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Construction and characterization of *Enterococcus faecalis* CG110/*gfp*/pRE25*, a tool for monitoring horizontal gene transfer in complex microbial ecosystems

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Abstract

Enterococci are among the most notorious bacteria involved in the spread of antibiotic resistance (ABR) determinants via horizontal gene transfer, a process that leads to increased prevalence of antibiotic-resistant bacteria. In complex microbial communities with a high background of ABR genes, detection of gene transfer is possible only when the ABR determinant is marked. Therefore, the conjugative multiresistance plasmid pRE25, originating from a sausage-associated *Enterococcus faecalis*, was tagged with a 34-bp random sequence marker spliced by *tet*(M). The plasmid constructed, designated pRE25*, was introduced into *E. faecalis* CG110/*gfp*, a strain containing a *gfp* gene as chromosomal marker. The plasmid pRE25* is fully functional compared with its parental pRE25, occurs at one to two copies per chromosome, and can be transferred to *Listeria monocytogenes* and *Listeria innocua* at frequencies of 6×10^{-6} to 8×10^{-8} transconjugants per donor. The markers on the chromosome and the plasmid enable independent quantification of donor and plasmid, even if ABR genes occur at high numbers in the background ecosystem. Both markers were stable for at least 200 generations, permitting application of the strain in long-running experiments. *Enterococcus faecalis* CG110/*gfp*/pRE25* is a potent tool for the investigation of horizontal ABR gene transfer in complex environments such as food matrices, biofilms or colonic models.

Introduction

Horizontal transfer of resistance genes and antibiotic-mediated selection pressure leads to a persistence and propagation of antibiotic-resistant bacteria in clinical environments, stock breeding, or in soil (Murray, 1990; Doucet-Populaire *et al.*, 1991; Showsh & Andrews, 1992; Agerso & Sandvang, 2005; Kazimierczak & Scott, 2007). Transfer of antibiotic resistance (ABR) determinants can cross the genus barrier and is mainly mediated by conjugative elements such as transposons and plasmids (Shoemaker *et al.*, 2001). Enterococci are Gram-positive, catalase-negative, oxidase-negative members of the functional related group of lactic acid bacteria predominantly encountered in the gastrointestinal tract (GI-tract) of humans and animals. Enterococci harbor a variety of mobile genetic elements such as conjugative plasmids and transposons and therefore the genus *Enterococcus* is supposed to be a main actor in the spreading of ABR genes (Clewell, 1990).

Characterization of the human microbial community has revealed a vast diversity of resistance genes, indicating that the human microbial community acts as a reservoir of ABR genes (Shoemaker *et al.*, 2001; Sommer *et al.*, 2009). So far, horizontal gene transfer (HGT) in the gut has mainly been observed after ingestion of a donor and a defined recipient in the presence of a complex background flora or between specific bacteria in gnotobiotic animals (Doucet-Populaire *et al.*, 1991; Licht *et al.*, 2002, 2003; Alpert *et al.*, 2003; Avrain *et al.*, 2004; Mater *et al.*, 2005, 2008; Hart *et al.*, 2006; Lester *et al.*, 2006; Jacobsen *et al.*, 2007; Moubareck *et al.*, 2007; Feld *et al.*, 2008; Boguslawska *et al.*, 2009). However, both experimental set-ups are limited in the selection of recipients against the microbial background and in the quantification of gene transfer.

The 50-kb plasmid pRE25 from *Enterococcus faecalis* RE25 encodes resistances against the structural antibiotic classes aminoglycosides, lincosamides, macrolides, chloramphenicol and streptothricin, and is transferrable to

E. faecalis, Lactococcus lactis and *Listeria innocua* (Schwarz, 2001; Schwarz *et al.*, 2001; Teuber *et al.*, 2003). The plasmid pRE25 belongs to the incompatibility group Inc18 of streptococcal plasmids, which replicate via the unidirectional θ mechanism (Bruand *et al.*, 1991; Ceglowski *et al.*, 1993; Le Chatelier *et al.*, 1993). Sequence comparison of pRE25 to other conjugative plasmids such as the *Streptococcus agalactiae* plasmid pIP501, the *Staphylococcus* plasmids pGO1 and pSK41, and the *Lactococcus* plasmid pMRC01 revealed that the modular organization of the transfer genes region is well-conserved, indicating common transfer potential of these plasmids (Grohmann *et al.*, 2003).

Here, we describe the construction and features of a chromosomally tagged *E. faecalis* strain harboring the multiresistant conjugative plasmid pRE25*, a derivative of pRE25 carrying a unique DNA sequence downstream of the erythromycin resistance gene. The two markers allow distinguishing between donor strain and recipient bacteria and the strain can therefore be used as a tool to monitor and quantify horizontal ABR gene transfer in complex microbial environments without defined recipients, such as the human GI-tract, food matrices, and biofilms.

