Elucidating the success of *Enterococcus faecium* as a nosocomial pathogen through functional genomics

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Elucidating the success of *Enterococcus faecium* as a nosocomial pathogen through functional genomics

Het succes van *Enterococcus faecium* als ziekenhuispathogeen ontrafeld, met behulp van functionele genomics

(met een samenvatting in het Nederlands)

Proefschrift

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Vuela libre pero con rumbo
Vuela alto pero nunca arrogante

- Mony, 1999

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Dedicated
to the beloved memory
of my mom & dad

Miryám Prieto Gil
RIP, 1954 – 2007

Ferney Guzmán Mondragón
RIP, 1950 – 2016

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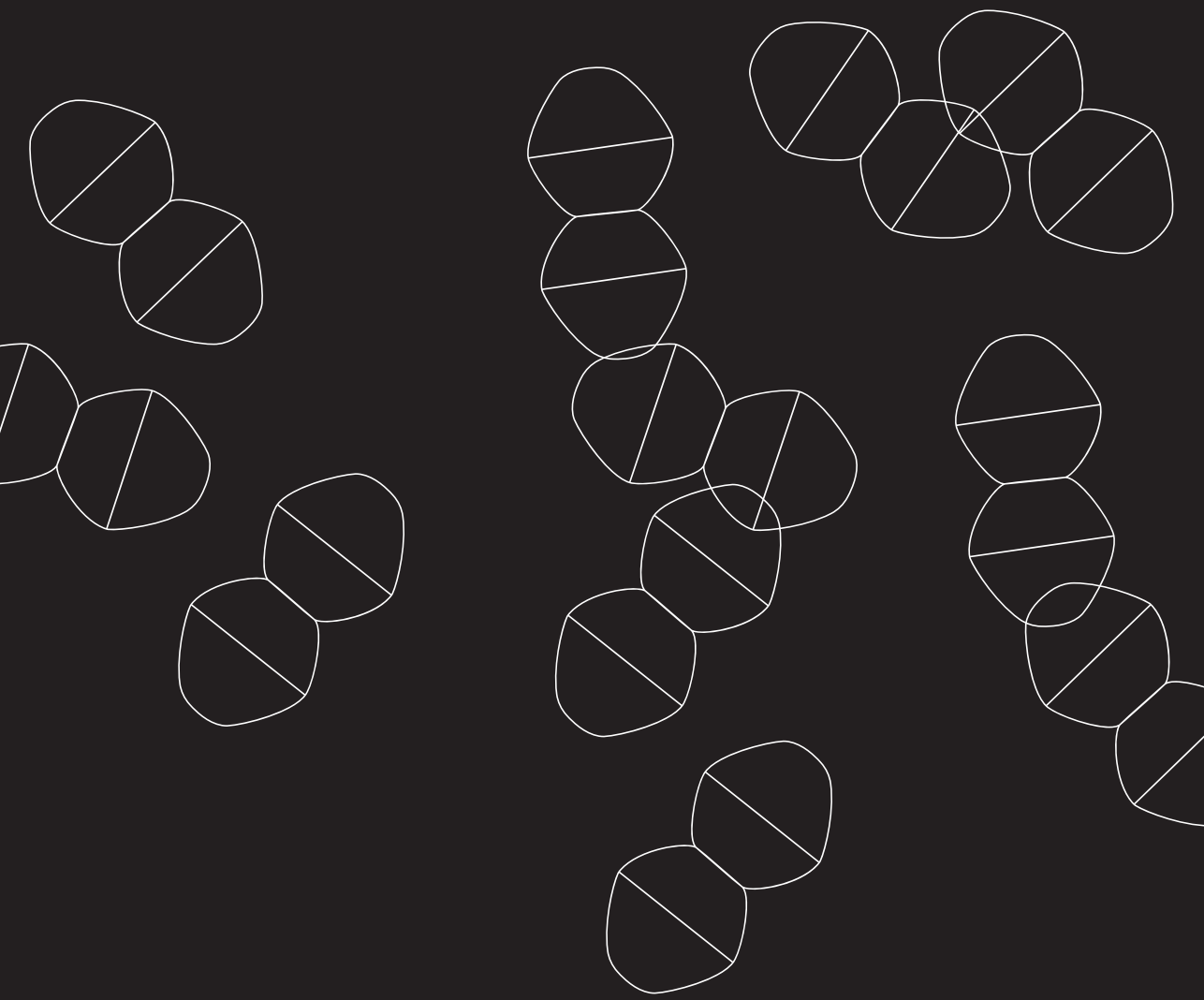

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General Introduction

Adapted from:
Global emergence and dissemination of enterococci as nosocomial pathogens: attack of the clones?

Ana M. Guzmán Prieto, Willem van Schaik, Malbert R.C. Rogers, Teresa M. Coque, Fernando Baquero, Jukka Corander, Rob J.L. Willems.

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Enterococci are low-GC Gram-positive ovococci that can form pairs and chains of diverse lengths. Bacteria from the genus *Enterococcus* are facultative anaerobic and grow optimally at 35ºC, but can tolerate temperatures ranging from 10ºC to 45ºC (Byappanahalli et al., 2012). The genus comprises 54 species (Parte, 2014), which are ubiquitously present in nature but the gastrointestinal tract (GIT) of animals, including mammals, reptiles, birds (Mundt, 1963) and insects (Martin and Mundt, 1972), is thought to be the largest reservoir of enterococci (Gilmore et al., 2013).

In humans, enterococci are common commensals of the GIT. In addition, enterococci have become ever more prominent as a causative agent of nosocomial infections since the 1970s (Arias and Murray, 2012). Two species, *Enterococcus faecalis* and *Enterococcus faecium*, cause the vast majority of hospital-acquired enterococcal infections in humans (Agudelo Higuita and Huycke, 2014). Of these two, *E. faecium* has rapidly acquired resistance to several classes of antibiotics. First, in the 1970s and 1980s, *E. faecium* gained high-level resistance to ampicillin (Galloway-Peña et al., 2009; Grayson et al., 1991) and since the 1980s it acquired resistance to aminoglycosides, fluoroquinolones and glycopeptides, particularly vancomycin (Leclercq et al., 1988; National Nosocomial Infections Surveillance System, 2004; Uttley et al., 1988). *E. faecalis* has also acquired resistance to aminoglycosides, but resistance to ampicillin and vancomycin is much rarer than in *E. faecium* (Edelsberg et al., 2014). Worryingly, resistance to antibiotics that are used to treat vancomycin resistant enterococci (VRE), like linezolid, tigecycline and daptomycin, has already been reported (Aksoy and Unal, 2008; Montero et al., 2008; Niebel et al., 2015; Scheetz et al., 2008).

The intrinsic resistance of enterococci to some antibiotics, including aminoglycosides, and the ability to acquire and disseminate antibiotic resistance determinants, like those involved in vancomycin resistance, only partly explain the recent emergence of these organisms as nosocomial pathogens. In addition, the plasticity of the enterococcal genomes allow enterococci to rapidly respond and adapt to selective constraints by acquiring genetic determinants that increase their ability to colonize or infect the host (Hendrickx et al., 2009; Lebreton et al., 2013; Palmer et al., 2012; Van Tyne and Gilmore, 2014). Other host or environmental factors, most notably exposure to antimicrobial agents, may favor an increase in colonization density of enterococci in the GIT of hospitalized patients (Donskey et al., 2000; Ruiz-Garbajosa et al., 2012; Ubeda et al., 2010). Antibiotic therapy that leads to the depletion of Gram-negatives, can reduce production of the antimicrobial peptide REGIIIγ by Paneth cells, and this may promote the outgrowth of VRE (Brandl et al., 2008).

Enterococci have become one of the most common causes of health care-associated infections with *E. faecalis* causing approximately 60% of infections and *E. faecium* the remainder (de Kraker et al., 2013; Sievert et al., 2013; Weiner et al., 2016). Patients undergoing transplants or with underlying diseases, such

as diabetes or renal failure, and patients with long-term catheter usage, are at higher risk of developing infections caused by multi-drug resistant (MDR) enterococci (Arias and Murray, 2012). High-density colonization of the patient GIT facilitates the transmission of MDR enterococci among hospital wards through fecal contamination (Arias and Murray, 2012). Therefore epidemiological surveillance and outbreak investigations, together with infection control polices and interventions, such as the use of protective barriers and proper disinfection, are key for infection control of these organisms in the nosocomial environment (Sydnor and Perl, 2011). High-level enterococcal GIT colonization can also lead to urinary tract infections (UTI) (De Vecchi et al., 2013; Neelakanta et al., 2015), which may progress to bloodstream infections or endocarditis (Fernández-Guerrero et al., 2002; Patterson et al., 1995). Enterococci from high-density intestinal populations may also directly translocate from the GIT into the bloodstream (Kamboj et al., 2014).

Emergence of antibiotic and disinfectant resistance in enterococci

E. faecalis and *E. faecium* exhibit intrinsic resistance to a broad range of antibiotics. Below we briefly describe the mechanisms that cause resistance to the classes of antibiotics that are currently used in the treatment of enterococcal infections.

Compared to other low-GC Gram-positive cocci, enterococci exhibit decreased susceptibility to β-lactam antibiotics. The β-lactams act through the inactivation of penicillin-binding proteins (PBPs), thereby interfering with synthesis of peptidoglycan. All enterococci display decreased susceptibility to β-lactam antibiotics due to the expression of PBPs with an intrinsic low affinity for this class of antibiotics. Resistance to β-lactams, most notably to ampicillin, is currently far more widespread in *E. faecium* than in *E. faecalis* (Cattoir and Giard, 2014). The most important determinants for β-lactam resistance in *E. faecium* are mutations in the genes encoding the penicillin-binding protein PBP5 (Rice et al., 2001; Zhang et al., 2012; Zorzi et al., 1996). There are clear indications that *E. faecium* progressed towards high-level ampicillin resistance in the 1970s and 1980s through the acquisition of specific mutations in the *pbp5* gene (Galloway-Peña et al., 2009, 2011; Grayson et al., 1991). It is of importance to note that these chromosomally encoded PBPs can be transferable (Dahl et al., 2000; Hanrahan et al., 2000; Rice et al., 2005), which indicates that dissemination of high-level ampicillin resistance can be the result of both clonal spread of strains with mutated *pbp5* genes and horizontal gene transfer. In addition to mutations in *pbp5*, production of β-lactamase has been described in both *E. faecalis* and *E. faecium* (Sarti et al., 2012; Tomayko et al., 1996). The expression level of beta-lactamase in these

species is, however, low and their impact on ampicillin susceptibility appears marginal.

Another group of antibiotics to which *E. faecium* and *E. faecalis* exhibit moderate intrinsic and high-level acquired resistance are the aminoglycosides. In *E. faecalis,* intrinsic resistance is thought to be caused by the inability of the antibiotic to enter the cytoplasm and inhibit ribosomal protein synthesis (Aslangul et al., 2006). In *E. faecium,* two chromosomally encoded genes, a 6'-N-aminoglycoside acetyltransferase (*aac(6')-Ii*) (Costa et al., 1993) and an rRNA methyltransferase (*efmM*) (Galimand et al., 2011), have been associated with intrinsic resistance to tobramycin and kanamycin. In addition to intrinsic resistance to aminoglycosides, the therapeutic success of these antibiotics is critically compromised by high-level resistance, due to the gain of aminoglycoside modifying enzymes, such as phosphotransferases, acetyltransferases and nucleotidyltransferases by *E. faecium* and *E. faecalis* (Chow, 2000; Miller et al., 2014).

The assessment of enterococci, particularly *E. faecium*, as important agents of multi-drug resistant nosocomial infections was definitively established when they acquired resistance to vancomycin. While vancomycin-resistant enterococci were virtually non-existent in hospitals in the USA before 1990, nowadays vancomycin resistance in *E. faecium* ranges from 58.4% in SSI to 82.2% in catheter associated urinary tract infections (CAUTI), while in *E. faecalis* this is only from 3.5% in surgical site infections (SSI) to 9.8% in central line associated bloodstream infections (CLABSI) (Weiner et al., 2016). Vancomycin is a glycopeptide antibiotic, which prevents cross-linking of peptidoglycan by binding to the D-alanine-D-alanine (D-Ala-D-Ala) moiety of the peptide chains that crosslink peptidoglycans. The mechanisms by which enterococci become resistant to vancomycin have been extensively reviewed elsewhere (Courvalin, 2006). In short, enterococci become resistant to vancomycin when the terminal amino acids of peptidoglycan precursors are altered from D-Ala-D-Ala to D-Ala-D-lactate (D-Ala-D-Lac) or to D-Ala-D-Serine (D-Ala-D-Ser), leading to high-level and low-level resistance to vancomycin, respectively. Nine gene clusters are currently known to be involved in vancomycin resistance in enterococci. These vancomycin resistance gene clusters are *vanA, vanB, vanD* and *vanM*, causing vancomycin resistance through the formation of D-Ala-D-Lac, and *vanC, vanE, vanG, vanL,* and *vanN* which catalyze the formation of D-Ala-D-Ser (Boyd et al., 2008; Courvalin, 2006; Lebreton et al., 2011; Xu et al., 2010). The most frequently found, and thus clinically most relevant, vancomycin resistance determinants are *vanA* and *vanB*, which are both part of larger gene clusters, which encode a two-component regulatory system and enzymes involved in the recycling of D-Ala-D-Ala peptidoglycan precursors to D-Ala-D-Lac. Both *vanA* and *vanB* are located on transposons that contribute to the dissemination of vancomycin resistance among enterococci (Courvalin, 2006). Recently, *vanM* was found to be the most important vancomycin-resistance

determinant among different *E. faecium* lineages in hospitals in Shanghai, China (Chen et al., 2015). Differences in the sequence diversity and prevalence of each *van* operon could be due to the different ecological origins of the *van* clusters. While *vanA* seems to have originated from soil organisms, *vanB, vanD,* and *vanG* have been reported in gut commensal microbiota (Domingo et al., 2007; Guardabassi et al., 2005; Howden et al., 2013)

While vancomycin resistance emerged and spread in USA hospitals in the 1990s, carriage of vancomycin-resistant *E. faecium* was rare among hospitalized patients in Europe. In contrast, VRE carriage among farm animals, and, to a lesser extent, in healthy humans was higher in Europe than in the USA (Devriese et al., 1996; Klare et al., 1995; Stobberingh et al., 1999). The widespread occurrence of VRE among farm animals was linked to the use of the vancomycin-analogue avoparcin as a growth promoter in Europe (Bager et al., 1997). The presence of indistinguishable vancomycin resistance transposons in both animal and human reservoirs provided the first indication that animal-derived enterococci could be a reservoir of antibiotic resistance genes that could be transmitted to humans (Arthur et al., 1993; Jensen et al., 1998; Willems et al., 1999; Woodford et al., 1998). After the ban on the use of avoparcin in 1997, the prevalence of VRE in animal husbandry declined in Europe (Aarestrup et al., 2001; van den Bogaard et al., 2000; Klare et al., 1999). In several European countries, the prevalence of VRE among hospitalized patients increased in the 21st century, but is still lower than the endemic levels reported in US hospitals (Bourdon et al., 2011; Gastmeier et al., 2014; Pinholt et al., 2015; Simner et al., 2015). Levels of VRE among hospitalized patients are also high in Australia, with 37% of *E. faecium* bacteremia isolates exhibiting resistance to vancomycin. Interestingly, vancomycin resistance in Australian *E. faecium* isolates is almost exclusively caused by *vanB*-type transposons, while *vanA* is the major vancomycin-resistance determinant in Europe and the USA (Coombs et al., 2014).

Due to the emergence and rapid spread of VRE, the antibiotics linezolid, daptomycin and tigecycline are increasingly used for the treatment of VRE infections. Their mode-of-action and the development of resistance to these antibiotics is discussed below.

Linezolid, the first oxazolidinone antibiotic, was introduced for clinical use in the USA in 2000. It acts on the ribosome by binding to a universally conserved site on 23S rRNA of the large 50S subunit of the ribosome (Colca et al., 2003), thereby inhibiting elongation of the polypeptide chain. The currently described linezolid resistance mechanisms alter the peptidyl transferase centre of 23S rRNA, by a mutation in the 23S rRNA gene, with the G2576T mutation being most prominent (Jones et al., 2008), mutations in the genes encoding ribosomal proteins L3 and L4 (Chen et al., 2013a) or methylation of the adenine at position 2503, which is catalyzed by the methyltransferase Cfr. The *cfr* gene is encoded on various conjugative and non-conjugative plasmids in enterococci (Diaz et al., 2012; Shen et al., 2013). Recently, the oxazolidinone resistance gene *optrA* has been identified in *E. faecalis* and *E.*

faecium isolates of human and animal origin (Cai et al., 2015; Wang et al., 2015). This gene codes for an ATP-binding cassette transporter, which contributes to reduced susceptibility for oxazolidinones (linezolid and tedizolid) and phenicols (chloramphenicol and florfenicol). The *optrA* gene was associated with a conjugative plasmid (Wang et al., 2015). Nevertheless, linezolid resistance is still rare. In a study using data from 19 US hospitals in the period 2007-2010, linezolid resistance was reported in 1.1 and 1.8% of *E. faecium* and *E. faecalis* isolates, respectively (Edelsberg et al., 2014).

Daptomycin is a lipopeptide antibiotic that targets the cell membrane through interactions with phospholipids (Humphries et al., 2013). The genetic basis for resistance in *E. faecalis* and *E. faecium* was first studied through genome sequencing of pairs of strains that developed resistance *in vitro* or *in vivo* (Arias et al., 2011; Palmer et al., 2011; Tran et al., 2013a). Additional genetic and biochemical work has established the role of the three-component regulatory system LiaFSR in contributing to daptomycin resistance in enterococci. The LiaFSR system is conserved in low-GC Gram-positive bacteria and governs the cell envelope stress response (Jordan et al., 2006). Mutations in the *liaF* gene of *E. faecalis* lead to a redistribution of cardiolipin-rich microdomains from the division septum to other regions of the cytoplasmic membrane, which affects the antimicrobial activity of daptomycin. In addition, other mutations in phospholipid biosynthesis genes, most notably in the cardiolipin synthase gene *cls*, are required for full expression of daptomycin resistance in *E. faecalis* (Davlieva et al., 2013; Tran et al., 2013b). Similar mechanisms have been associated with daptomycin resistance in *E. faecium* (Davlieva et al., 2013; Munita et al., 2012, 2014). Notably, emergence of daptomycin resistance in *E. faecium* during daptomycin therapy is not always linked to mutations in *liaFSR* and mutations in *cls* may already be sufficient (Kelesidis et al., 2013; Lellek et al., 2015). Daptomycin resistance among enterococci is infrequent, but is more common in *E. faecium* than in *E. faecalis*. A worldwide study in isolates from hospitalized patients over the period 2005 – 2012 showed levels of daptomycin resistance of 0.18% for *E. faecium* and 0.02% for *E. faecalis* (Sader et al., 2014). However, in certain settings daptomycin resistance may be considerably more frequent, as illustrated by a study including 4,274 *E. faecium* and 7,007 *E. faecalis* isolates from 19 US hospitals, which found daptomycin resistance among 3.9% and 0.2% of *E. faecium* and *E. faecalis* isolates, respectively (Edelsberg et al., 2014). Similar levels of daptomycin resistance in *E. faecium* were recently reported for a German hospital (Lübbert et al., 2015).

Tigecycline is a semisynthetic derivative of the broad-spectrum tetracycline antibiotic minocycline, which acts on the ribosome by inhibiting its association with aminoacyl-tRNAs. The emergence of resistance during tigecycline therapy was first observed in *E. faecalis* (Cordina et al., 2012; Werner et al., 2008) and has recently been described in *E. faecium* (Niebel et al., 2015). In enterococci,

resistance to tigecycline can be mediated through upregulation of tetracycline resistance determinants *tetL* (encoding an efflux pump) and *tetM* (providing ribosomal protection) (Fiedler et al., 2015) and mutations in the ribosomal protein *rpsJ* (Beabout et al., 2015; Cattoir et al., 2015; Niebel et al., 2015). Tigecycline resistance among *E. faecium* and *E. faecalis* is rare at 0.3% for both species (Hoban et al., 2015) and the antibiotic can still be used successfully to treat bacteremias caused by MDR enterococci, especially when it is used in combination with daptomycin (Polidori et al., 2011). The recovery of enterococcal isolates with decreased susceptibility to tigecycline in samples of animal origin is of concern, as extensive use with tetracyclines in the veterinary setting could select for tigecycline tolerant strains (Freitas et al., 2011).

