentation model. In the first fermentation, an *E. faecalis* donor harboring the marked 50-kb conjugative plasmid pRE25* and a chromosomal marker was co-immobilized with *L. monocytogenes* and infant feces. In this complex environment, the transfer of pRE25* to *L. monocytogenes* was observed. In a second fermentation, only the *E. faecalis* donor and feces were co-immobilized. Enumeration of pRE25* and the donor strain by quantitative PCR revealed an increasing ratio of pRE25* to the donor throughout the 16-day fermentation, indicating the transfer of pRE25*. An *Enterococcus avium* transconjugant was isolated, demonstrating that ABR gene transfer to gut commensals occurred. Moreover, pRE25* was still functional in both the *E. avium* and the *L. monocytogenes* transconjugant and transmittable to other genera in filter mating experiments. Our study reveals that the transfer of a multiresistance plasmid to commensal bacteria in the presence of competing fecal microbiota occurs in a colonic model, suggesting that commensal bacteria contribute to the increasing prevalence of antibiotic-resistant bacteria.

Introduction

The human gut microbiota is a complex ecosystem colonized by approximately 10^{14} bacterial cells, with *Bacteroides, Eubacterium, Bifidobacterium, Ruminococcus* and *Clostridium* as the predominant genera (Kurokawa *et al.*, 2007). The gut microbiota is supposed to have numerous beneficial effects on the host, for example production of nutrients, activation of the immune system and defense against pathogenic bacteria (Kurokawa *et al.*, 2007; Zhao, 2010). On the other hand, the huge diversity of antibiotic resistance (ABR) genes detected in the human gut microbiome suggests that antibiotic-resistant bacteria in the gastrointestinal tract (GIT) function as a reservoir of ABR genes (Salyers *et al.*, 2007; Sommer *et al.*, 2009). Moreover, there are serious concerns that ABR genes can be transmitted between transient and permanent colonizers of the GIT, because the highly dense microbial population favors horizontal gene transfer (HGT) via transposons and conjugative plasmids (Levy & Marshall, 2004; Kazimierczak & Scott, 2007).

A subdominant, but frequently encountered genus in the human gut is *Enterococcus*, occurring at 10^2–10^8 CFU g^{-1} of digestive content (Ogier & Serror, 2008). Enterococci exhibit low-level, usually nontransferable, intrinsic resistances to several antibiotics, for example β-lactams, aminoglycosides, clindamycin, lincomycin, ciprofloxacin and glycopeptides (Kak & Chow, 2002). Moreover, enterococci possess a remarkable ability to acquire and transmit extrinsic ABR genes via mobile genetic elements (Huycke *et al.*, 1998), and transferable ABR genes combined with the widespread use of antibiotics in human medicine and animal husbandry contribute to the high prevalence of antibiotic-resistant enterococci (ARE) worldwide (Levy & Marshall, 2004; Arias *et al.*, 2010; Palmer *et al.*, 2010). ARE are a major concern, because food-derived ARE transiently or permanently colonizing the human GIT might transfer ABR genes to the gut microbiota (Berchieri, 1999; Sørensen *et al.*, 2001; Lund *et al.*, 2002; Lester *et al.*, 2006). ARE typically carry resistances to all classes of antimicrobials and have been isolated

FEMS MICROBIOLOGY ECOLOGY

from a number of food products like dairy products, ready-to-eat products and processed meat (Teuber *et al.*, 1999; Flórez *et al.*, 2005). An example is the dry sausage-associated *Enterococcus faecalis* RE25, which harbors the conjugative multiresistance plasmid pRE25, a 50-kb plasmid belonging to the incompatibility group Inc18 of streptococcal plasmids. These plasmids can be found in a broad range of other Gram-positive microorganisms and require high cell densities for transfer (Schwarz *et al.*, 2001; Grohmann *et al.*, 2003). Because of their high abundance in the human microbiota, their frequent resistance to antibiotics and their capability to transfer these resistances, enterococci are considered to play a pivotal role in the spread of ABR genes in the human GIT via HGT (Arias *et al.*, 2010).

The demonstration of ABR gene transfer to commensal bacteria in the human gut is challenging due to the high prevalence of ABR genes in the gut microbiota itself and the complicated selection of transconjugants against the microbial background. Cultivation-independent approaches such as fluorescence-activated cell sorting have successfully been applied to demonstrate conjugative plasmid transfer from Gram-negative donors (Sørensen *et al.*, 2003; Musovic *et al.*, 2006). However, so far, most studies on HGT in complex colonic environments were performed using cultivation-dependent approaches with defined recipients and gnotobiotic or streptomycin-treated animals (Licht *et al.*, 2002, 2003; Moubareck *et al.*, 2003, 2007; Mater *et al.*, 2005, 2008; Jacobsen *et al.*, 2007; Feld *et al.*, 2008; Boguslawska *et al.*, 2009). In this study, we elucidated ABR gene transfer to commensal bacteria in the complex gut environment using a new molecular tool: the ABR donor strain *E. faecalis* CG110/*gfp*/pRE25* (Haug *et al.*, 2010). Strain CG110/*gfp*/pRE25* is chromosomally marked with a *gfp* gene and harbors the plasmid pRE25*, a functional pRE25 derivative that is marked with a specific sequence downstream of the erythromycin resistance gene. These two genetic markers allow the quantification and distinction of donor and transconjugants in complex environments (Haug *et al.*, 2010).