Materials and methods

Bacterial strains and media

Bacterial strains and growth conditions used in this study are listed in Table 1. Chemicals were routinely obtained from Sigma-Aldrich (Buchs, Switzerland), except when stated otherwise.

DNA isolation and manipulation

DNA manipulations were essentially performed as described previously (Sambrook & Russell, 2001). Oligonucleotides were obtained from Microsynth (Balgach, Switzerland) and are listed in Table 2. DNA for PCR amplification was extracted from single colonies using a trizol–lysozyme-based cell lysis and subsequent DNA isolation as described previously (Goldenberger *et al.*, 1995). DNA extraction for quantitative PCR was performed as follows: cells from 2-mL cultures were harvested and resuspended in 400 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The suspension was transferred to a screw cap tube containing 500 μL of phenol : chloroform : isoamylalcohol (25 : 24 : 1) and 500 mg of 0.1-mm zirconia/silica beads. Cells were disrupted by bead-beating four times 20 s at maximum speed in a FastPrep device (MP Biomedicals, Illkirch, France), interspaced by cooling on ice. The suspension was centrifuged at 20 000 ***g*** and 4 °C for 10 min and the supernatant was extracted once with chloroform to remove residual phenol. The DNA was precipitated with isopropanol, washed with 70% ethanol, dried and resuspended in TE buffer.

Plasmid DNA from *Escherichia coli* was isolated using the Plasmid Midi Kit (Qiagen). Plasmids from *E. faecalis* were purified according to the preparative protocol for large-scale plasmid isolation (Anderson & McKay, 1983) with slight modifications.

Construction of *E. faecalis* CG110/*gfp*/pRE25*

In order to insert a 34-bp random sequence interspaced by *tet*(M) into pRE25, the integration vector pMH401 was constructed (Fig. 1, Table 1). A 1-kb fragment directly upstream of the stopcodon of the *ermB* gene was amplified using the primer pair Ins_A2/B (Table 2), the proofreading Phusion polymerase (Finnzymes, Espoo, Finland), and DNA from *L. lactis* BuRE25 as template. Similarly, a 1-kb fragment downstream of the stopcodon of the *ermB* gene was amplified using the primers Ins_C and Ins_D. The two fragments were fused via splicing by overlap extension PCR using the 34-bp overlapping region introduced in the primers Ins_B and Ins_C (Table 2, underlined). PCR was then performed on the fused fragments using primers Ins_A2 and Ins_D and 2 × *taq* PCR Master Mix (Fermentas, Le-Mont-sur-Lausanne, Switzerland). The resulting 2120-bp fragment was cloned into the cloning vector pGEM®-T Easy (Promega, Madison) according to the manufacturer's instructions. The resulting plasmid was designated pMH400, a plasmid containing the 1-kb up- and downstream regions of the stopcodon of the *ermB* gene, interspaced with a 34-bp random sequence. Subsequently, *tet*(M) was amplified from *E. coli* CG120/pAM120 DNA using primers HP14 and HP15 and Phusion DNA polymerase. The 2678-bp fragment obtained was ligated into pMH400 linearized with SwaI. Correct plasmid construction was checked by restriction analyses and by PCR targeting *tet*(M) using primers HP14 and HP15 (Table 2). The obtained plasmid was designated pMH401 and harbors the 1-kb up- and downstream regions of the stopcodon of the *ermB* gene interspaced with *tet*(M) flanked by a 23-bp and an 11-bp random sequence (Fig. 1). Plasmid pMH401 was transferred into *L. lactis* BuRE25 (Table 1) by electroporation as described previously (Holo & Nes, 1989) and primary integrants were selected on streptococcal regeneration plates (Okamoto *et al.*, 1983) containing 10 μg mL^{-1} tetracycline. A double-cross-over event results in integration of *tet*(M) flanked by the two random sequences downstream of the *ermB* gene in pRE25 (Fig. 1). Therefore, integrants were streaked on brain–heart infusion (BHI) containing 10 μg mL^{-1} erythromycin and after incubation for 48 h at 30 °C, single colonies were checked for double-cross-over by PCR using the primer pairs Int401_A/F and Int401_G/D. An isolate showing correct PCR pattern was designated *L. lactis* BuRE25*, an *L. lactis* Bu2-60 derivative carrying pRE25 tagged with *tet*(M) flanked by two 23- and 11-bp random sequences in its chromosome. Next, pRE25* was