In addition to the antibiotics described above, the antibiotics tedizolid, telavancin, dalbavancin, and oritavancin have recently been approved by the Food and Drug Administration in the USA for the treatment of skin infections by Gram-positive bacteria (Crotty et al., 2016). Tedizolid is an oxazolidinone antibiotic that has improved in vitro activity compared to linezolid. Enterococcal strains that have acquired linezolid resistance due to the acquisition of the *cfr* gene or the G2576T mutation in the 23S rRNA gene, may still have relatively low MICs (≤4 µg/ml) for tedizolid (Barber et al., 2016; Silva-Del Toro et al., 2016). Telavancin, dalbavancin and oritavancin are semi-synthetic glycopeptides antibiotics that, similar to vancomycin, affect peptidoglycan stability by binding to the D-Ala-D-Ala moiety of the peptide chains that crosslink peptidoglycan chains. Interestingly, oritavancin is also active against *vanA*- and *vanB*-type vancomycin-resistant enterococci, while telavancin and dalbavancin have limited activity against enterococci carrying the *vanA*-type vancomycin resistance transposon but are active against enterococci with *vanB*-type vancomycin resistance (Jones et al., 2013; Karlowsky et al., 2015). Tedizolid, telavancin, dalbavancin, and oritavancin may be useful as alternatives to linezolid and vancomycin, but they do not have radically different modes of action and they may therefore suffer from the same resistance mechanisms that are threatening the efficacy of linezolid and vancomycin. Therefore, antibiotics that target other structures in the enterococcal cell, including teixobactin (Ling et al., 2015) and the acyldepsipeptides (Brötz-Oesterhelt et al., 2005), hold considerable promise as novel compounds for the treatment of infections with multi-drug resistant enterococci.

Due to the global rise of multi-drug resistant pathogens, the gradual loss of effectiveness of the current available antibiotics and the lack of new therapeutic alternatives, it is of considerable importance to prevent the spread of multi-drug resistant opportunistic pathogens, including VRE, in the nosocomial environment. For this purpose, disinfectants play an important role (Grare et al., 2010). Chlorhexidine (CHX) is a bisbiguanide agent that, depending on the concentration used, may disrupt the integrity of the bacterial cell membrane or lead to its

complete breakdown (Hugo and Longworth, 1964; Hugo and Longworth AR, 1965; Hugo and Longworth, 1966). CHX bathing in the form of prophylactic non-rise skin preparations, are common practice among clinical patients, to reduce the risk of central line-associated bloodstream infections (CLABSI) (Quach et al., 2014; Marschall et al., 2014; Kim HY et al., 2015). The use of CHX bathing can significantly reduce the colonization of VRE in patients (Chen et al., 2013b; Bass et al., 2013; Derde et al., 2012; Karki and Cheng, 2012). However, enterococcal isolates obtained from CLABSI in CHX-bathing wards had reduced susceptibility to CHX, compared to CLABSI isolates of wards in which CHX bathing was not implemented (Suwantarat et al., 2014). Recently, it has been suggested that the constant exposure of VRE to sub-inhibitory concentrations of CHX induces the expression of genes involved in vancomycin and daptomycin resistance in enterococci (Bhardwaj et al., 2016), raising concerns about the effect of the use of CHX in reduced antiseptic susceptibility and cross-resistance with antibiotics in clinical wards.

Molecular epidemiology and Population structure of *E. faecium*

As a consequence of the global rise of vancomycin-resistant *E. faecium* (VREF), a large number of molecular epidemiological studies have been performed to obtain insights into the dissemination of VREF clones in and between hospitals, in farm animals and healthy humans. However, methods like pulse-field gel electrophoresis (PFGE), soon proved to be insufficiently reproducible to study genetic relatedness of isolates (Morrison, Woodford et al. 1999).

A first insight into the existence of particular ecotypes in *E. faecium*, was obtained by using amplified fragment polymorphism (AFLP) to infer the genetic relatedness of strains from diverse hosts and environments (Willems et al., 2000). This study revealed that strains from hospitalized patients grouped in a specific sub-population that was distinct from groups of strains that were isolated from humans in the community and farm animals.

The use of AFLP for global studies showed the limitations of this technique for comparisons of data obtained from different laboratories. Therefore an alternative method, termed multi locus sequence typing (MSLT), was used in follow-up studies. In MLST, allelic profiles are based on the sequences of fragments of a number of housekeeping genes (seven in the case of the *E. faecium* and *E. faecalis* MLST schemes) (Homan et al., 2002; Ruiz-Garbajosa et al., 2006). Compared to AFLP, MLST has the distinct advantage that data are easily collated and shared through an on-line database. The first analyses of *E. faecium* MLST data were performed with the algorithm eBurst (Feil et al., 2004) and confirmed a distinct clustering of strains derived from the hospital environment. This cluster was

named clonal complex 17 (CC17) and soon CC17 was found to be disseminated throughout the world (Top et al., 2008; Willems et al., 2005). The later use of larger datasets and different algorithms for the analysis of MLST data (Didelot and Falush, 2007; Francisco et al., 2009), showed that eBurst can sometimes fail to correctly assign STs to clonal complexes, particularly when genetic variation in populations is largely driven by recombination rather than mutations, like is the case in *E. faecium* and *E. faecalis* (Turner et al., 2007).

Bayesian analysis of population structure (BAPS) has successfully been used to probabilistically infer the population structure and levels of recombination of several microbial pathogens (Corander et al., 2012; Thomas et al., 2014). When applied to *E. faecium* MLST data, BAPS allowed the partitioning of 519 STs of 1720 *E. faecium* isolates into 13 non-overlapping groups. Of these groups, BAPS 3-3 was significantly associated with isolates from hospitalized patients, while BAPS 2-1 and 2-4 were significantly associated with farm animals. This observation again confirmed that there exists structure in the *E. faecium* population, with a distinct subpopulation of isolates that are almost exclusively found in hospitalized patients (Willems et al., 2012). One of the important nodes in the previously described hospital-associated CC17, ST78 and its descendant STs, grouped in BAPS 2-1 together with farm animal isolates, while two other important CC17 nodes, ST17 and ST18 with their descendant STs, clustered in another BAPS group (BAPS 3-3). These findings indicate that nosocomial *E. faecium* isolates have not evolved from a single ancestor, like previously postulated, but rather the cumulative acquisition of adaptive elements in nosocomial isolates may have occurred multiple times in different genetic backgrounds. Another conclusion that could be drawn from Bayesian modeling of MLST data is that hospital isolates displayed a relative low-level of admixture, despite the high recombination rates in *E. faecium*, suggesting that once strains have adapted to the distinct hospital niche, they become ecologically isolated (including isolation by dominance) and recombination with other populations declines (Willems et al., 2012).

Despite the overt lack of reproducibility, PFGE long remained the "gold standard" for molecular typing of *E. faecium* until the introduction of whole genome sequence (WGS)-based epidemiology. Since the first complete whole genome in *E. faecium* was published in 2012 (Lam *et al.*, 2012), hundreds more *E. faecium* genomes have been added to different public data bases. In 2013, Howden and co-workers published a landmark study in which they used whole genome sequencing to track an outbreak of *vanB*-VREF in a large hospital in Australia (Howden et al., 2013). Interestingly, detailed phylogenomic analysis and precise mapping of the *vanB* gene revealed that 18 of the 36 *vanB*-VREF had acquired the *vanB* transposon during the outbreak period. This indicates that for *vanB*-VREF, frequent *de novo* generation of VREF through horizontal gene transfer may contribute to the emergence of VREF, in addition to clonal spread. This study

was followed by multiple other studies that used whole genome sequencing to investigate the molecular epidemiology of VREF with high resolution and high accuracy (Brodrick et al., 2016; van Hal et al., 2016; Reuter et al., 2013; Salipante et al., 2015). Bender and co-workers performed whole genome sequencing of 49 *vanB*-VREF, primarily of ST192 (39/49), from invasive infections from hospitals all across Germany and found that spread of the Tn*1549*-*vanB*-type resistance involved exchange of large chromosomal fragments between *vanB*-positive and *vanB*-negative enterococci rather than independent acquisition events of the *vanB* transposon alone (Bender et al., 2016).

WGS has become a tool in antibiotic resistance surveillance (Dunne Jr et al., 2012; Reuter et al., 2013; Schürch and van Schaik, 2017). WGS in clinical and public health laboratories is potentially useful when it is clinically relevant to rapidly elucidate the resistance mechanism of a particular multi-resistant isolate, in order to implement targeted infection control measures (Köser et al., 2014; Punina et al., 2015; Deurenberg et al., 2017). However, until now, WGS has mainly been used as a typing tool in clinical microbiology, and its use for global infection control is still far away.

WGS-based studies proposed that the *E. faecium* population was divided into two species-level subdivisions, based on phylogenetic analysis and the determination of average nucleotide identity (ANI) between the two sub-populations. The sub-populations were termed clade A, or hospital-associated clade, primarily containing isolates from hospitalized patients and clade B, or community-associated clade, mostly containing isolates from healthy, non-hospitalized individuals (Galloway-Peña et al., 2012; Palmer et al., 2012). The high level of diversity between these two clades indicates that the clade A-B split is ancient and precedes the modern antibiotic era. Further work provided additional evidence for this split and showed that the population of *E. faecium* in clade A had a second split of a more recent date (74 ± 30 years), with clade A1 containing the majority of clinical isolates and clade A2 mostly comprising animal-derived strains (Lebreton et al., 2013). WGS also confirmed that *E. faecium* was subject to high rates of recombination, leading to changes in MLST profiles in otherwise closely related strains, which invalidates the use of MLST for tracking transmission events (van Hal et al., 2016). Another finding of van Hal *et al.* was that other *E. faecium* strains are the most important donors of imported DNA fragments. Specifically, strains from clade B are an important reservoir for donating foreign DNA to clade A strains (de Been et al., 2013). These findings indicate that hospital-acquired clade A1 *E. faecium* strains have recently emerged from a background of human commensal and animal isolates.

Phylogenetic trees generated in WGS-based epidemiological studies, although providing high-resolution typing data, complicates comparisons of data between studies and hampers the construction of globally accessible databases.

This limitation may be overcome by using a genome-wide gene-by-gene comparison approach, as in classical MLST, but with an important extension of the number of analyzed genes from seven to the entire core genome of the species. This approach is denominated core genome MLST (cgMLST) (de Been et al., 2015; Maiden et al., 2013). Tree-like network representations of *E. faecium* genomic relatedness based on cgMLST data also revealed the distinct clustering of human commensal, animal and human clinical strains (Fig. 1). Notably, in this analysis the animal isolates do not group in a single clade A2 but form multiple distinct clusters that are located in the network, between the human commensal and clinical isolates, indicating that clade A2 may not be monophyletic, as previously postulated by (Lebreton et al., 2013). Interestingly, clade A1 *E. faecium* strains have lower fitness in natural environments, where they are out-competed by other *E. faecium* clones (Leclercq et al., 2013). Similarly, clade B strains outcompete clade A strains in an animal model of gut colonization in the absence of selection by antibiotics (Montealegre et al., 2015). These data highlight the exquisite niche specialization to the hospital environment of clade A1 strains, which may come at a fitness cost in non-hospital environments.

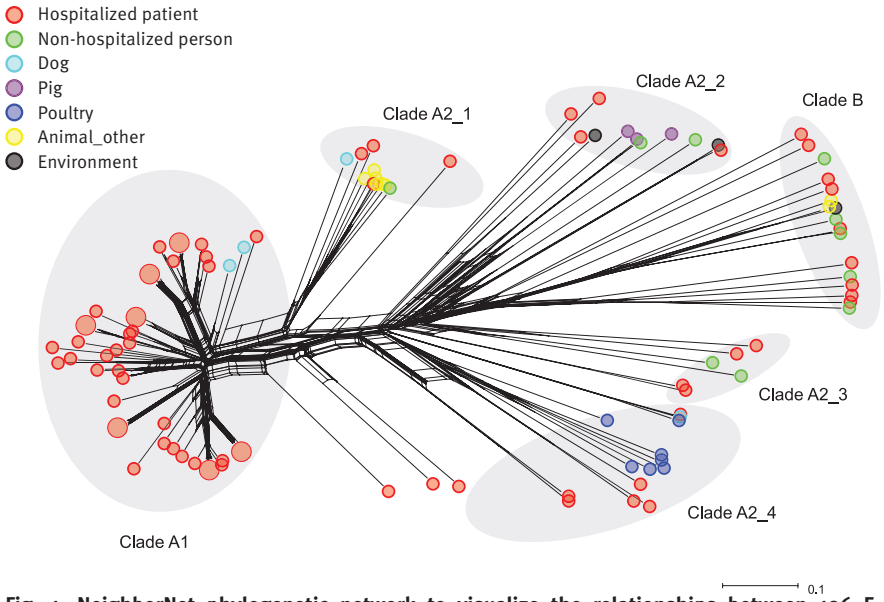

Fig. 1. NeighborNet phylogenetic network to visualize the relationships between 196 E. faecium isolates. The distance matrix underlying the network was built from all pairwise allelic profile comparisons of 1423 loci of the published *E. faecium* cgMLST scheme (de Been et al., 2015). Allelic profiles were extracted from SeqSphere. Small colored circles indicate the origin of isolates. *E. faecium* clades (A1 and B) inferred from the STs and based on Lebreton et al. (Lebreton et al., 2013) are indicated by large dotted circles. Notably, in this analysis clade A2 isolates do not group in a single clade but form at least four distinct clusters that are located in the network and are indicated clade A2_1, A2_2, A2_3 and A2_4.

Adaptive elements in nosocomial lineages of *E. Faecium*

Enterococci are ubiquitous in nature, where they act as commensals and opportunistic pathogens. A consequence of these different lifestyles is that enterococci need to adapt to different micro-environments, each one exerting strong selective pressures. A strategy for bacterial species to survive when confronted with a wide range of selective pressures is to specialize in particular fitness peaks. *E. faecium* probably followed such an evolutionary trajectory, resulting in the emergence of specific hospital-adapted lineages. Successful hospital-adapted clones in *E. faecium* have the ability to exchange mobile genetic elements, carrying antimicrobial resistance and virulence determinants, by horizontal gene transfer (Hegstad et al., 2010; Leavis et al., 2003; Palmer et al., 2010; van Schaik et al., 2010). The cumulative acquisition of adaptive elements has been named "genetic capitalism", in which the acquisition of a particular adaptive element by a particular clone enhances its fitness, thereby increasing the likelihood of acquiring a second adaptive element which finally can lead to the emergence of high-risk multidrug-resistant clones (Baquero, 2004). This cumulative acquisition of adaptive elements by the hospital-associated *E. faecium* clade A1 strains is reflected by their larger genome sizes (2,843 ± 159 genes; 2.98 ± 0.15 Mbp), compared to the genomes of strains in clade A2 (2,597 ± 153 genes; 2.75 ± 0.14 Mbp) or clade B (2,718 ± 120 genes; 2.84 ± 0.1 Mbp) (Lebreton et al., 2013).

The first adaptive element that was described as being specific for hospital-associated *E. faecium* strains was Esp (Willems et al., 2001), a surface protein with a signal sequence for transport and a LPxTG-like motif for cell wall anchoring. The *E. faecium esp* gene is located on an integrative conjugative element, called ICE*Efm1*, and contributes to biofilm formation, and urinary tract infections and endocarditis in animal models (Heikens et al., 2007, 2011; Leavis et al., 2004; Leendertse et al., 2009; Sava et al., 2010; van Schaik et al., 2010; Top et al., 2011). In addition to Esp, other determinants were found to be specific or significantly enriched among hospital-associated isolates. Characteristics of these genes have been previously discussed (Heikens et al., 2008; Hendrickx et al., 2009, 2013; Leavis et al., 2003; Paganelli et al., 2016; Zhang et al., 2013). Comparative genomic analyses of 73 *E. faecium* strains revealed major differences in gene content between clinical (clade A1), animal (clade A2) and non-clinical (clade B) strains, particularly through gain and loss of gene clusters with predicted roles in carbohydrate metabolism (Kim and Marco, 2013; Lebreton et al., 2013). Many of the clade B-specific genes have a predicted role in the utilization of complex carbohydrates from dietary sources, which were replaced with genes that were associated with the utilization of amino sugars (e.g. galactosamine), which occur on epithelial cell surfaces and in mucin. This metabolic switch may have contributed to the niche specialization of *E. faecium*.

In bacteria, mechanisms that preclude the acquisition of foreign DNA, include the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas system

(Horvath and Barrangou, 2010) and restriction modification and anti-restriction modification (RM-, antiRM-) systems (Clewell et al., 2014). In *E. faecium*, CRISPR-Cas is only found in a subset of clade B strains, again highlighting the general lack of barriers to horizontal gene exchange in this species (Lebreton et al., 2013). Restriction and anti-restriction systems located on mobile elements or enterococcal chromosomes can also determine the flow of adaptive traits among populations of the same and/or different species (Clewell et al., 2014).

High-throughput functional genomic technologies to study *E. faecium* biology

In bacterial genetics the process to discover genes able to explain the genetic mechanism of a particular phenotype of interest, has been a laborious process in which transposon mutagenesis became an essential tool (Reznikoff and Winterberg, 2008). Transposons are natural mobile genetic elements that have been used to perform random mutagenesis in many bacterial species (Hayes, 2003). However, the assessment of transposon mutant libraries through the study of loss of function of individual mutants, became a rate-limiting step. To overcome these challenges, new high-throughput applications were developed allowing parallel screening of a vast number of mutants (Kwon et al., 2016). Among these technologies, microarray-based screening methods (Mazurkiewicz et al., 2006; Bijlsma et al., 2007) were introduced. In a clinical *E. faecium* isolate, the development and implementation of Microarray-based Transposon insertion Mapping (M-TraM), allowed the identification of genes that play important roles in resistance to ampicillin (Zhang et al., 2012), bile (Zhang et al, 2013), and the development of endocarditis (Paganelli et al., 2016). Due to technological limitations, including the need to design a microarray for every strain and the relatively low precision due to background hybridization signals, microarray-based screening methods of transposon mutant libraries, are being discontinued.

The advent of next-generation sequencing (NGS) technologies (van Dijk et al., 2014) has not only revolutionized insights into the molecular epidemiology and population genetics of *E. faecium* (de Been et al., 2015; Lebreton et al., 2013; Salipante et al., 2015) but also boosted the development of new sequencing based transposon screening techniques (Goodman et al., 2009; Langridge et al., 2009; van Opijnen et al., 2015). NGS-based deep sequencing of individual transposon mutants in a pooled library, which was named Tn-seq (van Opijnen et al., 2009) or TRADIS (Langridge et al., 2009), results in a high depth of coverage and allows accurate quantification of such mutants (van Dijk et al., 2014). Tn-seq has facilitated research in the basic biology of microorganisms, including Gram-positive bacteria, by identifying genes that are essential in a broad array of

conditions (Le Breton et al., 2015; van Opijnen et al., 2015; Valentino et al., 2014). Sequencing technologies have also transformed research of bacterial mRNA profiling through the development of RNA-seq (Wang et al., 2009; Hrdlickova et al., 2017).

Outline of this thesis

The ubiquitous nature of *E. faecium*, the flexibility of its genome, allowing the accretion of plasmids, phages and conjugative transposons (Clewell et al., 2014; Werner et al., 2013), the ability to prosper in complex microbial communities (Barnes et al., 2012; Paganelli et al., 2013), and the widespread use of antibiotics in human and veterinary medicine, are important factors that drive the current emergence of *E. faecium* as multi-drug resistant nosocomial pathogen. The aim of this thesis is to characterize genetic determinants of *E. faecium* that are relevant for successful colonization and infection of the mammalian host. In **chapter 2** we performed comparative transcriptome profiling of an ampicillin-resistant *E. faecium* isolate at mammalian and environmental temperatures, to identify and characterize a novel protein that contributes to host-cell interactions, by binding to extracellular matrix components and platelets. **Chapter 3** describes a putative two-component system, identified through a microarray-based transposon screening method, that is implicated in tolerance to the disinfectant chlorhexidine. In **chapter 4** we sequenced the genome of a vancomycin-resistant clinical isolate and developed a high-throughput transposon sequencing approach, to perform a sequencing-based genome-wide functional profiling of the same *E. faecium* isolate, to identify genes that contribute to its growth in human serum. In **chapter 5**, we describe a novel resistance mechanism against the novel antibiotic tigecycline, using a combination of comparative genomic analysis of pairs of tigecycline-susceptible and -resistant *E. faecium* isolates, and functional genetic complementation. Finally **chapter 6** describes the conclusions and future perspectives of the research contained in this thesis.