To investigate HGT from *E. faecalis* CG110/*gfp*/pRE25* to the commensal human microbiota, a continuous colonic fermentation with immobilized infant feces (Cinquin *et al.*, 2004) was performed. This *in vitro* colonic model preserves the major bacterial populations from feces and closely mimics the highly diverse and dense colonic ecosystem (Cinquin *et al.*, 2004, 2006; Le Blay *et al.*, 2010), whereas the model also enables experiments with pathogenic and multiresistant bacteria without ethical restrictions (Le Blay *et al.*, 2009). A first colonic fermentation was performed to assess the suitability of *E. faecalis* CG110/*gfp*/pRE25* as a donor strain in a continuous colonic fermentation. A fresh fecal sample was co-immobilized with *E. faecalis* CG110/*gfp*/pRE25* and the foodborne pathogen *Listeria monocytogenes* as a recipient. In the second colonic fermentation, infant feces were co-immobilized with only *E. faecalis* CG110/*gfp*/pRE25*. HGT was demonstrated in both colonic fermentations, suggesting that HGT events can also occur in the human GIT despite the presence of competing gut microbiota. Our study therefore elaborates the role of the human gut microbiota as a reservoir of ABR determinants and the spread of ABR-resistant microorganisms.

Materials and methods

Bacterial strains, media and chemicals

The bacterial strains and plasmids used in this study are listed in Table 1. *Enterococcus* spp. and *Listeria* spp. were routinely grown aerobically in brain–heart infusion (BHI) broth (Labo-Life Sàrl, Pully, Switzerland) at 37 °C. Oligonucleotides are listed in Table 2 and were synthesized by Microsynth (Balgach, Switzerland). Antibiotics were obtained from Sigma-Aldrich (Buchs, Switzerland) and applied at the following concentrations: chloramphenicol 10 μg mL^{-1}, erythromycin 10 μg mL^{-1}, kanamycin 200 μg mL^{-1} and tetracycline 10 μg mL^{-1}.

Collection of fecal samples and the immobilization procedure

Fecal samples for the colonic fermentations were collected from infants (aged 4 months in fermentation 1 and 7 months in fermentation 2) never treated with antibiotics. Fresh samples were transported under anaerobiosis and immediately transferred into an anaerobic chamber under a nitrogen atmosphere containing 5% hydrogen (Coy Laboratories, Ann Arber, MI). The samples were diluted to a final concentration of 20% w/v in sterile peptone water (0.1% w/v, pH 7.0) prereduced with 0.6 g L^{-1} L-cysteine hydrochloride (Sigma-Aldrich) and the suspension was homogenized by vortexing at full speed for 2 min. Bacterial strains were prepared as follows: 2 mL of a fresh overnight culture were washed once with dilution solution (0.85% NaCl, 0.1% peptone from casein, pH 8.0) and resuspended in 2 mL of prereduced peptone water. The polymer solution was then inoculated with 10 mL of fecal slurries and 1 mL (approximately 10^9 CFU) of the tested bacterial cultures. Cell numbers were determined by plate counting. Immobilization of feces and bacterial cultures in gellan–xanthan beads, 1–2 mm in diameter, was performed as described previously (Cinquin *et al.*, 2004). Hardened beads (60 mL) were then transferred aseptically to a sterile 450-mL round-bottom fermenter (Sixfors, Infors, Bottmingen, Switzerland) containing 140 mL of a chyme-mimicking medium as described previously (Le Blay *et al.*, 2009).

In fermentation 1, the suitability of *E. faecalis* CG110/*gfp*/pRE25* as a donor in a colonic fermentation was

Table 1. Strains and plasmids used in this study

Material		Relevant features	Source
Strains			
Enterococcus faecalis	CG110/*gfp*/pRE25*	CG110/*gfp* (Scott *et al*., 2000) derivative harboring pRE25*; Cm^R, Em^R, Fus^R, Gen^R, Kan^R, Neo^R, Rif^R, Str^R, Tet^R	Haug *et al*. (2010)
Enterococcus faecalis	JH2-2	Derivative of the clinical isolate JH2, a recipient for filter mating; Fus^R, Rif^R	Jacob & Hobbs (1974)
Enterococcus avium	BT1/pRE25*	Transconjugant from colonic fermentation, gut isolate harboring pRE25*; Cm^R, Em^R, Gen^R, Kan^R, Neo^R, Str^R, Tet^R	This study
Listeria monocytogenes	10403S	Serovar 1/2a, derivative of the clinical isolate 10403, a recipient in fermentation 1 and in filter mating; Str^R	Bishop & Hinrichs (1987)
Listeria monocytogenes	10403S/pRE25*	Transconjugant from colonic fermentation, 10403S derivative harboring pRE25*; Cm^R, Em^R, Gen^R, Kan^R, Neo^R, Str^R, Tet^R	This study
Listeria monocytogenes	LM15	Serovar 1/2a, food isolate, a recipient for filter mating; Tet^R	Veterinary Hospital, Zurich
Listeria innocua	L19	Plasmid-free, a recipient for filter mating	Schwarz *et al*. (2001)
Pediococcus acidilactici	UVA-1	Fecal isolate, pediocin PA-1 producer	Von Ah (2006)
Plasmid			
pRE25*		52.9-kb; Cm^R, Em^R, Gen^R, Kan^R, Neo^R, Str^R, Tet^R, pRE25 derivative harboring a 34-bp random sequence spliced by *tet*(M)	Haug *et al*. (2010)