Table 1. Strains and plasmids used in this work

Material	Relevant features	Source
Strains[†]		
Escherichia coli		
CG120/pAM120	Harbors pAM120, a pBR211 derived plasmid containing *tet*(M) on Tn*916*	Gawron-Burke & Clewell (1984)
CG110/*gfp*	CG110 derivative harboring a chromosomal *gfp* in Tn*916* transposon; Fus^R, Rif^R, Tet^R	Scott *et al*. (2000)
CG110/*gfp*/pRE25*	CG110/*gfp* derivative harboring pRE25*; Cm^R, Em^R, Fus^R, Gen^R, Kan^R, Neo^R, Rif^R, Str^R, Tet^R	This work
Enterococcus faecalis		
RE25	Dry sausage isolate harboring pRE25; Cm^R, Em^R, Gen^R, Kan^R, Neo^R, Str^R, Tet^R	Perreten (1995)
1528	Clinical isolate, recipient for filter mating; Ery^R, Tet^R, Van^R	Klare *et al*. (1995)
Lactococcus lactis		
Bu2-60	Starter culture isolate, plasmid free; Fus^R, Rif^R, Str^R	Neve *et al*. (1984)
BuRE25	*L. lactis* Bu2-60 harboring a single copy of pRE25 integrated in chromosome; Cm^R, Em^R, Fus^R, Gen^R, Kan^R, Neo^R, Rif^R, Str^R	Perreten (1995)
BuRE25*	*L. lactis* Bu2-60 harboring pRE25* integrated in the chromosome; Cm^R, Em^R, Fus^R, Gen^R, Kan^R, Neo^R, Rif^R, Str^R, Tet^R	This work
Lactobacillus fermentum		
ROT1	Dairy isolate; Em^R, Nov^R, Tet^R	Gfeller *et al*. (2003)
Listeria innocua		
L19	Plasmid free, recipient for filter mating	Schwarz *et al*. (2001)
Listeria monocytogenes		
10403S	Derivative of the clinical isolate 10403, recipient for filter mating; Str^R	Bishop & Hinrichs (1987)
LM15	Food isolate, recipient for filter mating, Tet^R	Veterinary hospital, Zurich
10403S/pRE25*	Transconjugant from filter mating, strain 10403 harboring pRE25*; Cm^R, Em^R, Gen^R, Kan^R, Neo^R, Str^R, Tet^R	This work
Leuconostoc mesenteroides		
M7-1 (LMG19463)	Recipient for filter mating; Van^R	FBT culture collection
Staphylococcus aureus		
VG1	Dairy isolate; Gen^R, Neo^R, Pen^R	Perreten (1995)
Plasmids		
pGEM®-T Easy	3.0 kb, cloning vector	Promega
pMH400	5.1 kb, pGEM derivative harboring the 1-kb up- and downstream regions of the stopcodon of the *ermB* gene from pRE25 interspaced with 34-bp random sequence	This work
pMH401	7.7 kb, pMH400 derivative harboring the 1-kb up- and downstream regions of the stopcodon of the *ermB* gene from pRE25 interspaced with *tet*(M) flanked by two 23-bp and a 11-bp random sequences	This work
pRE25	50.2 kb, Cm^R, Em^R, Gen^R Kan^R, Neo^R, Str^R	Perreten (1995), Schwarz *et al*. (2001)
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)	This work

[†]*Staphylococcus aureus* and *Enterococcus* strains were cultivated aerobically at 37 °C in BHI (Biolife, Milano, Italy). *Lactobacillus fermentum* and *Leuconostoc mesenteroides* were grown anaerobically at 30 °C in MRS (Labo-Life Sàrl, Pully, Switzerland). *Listeria* spp. were grown in BHI at 37 °C without agitation. *Lactococcus lactis* strains were grown at 30 °C in GM17 medium [M17, Biolife, supplemented with 0.5% w/v glucose (VWR International, Dietikon, Switzerland)]. *Escherichia coli* strains were grown aerobically in lysogeny broth [LB (Sambrook & Russell, 2001), Becton Dickinson, Allschwil, Switzerland] at 37 °C, unless stated otherwise. Anaerobic conditions were maintained using AnaeroGen™ (Oxoid, Pratteln, Switzerland).

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; Van^R, vancomycin resistant; FBT, food biotechnology.

transferred to *E. faecalis* CG110/*gfp* via filter mating and transconjugants were selected on KF Streptococcus Agar (Becton Dickinson) supplemented with chloramphenicol ($10\,\mu g\,mL^{-1}$). The resulting strain was designated *E. faecalis* CG110/*gfp*/pRE25*, harboring a chromosomal *gfp* and pRE25* (Fig. 1). The presence of the *gfp* gene was confirmed by PCR using primers gfp_F and gfp_R (Table 2). Sequencing of the two regions overlapping the random sequence was performed by Microsynth using primer pairs seq1_fw/vr and seq2_fw/rv (Table 2) and confirmed that the random sequences flanking *tet*(M) were integrated downstream the *ermB* gene.

