

Construction and application of a novel pRE25-derived plasmid to monitor horizontal transfer of antibiotic resistance genes from *Enterococcus faecalis* to food and gut associated microbes in a colonic fermentation model

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**CONSTRUCTION AND APPLICATION OF A NOVEL pRE25-DERIVED PLASMID TO
MONITOR HORIZONTAL TRANSFER OF ANTIBIOTIC RESISTANCE GENES FROM
ENTEROCOCCUS FAECALIS TO FOOD AND GUT ASSOCIATED MICROBES IN A
COLONIC FERMENTATION MODEL**

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Doctor of Sciences

presented by

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ABBREVIATIONS

AA	Amino acid
ABR	Antibiotic resistance
ARE	Antibiotic resistant enterococci
BLAST	Basic local alignment search tool
CFB	<i>Cytophaga-Flavobacterium-Bacteroides</i>
CFU	Colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
Ct	Threshold cycle
DNA	Deoxyribonucleic acid
ESBL	Extended-spectrum β -lactamases
FACS	Fluorescence-activated cell sorting
FISH	Fluorescence <i>in situ</i> hybridization
GFP	Green fluorescent protein
GIT	Gastrointestinal tract
GRE	Glycopeptide-resistant enterococci
HGT	Horizontal gene transfer
HPLC	High-pressure liquid chromatography
IBD	Inflammatory bowel disease
MDR	Multidrug resistance
MGE	Mobile genetic element
MIC	Minimal inhibitory concentration
MLS	Macrolide-lincosamide-streptogramin
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
OD	Optical density
ORF	Open reading frame
PAI	Pathogenicity island
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SCFA	Short chain fatty acid
SNP	Single nucleotide polymorphism
SOE	Splicing by overlap extension
v/v	Volume per volume
w/v	Weight per volume

SUMMARY

The worldwide emergence of antibiotic resistances (ABR) in bacteria poses a serious risk for public health. Horizontal gene transfer (HGT) of ABR determinants, mainly via mobile genetic elements such as plasmids and transposons, contributes for a large extent to the increasing prevalence of bacteria resistant to a single or to multiple antibiotics. The genus *Enterococcus* has an exceptional ability to acquire and transmit ABR genes and is considered to be a major player in the dissemination of ABR genes worldwide. What makes this genus even more suspicious is its emergence as a significant cause of nosocomial and community-acquired infections. However, enterococci belong to the normal gut microflora of humans and animals and are frequently encountered in food products. In this thesis, the hypothesis that an antibiotic resistant *Enterococcus faecalis* strain can transmit its food-related ABR plasmid to *Listeria monocytogenes* and to commensal bacteria of the human gut microbiota in a continuous colonic fermentation system was investigated.

The gut ecosystem is continuously confronted with incoming bacteria from the environment, mainly via ingested food. Especially fermented food, e.g. cheese and sausages, can often harbor high concentrations of antibiotic resistant enterococci (ARE). The high tolerance of enterococci against adverse conditions enables ARE to survive the passage through the gastrointestinal tract. ARE reaching the large intestine might then transfer their resistance genes to members of the gut microbiota, thereby contributing to the spread of ABR genes.

The human gut microbiota, consisting of up to several hundred different species and 10^{14} bacterial cells, is assumed to serve as a reservoir of ABR genes. In Chapter 2, the prevalence of the tetracycline and erythromycin resistance genes *tet(M)* and *erm(B)* in infant fecal samples was determined using quantitative real-time PCR. Both genes were present at average log copy numbers of 7.12 and 6.58 per gram of feces, and even in infants younger than 2 weeks, both genes were detected at high numbers. This high background of ABR genes complicates monitoring of antibiotic gene transfer in the gut ecosystem.

Hence, a suitable ABR donor strain had to be evaluated. The dry sausage isolate *Enterococcus faecalis* SL5.7 was shown to encode the *tet(M)* gene on a 40-kb conjugative plasmid as well as in the chromosome (Chapter 2). The tetracycline resistance was transferable *in vitro* to *E. faecalis* JH2-2 at the high frequency of 7.04×10^{-3} transconjugants per donor cell. The nucleotide sequence of *tet(M)* and the flanking regions revealed 100% homology to Tn916-like elements in *Staphylococcus* and *Streptococcus* isolates.

The high homology of ABR genes in different genera as well as the high ABR gene background in infant feces thus requisite new molecular tools to enable monitoring the fate of a donor strain and the corresponding ABR determinant in complex environments, i.e. the human colonic microflora. The construction of such a tool is described in Chapter 3. The conjugative multiresistance plasmid pRE25 from the *E. faecalis* food isolate RE25 was used as model plasmid for this construction. Plasmid pRE25, previously transferred via filter mating to *Lactococcus lactis* Bu2-60 where it integrated in the chromosome, was genetically marked by the integration of *tet(M)* and two short random sequences. The marked plasmid, named pRE25*, was then transferred by filter mating to *E. faecalis* CG110/*gfp*, a strain genetically tagged in the chromosome with a *gfp* gene. The constructed strain, designated *E. faecalis* CG110/*gfp*/pRE25*, showed similar conjugation behavior as RE25 and, since both the chromosomal and the plasmid marker were stable for at least 200 generations, this strain is an applicable donor strain in horizontal ABR gene transfer experiments.

In Chapter 4 the HGT potential of pRE25* was assessed using a continuous intestinal fermentation model mimicking the infant proximal colonic ecosystem. In a first fermentation, the *E. faecalis* donor strain was co-immobilized with the human pathogen *Listeria monocytogenes* 10403S and feces from a healthy infant in gellan-xanthan beads. Plating of effluent samples during the 8 day fermentation revealed that plasmid pRE25* was transferred to *L. monocytogenes* in the presence of the competing microflora. Fermentation 2 was performed to investigate whether conjugal transfer of the multiresistance plasmid pRE25* occurs to commensal colonic bacteria. *E. faecalis* CG110/*gfp*/pRE25* was co-immobilized

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with feces from a healthy infant but without a defined recipient. To monitor the conjugational behavior of pRE25* during the 16-days fermentation, DNA was extracted from effluent daily. The *gfp* marker, only present in donor cells, and the marked plasmid pRE25* were quantified using real-time PCR during the fermentation and the ratio of pRE25* to the *gfp* gene was calculated to assess conjugal transfer of pRE25*. The pRE25*/*gfp* ratio increased 60% from day 1 to day 16, a clear indication for transfer of pRE25* to commensal fecal bacteria. Transconjugants were isolated on selective media and sequencing of the 16S rRNA genes revealed that pRE25* was transferred to the opportunistic pathogen *Enterococcus avium*. The results described in this thesis clearly confirm that horizontal ABR gene transfer from *E. faecalis* to *L. monocytogenes* and to commensal bacteria can occur in the presence of competing fecal microbiota in a colonic fermentation. Since this model mimics the dense and diverse microbial environment in the human gut, it can be assumed that gene transfer also takes place *in vivo*. Since fermented foods often contain a high load of antibiotic resistant bacteria, the food chain might play a crucial role in the dissemination of ABR genes and this clearly reinforces the requirements for starter- and protective cultures and probiotic products free from transferable ABR genes.

ZUSAMMENFASSUNG

Die weltweite Zunahme von Antibiotika-resistenten Bakterien stellt eine ernstzunehmende Bedrohung für die öffentliche Gesundheit dar. Gene, welche für Antibiotikaresistenz (ABR) kodieren, sind häufig auf mobilen genetischen Elementen wie Plasmiden und Transposons zu finden. Dies trägt massgeblich zur steigenden Verbreitung von Bakterien bei, welche einfach oder mehrfach gegen Antibiotika resistent sind. Der Genus *Enterococcus* besitzt besonders aussergewöhnliche Fähigkeiten, Resistenzgene aufzunehmen und auch weiterzugeben. Enterokokken werden deshalb als Hauptakteure in der Verbreitung von ABR-Genen verdächtigt. Was diesen Genus zusätzlich bedenklich macht, ist seine Bedeutung als Erreger von nosokomialen wie auch ambulant erworbenen Infektionen. Enterokokken gehören zur natürlichen Darmflora von Mensch und Tier. Der menschliche Darm wird stetig mit Bakterien aus der Umwelt, besonders aus der aufgenommenen Nahrung, konfrontiert. Antibiotika-resistente Enterokokken (ARE) sind sehr häufig in fermentierten Lebensmitteln wie Käse und Würsten zu finden. Die ausgeprägte Toleranz von Enterokokken gegenüber ungünstigen Umweltbedingungen befähigt diese Bakterien, die Passage durch den Verdauungstrakt zu überleben. Wenn ARE in den Dickdarm gelangen, können sie möglicherweise ihre Resistenzgene an andere Darmbewohner weitergeben, womit sie massgeblich zur Verbreitung von ABR Genen beitragen würden.

In dieser Doktorarbeit wurde deshalb folgende Hypothese untersucht: Nahrungsmittel-assoziierte ARE können ihre ABR-Gene an Krankheitserreger oder natürlich vorkommende Bakterien der menschlichen Darmflora in einem kontinuierlichen Darmmodell weitergeben.

Es wird vermutet, dass die menschliche Darmflora, bestehend aus mehreren hundert verschiedenen Spezies und bis zu 10^{14} Bakterien, ein Reservoir für ABR-Gene darstellt. In Kapitel 2 dieser Dissertation wurde daher die Verbreitung des Tetrazyklin-Resistenzgens *tet(M)* und des Erythromycin-Resistenzgens *erm(B)* in Fäzesproben von Säuglingen mittels quantitativer Echtzeit-Polymerasen-Kettenreaktion (englisch: real-time PCR) bestimmt. Beide Gene wurden mit durchschnittlichen Log-Kopienzahlen von 7.12 und 6.58 pro Gramm Fäzes

gefunden. Selbst in Stuhlproben von Säuglingen, welche weniger als 2 Wochen alt waren, wurden beide Gene in hoher Konzentration detektiert. Diese hohe Prävalenz von ABR Genen erschwert daher die Untersuchung von horizontalem Gentransfer (HGT) im intestinalen Ökosystem.

Als möglicher Donor für HGT Experimente wurde *E. faecalis* SL5.7, ein aus einer Rohwurst isolierter Stamm, evaluiert. Es konnte gezeigt werden, dass dieser Stamm das *tet(M)* Gen sowohl auf einem 40-kb grossen konjugativen Plasmid als auch integriert im Chromosom trägt. Die Resistenz gegen Tetrazyklin konnte *in vitro* auf *E. faecalis* JH2-2 mit einer hohen Frequenz von 7.04×10^{-3} Transkonjuganten pro Donor übertragen werden. Die Nukleotidsequenz des *tet(M)* Gens sowie der angrenzenden DNA-Regionen zeigte 100% Übereinstimmung mit Tn916-ähnlichen Elementen in Staphylokokken wie auch in Streptokokken. Die hohe Übereinstimmung von ABR Genen in verschiedenen Genera sowie die hohe Verbreitung von ABR Determinanten in Fäzesproben von Säuglingen verlangten deshalb neue molekulare Werkzeuge, welche das Verhalten eines ABR Donorstamms sowie des konjugativen Plasmids in der komplexen Darmflora ermöglichen. Die Konstruktion eines solchen Werkzeuges ist in Kapitel 3 beschrieben. Das konjugative Multiresistenz-Plasmid pRE25 aus dem Lebensmittelisolat *E. faecalis* RE25 wurde als Ausgangsplasmid für Konjugationsexperimente ausgewählt. Das Plasmid pRE25 wurde dafür durch das Einbringen des *tet(M)*-Gens sowie zwei kurzen DNA Fragmenten, welche eine Zufallssequenz aufweisen, genetisch markiert. Das markierte Plasmid mit dem Namen pRE25* wurde anschliessend durch Konjugation mittels "Filter-mating" in den Stamm *E. faecalis* CG110/*gfp* transferiert. Dieser Stamm ist genetisch durch ein chromosomal integriertes *gfp*-Gen markiert. Der neu konstruierte Stamm CG110/*gfp*/pRE25* zeigte ein dem Stamm RE25 vergleichbares Konjugationsverhalten. Zusätzlich waren sowohl der chromosomale Marker wie auch der Plasmidmarker über einen Zeitraum von 200 Generationen stabil. Daraus wurde gefolgert, dass der Stamm CG110/*gfp*/pRE25* ein geeigneter Donorstamm für Konjugationsversuche in komplexen Systemen ist.

In Kapitel 4 wird das HGT-Potenzial von pRE25* in einer kontinuierlichen Intestinalfermentation, welche den aufsteigenden Dickdarm eines Säuglings simuliert, untersucht. In einer ersten Fermentation wurde der *E. faecalis* Donorstamm mit einem humanpathogenen *Listeria monocytogenes* Stamm und der Fäkalflora eines gesunden Säuglings immobilisiert. Mittels Ausplattieren auf Selektivmedien während der 8-tägigen Fermentation konnte gezeigt werden, dass pRE25* trotz der konkurrierenden Fäkalflora auf *L. monocytogenes* übertragen worden ist. Das Ziel der zweiten Fermentation war es, konjugativen Transfer von pRE25* auf kommensale Darmbakterien zu untersuchen. Der Stamm CG110/*gfp*/pRE25* wurde mit Fäzes eines gesunden Säuglings immobilisiert. Um das Konjugationsverhalten des markierten Plasmids pRE25* während der 16-tägigen Fermentation zu überwachen, wurde täglich DNA aus den Fermenterproben extrahiert. Mittels quantitativer Echtzeit-PCR wurde sowohl das *gfp* Gen, welches nur im Donorstamm vorhanden ist, als auch das markierte Plasmid pRE25* quantifiziert. Der Verlauf des Verhältnisses von pRE25* zum *gfp*-Gen sollte folglich Aufschluss über den horizontalen Transfer von pRE25* geben. Es konnte gezeigt werden, dass sich das Verhältnis von pRE25* zu *gfp* zwischen Tag 1 und Tag 16 um 60% erhöht hat, was klar auf einen konjugativen Transfer von pRE25* auf Darmbakterien hinweist. Mittels Selektivmedien konnten Transkonjuganten isoliert werden, welche durch Sequenzierung der 16S rRNA Gene als *Enterococcus avium* identifiziert wurden.

Die in dieser Dissertation beschriebenen Resultate zeigen zum ersten Mal, dass horizontaler Transfer von ABR Genen von *E. faecalis* sowohl auf pathogene als auch auf kommensale Bakterien in Gegenwart einer kompetitiven Darmflora in einer Intestinalfermentation stattfinden kann. Da dieses Intestinalmodell das dichte und komplexe mikrobielle Ökosystem des Dickdarms simuliert, deuten diese Resultate darauf hin, dass ein solcher Gentransfer auch *in vivo* stattfinden kann. Die teilweise hohe Belastung von fermentierten Lebensmitteln mit Antibiotika-resistenten Bakterien lässt deshalb vermuten, dass die Nahrungskette eine bedeutende Rolle in der wachsenden Verbreitung von ABR Genen spielen könnte, was die

ZUSAMMENFASSUNG

Forderungen unterstreicht, dass Starter- und Schutzkulturen sowie probiotische Produkte frei von transferierbaren ABR Genen sein müssen.

1 INTRODUCTION

The discovery and application of antibiotics since the second half of the twentieth century has been a great success story leading to a dramatically decreased morbidity and mortality caused by bacterial infections (Overbye & Barrett, 2005). However, the imprudent use of antibiotics in therapeutics and as growth promoters in animal husbandry has led to the increasing prevalence of antibiotic resistant bacteria worldwide (Smith *et al.*, 2002). The Gram-positive bacterium *Enterococcus faecalis* represents an important example of a species with a high prevalence of antibiotic resistance (ABR) genes. *E. faecalis* is frequently encountered in food fermentations as well as in the gastrointestinal tract (GIT) of humans and animals and well-known for its capability to acquire and spread ABR genes via mobile genetic elements (MGEs). Enterococci in food might survive intestinal passage and the frequent isolation of antibiotic resistant enterococci (ARE) in fermented food products implies therefore a risk for the transmission of resistance genes into the human gut microbiota (Giraffa, 2002). Such transmission might result in an increase of the prevalence and lateral transfer of ABR genes in the gut, thereby constituting an impairment of human health.

1.1 Antibiotic resistance

Since the introduction of penicillin into clinical practice in the 1940s, a huge diversity of naturally occurring or synthetically derived antibiotics has been introduced in human and animal medicine and animal husbandry for treatment and prevention of infectious diseases (Aminov, 2009). Unfortunately, bacteria became rapidly resistant to several classes of clinically relevant antibiotics, thereby hampering effective treatment (Palumbi, 2001). Particularly in the hospital environment, the use of antibiotics leads to the selection of resistant organisms, resulting in difficulties in the treatment of nosocomial infections (Gold & Moellering, 1996). However, the increasing prevalence of ABR is not only restricted to pathogens. Commensal bacteria are developing the same resistances as pathogens, thereby

forming a reservoir of ABR genes (van den Bogaard & Stobberingh, 2000). These resistance genes can then be transferred to other commensals and to pathogens via horizontal gene transfer (HGT; Wang *et al.*, 2006).

Apart from ABR gene transfer, resistant strains themselves also proliferate in the environment. Worldwide dispersion of antibiotic resistant bacteria is often mediated by travelers (Hawkey & Jones, 2009). For example recently discovered multi-drug resistant *Enterobacteriaceae* are widely distributed in India and in the UK, probably due to spread by patients undergone surgeries in Indian hospitals (Kumarasamy *et al.*, 2010). The dissemination of ABR-strains and -genes has resulted in the prevalence of ABR resistance in practical all microbial environments (Hawkey & Jones, 2009).

1.1.1 Mechanisms of antibiotic resistance

There are several different mechanisms that enable bacteria to grow in the presence of antibiotics, encoded by hundreds of different genes (Mazel & Davies, 1999). A major mechanism is the degradation or enzymatic breakdown of the antibiotic, e.g. performed by β -lactamases, which hydrolyze antibiotics such as penicillins and cephalosporines. A second important resistance mechanism are efflux pumps, performing the active export of antibiotics, e.g. tetracyclines, fluoroquinolones, macrolide-lincosamide-streptogramin (MLS) antibiotics and aminoglycosides (Poole, 2007). The modification or replacement of the antimicrobial target is a further mechanism. A relevant example of this resistance mechanism is described for methicillin-resistant *Staphylococcus aureus* (MRSA), a widespread nosocomial pathogen causing very severe infections worldwide. Methicillin resistance in this pathogen is accomplished by the *mecA* gene, encoding a penicillin-binding protein, thereby inactivating methicillin (Hiramatsu *et al.*, 2001). Resistance to macrolides, e.g. erythromycin, azithromycin and clarithromycin, and to tetracycline and vancomycin, can be exhibited by ribosomal protection, thereby preventing the antibiotic from inhibiting protein synthesis (Chopra & Roberts, 2001, Walsh, 2003). Cell-wall modifying enzymes encoded by genes

such as *vanA* lead to resistance to vancomycin, an inhibitor of cell-wall synthesis and one of the last antibiotics to treat MRSA infections (Murray, 2000).

1.1.2 Intrinsic and acquired resistances

Bacteria can be intrinsically resistant to one or more antimicrobials, a trait that has evolved in bacteria before antibiotics were applied worldwide in medicine and husbandry (Tenover, 2006, Martinez *et al.*, 2009). In addition to intrinsic resistance, bacteria possess a remarkable ability to develop new resistances to antibiotics. A single nucleotide exchange can result in drug resistance, as shown for streptomycin resistance in *Mycobacterium tuberculosis* (Snider *et al.*, 1991, Gold & Moellering, 1996). In some bacteria, low-level drug resistance was an initial step of the development of high-level resistance. This was observed in *Enterobacteriaceae*, which have evolved as increasingly resistant to fluoroquinolones due to mutations in the target enzyme (Levy & Marshall, 2004). However, the majority of ABR genes are encoded on MGEs, e.g. conjugative plasmids and transposons (Mazel & Davies, 1999). MGEs are therefore accepted as main sources for horizontal spread of ABR genes, thereby leading the emergence of antibiotic resistant bacteria worldwide.

1.1.3 Antibiotic resistance gene transfer

Transfer of MGEs enables rapid acquisition and dissemination of ABR genes. This results in proliferation of a resistant population due to the continuing antibiotic mediated selection pressure (Ochman *et al.*, 2000, Frost *et al.*, 2005). The intercellular transmission of MGEs occurs via transformation, transduction or conjugation, enabling ABR gene transfer between different taxonomic groups (Levy & Marshall, 2004, Frost *et al.*, 2005). In some cases, sub-inhibitory antibiotic concentrations nonspecifically even favor the lateral transfer of resistance determinants, like e.g. transfer of the transposons *Tn916* and *Tn1545* in Gram-positive bacteria (Doucet-Populaire *et al.*, 1991). The overall use of antibiotics selects for resistant

bacteria, which can transmit their resistance genes further to other microbes (Salyers & Amábile-Cuevas, 1997). Besides ABR determinants, MGEs can also carry genes conferring resistance to mutagenic agents, disinfectants and heavy metals (Tennent *et al.*, 1985, Silver & Misra, 1988). Co-resistance, e.g. resistances to antibiotics as well as to metals encoded on the same MGE, can result in a co-selection process, thereby increasing the spread of ABR genes even in the absence of antibiotic pressure (Baker-Austin *et al.*, 2006, Skurnik *et al.*, 2010). Co-selection has mainly been described in Gram-negative bacteria from water- and soil environments, including aquaculture (McIntosh *et al.*, 2008, Malik & Aleem, 2010), but the phenomenon has also been observed in enterococci isolated from animals receiving copper-supplemented feed (Hasman & Aarestrup, 2002). The use of metals as feed-additives in farm animals may therefore contribute to the high prevalence of ABR in the normal flora of farm animals (Sørum & Sunde, 2001, Singer & Hofacre, 2006).

1.1.3.1 Conjugation

Conjugation is the most frequent mechanism involved in horizontal transfer of ABR genes among bacterial populations (Barlow, 2009). Conjugative transfer requires cell-to-cell contact and formation of interbacterial junctions that enable transfer of DNA (Thomas & Nielsen, 2005). The best-studied MGEs involved in conjugation are plasmids and transposons, which can have extremely wide host ranges and are discussed below (Salyers *et al.*, 1995, Meyer, 2009). Clinically important is ABR gene transfer via chromosomal MGEs, e.g. SCC*mec*, the Staphylococcal Cassette Chromosome *mec* which plays a crucial role in the horizontal spread of methicillin resistance in staphylococci (Bloemendaal *et al.*, 2010).

1.1.3.2 Conjugative plasmids

Horizontal gene spread by conjugative plasmids is the most efficient mechanism of lateral gene transfer (Kurenbach *et al.*, 2003). In Gram-negative bacteria, the formation of the mating-pair formation apparatus, mediated by sex pili, enables cell-to-cell contact and the

development of junctions and pores through which plasmid DNA, converted to single-stranded DNA, is transported (Thomas & Nielsen, 2005). Conjugative plasmids in Gram-negative bacteria often harbor resistances to fluoroquinolones, aminoglycosides, trimethoprim-sulfamethazole and tetracyclines (Lagacé-Wiens *et al.*, 2007), and even to third-generation cephalosporines mediated by extended-spectrum β -lactamases (ESBL).

An increasing problem is the frequent resistance of *Enterobacteriaceae* to extended-spectrum β -lactam antibiotics, complicating the treatment of urinary tract infections and pneumonia caused by e.g. *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. (Paterson, 2006). It is assumed that large multidrug resistance (MDR) plasmids have evolved by sequential addition of resistance determinants to a basic replicon (Dale & Park, 2004).

In Gram-positive bacteria, two types of conjugative plasmids are known: pheromone-responsive plasmids, exhibiting a narrow host range and mainly described in *Enterococcus faecalis*, and pheromone-independent plasmids, which have been found in all bacterial communities and are transferable to a wide host range (Table 1). Under laboratory conditions, transfer of these broad-host range plasmids usually occurs only on solid surfaces and not in liquid medium (Grohmann *et al.*, 2003, Sørensen *et al.*, 2005). It can be noticed that the majority of the ABR plasmids listed in Table 1 originate from *E. faecalis*, emphasizing once more the unique conjugative potential of this species. The emerging prevalence of antibiotic resistant Gram-positive bacteria poses serious health risks, especially in the clinical environment. Treatment of infections caused by Gram-positive pathogens is increasingly hampered by the emerging spread of ABR plasmids, especially in nosocomial settings (Tenover, 2006).

Table 1. Examples of large conjugative ABR plasmids in Gram-positive bacteria, their origin and their characteristics.