Cm^R, chloramphenicol resistant; Em^R, erythromycin resistant; Fus^R, fusidic acid resistant; Gen^R, gentamicin resistant; Kan^R, kanamycin resistant; Neo^R, neomycin resistant; Rif^R, rifampicin resistant; Str^R, streptomycin resistant; Tet^R, tetracycline resistant.

investigated using a defined recipient. Therefore, feces were co-immobilized with both *E. faecalis* CG110/*gfp*/pRE25* and the recipient strain *L. monocytogenes* 10403S (Table 1). In fermentation 2, gene transfer of pRE25* from *E. faecalis* to the commensal infant gut microbiota was investigated, and feces were co-immobilized only with the *E. faecalis* CG110/*gfp*/pRE25* donor.

Continuous intestinal fermentations

A single-stage continuous fermenter system was used to simulate the microbial ecosystem of the proximal infant colon (Cinquin *et al*., 2004). The microbiota of the proximal colon exhibits a very high cell density and physiological activity, which is assumed to influence the efficiency of HGT (Fanaro *et al*., 2003; Licht & Wilcks, 2006). A serial batch culture was performed for 72 h (fermentation 1) or 48 h (fermentation 2) until the beads were completely colonized. The nutritive medium was aseptically exchanged every 12 h and anaerobic conditions were maintained by continuously flushing the headspace with CO_2. Thereafter, the fermenter was connected to a stirred feedstock flask containing a sterile nutritive chyme medium (4 °C) purged with CO_2 to start the continuous fermentation. Fermentation 1 was continuously performed for 8 days, whereas fermentation 2 was run for 16 days. The medium flow rate was set at 40 mL h^{-1}, resulting in a retention time of 5 h. The pH was maintained at 5.7 using NaOH (5 N). Temperature (37 °C) and stirring conditions (120 r.p.m.) were automatically controlled during batch and continuous fermentation. To assess the stability of the system, the metabolic profile of the effluent was determined daily by HPLC analysis.

Sampling, DNA extraction and real-time quantitative PCR (qPCR)

During the continuous fermentations, effluent samples for DNA extraction were collected every 24 h from the fermenters as follows: 2 mL of effluent was centrifuged (10 000 ***g***, 5 min, 4 °C) and the pellet was stored at −20 °C until further use. DNA of effluent samples was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals Europe, Illkirch, France) according to the manufacturer's instructions. The *gfp* donor marker and pRE25* were quantified every 24 h, whereas the main bacterial populations were quantified every 48 h. Real-time qPCR using the SYBR Green and the TaqMan-based method and the determination of gene copy numbers were performed as described previously (Haug *et al*., 2010). Primer specificity was confirmed by performing a dissociation curve in the range of 60–95 °C. The primers and probes for the quantification of the total bacteria and the main bacterial populations found in the infant large intestine are listed in Table 2. In fermentation 2, the marked plasmid pRE25* (primer pair pRE25*_F2/R2 and Taqman probe pRE25*_TMP, Table 2) as well as the *gfp* donor marker (primer pair gfp_F/R, Table 2) were also quantified by qPCR. Copy numbers of pRE25* and the *gfp* gene per milliliter of effluent were normalized relative to the corresponding value at day 1 of the continuous fermentation. An increase in this ratio would thus