Table 2. Oligonucleotides used in this work

Primer	Sequence (5′ → 3′)	$T_{Annealing}$ (°C)*	Reference
Ins_A2	TGTATAATAGGAATTTGAAGTTA	48	This work
Ins_B	ATGACAG**ATTTAAAT**GGATCCGATCGAATTCCGAATAGAATTATTTCCTCCCGT		
Ins_C	TCGGAATTCGATCGGATCC**ATTTAAAT**CTGTCATGAGTCGCTTTTTTAAATTTG	48	This work
Ins_D	TAATGAGATCATAGTCACTT		
HP14	TTGAAGTCGACGGGAGTAATTGGAAG	60	Warren *et al*. (2004)
HP15	TAAAAGTCGACATACATAACGGAAAGAG		
Int401_A	CGAAATGATACACCAATC	56	This work
Int401_F	TTCCAATTACTCCCGT		
Int401_G	TCTTTCCGTTATGTATGT	56	This work
Int401_D	GGCGTTGGTACAGTATC		
seq1_fw	TCAATCGAGAATATCGTCA	56	This work
seq1_rv	AAGAGAGTACGTGATTACA		
seq2_fw	AAGCAGTTCAAAGTAACT	54	This work
seq2_rv	CCACACTTAGGACATT		
lmoF	CGCAAGAAGAAATTGCCATC	60	Huang *et al*. (2007)
lmoR	TCCGCGTTAGAAAAATTCCA		
linF2	TTGCTACTGAAGAAAAAGCA	60	Huang *et al*. (2007)
linR2	TCTGTTTTTGCTTCTGTAGC		
tufA_fw	GACAAACCATTCATGATGCCAG	60	Ke *et al*. (1999)
tufA_rv	CGTCACCAACGCGAACTTCA		
pRE25*_F	TCATCAAGCAATGAAACACG	54	This work
pRE25*_R	GCATATTTGTAAAGGAATCTCCA		
pRE25_F	CCGTTTACGAAATTGGAACA	60	This work
pRE25_R	TTGGTGAATTAAAGTGACACGA		
gfp_F	CTTTTCACTGGAGTTGTCC	51	Scott *et al*. (1998)
gfp_R	CCAGCAGCTGTTACAAACTC		
aph_F	AAATGACGGACAGCCGGTAT	60	This work
aph_R	CCTTTGGAACAGGCAGCTTT		
gfp_F2	TGGAAGCGTTCAATTAGCAGA	60	This work
gfp_R2	GGCAGATTGTGTGGACAGGT		
pRE25*_F2	GTACCATTACTTATGAGCAAGTATTGTC	60	This work
pRE25*_R2	CTATAATCTTCCAATTACTCCCGTC		
pRE25*_TMP	FAM-GGAAATAATTCTATTCGGAATTCGATCGGATC-TAMRA		

For primers Ins_B and Ins_C (SOE PCR), the random sequence (= overlapping sequence) is underlined and the restriction site for SwaI is depicted in bold letters. FAM, reporter dye (6-carboxyfluorescein); TAMRA, nonfluorescent quencher (carboxytetramethylrhodamine); SOE, splicing by overlapping extension.

*Annealing temperatures used in this study.

Conjugation experiments by filter mating technique

Overnight cultures of donor and recipient were mixed 1 : 3 and passed through a sterile 0.45-μm nitrocellulose filter (Millipore AG, Zug, Switzerland). The filter was incubated overnight cell-side up on nonselective plates under optimal conditions for the recipient. The filter was then washed by vortexing for 1 min in 2 mL of sterile dilution solution [0.85% NaCl, 0.1% peptone from casein (VWR), pH 8.0] and transconjugants were isolated by plating appropriate dilutions on selective medium. Correct plasmid transfer was confirmed by PCR using primer pairs pRE25*_F/R and pRE25_F/R (Table 2). *Listeria* transconjugants were verified using the primer pairs lmoF/R and linF2/R2. *Leuconostoc mesenteroides* transconjugants were identified by the absence of a *tuf*A gene according to a negative PCR using primer pair tufA_fw/rv (Table 2).