Plasmid	Original host	Origin	Resistance phenotype	Plasmid size	Host range	Reference
pKL0018	<i>Lc. garviae</i>	fish	MLS, Tet	20.0 kb	n.d.	Maki <i>et al.</i> , 2009
pSK41	<i>S. aureus</i>	clinical	Bm, Gen, Kan, Neo, Tob	46.4 kb	<i>Staphylococcus</i>	Firth <i>et al.</i> , 1993
pGO1	<i>S. aureus</i>	clinical	Bm, Gen, Kan, Neo, Tob, Trp	52.0 kb	<i>Staphylococcus</i>	Morton <i>et al.</i> , 1993
pLW1043	<i>S. aureus</i>	clinical	β -lactams, Gen, Kan Trp, Van	57.9 kb	<i>Staphylococcus</i>	Weigel <i>et al.</i> , 2003
pIP501	<i>S. agalactiae</i>	clinical	Cm, Gen, Kan, MLS, Neo, Str	30.2 kb	broad	Le Chatelier <i>et al.</i> , 1993
pIP816	<i>E. faecium</i>	clinical	Van	34.6 kb	broad	Sletvold <i>et al.</i> , 2010
pRUM	<i>E. faecium</i>	clinical	Cm, Ery, Str, Sth	24.9 kb	broad	Grady & Hayes, 2003
pAM61	<i>E. faecalis</i>	clinical	MLS	26.5 kb	broad	Bruand <i>et al.</i> , 1991
pSL40	<i>E. faecalis</i>	sausage	Tet	40 kb	<i>E. faecalis</i> ^a	Leisibach, 2004
pRE25	<i>E. faecalis</i>	salami sausage	Cm, MLS	50.2 kb	broad	Schwarz <i>et al.</i> , 2001
pRE39	<i>E. faecalis</i>	minced meat	MLS	26.5 kb	broad	Teuber <i>et al.</i> , 1999
pAMS1	<i>E. faecalis</i>	root canal	Cm, Str, Tet,	130 kb	<i>E. faecalis</i> (PR)	Flannagan <i>et al.</i> , 2008
pAM323	<i>E. faecalis</i>	clinical	Ery	~66 kb	<i>E. faecalis</i> (PR)	Murray <i>et al.</i> , 1988
pAM368	<i>E. faecalis</i>	clinical	Van	~107 kb	<i>E. faecalis</i> (PR)	Showsh <i>et al.</i> , 2001
pBEM10	<i>E. faecalis</i>	clinical	Gen, Kan, Pen, Tob	~70 kb	<i>E. faecalis</i> (PR)	Murray <i>et al.</i> , 1988
pCF10	<i>E. faecalis</i>	clinical	Tet	67.7 kb	<i>E. faecalis</i> (PR)	Dunny, 2007
pMG2200	<i>E. faecalis</i>	clinical	Van	106.5 kb	<i>E. faecalis</i> (PR)	Zheng <i>et al.</i> , 2009
pMG2201	<i>E. faecalis</i>	clinical	Ery	60 kb	<i>E. faecalis</i> (PR)	Zheng <i>et al.</i> , 2009

Abbreviations: *E.*: *Enterococcus*; *Lc.*: *Lactococcus*; *S.*: *Staphylococcus* or *Streptococcus*; Ami: amikacin; Amp: ampicillin; Bm: bleomycin; Cm: chloramphenicol; Ery: erythromycin; Gen: gentamicin; Kan: kanamycin; MGS: macrolide-lincosamide-streptogramin; Neo: neomycin; Pen: penicillin; Str: streptomycin; Sth: streptothricin; Tob: tobramycin; Trp: trimethoprim; Tet: tetracycline; Van: vancomycin; n.d.: no data available; PR: pheromone-responsive plasmid.
^a only genus tested

1.1.3.3 Conjugative transposons

Conjugative transposons are further MGEs contributing to horizontal spread of ABR between different genera (Salyers *et al.*, 1995). The common transfer mechanism includes excision of the transposon from the genome by site-specific recombination, integration of the resulting covalently closed circular form into a different site of the original genome, or conjugative transfer to a recipient cell and integration into the genome of the new host (Salyers *et al.*, 1995, Burrus *et al.*, 2002). Unlike plasmids, conjugative transposons do not harbor an origin of replication nor do they encode replicative genes (Burrus *et al.*, 2002).

One of the best described groups of such mobile elements is the Tn916-1545 family of conjugative transposons. Tn916, a 18-kb conjugative transposon harboring *tet(M)*, was first identified in *E. faecalis* DS16, a hemolytic multidrug resistant clinical isolate (Franke & Clewell, 1981). The genetic organization of Tn916 transfer regulation is depicted in Figure 1.

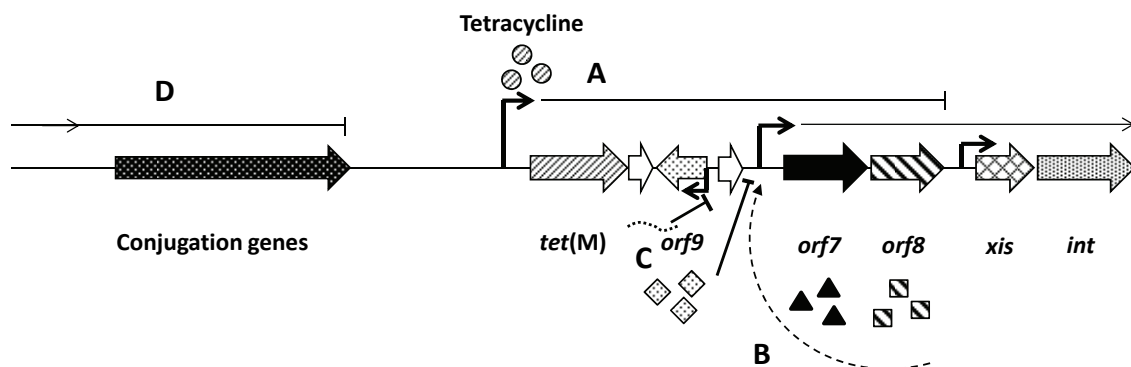


Figure 1. Regulation of transfer of Tn916 (18'032 bp). A) In the presence of tetracycline, the transcript starting at *tet(M)* and continuing to *orf7* is prolonged through to *orf8* (continuous line). B) Gene products of *orf7* and *orf8* then upregulate the expression of the promoter upstream of *orf7* (dashed line). C) Antisense RNA transcribed through *orf9* (dotted line) represses the translation of the *orf9* mRNA, which acts as a repressor of the promoter upstream of *orf7*. D) This leads then to the elongation of transcripts from *orf7* through *xis*, *int* and the conjugation genes in case that Tn916 is excised and in its circular form (adapted from Wozniak & Waldor, 2010).

The 25.3-kb conjugative transposon Tn1545 was first described in *Streptococcus pneumoniae* and harbors *tet(M)*, *erm(B)* and *aphA-3*, encoding resistances to tetracycline, erythromycin and kanamycin, respectively (Courvalin & Carlier, 1987). Conjugative transposons of the Tn916-Tn1545 family have an extremely wide host range, including many commensal and pathogenic bacteria belonging to the phyla Actinobacteria, Deinococcus-Thermus, Firmicutes, Fusobacterium, Proteobacteria and Tenericutes, and are therefore supposed to play a significant role in the development of multiple-drug-resistant bacteria (Clewell *et al.*, 1995, Roberts & Mullany, 2009). A concerning feature of Tn916-Tn1545 transposons is the occasional observation of elevated transfer frequency in the presence of subinhibitory tetracycline concentrations. This was observed in conjugation experiments between *E. faecalis* and *L. monocytogenes* and between *Bacillus* spp. (Doucet-Populaire *et al.*, 1991, Torres *et al.*, 1991, Showsh & Andrews, 1992). Tetracyclines, usually orally administered, are bacteriostatic antibiotics, which inhibit growth of sensitive bacteria but are not bactericidal (Chopra & Roberts, 2001). During antibiotic treatment, higher tetracycline concentrations in the intestine can lead to the proliferation of resistant bacteria whereas possible sensitive recipients remain viable. Decreasing tetracycline concentrations after tetracycline therapy might then result in transfer of Tn916-Tn1545-like elements to sensitive bacteria (Licht *et al.*, 2003, Moubareck *et al.*, 2003).

1.1.4 Challenges of horizontal antibiotic resistance gene transfer

The worldwide prevalence of antibiotic resistant bacteria has increased dramatically due to inappropriate use of antibiotics in human and animal medicine, in animal husbandry and in aquaculture (Aminov, 2009). ABR confers a selective growth advantage in the presence of antibiotics, enabling proliferation and eventually overgrowth of resistant strains (Gold & Moellering, 1996). Moreover, spontaneous mutations and the acquisition of ABR genes by HGT contribute further to the spread of antibiotic resistant bacteria (Levy & Marshall, 2004). Bacteria resistant to one antibiotic can acquire multiple resistance genes, especially after long-term use of a single antibiotic, thereby becoming even more difficult to treat (Levy & Marshall, 2004). As a worst case scenario, ABR genes can be transferred from commensal bacteria to pathogens, complicating treatment of certain bacterial infections (Tenover, 2006). The increasing prevalence of ABR in pathogenic bacteria leads to higher infectious morbidity and mortality in both humans and animals, thereby increasing the treatment costs (Hawkey & Jones, 2009). Another problem is that ABR determinants are maintained in nature, even in the absence of antibiotic-mediated selection pressure. Although antimicrobial resistance confers a fitness cost in bacteria, the loss of ABR plasmids is usually very low (Hegstad *et al.*, 2010). Plasmid stability is most probably assured by plasmid-encoded toxin-antitoxin systems, which assures stable plasmid inheritance, thereby facilitating the spread of antibiotic resistant bacteria also in antibiotic free environments (Levy & Marshall, 2004, Martinez, 2009). This can result in high ABR gene numbers in feces of people never treated with antibiotics and even in infants (Gueimonde *et al.*, 2006). The prevalence, spread and maintenance of ABR genes imply the importance of worldwide regulations of antibiotic use in all fields. Regulations should include guidelines to minimize the prevalence of antibiotic resistant bacteria in clinical settings by isolating colonized patients, the absence of transferable ABR genes in starter- and protective cultures and probiotic products, and compliance with specified limit values of antibiotics in food and animal feed (Levy & Marshall, 2004). To reduce the prevalence and development of ABR, the European Union has banned the use of antibiotics as

growth promoters in husbandry since 2006 (www.efsa.europa.eu). The U.S Food and Drug Administration (FDA; www.fda.gov) has recently issued a draft guidance concerning measurements to limit antimicrobial use in food-producing animals in order to reduce selective pressure leading to ABR. Moreover, there is a clear need in the development of new antimicrobial drugs against Gram-positive and Gram-negative MDR bacteria, as well as the development of alternative therapeutic measurements (Martinez *et al.*, 2007, Theuretzbacher, 2009, Gootz, 2010). These measurements and actions will be necessary to contain the current situation and assure control of bacterial infections in the 21st century.

1.2 The genus *Enterococcus*

1.2.1 Characteristics

The genus *Enterococcus* consists of Gram-positive, facultatively anaerobic and catalase negative cocci, which occur singly, in pairs or as short chains (Murray, 1990). Enterococci belong to the lactic acid bacteria, have a low G+C content (<50 mol%) and are well-known bacteriocin producers (Nes *et al.*, 2007, Fisher & Phillips, 2009, Birri *et al.*, 2010). The majority of enterococcal strains are homofermentative, characterized by lactic acid as the major end-product of glucose metabolism and the absence of gas production (Facklam *et al.*, 2002). Enterococci grow between 5°C and 45°C, with an optimum growth temperature of 35°C. All *Enterococcus* species are able to grow at a wide pH range from 4 to 9.6, at high salt concentration of 6.5% NaCl, and in the presence of 40% bile salt. This capability to growth under relative harsh conditions gives enterococci a selective growth advantage against many other bacteria (Facklam *et al.*, 2002, Franz *et al.*, 2003), which can also be observed under laboratory conditions on media claimed to be selective for other genera than enterococci. *E. faecalis* and *E. faecium*, the predominant enterococcal species involved in nosocomial infections (Sundsford & Willems, 2010), survive temperatures of 60°C for 30 min, emphasizing the stress tolerance of the genus (Foulquié Moreno *et al.*, 2006).

According to the German Collection of Microorganisms and Cell Cultures (DSMZ), the genus *Enterococcus* comprises 40 species (www.dsmz.de), with *E. thailandicus* as the most recently described species (Tanasupawat *et al.*, 2008). *E. faecalis* V583, a vancomycin-resistant pathogenic clinical isolate (Sahm *et al.*, 1989), was the first *Enterococcus* strain whose genome sequence was completely determined (Paulsen *et al.*, 2003). Remarkably, more than 25% of the 3.22-Mb genome of strain V583 consists of exogenously acquired DNA or MGEs including 7 phage-associated regions (Paulsen *et al.*, 2003, Yasmin *et al.*, 2010), whereas the completely sequenced strain OG1RF (2.74 Mb) possesses almost no MGEs (Bourgogne *et al.*, 2008). A partially sequenced *E. faecalis* isolate, Symbioflor 1, is applied as probiotic to

alleviate chronic bronchitis and sinusitis symptoms (Domann *et al.*, 2007). The genomic sequence of this strain revealed the absence of many virulence factors, e.g. enterococcal cytolysin, surface protein and gelatinase, commonly present in other strains (Domann *et al.*, 2007). The absence of such virulence determinants is an important feature before strains can be applied in probiotic products like Symbioflor 1 (Domann *et al.*, 2007, Wassenaar & Klein, 2008).

The main habitat of enterococci is the GIT of nearly all mammals and birds, with *E. faecalis* and *E. faecium* as most prevalent species (Aarestrup *et al.*, 2002, Palmer *et al.*, 2010). Enterococci are usually occurring at $10^2 - 10^8$ CFU/g of digestive content (Ogier & Serror, 2008). However, the remarkable ability of enterococci to withstand higher temperatures, a wide pH-range, oxidative stress and high osmotic pressure led to distribution of enterococci in numerous environment e.g. in fermented foods (Giraffa, 2002). The combination of this ubiquity with the ability to acquire and spread ABR genes renders the genus *Enterococcus* highly suspicious as a main player in the increasing prevalence of antibiotic resistant bacteria.

1.2.2 Virulence of enterococci

For a long time, enterococci have been considered as harmless commensals. Nowadays, they have arisen as important causes of clinical and community-acquired infections, mainly caused by the species *E. faecalis* and *E. faecium* (Giraffa, 2002, Tan *et al.*, 2010). Enterococcal infections can appear manifold, e.g. as urinary tract infections, neonatal sepsis, bacteremia, endocarditis, peritonitis, infections of the central nervous system, and wound infections (Murray, 1990, Sava *et al.*, 2010, Tan *et al.*, 2010). A significant part of enterococcal bacteremia and infective endocarditis are presumed to be endogenous, e.g. by enterococci translocating from the intestinal epithelial cells and via lymph nodes to other parts of the body (Franz *et al.*, 1999, Giraffa, 2002).

Adhesion to host tissue is considered as a fundamental trait for the ability to cause infections (Koch *et al.*, 2004), and the enterococcal aggregation substance is thought to increase

adherence to renal or intestinal epithelial cells and to macrophages, thereby rendering enterococci potent pathogens (Koch *et al.*, 2004). Two additional surface proteins, Ace (adhesion of collagen from *Enterococcus faecalis*; Nallapareddy *et al.*, 2000, Koch *et al.*, 2004) and Esp, frequently found in clinical isolates (Fisher & Phillips, 2009), seem to contribute to adhesion and colonization and to biofilm formation (Foulquié Moreno *et al.*, 2006). Cytolysin (hemolysin), a bacterial toxin causing eukaryotic cell lysis, is predominantly found in clinical isolates, especially in gentamicin resistant strains (Haas & Gilmore, 1999). Gelatinase, encoded by *gelE* (Su *et al.*, 1991) and frequently found in fecal, clinical and food isolates, is involved in the hydrolysis of gelatin, collagen and hemoglobin (Foulquié Moreno *et al.*, 2006). Gelatinase and other hydrolytic enzymes, e.g. hyaluronidase and serine protease, are involved in the pathogenesis of enterococcal infections, even though the mechanisms have not yet been elucidated (Fisher & Phillips, 2009).

Enterococcal infections seem to be caused by the interaction of various different virulence determinants rather than by one single essential virulence factor (Aakra *et al.*, 2007, Vebø *et al.*, 2010). Virulence in several *E. faecalis* strains can be linked to a large (>150 kb) and highly variable pathogenicity island (PAI), which harbors most determinants encoding enterococcal virulence traits such as cytolysin, aggregation substance and enterococcal surface protein (Shankar *et al.*, 2002). The enterococcal PAI was originally identified in the vancomycin resistant *E. faecalis* strain V583 and in the high-level gentamicin resistant *E. faecalis* strain MMH594, the latter being a clinical isolate that had caused severe infections in the mid-1980s (Shankar *et al.*, 2002). The complete PAI has been conjugatively transferred from strain V583 to the laboratory strain OG1RF, and transfer seemed to be dependent on the presence of pheromone-responsive plasmids in the donor strain (Manson *et al.*, 2010).

However, the detection of virulence determinants in various nonclinical *E. faecalis* isolates indicates that the presence of these determinants alone does not necessarily result in an infectious phenotype (Abriouel *et al.*, 2008, Solheim *et al.*, 2009, van Schaik & Willems, 2010).

1.2.3 Antibiotic resistance in enterococci

Enterococci are intrinsic resistant to a wide variety of antimicrobials including cephalosporins and low levels of aminoglycosides, whereas also lincosamides resistance is found in many strains (Murray, 1990, Shepard & Gilmore, 2002). However, enterococci efficiently acquire and transmit ABR determinants via MGEs, e.g. conjugative plasmids and transposons (Clewell, 1990, Huycke *et al.*, 1998, Klare *et al.*, 2003). Bacteriophages are supposed to play an additional role in the horizontal transfer of ABR as well as virulence genes (Yasmin *et al.*, 2010).

The high prevalence of transferable ABR determinants in enterococci combined with the increasing involvement of enterococci in nosocomial infections indicates a major hazard for human health (Huycke *et al.*, 1998). The most prevalent acquired resistance in enterococci strains isolated from various sources is tetracycline resistance (Teuber *et al.*, 1999, Peters *et al.*, 2003). The ABR profile of 106 *E. faecalis* isolates from food, fecal and animal samples revealed tetracycline resistance as the most frequent resistant phenotype (58% of the isolates) followed by erythromycin resistance (33% of the isolates) (McBride *et al.*, 2007). Furthermore, tetracycline resistance was also the most frequent resistance phenotype in *E. faecalis* isolates from 11 healthy Norwegian infants (Solheim *et al.*, 2009). Tetracycline resistance in enterococci is often mediated by the ribosomal protection gene *tet(M)* that also confers resistance to doxycycline and minocycline (Chopra & Roberts, 2001). The high frequency of *tet(M)*-mediated tetracycline resistance in enterococci is due to the wide distribution of *tet(M)* harboring Tn916-1545-type transposons in this genus (Huys *et al.*, 2004). Another common ABR gene in enterococci is *erm(B)*, encoding an rRNA methylase that leads to the modification of the target site for MLS antibiotics (Seppälä *et al.*, 1998). This resistance gene is found on Tn1545 and other transposons, as well as on conjugative plasmids (Jensen *et al.*, 1999, Schmitz *et al.*, 2000, Huys *et al.*, 2004, Jackson *et al.*, 2007).

A conjugation system mainly described in *E. faecalis* is the sex pheromone system mediating conjugative plasmid transfer (Clewell, 1990). Conjugal transfer of pheromone-responsive plasmids occurs at high transfer rates in liquid medium. Potential recipient cells excrete sex pheromones that induce synthesis of a surface adhesion compound, termed aggregation substance, in the *E. faecalis* donor strain. The aggregation substance enables clumping of the cells and cell-to-cell contact that is essential for conjugative plasmid transfer (Wirth, 1994). Several sex pheromone plasmids carry ABR genes, thereby contributing to the increasing ABR in *E. faecalis* (Table 1; Wirth, 1994, Dunny, 2007). Moreover, aggregation substance contributes to the virulence of *E. faecalis* since it is involved in the adhesion of *E. faecalis* to the host tissue (Hirt *et al.*, 2005, Chuang *et al.*, 2009). The ABR and the virulence factors on pheromone-responsive plasmids indicate that these plasmids are a major problem in treatment of *E. faecalis* infections.

Beside conjugative transposons and pheromone responsive plasmids, conjugative plasmids of the incompatibility group Inc18 are important determinants in dissemination of ABR genes. In laboratory, these plasmids are transferred only on solid media and exhibit a broad host range (Weaver *et al.*, 1996). Plasmids belonging to the Inc18 family, e.g. the two *Streptococcus* plasmids pIP501 and pSM19035 and the two *Enterococcus* plasmids pAM β 1 and pRE25 (Table 1), are large plasmids with low-copy numbers that replicate by the unidirectional θ -mechanism (Clewell *et al.*, 1974, Bruand *et al.*, 1991, Ceglowski *et al.*, 1993b, Schwarz *et al.*, 2001). Inc18-plasmids frequently harbor resistances to the MLS antibiotics, chloramphenicol, aminoglycosides, streptomycin, methicillin and vancomycin (Schwarz *et al.*, 2001, Flanagan *et al.*, 2003, Zhu *et al.*, 2008, Lioy *et al.*, 2010). Inc18-plasmids are transferable to *E. coli* and to a wide variety of Gram-positive bacteria, including *Streptococcus*, *Enterococcus*, *Staphylococcus* and *Listeria* (Abajy *et al.*, 2007).

The Inc18-plasmid pRE25 was identified in a multiresistant *E. faecalis* strain isolated from a dry sausage (Table 1, Figure 2; Perreten, 1995). This 50-kb plasmid encodes 58 open reading frames including the ABR genes *cat*, *erm(B)*, *aadK*, *sat4* and *aph(3')-III*, encoding resistance to chloramphenicol, erythromycin and high levels of aminoglycosides (Figure 2; Schwarz *et al.*, 2001).

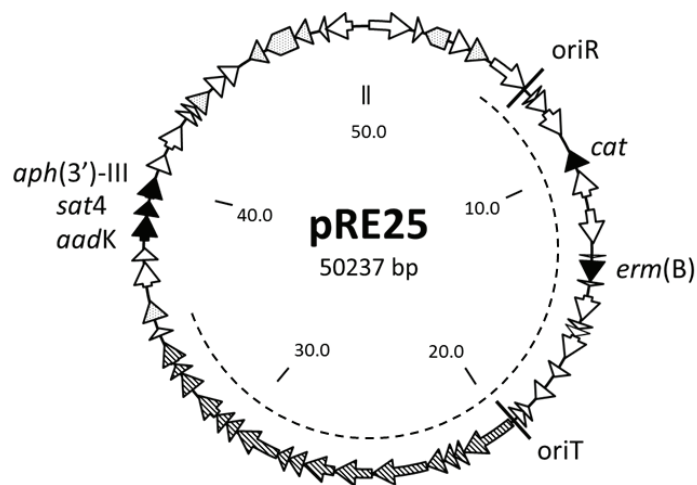


Figure 2. Circular map of the conjugative plasmid pRE25. ABR genes are depicted as solid arrows, IS elements as dotted arrows, and genes involved in plasmid transfer as shaded arrows. White arrows represent genes not involved in gene transfer or ABR, most of them with hypothetical functions. The dashed semicircle indicates regions almost identical to the *Streptococcus agalactiae* plasmid pIP501. OriT is origin of transfer, oriR is origin of replication. For functions of ABR genes see text. The nucleotide sequence of pRE25 is available under the GenBank/EMBL accession number X92945 (picture drawn based on information from Schwarz *et al.*, 2001).

Plasmid pRE25 was transferred *in vitro* to *E. faecalis* JH2-2, *L. innocua* L19 and *L. lactis* Bu2-60 (Schwarz *et al.*, 2001). A 30.5-kb region of pRE25, flanked by two insertion elements, is almost 100% identical to the streptococcal broad-host range plasmid pIP501, presuming that pRE25 derived from pIP501 (Teuber *et al.*, 2003). Moreover, the regions encoding the transfer (*tra*) genes in pIP501 and pRE25 are highly similar to the corresponding regions in the *Staphylococcus* plasmids pGO1 and pSK41 and the *Lactococcus* plasmid pMRC01, suggesting an ancestrally related transfer mechanism (Kurenbach *et al.*, 2003).

The diverse mechanisms in enterococci to acquire and disseminate ABR genes result in the increasing clinical importance of this opportunistic pathogen (Cantón *et al.*, 2010). Nowadays, the most relevant medical impact arises from glycopeptide resistant enterococci (GRE; Tacconelli & Cataldo, 2008), since glycopeptide therapy is the standard treatment in severe nosocomial *Enterococcus* infections (Klein, 2003). Infections with GRE are associated with high mortality and morbidity rates (Tacconelli & Cataldo, 2008). The oral administration of glycopeptides, e.g. vancomycin, probably caused the emerging vancomycin resistance in enterococci (Rice, 2001). The high-potential of gene transfer by enterococci is a serious concern in public health. Vancomycin is the first-line therapy in severe MRSA infections (DeLeo *et al.*, 2010, Welte & Pletz, 2010). Because plasmid-mediated transfer of the vancomycin resistance gene *vanA* to MRSA has already been observed (Sievert *et al.*, 2008, Palmer *et al.*, 2010), it is plausible that treatment of MRSA infections will become even more complicated (DeLeo *et al.*, 2010, Palmer *et al.*, 2010). It is therefore essential to understand the frequencies and basics of ABR transfer by enterococci, to minimize the occurrence of ABR strains and to control bacterial infections in the future.