Table 2. Oligonucleotides used in this study

Primer/probe	Target	Purpose	Sequence (5′ → 3′)	$T_{Annealing}$ (°C)	Reference(s)
eub338F eub518R	Total bacteria	qPCR	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	60	Guo *et al*. (2008)
xfp_fw xfp_rv	*Bifidobacterium* spp.	qPCR	ATCTTCGGACCBGAYGAGAC CGATVACGTGVACGAAGGAC	60	Cleusix *et al*. (2010)
bac303F bfr-Fmrev	*Bacteroides* spp.	qPCR	GAAGGTCCCCCACATTG CGCKACTTGGCTGGTTCAG	60	Bartosch *et al*. (2004), Ramirez-Farias *et al*. (2009)
Firm934F Firm1060R	*Firmicutes*	qPCR	GGAGYATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC	60	Guo *et al*. (2008)
RrecF Rrec630mR	*Roseburia* spp./ *Eubacterium rectale*	qPCR	GCGGTRCGGCAAGTCTGA CCTCCGACACTCTAGTMCGAC	60	Ramirez-Farias *et al*. (2009)
Eco1457F Eco1652R	*Enterobacteriaceae*	qPCR	CATTGACGTTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC	60	Bartosch *et al*. (2004)
F_Lacto 05 R_Lacto 04	*Lactobacillus/ Leuconostoc/Pediococcus* spp.	qPCR	AGCAGTAGGGAATCTTCCA CGCCACTGGTGTTCYTCCATATA	60	Furet *et al*. (2009)
hly_fw hly_rv	*Listeria monocytogenes*	qPCR/ normal PCR	GGGAAATCTGTCTCAGGTG CGATGATTTGAACTTCATCTTTTGC	60	Guilbaud *et al*. (2005)
linF2 linR2	*Listeria innocua*	normal PCR	TTGCTACTGAAGAAAAAGCA TCTGTTTTTGCTTCTGTAGC	60	Huang *et al*. (2007)
tufA_fw tufA_rv tufA_TMP	*Enterococcus faecalis*	qPCR	GACAAACCATTCATGATGCCAG CGTCACCAACGCGAACTTCA FAM-TTCTCAATYACTGGWCGTGGTACTGTTGC-TAMRA	60	Ke *et al*. (1999)
FL1 FL2	*Enterococcus faecalis*	normal PCR	ACTTATGTGACTAACTTAACC TAATGGTGAATCTTGGTTTGG	55	Jackson *et al*. (2004)
gfp_F gfp_R	*gfp*	qPCR/ normal PCR	TGGAAGCGTTCAATTAGCAGA GGCAGATTGTGTGGACAGGT	60	This study
pRE25*_F pRE25*_R	Marker sequence of pRE25*	normal PCR	TCATCAAGCAATGAAACACG GCATATTTGTAAAGGAATCTCCA	54	This study
pRE25*_F2 pRE25*_R2 pRE25*_TMP	Marker sequence of pRE25*	qPCR	GTACCATTACTTATGAGCAAGTATTGTC CTATAATCTTCCAATTACTCCCGTC FAM-GGAAATAATTCTATTCGGAATTCGATCGGATC-TAMRA	60	This study
bak11w bak4	16S rRNA genes	normal PCR	AGGAGGTGATCCARCCGCA AGTTTGATCMTGGCTCAG	58	Dasen *et al*. (1998)

FAM, reporter dye [5(6)-carboxyfluorescein]; TAMRA, nonfluorescent quencher (carboxytetramethylrhodamine).

indicate lateral transfer of pRE25* from its *E. faecalis* host to commensal bacteria.

Metabolite analysis

Acetate, formate, propionate, butyrate, isovalerate, isobutyrate and lactate in cell-free effluent supernatants were determined by HPLC using an Aminex HPX-87 H column as described previously (Cleusix *et al*., 2008). Analyses were performed in duplicate.

Selection of transconjugants from colonic fermentations

In the continuous stage of fermentation 1, *L. monocytogenes* 10403S transconjugants harboring pRE25* (fermentation 1) were selected by plating effluent samples daily onto a Palcam agar base containing a Palcam selective supplement (Oxoid) and erythromycin (10 μg mL^{-1}). Plates were incubated at 37 °C for 48 h. To isolate putative transconjugants harboring pRE25* from fermentation 2, effluent samples (10 mL) were taken on days 5, 10 and 15. Transconjugants were selected by plating appropriate dilutions either directly or after two 8–16-h enrichment culturing steps according to Table 3 onto selective media for *Enterobacteriaceae*, lactobacilli and bifidobacteria, staphylococci, anaerobic Gram-negative bacteria and anaerobic Gram-positive bacteria (Table 3). All selective media contained 10 μg mL^{-1} chloramphenicol (Cm_{10}), 10 μg mL^{-1} erythromycin (Ery_{10}) and 10 μg mL^{-1} tetracycline (Tet_{10}) to select bacteria harboring pRE25*. To extract DNA, cell material of a single colony was smeared into a

Table 3. Selective media for the detection of putative transconjugants in fermentation 2

Organisms	Enrichment broth	Media for plating	Incubation
Enterobacteriaceae	–	VRBD* (Mossel *et al.*, 1962)	Aerobic
Lactobacillus spp.	–	LAMVAB* (Hartemink *et al.*, 1997)	Anaerobic jar†
Bifidobacterium spp.	–	Beerens* (Beerens, 1991)	Anaerobic chamber‡
Anaerobic Gram-negative bacteria	YCFAG§ + nisin (800 IU mL^{-1})	Wilkins-Chalgren* (Wren, 1977)	Anaerobic chamber‡
Anaerobic Gram-positive bacteria	YCFAG§ + polymyxin B¶ + pediocin PA-1‖	RCA(CN)*,**	Anaerobic chamber‡
Staphylococcus spp.	BHI + polymyxin B¶ + pediciocin PA-1‖	Baird-Parker* (Baird-Parker, 1962)	Aerobic

Enrichment culturing was performed by inoculating 1 mL of an effluent sample into 50 mL of the appropriate enrichment broth. Agar plates contained chloramphenicol (10 μg mL^{-1}), erythromycin (10 μg mL^{-1}) and tetracycline (10 μg mL^{-1}) for the selection of cells harboring pRE25*. All media were incubated at 37 °C and media for strict anaerobes were pre-reduced 24 h before use in the anaerobic chamber.