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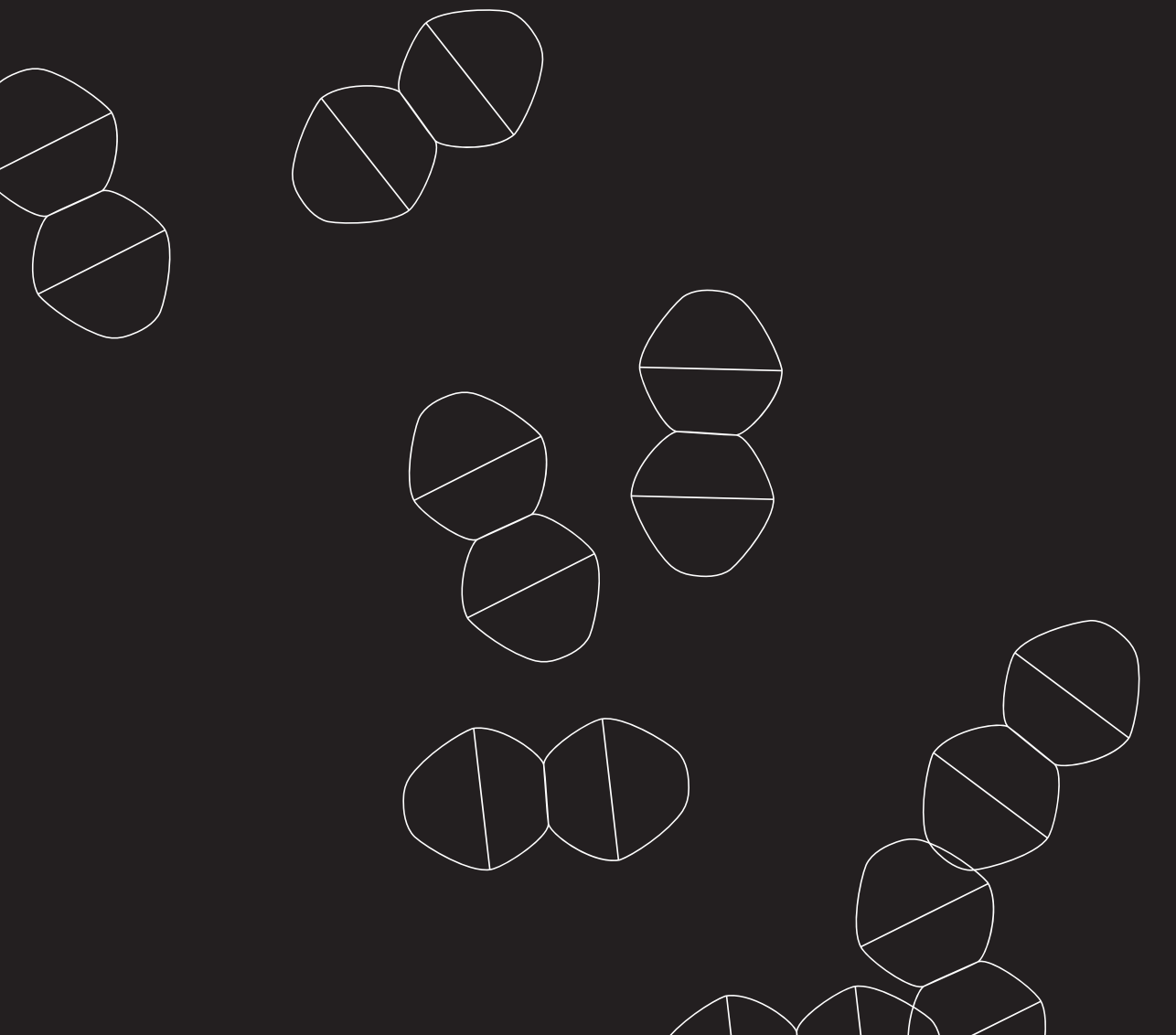

2

The N-terminal domain of the thermo-regulated surface protein PrpA of *Enterococcus faecium* binds to fibrinogen, fibronectin and platelets

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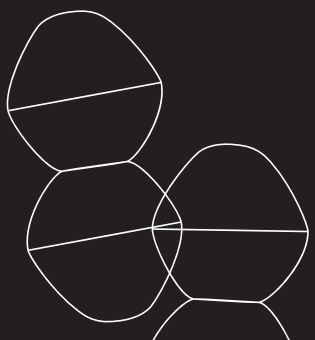


Abstract

Enterococcus faecium is a commensal of the mammalian gastrointestinal tract, but is also found in non-enteric environments where it can grow between 10°C and 45°C. *E. faecium* has recently emerged as a multi-drug resistant nosocomial pathogen. We hypothesized that genes involved in the colonization and infection of mammals exhibit temperature-regulated expression control and we therefore performed a transcriptome analysis of the clinical isolate *E. faecium* E1162, during mid-exponential growth at 25°C and 37°C. One of the genes that exhibited differential expression between 25°C and 37°C, was predicted to encode a peptidoglycan-anchored surface protein. The N-terminal domain of this protein is unique to *E. faecium* and closely related enterococci, while the C-terminal domain is homologous to the *Streptococcus agalactiae* surface protein BibA. This region of the protein contains proline-rich repeats, leading us to name the protein PrpA for proline-rich protein A. We found that PrpA is a surface-exposed protein which is most abundant during exponential growth at 37°C in *E. faecium* E1162. The heterologously expressed and purified N-terminal domain of PrpA was able to bind to the extracellular matrix proteins fibrinogen and fibronectin. In addition, the N-terminal domain of PrpA interacted with both non-activated and activated platelets.

Introduction

Enterococci are Gram-positive, facultative anaerobic bacteria that are ubiquitously present in nature and which can grow in a wide range of temperatures between 10°C and 45°C[1]. The genus *Enterococcus* comprises around forty different species, including *Enterococcus faecium* and *Enterococcus faecalis*[2]. These two species are common commensals of the mammalian gastrointestinal tract, but in the last decades they have become important causes of nosocomial infections, ranging from urinary tract infections to infective endocarditis[3].

In the late 1970s, *E. faecalis* was responsible for almost 95% of the *Enterococcus*-associated infections, with *E. faecium* being a comparatively rare cause of disease. In the last three decades this pattern has shifted, and now *E. faecium* is becoming an increasingly frequent cause of hospital-associated infections[4,5]. Furthermore, the infections caused by *E. faecium* are often more difficult to treat than those caused by *E. faecalis*. This is due to the antibiotic resistance determinants that have recently accumulated in nosocomial *E. faecium* strains and which confer resistance to clinically important antibiotics, including β-lactams and vancomycin[6].

E. faecium hospital-acquired bloodstream infections are often associated with the use of invasive medical devices such as catheters and implants, which disrupt the continuity of the epithelium. Notably, patients with a bacteremia caused by *E. faecium* have a worse prognosis than patients with bacteremias caused by other enterococci[7,8]. Bacterial surface-exposed proteins which can potentially interact with exposed host tissues, for instance due to the use of indwelling medical devices, have been studied in *E. faecium* to clarify the interactions of the bacterial cell with its host[9]. Several *E. faecium* surface proteins that are anchored to the peptidoglycan through a motif composed of leucine-proline-X-threonine/serine/alanine-glycine[10,11], have been identified and characterized. These include pili[12,13], surface proteins involved in biofilm formation[14,15] and proteins that recognize the components of the extracellular matrix (ECM)[10], including microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)[16,17].

Platelets, which are normally responsible for maintaining the balance in blood between fluidity and coagulation[18], can contribute to binding of bacteria to host tissues which promote disease[19]. Gram-positive bacteria can attach to platelets either through direct binding of bacterial surface proteins to platelets[20-22] or by indirect interactions mediated through plasma molecules like fibrinogen and fibronectin[23]. Fibrinogen and fibronectin are not only essential in the coagulation cascade for the clotting of blood, but can also be found as constituents of the host ECM[24,25]. In enterococci, several proteins have previously been described to interact with fibrinogen and fibronectin[26-28], but only the Ebp-type pilus of *E. faecalis* has been shown to have a role in adherence to platelets[29].

In this study we aimed to identify and characterize novel *E. faecium* proteins

that may contribute to host-cell interactions. In order to successfully establish colonization or an infection, commensal and pathogenic bacteria need to adapt to the conditions that prevail in the host. Temperature is an important environmental signal that is frequently linked to the differential expression of bacterial virulence genes[30,31]. Consequently, we first performed comparative transcriptome profiling of *E. faecium* growing at 25°C and 37°C, to identify genes that exhibited higher expression at mammalian temperatures. We subsequently focused on a gene, encoding a surface protein (termed PrpA), that exhibited higher expression at 37°C than at 25°C. PrpA was further characterized in terms of its capacity to bind to ECM components and platelets.

Results

Thermo-regulated expression of a gene encoding a previously uncharacterized *E. faecium* surface protein.

To identify *E. faecium* genes which exhibit thermo-regulated gene expression and consequently may have a role in virulence or colonization, we performed a transcriptome analysis of *E. faecium* E1162 during mid-exponential growth at 25°C and 37°C. In the transcriptome analysis, the differences in gene expression observed between 25°C and 37°C were relatively limited, reflecting the homeostatic nature of enterococcal physiology under permissive growth conditions. Nevertheless, thirty-three genes showed significantly higher expression at 37°C compared to 25°C. Supplementary Table 1 shows the genes that were up-regulated at 37°C. The transcriptome analysis results were confirmed by qPCRs, in which we measured the expression of the four genes that exhibited the largest fold-difference in their expression at 37°C versus 25°C, as determined by the microarray analysis (Fig 1).

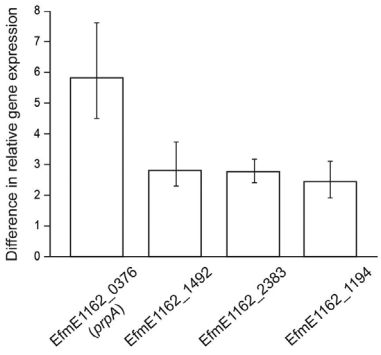


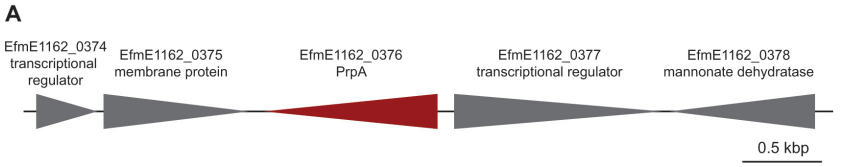
Fig. 1. Differences in relative gene expression of *E. faecium* E1162 between 25°C and 37°C. Expression levels of four genes exhibiting higher expression (in the transcriptome analysis) during mid-exponential growth at 37°C compared to 25°C were determined by qRT-PCR. The data from the qRT-PCR were normalized using *tufA* as housekeeping gene. The differences in gene expression between 37°C and 25°C are shown. Error bars correspond to the standard deviation of four biological replicates.

One of the genes that were differentially regulated during growth at 37°C (4.4-fold, compared to growth at 25°C), is predicted to encode a surface protein (locus-tag: EfmE1162_0376). The up-regulated expression of EfmE1162_0376 at 37°C, combined with the observation that surface-exposed proteins frequently have a role in host-bacterial cell interactions in enterococci and other Gram-positives[11,32], led us to further study the function of EfmE1162_0376.

After PCR amplification, cloning and sequencing of EfmE1162_0376 from *E. faecium* E1162 in an overexpression construct (see below), we noticed that the sequence that was originally deposited to Genbank for *E. faecium* E1162 (accession number: EFF35774) was inaccurate as one of the repeats in EfmE1162_0376 was

lacking. Presumably, DNA repeats in the coding sequence of EfmE1162_0376 could not be correctly resolved using the pyrosequencing technology that was previously used to sequence the *E. faecium* E1162 genome[33]. The corrected sequence of EfmE1162_0376 has been deposited to Genbank (accession number: KF475784). EfmE1162_0376 (which was previously designated *orf884* by Hendrickx *et al.*[34]) is present ubiquitously in *E. faecium* strains from different environments, including bloodstream infections and feces of hospitalized patients, healthy individuals and farm animals[34].

The genes that flank EfmE1162_0376 are transcribed in opposite directions, suggesting that EfmE1162_0376 is transcribed monocistronically (Fig. 2A). An overview of the protein encoded by EfmE1162_0376 is provided in Fig. 2B.



B

MRYSTILISTFAALSLSLTINQTVHT**EEIETAKWTETKWANVNRITFQDEFSSFNSGVAKLRKGQTATIK**
AKIDYSGDLAPILQSAVTFENVDPALSIGYDENTLTIQHKQAIFTFTVTLNQDLLKPALFTVKVSDGLPN
DHHHLTPYSQRQTIEQDSAIDSGGDLVEEPTDKPENENKPEVPPTENPDGEQKPEIEPGEEPDTETQPEP
DNESKPEITPGEKPDVDPEEKPDVTPEPDTDSGNQTVPETNPDTDNETENPEKPEVAPEEKPDVTPEPDT
DSGNQTVPETNPDTDNETENPEKLEVDPEEKPDVTPEPDTDARDQGIPEKINKKTIQEDGKKESKKSNLA
ILKINEEQLNKKSRIFDSAQSAETLKSSKDTTFASPETKNKQLPKSGESQNKVILWSGIILLSIATMLSA
KRFKQNRSL

Fig. 2. The genetic context of *prpA* and overview of the PrpA protein. Panel A shows the genetic environment of the *prpA* gene (in red). Arrows indicate the direction of transcription. The amino acid sequence of the PrpA protein is shown in Panel B. The N-terminal signal sequence is underlined and the LPxSG-motif is boxed. The position of the overexpressed and purified $PrpA_{27\text{-}167}$ protein fragment is indicated in bold. The region of the protein that is highlighted in yellow is homologous to BibA of *S. agalactiae* (further details in the text) and repeats in this region are in italics. Proline residues in PrpA are indicated in orange.

The N-terminal domain of the protein encoded by EfmE1162_0376 contains a signal sequence, which is predicted to be required for the targeting of the protein to the cell wall. Based on currently available genome sequences, orthologous domains of the N-terminal region of this protein only exist in *E. faecium* and closely related species, like *E. hirae, E. mundtii* and *E. durans*, but not in *E. faecalis* or other bacteria. The C-terminal part of EfmE1162_0376 contains a predicted LPxSG-type anchor (LPKSG), which is expected to be required for the cell-wall anchoring

of the protein to peptidoglycan by sortases[32]. Furthermore, the C-terminal part contains repeat regions that are rich in proline, aspartic acid and glutamic acid residues. The high number of proline residues (41 of 370 amino acids in the surface-anchored protein) present in the protein led us to name the protein PrpA, for proline-rich protein A. The C-terminal region of PrpA is similar (35% amino acid identity) to the C-terminal repeat motif of BibA, an adhesin of *Streptococcus agalactiae*[35]. Like in BibA, the proline-rich repeats of PrpA might serve to protrude the N-terminal domain to the outside.

The N-terminal domain of surface exposed proteins is of primary importance for their function in enterococci[36, 37] and other gram positive bacteria[38]. Therefore, we decided to focus on the role of the of the previously uncharacterized N-terminal domain of PrpA and its ability to interact with host components.

Determination of the transcriptional start site of *prpA*

In order to better understand the mechanism of the thermo-regulation of *prpA* expression, we performed 5' rapid amplification of cDNA ends (5' RACE) to map the 5'-end of *prpA* and to identify the promoter region of *prpA*. Upon gel electrophoresis of the 5' RACE reactions, a band was observed at approximately 520 bp in the reaction that was performed with RNA isolated at 37°C (Fig. 3A). The 5' RACE reaction performed on RNA isolated during mid-exponential growth at 25°C was considerably weaker, perhaps reflecting the lower expression of *prpA* at this temperature. Cloning and sequencing of the product revealed the transcriptional start site of *prpA* (Fig. 3B). Upstream of the *prpA* promoter, we identified two inverted repeats that may play a role in the transcriptional regulation of *prpA*.

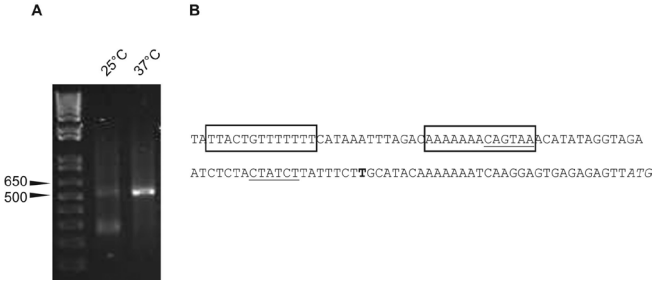


Fig. 3. Mapping of the transcriptional start site of *prpA* by 5' RACE. A) Agarose gel electrophoresis of 5' RACE PCRs shows the product at approximately 520 bp obtained during 5'RACE after nested amplification of cDNA (reverse transcribed from RNA isolated at 25°C and 37°C). B) The upstream region of the *prpA* gene is shown. The transcriptional start site determined by 5' RACE is indicated in bold. The boxes indicate the inverted repeats that are discussed in the text. Putative -35 and -10 promoter regions are underlined. The ATG start codon of *prpA* is shown in italics.

PrpA is a surface protein that is most abundant during exponential growth at 37°C

In order to further characterize the function of the N-terminal fragment of PrpA, we heterologously overexpressed and purified $PrpA_{27\text{-}167}$, corresponding to the N-terminal domain of PrpA, minus the signal sequence. We also constructed a markerless *prpA* mutant (*ΔprpA*) and a strain in which the mutation was complemented *in trans* (*ΔprpA+prpA*). No growth defect was observed in the mutant and complemented strain compared to the E1162 wild-type (Fig. S1A and S1B) when grown at 25°C and 37°C. Subsequently, we quantified surface-exposed levels of PrpA in exponential, stationary phase and overnight cultures of E1162 at 37°C by flow cytometry, using polyclonal anti-PrpA antibodies. Levels of surface-exposed PrpA were highest in E1162 during early stages of exponential growth, when cells were harvested at A_{660} = 0.3 during growth at 37°C. Quantities of surface-exposed PrpA declined at later stages of growth (Fig. 4A).

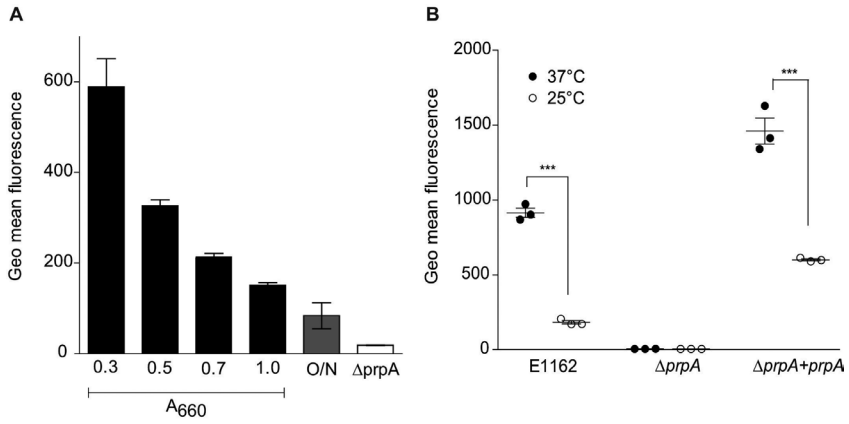


Fig. 4. Analysis of surface-exposed PrpA levels. PrpA was detected on the surface of the bacterium by flow cytometry using rabbit anti-PrpA IgG and FITC-labeled goat anti-rabbit IgG. Panel A shows PrpA-levels on the surface of wild-type *E. faecium* E1162 grown at 37°C. Samples were taken throughout exponential phase (A_{660} 0.3, A_{660} 0.5), transition phase (A_{660} 0.7) and early stationary phase (A_{660} 1.0). Levels of surface-exposed PrpA in an overnight culture of E1162 and during mid-exponential growth of Δ*prpA* are also shown. Panel B shows levels of surface-exposed PrpA in *E. faecium* E1162, Δ*prpA* and Δ*prpA*+*prpA* at 25°C and 37°C during mid-exponential growth (A_{660} 0.3). The data presented were obtained in three independent experiments and significant differences ($p < 0.01$, Student's t-test) are indicated by three asterisks. The geometrical mean of the fluorescence detected in the FITC channel is shown on the y-axis in both panels.