1.2.4 Enterococci in food

E. faecalis and *E. faecium* are frequently encountered in food from animal origin, e.g. cheese, sausages and other meat products, and fish (Foulquié Moreno *et al.*, 2006). The role of enterococci in dairy products is rather ambiguous. In Mediterranean-type cheeses, enterococci

contribute to flavor development and organoleptic properties (Ogier & Serror, 2008). Due to their beneficial role in ripening, enterococci are also applied as starter cultures in various European cheeses (Franz *et al.*, 2003). Furthermore, dairy product associated enterococci frequently produce bacteriocins that are active against food pathogens, e.g. *Listeria monocytogenes*, thereby contributing to fermentation stability and shelf-life of the end-product (Leroy *et al.*, 2003, Izquierdo *et al.*, 2009, Bayoub *et al.*, 2010).

In fermented sausages, enterococcal cell numbers usually reach 10^2 to 10^5 CFU/g (Teuber *et al.*, 1996). The most prevalent *Enterococcus* species in raw and fermented meat are *E. faecalis* and *E. faecium* (Franz *et al.*, 2003, Rizzotti *et al.*, 2005, Gomes *et al.*, 2008). The application of enterococci as adjunct starter culture in meat fermentation is less studied compared to cheese, but *E. faecalis* and *E. faecium* are able to establish during sausage fermentation, to acidify the product and to inhibit growth of *Listeria* spp. during fermentation (Callewaert *et al.*, 2000). However, adjunct *Enterococcus* cultures must not contain transferable ABR genes and should not produce biogenic amines.

Since the animal GIT is a main habitat of enterococci, the risk of meat contamination during slaughtering is generally high (Franz *et al.*, 2003) and in many countries, enterococci are considered as indicator organisms for hygienic food production (Burdychova & Komprda, 2007). Furthermore, enterococci are also food spoilers and many strains produce biogenic amines that are potentially toxic for the consumer (Burdychova & Komprda, 2007). Due to their aptitude to grow in a wide range of environmental conditions, enterococci have also been detected in fruits and vegetables, where *E. casseliflavus* seems to be a predominant species (McGowan *et al.*, 2006). In Spanish-style olives, *E. faecalis* and *E. faecium* might play a role during fermentation (Foulquié Moreno *et al.*, 2006). Taken together, *Enterococcus* is a versatile genus encountered in many food-products where it can be involved in food-spoilage as well as contribute to food quality.

1.2.5 Antibiotic resistant enterococci in food

The frequent presence of enterococci in animal-derived food and the high prevalence of antimicrobial resistance in this genus inevitably leads to the presence of ARE in foods (Giraffa, 2002). ARE can reach high cell numbers in food and harbor resistance determinants against a wide range of antibiotics, including ampicillin, penicillin, chloramphenicol, tetracycline, erythromycin, ciprofloxacin, streptomycin, gentamicin, quinupristin/dalfopristin, nitrofurantoin, kanamycin, and vancomycin (Teuber *et al.*, 1996, Franz *et al.*, 2001, Cocconcelli *et al.*, 2003, Peters *et al.*, 2003, Huys *et al.*, 2004, Garofalo *et al.*, 2007, Templer & Baumgartner, 2007, Barbosa *et al.*, 2009). Due to the ability of enterococci to withstand a wide range of adverse conditions, they can survive and proliferate during food fermentations. This raises the question whether transfer of ABR genes can also occur during food processing. Indeed, conjugative transfer of the pheromone plasmid pCF10, harboring *tet(M)*, and of a conjugative plasmid carrying the *vanA* gene from clinical *E. faecalis* isolates to *E. faecalis* food isolates occurred in cheese and sausage fermentation models. Even in the absence of antibiotic pressure, high transfer rates of up to 10^{-3} transconjugants per recipient were obtained (Cocconcelli *et al.*, 2003). These observations indicate a potentially important role of ARE in the spread of ABR genes. Moreover, they emphasize the need for starter strains free of transferable ABR genes. If ARE from food survive the passage through the GIT and transiently colonize the intestine, resistance genes might be transferred to commensals, resulting in an increasing ABR gene pool in the human gut microbiota (Berchieri, 1999, Sørensen *et al.*, 2001, Lund *et al.*, 2002, Franz *et al.*, 2003). ABR gene transfer from commensal to pathogenic bacteria would then enhance the risk of ineffective antibiotic treatment of infections. To understand such risk, the incidence of ABR genes in the GIT including transfer rates and possible transfer routes should be determined. However, the GIT is a highly complex ecosystem and such analyses are hampered by the high background of ABR-genes, the innumerable number of strains residing in the gut, and the complicated accessibility of the GIT.

1.3 The human gastrointestinal tract

1.3.1 Physiological functions of the gastrointestinal tract

The human GIT, a coiled, muscular tube, is subdivided into seven functional parts: mouth, pharynx, esophagus, stomach, small intestine, large intestine and anus. Each section has specific functions in digestion, absorption, secretion and fermentation (Saulnier *et al.*, 2009). The human GIT has several important physiological functions. Food particles are chemically and mechanically digested and transported through the GIT. Substances important for human metabolism are released during this degradation and are absorbed by epithelial cells. Finally, the gut microbiota contributes to the breakdown of carbohydrates and proteins, thereby releasing short chain fatty acids (SCFA) which are also absorbed by epithelial cells and contribute to approximately 10% of the energy uptake of the body (Saulnier *et al.*, 2009).

1.3.2 The microbiota of the gastrointestinal tract

The gastrointestinal microbiota outnumbers the number of eukaryotic cells in the human body by at least a factor of 10 (Manson *et al.*, 2008). It is assumed that the human gut microbiota consists of up to 1000 different species (Zhao, 2010) that can be divided into two groups, the autochthonous flora comprising residing bacteria occupying an ecological niche, and the allochthonous flora either passing through or transiently colonizing the GIT (Manson *et al.*, 2008).

Each functional part of the GIT harbors a typical microbial composition which is influenced by disease, antibiotic therapy, diet or age (Manichanh *et al.*, 2006, Macfarlane & Macfarlane, 2009, Saulnier *et al.*, 2009). The first cavities of the GIT, mouth and pharynx, harbor a highly diverse microbiota consisting of more than 600 bacterial species, of which a third has not yet been cultivated (Dewhirst *et al.*, 2010). In the esophagus, the food transit time is usually below 1 min, leading to a decrease in bacterial concentrations to 10^2 per ml (Manson *et al.*, 2008, Macfarlane & Macfarlane, 2009). After the esophagus, the food enters the stomach

where secretion of HCl can decrease the pH to as low as 2 (Blaut, 2002). This results in sparse colonization with cell counts usually reaching not more than 10^3 per ml content (Manson *et al.*, 2008). Two recent studies using 16S rRNA gene sequencing to analyze the stomach microbiota showed that the predominant phyla are Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria and Fusobacteria, and approximately 40% of all clones belonged to the two genera *Streptococcus* and *Prevotella* (Bik *et al.*, 2006, Li *et al.*, 2009). Thereafter, in the small intestine, the bacterial population increases again, reaching cell numbers of approximately 10^6 per ml of content (Saulnier *et al.*, 2009). Aerotolerant and facultatively anaerobic bacteria, e.g. lactobacilli, streptococci and staphylococci, are the main inhabitants of the upper part of the small intestine (Saulnier *et al.*, 2009). Finally, in the large intestine, the highest colonization of the GIT occurs, with cell numbers of up to 10^{12} bacteria per gram of content and a number of species estimated between 500 and several thousands (Frank *et al.*, 2007, Sekirov *et al.*, 2010).

1.3.2.1 The microbiota of the large intestine

The large intestine is divided into cecum, proximal colon, transverse colon, distal colon and sigmoid colon, and performs important functions such as absorption of water and electrolytes and the storage and excretion of waste (Bharucha, 2008). It is the most heavily colonized cavity in the human GIT and the predominant species belong to two different phyla, Firmicutes and the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group (Eckburg *et al.*, 2005, Manson *et al.*, 2008, Qin *et al.*, 2010). It is suggested that 60-80% of colonic or fecal bacteria belong to these two phyla (Duncan *et al.*, 2007). The genus *Bifidobacterium*, belonging to the phylum Actinobacteria, is assumed to represent a subdominant population in adults, whereas it constitutes a predominant genus in infants (Penders *et al.*, 2007, Manson *et al.*, 2008, Furet *et al.*, 2009, Fallani *et al.*, 2010).

Most Firmicutes residing the large intestine belong to the *Clostridium coccoides* group (*Clostridium* subcluster XIVa) and *Clostridium leptum* group (*Clostridium* subcluster IV). Bacteria belonging to the *C. coccoides* group include members of the genera *Clostridium*, *Eubacterium*, *Ruminococcus*, *Coprococcus*, *Dorea*, *Lachnospira*, *Roseburia* and *Butyrivibrio* (Manson *et al.*, 2008). Many species of this *Clostridium* cluster, e.g. *Roseburia intestinalis* and *Eubacterium rectale*, produce butyrate which is assumed to have health-promoting effects (Duncan *et al.*, 2002a, Louis & Flint, 2009). *R. intestinalis* and many other species of the *C. coccoides* group are extremely sensitive to oxygen, surviving for less than 2 min if exposed to oxygen on an agar surface (Duncan *et al.*, 2006, Flint *et al.*, 2007), thereby complicating cultivation of such bacteria. The *C. leptum* cluster comprises approximately 25% of the colonic microbiota (Flint, 2006). The most prevalent species of this group is *Faecalibacterium prausnitzii* (Lay *et al.*, 2005, Louis & Flint, 2009). *F. prausnitzii* is able to degrade a wide variety of carbohydrates, including inulin and starch, and belongs to the main butyrate producers in the human gut (Duncan *et al.*, 2007). Many *Ruminococcus* species of the *C. leptum* group are able to degrade starch or complex cell wall materials that are metabolized mainly to the SCFA acetate (Flint, 2006, Duncan *et al.*, 2007).

The most frequently encountered genera of the second important phylum, the CFB group, are *Bacteroides*, *Prevotella* and *Porphyromonas* (Manson *et al.*, 2008). Members of the CFB division comprise approximately 25% of the colonic microbiota (Duncan *et al.*, 2007). *Bacteroides* phlotypes can vary largely between subjects (Hayashi *et al.*, 2002, Hold *et al.*, 2002, Eckburg *et al.*, 2005). *Bacteroides thetaiotaomicron* is a frequently detected species associated with beneficial functions such as nutrient absorption and maturation of epithelial cells (Hooper *et al.*, 2001, Eckburg *et al.*, 2005).

Investigating 16S rRNA gene diversity of the human gut microbiota revealed that more than 75% of the phlotypes did not correspond to cultured species (Eckburg *et al.*, 2005, Flint *et al.*, 2007). Therefore, culture-independent approaches using molecular techniques like microarrays and metagenomic sequencing are nowadays essential tools in the investigation of

the human core microbiome (Eckburg *et al.*, 2005, Rajilić-Stojanović *et al.*, 2009, Candela *et al.*, 2010, Qin *et al.*, 2010).

1.3.2.2 Development of the infant colonic microbiota

The GIT of a healthy fetus is sterile and bathed in amniotic fluid (Fanaro *et al.*, 2003). The first determinant influencing the intestinal colonization is the mode of delivery. During vaginal delivery, the fetus is mainly inoculated with bacteria from the mother's microflora during passage through the birth canal. Newborns delivered by Caesarean sections are firstly colonized by bacteria originating from the hospital environment, including health care workers (Penders *et al.*, 2006).

Intestinal colonization is considered as an important step in maturation of the immune system (Adlerberth *et al.*, 1999). The first bacteria appearing in infant feces are *E. coli* and clostridia, followed by members of the genera *Bifidobacterium*, *Bacteroides*, *Streptococcus*, *Enterococcus* and *Actinomyces* (Favier *et al.*, 2003). Oxygen-consumption by aerobic and facultative anaerobic bacteria leads then to a more reduced environment, enabling obligate anaerobes including *Bifidobacterium*, *Clostridium* and *Bacteroides* to proliferate (Bezirtzoglou, 1997, Adlerberth, 2008). Infants delivered by Caesarean section seem to exhibit a delayed colonization by bifidobacteria and *Bacteroides* (Penders *et al.*, 2006, Fallani *et al.*, 2010). Enterococci are isolated from most neonates, and numbers can reach levels of $10 \log_{10}$ per gram of feces, albeit their origin has not yet been elucidated (Adlerberth *et al.*, 1999). Lactobacilli, generally considered to have beneficial effects on human health (see 1.3.4), are less frequently isolated from infant feces (Ahrné *et al.*, 2005).

The further colonization of the neonate's intestinal tract is affected by several factors including diet, environment or antibiotic treatment (Penders *et al.*, 2006, Fallani *et al.*, 2010). Analysis of the main bacterial groups in the microbiota of 606 six-week-old infants from several European countries by fluorescence in situ hybridization (FISH) combined with flow cytometry revealed that bifidobacteria, *Bacteroides fragilis* and *E. coli* were the most

prevalent bacterial populations (Fallani *et al.*, 2010). These results were confirmed by a study investigating fecal samples of 1032 one-month infants. Total numbers of bacteria in these infants ranged between 9.43 and 12.14 log₁₀ per gram of feces (Penders *et al.*, 2006). Genera frequently encountered in adults, including *Ruminococcus* and *Fusobacterium* are rarely isolated from infant feces (Adlerberth, 2008) and the colonization of the infant GIT with obligate anaerobes is a successive process proceeding during several years (Adlerberth *et al.*, 1999).

1.3.2.3 Antibiotic treatment in infancy

Antibiotics are the most prescribed medicine in neonatal intensive care units, leading to an altered colonization pattern (Adlerberth, 2008, Liem *et al.*, 2010). Obligate anaerobes are repressed, and total bacterial diversity is reduced. Especially bifidobacteria are known to be sensitive to antibiotics (Delgado *et al.*, 2005). Facultative anaerobic bacteria, mainly *Enterobacteriaceae* such as *Klebsiella* and *Enterobacter*, coagulase-negative staphylococci and enterococci reach higher levels in infants treated with antibiotics (Adlerberth, 2008). In pediatric intensive care, the increasing occurrence of nosocomial infections caused by enterococci combined with the emerging prevalence of ABR in this genus therefore poses a severe clinical challenge (Butler, 2006). Preterm newborns seem to have a higher risk for colonization by ARE, and the prepartal use of antibiotics favors colonization by multidrug resistant *Enterococcus* strains (Hufnagel *et al.*, 2007). These observations are even more alarming if considering that the infant gut microbiota is not fully diversified and therefore not capable to suppress the proliferation of incoming potential pathogens (Adlerberth *et al.*, 1999).

1.3.3 Functions of the gut microbiota

The colonic microbiota can be considered as a distinct organ, fulfilling metabolic functions, influencing the development and the differentiation of epithelial cells and accounting for host immunity (Collignon & Butel, 2006). The huge diversity of bacteria in the large intestine provides a wide variety of metabolic pathways used for the degradation of molecules that are absorbed in the small intestine (Bharucha, 2008). These metabolic activities deliver nutrients and energy for the host as well as for the gut microbiota (Guarner & Malagelada, 2003). The anaerobic microbiota of the large intestine serves a fundamental role in supplying energy through fermentations, mainly of carbohydrates (Duncan *et al.*, 2007). The main metabolites produced by anaerobes in the large intestine are the SCFA acetate, butyrate and propionate, which can count for up to 10% of the host's energy requirements (McNeil, 1984, Duncan *et al.*, 2007). Protein and peptide degradation also releases SCFA, but additionally potentially toxic substances such as ammonia, amines, phenols, thiols and indols (Macfarlane *et al.*, 1986, Smith & Macfarlane, 1996). Further important metabolic functions of the gut microbiota include synthesis of vitamins and absorption of calcium, magnesium and iron (Guarner & Malagelada, 2003). Due to the high supply of nutrients, bacterial growth and carbohydrate fermentation is very intense in the proximal colon, whereas substrate availability is decreased in the distal colon, leading a decelerated bacterial growth (Fanaro *et al.*, 2003, Guarner & Malagelada, 2003). The microflora in the proximal colon is also more variable over time compared to the distal colon (Marteau, 2006).

1.3.4 Gut microbiota in health and disease

Gut bacteria can have beneficial effects on human health via the production of e.g. anti-inflammatory factors, antioxidants and vitamins (Zhao, 2010). Moreover, the gut microbiota plays an important role in the development of the host immune system by influencing the gut-associated lymphoid tissue (Guarner & Malagelada, 2003, Kleerebezem & Vaughan, 2009). Additionally, resident bacteria serve as a barrier for the proliferation of exogenous bacteria, a

feature known as colonization resistance, thereby also decreasing the establishment and invasion of pathogenic bacteria (Guarner & Malagelada, 2003, Collignon & Butel, 2006). Bifidobacteria and some *Lactobacillus* species are assumed to contribute to a large extent to the host well-being. They are associated with colonization resistance, decreased affection by diarrhea and infections, stimulation of host immune system and rehabilitation of the gut microbiota after antibiotic treatment (Liévin *et al.*, 2000, Macfarlane *et al.*, 2008, Saulnier *et al.*, 2009).

On the other hand, compositional imbalances in the human core microbiota are suggested to negatively impact on the host (Candela *et al.*, 2010). Such features include the production of toxins and mutagenic compounds, thereby negatively affecting the host immune and nervous system (Zhao, 2010). Disturbed function of the intestinal mucosa can also result in the translocation of microbes, mainly aerobic Gram-negatives, into the lymph system, thereby reaching other organs, e.g. mesenteric lymph nodes, spleen and liver, causing potentially life-threatening infections (Guarner & Malagelada, 2003).

Gut bacteria also play a crucial role in the pathogenesis of inflammatory bowel diseases (IBDs), e.g. Crohn's disease and ulcerative colitis (Sartor, 2008, Nell *et al.*, 2010). The commensal microbiota directly interacts with the intestinal mucosa and is supposed to influence the initiation and maintenance of IBDs. A recent study comparing abundance of bacterial species in healthy individuals and patients suffering from IBDs revealed a clear disparity between these two groups, with reduced Firmicutes ribotypes and differences in *Prevotella* and *Bacteroides fragilis* subgroups in IBD-patients (Manichanh *et al.*, 2006, Qin *et al.*, 2010). There is a high interest in the development of probiotic and prebiotic food supplements in order to modulate the gut microbiota, thereby influencing host health and well-being. However, the impact of probiotic bacteria, e.g. lactobacilli and bifidobacteria, on the gut microbiota has not yet been completely elucidated and mechanisms are poorly known (Kleerebezem & Vaughan, 2009). The investigation of the presence of molecular biomarkers as indicators of the health state of an organism has now gained importance. Biomarkers can

be identified by new technologies including genomics, transcriptomics, proteomics, as well as metabolomics and are assumed to be useful tools in the prediction and prevention of allergies or IBDs, as well as in the investigation of the impact of probiotics on the gut microbiota (Lyra *et al.*, 2010, Oozeer *et al.*, 2010).

1.3.5 Antibiotic resistance gene transfer in the gut

The inappropriate use of antibiotics and the HGT of ABR determinants contribute to the worldwide dissemination of antibiotic resistant bacteria (Bloemendaal *et al.*, 2010). The resistance problem is not only restricted to pathogens, although the clinical relevance is clearly focused on these bacteria. However, commensal bacteria are now considered as main players in the dissemination of ABR genes (Wang *et al.*, 2006).

Via the food chain, especially via fermented animal-derived food, the human intestinal tract is continuously challenged with incoming antibiotic resistant bacteria (Furuya & Lowy, 2006). If antibiotic resistant bacteria succeed in transiently colonizing the GIT, horizontal transfer of ABR genes is very likely to occur (Sørensen *et al.*, 2001). The GIT is therefore assumed to act as a reservoir of ABR genes, which can be disseminated to resident or incoming bacteria or vice versa (van den Bogaard & Stobberingh, 2000, Salyers & Shoemaker, 2006). In infants, the colonic microbiota is not yet fully developed and incoming bacteria might therefore have a higher impact on the microbial balance in the GIT (Lindberg *et al.*, 2004, Zoetendal *et al.*, 2006), thereby presumably increasing the risk of lateral transfer of ABR genes between commensals and pathogens in the infant colon (Karami *et al.*, 2006).

ABR gene transfer in complex colonic microbial background has been demonstrated in several studies. However, most of these studies used gnotobiotic animals and defined recipient strains (Table 2). The only study demonstrating ABR gene transfer between two members of the gut environment described conjugal transfer of an ampicillin resistance plasmid between two *E. coli* strains co-residing the gut of an 8-day old infant (Karami *et al.* 2007; Table 2).

Table 2. Examples of HGT of ABR determinants in the gastrointestinal environment.

Donor	Recipient	Transferred resistance(s)	Genetic element	Environment	Reference
<i>C. jejuni</i>	<i>C. jejuni</i>	Tet	plasmid	chicken	Avrain <i>et al.</i> , 2004
<i>E. coli</i>	<i>E. coli</i>	Tet	plasmid	streptomycin-treated mice	Licht <i>et al.</i> , 2003
	<i>E. coli</i>	Tet	plasmid	mice and chicken	Hart <i>et al.</i> , 2006
	<i>E. coli</i>	Amp	plasmid	infant gut	Karami <i>et al.</i> , 2007
	<i>E. coli</i>	Sul	plasmid	human volunteers	Trobos <i>et al.</i> , 2009
<i>E. faecalis</i>	<i>L. monocytogenes</i>	Ery, Kan, Tet	transposon	gnotobiotic mice	Doucet-Populaire <i>et al.</i> , 1991
	<i>E. faecalis</i>	Tet	plasmid	streptomycin-treated mini-pig	Licht <i>et al.</i> , 2002
	<i>E. faecalis</i>	Tet	transposon	gnotobiotic rats	Bahl <i>et al.</i> , 2004
<i>E. faecium</i>	<i>E. faecium</i>	Van	transposon	mice with enterococci-free human microbiota	Mater <i>et al.</i> , 2005
	<i>E. faecium</i>	Van	transposon	human volunteers	Lester <i>et al.</i> , 2006
	<i>E. faecium</i>	Ery, Van	plasmid	gnotobiotic mice	Moubareck <i>et al.</i> , 2003
<i>K. pneumoniae</i>	<i>Lb. acidophilus</i>	Van	transposon	gnotobiotic mice	Mater <i>et al.</i> , 2008
	<i>E. coli</i>	Kan, ESBL	plasmid	gnotobiotic mice	Moubareck <i>et al.</i> , 2007
<i>Lc. lactis</i>	<i>E. faecalis</i>	Tet	transposon	gnotobiotic rats	Boguslawska <i>et al.</i> , 2009
<i>Lb. plantarum</i>	<i>E. faecalis</i>	Ery, Tet	plasmid	gnotobiotic rats	Jacobsen <i>et al.</i> , 2007
	<i>E. faecalis</i>	Ery	plasmid	gnotobiotic rats	Feld <i>et al.</i> , 2008
<i>S. Typhimurium</i>	<i>E. coli</i>	Tet, ESBL	plasmid	gnotobiotic mice	Moubareck <i>et al.</i> , 2007

Abbreviations: C.: *Campylobacter*; E.: *Escherichia* or *Enterococcus*; K.: *Klebsiella*; L.: *Listeria*; Lc.: *Lactococcus*; Lb.: *Lactobacillus*; S.: *Salmonella*; Amp: ampicillin, Ery: erythromycin, ESBL: extended-spectrum β -lactam antibiotics, Kan: kanamycin, Sul: sulfonamides, Tet: tetracycline, Van: vancomycin.

However, the infant was suffering from a urinary tract infection and was treated with ampicillin and amoxicillin. The antibiotic therapy resulted in increasing colonization density of the ampicillin resistant donor from log 6.4 CFU/g feces before the treatment to log 11 CFU/g feces after ampicillin and amoxicillin treatment. It was therefore assumed that HGT of the ampicillin resistance gene was only possible due to the unnatural huge donor population density (Karami *et al.*, 2007). Horizontal ABR gene transfer from food-originating bacteria to the gut microbiota would clearly contribute to the public health problem of increasing prevalence of antibiotic resistant bacteria. As a worst-case scenario, resistances might be transmitted to incoming pathogens, thereby hampering the efficient treatment of bacterial infections with antibiotics. However, the demonstration of ABR gene transfer in the GIT is challenging due to the complex microbial background, the high prevalence of antibiotic resistant bacteria and ABR genes and, with respect to humans and animals, ethical reasons. Ethical concerns can be avoided by using *in vitro* models mimicking the colonic ecosystem (Cinquin *et al.*, 2004). Colonic fermentation models allow controlled and reproducible experiments under standard conditions and they have several advantages, including reduced costs, simple handling and sampling, the application of pathogenic and multi-drug resistant bacteria as well as the possibility to investigate the effect of one or more parameters on fermentation processes by the colonic microbiota (Cinquin *et al.*, 2004, Cinquin *et al.*, 2006, Egert *et al.*, 2006, Le Blay *et al.*, 2009). Moreover, colonic fermentation models are suitable tools to mimic colonization by pathogenic bacteria (Le Blay *et al.*, 2009). A system using immobilized feces and continuous culture has been validated by Cinquin *et al.* (Cinquin *et al.*, 2004). It has been demonstrated that immobilization of fecal samples in polymer beads allowed the preservation of the bacterial concentration and diversity of the fecal inoculum. Furthermore, the microbial and metabolic stability was maintained in long-term experiments (Cinquin *et al.*, 2004, Cinquin *et al.*, 2006, Le Blay *et al.*, 2009). Despite some limitations including the absence of host cells and immune response, the inoculation with fecal microbiota and the lack of a vertical gradient in microbial colonization (Egert *et al.*, 2006), *in*

vitro colonic fermentation models are useful tools to investigate gut-microbiota-related processes, including horizontal ABR gene transfer in a complex fecal background.