*These media contained antibiotics at the following concentrations: chloramphenicol 10 μL mL^{-1}; erythromycin 10 μg mL^{-1} and tetracycline 10 μg mL^{-1}.

†AnaeroGen™ (Oxoid, Pratteln, Switzerland).

‡Nitrogen atmosphere containing 5% hydrogen (Coy Laboratories, Ann Arber, MI).

§YCFA medium (Duncan *et al.*, 2002) + 1 g L^{-1} glucose (VWR) + 1 g L^{-1} starch from potato.

¶70 IU mL^{-1} final concentration.

‖50 μL mL^{-1} cell-free supernatant of a *Pediococcus acidilactici* UVA-1 culture (Table 1), 10 × concentrated by freeze–drying.

**Reinforced clostridial agar (VWR) + 8 mg L^{-1} colistin + 8 mg L^{-1} novobiocin.

sterile Eppendorf tube and heated in a microwave oven at 600 W for 3 min. The DNA released was resuspended in 50 μL of autoclaved water. A PCR was performed using 2 × PCR Master Mix (Fermentas, Le-Mont-sur-Lausanne, Switzerland) and the primer pairs pRE25*_F/R for pRE25* and gfp_F/R for the *gfp* gene (Table 2). Detection of pRE25* and no detection of *gfp* indicates transconjugants harboring pRE25*. *Listeria monocytogenes* transconjugants in fermentation 1 were additionally verified by PCR targeting the *L. monocytogenes*-specific *hly* gene (primer pair hly_fw/rv; Table 2). Transconjugants from fermentation 2 were identified by amplifying the 16S rRNA genes using the primers bak11w and bak4 and subsequent sequencing of the product at Microsynth. A BLAST analysis (Altschul *et al.*, 1990) was performed with the obtained nucleotide sequence against the microbial database at NCBI (http://blast.ncbi.nlm.nih.gov/).

Conjugation experiments using the filter mating technique

To examine the functionality of plasmid replication in transconjugants isolated from the continuous colonic fermentations, filter mating experiments were performed as described previously (Haug *et al.*, 2010). Plasmid transfer was confirmed by PCR using the primer pairs pRE25*_F/R (Table 2). Transconjugants were verified by PCR using the primer pairs hly_fw/rv for *L. monocytogenes*, linF2/R2 for *Listeria innocua* and FL1/2 for *E. faecalis* (Table 2).

Results

ABR gene transfer to *L. monocytogenes* 10403S

In order to test the suitability of *E. faecalis* CG110/*gfp*/pRE25* as an ABR donor strain in a continuous colonic fermentation, the donor (1.49×10^9 CFU mL^{-1}) was co-immobilized with the recipient *L. monocytogenes* 10403S (6.60×10^9 CFU mL^{-1}) and fecal microbiota of a 4-month-old infant (fermentation 1). Real-time qPCR targeting the main bacterial groups typically present in the infant gut microbiota (Hopkins *et al.*, 2005; Kurokawa *et al.*, 2007) as well as *E. faecalis* CG110/*gfp*/pRE25* and *L. monocytogenes* was performed with fermenter effluent samples on days 1, 3, 5, 7 and 8. The real-time PCR analyses confirmed the complex background of the immobilized fecal microbiota, the colonization of the beads with *L. monocytogenes* and the *E. faecalis* donor as well as the stability of the bacterial composition throughout the fermentation (Table 4). Members of the *Firmicutes* and *Enterobacteriaceae* family were the most dominant populations, with mean genus-specific 16S rRNA gene copy numbers of log 10.45 ± 0.17 and log 9.59 ± 0.23 mL^{-1} effluent, respectively (Table 4). Lactobacilli and *E. faecalis* were present at subdominant levels with corresponding gene copy numbers of log 5.37 ± 0.39 and log 7.98 ± 0.25 mL^{-1} effluent. The co-immobilized *L. monocytogenes*, detected via *hly* copy numbers, was present at log 6.03 ± 0.18 mL^{-1} effluent. The *E. faecalis* donor strain was detected at slightly lower numbers of log 5.08 ± 0.18 mL^{-1} effluent (Table 4). *Roseburia* spp./*Eubacterium rectale* and *Bacteroides* spp. were detected at low copy numbers of 3.13 ± 0.30 and 3.56 ± 0.15 log 16S rRNA gene copies mL^{-1} effluent (Table 4). The total metabolite concentration was 83.22 ± 5.81 mM, with acetate being the main product, followed by butyrate and propionate (Table 5). Isobutyrate and formate were present at low concentrations (≤ 10 mM), whereas lactate was not detected.