Additionally, we measured the production of PrpA in E1162, *ΔprpA* and *ΔprpA+prpA* at 25˚C and 37˚C. Since we observed the highest levels of surface-exposed PrpA in E1162 at A_{660} = 0.3, we chose this condition for our subsequent experiments. In Figure 4B we show that cells of E1162 grown at 25°C had significantly lower levels of PrpA at the surface than cells grown at 37°C, indicating that the lower expression of *prpA* at 25°C observed in the transcriptome analysis translates to lower levels of surface-associated PrpA protein. As expected, in *ΔprpA* no signal was detected on the surface of cells. PrpA production is restored in the complemented strain, but also in this strain, levels of PrpA are higher at 37°C than at 25°C. Subsequently, we used transmission electron microscopy (TEM), to visualize the surface localization of PrpA in *E. faecium*, using polyclonal anti-PrpA antibodies and a protein A-10 nm gold particle conjugate (Fig. 5A-5D). TEM showed that PrpA localized to the 'old' hemispheres of dividing cells of E1162 (Fig. 5B) and of *ΔprpA+prpA* (Fig. 5D). No apparent change in cellular morphology due to either the deletion or the overexpression of *prpA* was observed.

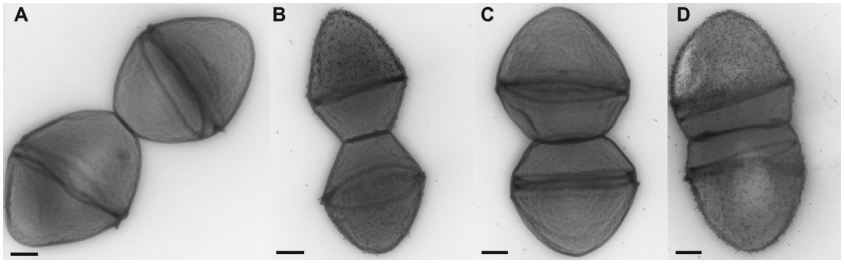


Fig. 5. Cellular localization of PrpA assessed by transmission electron microscopy. *E. faecium* E1162, Δ*prpA* and Δ*prpA+prpA* were grown at 37°C to A_{660} 0.3. Panel A shows cells incubated with pre-immune serum (negative control). Figures B to D show the localization of PrpA in the wild type, Δ*prpA* and complemented strain, respectively. PrpA was detected using rabbit anti-PrpA IgG and a subsequent incubation with 10 nm protein A-gold beads. The scale bar corresponds to 200 nm.

Thermo-regulation of PrpA is observed in other *E. faecium* strains

After characterizing the temperature-dependent production of PrpA in strain E1162, we studied the production of PrpA in fourteen *E. faecium* strains, of which the genomes were previously sequenced and which together covered the different phylogenetic clades of *E. faecium*, i.e. clade A1 representing clinical isolates, clade A2 representing animal strains and clade B representing human commensals[39]. All tested strains contained a copy of the *prpA* gene that was ≥79% identical to

prpA in *E. faecium* E1162, with the difference mainly being introduced through the variable number of proline-rich repeats. We found that none of the clade B strains produced PrpA during mid-exponential growth (A_{660} = 0.3) at 25°C or at 37°C (Fig. 6). Besides E1162 (a clade A1 strain), we found that one additional strain from clade A1 and three strains from clade A2, were able to produce PrpA when grown at 37°C. Interestingly, in all these strains the production of PrpA was found to be thermo-regulated, with higher levels at 37°C, as observed before for E1162, when compared with 25°C (Fig. 6). In the strains (n = 10) in which we were not able to demonstrate production of PrpA at the cell surface, sequence analysis of the *prpA* gene revealed that four strains (E2560, E1575, E2620 and E1007) contained mutations in *prpA* that led to a truncation of the gene.

Alignment of the *prpA* promoter regions of the clade A strains did not provide evidence to why E1644, E1731 and E1636, which contain an intact copy of *prpA,* failed to produce this protein. The promoter sites, including the inverted repeat upstream of *prpA* promoter in *E. faecium* E1162, were completely identical to all of these three strains. In contrast to clade A strains, the same region in clade B strains contained several SNPs, which may affect the transcriptional regulation of the *prpA* gene in the clade B strains (Fig. S2).

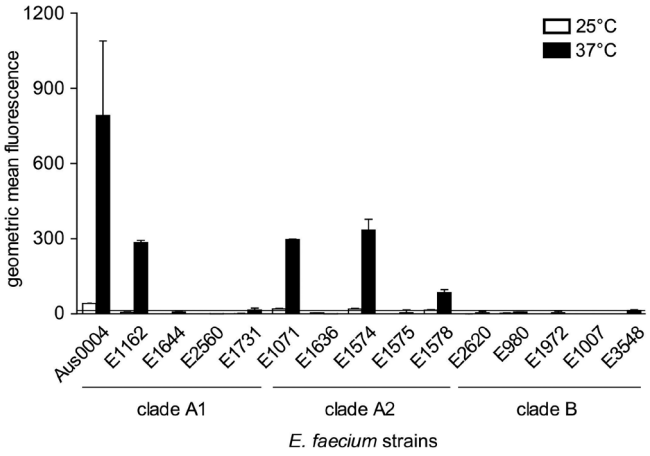


Fig. 6. Production of PrpA in *E. faecium* strains. Production of PrpA is shown in fifteen different *E. faecium* strains isolated from diverse environments and belonging to different clades of the *E. faecium* species. Bacteria were grown to mid-exponential phase at either 25°C or 37°C and PrpA production was determined by flow cytometry. White and black bars indicate the production of PrpA at 25°C and 37°C, respectively. The horizontal line indicates the signal obtained from Δ*prpA*, where no PrpA is produced. Error bars indicate the standard deviation of the results in three independent experiments.

PrpA$_{27\text{-}167}$ binds to fibrinogen and fibronectin

In Gram-positive bacteria, surface-exposed proteins can mediate adherence to host tissues, often involving interactions with ECM components[40-42]. Therefore we studied whether PrpA could have a role in the adhesion to ECM proteins. Using ELISA, we found that PrpA$_{27\text{-}167}$ binds to immobilized fibrinogen and fibronectin (Fig. 7A). Binding of PrpA$_{27\text{-}167}$ to collagen types I, II and IV was also observed but at approximately three-fold lower levels compared to binding to fibrinogen and fibronectin. By ligand affinity blotting, we confirmed that PrpA$_{27\text{-}167}$ binds to fibrinogen and fibronectin (Fig. 7B), but binding to collagen I could not be demonstrated (Fig. 7B) and identical, negative results were observed for collagen II and collagen IV (data not shown). Based on these data, we conclude that, among the tested ECM proteins, the N-terminal domain of PrpA only mediates binding to fibrinogen and fibronectin. Binding of *E. faecium* E1162 and Δ*prpA* to fibrinogen and fibronectin was also assessed by ELISA. E1162 was able to bind both fibrinogen and fibronectin however no significant difference between wild-type and mutant was found (data not shown), which could be due to the functional redundancy of multiple fibrinogen binding proteins in *E. faecium* [43].

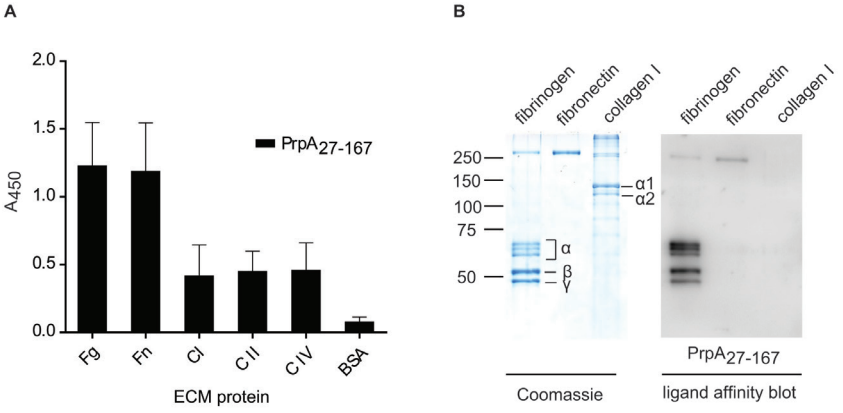


Fig. 7. Binding of PrpA to ECMs proteins. Panel A shows the binding of PrpA$_{27\text{-}167}$ to immobilized extracellular matrix (ECM) proteins fibrinogen (Fg), fibronectin (Fn) and collagen type I (C I), II (C II) and IV (C IV) as determined by ELISA. These data are averages of two independent experiments performed with two technical replicates each. Panel B shows ligand-affinity blots of the binding of PrpA$_{27\text{-}167}$ to ECMs proteins transferred to nitrocellulose membranes. Binding of PrpA$_{27\text{-}167}$ to ECMs proteins was detected using anti-PrpA IgG and HRP-goat anti-rabbit IgG. ECM proteins were also visualized after SDS – PAGE by staining with Coomassie. The different subunits of fibrinogen, collagen I and marker sizes (in kDa) are indicated.

$PrpA_{27-167}$ binds to human platelets

Apart from acting as extracellular matrix components, fibrinogen and fibronectin are also found in soluble forms in blood where they play an important role in the coagulation cascade [25]. Since the N-terminal domain of PrpA was found to mediate binding to fibrinogen, we examined the possibility whether $PrpA_{27-167}$ could interact with platelets. The binding of FITC-labeled $PrpA_{27-167}$ to resting platelets, and platelets that were activated using the thrombin receptor-activating peptide TRAP, was assayed by flow cytometry. These experiments were performed with washed platelets, thereby minimizing the amount of fibrinogen in the assay, and with resting and activated platelets in whole blood (RP-WP and AP-WB, respectively). Levels of surface-bound fibrinogen in each condition are shown in Fig. S3A for washed platelets and in Fig. S3B for platelets in whole blood. Activation of the washed platelets (Fig. S3C) and platelets in whole blood (Fig. S3D) was measured by the detection of the activation marker P-selectin on the surface of the platelets. $PrpA_{27-167}$ was able to bind to RP (Fig. 8A) and to RP-WB (Fig. 8B), but activation of platelets considerably increased binding of $PrpA_{27-167}$ to platelets, resulting in 3.0-fold higher binding to AP and 10.8-fold to AP-WB, compared to resting platelets in both conditions (Fig. 8A and 8B).

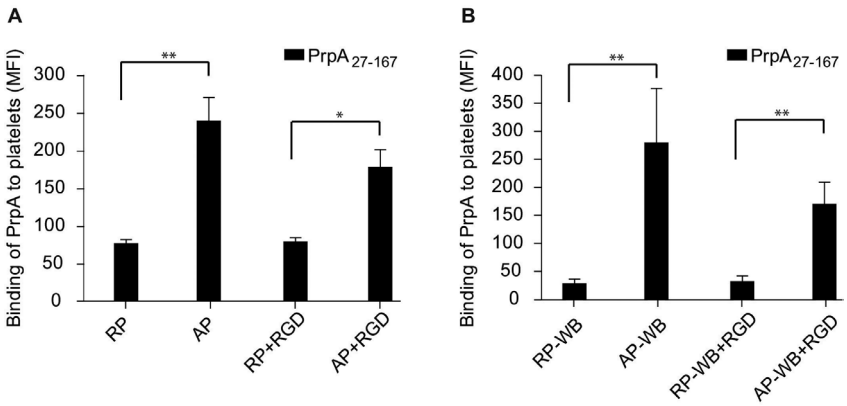


Fig. 8. Binding of $PrpA_{27-167}$ to platelets. Binding of the protein to platelets was measured by flow cytometry. $PrpA_{27-167}$ was labeled with FITC. Binding of $PrpA_{27-167}$ to resting (RP) and TRAP-activated washed platelets (AP) is shown in panel A and to resting (RP-WB) and TRAP-activated platelets (AP-WB) in whole blood in panel B. In panels A and B, D-arginyl-glycyl-L-aspartyl-L-tryptophane (RGD) was used to block the fibrinogen receptor (GPIIb-IIIa $\alpha_{IIb}\beta_3$) in resting (RP+RGD) and activated (AP+RGD) washed platelets and in resting (RP-WB+RGD) and activated platelets (AP-WB+RGD) in whole blood. The mean fluorescence intensity (MFI) is shown. The figures represent data from three independent experiments and the error bars indicate the standard deviation. Data were analyzed by Student's t-test and significant differences are indicated by one ($p < 0.05$) or two ($p < 0.01$) asterisks.

The increased binding of $PrpA_{27-167}$ to activated platelets could be due to the increase in surface area of platelets upon activation[44,45]. Alternatively, binding of $PrpA_{27-167}$ to activated platelets may be mediated by the release and subsequent binding of fibrinogen to the platelet surface during activation, which would reflect the ability of PrpA to bind this ECM protein. Blocking the fibrinogen receptor α(IIb)β3 on the platelet surface using D-arginyl-glycyl-L-aspartyl-L-tryptophane (RGD)[46], did not affect binding of $PrpA_{27-167}$ to RP or RP-WB. Blocking the fibrinogen receptor upon platelet activation (AP+RGD and AP-WB+RGD) decreased, but did not abolish, binding of $PrpA_{27-167}$ to platelets (Fig. 8A and 8B). In AP+RGD, no fibrinogen was detected on the platelet surface (Fig. S3A), but binding of $PrpA_{27-167}$ to the platelet surface was still observed. These observations suggest that both direct and fibrinogen-mediated interactions of $PrpA_{27-167}$ contribute to binding of this protein to platelets.

The ability of *E. faecium* E1162 cells and the *prpA* deletion mutant to bind to platelets was also assayed. Both E1162 and the *prpA* deletion mutant were able to bind to platelets but no differences were observed (data not shown). This result probably reflects redundancy of multiple platelet-binding proteins that are important for the interaction of *E. faecium* with platelets, as previously described for *E. faecalis* [29,47,48].

Discussion

E. faecium is a commensal of the gastrointestinal tract but due to the accumulation of antibiotic resistance determinants and other adaptive elements, it has become an important nosocomial pathogen[3,5,39,49]. For opportunistic pathogens like *E. faecium*, which live in a wide variety of ecological niches ranging from plants and insects to the gastrointestinal tract of mammals, temperature is a particularly important environmental cue[1,30,50]. Under these different conditions, *E. faecium* needs to adjust its gene expression to survive in the environment and to be successful when interacting with the host[31]. Surface-exposed proteins in enterococci can facilitate bacterial interactions with host factors[9], that might become exposed due to the use of indwelling medical devices in hospitalized patients. In the present study, we describe a thermo-regulated surface-exposed protein in *E. faecium*, termed PrpA, of which the N-terminal domain interacts with ECM components.

The presence of the C-terminal LPKSG motif in PrpA is predicted to allow anchoring of this protein to peptidoglycan. We hypothesized that in its peptidoglycan-anchored form, the proline-rich repeat region of PrpA may adopt a poly-proline helix-like conformation, similar to surface proteins with proline-rich repeat regions of streptococci[35,51], thereby extending the functional N-terminal domain to the exterior milieu. For this reason, we focused on the function of the

N-terminal domain of PrpA. However, the exact contribution of the C-terminal domain still needs to be investigated.

In accordance with the prediction that PrpA is a surface protein, we were able to detect surface-exposed PrpA on *E. faecium* E1162 cells, at higher levels when cultured at 37°C than at 25°C. *E. faecium* strains originating from healthy individuals that were previously assigned to clade B[39] did not produce PrpA under the conditions tested. On the other hand, strains from clade A[39], which covers strains of clinical and animal origins, can produce PrpA in a thermo-regulated fashion. Strains from *E. faecium* clade A are genetically distinct from the human commensal clade B strains and have been following distinct evolutionary trajectories[39]. In clade B strains, the role of PrpA remains to be determined and the expression of *prpA* may be regulated by environmental triggers other than temperature.

Using ELISA and ligand affinity blotting, we showed that the N-terminal domain of PrpA ($PrpA_{27\text{-}167}$) binds to fibrinogen and fibronectin. We also observed that $PrpA_{27\text{-}167}$ is able to interact with activated and resting platelets. Upon activation, fibrinogen bound to the platelet surface seems to facilitate the interaction of $PrpA_{27\text{-}167}$ with the platelets, congruent with the ECM-binding characteristic of this protein. However, the binding of $PrpA_{27\text{-}167}$ to activated washed platelets upon treatment with RGD, blocking the fibrinogen receptor, suggests that $PrpA_{27\text{-}167}$ can also bind directly to platelets, in a fibrinogen-independent manner as has previously been described for other bacterial proteins[29,47,48]. The observation that deletion of *prpA* does not impair binding of *E. faecium* E1162 to fibrinogen, fibronectin and platelets, could reflect the functional redundancy of surface-exposed proteins of *E. faecium* in binding ECM components[15,27,28].

Surface proteins of *E. faecium* are studied to understand the roles these proteins play in mediating interactions between the bacterial cell and its environment. PrpA is a surface protein that is characteristic for *E. faecium* and closely related enterococci, as it contains a unique N-terminal domain. In this study, we show that the N-terminal domain of PrpA interacts with fibrinogen, fibronectin and platelets. The thermo-regulated production of PrpA by clinical *E. faecium* isolation suggests that this protein may have a specific, but as yet undetermined, role in the colonization and infection of mammals.

Materials and methods

Bacterial isolates, construction of a markerless *prpA* deletion mutant and *in trans* complementation.

Information on the strains and plasmids that were used in this study is provided in Table S2. *E. faecium* was grown in Brain Heart Infusion (BHI; Oxoid, Basingstoke, United Kingdom) broth, unless otherwise noted. *Escherichia coli* was grown in Luria-Bertani Broth (LB; Oxoid). When needed, appropriate antibiotics were used at the following concentrations: gentamicin 300 µg ml^{-1} for *E. faecium* and 25 µg ml^{-1} for *E. coli*, spectinomycin 300 µg ml^{-1} for *E. faecium* and 100 µg ml^{-1} for *E. coli*, and erythromycin 25 µg ml^{-1} for *E. faecium* and 150 µg ml^{-1} for *E. coli*. All antibiotics were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

To generate a markerless *prpA* deletion mutant in *E. faecium* E1162, we used a previously described technique[52]. In brief, the 5' and 3' flanking regions (approximately 500 bp each) of the *prpA* gene were PCR amplified with two sets of primers: Up-PrpA-F-XhoI and Up-PrpA-R- EcoRI at the 5' end, and down-PrpA-F-EcoRI and down-PrpA-R-SmaI at the 3' end of *prpA* (primer sequences are listed in Table S3). The two flanking regions were then fused together by PCR and cloned into pWS3[53]. A gentamicin-resistance cassette flanked by *lox66* and *lox71* sites was PCR amplified (oligonucleotides listed in Table S3) and cloned into the EcoRI site that was generated between the 5' and 3' flanking regions of *prpA* in the pWS3 construct as described previously[52]. Finally, the construct, named pMP1, was electrotransformed into *E. faecium* E1162 and a markerless deletion of *prpA* (termed Δ*prpA*) was generated as described before[52].

For *in trans* complementation of Δ*prpA*, *prpA* with its native promoter was amplified by PCR using Accuprime High Fidelity Taq Polymerase (Life Technologies, Bleiswijk, The Netherlands) with primers Comp-prpA-F-BamHI and Comp-prpA-R-PstI, which introduced BamHI and PstI restriction sites. The resulting product was cloned into pMSP3535[54]. The construct was sequenced to confirm the absence of mutations in *prpA*. This plasmid was electrotransformed into Δ*prpA* as described previously[52], generating the complemented strain Δ*prpA+prpA*. The expression of *prpA* in Δ*prpA+prpA* is under the control of its native promoter, as the inducible promoter on pMSP3535 is not activated by the addition of nisin in these experiments.

Determination of growth curves.

E. faecium E1162, its isogenic Δ*prpA* mutant, the *in trans* complemented Δ*prpA*+*prpA* strain and E1162 carrying the vector pMSP3535[54], which was used for complementation, were grown overnight at 37°C and 25°C in BHI containing appropriate antibiotics. Cultures were diluted 1:50 in pre-warmed BHI at the appropriate temperature and the A_{660} was recorded for every 30 min until stationary phase was reached. Each experiment was performed in triplicate.

Transcriptome analysis of *E. faecium* E1162.