1.4 Aim of the thesis

If ingested ARE reach the human gut, they might be able to laterally transfer ABR genes to the commensal bacteria of the gut microflora, thereby increasing the ABR reservoir in the gut as well as the risk of HGT to pathogenic bacteria. The aim of this study was therefore the demonstration of ABR gene transfer from *E. faecalis* to human commensal bacteria and to the human pathogen *Listeria monocytogenes* using an *in vitro* continuous colonic fermentation model with immobilized fecal microbiota closely mimicking the infant proximal colon.

In order to first monitor the prevalence of ABR genes in fecal samples, a suitable DNA-extraction method as well as a quantitative real-time PCR protocol had to be evaluated. Secondly, an appropriate ABR model plasmid to monitor HGT in a complex ecosystem had to be selected. Such a plasmid should have a broad host range, high conjugation frequency as well as unique markers in order to be detectable in the complex background. The completely sequenced conjugative multiresistant plasmid pRE25, originating from an *E. faecalis* strain isolated from a dry sausage, was chosen for this purpose. However, the high background of ABR genes in the human gut microbiota made it inevitable to introduce a molecular marker into pRE25. Moreover, *E. faecalis* is a natural inhabitant of the human GIT and one of its early colonizers and to distinguish the donor strain from commensal *E. faecalis* strains, the donor had to be tagged as well. Both genetic markers should not affect the conjugal characteristics of the model plasmid. Furthermore, they should be genetically stable over a long time in order to be applicable in a continuous colonic fermentation. A genetically marked pRE25-derivative was constructed and introduced into a chromosomally tagged *E. faecalis* strain. This strain was subsequently applied in two colonic fermentations in order to investigate its potential to transfer the marked multiresistance plasmid to either *L. monocytogenes* or commensal bacteria co-immobilized with infant feces.

1.4.1 Hypothesis

E. faecalis is able to transfer plasmid-mediated ABR to *L. monocytogenes* and to commensal bacteria of the infant fecal microbiota in an *in vitro* continuous colonic fermentation with immobilized fecal microbiota as model of the infant proximal colon.

1.4.2 Specific objectives

In order to verify the hypothesis of this work, the following specific objectives were defined:

- Investigation of the prevalence of ABR genes in infant feces
- Selection and characterization of a suitable conjugative ABR model plasmid
- Construction of a genetically marked conjugative plasmid exhibiting similar characteristics as the multiresistance plasmid pRE25 of *E. faecalis* and transfer of this plasmid into a chromosomally marked *E. faecalis* donor strain
- Characterization of the new constructed strain concerning stability, conjugation potential, copy number, and ABR profile.
- Evaluation of a quantitative real-time PCR system to detect donor and plasmid in complex environments
- Application of the new donor strain in continuous colonic fermentations to monitor the fate of the multiresistance plasmid and the *E. faecalis* donor strain and to investigate horizontal ABR gene transfer to *L. monocytogenes* and to commensal gut bacteria

2 Suitability of the *Enterococcus faecalis* Conjugative Antibiotic Resistance Plasmid pSL40 for Monitoring Gene Transfer in Fecal Microbiota Exhibiting a High Background of Antibiotic Resistance Genes

2.1 Abstract

Antibiotic resistant enterococci (ARE) frequently harbor antibiotic resistant determinants on mobile genetic elements such as conjugative transposons and plasmids. ARE are widespread in foodstuffs, e.g. fermented sausages and cheese. This implies a potential risk for human health, since ARE reaching the human gut might transfer their antibiotic resistance (ABR) genes to commensal or transiently colonizing bacteria.

To elucidate the prevalence of ABR genes in fecal samples, a quantitative real-time PCR approach to detect ABR determinants in fecal samples was optimized. Subsequently, the prevalence of the two ABR genes *tet(M)* and *erm(B)* in 22 fecal samples from infants aged 5 days to 9 months was determined. An average gene copy number of 7.12 and 6.58 log per g feces respectively demonstrated a high prevalence of ABR genes even in healthy infants. Investigation of ABR gene transfer in complex fecal environments therefore requires a suitable *E. faecalis* donor. A candidate, *E. faecalis* strain SL5.7, isolated from a dry sausage, harbors the tetracycline resistance gene *tet(M)* on the chromosome and on the 40-kb plasmid pSL40. The plasmid was transferable to *E. faecalis* JH2-2 at a high frequency of 7.04×10^{-3} transconjugants per donor. Sequencing of *tet(M)* and the flanking regions revealed 100% nucleotide homology to the corresponding genes in *Staphylococcus rostri*, *Streptococcus parauberis* and *Streptococcus agalactiae*.

The high homology of *tet(M)* of strain SL5.7 to other *tet(M)* genes in the database as well as the high ABR background in infant's feces necessitates the construction of genetically marked ABR determinants to enable demonstration of ABR gene transfer of foodborne enterococci to commensal gut bacteria in a colonic fermentation.

2.2 Introduction

The increasing prevalence of antibiotic resistant bacteria poses a health risk for humans and animals since it hinders the effective treatment of bacterial infections (Singer *et al.*, 2003, Sommer *et al.*, 2009). Two distinct antibiotic resistance (ABR) types occur in nature: intrinsic resistance usually arising by mutations, and extrinsic resistances acquired by horizontal gene transfer (HGT). Conjugative plasmids and transposons are the major mobile genetic elements (MGEs) involved in transfer of such extrinsic resistances (Levy, 1998). The genus *Enterococcus* has a potent ability to exchange genetic material via MGEs. MGEs carrying resistance genes are frequently detected in enterococci, which are therefore considered to be main players in the dissemination of ABR genes (Clewell, 1990, Jett *et al.*, 1994, Arias *et al.*, 2010). Enterococci are lactic acid bacteria frequently encountered in the mammalian gastrointestinal tract (GIT). However, enterococci have also evolved as important causes of nosocomial and community-acquired infections (Tan *et al.*, 2010). Their ability to withstand a wide range of adverse environmental conditions enables them to occupy various ecological niches including fermented foodstuffs, e.g. cheese, fermented sausages and fermented olives (Teuber *et al.*, 1996, Giraffa, 2002, Franz *et al.*, 2003, Foulquié Moreno *et al.*, 2006). Levels of enterococci range from 10^2 to 10^5 CFU/g in fermented meat products, and 10^2 and 10^8 CFU/g in cheeses, dependent on the cheese type (Teuber *et al.*, 1996, Templer & Baumgartner, 2007, Martín-Platero *et al.*, 2009, Fuka *et al.*, 2010, Özmen Toğay *et al.*, 2010). Enterococci contribute to the organoleptic properties and preservation of food. However, food associated enterococci frequently harbor the virulence genes *asaI* and *gelE*, associated to adherence and damage of host tissue (Gilmore *et al.*, 2002) and extrinsic ABR determinants against a wide range of antibiotics including chloramphenicol, erythromycin, streptomycin, tetracycline, vancomycin, gentamicin, kanamycin, penicillin, ampicillin and ciprofloxacin (Franz *et al.*, 2001, Peters *et al.*, 2003, Messi *et al.*, 2006, Garofalo *et al.*, 2007, Devirgiliis *et al.*, 2010, Özmen Toğay *et al.*, 2010). If food-derived antibiotic resistant enterococci (ARE) end up in the human GIT, resistances are potentially transferred to commensal or transient

bacteria of the gut via HGT (Berchieri, 1999, Sørensen *et al.*, 2001, Lund *et al.*, 2002, Lester *et al.*, 2006, Toomey *et al.*, 2010).

New technologies including metagenomic sequencing revealed a vast diversity of ABR genes in the human microbiome, indicating that the intestinal microbiota may act as a reservoir of ABR genes (Sommer *et al.*, 2009). The diverse and dense microbial population in the human GIT is assumed to favor dissemination of ABR genes between different members of the human microbiota (Kazimierczak & Scott, 2007). Enterococci isolated from human stool frequently harbor the resistance determinants *tet(M)*, encoding ribosomal protection against tetracycline, and *erm(B)*, encoding erythromycin ribosome methylase (Barreto *et al.*, 2009, Seville *et al.*, 2009). Both genes have a diverse host range and are frequently found in human feces (Seville *et al.*, 2009).

In order to monitor HGT from *E. faecalis* in complex environments, e.g. the fecal microbiota, the prevalence of ABR genes in infant feces as well the evaluation of a suitable *E. faecalis* ABR donor strain has to be investigated. For this purpose, two different methods for fecal DNA extraction were compared for their efficiency to quantitatively extract *E. faecalis* DNA from feces. The most accurate DNA extraction method was subsequently applied to determine *tet(M)*, *erm(B)*, and *E. faecalis*, *Bacteroides* spp., and *Bifidobacterium* spp. specific gene copy numbers in 22 fecal samples from infants between 5 days and 9 months by an optimized real-time PCR approach. Secondly, the dry sausage isolate *E. faecalis* SL5.7 which harbors the tetracycline resistance gene *tet(M)* and the conjugative transposon Tn916 integrase gene *int* was characterized. Furthermore, the presence of MGEs in strain SL5.7 and their transfer capability to other genera was determined in order to explore suitable tools for quantitatively monitoring gene transfer under gut conditions.

2.3 Material and methods

2.3.1 Bacterial strains and media

Chemicals used in this study were routinely obtained from Sigma-Aldrich (Buchs, Switzerland), except when stated otherwise. Bacterial strains used in this work are listed in Table 3. Oligonucleotides were obtained from Microsynth (Balgach, Switzerland) and are listed in Table 4. *E. faecalis* was cultivated aerobically at 37°C in brain heart infusion (BHI, Biolife, Milano, Italy).

Table 3. Strains and plasmids used in this work.

Material		Relevant features	Source
Strains			
<i>E. faecalis</i>	SL5.7	harbors pSL40 (40 kb) and pSL5 (5 kb), <i>tet</i> (M)	Leisibach, 2004, this work
	89.10	<i>tet</i> (M)	Leisibach, 2004
	JH2-2	derivative of the clinical isolate JH2, recipient for filter mating; Fus ^R , Rif ^R	Jacob & Hobbs, 1974
	DSM 20478T	type strain	DSMZ
	JH2-2×SL5.7	transconjugant from filter mating, carries <i>tet</i> (M) in pLS40 and integrated in the chromosome	this work
<i>S. agalactiae</i>	2364	reference strain for positive β-hemolysis assay	FBTSC
Plasmids			
	pSL5	5 kb; non-mobile plasmid isolated from <i>E. faecalis</i> SL5.7	Leisibach, 2004, this work
	pSL40	40 kb; <i>tet</i> (M); conjugative plasmid isolated from <i>E. faecalis</i> SL5.7	Leisibach, 2004, this work

Abbreviations: *E.*: *Enterococcus*; *S.*: *Streptococcus*; DSMZ: Deutsche Sammlung für Mikroorganismen und Zellkulturen; FBTSC: Food Biotechnology strain collection, ETH Zurich

Table 4. Oligonucleotides used in this work.

Primer/Probe	Target gene	Sequence (5'→3')	T _{Ann.} ^a	Reference
ermBfw95	<i>erm(B)</i> (qPCR)	cggttacgaaatggaaca	60°C	Leisibach, 2004
ermBrv246		ttggtgaattaaagtgacacga		
ermBTMP121		FAM-agccagtttcgctgtaaatgccc-TAMRA		
tetM137F	<i>tet(M)</i> (qPCR)	cgcttttagaacgctcagagagga	60°C	Leisibach, 2004
tetM299R		gaaatcagtagaattgccccatct		
tetM205T		FAM-aagtgaaacatcatagacagcca-TAMRA		
tufA_fw	<i>tufA</i> (qPCR)	gacaaaccattcatgatgccag	60°C	Ke <i>et al.</i> , 1999, Leisibach, 2004
tufA_rv		cgtcaccaaacggaacttca		
tufA_TMP		FAM-ttctcaatyactggwgcggtgactgttgc-TAMRA		
bac303F	<i>Bacteroides</i> spp. (qPCR)	gaaggtccccacattg	60°C	Bartosch <i>et al.</i> , 2004, Ramirez-Farias <i>et al.</i> , 2009
bfr-Fmrev		cgckacttggctggttcag		
xfp_fw	<i>Bifidobacterium</i> spp. (qPCR)	atcttcggaccbgyagagac	60°C	Cleusix <i>et al.</i> , 2010
xfp_rv		cgatvacgtgva cgaaggac		
tetM1	<i>tet(M)</i> (normal PCR)	gctcatgttgatgcagga	54°C	Leisibach, 2004
tetM2		aggattnnccggcacttcgac		
int_F	integrase gene of Tn916	gggtgatgtatctcact	50°C	Doherty <i>et al.</i> , 2000
int_R		gacgiccctgttctct		
gelE_fw	<i>gelE</i>	acccgtatcattggttt	52°C	Creti <i>et al.</i> , 2004
gelE_rv		acgcattgctttccatc		
asa1_fw	<i>asa1</i>	ccagccaactatggcggaaac	58°C	Creti <i>et al.</i> , 2004
asa1_rv		cctgtcgaagatcgacigta		
tetM_S1	<i>tet(M)</i> (sequencing primers)	acgacgttctcaagctctatcct	53°C	This work
tetM_S2		cgaatttgtgctgtgta cggccatc		
tetM_S3	<i>tet(M)</i> (sequencing primers)	gacagccaggacatatgg	52°C	This work
tetM_S4		ccgagctctcatctgcatcc		
tetM_S5	<i>tet(M)</i> (sequencing primers)	tcgaaagtccgccaatccc	53°C	This work
tetM_S6		cattttatcggctctgctttt		

Abbreviations: FAM: reporter dye (6-Carboxyfluorescein); TAMRA: non-fluorescent quencher (Carboxytetramethylrhodamine)

^a Annealing temperatures used in this work.

2.3.2 Validation of the DNA extraction method and quantitative real-time PCR

Two DNA extraction methods were compared in their performance to extract *E. faecalis* DNA from spiked fecal samples. Ethical approval for the analysis of fecal samples was obtained from the Ethics Committee of the Canton of Zurich and the University Children's Hospital of Zurich (project StV31/05).

Spiking of fecal samples with E. faecalis

A fresh fecal sample from a 6-months healthy infant was aliquoted into portions of 200 mg. To minimize *E. faecalis* DNA background in the fecal samples before spiking, aliquots were autoclaved 5 times and treated in a sonicator for 20 min to shear DNA. The feces were then spiked with 100 µl of different dilutions of a fresh overnight culture of *E. faecalis* DSM 20478T or 100 µl autoclaved water as negative control. Appropriate dilutions of the overnight culture were additionally plated onto BHI agar plates and incubated for 24 h at 37°C to determine viable cell counts.

DNA extraction

DNA was extracted either using the FastDNA® SPIN Kit for Soil (MP Biomedicals Europe, Illkirch, France) or a DNA extraction method according to Godon *et al.* (1997). Using the FastDNA® SPIN Kit for Soil, DNA was extracted from 300 mg of spiked feces according to the manufacturer's instructions and eluted from the column with 200 µl of ultrapure water. Using the protocol of Godon *et al.*, DNA was extracted from 300 mg of spiked feces as follows: to weaken the bacterial cells, 250 µl of 4 M guanidinium thiocyanate, 40 µl of N-lauroyl sarcosine (10% w/v, in H₂O) and 500 µl of N-lauroyl sarcosine (5% w/v, in 0.1 M sodium phosphate buffer, pH 8.0) were added to the frozen sample. After homogenizing by vortexing, the mixture was spun down and incubated at 70°C during 1 h. For cells lysis, 750 µl of 0.1-mm zirconia/silica beads (BioSpec Products Inc. Bartlesville, USA) were added

to the sample and cells were mechanically broken during 2×5 min using a FastPrep device (MP Biomedicals, Illkirch, France). Polyvinylpyrrolidone (15 mg, PVPP) was added and the lysate was centrifuged for 3 min at 12'000 g. The supernatant was transferred to a clean tube and the pellet was washed three times with 500 µl of TENP (50 mM Tris, 20 mM EDTA, 100 mM NaCl, 1% w/v PVPP, pH 8.0). The supernatants from the first centrifugation and the three washing steps were pooled together and nucleic acids were precipitated using 1 volume of isopropanol. After incubation at room temperature for 10 min, DNA was precipitated by centrifugation for 15 min at 20'000 g. The pellet was resuspended with 225 µl of 0.1 M sodium phosphate buffer (pH 8.0) and 25 µl of 3 M potassium acetate and incubated on ice during 90 min in order to precipitate proteins. The suspension was centrifuged at 16'000 g during 30 min and the supernatant was transferred to a clean tube. RNA was digested using 2 µl of RNase A (10 mg/ml) followed by incubation at 37°C during 1-2 h. DNA was then precipitated by the addition of 50 µl of 3 M sodium acetate (pH 5.2) and 1 ml of 100% ethanol for 5 min at room temperature. DNA was recovered by centrifugation at 20'000 g during 10 min. The pellet was finally washed with 70% ethanol, dried and resuspended in 200 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Quantitative real-time PCR

All reactions were performed in a reaction volume of 25 µl containing 5 µl of template DNA in appropriate dilutions, 5 µl of the negative control for extraction or 5 µl of autoclaved water as no template control. Each sample was measured in triplicate. For real-time PCR using 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 200 nM were used. For real-time PCR using the SYBR Green method, 12.5 µl of 2×SYBR® Green PCR Master Mix (Applied Biosystems, Zug, Switzerland) and 200 nM of each primer were used. Amplification was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) with the following cycling profile: 50°C for 2 min; 95°C for 10 min; 40 cycles of

95°C for 15 s and 60°C for 1 min. Data were analyzed using the 7500 Fast SDS software (Applied Biosystems). Total copy numbers were quantified using a DNA calibration curve obtained by plotting Ct values from serial dilutions containing known gene copy numbers, obtained in the same qPCR run.

2.3.3 Quantification of antibiotic resistance genes in fecal samples

The tetracycline resistance gene *tet(M)* and the erythromycin resistance gene *erm(B)* were quantified using quantitative real-time PCR in 22 fecal samples of infants between 5 days and 9 months. In addition, copy numbers of *tufA* of *E. faecalis*, the 16S rRNA gene of *Bacteroides* spp. and the *xfp* gene of *Bifidobacterium* spp. were quantified. Feces were transported at 4°C and stored at -20°C until further processing. DNA was extracted from 200 mg fecal sample and from 200 µl autoclaved water (no extraction control) using the FastDNA® SPIN Kit for Soil (MP Biomedicals). Real-time PCR was conducted as described above and using nuclease free water as negative control. Primers and probes are listed in Table 4.

2.3.4 Characterization of *Enterococcus faecalis* SL5.7

Determination of gene transfer by filter mating

The donor *E. faecalis* SL5.7, a fermented sausage isolate, was grown in BHI + 10 µg/ml tetracycline (Tet₁₀) overnight. The recipient strain JH2-2 was cultivated in BHI + 50 µg/ml rifampicin (Rif₅₀) + 100 µg/ml fusidic acid (Fus₁₀₀) overnight. The next day, both overnight cultures were washed once with BHI and 0.5 ml of donor culture and 1.5 ml of recipient culture were mixed and passed through a sterile 0.45-µm nitrocellulose filter (Millipore AG, Zug, Switzerland). The filter was incubated cell-side up on non-selective BHI plates at 37°C for 24 h. Cells were then washed from the filter by vortexing for 1 min in 2 ml of sterile dilution solution [0.85% NaCl, 0.1% peptone from casein, (VWR), pH 8.0]. Transconjugants were isolated by plating appropriate dilutions on BHI+Tet₁₀+Rif₅₀+Fus₁₀₀. To exclude

spontaneous mutations, overnight cultures of donor and recipient were plated individually on BHI+Tet₁₀+Rif₅₀+Fus₁₀₀, selective for transconjugants.

DNA extraction

DNA for PCR amplification and for probe construction was extracted from single colonies using a trizol-lysozyme based cell lysis and subsequent DNA extraction as described previously (Goldenberger *et al.*, 1997). Genomic DNA of *E. faecalis* for microarray hybridization was isolated as follows: cells from 4 ml of a fresh overnight culture were lysed using a guanidium thiocyanate method (Pitcher *et al.*, 1989). DNA was purified from the cell lysate using a phenol-chloroform-based method as described previously (Perreten *et al.*, 2005).

Plasmid DNA was purified from *E. faecalis* according to the protocol of Anderson and McKay (Anderson & McKay, 1983) with the following modifications: cells were grown in 600 ml of BHI supplemented with 10 µg/ml tetracycline until the late exponential growth phase. The pelleted cells were stored at -20°C for 16 h to facilitate cell disruption. After thawing, the cells were resuspended in 30 ml lysis buffer and cell walls were degraded by addition of 80 µl of mutanolysin (2500 units/ml) and 20 mg/ml lysozyme followed by incubation at 37°C for 1 hour. Chromosomal DNA was precipitated by the addition of 5 M NaCl, followed by incubation on ice during 1 hour. After centrifugation at 23'000 g at 4°C for 45 min, nucleic acids were purified using phenol/methylenchloride/isoamyl alcohol (25:24:1), and subsequently precipitated with isopropanol. The resulting pellet was washed twice with ice-cold 70% EtOH, dried at room temperature and resuspended in TE buffer. RNA was digested using 10 mg/ml RNase A at 37°C for 15 min.

Southern blot

The probe for the detection of the *tet(M)* gene by Southern blot was constructed using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Rotkreuz, Switzerland), primers tetM1

and tetM2 (Table 4) and genomic DNA of *E. faecalis* 89.10 as template (Table 3). DNA transfer and hybridization (16 h at 40°C) were performed using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics). Chemiluminescence was detected by exposure of the blot to the Kodak Image Station for 60 min.

Microarray hybridization

ABR genes were detected using a microarray harboring probes for 90 different ABR genes mainly found in Gram-positive bacteria (Perreten *et al.*, 2005). Hybridization and microarray analysis was performed as described previously (Perreten *et al.*, 2005).

Disk diffusion assay

To determine the ABR profile of *E. faecalis* SL5.7 and its JH2-2 transconjugant, a pre-culture was grown overnight in 10 ml BHI at 37°C. The cultures were diluted with sterile dilution solution to an optical density at 600 nm of 0.25 and plated on Mueller-Hinton agar (Labo-Life Sàrl, Pully, Switzerland). Commercially available antibiotic discs were placed on the surface of the agar and the plates were incubated at 37°C for 24 h. The diameter of the inhibition zone including the disk diameter (8 mm) were measured in mm and compared to guidelines of the Clinical and Laboratory Standards Institute (CLSI) for *Enterococcus* spp. (CLSI 2008).

Sequencing of tet(M)

Sequencing of the tetracycline resistance gene *tet(M)* and the flanking regions was performed by Sanger sequencing at Microsynth using primer pairs tetM_S1/S2, tetM_S3/S4 and tetM_S5/S6 (Table 4). Primers were designed based on the sequence of the cloning vector pAM120 (GenBank U49939.1, Table 3).

Virulence determinants

The virulence genes *gelE* (gelatinase) and *asa1* (aggregation substance) were detected by PCR using primers *gelE_fw/rv* and *asa1_fw/rv* (Table 4).

Phenotypic assays

Production of gelatinase was examined by streaking a single colony onto BHI agar plates containing 3% w/v gelatin (VWR) followed by incubation at 37°C for 48 h. A clear lysis zone around colonies indicates a positive result (Creti *et al.*, 2004). To determine the production of aggregation substance, a clumping assay was performed (Donelli *et al.*, 2004). Shortly, exponential and stationary phase supernatants of the pheromone producer *E. faecalis* JH2-2 were filter sterilized and used directly or after autoclaving at 121°C for 15 min. Clumping was evaluated by mixing 50 µl of stationary-phase *E. faecalis* SL5.7 with 2 ml of the JH2-2 supernatant and incubation at 37°C for 3 h under moderate shaking (65 rpm). The clumping ability was then examined using phase-contrast microscopy. To determine hemolysin production, a single colony of *E. faecalis* SL5.7 was streaked onto Columbia agar plates containing 5% w/v sheep blood (Oxoid, Pratteln, Switzerland). *S. agalactiae* 2364 was used as a positive control for β-hemolysis, indicated by clearing zones around colonies after 24 h at 37°C (Franz *et al.*, 2001).