During the continuous fermentation, *L. monocytogenes* 10403S transconjugants, isolated by plating effluent samples daily onto *Listeria* spp. selective medium supplemented with

Table 4. Bacterial populations in effluent samples of fermentations 1 and 2

Bacterial population	Ferm. 1 ($\log_{10}$ gene copies mL^{-1})	Ferm. 2 ($\log_{10}$ gene copies mL^{-1})
Total 16S rRNA genes	11.04 ± 0.11	10.75 ± 0.15
Bacteroides spp.	3.56 ± 0.15	9.90 ± 0.23
Bifidobacterium spp.	ND*	7.46 ± 0.27
Firmicutes	10.45 ± 0.17	10.01 ± 0.09
Enterobacteriaceae	9.59 ± 0.23	8.36 ± 0.50
Roseburia spp./*Eubacterium rectale*	3.13 ± 0.30	8.03 ± 2.47
Total *E. faecalis*	7.98 ± 0.25	8.04 ± 0.59
E. faecalis CG110/*gfp*/pRE25*	5.08 ± 0.18	5.98 ± 0.46
Lactobacillus/*Leuconostoc*/*Pediococcus* spp.	5.37 ± 0.39	ND*
L. monocytogenes	6.03 ± 0.18	Not determined

Total copy numbers were determined using qPCR (Table 2). The total gene copy numbers were quantified using a DNA calibration curve obtained by plotting the C_t values from serial dilutions of the corresponding target, obtained in the same qPCR run. 16S rRNA genes were used as a specific target gene for the different groups, except for *Bifidobacterium* spp. (*xfp* gene), *Enterococcus faecalis* (*tufA* gene), the donor strain *E. faecalis* CG110/*gfp*/pRE25* (*gfp* gene) and *Listeria monocytogenes* (*hly* gene; Table 2). Values are means ± SD of effluent samples analyzed every 48 h.

*ND, not detected, below the detection limit of log 3.40 mL^{-1} effluent.

Table 5. Metabolites in fermentations 1 and 2 determined by HPLC

	Metabolite concentration (mM)	
Metabolite	Fermentation 1	Fermentation 2
Acetate	42.81 ± 3.95	67.80 ± 12.03
Butyrate	25.80 ± 3.03	35.10 ± 4.95
Propionate	10.18 ± 0.61	18.29 ± 2.36
Formate	5.55 ± 1.75	ND*
Isobutyrate	0.97 ± 0.11	8.04 ± 1.51
Isovalerate	ND*	3.93 ± 1.27
Lactate	ND*	ND*
Total metabolites	83.22 ± 5.81	133.90 ± 6.95

Data are means ± SD for days 1–8 (fermentation 1) and days 1–16 (fermentation 2).

*ND, not detected, below the detection limit of 0.2 mM for formate, 0.17 mM for isovalerate and 0.03 for lactate.

erythromycin (10 μg mL^{-1}), were detected on days 1–6 and 8 at an average cell count of log 1.4 ± 0.5 CFU mL^{-1}.

ABR gene transfer to commensal bacteria

A second *in vitro* colonic fermentation was performed to investigate the transfer potential of pRE25* from *E. faecalis* to commensal bacteria of the complex human gut microbiota. *E. faecalis* CG110/*gfp*/pRE25* (2.11×10^9 CFU mL^{-1}) was immobilized with the fecal microbiota of a 7-month-old donor and the continuous colonic fermentation was performed for 16 days. Similar to fermentation 1, the main bacterial groups normally encountered in infant feces as well as the *E. faecalis* donor strain were quantified by qPCR (Table 2). *Bacteroides* spp. and *Firmicutes* were the predominant groups, with log 9.90 ± 0.23 and log 10.01 ± 0.09 16S rRNA gene copy numbers per milliliter of effluent. *Roseburia* spp./*E. rectale* 16S rRNA gene copies were low at the beginning of fermentation 2, but reached an average number of log 9.53 ± 0.43 from day 7 to day 16 (data not shown). *Enterobacteriaceae*, *E. faecalis* and *Bifidobacterium* spp. target gene copies were slightly lower, whereas *Lactobacillus*/*Leuconostoc*/*Pediococcus* spp. 16S rRNA genes were not detected in the effluent (Table 4). *Enterococcus faecalis* CG110/*gfp*/pRE25* was present at an average gene copy number of log 5.98 ± 0.46 mL^{-1} effluent (Table 4). The total metabolite concentration determined by HPLC was 133.90 ± 6.95 mM (Table 5). Acetate was the main metabolite, with an average concentration of 67.80 ± 12.03 mM (Table 5). Butyrate and propionate concentrations were lower, with 35.10 ± 4.95 and 18.29 ± 2.36 mM (Table 5).