Marker stability

Marker stability in *E. faecalis* CG110/*gfp*/pRE25* was examined by cultivating the cells serially in BHI broth at 37 °C for at least 200 generations. The presence of pRE25* was confirmed by plating daily on BHI agar supplemented with chloramphenicol (10 μg mL^{-1}). The stability of *gfp* was verified by PCR with primers gfp_F and gfp_R.

Quantitative real-time PCR

All reactions were performed in a reaction volume of 25 μL. For real-time PCR using the SYBR Green method, 12.5 μL of

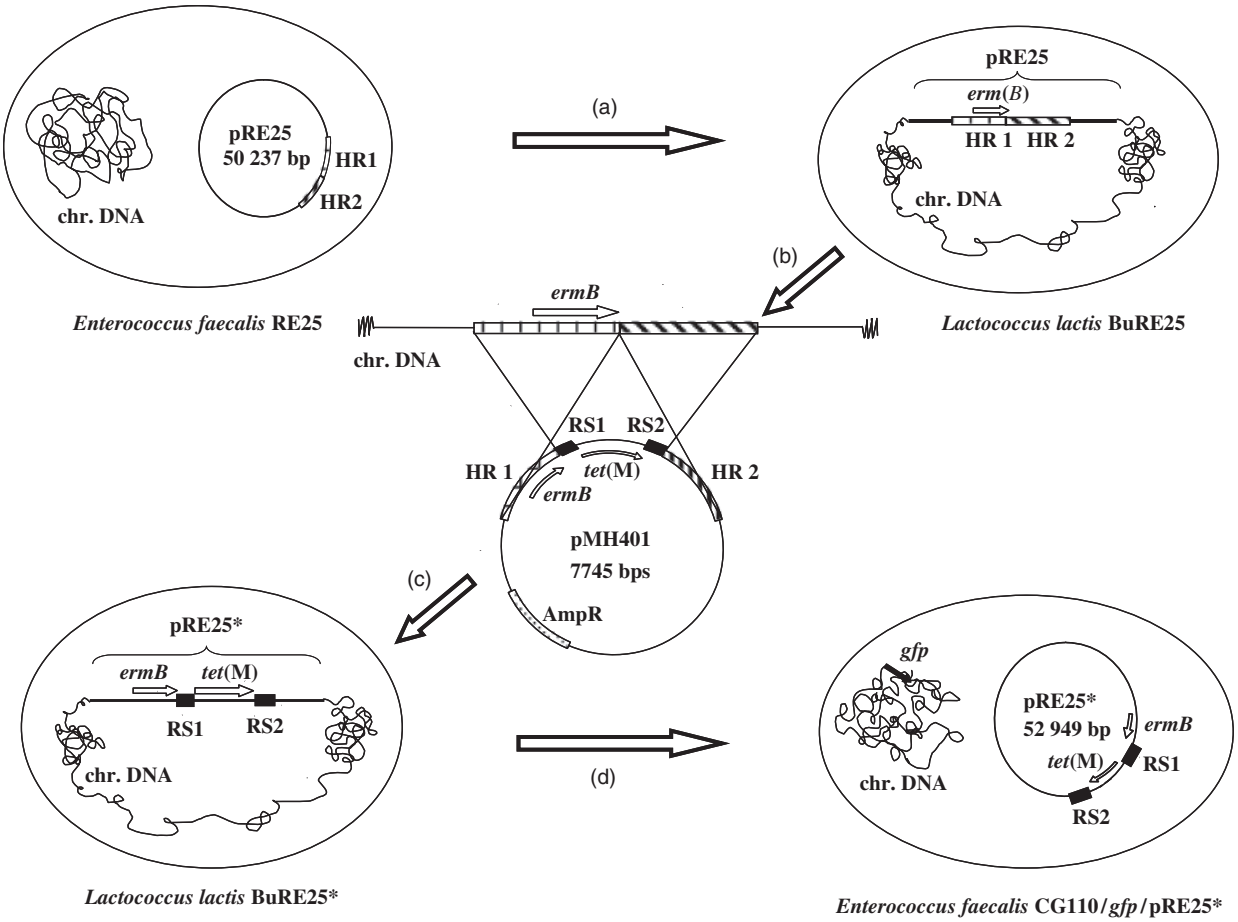

Fig. 1. Construction of *Enterococcus faecalis* CG110/*gfp*/pRE25*. (a) Transfer of pRE25 from *E. faecalis* RE25 to *Lactococcus lactis* Bu2-60 by filter mating (Perreten, 1995); (b) transformation of *L. lactis* BuRE25 (*L. lactis* Bu2-60 harboring pRE25 integrated in the chromosome) with pMH401; (c) homologous recombination leading to the integration of *tet*(M) flanked by the two random sequences RS1 and RS2 downstream of *ermB* of pRE25, resulting in pRE25*; (d) mobilization of pRE25* from *L. lactis* to *E. faecalis* CG110/*gfp* by filter mating. HR, homologous regions; RS, random sequences; chr., chromosomal. Nucleotide sequences (5′ → 3′): RS1, TCGGAATTCGATCGGATCCATTT; RS2, AAATCTGTCAT.