2.4 Results

2.4.1 Validation of the DNA extraction method and quantitative real-time PCR

To enumerate ABR genes in complex environments like fecal samples, the DNA has to be quantitatively extracted and therefore two different DNA isolation protocols were compared. Fecal samples were 5 times autoclaved and sonicated, which was necessary to degrade all DNA (data not shown). Next, several dilutions of a fresh overnight culture of *E. faecalis* DSM 20478T were mixed with the fecal samples. DNA was extracted from the samples using the FastDNA® SPIN Kit for Soil or a method previously described (Godon *et al.*, 1997). The single copy chromosomal *tufA* gene was used as target gene to deduce *E. faecalis* cell numbers via real-time PCR. The *tufA* copy numbers in the spiked samples determined by qPCR correlated well with CFU of *E. faecalis* in each spiked sample ($R^2 > 0.99$, Figure 3) using the FastDNA® SPIN Kit for Soil. The detection limit of the method was estimated at 10^3 copies per g of feces and the linearity between the plate counts and qPCR was given within the range from 10^3 to 10^9 copies of *tufA* per g of fecal slurry (Figure 3). The correlation using the method of Godon, R^2 was significantly lower ($R^2 = 0.92$) for the same range of sampling points (Figure 3). Furthermore, *tufA* was neither detected in the negative extraction control nor in the no template control when using the FastDNA® SPIN Kit for Soil. By using this simple test protocol it could be shown that the FastDNA® SPIN Kit for Soil is a suitable kit for DNA extraction from fecal samples.

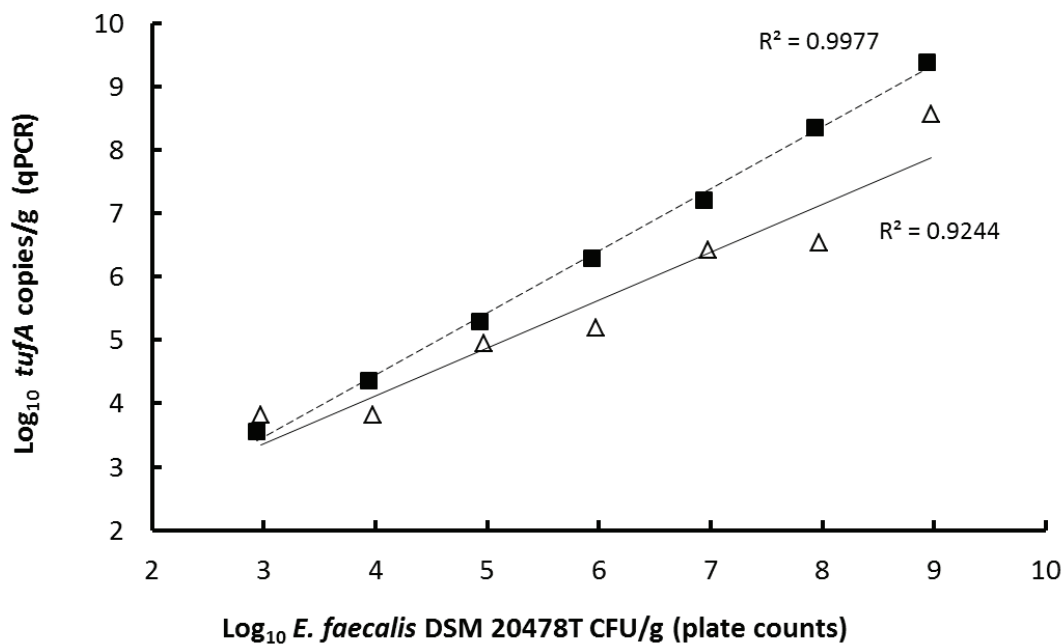


Figure 3. Correlation between *tufA* gene copies/g of spiked fecal sample determined by qPCR and CFU of *E. faecalis* DSM 20478T per g of spiked sample determined by plate counts. DNA was extracted either using the FastDNA® SPIN Kit for Soil (filled rectangles; dashed trendline) or a method according to Godon *et al.* (1997; empty triangles; continuous trendline). Correlation coefficients are indicated in the plot area. Linearity of both parameters between 10^3 and 10^9 copies per g sample indicates the detection limit of 10^3 copies of *tufA* per g of feces.

2.4.2 Distribution of *tet(M)* and *erm(B)* in environmental samples

It is assumed that the human intestinal microbiota acts as a reservoir of ABR genes (Salyers *et al.*, 2007). To determine the prevalence of ABR genes in young infants, a total of 22 fecal samples originating from children aged between 5 days and 9 months were investigated for the presence of the two ABR genes *tet(M)* and *erm(B)*. Both genes were present in all fecal samples (Table 5). Gene copies of *tet(M)* ranged from 5.3 to 9.6 log copy numbers/g of feces, whereas copies of *erm(B)* ranged from 4.8 and 9.3 log copy numbers/g of feces (Table 5). *E. faecalis* cells, quantified via *tufA* copy numbers, reached an average number of 6.8 ± 1.4 log copy numbers/g of feces, whereas *Bifidobacterium* spp. *xfp* gene copies and *Bacteroides* spp.

16S rRNA gene copies reached higher values of 8.5 and 8.1 log copy numbers/g of feces (Table 5), comparable to determined fecal counts of the latter two genera obtained in previous studies (Adlerberth *et al.*, 1999, Orrhage & Nord, 1999, Penders *et al.*, 2006, Penders *et al.*, 2007). This shows that ABR determinants are widespread even in healthy newborns.

Table 5. Log copy numbers of ABR genes, *tufA* of *E. faecalis*, 16S rRNA gene of *Bacteroides* spp. and *xfp* gene of *Bifidobacterium* spp per gram of feces.

Sample	Age/remarks	<i>tet</i> (M)	<i>erm</i> (B)	<i>E. faecalis</i>	<i>Bacteroides</i> spp.	<i>Bifidobacterium</i> spp.
F1	6 m	5.96	4.77	6.95	5.32	7.03
F2	7 m	5.80	5.03	8.18	5.19	9.06
F3	5 d	5.56	5.67	5.59	9.87	6.46
F4	14 d	7.48	6.24	6.05	9.66	8.24
F5	3 m	5.86	6.03	7.80	8.23	9.59
F6	5 m	5.31	5.16	6.46	8.07	7.41
F7	4 m	5.62	5.14	6.29	5.33	9.44
F8	2 m	6.62	5.35	6.09	4.75	9.82
F9	14 d	6.58	5.33	5.71	8.31	7.14
F10	3 m	6.94	5.96	8.21	5.11	9.08
F11	1 m	6.67	9.26	8.98	7.97	8.47
F12	1 m	5.02	5.75	5.15	8.95	9.08
F13	5 m	5.98	5.70	8.29	10.96	9.39
F14	2 m	7.84	7.34	6.79	10.92	9.69
F15	6 m	8.19	8.52	7.65	10.11	9.42
F16	2 m	7.85	5.26	5.50	9.40	9.60
F17	13 d	9.32	6.89	8.59	3.71	2.92
F18	9 m	9.59	8.84	4.57	9.50	9.78
F19	9 m	9.00	8.54	8.46	4.41	9.89
F20	2 w	7.13	8.37	5.97	9.79	9.12
F21	15 d	8.70	6.41	7.62	9.56	6.24
F22	7 m	8.19	7.63	4.14	10.63	9.16
Average ± SD		7.12 ± 1.38	6.58 ± 1.43	6.83 ± 1.38	8.08 ± 2.36	8.50 ± 1.65

^aAbbreviations: d: days; m: months; w: weeks; SD: standard deviation. Primers are listed in Table 4.

2.4.3 Characterization of *E. faecalis* SL5.7 and its potential as gene transfer model organism

To identify an appropriate *Enterococcus* donor strain to monitor gene transfer in complex fecal environments, we characterized the dry sausage isolate *E. faecalis* SL5.7 which harbors the tetracycline resistance gene *tet(M)* and the conjugative transposon Tn916 integrase gene *int* (Leisibach, 2004). In order to investigate the transfer potential of the tetracycline resistance in strain SL5.7, the average transfer frequency to the plasmid-free recipient *E. faecalis* JH2-2 was determined by filter mating technique. The frequency was 7.0×10^{-3} transconjugants per donor, slightly lower than the previously described frequency of 2.1×10^{-2} transconjugants per donor (Leisibach, 2004). No spontaneous mutations were observed. To identify the localization of the *tet(M)* gene in *E. faecalis* SL5.7 and in the transconjugant *E. faecalis* JH2-2×SL5.7, total DNA was isolated from donor and transconjugant according to Anderson and McKay (Anderson & McKay, 1983). Plasmid profiling revealed that *E. faecalis* SL5.7 harbors a large plasmid of approximately 40 kb, designated pSL40, and a smaller plasmid of approximately 5 kb, named pSL5 (Table 3, Figure 4). *E. faecalis* JH2-2×SL5.7 showed a similar 40-kb band but no 5-kb band (Figure 4). As the recipient *E. faecalis* JH2-2 is plasmid-free, pSL40 was transferred from SL5.7 to JH2-2 whereas pSL5 was not transferred. Southern blot analysis revealed that *tet(M)* is located on the chromosome as well as on the large 40-kb plasmid in both the donor strain and the transconjugant. No hybridization signal with the *tet(M)* probe was obtained for the smaller plasmid of strain SL5.7 (Figure 4).

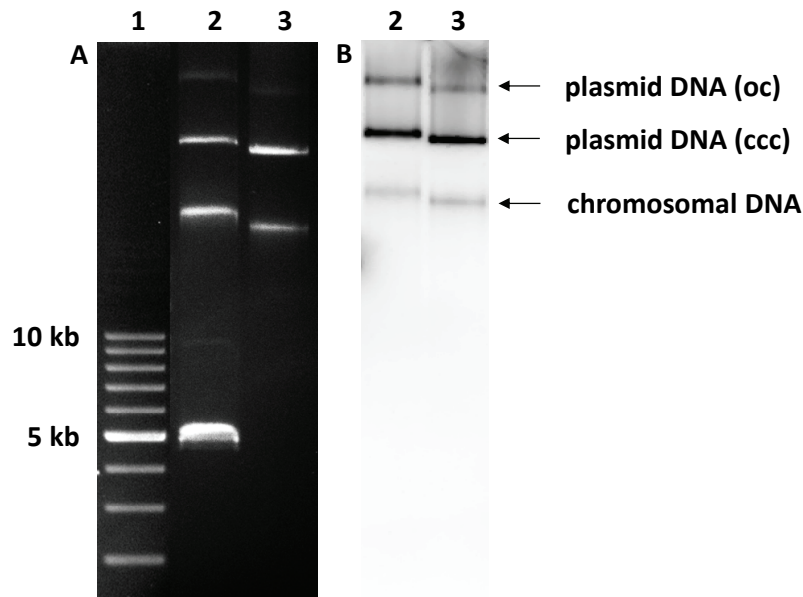


Figure 4. Detection of genetic elements in *E. faecalis* SL5.7 and transconjugant JH2-2×SL5.7 by gel electrophoresis and Southern blot hybridization. (A) 0.8% agarose gel of DNA extracted according to the protocol of Anderson and McKay (Anderson & McKay, 1983) stained with ethidium bromide. (B) Southern blot hybridization using a DIG-labeled *tet(M)* probe. 1: supercoiled DNA ladder (Promega). 2: plasmid and chromosomal DNA from *E. faecalis* SL5.7. 3: plasmid and chromosomal DNA of transconjugant *E. faecalis* JH2-2×SL5.7.

2.4.4 Nucleotide sequence of *tet(M)* in *E. faecalis* SL5.7

The *tet(M)* gene including the 629 bp upstream and the 291 bp downstream region of *E. faecalis* SL5.7 obtained by PCR (Table 4) was sequenced and analyzed using the BLAST tool (www.ncbi.nlm.nih.gov). The obtained 2840-bp nucleotide sequence revealed 100% homology to *tet(M)* and flanking regions on the Tn916-like elements of *Staphylococcus rostri*, *Streptococcus parauberis* and *Streptococcus agalactiae* (data not shown). Alignment with the corresponding regions on the original Tn916 of *E. faecalis* DS16 revealed 27 single nucleotide polymorphisms (SNPs), 15 SNPs upstream of *tet(M)* and 12 SNPs in the coding region. *In silico* analysis revealed that the 12 SNPs in the coding sequence result in 4 amino acid (AA) exchanges in the 639 AA sequence of the ribosomal protection protein of strain SL5.7 compared to Tet(M) of strain DS16 (data not shown; Flannagan *et al.*, 1994). The high homology of the nucleotide sequence of *tet(M)* and the flanking regions to Tn916-like

elements as well as the presence of the integrase gene *int* in *E. faecalis* SL5.7 determined by PCR indicate that this food isolate harbors at least one copy of a conjugative transposon of the Tn916-1545 family.

2.4.5 Antibiotic resistance pattern of *E. faecalis* SL5.7

Microarray hybridization using a microchip for the detection of up to 90 different ABR genes and the virulence gene *gelE* confirmed once more the presence of the *tet(M)* gene in strain SL5.7 (Figure 5). No other ABR gene was detected.



Figure 5. Microarray hybridization of *E. faecalis* SL5.7. Clear signals for the virulence gene *gelE* and the tetracycline resistance gene *tet(M)* are indicated. The dots marked with an asterisk are biotin markers (positive control for the hybridization).

To assess the activity of the identified ABR genes, the phenotypic resistance patterns of *E. faecalis* SL5.7 and its JH2-2 transconjugant were compared in a disk diffusion assay according to CLSI guidelines (CLSI, 2008). Both donor and transconjugants were sensitive to ampicillin, penicillin and vancomycin and resistant to tetracycline (Table 6). Both strains showed an intermediate resistance to erythromycin (Table 6).

Table 6. ABR profile of *E. faecalis* SL5.7 and JH2-2×SL5.7 determined by disk diffusion test according to CLSI guidelines.

Antibiotic (quantity/disc)	<i>E. faecalis</i> SL5.7		<i>E. faecalis</i> JH2-2×SL5.7	
	ID (mm)	Resistance phenotype	ID (mm)	Resistance phenotype
Ampicillin (10 µg)	24 ± 1	sensitive	25 ± 1	sensitive
Chloramphenicol (30 µg)	18 ± 1	sensitive	15 ± 1	intermediate
Erythromycin (15 µg)	22 ± 2	intermediate	21.5 ± 1.5	intermediate
Penicillin (10 units)	19 ± 2	sensitive	16 ± 2	sensitive
Tetracycline (30 µg)	8 ± 0	resistant	8 ± 0	resistant
Vancomycin (30 µg)	17 ± 2	sensitive	17 ± 1	sensitive

Abbreviation: ID: inhibition diameters; numbers are clearing zone diameters in mm, including disc diameter (8 mm).

2.4.6 *E. faecalis* SL5.7 virulence determinants and expression

Since virulence factors in *E. faecalis* might be involved in colonization ability (Jett *et al.*, 1994), the presence of the two most frequent virulence determinants in enterococcal food isolates, *gelE* and *asaI* (Franz *et al.*, 2001), was investigated using PCR (Table 4). The *gelE* gene, encoding gelatinase, is associated to the damage of host tissue (Gilmore *et al.*, 2002) whereas *asaI* encodes an aggregation substance that is associated to adherence to eukaryotic cells (Gilmore *et al.*, 2002). This aggregation substance is also involved in the transfer of conjugative pheromone-responsive plasmids through agglutination of *Enterococcus faecalis* cells (Galli *et al.*, 1990). Both genes were detected in *E. faecalis* SL5.7 by PCR using primers indicated in Table 4 (data not shown). The presence of the *gelE* gene was additionally confirmed on the microarray (Figure 5). However, a gelatinase assay showed that gelatinase was not produced by strain SL5.7 *in vitro* (data not shown), and no agglutination of *E. faecalis* SL5.7 in the presence of the pheromone produced by *E. faecalis* JH2-2 was observed, suggesting that the corresponding AsaI protein is not active. Additionally, no hemolysin production could be detected in *E. faecalis* SL5.7 (data not shown), demonstrating the absence of a further virulence factor in this food-isolate.

2.5 Discussion

The use of antibiotics in medicine and animal husbandry has resulted in the increasing prevalence of antibiotic resistant strains in members of the normal gut microbiota. It is assumed that ABR genes can be horizontally transferred between resident and incoming bacteria in the human gut (van den Bogaard & Stobberingh, 2000, Salyers & Shoemaker, 2006). To investigate the prevalence of ABR genes in infants, the two ABR genes *tet(M)* and *erm(B)* were quantified in 22 infant fecal samples. The mean *tet(M)* copy number was 7.1 log per g feces (Table 5). This gene is thought to have the widest host range of all *tet* genes in Gram-positive bacteria (Chopra & Roberts, 2001, Roberts, 2005) and is even widely distributed in breast-fed infants that had neither direct nor indirect contact with antibiotics (Gueimonde *et al.*, 2006). Log copy numbers of the *erm(B)* gene in infant fecal samples were between 4.8 and 9.2 per g (Table 5). The *erm(B)* gene has been described as the most prevalent erythromycin resistance gene in fecal samples and the most widely distributed *erm* determinant in streptococci and enterococci (Min *et al.*, 2003, Seville *et al.*, 2009). Even in the feces of the 5 infants not older than 14 days, the average *tet(M)* and *erm(B)* copy numbers were high (Table 5). In the hospital environment including maternity wards, the application of antibiotics favors the survival and spread of antibiotic resistant bacteria even in people not treated with antibiotics (Levy, 1998, Furuya & Lowy, 2006). Since colonization of the newborn GIT begins shortly after delivery (Favier *et al.*, 2003), the colonization of neonates with antibiotic resistant bacteria is likely and our data showed that early colonization with ABR resistant bacteria occurs in humans.

Enterococcus strains are normal gut inhabitants in mammals and belong to the first colonizers of the human GIT. Additionally, enterococci are frequently found in several types of foods, e.g. fermented olives, sausages and cheese, as spoilage organisms or as essential microflora contributing to flavor and shelf-life (Giraffa, 2002). ABR genes are highly prevalent in food-associated enterococci. ARE can enter the GIT via the food chain, potentially resulting in the spread of resistance genes in the dense and complex gut microflora (Franz *et al.*, 2003). In this

study, the *Enterococcus faecalis* strain SL5.7, isolated from a dry fermented sausage and harboring the tetracycline resistance gene *tet(M)*, was characterized.

In *in vitro* conjugation experiments, strain SL5.7 transmitted its tetracycline resistance to the plasmid-free recipient strain *E. faecalis* JH2-2 at a transfer frequency of 7.04×10^{-3} transconjugants per donor cell. This frequency lies in the typical range of 10^{-3} to 10^{-6} reported for broad-host range plasmids (Grohmann *et al.*, 2003). Plasmid isolation and electrophoresis revealed that the donor SL5.7 harbors two plasmids, a ~40-kb plasmid, designated pSL40, which was shown to be transferable to JH2-2, and a 5-kb plasmid, named pSL5 (Table 3, Figure 4). Albeit larger conjugative plasmids can mobilize smaller non-conjugative plasmids, as it was observed for the conjugative enterococcal plasmid pRE25 and the non-conjugative pESP91 (Schwarz *et al.*, 2001), the cryptic 5-kb plasmid pSL5 was not mobilized from strain SL5.7 to JH2-2 (Figure 4). Southern blot and hybridization demonstrated that *tet(M)* lies on the chromosome as well as on the large conjugative plasmid pSL40 in both the donor strain and its JH2-2 transconjugant (Figure 4). Albeit the protocol is designed to isolate plasmid DNA, the final DNA solution still contained chromosomal DNA, as indicated in Figure 4. However, the lower bands in lanes 2 and 3 indicated as chromosomal DNA have much lower signal intensities in the Southern blot, indicating that they do not represent plasmid DNA.

Tetracycline resistance encoded by *tet(M)* is common in enterococci and often linked to conjugative transposons of the Tn916-1545 family (Huys *et al.*, 2004, Rizzotti *et al.*, 2009). Members of this family have been found in a wide variety of Gram-positive and Gram-negative bacteria (Rice, 1998). BLAST analysis of the *tet(M)* gene and the flanking sequences of SL5.7 revealed 100% nucleotide homology to the corresponding regions of Tn916-like transposons in *S. rostri*, *S. parauberis* and *S. agalactiae* (data not shown). *S. rostri* is a newly described *Staphylococcus* species isolated from pig's noses (Riesen & Perreten, 2009). *S. parauberis* harboring Tn916-like elements was isolated from diseased flounders in Japan usually treated with tetracycline (Meng *et al.*, 2009). The high prevalence of *tet(M)*-positive strains in this animal pathogen is therefore assumed to result from the dissemination of

tetracycline resistance via Tn916-like elements (Meng *et al.*, 2009). The Tn916-like element in the genome of *S. agalactiae* 2603V/R showed atypical nucleotide composition, indicating that this element was acquired by lateral gene transfer (Tettelin *et al.*, 2002). Strain SL5.7 most probably harbors Tn916, since Tn1545 also encodes resistances to erythromycin and kanamycin (Clewell *et al.*, 1995), which was not observed in SL5.7 (Figure 5, Table 6). Tn916 is mostly chromosomally integrated, even though it has occasionally been found in low-copy-number plasmids (Christie *et al.*, 1987). However, the precise localization of Tn916 in *E. faecalis* SL5.7 remains to be established.

None of the two virulence determinants detected in *E. faecalis* SL5.7, namely *gelE* and *asa1*, were active. The *gelE* gene is common in *E. faecalis* food-, starter- and medical strains (Eaton & Gasson, 2001). However, *gelE* is sometimes present as a silent gene. These genes may become activated *in vivo*, e.g. in the GIT (Eaton & Gasson, 2001, Creti *et al.*, 2004). The absence of the gelatinase phenotype *in vitro* might therefore not reflect behavior *in vivo*, since sublethal stresses encountered during food processing or digestion may significantly up-regulate *E. faecalis* expression of virulence genes (Lenz *et al.*, 2010). The gene *asa1*, encoding an aggregation substance, is frequently detected in *E. faecalis* (Creti *et al.*, 2004). Agglutination enhances the transfer of sex pheromone plasmids into recipient cells producing small pheromone peptides into liquid medium (Francia & Clewell, 2002). The absence of agglutination therefore indicates that pSL5.7 belongs to the pheromone-independent plasmids. The high prevalence of ABR genes in fecal samples indicates that the human gut microbiota may serve as a reservoir of resistance determinants. Foods containing viable antibiotic resistant bacteria, e.g. fermented sausages and cheeses, are assumed to contribute to the transmission of ABR genes into the GIT. The high tolerance to adverse environmental conditions might favor enterococci to survive during transit in the stomach and lower intestine. If they can reach the colon, resistance genes might be readily transmitted to residing or transient bacteria of the highly diverse and dense colonic microbiota.

Both the high background of ABR genes even in newborns and the high homology of the *tet(M)* gene from SL5.7 to *tet(M)* genes of various Gram-positive bacteria currently present in nucleotide databases reveal that analyses of horizontal ABR gene transfer in complex environments requests the construction of genetically marked mobile ABR determinants. For example, a chromosomally marked donor strain harboring a tagged conjugative ABR plasmid would be a suitable tool, allowing monitoring of HGT in conjugation experiments and distinguishing between donor and recipients in a complex colonic environment. The construction and characterization of such a tool is described in Chapter 3.

2.6 Acknowledgements

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3 Construction and Characterization of *Enterococcus faecalis* CG110/*gfp*/pRE25*, a Tool for Monitoring Horizontal Gene Transfer in Complex Microbial Ecosystems¹

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3.1 Abstract

Enterococci are among the most notorious bacteria involved in spread of antibiotic resistance (ABR) determinants via horizontal gene transfer (HGT), leading to increased prevalence of antibiotic resistant bacteria. In complex microbial communities with a high background of ABR genes, detection of gene transfer is only possible when the ABR determinant is marked. Therefore the conjugative multiresistance plasmid pRE25, originating from a sausage-associated *E. faecalis*, was tagged with a 34-bp random sequence marker spliced by *tet(M)*. The constructed plasmid, 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 to its parental pRE25, occurs at 1-2 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. *E. 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.

3.2 Introduction

Horizontal transfer of resistance genes and antibiotic mediated selection pressure leads to a persistence and propagation of antibiotic resistant bacteria in clinical environment, 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 like 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 (GIT) of humans and animals. Enterococci harbor a variety of mobile genetic elements (MGEs) like 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 microflora has revealed a vast diversity of resistance genes, indicating that the human microflora 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, Alpert *et al.*, 2003, Licht *et al.*, 2003, Avrain *et al.*, 2004, Mater *et al.*, 2005, Hart *et al.*, 2006, Lester *et al.*, 2006, Jacobsen *et al.*, 2007, Moubareck *et al.*, 2007, Feld *et al.*, 2008, Mater *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*, *L. lactis* and *L. 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.*, 1993a, Le Chatelier *et al.*, 1993). Sequence comparison of pRE25 to other conjugative plasmids like 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 GIT, food matrices and biofilms.

3.3 Material and methods

3.3.1 Bacterial strains and media

Bacterial strains used in this study are listed in Table 7. Chemicals were routinely obtained from Sigma-Aldrich (Buchs, Switzerland), except when stated otherwise. *Staphylococcus aureus* and *Enterococcus* strains were cultivated aerobically at 37°C in brain heart infusion (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)]. *E. coli* strains were grown aerobically in lysogeny broth (LB, Becton Dickinson, Allschwil, Switzerland) at 37°C, unless stated otherwise. *Bacteroides thetaiotaomicron* was grown anaerobically in YCFA medium (Duncan *et al.*, 2002b) at 37°C. Anaerobic conditions were maintained using AnaeroGen™ (Oxoid, Pratteln, Switzerland).