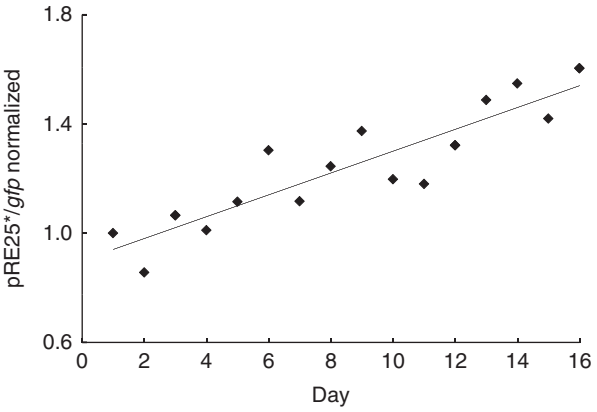

Fig. 1. Ratio of plasmid pRE25* to the chromosomal donor marker *gfp* in effluent samples of fermentation 2. Copy numbers of pRE25* and the *gfp* gene were determined by qPCR and normalized to copy numbers on day 1. The trendline is depicted in the chart area.

To monitor the conjugation of pRE25*, both the *E. faecalis* donor (via the *gfp* gene) and pRE25* were quantified by qPCR during the continuous fermentation. The ratio of pRE25* to *gfp* in the effluent increased constantly from day 1 to day 16 (Fig. 1), indicating that HGT has occurred. On days 5, 10 and 15, we attempted to isolate transconjugants by plating effluent samples onto different selective media (Table 3). No colonies were detected on *Lactobacillus* spp. selective media. On a number of selective plates, all colonies showed a swarming morphology and a very distinct odor. Sequencing of the 16S rRNA genes of one of these colonies revealed an overall presence of *Proteus mirabilis*, a species with a natural resistance to chloramphenicol, erythromycin and tetracycline (Franklin & Rownd, 1973;

Charles *et al.*, 1985; Arthur *et al.*, 1987). The antibiotic spectrum for the selection of transconjugants was then extended to 200 μg mL^{-1} kanamycin (Kan_{200}), because *P. mirabilis* was sensitive to kanamycin (data not shown), and the kanamycin resistance gene *aph*(3′)-III is encoded on pRE25*. No colonies were obtained on *Enterobacteriaceae*-specific agar with Cm_{10}, Ery_{10}, Kan_{200} and Tet_{10}. Colonies growing on selective agar for *Staphylococcus* spp., *Clostridium* spp. and anaerobic Gram-negative bacteria (Table 3) were streaked onto fresh selective media to obtain single colonies. However, PCR targeting pRE25* and *gfp* revealed that all examined colonies were *E. faecalis* donor colonies and no transconjugants could be found on these selective media.

Because *E. faecalis* CG110/*gfp* is not able to establish itself in a free cell continuous colonic fermentation (Scott *et al.*, 2000), we attempted to enrich transconjugants by serially culturing effluent samples from day 16 in 50 mL BHI+Cm_{10}, Ery_{10}, Kan_{200} and Tet_{10} at 37 °C. Multiple antibiotics were applied to ensure that only cells harboring pRE25* were able to grow. After enrichment culturing, appropriate dilutions were plated onto BHI+Cm_{10}, Ery_{10}, Kan_{200} and Tet_{10}. All 96 tested colonies were positive for pRE25*, whereas the *gfp* donor marker was only detected on 89 out of 96 tested colonies. Sequencing of the 16S rRNA genes of the seven colonies deficient in the *gfp* gene, followed by BLAST analysis revealed only one single nucleotide polymorphism and 99.93% identity to the nucleotide sequence of the *Enterococcus avium* 16S rRNA gene, showing that pRE25* was transferred into the gut commensal *E. avium*.

Characterization of transconjugants

To investigate the functionality of pRE25* in the isolated transconjugants, conjugation capacity was tested in filter mating experiments. *L. monocytogenes* 10403S/pRE25* (fermentation 1) transferred pRE25* to *E. faecalis* JH2-2 at a transfer frequency of 5.7×10^{-6} transconjugants per donor. *Enterococcus avium* BT1/pRE25* (fermentation 2) was able to transfer pRE25* to *L. monocytogenes* 10403S and LM15, *L. innocua* L19 and *E. faecalis* JH2-2 at transfer frequencies of 4.6×10^{-7}, 1.3×10^{-7}, 5.6×10^{-4} and 7.5×10^{-4} transconjugants per donor.

Discussion

In this study, the transfer characteristics of the genetically marked conjugative multiresistance plasmid pRE25* from the chromosomally tagged *E. faecalis* strain CG110/*gfp* were monitored in two continuous colonic fermentations mimicking the infant proximal colon. Transfer of pRE25* was demonstrated to the co-immobilized *L. monocytogenes* and to the fecal commensal *E. avium* in the presence of a competing fecal microbiota.