2 × SYBR® Green PCR Master Mix (Applied Biosystems, Zug, Switzerland) and each primer at a concentration of 200 nM was used. In the TaqMan-based method, 12.5 μL of qPCR MasterMix Plus Low ROX w/o UNG (Eurogentech, Seraing, Belgium), each primer at a concentration of 300 nM and the TaqMan probe at a concentration of 200 nM was used. Amplification was performed in a 7500 Fast Real-time PCR System (Applied Biosystems) and data were analyzed using the 7500 FAST SDS software (Applied Biosystems). Total gene copy numbers were quantified using a DNA calibration curve obtained by plotting C_t values from serial dilutions of the corresponding target, obtained in the same qPCR run.

Validation of quantitative real-time PCR for the detection of HGT in complex microbiota

To test whether pRE25* and *gfp* copy numbers can be quantified by qPCR in a complex ecosystem, fresh overnight cultures of *E. faecalis* CG110/*gfp*/pRE25*, *Listeria monocytogenes* 10403S, and *L. monocytogenes* 10403S/pRE25* (Table 1) were mixed at six different transconjugants to donor ratios with complex infant microbiota from a continuous colonic fermentation mimicking the ecosystem of the infant proximal colon (Le Blay *et al.*, 2009). DNA was immediately extracted from 1 mL of this cell suspension and the *gfp* gene was quantified using the primer pair gfp_F2/R2, and pRE25* using the primer set pRE25*_F2/R2 and the TaqMan probe pRE25*_TMP.

Results and discussion

Construction of *E. faecalis* CG110/*gfp*/pRE25*

The *ermB* gene on pRE25 plasmid was marked to allow quantification of the gene and to monitor transmission routes in conjugation experiments in complex genetic

Table 3. Minimal inhibitory concentrations (MIC) (μg mL^{-1}) of *Enterococcus faecalis* CG110/*gfp*/pRE25* compared with *E. faecalis* RE25

E. faecalis strains	Cm	Amo	Amp	Em	Gen	Kan	Nov	Cip	Clin	Pen	Rif	Sm	Tet	Tri	Van	Nal
CG110/*gfp*/pRE25*	64	0.5	1	32*	16	> 256	4	2	32*	2	32	64	64†	0.25	1	32*
RE25	> 256	ND	0.38	> 256	6	> 256	ND	ND	> 256	ND	0.75	> 1024	96	ND	ND	> 256

*Highest concentration tested in microdilution test.

†Next highest concentration tested: 128 μg mL^{-1}.

MIC for RE25 were performed with *E*-test (Schwarz, 2001).

Cm, chloramphenicol; Amo, amoxicillin; Amp, ampicillin; Em, erythromycin; Gen, gentamicin; Kan, kanamycin; Nov, novobiocin; Cip, ciprofloxacin; Clin, clindamycin; Pen, penicillin; Rif, rifampicin; Sm, streptomycin; Tet, tetracycline; Tri, trimetroprim; Van, vancomycin; Nal, nalidixic acid; ND, not determined.

backgrounds as for example the GI-tract. Therefore, *L. lactis* BuRE25 (Table 1), harboring pRE25 in its chromosome, was transformed with the integration vector pMH401 (Fig. 1), and a double-cross-over event resulted in pRE25*, a pRE25-derivative harboring *tet*(M) flanked by two random sequences. Next, pRE25* was transferred to *E. faecalis* CG110/*gfp* (Scott *et al.*, 2000; Table 1) via filter mating, resulting in strain *E. faecalis* CG110/*gfp*/pRE25*, a new tool for monitoring and quantification gene transfer in complex microbial environments.

Even in the postgenomic era, classical manipulation of large DNA molecules is still inefficient due to technical limitations in purification, size separation, and handling, (Gibson *et al.*, 2010.), and initial attempts to manipulate the 50-kb plasmid pRE25 directly were not successful. *Lactococcus lactis* BuRE25 harbors pRE25 in its chromosome (Perreten, 1995) and this allowed a relatively easy manipulation of the plasmid via homologous recombination. Moreover, *L. lactis* BuRE25 is tetracycline sensitive, thus providing use of the additional selection marker *tet*(M).