3.3.2 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 8.

Table 7. Bacterial strains and plasmids used in this work.

Material		Relevant features		Source
Strains				
<i>Escherichia coli</i>	XL1-blue	Cloning host		Stratagene Amsterdam
	DH5 α	Cloning host		Woodcock <i>et al.</i> , 1989;
	S17-1	Str ^R , Trp ^R		Invitrogen, Basel, Switzerland
	CG120/pAM120	Harbors pAM120, a pBR211 derived plasmid containing tet(M) on Tn916		Simon <i>et al.</i> , 1983
<i>Enterococcus faecalis</i>	CG110/ <i>gfp</i>	CG110 derivative harboring a chromosomal <i>gfp</i> in Tn916 transposon; Fus ^R , Rif ^R , Tet ^R		Gawron-Burke & Clewell, 1984
	CG110/ <i>gfp</i> /pRE25*	CG110/ <i>gfp</i> derivative harboring pRE25*; Cm ^R , Ery ^R , Fus ^R , Gen ^R , Kan ^R , Neo ^R , Rif ^R , Str ^R , Tet ^R		Scott <i>et al.</i> , 2000
	RE25	Dry sausage isolate harboring pRE25; Cm ^R , Ery ^R , Gen ^R , Kan ^R , Neo ^R , Str ^R , Tet ^R		this work
	1528	Clinical isolate, recipient for filter mating; Ery ^R , Tet ^R , Van ^R		Perreten, 1995
	Bu2-60	Starter culture isolate, plasmid free; Rif ^R , Str ^R ,		Klare <i>et al.</i> , 1995
<i>Lactococcus lactis</i>	BuRE25	<i>L. lactis</i> Bu2-60 harboring a single copy of pRE25 integrated in chromosome; Cm ^R , Ery ^R , Fus ^R , Gen ^R , Kan ^R , Neo ^R , Rif ^R , Str ^R		Neve <i>et al.</i> , 1984
	BuRE25*	<i>L. lactis</i> Bu2-60 harboring pRE25* integrated in the chromosome; Cm ^R , Ery ^R , Fus ^R , Gen ^R , Kan ^R , Neo ^R , Rif ^R , Str ^R , Tet ^R		Perreten, 1995
	ROT1	Dairy isolate; Ery ^R , Nov ^R , Tet ^R ,		this work
<i>Lactobacillus fermentum</i>	L19	Plasmid free, recipient for filter mating		Gfeller, 2003
<i>Listeria monocytogenes</i>	10403S	Derivative of the clinical isolate 10403, recipient for filter mating; Str ^R		Schwarz <i>et al.</i> , 2001
	LM15	Food isolate, recipient for filter mating, Tet ^R		Bishop & Hinrichs, 1987
				Veterinary hospital, Zurich

Table 7. continued.

Material	Relevant features		Source
Strains			
<i>Listeria monocytogenes</i>	10403S/pRE25*	Transconjugant from filter mating, strain 10403 harboring pRE25*; Cm ^R , Ery ^R , Gen ^R , Kan ^R , Neo ^R , Str ^R , Tet ^R	this work
<i>Bacteroides thetaiotaomicron</i>	BT4100	Derivative of the fecal isolate VPI-5482; recipient for filter mating; Gen ^R	Shoemaker <i>et al.</i> , 1992
<i>Leuconostoc mesenteroides</i>	M7-1 (LMG19463)	Recipient for filter mating; Van ^R	FBTSC
<i>Staphylococcus aureus</i>	VG1	Dairy isolate; Gen ^R , Neo ^R , Pen ^R	Perreten, 1995
Plasmids			
pGEM [®] -T Easy		3.0 kb, cloning vector	Promega, Madison, USA
pMH400		5.1 kb, pGEM derivative harboring the 1-kb up- and downstream regions of the stopcodon of the <i>erm(B)</i> 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 <i>erm(B)</i> gene from pRE25 interspaced with <i>tet(M)</i> flanked by two 23-bp and a 11-bp random sequences	this work
pRE25		50.2 kb, Cm ^R , Ery ^R , Gen ^R Kan ^R , Neo ^R , Str ^R	Perreten, 1995, Schwarz <i>et al.</i> , 2001
pRE25*		52.9 kb, Cm ^R , Ery ^R , Gen ^R , Kan ^R , Neo ^R , Str ^R , Tet ^R , pRE25 derivative harboring a 34-bp random sequence spliced by <i>tet(M)</i>	This work

Abbreviations: Cm^R: chloramphenicol resistant; Ery^R: erythromycin resistant; Fus^R: fusidic acid resistant; Gen^R: gentamicin resistant; Kan^R: kanamycin resistant; Neo^R: neomycin resistant; Rif^R: rifampicin resistant; Sp^R: spectinomycin resistant; Str^R: streptomycin resistant; Tet^R: tetracycline resistant; Trp^R: trimethoprim resistant; Van^R: vancomycin resistant; FBTSC: Food Biotechnology strain collection, ETH Zurich.

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Table 8. Oligonucleotides used in this work.

Primer	Sequence (5'→3')	T _{Ann.} ^a	Reference
CatpIP501_1	ggatatgaaatttatccctc	53°C	Aarestrup <i>et al.</i> , 2000
CatpIP501_2	caatcatctaccctatgaat		
Ins_A2	tgtataataggaatttgaagtta	48°C	This work
Ins_B	<u>atgacagatt</u> aaat ggatccgatcgaattccgaatagaattatttctcccg		
Ins_C	<u>tcggaattcgatcggatcc</u> att aaatctgtcatgagtcgctttttaaattg	48°C	This work
Ins_D	taatgagatcatagtcaact		
HP14	ttgaagtcgacgggagtaattggaag	60°C	Warren <i>et al.</i> , 2004
HP15	taaaagtcgacatacataacggaaagag		
Int401_A	cgaaatgatacacaatc	56°C	This work
Int401_F	ttccaattactcccgt		
Int401_G	tctttccgttatgtatgt	56°C	This work
Int401_D	ggcgttggtacagtatc		
seq1_fw	tcaatcgagaatatacgta	56°C	This work
seq1_rv	aagagagtacgtgattaca		
seq2_fw	aagcagttcaaagtaact	54°C	This work
seq2_rv	ccacacttaggacatt		
lmoF	cgcaagaagaaattgcatc	60°C	Huang <i>et al.</i> , 2007
lmoR	tccgcgttagaaaaattcca		
linF2	ttgctactgaagaaaaagca	60°C	Huang <i>et al.</i> , 2007
linR2	tctgttttgcttctgtagc		
tufA_fw	gacaaccattcatgatgccag	60°C	Ke <i>et al.</i> , 1999
tufA_rv	cgtcaccaacgcgaactca		
pRE25*_F	tcatcaagcaatgaaacacg	54°C	This work
pRE25*_R	gcatattgtaaaggaatctcca		
pRE25_F	ccgtttacgaaattggaaca	60°C	This work
pRE25_R	ttggtgaattaaagtgacacga		
gfp_fw	ctttcactggagttgtcc	51°C	Scott <i>et al.</i> , 1998
gfp_rv	ccagcagctgttacaactc		
aph_F	aatgacggacagccggtat	60°C	This work
aph_R	cctttggaacaggcagcttt		
hly_fw	gggaaatctgtctcaggtg	60°C	Guilbaud <i>et al.</i> , 2005
hly_rv	cgatgattgaactcatctttgc		
gfp_F2	tggaagcgttcaattagcaga	60°C	This work
gfp_R2	ggcagattgtgtggacaggt		
pRE25*_F2	gtaccattactatgagcaagtattgtc	60°C	This work
pRE25*_R2	ctataatctccaattactcccgtc		
pRE25*_TMP	FAM-ggaaataattctattcgaattcgatcggatc-TAMRA		

For primers Ins_B and Ins_C (SOE PCR), the random sequence (= overlapping sequence) is underlined and the restriction site for *Sma*I is depicted in bold letters. All primers were synthesized by Microsynth (Balgach, Switzerland).

Abbreviations: FAM: reporter dye (6-Carboxyfluorescein); TAMRA: non-fluorescent quencher (Carboxy-tetramethylrhodamine); SOE: splicing by overlap extension.

^a Annealing temperatures used in this study

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 plasmid copy number determination in *E. faecalis* and for the quantification of donor and transconjugants in complex microbiota was performed as follows: 2 ml of culture were harvested and resuspended in 400 μ l of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The suspension was transferred to a 2 ml screw cap tube (Sarstedt, Sevelen, Switzerland) containing 500 μ l of PCIA solution (Phenol:Chloroform:Isoamylalcohol 25:24:1) and 500 mg of 0.1 mm zirconia/silica beads (BioSpec Products Inc. Bartlesville, USA). The cells were disrupted mechanically by bead-beating four times 20 s at maximum speed in a FastPrep device (MP Biomedicals, Illkirch, France), interspaced by cooling for 1 minute on ice. The suspension was centrifuged at 20'000 g at 4°C for 10 min and the supernatant was extracted once with chloroform to remove residual phenol. The DNA was precipitated with 1 volume isopropanol, washed once with 70% v/v ethanol, dried and resuspended in TE buffer. Plasmid DNA from *E. coli* was isolated using the Plasmid Midi Kit (QIAGEN) using 100 ml of fresh overnight cultures. Plasmids from *E. faecalis* were purified according to the preparative protocol for large scale plasmid isolation (Anderson & McKay, 1983), with following modifications: the pelleted cells from 600 ml of an overnight culture were stored at -20°C for 16 h to facilitate cell disruption. The next day, cells were resuspended in 30 ml lysis buffer and cell walls were degraded by the addition of 80 μ l of mutanolysin (2500 units/ml) and 20 mg/ml lysozyme followed by incubation at 37°C for 1 hour. Chromosomal DNA was precipitated by the addition of 5 M NaCl (J.T. Baker, Deventer, the Netherlands), incubation on ice during 1 hour and centrifugation at 23'000 g, 4°C for 45 min. After purification using phenol/methylenchloride/isoamyl alcohol (25:24:1), nucleic acids were precipitated with isopropanol. The resulting pellet was washed twice with ice-cold 70% EtOH, dried at room temperature and resuspended in TE buffer. RNA was subsequently digested using 10 mg/ml RNase A at 37°C for 15 min.

3.3.3 Southern blot analysis

The probe for the detection of the *cat* gene by Southern blot was constructed using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Rotkreuz, Switzerland) and pRE25* as template combined with the primer set CatpIP501_1/2 (Table 8). DNA transfer and hybridization (16 h at 36°C) were performed using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics). Chemiluminescence was detected by exposure of the blot to the Kodak Image Station for 60 min. Digestion of chromosomal and plasmid DNA by restriction endonucleases was performed following standard protocols according to the manufacturer's instructions (New England Biolabs, Boston, USA).

3.3.4 Microarray analysis

ABR genes were detected using a microarray harboring probes for 90 different ABR genes (Perreten *et al.*, 2005). Genomic DNA of *E. faecalis* for microarray hybridization was isolated as follows: cells from 4 ml of a fresh overnight culture were lysed using a guanidium thiocyanate method (Pitcher *et al.*, 1989). DNA was extracted from the cell lysate as described previously (Perreten *et al.*, 2005). Hybridization and microarray analysis were performed as described previously (Perreten *et al.*, 2005).

3.3.5 Construction of *Enterococcus 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 (Figure 6, Table 7). A 1-kb fragment directly upstream of the stopcodon of the *erm*(B) gene was amplified using the primer pair Ins_A2/B (Table 8), the proof reading Phusion polymerase (Finnzymes, Espoo, Finland), and DNA from *L. lactis* BuRE25 as template (Table 7). Similarly, a 1-kb fragment downstream of the stopcodon of the *erm*(B) gene was amplified using the primers Ins_C and Ins_D. The two fragments were fused via splicing by overlap extension (SOE) PCR using the 34-bp overlapping region

introduced in the primers (Table 8, underlined). To this end, the fragments (50 ng each) were mixed and a PCR-step was performed using the primers *Ins_A2* and *Ins_D* and 2×PCR Master Mix (Fermentas, Le-Mont-sur-Lausanne, Switzerland). The resulting 2120-bp fragment was ligated into the cloning vector pGEM®-T Easy (Table 7, Promega, Madison, USA) according to the manufacturer's instructions. *E. coli* XL1-Blue electrocompetent cells were transformed with the ligation mix using 2 mm electrocompetent cuvettes (Cellprojects, Kent, UK) and an Equibio electrocompetent device (Witec AG, Littau, Switzerland) according to the manufacturer's instructions. Transformants were selected on LB agar supplemented with 100 µg/ml ampicillin, 0.5 mM IPTG (Axon Lab, Dättwil, Switzerland) and 80 µg/ml X-Gal (Axon Lab). Plasmids were isolated from colonies displaying correct growth pattern and correct plasmid construction was confirmed by restriction analysis. The resulting plasmid was designated pMH400, a plasmid containing the 1-kb up- and downstream regions of the stopcodon of the *erm(B)* gene, interspaced with a 34-bp random sequence containing a *SwaI* site (Table 7). Subsequently, *tet(M)* was amplified from *E. coli* CG120/pAM120 DNA using primers HP14 and HP15 and Phusion DNA polymerase. The obtained 2678-bp fragment was ligated into pMH400 which had been linearized with *SwaI* (NEB) using T4 DNA Ligase (2'000 units/µl, NEB). Competent *E. coli* DH5α (Invitrogen, Basel, Switzerland) were transformed with the ligation mix by heat shock and colonies were selected on LB agar supplemented with 10 µg/ml tetracycline. A plasmid from one transformant was checked by restriction analysis and by PCR targeting *tet(M)* using primers HP14 and HP15 (Table 8). The obtained plasmid was designated pMH401 and harbors the 1-kb up- and downstream regions of the stopcodon of the *erm(B)* gene interspaced with *tet(M)* flanked by 23-bp and a 11-bp random sequences (Figure 6). Additional restriction analysis using *BsoBI* and *HindIII* revealed that *tet(M)* is orientated in the same direction as *erm(B)*. Plasmid pMH401 was transferred into *L. lactis* BuRE25 (Table 7) by electrocompetent as described previously (Holo & Nes, 1989) with slight modifications: cells were grown in 200 ml of GM17 medium with 2% of glycine, but without sucrose. Primary integrants were

selected by plating on selective streptococcal (SR) plates (Okamoto *et al.*, 1983) containing 10 µg/ml tetracycline. A double-cross-over event results in integration of *tet*(M) flanked by the two random sequences downstream of the *erm*(B) gene in pRE25 (Figure 6). Therefore integrants were streaked on BHI containing 10 µg/ml 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*, a *L. lactis* Bu2-60 derivative carrying pRE25 tagged with *tet*(M) flanked by two 23- and 11-bp random sequences, i.e. pRE25*, in its chromosome. Next, pRE25* was transferred to *E. faecalis* CG110/*gfp* via filter mating and transconjugants were selected by plating on KF Streptococcus Agar (Becton Dickinson) supplemented with chloramphenicol (10 µg/ml). Sequencing of the two regions overlapping the random sequence was performed by Microsynth using primer pairs seq1_fw/rv and seq2_fw/rv (Table 8). The presence of the *gfp* gene was confirmed using primers *gfp*_fw and *gfp*_rv (Table 8). The resulting strain was designated *E. faecalis* CG110/*gfp*/pRE25*, harboring a chromosomal *gfp* and pRE25* (Figure 6).

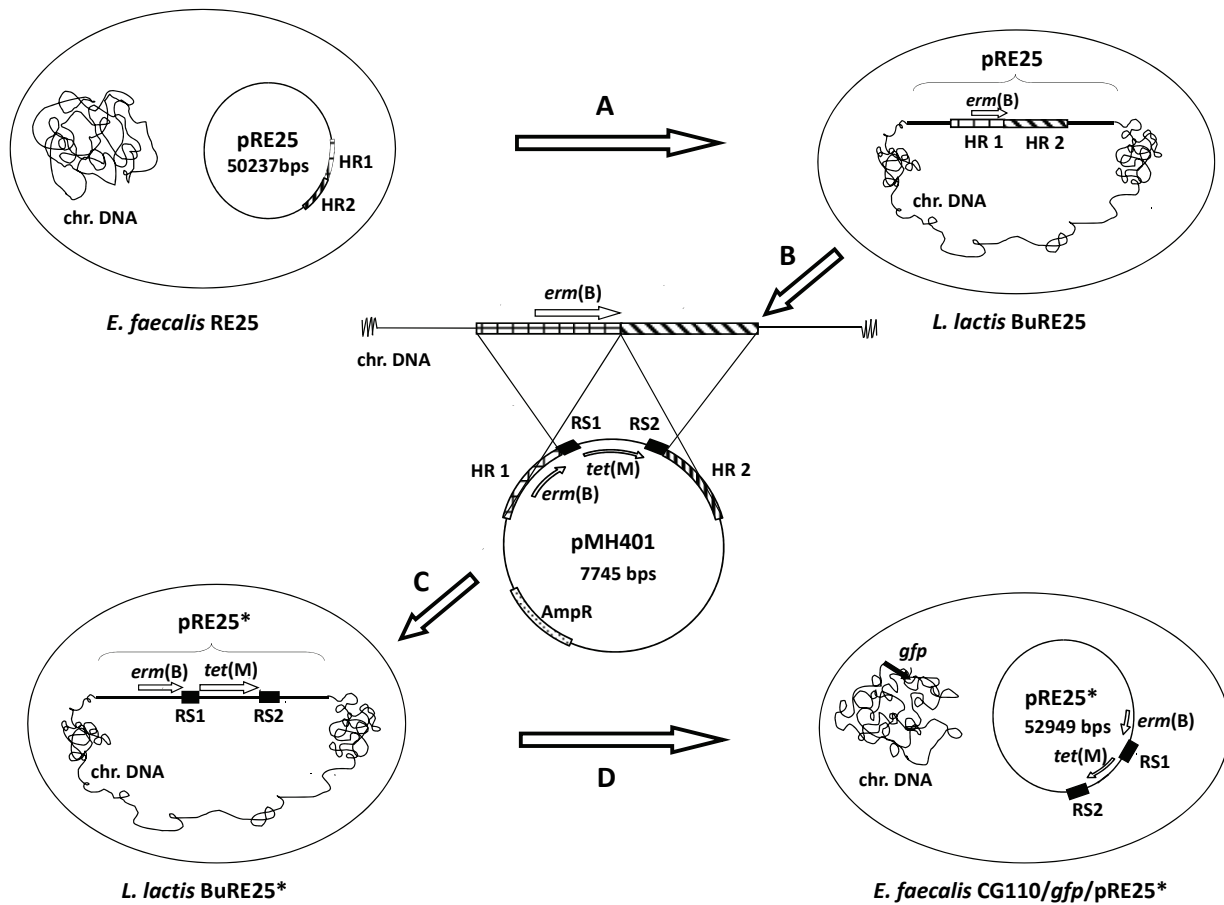


Figure 6. Construction of *E. faecalis* CG110/*gfp*/pRE25*. A) transfer of pRE25 from *E. faecalis* RE25 to *L. lactis* Bu2-60 by filter mating (Perreten, 1995); B) transformation of *L. lactis* BuRE25 (*L. lactis* Bu2-60 harboring pRE25 integrated in the chromosome) with pMH401; C) homologous recombination leading to the integration of *tet(M)* flanked by the two random sequences RS1 and RS2 downstream of *erm(B)* 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: TCGGAATTCGATCGGATCCATT; RS2: AAATCTGTCAT.

3.3.6 Conjugation experiments by filter mating technique

Overnight cultures of donor culture and recipient were mixed 1:3 and passed through a sterile 0.45- μ m nitrocellulose filter (Millipore AG, Zug, Switzerland). The filter was incubated overnight cell-side up on non-selective 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 8). *Listeria* transconjugants were verified by using the primer pairs lmoF/R and linF2/R2. *L. mesenteroides* transconjugants were identified by the absence of a *tufA* gene according to a negative PCR using primer pair *tufA_fw/rv* (Table 8).

3.3.7 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 μ g/ml). The stability of *gfp* was verified by PCR with primers *gfp_fw* and *gfp_rv*.

3.3.8 Quantitative real-time PCR

All reactions were performed in a reaction volume of 25 μ l. For real-time PCR using the SYBR Green method, 12.5 μ l of 2 \times 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 μ l 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 Ct values from serial dilutions of the corresponding target, obtained in the same qPCR run.

3.3.9 Determination of plasmid copy number

To determine the number of plasmids pRE25 and pRE25* relative to their chromosome by qPCR, DNA from 2 ml of both an exponentially growing and a stationary phase cell-culture of *E. faecalis* RE25 and CG110/*gfp*/pRE25* was extracted as described above. The *tufA* gene was used as *E. faecalis* chromosomal target gene and *aph(3')*-III as the plasmid target gene for pRE25 and pRE25*. Both targets are single copy genes in their respective DNA molecules. Copy numbers of both targets were calculated via a standard curve as described above.

3.3.10 Validation of quantitative real-time PCR for the detection of horizontal gene transfer 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*, *L. monocytogenes* 10403S, and *L. monocytogenes* 10403S/pRE25* (Table 7) were mixed at 6 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 (Table 8).

3.4 Results

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

E. faecalis CG110/*gfp*/pRE25* was first checked for correct genetic profile. The presence of the *gfp* gene in the chromosome was confirmed by PCR. Determination of the nucleotide sequences of the two inserted fragments confirmed that the random sequences flanking *tet*(M) were integrated downstream the *erm*(B) gene (data not shown). Correct genetically rearrangement of pRE25* in *E. faecalis* CG110/*gfp* was additionally checked by Southern analysis. Restriction of both pRE25 and pRE25* with *Eco*RI and subsequent targeting of the *cat* gene revealed a 26-kb fragment for pRE25 and a 19-kb fragment for pRE25* (Figure 7), confirming conjugation of a correct pRE25* to *Enterococcus faecalis* CG110/*gfp*.

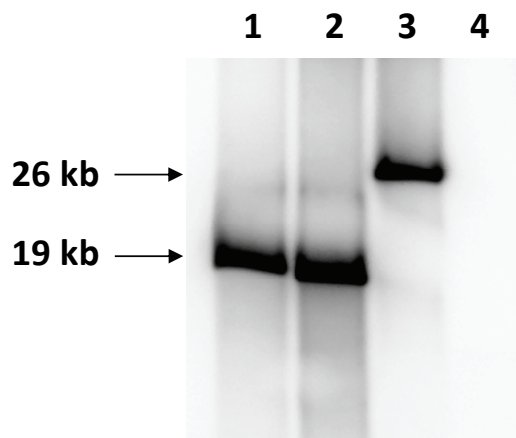


Figure 7. Southern blot hybridization of digested plasmid DNA of pRE25* and pRE25. Lanes: 1) *Eco*RI digested pRE25* (clone 1); 2) *Eco*RI digested pRE25* (clone 2); 3) *Eco*RI digested pRE25; 4) *Eco*RI digested chromosomal DNA of *E. faecalis* RE25; Arrows indicate the 19 kb fragment of pRE25* and the 26 kb fragment of pRE25 hybridizing with the DIG labeled *cat* probe.

3.4.2 Antibiotic resistance in *E. faecalis* CG110/*gfp*/pRE25*

Genetic and phenotypic ABR in *E. faecalis* CG110/*gfp*/pRE25* were determined by microarray analysis and microdilution test. Microarray hybridization confirmed the presence of the virulence factor *gelE* and the ABR genes *tet*(M), *erm*(B), *sat4*, *aph*(3')-III, and *ant6-Ia*, encoding resistances to tetracycline, erythromycin, streptothricin, kanamycin and streptomycin (Figure 8). CG110/*gfp*/pRE25* is a derivative of the laboratory strain JH2-2, which harbors *gelE*, although it does not produce gelatinase (Zeng *et al.*, 2005), explaining the presence of the *gelE* signal. Remarkably, no signal for the *cat* gene, encoding chloramphenicol transferase, was detected in the microarray hybridization. However, the same gene in *E. faecalis* JHRE25-2 and *B. anthracis* BR4253, both harboring pRE25, did not give a specific signal on the same array (Perreten *et al.*, 2005) and therefore the presence of *cat* in strain CG110/*gfp*/pRE25* was confirmed by PCR (data not shown).