To determine thermo-regulated gene expression, RNA was isolated from *E. faecium* E1162 grown in BHI until the mid-exponential growth phase (A_{660} = 0.3) at 25°C and 37°C in a shaking (200 rpm) water bath. Further details of growth conditions, RNA isolation, cDNA synthesis and labeling, microarray hybridization and data analysis have been described previously[52].Microarray data have been deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-3941.

Quantitative real-time RT-PCR (qRT-PCR) analysis of *prpA* expression.

Total RNA was isolated as described before and used to confirm the transcriptome analysis by qRT-PCR[55]. cDNA was synthesized using the Superscript III First-Strand Synthesis System (Life Technologies, Breda, The Netherlands) according to the manufacturer's instructions. Using synthesized cDNAs, qRT-PCR was performed using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Breda, The Netherlands) and a StepOnePlus instrument (Life Technologies). The expression of *tufA* was used as a housekeeping control. Ct values were calculated using the StepOne analysis software v2.2. REST 2009 V2.0.13 (Qiagen, Venlo, The Netherlands) was used to determine the transcript levels, relative to *tufA,* of the assayed genes and for statistical analysis. We also performed qPCRs on reaction mixtures that lacked reverse transcriptase. In these negative control samples Ct values were consistently higher (›34) than in the samples in which reverse transcriptase was added (Ct values ranging between 15 and 26), indicating that residual or contaminant DNA was present at minimal quantities and did not influence the determination of gene expression levels by qPCR. This experiment was performed with four biological replicates.

Determination of the 5' end of the *prpA* transcript.

We used 5'RACE (Life Technologies) to map the 5'-end of the *prpA* mRNA, following the manufacturer's instructions using two *prpA*-specific primers (GSP1 and GSP2) (Table S3). The amplified product was cloned using the CloneJET PCR cloning kit (Thermo Scientific) and sequenced by Sanger sequencing.

Heterologous overexpression and purification of PrpA.

A gene fragment of *prpA*, encoding the N-terminal domain of PrpA excluding the N-terminal signal sequence, was amplified with the primers PrpA-BamHI and $PrpA_{27\text{-}167}$-R-NotI-STOP. The purified protein is referred to in this manuscript as $PrpA_{27\text{-}167}$. The PCR products were digested with BamHI and NotI and then cloned into the similarly digested overexpression vector pEF110[56], resulting in pMP2 which is an overexpression construct encoding the proteins with an N-terminal polyhistidine tag. The overexpression construct was transformed into *E. coli* BL21 (DE3) and further overexpression and purification of the recombinant proteins was performed as described previously[56].

Production of anti-PrpA polyclonal antibodies.

Polyclonal antibodies against PrpA were raised by Eurogentec (Belgium) according to their rabbit immunization protocol. From the rabbit serum, IgG was purified using a 1-ml HiTrap Protein G HP column (GE Healthcare, Zeist, The Netherlands) and dialyzed overnight in phosphate buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 140 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH 7.4 with HCl).

Quantification of surface-exposed PrpA by flow cytometry.

Levels of surface-exposed PrpA were determined by flow cytometry, essentially as described previously[57] with some minor modifications. In brief, *E. faecium* strains were grown overnight in BHI at 37˚C and 25˚C. The cells were then diluted (1:50) in pre-warmed media and cultured further at the appropriate temperature. Samples of the cultures were taken during different time points of the growth curve (A_{660} = 0.3, 0.5, 0.7, 1.0) and pelleted by centrifugation at 6,500 *g* for 1 min. PrpA was detected using 1:100 anti-PrpA rabbit IgG and 1:50 FITC-labeled goat anti rabbit IgG (Sigma-Aldrich). Flow cytometry was performed using the FACS Calibur system (BD Biosciences, Breda, The Netherlands). The geometric mean fluorescence was used as a measure for cell surface-exposed PrpA. This experiment was performed with three biologically independent replicates and statistical analysis of the data was performed using a two-tailed Student's t-test.

Sequence analysis of *prpA* in *E. faecium* strains.

The *prpA* gene of different *E. faecium* strains was amplified by PCR and sequenced using the primers PrpA_Fw and PrpA_Rv (Table S3). Analysis of the sequences was performed using Serial Cloner version 2.6 and alignments were made using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The sequences have been deposited in Genbank under the following accession numbers: KP030673 (E980), KP030674 (E1007), KP030675 (E1071), KP030676 (E1574), KP030677 (E1575), KP030678 (E1578), KP030679 (E1636), KP030680 (E1644), KP030681 (E1731), KP030682 (E1972), KP030683 (E2560), KP030684 (E2620), KP030685 (E3548), KP030686 (E7345).

Determination of PrpA localization by transmission electron microscopy (TEM).

The cellular localization of PrpA in *E. faecium* E1162 was determined by transmission EM with immunogold labeling as described previously[58]. Bacteria were grown to mid-exponential phase (A_{660} = 0.3) and PrpA was detected using purified anti-PrpA rabbit IgG labeled with 1:60 diluted Protein A-gold (10 nm). Microscopy was performed on a JEOL 1010 transmission electron microscope (JEOL Europe, Nieuw-Vennep, The Netherlands).

ELISAs to determine binding of PrpA to ECM components.

ELISAs to determine binding of $PrpA_{27\text{-}167}$ to ECM components were performed as previously described[15]. After coating with ECM proteins, 50 µl of a 25 µg/ml solution in PBS of $PrpA_{27\text{-}167}$ was used in the binding experiment. All ECMs proteins were obtained from Sigma-Aldrich. Binding of PrpA to ECM proteins was detected by anti-PrpA IgG (1:1000) and peroxidase-conjugated goat anti-rabbit IgG-horseradish peroxidase 1:10000 from Southern Biotech (Birmingham, AL; USA) and measuring absorbance at 450 nm.

Ligand affinity blotting.

Ligand affinity blotting was carried out as described by Hendrickx *et al.*[15]. 1 µg of ECM protein was loaded per well onto a 7.5% SDS gel. PVDF membranes (Merck Millipore) were incubated with purified $PrpA_{27\text{-}167}$ at a concentration of 1 nM.

FITC labeling of PrpA.

FITC was dissolved at a concentration of 1 mg/ml in 1 M sodium carbonate buffer pH 9.6. A 2 mg/ml solution of $PrpA_{27-167}$ was prepared in the same buffer and 500 µl of this solution was mixed with 55 µl of FITC solution. This mixture was incubated for 2 h at 4°C in the dark. After labeling, the solution was desalted using Polyacrylamide Spin Desalting Columns (Thermo Scientific). FITC labeling of the protein was assessed by SDS-PAGE, running both labeled and unlabeled protein onto a 12% gel and imaging the gel under the GFP channel of an Image Quant LAS4000. FITC was obtained from Sigma-Aldrich.

Binding of $PrpA_{27-167}$ to human platelets.

Whole blood was collected from three healthy volunteers using vacuum blood collection system tubes containing 3.2% sodium citrate. Platelets were isolated and washed as previously described[59]. The protocol for blood collection was approved by the Institutional Review Board of the University Medical Center Utrecht. Written informed consent was obtained from all donors in accordance with the declaration of Helsinki. For each donor, the mean platelet volume (MPV) was measured using the cell analyzer CELL-DYN 1800 (Abbott) in both whole blood and in washed platelets (WP) to exclude that activation of the platelets had occurred during isolation. Platelets were left at room temperature for at least 30 min to ensure a resting state before they were used in the experiments.In a final volume of 200 µl, FITC-labeled $PrpA_{27-167}$ (50 µg/ml) were mixed with 20 µl of either whole blood or washed platelets (adjusted to 200,000 platelets/µl). Whole blood and platelets were activated with 200 µM of the thrombin receptor-activating peptide SFLLRN (TRAP-6) from Bachem (Bubendorf, Switzerland). Platelet activation was monitored through binding of phycoerythrin-labelled mouse anti-human P-selectin antibodies (diluted 1:50; BD Biosciences) to the activation marker P-selectin on the platelet surface[60]. Binding of fibrinogen to platelets was detected using FITC labeled rabbit anti-human fibrinogen antibodies (Dako - Agilent Technologies, Heverlee, Belgium, diluted 1:50). The fibrinogen receptor ($\alpha_{IIb}\beta_3$) was blocked using 100 µM of D-arginyl-glycyl-L-aspartyl-L-tryptophane (RGD)[46], synthesized at the Department of Membrane Enzymology, Faculty of Chemistry, Utrecht University (Utrecht, The Netherlands). All samples were incubated for 30 min at room temperature and then fixed in a 0.2% paraformaldehyde, 0.9% NaCl solution. Flow cytometric detection of the binding of $PrpA_{27-167}$ to platelets was performed on a FACSCanto II system (BD Biosciences). Statistical analysis of the data was performed using a two-tailed Student's *t*-test.

Acknowledgments

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Author contributions

W.v.S. designed the study. A.M.G.P., R.T.U., X.Z., D.B., A.K., M.v.L.A., J.P.O, M.P., F.L.P., D.W. and J.H. performed experiments. All authors contributed to data interpretation. The manuscript was written by A.M.G.P., A.P.A.H., M.J.M.B., R.J.L.W. and W.v.S.

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Supplementary information

Table S1: List of genes that exhibit higher expression during mid-exponential growth at 37°C than at 25°C as determined by transcriptome analysis.[a]

Locus tag	Annotation	Expression ratio 37°C / 25°C
EfmE1162_1194	hypothetical protein	4,5
EfmE1162_0376	surface protein, putative	4,4
EfmE1162_2382	hypothetical protein	3,2
EfmE1162_1492	tyrosine decarboxylase	3,2
EfmE1162_1491	antiporter	3,0
EfmE1162_1284	Fmu	2,9
EfmE1162_2341	conserved hypothetical protein	2,5
EfmE1162_1038	PspC domain family	2,4
EfmE1162_2340	ribosomal protein L32	2,4
EfmE1162_1737	conserved hypothetical protein	2,4
EfmE1162_2343	cobalamin synthesis protein/P47K family protein	2,3
EfmE1162_1490	Na+/H+ antiporter NhaC	2,3
EfmE1162_1702	conserved hypothetical protein	2,3
EfmE1162_0259	DltD protein	2,3
EfmE1162_0769	hypothetical protein	2,3
EfmE1162_2266	methionine gamma-lyase	2,2
EfmE1162_2342	ribosomal protein L33	2,2
EfmE1162_0678	hypothetical protein	2,2
EfmE1162_1816	branched-chain amino acid transport protein AzlD	2,2
EfmE1162_1815	AzlC family protein	2,2
EfmE1162_0261	protein DltB	2,1
EfmE1162_1037	conserved hypothetical protein	2,1
EfmE1162_1704	collagen adhesin	2,1
EfmE1162_0446	hypothetical protein	2,1
EfmE1162_2327	manganese transport system ATP-binding protein MntA	2,1
EfmE1162_0770	conserved hypothetical protein	2,1
EfmE1162_0644	shikimate kinase	2,1
EfmE1162_0260	D-alanyl carrier protein	2,1
EfmE1162_0263	putative D-Ala-teichoic acid biosynthesis protein	2,1
EfmE1162_0601	hydrolase	2,0
EfmE1162_2339	30S ribosomal protein S14	2,0
EfmE1162_2639	hypothetical protein	2,0
EfmE1162_1521	hypothetical protein	2,0
EfmE1162_0318	hypothetical protein	2,0

[a] The genes which exhibit a differential expression >2 between 37°C and 25°C are shown here.

Table S2: Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source
E. faecium		
E1162	Clinical isolate (blood infection); Amp^r, Tet^r	33
Δ*prpA*	Markerless deletion mutant of *prpA* in E1162	This study
Δ*prpA*+*prpA*	*In trans* complementation strain of Δ*prpA*, Δ*prpA* harboring pMSP3535-*prpA*	This study
Aus0004	Clinical isolate (bloodstream infection)	61
E1644	Hospital outbreak, identical to EnGen0051	39
E2560	Clinical isolate (bloodstream infection), identical to strain EnGen0046	39
E1731	Clinical isolate (bloodstream infection), identical to strain EnGen0036	39
E1071	Hospital surveillance (feces)	33
E1636	Clinical isolate (bloodstrean infection)	33
E1574	Isolated from feces of a dog, identical to strain EnGen0020	39
E1575	Isolated from feces of a chicken, identical to strain EnGen0001	39
E1578	Isolated from feces of a mini pig, identical to strain EnGen0007	39
E2620	Clinical isolate (bloodstream infection), identical to strain EnGen0038	39
E980	Isolated from feces of a non-hospitalized individual	33
E1972	Clinical isolate (bloodstream infection), identical to strain EnGen0033	39
E1007	Isolate from feces of a non-hospitalized individual, identical to strain EnGen0015	39
E3548	Clinical isolate (bloodstream infection), identical to strain EnGen0047	39
E. coli		
BL21 (DE3)	Strain for recombinant protein expression	Invitrogen
Plasmids		
pEF110	Overexpression vector for *E. coli*, contains a sequence for an N-terminal histidine tag	Laboratory stock
pWS3	Gram-positive thermosensitive origin; Spc^r	53
pMP1	pWS3 derivative containing fused 5'and 3'flanking regions of *prpA*	This study
pMP2	pEF110 derivative carrying constructs for overexpression of $PrpA_{27\text{-}396}$ or $PrpA_{27\text{-}167}$	This study
pWS3-Cre	Derivative of pWS3 expressing cre recombinase in *E. faecium*	52
pMSP3535	pAMβ1 (from pIL252), ColE1 replicon, Erm^r, nisRK, PnisA	54
pMSP3535-*prpA*	Plasmid for the complementation of Δ*prpA*, pMSP3535 carrying *prpA*	This study

Amp: ampicillin; Tet: Tetracycline; Spc: Spectinomycin; Erm: Erythromycin; nisRK: nisin two-component system; PnisA: nisin inducible promoter

Table S3: Oligonucleotides used in this study

Primer	Sequence (5' – 3')
Up-PrpA-F-XhoI	CCGCTCGAGCGAATTTGTCTGACAGCTGAA
Up-PrpA-R-EcoRI	GATTTTGGTAGAATTCGATAAGCTTAAGGCTGCAAA
Down-PrpA-F-EcoRI	AGCTTATCGAATTCTACCAAAATCCGGAGAGTCACAA
Down-PrpA-R-SmaI	GTCGCCCGGGGGCGTTTGGGGCTTGATTGTCGG
Comp prpA F BamHI	ACGGGATCCCGATGTTCAGATAGGAAAAGAAAAA
Comp prpA R PstI	CAACTGCAGTTGGGGTCGTTTTCTGGATA
PrpA check dn	GAGGCATCCGAGATGTTGTT
PrpA check up	AAACCGTACAATGCTAAAAATGC
PrpA_Fw	AATCAGACAGTCCACACAGAG
PrpA_Rv	AATGATTCCGCTCCACAGTA
PrpA-BamHI	GTGGGGATCCGAGGAGATAGAAACAGCCAAATGG
$PrpA_{27-167}$-R-NotI-STOP	GGTCGCGGCCGCCTAAACAAGATCTCCTCCTGAGTC
pAT392_EcoRI_lox66_genta_F	GAGGGAATTCTACCGTTCGTATAGCATACATTATACGA AGTTATGATAAACCCAGCGAACCATTTGAGG
pAT392_EcoRI_lox71_genta_R	CTCCGAATTCTACCGTTCGTATAATGTATGCTATACGAA GTTATTCAATCTTTATAAGTCCTTTTATAA
qPCR Fw E1162_1194	CGACCACTTTGAAAACCCAGA
qPCR Rv E1162_1194	TCTCTTTGAGTTTCTGATCCATTGC
qPCR Fw E1162_0376	AATCGACAAAGCGGGATCAA
qPCR Rv E1162_0376	CAGCGACCATCAAAGCAAAA
qPCR Fw E1162_2382	TTTCCCATGCTGATTCGACA
qPCR Rv E1162_2382	CTACGCCAGCAAGTGTGTGC
qPCR Fw E1162_1492	CCAATCGCTTTTTCCAGCAA
qPCR Rv E1162_1492	CTTTGGTATGCCCGCAACAT
GSP1	CGTCTGGATTTTCTGTAGGCGG
GSP2	GGTTCTTCAACAAGATCTCCTCC

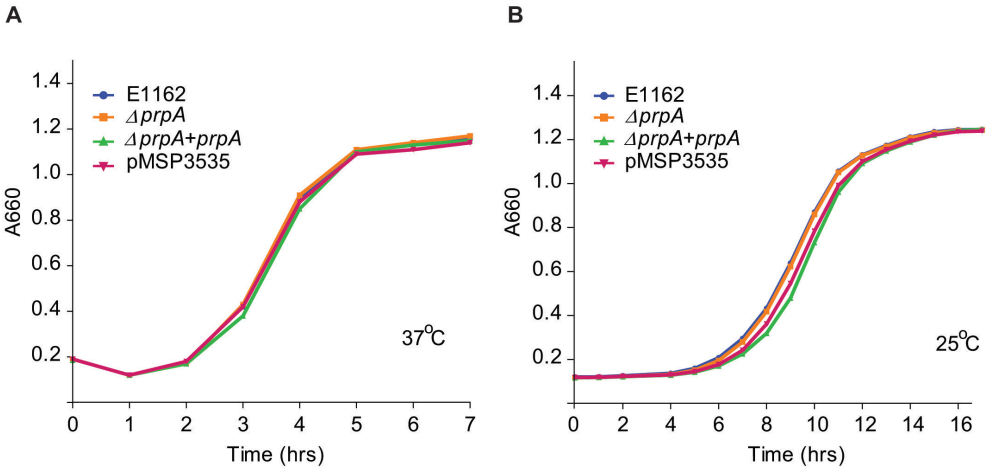


Fig S1. Growth curves of *E. faecium* strains. Panel A and panel B show the growth curves for E1162, Δ*prpA*, Δ*prpA+prpA* and E1162 with the vector (pMSP3535) used for *in trans* complementation at 25°C and 37°C respectively. The growth curves represent the averages of three independent experiments.

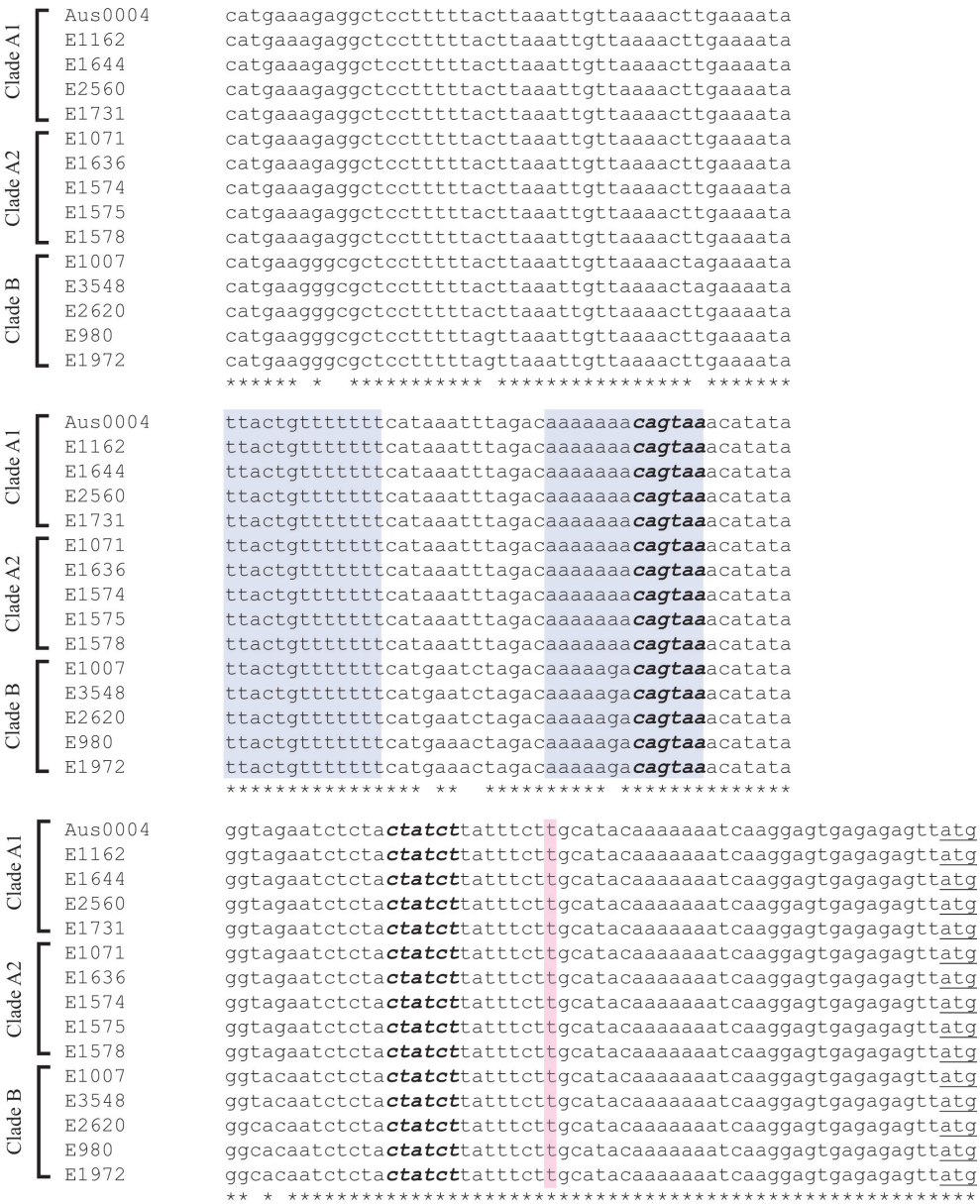


Fig S2. Alignment of the promoter region of *prpA* in different *E. faecium* strains. Fifteen *E. faecium* strains from different environments, clinical isolates (clade A1), animal strains (clade A2) and human commensals (clade B) were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The blue boxes indicated the inverted repeats. The red box depicts the transcriptional start site of *prpA*. Putative -35 and -10 promoter regions are shown in bold italics and the start of the *prpA* gene is underlined.