The inoculums immobilized in the two fermentations derived from different donors, which explains the differences observed in the microbial and metabolic composition of the fermentation effluents. However, qPCR targeting the main bacterial groups commonly encountered in infant feces demonstrated that the microbiota in the effluent was complex and representative of infant fecal samples throughout both fermentations (Table 4). Remarkably, no bifidobacteria were detected in fermentation 1, whereas *Bacteroides* numbers were low (Table 4). The 4-month-old feces donor for fermentation 1 was delivered by Cesarean section, and even though bifidobacteria are frequently found as colonizers of the infant gut, colonization can be delayed after Cesarean section (Penders *et al.*, 2006, 2007). Such a delay is also observed for *Bacteroides* spp. colonization (Penders *et al.*, 2006; Fallani *et al.*, 2010). Donor and recipient populations were stable throughout fermentation 1 at a subdominant level, as intended (Table 4). The dominant groups in fermentation 2 (Table 4) were in accordance with major populations commonly detected in infant feces (Hopkins *et al.*, 2005; Kurokawa *et al.*, 2007), albeit no lactobacilli were detected. However, persistent colonization by lactobacilli is not very common in infants (Ahrné *et al.*, 2005; Penders *et al.*, 2007). The qPCR data therefore demonstrated a high and stable colonization of the beads, the presence of common infant gut colonizers as well as a complex bacterial composition in both fermentations (Table 4), attributes that are assumed to impact colonization as well as HGT *in vivo* (Licht & Wilcks, 2006). The multiresistance plasmid pRE25* was transferred from *E. faecalis* CG110/*gfp*/pRE25* to *L. monocytogenes* 10403S in the presence of the competing microbiota, confirming the suitability of *E. faecalis* CG110/*gfp*/pRE25* as an applicable donor in a colonic fermentation model. Moreover, these transfer events imply that genes conferring resistances against antibiotics used in the treatment of listeriosis in humans, such as gentamicin, clindamycin and erythromycin (Chen *et al.*, 2010; Johnsen *et al.*, 2010), can be transferred in a colonic fermentation mimicking the GIT. Because the GIT is the most probable meeting point of *E. faecalis* and *L. monocytogenes* (Doucet-Populaire *et al.*, 1991), horizontal ABR gene transfer from *E. faecalis* to *L. monocytogenes* could exacerbate the effective treatment of listeriosis.

A clear indication that HGT has occurred in the second fermentation is the increase in the ratio of pRE25* to the *gfp* gene (Fig. 1). The *gfp* gene is stably integrated in the chromosome (Haug *et al.*, 2010), and, moreover, pRE25* and *gfp* copy numbers relative to the chromosome in the donor determined by qPCR were identical before and after the fermentation (data not shown). The observed increase in the pRE25*/*gfp* ratio therefore strongly suggests the

transmission of pRE25* to commensal bacteria. Indeed, an *E. avium* strain harboring pRE25*, designated *E. avium* BT1/pRE25*, could be isolated, exemplifying the transfer capability of pRE25* from its host to commensal bacteria. However, it is not clear whether pRE25* was transferred by a single event or whether HGT has occurred more than once throughout the fermentation. The plasmid pRE25* was still functional in *E. avium* BT1/pRE25*, as demonstrated by its transferability to other species. *E. avium* has originally been isolated from human feces and has occasionally been detected as the predominant *Enterococcus* species in the feces of healthy adults (Kubota *et al.*, 2010). Although the majority of *Enterococcus* infections are caused by *E. faecalis* and *E. faecium* (Tan *et al.*, 2010), transfer of the complete and functional pRE25* to *E. avium* is of concern, because *E. avium* is also considered as an opportunistic pathogen (Tan *et al.*, 2010).

E. avium BT1/pRE25* was the only transconjugant that could be isolated. However, the presence of further transconjugants cannot be excluded, in case pRE25* has been transferred to other enterococci, which could not be uniquely selected for, or to bacteria of the fecal microbiota that are not cultivable with the selective media used in this study. Transfer of an incomplete pRE25* that does not encode resistances to the antibiotics used for the selection could also have occurred.

We demonstrated that an antibiotic-resistant *E. faecalis* strain in a continuous colonic fermentation mimicking the gut ecosystem can transfer its multiresistance plasmid to commensal bacteria colonizing the same site. Because the colonic model reflects the situation in nature of high cell density and diversity, our results provide an important step towards understanding the behavior of transferable ABR genes in a complex microbial ecosystem. ARE are highly prevalent in food, particularly in animal-derived food like fermented meat products and cheeses (Teuber, 1999). Moreover, cheese-derived *Enterococcus* strains can dominate the enterococcal population in the human intestine during a cheese consumption period, even if the strain is present at only low numbers in the cheese (Gelsomino *et al.*, 2003). If ARE from food can transiently or permanently colonize the GIT, the risk of lateral ABR gene transfer to the gut microbiota increases (Berchieri, 1999). This study therefore suggests that high abundances of food-derived enterococci carrying transferable ABR determinants are a concern for human health because they could, as a worst-case scenario, seriously limit the effectiveness of antibiotic therapy in case of intestinal infections.

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