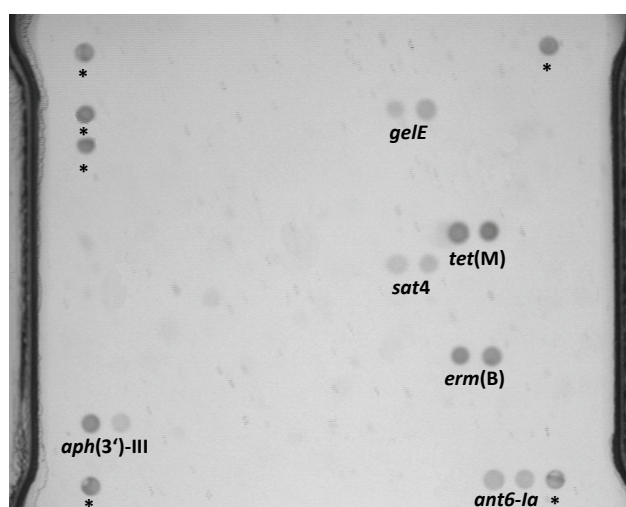


Figure 8. Microarray hybridization of *E. faecalis* CG110/*gfp*/pRE25*. Clear signals for the virulence gene *gelE* and the ABR genes *tet*(M), *sat4*, *erm*(B), *aph*(3')-III and *ant6-Ia* are indicated. The dots marked with asterisks are biotin markers (positive control for the hybridization).

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 to RE25 (Table 9). 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 in strain CG110/*gfp*/pRE25* is encoded on the chromosome and on the plasmid, whereas the other resistance genes are encoded only on pRE25* (Table 7). The microarray analysis and the resistance pattern of CG110/*gfp*/pRE25* indicate that the strain harbors a complete pRE25*.

3.4.3 Determination of plasmid copy number

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 8). The *tufA* gene was used as 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 1-2 copies per chromosome, independent of the growth phase (Table 10), indicating that the 2.7-kb insertion in pRE25* had no significant impact on the plasmid copy number.

3.4.4 Marker stability

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* were tested. The serial culture test revealed that replication of pRE25* as well as the integration of *gfp* were stable for at least 200 generations (data not shown).

Table 9. Minimal inhibitory concentrations (MIC) ($\mu\text{g/ml}$) of *E. faecalis* CG110/*gfp*/pRE25* compared with *E. faecalis* RE25.

<i>E. faecalis</i> strain	Amo	Amp	Cip	Clin	Cm	Ery	Gen	Kan	Nal	Nov	Pen	Rif	Str	Tet	Tri	Van
CG110/ <i>gfp</i> /pRE25*	0.5	1	2	32*	64	32*	16	>256	32*	4	2	32	64	64**	0.25	1
RE25	n.d.	0.38	n.d.	>256	>256	>256	6	>256	>256	n.d.	n.d.	0.75	>1024	96	n.d.	n.d.

Abbreviations: Cm: chloramphenicol; Amo: amoxicillin; Amp: ampicillin; Ery: erythromycin; Gen: gentamicin; Kan: kanamycin; Nov: novobiocin; Cip: ciprofloxacin; Clin: clindamycin; Pen: penicillin; Rif: rifampicin; Str: streptomycin; Tet: tetracycline; Tri: trimethoprim; Van: vancomycin; Nal: nalidixic acid.
n.d.: not determined

*: highest concentration tested in microdilution test

** : next higher concentration tested: 128 $\mu\text{g/ml}$

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

Table 10. Determination of copy numbers of pRE25* and pRE25 relative to the chromosome.

<i>E. faecalis</i> strain	Growth phase	Culture density (OD ₆₀₀)	<i>tufA</i> (average \pm SD copies/ml)	<i>aph(3')</i> -III (average \pm SD copies/ml)	Plasmids/chromosome
CG110/ <i>gfp</i> /pRE25*	exponential	0.622	$1.8 \times 10^{10} \pm 2.2 \times 10^9$	$2.8 \times 10^{10} \pm 2.3 \times 10^9$	1.51
	stationary	2.878	$2.8 \times 10^{10} \pm 2.6 \times 10^9$	$3.6 \times 10^{10} \pm 4.1 \times 10^9$	1.25
RE25	exponential	0.924	$3.3 \times 10^{10} \pm 1.5 \times 10^9$	$4.0 \times 10^{10} \pm 5.7 \times 10^9$	1.22
	stationary	2.838	$6.0 \times 10^{10} \pm 3.2 \times 10^9$	$7.3 \times 10^{10} \pm 6.6 \times 10^9$	1.21

The genes *tufA* (single copy gene on the chromosome) and *aph(3')*-III (single copy on pRE25* and pRE25) were quantified using real-time qPCR.

SD: standard deviation

3.4.5 Conjugation potential

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 to pRE25 in *E. faecalis* RE25. Therefore 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 11). Conjugal transfer to *Leuconostoc mesenteroides* M7-1 was only obtained with pRE25, albeit at very low frequency. No conjugation was observed to *E. faecalis* 1528, *Lb. fermentum* ROT1, *S. aureus* VG1, *B. thetaiotaomicron* BT4100 and *E. coli* S17-1 for both plasmids.

Table 11. Transconjugation rates of filter mating experiments.

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

Transconjugation efficiencies are calculated as transconjugants per donor cells. Averages of two independent experiments are shown.

n.d.: not detected.

The comparison of pRE25* to 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.

3.4.6 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 7) were mixed at different transconjugants to donor ratios ranging from 0.2:1 to 2000:1 in a complex microbial 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 (R^2 of >0.99) over a pRE25*/*gfp* ratio of more than 3 orders of magnitude (Figure 9).

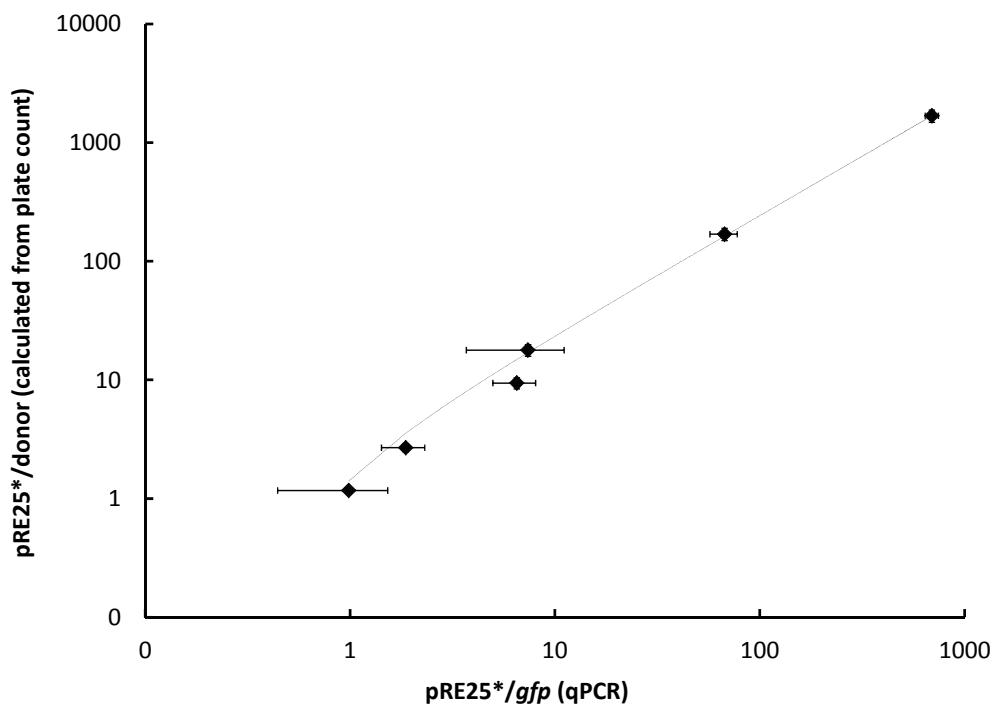


Figure 9. Correlation of the ratios of total pRE25* to donor cells calculated from plate count to the ratio of pRE25* to *gfp* quantified by quantitative real-time PCR (qPCR). The trendline is depicted in the chart area. R-squared value is >0.99 .

RESULTS

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.

3.5 Discussion

Enterococcus is one of the best studied bacterial genera associated with the acquisition and spread of ABR genes that predominately reside on transposons or large conjugative plasmids. Enterococci are ubiquitous bacteria and there is evidence that enterococci derived from food can survive ingestion and establish themselves in the GIT (Berchieri, 1999, Sørensen *et al.*, 2001, Lund *et al.*, 2002, Lester *et al.*, 2006, Egervärn *et al.*, 2010). If ARE can colonize the GIT, resistance genes are potentially transmitted to the microbiota or, as a worst case, to transient pathogenic bacteria as e.g. *L. monocytogenes* (Doucet-Populaire *et al.*, 1991). The strain *E. faecalis* CG110/*gfp*/pRE25* was constructed as a tool for the monitoring and quantification of horizontal ABR gene transfer events in complex microbial ecosystems like the human intestine. The two markers, *gfp* and the random sequences on pRE25*, are usually not occurring in the human intestine, allowing distinguishing of the donor strain from transconjugants in the complex background flora by molecular methods such as quantitative real-time PCR.

Even in the post-genomic 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. *L. lactis* BuRE25 harbors pRE25 in its chromosome (Perreten, 1995) and this allowed relative 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).

Genetic analyses confirmed correct construction and transfer of pRE25* to *E. faecalis* CG110/*gfp*. Additional characterization of pRE25* revealed that the insertion of a 2.7-kb DNA fragment did not affect characteristics nor functionality of the constructed plasmid. The plasmid copy numbers for pRE25 and pRE25* were 1-2 plasmids per chromosome (Table 10), which is in agreement with the assumption that large plasmids are present in the cell at low copy numbers (Dale & Park, 2004). Serial culturing testing established stability of both

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markers for at least 200 generations (data not shown). The stable integration of the *gfp* gene was already reported 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, usually associated with stable plasmids (Janni re *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), like the ϵ/ζ -system on pSM19035 from *Streptococcus pyogenes* (Ceglowski *et al.*, 1993a). Sequences of the proteins encoded by ORF18 and ORF49 of pRE25 are highly homologous to the ϵ -protein (unstable antitoxin), ORF19 and ORF50 to the ζ -protein (stable toxin) of pSM19035 (Meinhart *et al.*, 2003), indicating that a toxin-antitoxin system is present on pRE25 and secures its stability.

Transcription and translation of the *gfp* gene would enable easy distinguishing between *E. faecalis* transconjugants and the donor by simply plating and identifying fluorescent colonies. However, fluorescence microscopy of *E. faecalis* CG110/*gfp* and CG110/*gfp*/pRE25* revealed that only a small portion of the cells were fluorescent (data not shown), which has already been observed in strain CG110/*gfp* before (K. P. Scott, personal communication).

Conjugal transfer of pRE25* and pRE25 was demonstrated to the recipients *L. monocytogenes* LM15 and 10403S and to *L. innocua* L19 (Table 11). The transfer of pRE25 to *L. innocua* L19 has been observed before at a frequency of 10^{-5} per donor (Schwarz *et al.*, 2001). Transfer rates of pRE25* were only slightly lower compared to pRE25, which is probably due to the different host strain or the slightly increased plasmid size of pRE25* (Table 7). Transfer of both pRE25 and pRE25* to *L. monocytogenes* LM15 was rather low (Table 11), whereas the transfer frequency of 10^{-6} for *L. monocytogenes* 10403S was in the range of conjugative transfer for broad-host range plasmids (Grohmann *et al.*, 2003). No transconjugants were obtained with *E. faecalis* 1528, *Lb. fermentum* ROT1 and *S. aureus* VG1 as recipients, most probably due to plasmids incompatible to pRE25 and pRE25* present in those strains. Gene transfer from RE25 to *L. mesenteroides* M7-1 has been

observed before, albeit at low frequencies (Devirgiliis *et al.*, 2009), so the unsuccessful transfer of pRE25* from *E. faecalis* to *L. mesenteroides* is probably due to the natural occurring low efficiency of gene transfer between these species. Transconjugants of Gram-negative *Bacteroides thetaiotaomicron* BT4100 and *Escherichia coli* S17-1 were not detected. Establishment of a conjugative plasmid in the recipient strain is determined by the functionality of the replicon in the new host. Gram-negative and Gram-positive replicons are commonly not exchangeable, explaining the non-transferability of pRE25-derivatives to these strains (Courvalin, 1994).

To validate the quantitative real-time PCR assay for the detection of transconjugants harboring pRE25* in complex background, samples with different transconjugant to donor ratios were subjected to qPCR targeting the *gfp* gene and pRE25*. The quantification of both targets pRE25* and *gfp* highly correlated with the viable cell counts of donor and transconjugants (Figure 9), demonstrating that the constructed tool allows quantification of donor and transconjugants in complex microbiota.

Even though new technologies e.g. metagenomic sequencing yield a deep insight into the human microbiome (Qin *et al.*, 2010), general links between DNA sequences and their transmission route within the microbiota cannot be established using such methods, making the 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.

3.6 Acknowledgements

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4 Monitoring Horizontal Antibiotic Resistance Gene Transfer in a Colonic Fermentation Model¹

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¹Submitted for publication.

4.1 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, 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, 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-days fermentation, indicating 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 *L. monocytogenes* transconjugant and transmittable to other genera in filter mating experiments. Our study reveals that transfer of a multiresistance plasmid from *E. faecalis* 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.

4.2 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 pre-dominant genera (Kurokawa *et al.*, 2007). The gut microbiota is supposed to have numerous beneficial effects on the host, e.g. production of nutrients, activation of the immune system and defense against pathogenic bacteria (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 reservoir of ABR genes (Salysers *et al.*, 2007, Sommer *et al.*, 2009). Moreover, there are serious concerns that ABR genes can be transmitted between transient or permanent colonizers of the GIT, since 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 of digestive content (Ogier & Serror, 2008). Enterococci exhibit low-level, usually non-transferable, intrinsic resistance to several antibiotics, e.g. 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, since 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 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 *E. faecalis* RE25 which harbors the conjugative multiresistance plasmid pRE25, a 50-kb plasmid belonging to the incompatibility group Inc18 of streptococcal plasmids. These plasmids exhibit a broad range of other Gram-positive microorganisms and require high cell densities for transfer (Schwarz *et al.*, 2001). Due to 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 gut via HGT (Arias *et al.*, 2010).

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. Most approaches to study HGT in complex colonic environments so far have used defined recipients and gnotobiotic or streptomycin-treated animals (Licht *et al.*, 2002, Licht *et al.*, 2003, Moubareck *et al.*, 2003, Mater *et al.*, 2005, Jacobsen *et al.*, 2007, Moubareck *et al.*, 2007, Feld *et al.*, 2008, Mater *et al.*, 2008, Boguslawska *et al.*, 2009, Fallani *et al.*, 2010). 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 unique 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, Cinquin *et al.*, 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 *L. monocytogenes* as 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.

4.3 Material and methods

4.3.1 Bacterial strains, media and chemicals

Bacterial strains and plasmids used in this study are listed in Table 12. *Enterococcus* spp. and *Listeria* spp. were routinely grown aerobically in BHI broth (Labo-Life Sàrl, Pully, Switzerland) at 37°C. Oligonucleotides are listed in Table 13 and were synthesized by Microsynth (Balgach, Switzerland). Antibiotics were obtained from Sigma-Aldrich (Buchs, Switzerland).

4.3.2 Collection of fecal samples and 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 Arbor, MI). The samples were diluted to a final concentration of 20% w/v in sterile peptone water (0.1% w/v, pH 7.0) pre-reduced with 0.06 g/l L-cystein hydrochloride (Sigma-Aldrich) and the suspension was homogenized by vortexing at full speed for two minutes. 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 pre-reduced peptone water. The polymer solution was then inoculated with 2% fecal slurries and 0.2% of the tested bacterial cultures.

Table 12. Strains and plasmids used in this work.

Material	Relevant features		Source
Strains			
<i>E. faecalis</i>	CG110/ <i>gfp</i> /pRE25*	CG110/ <i>gfp</i> (Scott <i>et al.</i> , 2000) derivative harboring pRE25*; Cm ^R , Ery ^R , Fus ^R , Gen ^R , Kan ^R , Neo ^R , Rif ^R , Str ^R , Tet ^R	Haug <i>et al.</i> , 2010
<i>E. faecalis</i>	JH2-2	Derivative of the clinical isolate JH2, recipient for filter mating; Fus ^R , Rif ^R	Jacob & Hobbs, 1974
<i>E. avium</i>	BT1/pRE25*	Transconjugant from colonic fermentation, gut isolate harboring pRE25*; Cm ^R , Ery ^R , Gen ^R , Kan ^R , Neo ^R , Str ^R , Tet ^R	This work
<i>L. monocytogenes</i>	10403S	Derivative of the clinical isolate 10403, recipient for filter mating; Str ^R	Bishop & Hinrichs, 1987
<i>L. monocytogenes</i>	10403S/pRE25*	Transconjugant from colonic fermentation, strain 10403S harboring pRE25*; Cm ^R , Ery ^R , Gen ^R , Kan ^R , Neo ^R , Str ^R , Tet ^R	This work
<i>L. monocytogenes</i>	LM15	Food isolate, recipient for filter mating; Tet ^R	Veterinary Hospital, Zurich
<i>L. innocua</i>	L19	Plasmid-free, recipient for filter mating	Schwarz <i>et al.</i> , 2001
<i>P. acidilactici</i>	UVA-1	fecal isolate, produces pediocin PA-1	Von Ah, 2006
Plasmids			
pRE25*		52.9 kb; Cm ^R , Ery ^R , Gen ^R , Kan ^R , Neo ^R , Str ^R , Tet ^R , pRE25 derivative harboring a 34-bp random sequence spliced by <i>tet</i> (M)	Haug <i>et al.</i> , 2010

Abbreviations: *E.*: *Enterococcus*; *L.*: *Listeria*; *P.*: *Pediococcus*; Cm^R: chloramphenicol resistant; Ery^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.

Table 13. Oligonucleotides used in this work.

Primer/probe	Target	Sequence (5'→3')	T _{Ann.} ^a	Reference
eub338F	total bacteria	actctacggggagccagcag	60°C	Guo <i>et al.</i> , 2008
eub518R		attaccggctgctgg		
xfp_fw	<i>Bifidobacterium</i> spp.	atcttcggaccbgaagagac	60°C	Cleusix <i>et al.</i> , 2010
xfp_rv		cgatvacgtgvacgaaggac		
bac303F	<i>Bacteroides</i> spp.	gaaggtccccacattg	60°C	Bartosch <i>et al.</i> , 2004, Ramirez-Farias <i>et al.</i> , 2009
bfr-Fmrev		cgckacttggctggttcag	60°C	Guo <i>et al.</i> , 2008
Firm934F	Firmicutes	ggagyatgtggtttaattcgaagca		
Firm1060R		agctgacgacaaccaatgcac	60°C	
RrecF	<i>Roseburia</i> spp./ <i>Eubacterium rectale</i>	gcggtcggcaagtctga	60°C	Ramirez-Farias <i>et al.</i> , 2009
Rrec630mR		cctcgcactctagtmcgac		
Eco1457F	<i>Enterobacteriaceae</i>	catgacgttaccgcagaagaagc	60°C	Bartosch <i>et al.</i> , 2004
Eco1652R		ctctacgagactcaagcttgc		
F_Lacto 05	<i>Lactobacillus</i> spp.	agcagtagggaatctcca	60°C	Furet <i>et al.</i> , 2009
R_Lacto 04		cgccactgggttcytccatata		
hly_fw	<i>Listeria monocytogenes</i>	gggaaatcigtctcaggtg	60°C	Guilbaud <i>et al.</i> , 2005
hly_rv		cgatgatttgaacttcactcttttgc		
linF2	<i>Listeria innocua</i>	ttgtactgaagaaaagca	60°C	Huang <i>et al.</i> , 2007
linR2		tctgttttgcctctgtagc		
tufA_fw	<i>Enterococcus faecalis</i> (qPCR)	gacaaccattcatgatgccag	60°C	Ke <i>et al.</i> , 1999
tufA_rv		cgtcaccaacggcaactca		
tufA_TMP		FAM-ttctcaatvacitggwctggctactgttgc-TAMRA		
FL1	<i>Enterococcus faecalis</i> (normal PCR)	acttatgtactaacttaacc	55°C	Jackson <i>et al.</i> , 2004
FL2		taatggtgaactctgttttgg		
gfp_F	<i>gfp</i>	tggaagcgttcaattagcaga	60°C	This work
gfp_R		ggcagattgttggacaggt		
pRE25*_F	marker sequence of pRE25* (normal PCR)	tcataagcaatgaaacacg	54°C	This work
pRE25*_R		gcataattgtaaaaggaaatctcca		
pRE25*_F2	marker sequence of pRE25* (qPCR)	gtaccattacttatgacaaagtattgtc	60°C	This work
pRE25*_R2		ctataatctccaattactctccgctc		
pRE25*_TMP		FAM-ggaaataattcttattcgggaattcggatc-TAMRA		
bak11w	universal primers for sequencing	aggaggtagtccarccgca	58°C	Dasen <i>et al.</i> , 1998
bak4		agttgtcmtggctcag		

Abbreviations: FAM: reporter dye (6-Carboxyfluorescein); TAMRA: non-fluorescent quencher (Carboxytetramethylrhodamine)

^a Annealing temperatures for PCR used in this work.

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 chyme-mimicking medium as described previously (Le Blay *et al.*, 2009).

In fermentation 1, the suitability of *E. faecalis* CG110/*gfp*/pRE25* as donor in a colonic fermentation was 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 12). 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.

4.3.3 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). To colonize the beads, a serial batch culture was performed. The nutritive medium was aseptically exchanged every 12 h and anaerobic conditions were maintained by continuously flushing the headspace with CO₂. After 72 h (fermentation 1) or 48 h (fermentation 2), the fermenter was connected to a stirred feedstock flask containing sterile nutritive chyme medium (4°C) purged with CO₂ to start the continuous fermentation. The medium flow rate was set at 40 ml/h, 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 rpm) were automatically controlled during batch and continuous fermentation. To assess the stability of the system, the metabolic profile of the effluent was daily determined by HPLC analysis.

4.3.4 Sampling, DNA extraction and quantitative real-time PCR

During the continuous fermentations, effluent samples for DNA extraction were collected daily from the fermenters as follows: 2 ml of effluent were 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. Quantitative real-time PCR using the SYBR Green and the TaqMan-based method and determination of gene copy numbers was performed as described in Chapter 3 (section 3.3.8). Primers and probes for the quantification of total bacteria and main bacterial populations found in the infant large intestine are listed in Table 13. In fermentation 2, the marked plasmid pRE25* as well as the *gfp* donor marker were also quantified by real-time PCR (Table 13). Copy numbers of pRE25* and the *gfp* gene per ml effluent were normalized relative to the corresponding value at day 1 of the continuous fermentation. An increase in this ratio would thus indicate lateral transfer of pRE25* from its *E. faecalis* host to commensal bacteria.

4.3.5 Metabolite analysis

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

4.3.6 Selection of transconjugants from colonic fermentations

L. monocytogenes 10403S transconjugants harboring pRE25* (fermentation 1) were selected by plating daily onto Palcam agar base containing Palcam selective supplement (Oxoid) and erythromycin (10 µg/ml). 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 day 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 14 onto selective media for *Enterobacteriaceae*, lactobacilli, bifidobacteria, staphylococci, anaerobic Gram-negatives and anaerobic Gram-positives. All selective media contained 10 µg/ml chloramphenicol (Cm₁₀), 10 µg/ml erythromycin (Ery₁₀) and 10 µg/ml tetracycline (Tet₁₀) in order to select bacteria harboring pRE25*. To extract DNA, cell material of a single colony was smeared into a sterile Eppendorf tube and heated in a microwave oven at 600 W for 3 min. The released DNA 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 and gfp_R for *gfp* (Table 13). Detection of pRE25* and no detection of *gfp* indicates transconjugants harboring pRE25*. *L. monocytogenes* transconjugants in fermentation 1 were additionally verified by PCR targeting the *L. monocytogenes* specific *hly* gene (primer pair hly_fw/rv, Table 13). Transconjugants from fermentation 2 were identified by sequencing the 16S rRNA genes using primers bak11w and bak4 (Table 13).

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Table 14. Selective media for the detection of putative transconjugants in fermentation 2.

Organism	Enrichment broth	Media for plating	Incubation
<i>Enterobacteriaceae</i>		VRBD+ (Mossel <i>et al.</i> , 1962)	Aerobic
<i>Lactobacillus</i> spp.	-	LAMVAB+ (Hartemink <i>et al.</i> , 1997)	Anaerobic jar ^a
<i>Bifidobacterium</i> spp.	-	Beerens+ (Beerens, 1991)	Anaerobic chamber ^b
Anaerobic Gram-negatives	YCFA ^c + nisin (800 IU/ml)	Wilkins-Chalgren+(Wren, 1977)	Anaerobic chamber ^b
Anaerobic Gram-positives	YCFA ^c + polymyxin B ^d + pediocin PA-1 ^e	RCA(CN) ^f +	Anaerobic chamber ^b
<i>Staphylococcus</i> spp.	BHI (Biolife, Milan, Italy) + polymyxin B ^d + pediocin PA-1 ^e	Baird-Parker+ (Baird-Parker, 1962)	Aerobic

Enrichment culturing was performed by inoculating 1 ml of effluent sample into 50 ml of the appropriate enrichment broth. Agar plates and enrichment broth contained chloramphenicol (10 µg/ml), erythromycin (10 µg/ml) and tetracycline (10 µg/ml) 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.