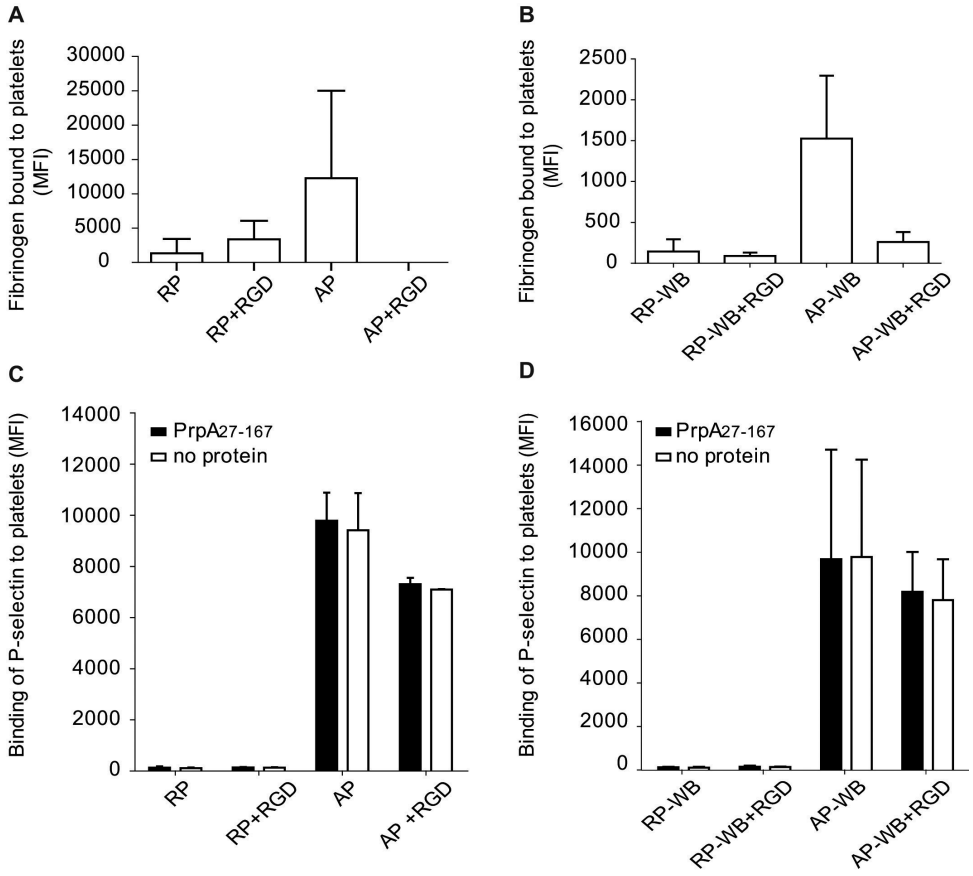
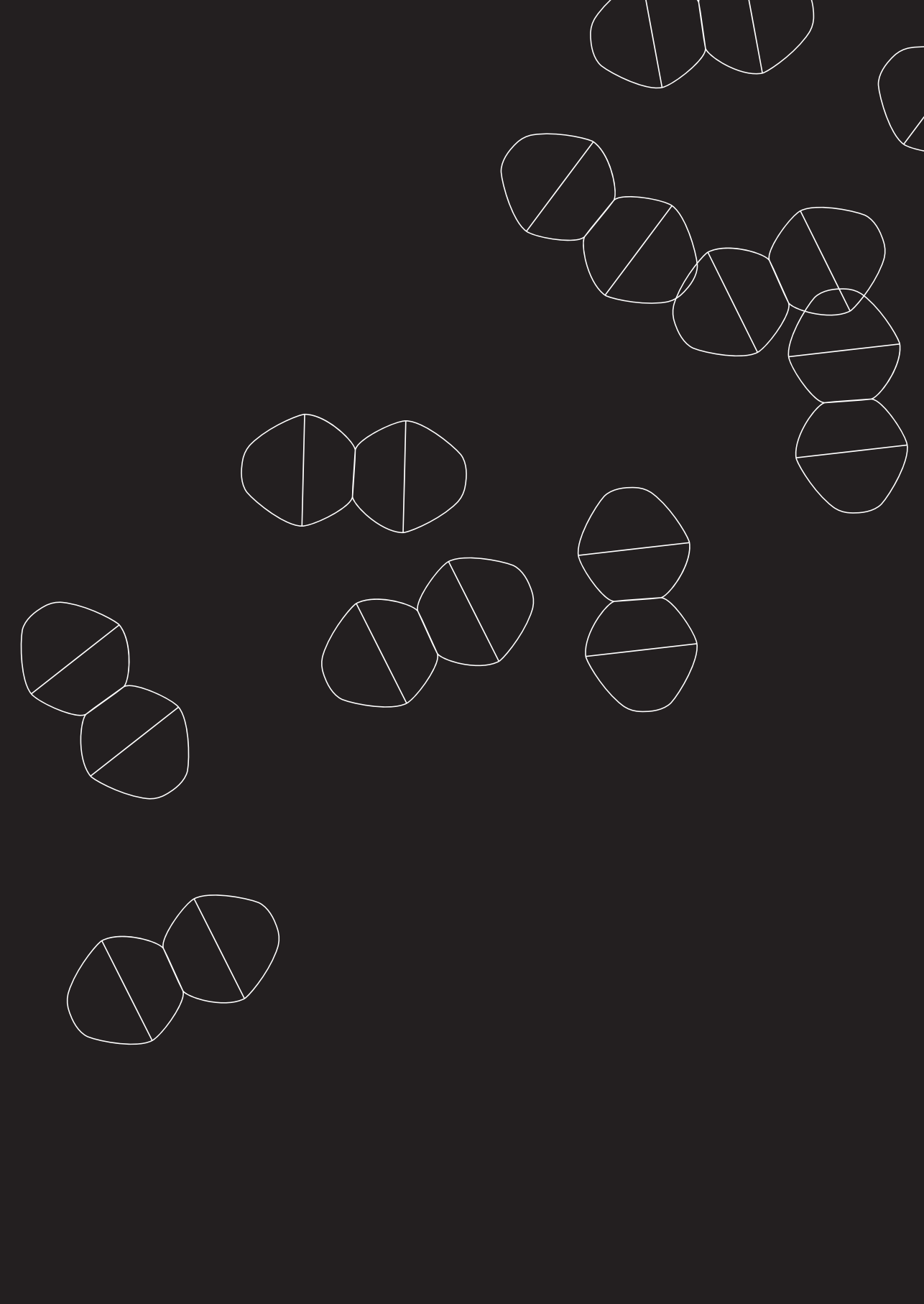
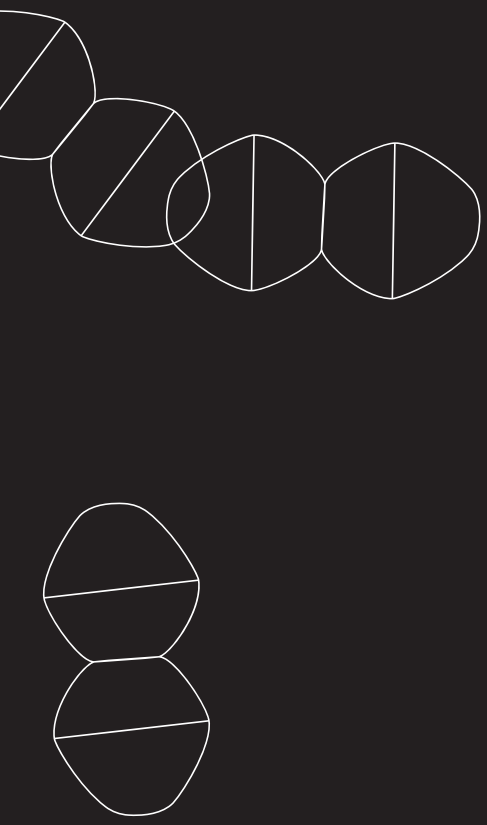


Fig S3. Fibrinogen levels and platelet activation in washed platelets and in whole blood. Panel A shows the levels of fibrinogen bound to resting (RP) and TRAP-activated (AP) washed platelets and to resting (RP-WB) and TRAP activated platelets (AP-WB) in whole blood in panel B. Fibrinogen bound to platelets was measured by flow cytometry in the absence of PrpA using an α-fibrinogen antibody labeled with FITC. D-arginyl-glycyl-L-aspartyl-L-tryptophane (RGD) was used to block the fibrinogen receptor (GPIIb-IIIa) in resting (RP+RGD) and activated (AP+RGD) washed platelets and in resting (RP-WB+RGD) and TRAP activated platelets (AP-WB+RGD) in whole blood. Activation of platelets is demonstrated by the binding of P-selectin to the surface of washed platelets (panel C) and to platelets in whole blood (panel D). P-selectin was detected using phycoerythrin-labelled mouse anti-human P-selectin antibodies. For all panels, the Mean fluorescence intensity (MFI) is shown. The results are data of three biological replicates.





3

The two component system ChtRS contributes to chlorhexidine tolerance in *Enterococcus faecium*

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Abstract

Enterococcus faecium is one of the primary causes of nosocomial infections. Dinsinfectants are commonly used to prevent infections with multidrug-resistant *E. faecium* in hospitals. Worryingly, *E. faecium* strains that exhibit tolerance to disinfectants have already been described. We aimed to identify and characterize *E. faecium* genes that contribute to tolerance to the disinfectant chlorhexidine (CHX).

We used a transposon mutant library, constructed in a multi-drug resistant *E. faecium* bloodstream isolate, to perform a genome-wide screen to identify genetic determinants involved in tolerance to CHX. We identified a putative two-component system (2CS), composed of a putative sensor histidine kinase (ChtS) and a cognate DNA-binding response regulator (ChtR), which contributed to CHX tolerance in *E. faecium*. Targeted deletion mutants in *chtR* and *chtS* exhibited compromised growth in the presence of CHX. Growth of the *chtR* and *chtS* mutants was also affected in the presence of the antibiotic bacitracin. The CHX- and bacitracin-tolerant phenotype of *E. faecium* E1162 was linked to a unique, non-synonymous single nucleotide polymorphism in *chtR*. Transmission electron microscopy showed that upon challenge with CHX, Δ*chtR* and Δ*chtS* failed to divide properly and formed long chains. Normal growth and cell morphology was restored when the mutations were complemented *in trans*. Morphological abnormalities were also observed upon exposure of Δ*chtR* and Δ*chtS* to bacitracin.

The tolerance to both chlorhexidine and bacitracin provided by ChtRS in *E. faecium* highlights the overlap between responses to disinfectants and antibiotics and the potential for the development of cross-tolerance for these classes of antimicrobials.

Introduction

Enterococcus faecium is a facultative anaerobic Gram-positive bacterium, which naturally colonizes the gastrointestinal tract of humans and animals. Since the 1990s *E. faecium* has also emerged as one of the leading causes of nosocomial infections (1, 2). The population of *E. faecium* is currently divided into clade A-1, containing most clinical isolates, clade A-2 with most animal-derived strains and clade B in which most isolates of healthy humans are clustered (3). Whether clade A-1 and clade A-2 are monophyletic and can be reliably distinguished from each other, has recently been questioned (4). Nosocomial *E. faecium* strains are frequently resistant to glycopeptides and β-lactam antibiotics (5, 6), complicating the treatment of clinical infections. Since the late 1990s, and despite the worldwide spread of vancomycin-resistant enterococci (VRE), only two antibiotics (daptomycin and linezolid) have been approved by the FDA for use against VRE. Other antibiotics (quinupristin-dalfopristin, tigecycline, oritavancin tedizolid, telavancin and dalbavancin) have been suggested as alternatives for treatment of infections caused by VRE in clinical practice. They have, however not been approved by the FDA for the treatment of VRE infections (7–9). The use of the polypeptide antibiotic bacitracin, in combination with other antibiotics, in the treatment of VRE infections has also been proposed (10).

While antibiotics are gradually losing their effectiveness against *E. faecium*, antiseptics and disinfectants are becoming increasingly important to prevent the spread of multi-drug resistant *E. faecium* in health care settings (11). Chlorhexidine (CHX) is a bisbiguanide agent and has diverse applications as disinfectant for surfaces and as an antiseptic for topical applications (12). The mode of action of CHX is poorly understood. CHX, which is positively charged at neutral pH, is thought to be attracted to the bacterial cell surface where it may electrostatically interact with negatively charged phospholipids. Depending on the concentration of CHX, it can reduce bacterial membrane fluidity or disrupt the structural integrity of the membrane, causing increased permeability and leakage of cell contents and, ultimately, cell death (13–15). In healthcare, CHX is often used in surgical scrubs for preoperative skin preparation, impregnated wash cloths for post-operative wound care, daily patient bathing and oral care of intubated patients (16, 17).{Jones, 1997 #126} Regular bathing of patients with CHX significantly reduces the colonization by VRE and other multi-resistant organisms in intensive care units and general medicine wards (18–23). Recently, increased tolerance to these compounds has been reported for Gram-positive cocci and this could contribute to future co- or cross selection for antibiotic resistance (24–30). In addition, sub-inhibitory concentrations of CHX induce the expression of genes involved in vancomycin and daptomycin resistance in enterococci (31).

In this study, we used Microarray-based Transposon Mapping (M-TraM (32)) to perform a genome-wide screening of a transposon mutant library, to identify

genes involved in the tolerance to CHX in *E. faecium*. Two genes that were identified in the M-TraM screening were predicted to encode a two-component regulatory system (2CS). 2CS are signal transduction systems in bacteria consisting of a sensor histidine kinase and its response regulator. They play important roles in the adaptation of bacteria to changes in the environment and have been implicated in orchestrating cellular responses that lead to increase of tolerance to antimicrobials in different gram-positive bacteria, including enterococci (33–35). The two genes encoding the 2CS were further characterized to define their role in tolerance to CHX.

Results

E. faecium strains from different phylogenetic backgrounds differ in their tolerance to CHX.

First, we assayed the tolerance to CHX of the *E. faecium* strain E1162, a multi-drug resistant bloodstream isolate, previously assigned to clade A-1 (3, 32), by measuring growth in MHB supplemented with different concentrations of CHX (Fig. S1). We found that at CHX concentrations of 1.7 μg ml^{-1} or higher, growth was essentially inhibited completely. In follow-up experiments, CHX was used at 1.2 μg ml^{-1}, as this concentration led to an extended lag phase and lower growth rate of *E. faecium* E1162. Next, we compared the ability of seven other *E. faecium* strains (two strains from clade A-1, two strains from clade A-2 and three strains from clade B (3)), to grow in the presence of CHX (Fig. 1). We found that the strains from clade A-1, all of which were isolated from bloodstream infections in hospitalized patients, were able to grow in medium containing 1.2 μg ml^{-1} CHX, while the strains from clade A-2 or clade B could not (Fig. 1). E1162 had the highest growth rate in the presence of the disinfectant and therefore it was chosen for the follow-up experiments into the mechanism of CHX tolerance in *E. faecium*.

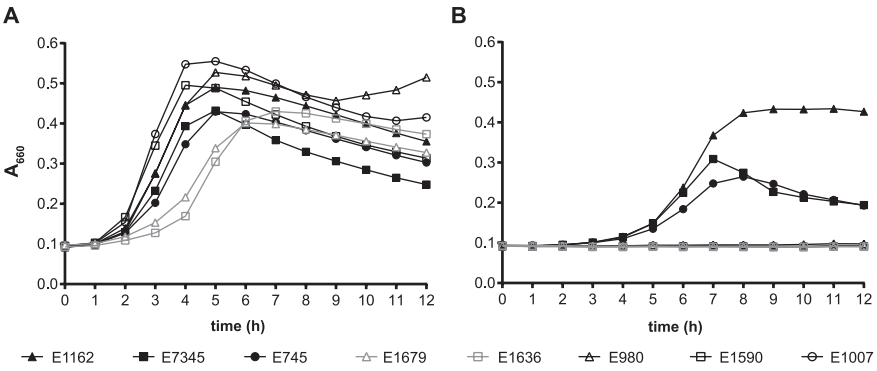

Fig. 1. Growth of *E. faecium* strains challenged with CHX. The growth curves for different *E. faecium* strains in MHB (panel A) and MHB in the presence of 1.2 μg ml^{-1} CHX (panel B). Clade A-1 strains are shown in filled black symbols, clade A-2 and clade B are shown in open grey and open black symbols, respectively. The growth curves represent the averages of three replicates.

Identification of a CHX tolerance locus in *E. faecium* E1162 by M-TraM.

We identified conditionally essential genes in the *E. faecium* E1162 transposon mutant library, during growth in the presence of 1.2 µg ml^{-1} CHX through a microarray-based transposon mapping approach (32). While the M-TraM analysis hinted at a functional contribution of several genes in CHX tolerance (Table 1), we focused on the gene with locus tag EfmE1162_2203, of which the transposon mutant was significantly affected (8.7-fold lower abundance in the CHX-exposed library compared to the untreated library) during growth in the presence of the disinfectant. EfmE1162_2203 is annotated as encoding a DNA binding response regulator. EfmE1162_2203 is located adjacent to a gene (Efm E1162_2202) encoding a histidine kinase of which transposon mutant abundance was moderately (3.0-fold) reduced upon exposure to CHX. The proteins encoded by EfmE1162_2202 and EfmE1162_2203 likely form a 2CS in *E. faecium* E1162. We have renamed EfmE1162_2203 and EfmE1162_2202 to *chtR* and *chtS*, for chlorhexidine tolerance response regulator and sensor histidine kinase, respectively.

Table 1: *E. faecium* genes implicated in tolerance to chlorhexidine by M-TraM analysis

LocusTag[a]	Gene Name	Annotation	Average fold change[b]
EfmE1162_2203	***chrR***	**DNA-binding response regulator**	**8.7**
EfmE1162_0264		permease protein, putative	4.6
EfmE1162_0996		hypothetical protein	4.2
EfmE1162_2026		lactose phosphotransferase system repressor	3.9
EfmE1162_0997		conserved hypothetical protein	3.9
EfmE1162_2510		holliday junction DNA helicase RuvA	3.8
EfmE1162_0300		PrgW	3.6
EfmE1162_2635		Esterase	3.6
EfmE1162_0431		conserved hypothetical protein	3.2
EfmE1162_0394		PrgO	3.2
EfmE1162_2202	***chrS***	**sensor histidine kinase**	**3.0**

[a] indicates the gene containing the transposon insertion
[b] indicates the fold-change of the gene as determined by a ratio of the library grown in the control condition to the CHX challenged growth condition. Results are averaged from four replicates.
*Bold type indicates the *chtRS* system targeted for further analysis

To determine the distribution of *chtR* and *chtS* among *E. faecium*, we assessed the presence of these two genes in all the strains tested for CHX tolerance and in 85 previously published genome sequences, which were previously assigned to clade A-1 (n = 41), clade A-2 (n = 31) and, clade B (n = 13) (3, 40). The analysis showed that the ChtRS 2CS is conserved in all analyzed *E. faecium* strains. By analyzing the nucleotide sequences of the *chtR* and *chtS* genes of the eight *E. faecium* strains tested for their tolerance to CHX (Fig. 1), a single non-synonymous nucleotide change, leading to an amino acid substitution (P102H) , was found in the ChtR protein of all CHX-tolerant clade A-1 strains, compared to the non-CHX-tolerant clade A-1 and B strains. This amino acid is located in a predicted dimerization interface located in the signal receiver domain of ChtR.