The two markers in strain *E. faecalis* CG110/*gfp*/pRE25*, *gfp* and the random sequences on pRE25*, are usually not present in the human intestine, allowing one to distinguish a donor strain from transconjugants in complex background flora by molecular methods such as quantitative PCR.

Characterization of pRE25* in *E. faecalis* CG110/*gfp*/pRE25*

Strain *E. faecalis* CG110/*gfp*/pRE25* harbors a number of ABR genes, and we initially analyzed the presence and function of these genes to characterize the strain. Hybridization using a microarray harboring probes for 90 different ABR genes confirmed the presence of resistance genes against tetracycline, erythromycin, streptothricin, kanamycin, and streptomycin, whereas the presence of *cat* in strain CG110/*gfp*/pRE25* was confirmed by PCR (data not shown). Microdilution test showed phenotypic resistance of strain CG110/*gfp*/pRE25* to chloramphenicol, erythromycin, gentamicin, kanamycin, rifampicin, streptomycin, and tetracycline, with a lower MIC for chloramphenicol and streptomycin compared with *E. faecalis* RE25 (Table 3). Phenotypic resistance of CG110/*gfp*/pRE25* to rifampicin is due to the chromosomally encoded resistance of the host strain CG110 (Jacob & Hobbs, 1974). Tetracycline resistance is encoded on the chromosome and on the plasmid, whereas the other resistance genes are encoded only on pRE25*. The microarray analysis and the resistance pattern of CG110/*gfp*/pRE25* indicates that the strain harbors a complete pRE25*.

The insertion of a 2.7-kb sequence in pRE25* might have an impact on the relative copy number and therefore the copy numbers of pRE25* and pRE25 were determined by qPCR using primer pairs tufA_fw/rv and aph_F/R (Table 2). The *tuf*A gene was used as a chromosomal target gene and *aph*(3′)-III as the plasmid target gene for pRE25 and pRE25*. The copy number of both pRE25* and pRE25 was one to two copies per chromosome, independent of the growth phase (data not shown), indicating that the 2.7-kb insertion in pRE25* had no significant impact on the copy number. This relative low copy number is in agreement with the assumption that large plasmids are present in the cell at low copy numbers (Dale & Park, 2004).

To ensure the genetic stability of the constructed strain, the stable integration of the *gfp* gene and the stable replication of pRE25* in *E. faecalis* CG110/*gfp*/pRE25* was tested. The serial culture test revealed that the integration of *gfp* was stable for at least 200 generations (data not shown), confirming previously described stability for 30 generations (Scott *et al.*, 2000). Replication of pRE25* was also stable, which was expected because plasmids of the Inc18 family, including pRE25, replicate unidirectional by a theta (θ) mechanism, which is usually associated with stable plasmids (Jannière *et al.*, 1990; Bruand *et al.*, 1991). Furthermore, stability of low-copy plasmids in prokaryotes is often secured by a toxin–antitoxin system (Magnuson, 2007), such as the ε/ζ-system on pSM19035 from *Streptococcus pyogenes* (Ceglowski *et al.*, 1993). Sequences of the proteins encoded by ORF18 and ORF49 of pRE25 are highly homologous to the ε-protein (instable antitoxin), ORF19 and

ORF50 to ζ-protein (stable toxin) of pSM19035 (Meinhart *et al.*, 2003), indicating that a toxin–antitoxin system is present on pRE25 and secures its stability.

Although the inserted sequence did not affect copy number and stability of pRE25*, the conjugation potential of pRE25* in *E. faecalis* CG110/*gfp* could be altered compared with pRE25 in *E. faecalis* RE25. Therefore the conjugation potential of both pRE25* in *E. faecalis* CG110/*gfp* and pRE25 in RE25 to other Gram-positive bacteria was examined.

Similar conjugational transfer of pRE25* and pRE25 was observed to *L. monocytogenes* strains LM15 and 10403S, and to *L. innocua* L19 (Table 4). The transfer of pRE25 to *L. innocua* L19 has already been observed at a frequency of 10^{-5} per donor (Schwarz *et al.*, 2001), paralleling our results. Transfer rates of pRE25* were only slightly lower compared with pRE25, which is probably due to the different host strain or the slightly increased plasmid size of pRE25* (Table 1). Transfer of both pRE25 and pRE25* to *L. monocytogenes* LM15 was rather low (Table 4), whereas the transfer frequency of 10^{-6} for *L. monocytogenes* 10403S was in the range of conjugative transfer of broad-host range plasmids (Grohmann *et al.*, 2003).