^a AnaeroGen™ (Oxoid, Pratteln, Switzerland)

^b Nitrogen atmosphere containing 5% hydrogen (Coy Laboratories, Ann Arbor, MI)

^c YCFA medium (Duncan *et al.*, 2002b) + 1 g/l glucose (VWR) + 1 g/l starch from potato

^d 70 IU/ml final concentration

^e 50 µl/ml cell-free supernatant of a *P. acidilactici* UVA-1 culture (Table 12), 10x concentrated by freeze-drying

^f Reinforced clostridial agar (VWR) + 8 mg/l colistin + 8 mg/l novobiocin

+: chloramphenicol 10 µl/ml, erythromycin 10 µg/ml and tetracycline 10 µg/ml

4.3.7 Conjugation experiments by filter mating technique

To examine the functionality of plasmid replication in transconjugants isolated from the continuous colonic fermentation, filter mating experiments were performed as described in Chapter 3 (section 3.3.6). Correct plasmid transfer was confirmed by PCR using primer pairs pRE25*_F/R (Table 13). Transconjugants were verified by PCR using the primer pairs hly_fw/rv for *L. monocytogenes*, linF2/R2 for *L. innocua* and FL1/2 for *E. faecalis* (Table 13).

4.4 Results

4.4.1 ABR gene transfer to *L. monocytogenes* 10403S

First, transfer of ABR genes encoded on the conjugative multiresistance plasmid pRE25* from *E. faecalis* to the human pathogen *L. monocytogenes* in the presence of a competing infant gut microbiota was investigated (fermentation 1). Quantitative real-time PCR targeting the main bacterial groups typically present in the infant gut microbiota (Hopkins *et al.*, 2005, Kurokawa *et al.*, 2007) was performed with fermenter effluent samples and confirmed the complex background of the immobilized fecal microbiota and the colonization with *L. monocytogenes* (Table 15). Members of the Firmicutes and *Enterobacteriaceae* family were the most dominant populations with genus-specific 16S rRNA gene copy numbers of $\log 10.45 \pm 0.17$ and $\log 9.59 \pm 0.23$ per ml effluent respectively (Table 15). Lactobacilli and *E. faecalis* were present at subdominant levels with corresponding gene copy numbers of $\log 5.37 \pm 0.39$ and $\log 7.98 \pm 0.25$ per ml effluent. *L. monocytogenes*, detected via *hly* copy numbers, were present at $\log 6.03 \pm 0.18$ per ml effluent. *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 copies per ml effluent (Table 15). Total metabolite concentration was 83.22 ± 5.81 mM, with acetate being the main product, followed by butyrate and propionate. Isobutyrate and formate were present at low concentrations (≤ 10 mM), whereas lactate was not detected (Table 16). During the continuous fermentation, *L. monocytogenes* 10403S transconjugants were isolated daily by plating effluent samples onto *Listeria* spp. selective medium supplemented with erythromycin (10 $\mu\text{g/ml}$), except on day 7.

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Table 15. Bacterial populations in effluent samples of fermentation 1 and 2.

Bacterial population	Ferm. 1 (log ₁₀ gene copies/ml)	Ferm. 2 (log ₁₀ gene copies/ml)
Total 16S rRNA genes	11.04 ± 0.11	10.78 ± 0.12
<i>Bacteroides</i> spp.	3.56 ± 0.15	9.89 ± 0.12
<i>Bifidobacterium</i> spp.	ND ^a	7.42 ± 0.23
Firmicutes	10.45 ± 0.17	10.06 ± 0.09
<i>Enterobacteriaceae</i>	9.59 ± 0.23	8.24 ± 0.33
<i>Roseburia</i> spp./ <i>E. rectale</i>	3.13 ± 0.30	9.68 ± 0.27
<i>E. faecalis</i>	7.98 ± 0.25	8.27 ± 0.11
<i>Lactobacillus</i> spp.	5.37 ± 0.39	ND ^a
<i>L. monocytogenes</i>	6.03 ± 0.18	not determined

Total copy numbers were determined using quantitative real-time PCR. Total gene copy numbers were quantified using a DNA calibration curve obtained by plotting Ct values from serial dilutions of the corresponding target, obtained in the same qPCR run. 16S rRNA genes were used as specific target gene for the different groups, except for *Bifidobacterium* spp. (*xfp* gene), *E. faecalis* (*tufA* gene) and *L. monocytogenes* (*hly* gene; Table 13). Values are means ± SD of daily analyzed effluent samples.

^aND: not detected, below detection limit.

Table 16. Metabolites in fermentation 1 and 2 determined by HPLC.

Metabolite	Metabolite concentration (mM)	
	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 ^a
Isobutyrate	0.97 ± 0.11	8.04 ± 1.51
Isovalerate	ND ^a	3.93 ± 1.27
Lactate	ND ^a	ND ^a
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).

^aND, not detected, below detection limit of the method.

4.4.2 ABR gene transfer to commensal bacteria

The second *in vitro* colonic fermentation was performed to investigate the transfer potential of pRE25* to commensal bacteria of the complex human gut microbiota. The main bacterial groups normally encountered in infant feces were quantified by real-time PCR (Table 15). *Bacteroides* spp., Firmicutes and *Roseburia* spp./*E. rectale* were the predominant groups with $\log 9.89 \pm 0.12$, $\log 10.06 \pm 0.09$ and $\log 9.68 \pm 0.27$ 16S rRNA gene copy numbers per ml effluent, whereas *Enterobacteriaceae*, *E. faecalis* and *Bifidobacterium* spp. target gene copies were slightly lower (Table 15). The *Lactobacillus* spp. 16S rRNA gene was not detected (Table 15). Total metabolite concentration determined by HPLC was 133.90 ± 6.95 mM (Table 16). Acetate was the main metabolite with an average concentration of 67.80 ± 12.03 mM. Butyrate and propionate concentrations were lower with 35.10 ± 4.95 and 18.29 ± 2.36 mM (Table 16). To monitor conjugation of pRE25* in fermentation 2, donor (via *gfp* copy numbers) and pRE25* were both quantified by real-time PCR. The ratio of pRE25* to *gfp* in the effluent increased constantly from day 1 to day 16 (Figure 10), indicating that HGT has occurred.

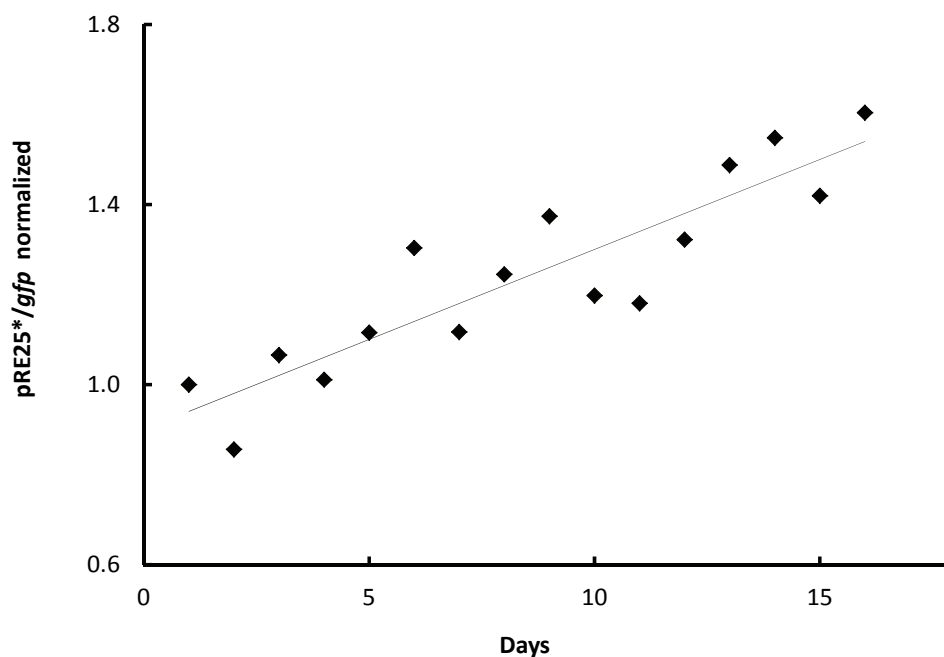


Figure 10. Ratio of plasmid pRE25* to the chromosomal donor marker *gfp* in effluent samples. Copy numbers of pRE25* and *gfp* gene were determined by qPCR and normalized to copy numbers on day 1. The trendline is depicted in the chart area.

To isolate transconjugants, effluent samples were plated onto different selective media (Table 14). 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 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). Therefore, the antibiotic spectrum for selection of transconjugants was extended with 200 µg/ml kanamycin (Kan₂₀₀), since *P. mirabilis* was sensitive to kanamycin (data not shown), and the kanamycin resistance gene *aph(3')*-III is encoded on pRE25* (Figure 2). No colonies were obtained on *Enterobacteriaceae*-specific agar with Cm₁₀, Ery₁₀, Kan₂₀₀ and Tet₁₀. Colonies growing on selective agar for *Staphylococcus* spp., *Clostridium* spp. and anaerobic Gram-negatives (Table 14) were streaked onto fresh selective media to obtain single colonies.

However, PCR targeting pRE25* and *gfp* revealed that all examined colonies were donor colonies.

Since *E. faecalis* CG110/*gfp* is not able to establish itself in a free cell continuous colonic fermentation (Scott *et al.*, 2000), we tried to enrich transconjugants by serially culturing effluent samples from day 16 in 50 ml BHI + Cm₁₀, Ery₁₀, Kan₂₀₀ and Tet₁₀ at 37°C. To release cells from beads, a portion of ~0.5 g was diluted 1:20 (w/v) in EDTA (1% w/v, pH 7, Sigma-Aldrich, Buchs, Switzerland) and mechanically disrupted using a Stomacher® at the highest setting for 5 min. After enrichment culturing for approximately 100 generations, appropriate dilutions were plated onto BHI + Cm₁₀, Ery₁₀, Kan₂₀₀ and Tet₁₀. From a total of 96 tested colonies, 96 colonies harbored pRE25*, whereas in 7 colonies the *gfp* gene was not detected, an indication for transconjugants. Sequencing of the 16S rRNA genes and BLAST analysis revealed only one single nucleotide polymorphism and 99.93% identity to the nucleotide sequence of the *Enterococcus avium* 16S ribosomal RNA gene (data not shown), demonstrating that pRE25* was transferred into the gut commensal *E. avium*.

4.4.3 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 conjugation frequency of 5.7×10^{-6} transconjugants per donor. *E. 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.

4.5 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. Conjugative 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.

Quantitative real-time PCR targeting the main bacterial groups commonly encountered in infant feces demonstrated that the microbiota in the effluent was complex and representative for infant fecal samples throughout both fermentations (Table 15). Remarkably, no bifidobacteria were detected in fermentation 1, whereas *Bacteroides* numbers were low (Table 15). The 4-months old feces donor for fermentation 1 was delivered by Caesarean section, and even though bifidobacteria are frequently found as colonizers of the infant gut, colonization can be delayed after Caesarean section (Penders *et al.*, 2006, Penders *et al.*, 2007). Similar delay is also observed for *Bacteroides* spp. colonization (Penders *et al.*, 2006, Fallani *et al.*, 2010). The dominant groups in fermentation 2 (Table 15) were in accordance with major populations commonly detected in infant feces (Hopkins *et al.*, 2005), albeit no lactobacilli were detected. However, persistent colonization of lactobacilli is not very common in infants (Ahrné *et al.*, 2005, Penders *et al.*, 2007). Real-time PCR data therefore could demonstrate a high colonization, the presence of common infant gut colonizers as well as complex bacterial composition in both fermentations (Table 15). These attributes are assumed to influence colonization as well as HGT *in vivo* (Licht & Wilcks, 2006).

The multiresistance plasmid pRE25* was conjugatively transferred from *E. faecalis* CG110/*gfp* to *L. monocytogenes* 10403S in the presence of the competing microbiota. *L. monocytogenes* is the causative agent of severe foodborne infections and considered as a common transient colonizer of the human gut (Schlech, 2000). The GIT is the most probable meeting point of *E. faecalis* and *L. monocytogenes* (Doucet-Populaire *et al.*, 1991) and lateral ABR gene transfer from *E. faecalis* to *L. monocytogenes* would exacerbate effective treatment

of listeriosis, since pRE25* harbors resistance genes against antibiotics that are used in the treatment of listeriosis in humans, such as gentamicin, clindamycin, erythromycin and tetracycline (Chen *et al.*, 2010, Johnsen *et al.*, 2010). A clear indication for HGT events was shown in the second fermentation, where the ratio of pRE25* to the donor strain increased continuously (Figure 10). Because the *gfp* gene is stably integrated in the donor strain (Haug *et al.*, 2010), pRE25* must have been transmitted to other 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. The plasmid pRE25* was still functional in the transconjugants, 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). Even though the majority of *Enterococcus* infections are caused by *E. faecalis* and *E. faecium*, species frequently harboring ABR genes (Tan *et al.*, 2010), transfer of pRE25* to *E. avium* is however concerning, 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, although the increase in the pRE25*/*gfp* ratio suggests higher abundance of transconjugants. This could be due to several reasons: transfer of pRE25* to other enterococci which cannot be uniquely selected for, or transfer to bacteria of the fecal microbiota not cultivable with the selective media used in this study. Other reasons are transfer to bacteria with a growth disadvantage compared to the donor strain or transfer of the incomplete pRE25* that does not encode resistances to the antibiotics used for the selection.

ARE are highly prevalent in food, particularly in animal-derived food like fermented meat products and cheeses (Teuber, 1999, Gevers *et al.*, 2003, Rizzotti *et al.*, 2005). 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 demonstrates 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 concerning high cell density and diversity, it is assumable that such ABR gene transfer also occurs in the human GIT. This would, as a worst-case scenario, seriously limit the effectiveness of antibiotic therapy in case of intestinal infections. This study therefore shows that high abundance of enterococci carrying transferable ABR determinants in food are a potential concern for human health when they can establish in the GIT.

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5 General conclusions and perspectives

5.1 General conclusions

The increasing prevalence of antibiotic resistant bacteria implies a serious risk for public health worldwide. Antibiotic resistance (ABR) genes are easily spread via horizontal gene transfer (HGT), leading to severe complications in the treatment of infections caused by multidrug resistant bacteria (Tenover, 2006). However, ABR is not restricted to pathogens, and the role of commensal bacteria in the dissemination of ABR genes has gained more attention in the last decade (Wang *et al.*, 2006). Especially members of the genus *Enterococcus* frequently harbor transferable ABR genes and are assumed to play a pivotal role in the horizontal spread of ABR genes (Hegstad *et al.*, 2010). Antibiotic resistant enterococci (ARE) ingested via food can transiently colonize the human gut and might transmit ABR genes to commensal or ingested bacteria in the colon.

In this dissertation, the hypothesis that foodborne ABR genes are transferable from *E. faecalis* to *L. monocytogenes* or to commensal bacteria in the human gut was tested after setting up the model and the construction of suitable molecular tools.

First, the abundance of the ABR genes *tet(M)* and *erm(B)* in infant fecal samples was investigated and revealed that both genes are encountered in high numbers, even in newborns (Chapter 2). The investigation of HGT in a complex ecosystem exhibiting such a high ABR background therefore required a suitable ABR donor strain. For this purpose, *E. faecalis* SL5.7, resistant to tetracycline and isolated from a sausage, was evaluated. *E. faecalis* SL5.7 harbors a 40-kb conjugative tetracycline resistance plasmid which was transferable to *E. faecalis* JH2-2 with a high frequency of 7×10^{-3} transconjugants per donor. However, sequencing of the *tet(M)* gene and the flanking regions in strain SL5.7 revealed 100% nucleotide homology to the corresponding regions in other genera (Chapter 2). This high homology as well as the high amount of ABR genes in infant feces necessitated the genetic labeling of a transferable plasmid to enable distinguishing of the plasmid from the

background and to monitor HGT. Since it is completely sequenced, the conjugative multiresistance plasmid pRE25 was chosen as a model plasmid for this work. The plasmid was marked by introducing *tet(M)* flanked by two 23- and 11-bp random sequences (Chapter 3). The new plasmid, designated pRE25*, was transferred to the chromosomally *gfp*-tagged *E. faecalis* strain CG110/*gfp* resulting in the donor strain *E. faecalis* CG110/*gfp*/pRE25*. Such chromosomal marker was essential for monitoring and quantifying the *E. faecalis* donor strain during gene transfer experiments in the complex fecal background since enterococci are common residents of the human gut microbiota. Plasmid copy number as well as conjugation behavior of pRE25* was comparable to its parental plasmid pRE25. Moreover, the chromosomal *gfp* marker and the marker sequence on pRE25* were stable for at least 200 generations under non-selective conditions. Quantification of the donor and plasmid in the fecal background was shown to be possible by qPCR analyses with a detection limit of 0.2 transconjugants per donor (Chapter 3). The constructed strain is therefore applicable as donor strain to monitor HGT events in complex microbial ecosystems as e.g. a continuous colonic fermentation with immobilized feces. Horizontal transfer of the enterococcal multiresistance plasmid pRE25* was then investigated using a continuous colonic fermentation model that mimics the microbial ecosystem in the human proximal colon. In a first colonic fermentation with immobilized feces from a healthy 4-month infant, pRE25* was shown to be transferable from *E. faecalis* to the co-immobilized human pathogen *L. monocytogenes* 10403S (Chapter 4). Since the human GIT is one of the most probable sites for HGT between enterococci and listeria (Zhang *et al.*, 2007), the conjugal transfer of this multiresistance plasmid from *E. faecalis* to *L. monocytogenes* in the presence of the competing fecal microbiota implies a serious health risk. Listeria can cause life-threatening infections, e.g. sepsis and meningitis (Kvistholm Jensen *et al.*, 2010), and the effective treatment of these infections would clearly be hampered if multiresistant *L. monocytogenes* are causing the illness.

In a second fermentation, horizontal transfer of pRE25* to the commensal colonic microbiota was investigated. During the 16-day fermentation, the ratio of pRE25* to *gfp* copy numbers increased constantly, clearly indicating that lateral transfer of pRE25* occurred. Enrichment and identification of transconjugants revealed that pRE25* was transferred from *E. faecalis* to the gut commensal *Enterococcus avium* (Chapter 4). *E. avium* is an opportunistic pathogen occasionally causing bacteremia, mainly in the hospital environment (Tan *et al.*, 2010). This species is usually susceptible to a large number of antibiotics, although two recently isolated *E. avium* strains harbored a plasmid from the Inc18 family (which also contains pRE25), which encoded the vancomycin resistance gene *vanA* (Zhu *et al.*, 2010).

The transfer of the multiresistance plasmid pRE25* to *L. monocytogenes* and *E. avium* emphasizes the important role of commensal bacteria, especially enterococci, in the dissemination of ABR genes. Moreover, the pRE25* replicon retained its functionality in both transconjugants, indicating that transconjugants harboring a multiresistance plasmid can further act as donor strains, thereby increasing the probability and frequency of horizontal transmission of ABR determinants in the colonic environment.

In infants, the gut microbiota is not yet fully diversified, still unstable and adaptable (Kurokawa *et al.*, 2007). Ingested microbes deriving from food might therefore establish easier in the GIT. A similar situation might occur after antibiotic therapy or in immunocompromised people. Administration of antibiotics usually has major impacts on the gut microbiota, and although the microbiota is usually recovering after discontinuing medication, it can be imbalanced for several years (Jakobsson *et al.*, 2010). The combination of reduced colonization resistance and a higher risk of bacterial infections, as e.g. in infants and immunocompromised people, could increase the health hazard arising from horizontal spread of ABR genes via the food chain into the gut. Since food products are main vehicles for transferring bacteria into the human gut microbiota, the load of antibiotic resistant bacteria in ready-to-eat products has to be reduced, e.g. by heat-treatment of the raw material or the

final product. Moreover, it is essential to screen bacteria in probiotic products, starter cultures and protective cultures for transferable ABR genes (Kastner *et al.*, 2006).

The results described in this thesis validated the hypothesis that transfer of foodborne resistances to commensal or pathogenic bacteria in an *in vitro* continuous colonic fermentation model with immobilized fecal microbiota occurs. The fermentation model allowed the maintenance of a complex microbial ecosystem that is representative for infant fecal samples. Demonstration of horizontal ABR gene transfer in this model extends the suitability of the model for investigation of gut-microbiota-related processes to the level of genetic exchange. Furthermore, the constructed *E. faecalis* CG110/*gfp*/pRE25* was shown to be a potent tool to monitor ABR gene transfer in a complex microbial ecosystem and can be used in further HGT experiments in various complex microbial environments.

5.2 Perspectives

Using a colonic model, horizontal ABR gene transfer was demonstrated from *E. faecalis* to *L. monocytogenes* and to the natural fecal commensal *E. avium*. Colonic models are valuable tools for investigating different processes mediated by the colonic microbiota and several parameters such as probiotics and prebiotics, micronutrients or antimicrobial compounds can be tested for their impact on the gut microbiota. But even though continuous cultures closely simulate colonic conditions, such models have limitations, including absence of immune response and host cells (Egert *et al.*, 2006). However, HGT experiments in humans are difficult to conduct due to ethical restrictions. To overcome these restrictions, HGT of pRE25* could be studied in animal models, preferably in gnotobiotic mice harboring a human gut microbiota. Such a model might represent the situation in the GIT more closely compared to colonic fermentation models and potentially provide more information about ABR gene transfer *in vivo*.

In addition, the effect of different concentrations of antibiotics against which pRE25* carries resistances on the horizontal transfer of pRE25* could be investigated. Antibiotics strongly affect the composition of the gut microbiota, which presumably affects the establishment of resistant strains as well as the frequency of ABR gene transfer (Licht *et al.*, 2003). Such effect might temporally remain after antibiotic treatment, especially in case antibiotics are not completely absorbed during intestinal passage and sub-inhibitory levels are still present in the heavily colonized colon (Khoder *et al.*, 2010).

Beside the human GIT, several other environments exhibit favorable conditions for ABR gene transfer. In food producing animals, e.g. cattle and poultry, antibiotics are applied not only for therapy but also as growth promoters and for prophylaxis (Baurhoo *et al.*, 2009). This favors the lateral transfer of ABR genes in these animals but also the establishment of resistant bacteria which might then be transmitted into food products (Rizzotti *et al.*, 2005, Aslam *et al.*, 2010). The constructed strain CG110/*gfp*/pRE25* could be useful to elucidate ABR gene transfer in livestock ecosystems.

Furthermore, fermented foods may also represent a favorable site for resistance gene transfer, since the microbial diversity and density can reach high numbers in such food (Giraffa, 2002). Enterococci are frequently found in cheese and sausages, which are well-known source of listeriosis. Therefore, ripening experiments with *E. faecalis* CG110/*gfp*/pRE25* as natural adjunct culture and *L. monocytogenes* as contaminant could provide insights in HGT events during food fermentations.

To mimic the *in vivo* situation more closely, a foodborne *E. faecalis* strain could be used as pRE25* donor in HGT experiments. Plasmid pRE25* can be easily transferred from *L. lactis* BuRE25* to probably any foodborne *Enterococcus* strain although the strain itself still has to be tagged chromosomally, e.g. with the *gfp* gene. Due to the broad host-range of pRE25*, it can even be transferred to other Gram-positive food-associated bacteria, such as *Leuconostoc* or *Listeria*, which can then be applied as donor in HGT experiments.

In fermentation 2, only one transconjugant strain was isolated, even though the pRE25*/*gfp* ratio increased in 60% from day 1 to day 16 (Chapter 4). This indicates that the approach for the isolation of transconjugants from complex background still can be optimized. A promising approach to enrich transconjugants could be by counter-selection, e.g. by the application of bacteriophages (Monk *et al.*, 2010). Phages exhibit an extremely narrow infection spectrum, which can be species- or even strain-specific (Santiago-Rodríguez *et al.*, 2010, Son *et al.*, 2010). The addition of a highly donor-specific *Enterococcus* phage to enrichment cultures would result in lysis of the *E. faecalis* donor without affecting transconjugants, which can subsequently be isolated and identified using established methods such as sequencing of 16S rRNA genes.

Another possible approach to identify transconjugants is by separating donor cells from transconjugants by fluorescence-activated cell sorting (FACS). FACS is a flow cytometry-based technique which allows the separation of cells on the basis of their differential fluorescing characteristics. However, since strain CG110/*gfp*/pRE25* cells do not fluoresce uniformly, the GFP signal intensity in donor cells has to be elevated. This could possibly be

achieved by the introduction of a stronger promoter upstream of the *gfp* gene, thereby increasing *gfp* expression and as a consequence the GFP signal. A second approach to attain increased fluorescence could be the application of quantum dot-labeled GFP-antibodies. Quantum dots are nanocrystals which are brighter and more stable than organic dyes (Walling *et al.*, 2009), and therefore potential labels that permit the separation of fluorescing donor cells from transconjugants by FACS (Tracy *et al.*, 2010). Transconjugants can be isolated subsequently and identified on basis of their 16S rRNA gene sequence. Although the selection of transconjugants is a technical challenge as still a huge number of bacterial strains in the GIT is not yet cultivable (Rajilić-Stojanović *et al.*, 2007) and detection methods for single-cell identification are still in their infancy, identification of transconjugants will provide a much deeper and detailed insight in ABR transfer in the GIT.

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