The ChtRS 2CS contributes to CHX and bacitracin tolerance.

To validate the M-TraM results, we constructed markerless deletion mutants in *chtR* and *chtS*, which were termed Δ*chtR* and Δ*chtS*. We also constructed two strains in which these mutations were complemented *in trans*, which were named Δ*chtR*+*chtR* and Δ*chtS*+*chtS*. No differences in growth were observed between E1162 and the two targeted mutants or the complemented strains when grown in MHB (Fig. 2A). In the presence of 1.2 μg ml^{-1} CHX, E1162 had a lag-phase of approximately 4 hours. Under the same conditions, the mutants Δ*chtR* and Δ*chtS*, had a lag-phase of almost 8 hours and exhibited slower exponential growth than E1162 (Fig. 2B). The complemented strains Δ*chtR*+*chtR* and Δ*chtS*+*chtS*, exhibited wild-type levels of growth in the presence of CHX. These results confirm that both *chtR* and *chtS* are involved in CHX tolerance of *E. faecium* E1162. When the *in trans* copy of *chtR* was mutated to engineer a proline at position 102 of ChtR, as is characteristic for ChtR in CHX-susceptible strains, it could no longer complement the growth defect caused by the *chtR* deletion. In addition, we decided to test the effect of the deletions in *chrR* and *chrS* on the tolerance of *E. faecium* E1162 to the antibiotic bacitracin, as a homologous 2CS (EF0926-EF0927) has previously been described to have a minor role in bacitracin tolerance in *E. faecalis* V583 (41). Upon exposure of Δ*chtR* and Δ*chtS* to 4 μg ml^{-1} bacitracin, the growth of both mutants is completely inhibited, while in the *in trans* complemented strains growth was restored to near wild-type levels (Fig. 2C). The *chtR* deletion mutant complemented with the *chtR** allele remained inhibited in its growth in the presence of bacitracin.

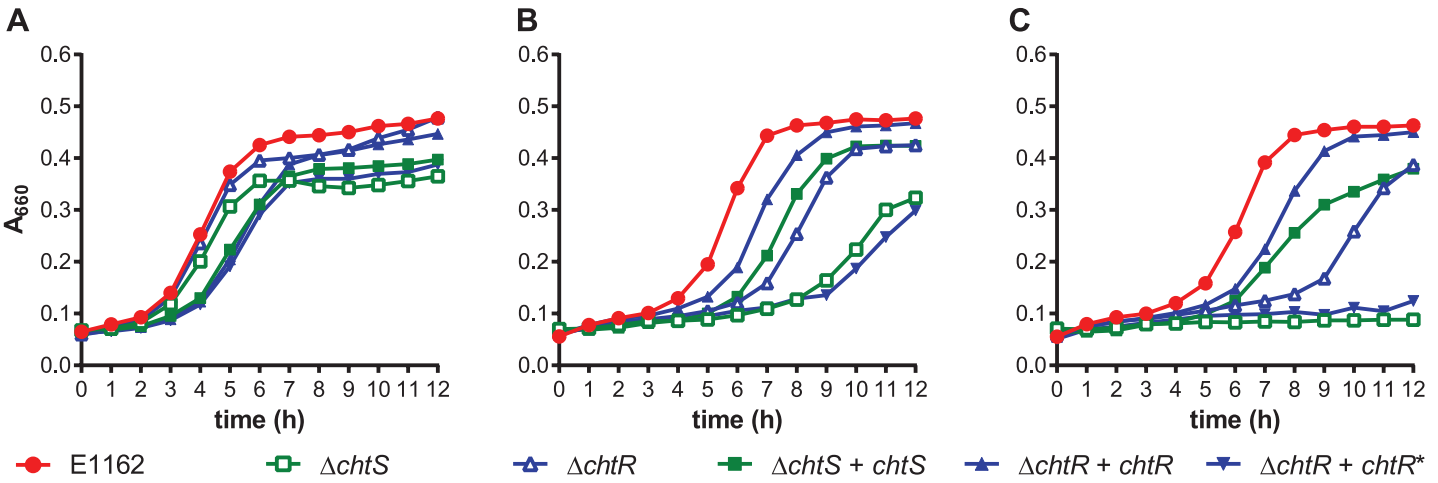

Fig 2. Effect of targeted mutations in *ΔchtR and ΔchtS* upon challenge with CHX and bacitracin. The growth curves shown in panel A correspond to strains growing in MH broth. Panels B and C correspond to the same strains growing in MH broth supplemented with 1.2 μg ml^{-1} CHX and 4 μg ml^{-1} bacitracin, respectively. Wilde type E1162 is shown in red, while the targeted deletions mutants in *chtS* and *chtR* are shown in green and blue open symbols, respectively, Closed green and blue symbols represent the growth curves of the *in trans* complemented strains (Δ*chtS*+*chtS*, Δ*chtR*+*chtR* and Δ*chtR*+*chtR**). The growth curves represent the averages of three experiments.

CHX and bacitracin challenge affects cellular morphology in Δ*chtR* and Δ*chtS* but not in *E. faecium* E1162.

In order to further characterize the effects of CHX and bacitracin on *E. faecium* E1162 and its *chtR and chtS* mutants, cells were analyzed by scanning electron microscopy (Fig. 3). In these experiments, bacitracin was added to the medium at 1 µg ml^{-1}, as this concentration is permissive for growth of the *chtR* and the *chtS* deletion mutants. No apparent changes in cellular morphology were found when E1162 was challenged with CHX or bacitracin, compared to growth in MHB. However, when Δ*chtS* and Δ*chtR* were challenged with CHX, the cells failed to divide properly and formed chains. Exposure of Δ*chtS* and Δ*chtR* to bacitracin, resulted in swollen cells with various cellular abnormalities. The chaining phenotype and the cellular abnormalities found in CHX and bacitracin-challenged Δ*chtS* and Δ*chtR*, respectively, were not observed in the *in trans* complemented strains Δ*chtR+chtR* and Δ*chtS+chtS*.

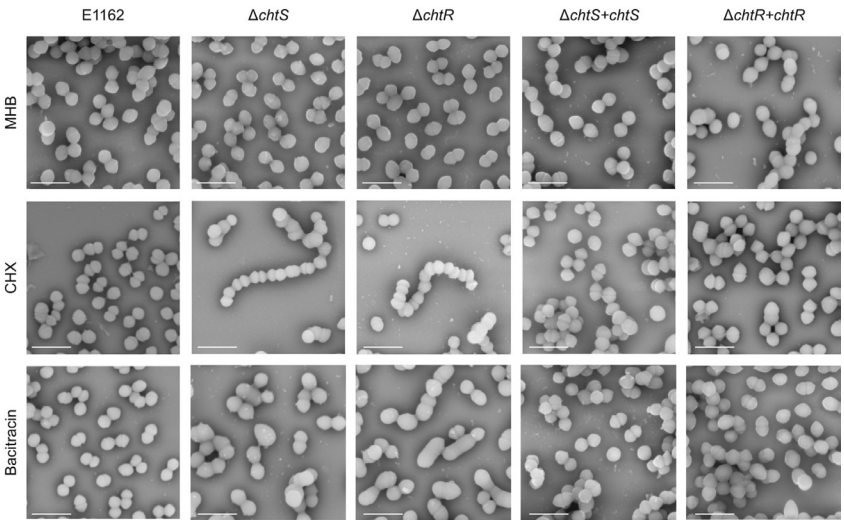

Fig 3. Cell morphology of *E. faecium* E1162, *ΔchtS, ΔchtR*, Δ*chtS+chtS*, and Δ*chtR+chtR* upon exposure to CHX and bacitracin. E1162, *ΔchtS, ΔchtR* and the complemented strains Δ*chtS+chtS* and Δ*chtR+chtR*, were grown to OD_{660} = 0.2. The cells were grown in Mueller-Hinton Broth (MHB), in MHB with 1.2 µg ml^{-1} chlorhexidine (CHX) or in MHB with 1 µg ml^{-1} bacitracin. Specimens were coated with 1 nm gold particles. Images were taken at a magnification of 35.000X. The scale bar corresponds to 2 µm.

Discussion

Enterococci have recently become important nosocomial pathogens (4). The ability of *E. faecium* to rapidly acquire drug resistance determinants threatens the treatment of infections caused by this organism. Antiseptics and disinfectants, including CHX, have been used for decades to prevent the spread of multidrug resistance pathogens, including enterococci, in health care settings (19). In the present study, we found that clinical multidrug-resistant *E. faecium* isolates, belonging to a distinct subpopulation of hospital-associated strains that are contained in clade A-1, were able to tolerate CHX, while *E. faecium* strains belonging to the other clades (A-2 and B), were more susceptible to CHX. The increased tolerance to CHX in clade A-1 strains, compared to strains from other *E. faecium* clades, may have been selected by the exposure to antiseptics and disinfectants, which are commonly used in healthcare settings. As clade A-1 strains appear to have specifically evolved to thrive in hospitalized patients, the increased tolerance to disinfectants may form an additional adaptation to this specific niche and could contribute to the success of these isolates as hospital acquired-opportunistic pathogens. Using the high-throughput M-TraM technique, we identified the 2CS ChtRS as being essential for CHX tolerance in the drug-resistant clinical isolate *E. faecium* E1162.

The *chtS* and *chtR* genes putatively encode a histidine kinase and a response regulator, together forming a 2CS. 2CSs regulate the expression of genes as a response to environmental cues (42–44). The signal is received at the extracellular sensor domain and its transduction occurs via ATP-depended phosphorylation. The phosphoryl group is transferred from the histidine phosphotransfer domain to the conserved signal receiver domain domain of the response regulator (45). The single amino acid substitution (P102H) that distinguishes ChtR in CHX-tolerant clade A-1 strains from ChtR in CHX-susceptible clade A-2 and clade B strains, is located in the predicted dimerization interface of the REC domain in ChtR. The activation and regulation of response regulators by dimerization through receiver domains, has previously been studied in other bacteria (46–48), including gram positives (49) and changes in the dimerization interface of the signal receiver domain of ChtR, could affect the function of the response regulator, thereby altering the control of gene expression upon CHX exposure by ChtRS. The inability of the construct with the mutated allele of *chtR* (encoding the ChtR protein with a proline residue at position 102) to complement a *chtR* deletion, suggests a crucial role for this SNP in the CHX-tolerant phenotype of clade A-1 strains. Mechanistic studies of the proteins encoded by the different *chtR* alleles, including their dimerization and phosphotransfer characteristics, may be the topic of future studies.

In the present study, E1162 mutants deficient in *chtRS* exhibited decreased tolerance to CHX and in addition, were also more susceptible to bacitracin. In other gram-positives, including *E. faecalis*, 2CSs also contribute to the protective

response against low concentrations of bacitracin (50–54). Bacitracin is an antibiotic that targets peptidoglycan biosynthesis by binding to undecaprenol pyrophosphate (UPP), blocking its recycling during peptidoglycan synthesis. This, in turn, interferes with the transport of new peptidoglycan building blocks, leading to disruption of cell wall synthesis (55). Since 2CSs exert their effect through the regulation of expression of effector genes, the observed loss of CHX and bacitracin tolerance in the *chtR* and *chtS* deletion mutants, is most likely due to altered expression of genes regulated by ChtSR. The effector genes regulated by ChtSR still remain to be elucidated.

Bacitracin can decrease colonization by vancomycin-resistant *E. faecium* in the gastrointestinal tract (56, 57) and may be used orally to control the dissemination of vancomycin-resistant enterococci (10). However, therapeutic failure of bacitracin to treat VRE colonization has also been reported (58, 59) and may be caused by intrinsic bacitractin resistance in enterococci, to which ChtRS contributes in *E. faecium*. *E. faecium* has a multitude of intrinsic and acquired resistance mechanisms that allow it to survive the selective pressures imposed by the nosocomial environment, including antibiotic therapy and the use of disinfectants. Further studies should be performed to characterize the mechanisms by which different antiseptics and disinfectants may cross- or co-select for clinically relevant antibiotics. Information resulting from this line of inquiry may be used to develop efficient disinfection protocols, while minimizing the risk of further resistance development in *E. faecium*.

Materials and methods

Bacterial strains and growth conditions.

The strains and plasmids used in this study are listed in Table S1. *Escherichia coli* was grown in Lysogeny Broth (LB) and *E. faecium* strains were grown in Muller-Hinton broth (MHB) at 37°C with shaking at 200 rpm, unless mentioned otherwise. When appropriate, antibiotics were added at the following concentrations: spectinomycin at 300 µg ml^{-1} for *E. faecium* and 100 µg ml^{-1} for *E. coli*, gentamicin at 300 µg ml^{-1} for *E. faecium* and 25 µg ml^{-1} for *E. coli*. CHX was used at 1.2 µg ml^{-1}, unless mentioned otherwise. Media were obtained from Oxoid (Basingstoke, United Kingdom). Antibiotics and disinfectants were obtained from Sigma Aldrich (Saint Louis, MO).

Microarray-based Transposon mapping (M-TraM) to identify genes involved in CHX tolerance.

The *mariner* transposon mutant library of *E. faecium* E1162 and the M-TraM method have been described previously (32). In brief, four overnight cultures of the E1162 mutant library were cultured at 37°C in MHB and then diluted to OD_{660} 0.025 in 20 ml of pre-warmed MHB supplemented with CHX and then grown at 37°C until mid-exponential phase (OD_{660} = 0.3). M-TraM was performed with four biological replicates following previously described procedures (32). The microarray data generated in the M-TraM experiment is deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4173. Statistical analysis of hybridization signals between the conditions was performed using Cyber-T (36). Genes were considered differentially expressed when all four probes showed a Bayesian P-value ‹0.001 and abundance of a gene was ≤0.2 or ≥5.0, compared to the untreated control (32).

Construction of markerless deletion mutant in *chtS* and *chtR* and *in trans* complementation.

Markerless deletion mutants where generated in *chtS* and *chtR* using previously described methods (32). Brain heart infusion broth or agar was used as growth medium for *E. faecium* during all genetic manipulations. In brief, the 5' and 3' flanking regions (approximately 500 bp) of each gene were PCR amplified with two sets of primers as follows: Up-chtS_2202-F-XhoI and Up-chtS_2202-R-EcoRI for the upstream fragment of *chtS*, and Down-chtS_2202-F-EcoRI and Down-chtS_2202-R-XmaI for the downstream fragment of *chtS*; Up-chtR_2203-F-XhoI and Up-chtR_2203-R-EcoRI for the upstream fragment of *chtR*, and Down-chtR_2203-F-EcoRI and Down-chtR_2203-R-XmaI for the downstream fragment of *chtR*. The primer sequences are listed in Table S2. The two flanking regions of each gene were then fused together by PCR and cloned into pWS3 (37). A gentamicin-resistance cassette flanked by lox66 and lox71 sites was amplified by PCR using the primers pAT392_EcoRI_lox66_genta_F and pAT392_EcoRI_lox71_genta_R and cloned into the EcoRI site that was generated between the 5' and 3' flanking regions of each gene in the pWS3 construct, as described previously (32). Finally, the two constructs, named pWJ1 and pWJ2, were electrotransformed individually into *E. faecium* E1162 and markerless deletions of *chtS* (termed Δ*chtS*) and *chtR* (termed Δ*chtR*), were generated as described before (32). For *in trans* complementation of Δ*chtS* and Δ*chtR*, the full-length genes and an upstream region of 356 and 390 nucleotides respectively, were amplified by PCR using Accuprime High Fidelity Taq Polymerase (Life Technologies, Bleiswijk, The Netherlands). The primers for these PCRs were named Comp2202_Fw_SacI and Comp2202_Rv_SmaI for the amplification of *chtS* and Comp2203_Fw_SacI and Comp2203_Rv_SmaI for the amplification of *chtR*. These primers introduce SacI and SmaI restriction sites

and after digestion with these enzymes, the resulting products were cloned into pEF25. The constructs were sequenced, to confirm the absence of mutations, and electrotransformed into Δ*chtS* and Δ*chtR* as described previously (32), generating the complemented strains Δ*chtS+chtS and* Δ*chtR+chtR*. To determine the role of a non-synonymous SNP in *chtR*, leading to a P102H amino acid substitution in ChtR in clade A-1 strains, we ordered the *chtR* gene of E1162 and its promoter as a gBlock (Integrated DNA Technologies; Leuven, Belgium) but made a specific base change leading to a proline residue at position 102 in the translated protein. The construct was otherwise identical (confirmed by sequencing) to the PCR product used to complement the Δ*chtR* mutant in E1162. SacI and SmaI sites at the end of the gBlock were used to clone the fragment into pEF25. The construct was then electrotransformed into Δ*chtR*, resulting in strain Δ*chtR+chtR**.

Growth inhibition assays.

The minimum inhibitory concentration (MIC) of CHX was determined by broth microdilution in MHB, according to standard methodologies (38). MICs of CHX were not more than one dilution step different from each other for all strains in this study (data not shown). For this reason, we focused on kinetic growth assays, which provide more quantitative information than the endpoint measurements used in MIC determinations. Growth curves were determined using a BioScreen C instrument (Oy Growth Curves AB, Helsinki, Finland). Cultures of *E. faecium* E1162, Δ*chtR*, Δ*chtS* and the *in trans* complemented strains, were inoculated in MHB, with appropriate antibiotics, and incubated overnight at 37ºC. Overnight cultures were then diluted to an OD_{660} of 0.0025 in 300 µl MHB and challenged with CHX or bacitracin. The cultures were incubated in the BioScreen C system at 37ºC with continuous shaking. The absorbance at 660 nm (A_{660}) was recorded every 15 minutes for 12 hours. Each experiment was performed in triplicate.

Scanning electron microscopy (SEM).

E1162, *ΔchtS and ΔchtR* were grown overnight in MHB. Subsequently, they were diluted to OD_{660} 0.0025 in MHB and MHB supplemented with 1.2 µg ml^{-1} CHX or 1 µg ml^{-1} bacitracin and further grown until OD_{660} = 0.2. Bacteria were immediately fixed with 1% glutaraldehyde (Sigma) onto poly-L-lysine coated glass slides and prepared for SEM, as previously described (39). In brief, the cells were serially dehydrated by consecutive incubations of 5 min in 25% ethanol-in phosphate buffered saline (PBS: 137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4; pH 7.4)

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Author contributions

A.M.G.P, R.J.L.W. and W.v.S. designed the study. A.M.G.P., J.W., J.C.B., M.R., E.C.B., E.M. and X.Z. performed experiments. J.R.B. contributed bioinformatic analyses. All authors contributed to data interpretation. The manuscript was written by A.M.G.P., M.J.M.B., R.J.L.W. and W.v.S.

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Supplementary information

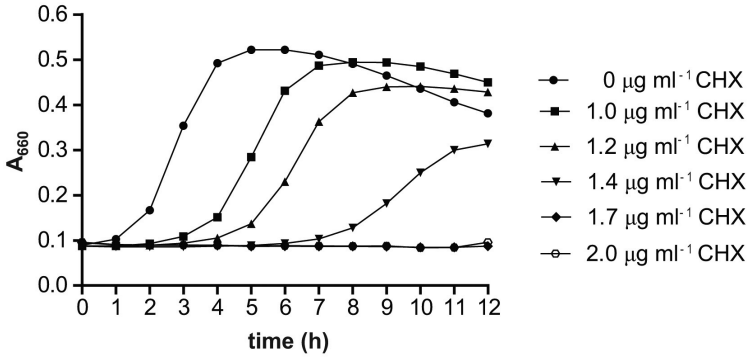

Fig S1. Growth of *E. faecium* E1162 in different concentrations of CHX. Growth curves of *E. faecium* E1162 challenged with different concentrations of CHX. The growth curves represent the averages of three replicates.