Conjugal transfer to *L. mesenteroides* M7-1 was only obtained with pRE25, albeit at very low frequency (Table 4). Gene transfer from RE25 to *L. mesenteroides* M7-1 has been observed before at low frequencies (Devirgiliis *et al.*, 2009), and so the unsuccessful transfer of pRE25* from *E. faecalis* to *L. mesenteroides* is probably due to the naturally occurring low efficiency of gene transfer between these species

No transconjugants were obtained with *E. faecalis* 1528, *Lactobacillus fermentum* ROT1, and *Staphylococcus aureus* VG1 as recipients (Table 1), most probably due to plasmids incompatible to pRE25 present in those strains.

The comparison of pRE25* with its parental plasmid pRE25 revealed that the inserted 2.7-kb sequence did not affect the copy number of pRE25*, nor did it have a major impact on its conjugational potential. Furthermore, both pRE25* and the *gfp* marker were stable, showing that *E. faecalis* CG110/*gfp*/pRE25* is suitable as a marker tool to examine horizontal ABR gene transfer in complex microbial communities using elevated experimental durations.

Table 4. Transconjugation rates of filter mating experiments

	Donor	
Recipient	*E. faecalis* RE25	*E. faecalis* CG110/*gfp*/ pRE25*
L. monocytogenes LM15	8.4×10^{-7}	8.2×10^{-8}
L. monocytogenes 10403S	7.2×10^{-6}	5.8×10^{-6}
L. innocua L19	4.6×10^{-5}	9.0×10^{-6}
L. mesenteroides M7-1	7.2×10^{-7}	ND

The transconjugation efficiencies are calculated as transconjugants per donor cells. Averages of two independent experiments are shown.
ND, not detected.

Quantification of transconjugants in complex microbiota

After construction and characterization of *E. faecalis* CG110/*gfp*/pRE25*, the tool was tested in a complex microbial background for its functionality. Fresh overnight cultures of the donor strain *E. faecalis* CG110/*gfp*/pRE25*, the recipient strain *L. monocytogenes* 10403S, and the transconjugant *L. monocytogenes* 10403S/pRE25* (Table 1) were mixed at different transconjugants to donor ratios ranging from 0.2 : 1 to 2000 : 1 in complex microbiota background. The composition of this microbiota was determined by qPCR and consistent with the main groups usually encountered in infant feces (Laboratory of Food Biotechnology, ETH Zurich, unpublished data). Subsequently, donor and transconjugants were quantified by real-time PCR and plate counts. The ratio of pRE25* to *gfp* quantified by real-time PCR was plotted against the ratio calculated from plate counts and showed linear correlation coefficient (R^2 of > 0.99) over a pRE25*/*gfp* ratio of more than three orders of magnitude (Fig. 2). Furthermore, differences as low as 0.2 transconjugants per donor were detectable by qPCR, thereby elaborating the detection limit of the method. This demonstrates that the genetic markers of *E. faecalis* CG110/*gfp*/pRE25* can be quantified in complex backgrounds by qPCR and that *E. faecalis* CG110/*gfp*/pRE25* is indeed a suitable tool for quantification of HGT.

Even though new technologies, for example metagenomic sequencing, yield a deep insight into the human microbiome (Qin *et al.*, 2010), general links between DNA

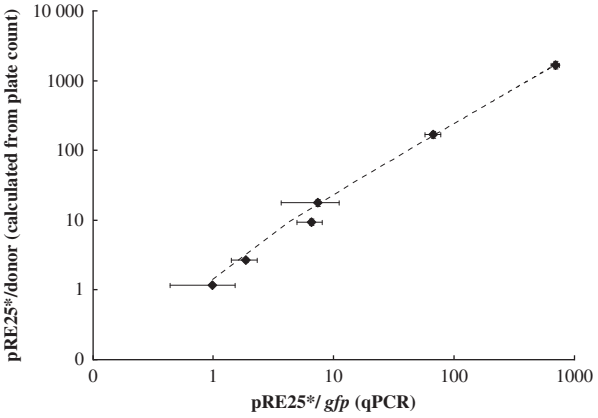

Fig. 2. Correlation of the ratios of total pRE25* to donor cells calculated from plate count to the ratio of pRE25* to *gfp* quantified by quantitative real-time PCR (qPCR). The trendline is depicted in the chart area. R^2 value is > 0.99.

sequences and their transmission route within the microbiota cannot be established using such methods, making use of tagged strains and genes insurmountable for mechanistic studies. The novel strain *E. faecalis* CG110/*gfp*/pRE25* can be applied for investigation of horizontal ABR gene transfer in complex environments in which enterococci and ABR genes are encountered in large numbers, such as food matrices, biofilms or colonic models. The two genetic markers allow the detection and quantification of donor and transconjugant cells independently from the bacterial or ABR gene load in the background flora.

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