Table S1: Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)		Reference
E. faecium		Clade	
E1162	Clinical isolate (blood infection); Amp^r, Tet^r	A-1	61
E980	Isolated from feces of a non-hospitalized individual	B	61
E1590	Isolated from feces of a non-hospitalized individual	B	3
E7345	Clinical isolate (bloodstream infection) Aus0004; Van^r	A-1	60
E745	Clinical isolate (bloodstream infection) ; Van^r	A-1	unpublished
E1007	Isolate from feces of a non-hospitalized individual, identical to strain EnGen0015	B	61
E1679	Hospital outbreak (catheter tip)	A-2	61
E1636	Clinical isolate (bloodstream infection)	A-2	61
Δ*chtS*	Markerless deletion mutant of *chtS* in E1162		This study
Δ*chtR*	Markerless deletion mutant of *chtR* in E1162		This study
Δ*chtS+chtS*	*In trans* complementation strain of Δ*chtS*, Δ*chtS* harboring pEF25-*chtS*		This study
Δ*chtR+chtR*	*In trans* complementation strain of Δ*chtR*, Δ*chtR* harboring pEF25-*chtR*		This study
Δ*chtS*+pEF25	Markerless deletion mutant of *chtS* in E1162 carrying empty pEF25		This study
Δ*chtR*+pEF25	Markerless deletion mutant of *chtP* in E1162 carrying empty pEF25		This study
E. coli			
Ec1000	MC1000 *glgB*::repA		62
Plasmids			
pWS3	Gram-positive thermosensitive origin; Spc^r		41
pWJ1	pWS3 derivative containing fused 5'and 3'flanking regions of *chtS*		This study
pWJ2	pWS3 derivative containing fused 5'and 3'flanking regions of *chtR*		This study
pWS3-Cre	Derivative of pWS3 expressing *cre* recombinase in *E. faecium*		31
pEF25	pAT18 derivative carrying spectinomycin cassette		63
pEF25-*chtS*	Plasmid for the complementation of Δ*chtS*, pEF25 carrying *chtS*		This study
pEF25-*chtR*	Plasmid for the complementation of Δ*chtR*, pEF25 carrying *chtR*		This study

Amp: ampicillin; Tet: tetracycline; Van: vancomycin; Spc: spectinomycin.

Table S2: Oligonucleotides used in this study

Primer	Sequence
Up-chtS_2202-F-XhoI	CCGCTCGAGGGAAGAAAACGGCTCCCG
Up-chtS_2202-R- EcoRI	GCGCCAAACATCGAATTCGACGGCTGAATCAACTG
Down-chtS_2202-F- EcoRI	CCGTCGAATTCGATGTTTGGCGCCAGCCAC
Down-chtS_2202-R-XmaI	CCCCCCGGGCATTTCCAAATCGATGTACTG
Up-chtR_2203-F-XhoI	CCGCTCGAGGAAGCGGAAAGTGGCACTTTG
Up-chtR_2203-R- EcoRI	CAATTGATCGAATTCCAACATCAATCGGTTACGCCG
Down-chtR_2203-F- EcoRI	CGATTGATGTTGGAATTCGATCAATTGTCGAATGATCTCTTCG
Down-chtR_2203-R-XmaI	CCCCCCGGGATACATTAGCAATTAGAGAAAAGC
pAT392_EcoRI_lox66_genta_F	GAGGGAATTCTACCGTTCGTATAGCATACATTATACGAAGTTAT GATAAACCCAGCGAACCATTTGAGG
pAT392_EcoRI_lox71_genta_R	CTCCGAATTCTACCGTTCGTATAATGTATGCTATACGAAGTTATT CAATCTTTATAAGTCCTTTTATAA
Comp2202_Fw_SacI	CCGGAGCTCTCCAAGCTAAGCCGGATAAA
Comp2202_Rv_SmaI	TTGCCCGGGAATGCATTACTTCGCCGTTC
Comp2203_Fw_SacI	CCGGAGCTCTGGACCCATGAGTCAATATAATC
Comp2203_Rv_SmaI	TTGCCCGGGACCGTGAACACTCACCGATT

Restriction sites are underlined.

Supplemental references

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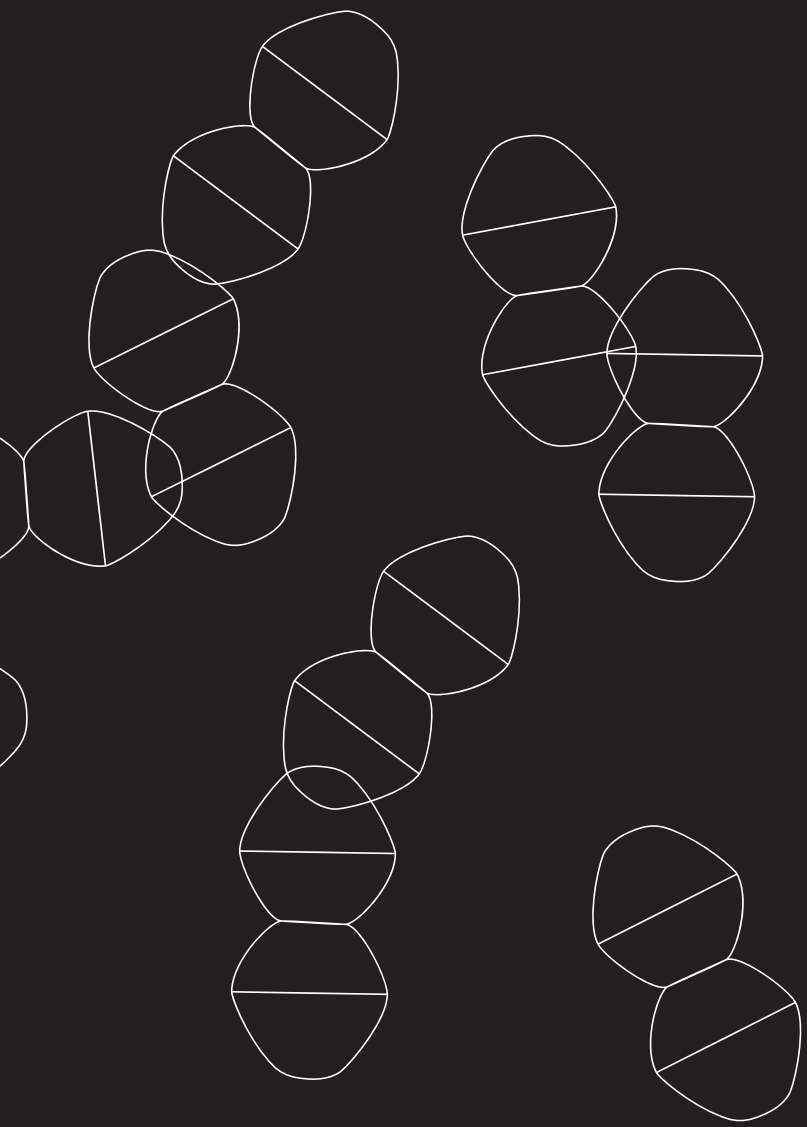

4

Fitness determinants of vancomycin-resistant *Enterococcus faecium* during growth in human serum

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Submitted for publication

Abstract

Enterococcus faecium is a ubiquitous gut commensal and a frequent cause of bloodstream infections in hospitalized patients. Here, we identify genes that contribute to growth of *E. faecium* in human serum. We sequenced the genome of *E. faecium* E745, a vancomycin-resistant clinical isolate, to completion and then compared its transcriptome during exponential growth in rich medium and in human serum by RNA-seq. This analysis revealed that 27.8% of genes on the *E. faecium* E745 genome were differentially expressed, with a purine biosynthesis gene cluster exhibiting particularly strong upregulated expression during growth in serum. High-throughput transposon sequencing (Tn-seq) was then used to identify conditionally essential genes in *E. faecium* E745 during growth in serum. Genes involved in nucleotide biosynthesis (including *pyrK_2, pyrF, purD, purH*) and a gene encoding a phosphotransferase system subunit (*manY_2*) were thus identified to be contributing to growth in human serum. Transposon mutants in *pyrK_2, pyrF, purD, purH* and *manY_2* were isolated from the library and their impaired growth in human serum was confirmed. In addition, the *pyrK_2* and *manY_2* mutants exhibited significantly attenuated virulence in a zebrafish model. We conclude that genes involved in carbohydrate and nucleotide metabolism of *E. faecium* are essential for growth in human serum and contribute to pathogenesis.

Introduction

Enterococci are commensals of the gastrointestinal tract of humans and animals, but some enterococcal species, particularly *E. faecium* and *E. faecalis,* are also common causes of hospital-acquired infections in immunocompromised patients (1). While *E. faecalis* has been recognized as an important nosocomial pathogen for over a century, *E. faecium* has emerged as a prominent cause of hospital-acquired infections over the last two decades (2). Since the 1980s, *E. faecium* acquired resistance to multiple antibiotics, including β-lactams, aminoglycosides and finally, to the glycopeptide vancomycin (3). Nosocomial infections are almost exclusively caused by a specific sub-population of *E. faecium,* termed clade A-1, which has emerged from a background of human commensal and animal *E. faecium* strains (4). Strains in clade A-1 carry genetic elements that are absent from animal or human commensal isolates and which contribute to gut colonization or pathogenicity (5–9).

E. faecium bloodstream infections frequently develop in patients undergoing immunosuppressive therapy, and can result from translocation of strains from the intestinal tract to the bloodstream (10). In addition, the use of intravenous catheters in hospitalized patients, is another risk factor for the introduction of *E. faecium* into the bloodstream (3, 11, 12). Currently, *E. faecium* causes approximately 40% of enterococcal bacteremias. Due to the accumulation of

antibiotic resistance determinants in clade A-1 strains, *E. faecium* infections are more difficult to treat than infections caused by *E. faecalis* or other enterococci (13–15). To cause bloodstream infections, *E. faecium* needs to be able to survive and multiply in blood, but the mechanisms by which it can do so, have not yet been studied. To thrive in the bloodstream, an opportunistic pathogen has to evade host immune mechanisms and to adjust its metabolism to an environment that is relatively poor in nutrients (16).

To identify genes that are conditionally essential in bacteria, high-throughput screening methods for transposon mutant libraries have been developed and optimized for many different bacterial species (17, 18). To perform high-throughput functional genomics in ampicillin-resistant, vancomycin-susceptible clinical *E. faecium* strains, we previously developed a microarray-based transposon mutagenesis screening method which was used to identify genes involved in the development of endocarditis (7), resistance to ampicillin (19), bile (20) and disinfectants (Guzmán Prieto *et al.*, submitted for publication). However, microarray-based methods for transposon mutant library screening are limited in their accuracy and can only be used in strains for which the microarray was designed. To address these limitations, several methods, including Tn-seq (21) and TraDIS (22), which are based on high-throughput sequencing of the junctions of the transposon insertion sites and genomic DNA, have been developed (23).

In this study, we set-up Tn-seq in the clinical *E. faecium* isolate E745 to identify genes that contribute to survival and growth in human serum. In addition, we determined the transcriptional response of *E. faecium* E745 in that same environment. Finally, we substantiated the role of several *E. faecium* genes that contribute to growth in serum and in virulence, in a high-throughput zebrafish infection model. Collectively, our findings show that metabolic adaptations are key to *E. faecium* growth in serum and contribute to virulence.

Results

The complete genome sequence of *E. faecium* E745

In this study, we implemented RNA-seq and Tn-seq analyses in *E. faecium* strain E745, an ampicillin- and vancomycin-resistant clinical isolate that was isolated from faeces of a hospitalized patient during an outbreak of VRE in the nephrology ward of a Dutch hospital in 2000 (24, 25). To allow the application of RNA-seq and Tn-seq in *E. faecium* E745, we first determined the complete genome sequence of this strain through a combination of short-read Illumina sequencing and long-read sequencing on the RSII Pacific Biosciences and Oxford NanoPore's MinION systems. This resulted in a circular chromosomal sequence of 2,765,010 nt and 6 plasmids, with sizes ranging between 9.3 kbp and 223.7 kbp (Supplementary table 1). Together, the chromosome and plasmids have 3,095 predicted coding sequences.

Transcriptome of *E. faecium* E745 during growth in rich medium and in human serum

The transcriptional profile of E745 was determined using RNA-seq during growth in rich medium (BHI) and in heat-inactivated human serum. A total of 99.9 million (15.6 - 17.6 million per sample) 100 bp paired-end reads were successfully aligned to the genome, allowing the quantification of rare transcripts (Fig. 1). A total of 3217 transcription units were identified, including 651 predicted multi-gene operons, of which the largest contains 22 genes (Fig. 1A and Supplementary table 2).

A comparative analysis of E745 during growth in BHI and in human serum, showed that 860 genes exhibited significantly (q<0.001 and a fold change in expression of >2 or <0.5 between cultures grown in BHI versus heat-inactivated serum) different expression between these conditions (Supplementary table 3). The large number (27.8% of genes on the *E. faecium* E745 genome) of differentially expressed genes, indicates that growth in human serum leads to a dramatic reprogramming of global *E. faecium* gene expression, involving both chromosomal and plasmid-located genes. Among the genes with the highest difference in expression between growth in serum and in rich medium, we identified a gene cluster with a role in purine biosynthesis (Fig. 1B). In addition, we found a 58.4 kbp prophage-like gene cluster that exhibited higher expression in E745 during growth in serum (Fig. 1C).

To confirm the RNA-seq analysis, we independently determined expression levels of eight genes during growth in serum versus growth in BHI by qPCR (Supplementary Fig. 1). RNA-seq and qPCR data were highly concordant ($r^2 = 0.98$).

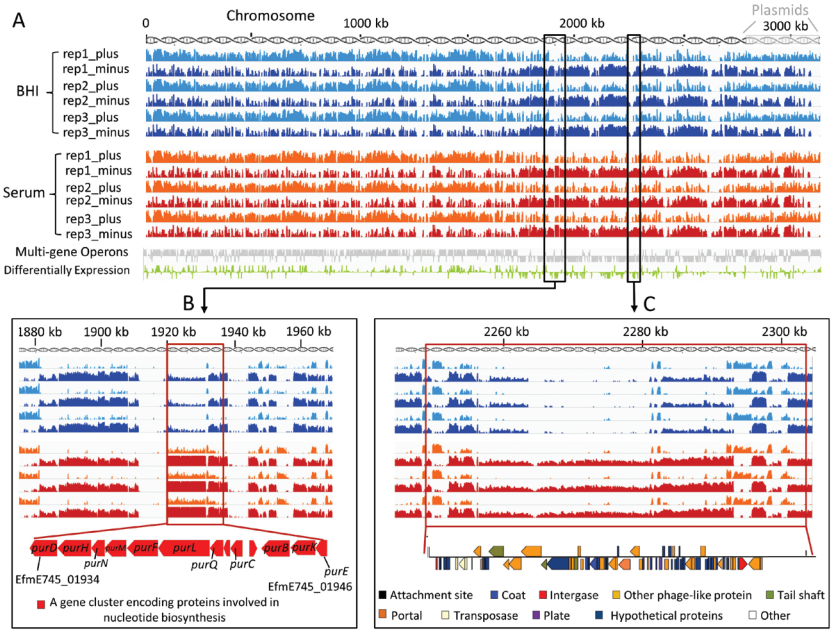

Fig. 1: Coverage plots of RNA-seq data aligning to chromosome and plasmid DNA. The *y* axis of each track indicates reads coverage and is represented on a log scale, ranging from 0 to 10000. The *x* axis represents the genomic location. Light blue (BHI) or orange (serum) tracks correspond to sequencing reads aligned to the plus strand of the replicon, and dark blue (BHI) or dark red (serum) tracks correspond to sequencing reads aligned to the minus strand of the replicon. The grey track corresponds to multi-gene operons. The green track corresponds to differentially expressed genes (BHI vs serum), with the height of the green bars indicative of differential expression. In panels B and C, two serum-induced regions are shown, i.e. a gene cluster involved in nucleotide biosynthesis (panel B) and a prophage (panel C). The RNA-seq experiments were performed using three biological replicates.

Construction and analysis of a transposon mutant library in *E. faecium* E745

A *mariner*-based transposon mutant library was generated in *E. faecium* E745 and Tn-seq (21) was performed on ten replicate transposon mutant libraries, that were grown overnight in BHI at 37°C, resulting in an average of 15 million Tn-seq reads for each library. To analyze the Tn-seq data, we divided the E745 genome in 25-nt windows. Of a total of 110,601 windows, 49,984 (45%) 25-nt windows contained one or more sequence reads. No positional bias was observed in the transposon insertion sites in the chromosome and plasmids of *E. faecium* E745 (Supplementary Fig. 2A). The genome-wide coverage of this transposon mutant

library allowed the identification of genes that are conditionally essential in *E. faecium*. A total of 455 chromosomal genes were determined to be essential for growth of *E. faecium* E745 in BHI (Supplementary Table 4). An example of a gene identified as essential is shown in Supplementary Fig. 2B. Among the genes that were essential for growth of *E. faecium* E745 in BHI, 87% were present in all genome sequences of a set of 74 previously sequenced *E. faecium* strains (4) that represented the genetic and ecological diversity of the species. An additional 7% of the essential genes were present in 73 out of 74 *E. faecium* genomes (data not shown). The conserved presence of these essential genes among diverse *E. faecium* strains is in line with their crucial role in *E. faecium* viability. The *E. faecium* E745 transposon mutant library was then used to identify genes that were specifically required for growth of *E. faecium* in serum.

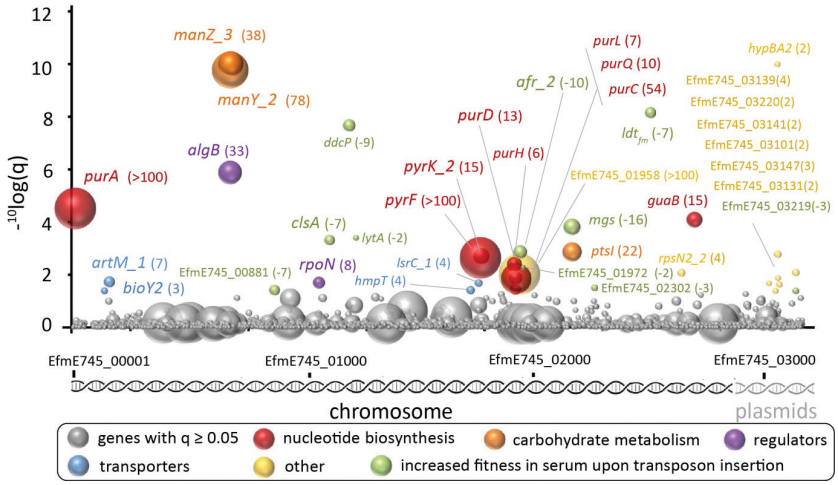

Fig. 2. Tn-seq analysis for *E. feacium* genes required for growth in human serum. Bubbles represent genes, and bubble size corresponds to the fold-changes (for visual reasons, a 100-fold change in transposon mutant abundance is set as a maximum) derived from the read-count ratio of libraries grown in BHI to libraries grown in human serum. On the *x* axis genes are shown in order of their genomic location and the chromosome and plasmids are indicated. The outcome of statistical analysis of the Tn-seq data is indicated on the *y* axis. Genes with a significant change ($q < 0.05$) in fitness in serum versus BHI are grouped by function and are labelled with different colors, and the name or locus tag and the change in abundance between the control condition and growth in serum is indicated next to the bubbles. Negative values indicate that mutants in these genes outgrow other mutants in serum, suggesting that these mutants, compared to the wild-type E745, have a higher fitness in serum.

E. faecium E745 genes required for growth in human serum

In order to identify genes that contribute to growth of *E. faecium* E745 in human serum, we performed Tn-seq on cultures of the *E. faecium* E745 transposon mutant libraries upon growth in rich medium (BHI) and in human serum. The human serum was either used natively or heat-treated. The serum showed effective complement activity in both classical and alternative pathways, as determined by hemolytic assays (40), while this activity was abolished by heat-inactivation (data not shown). Minor differences were observed among conditionally essential genes between the experiments performed in native human serum or heat-inactivated serum (Supplementary Table 5) and the following results correspond to the experiments obtained with heat-inactivated serum. This condition was chosen because it may be a more reproducible *in vitro* environment, particularly since the interaction between the complement system and Gram-positive bacteria remains to be fully elucidated (41, 42).

We identified 37 genes that significantly contributed to growth of E745 in human serum (Fig. 2 and Supplementary table 6): twenty-nine genes were located on the chromosome and eight genes were present on plasmids (six genes on pE745-5, two genes on plasmid pE745-6). The relatively large number of genes identified indicates that growth of *E. faecium* in human serum is a multi-factorial process. The genes that conferred the most pronounced effect on growth of *E. faecium* in serum included genes that are part of a phosphotransferase systems (PTS) involved in carbohydrate uptake (*manZ_3, manY_2*, *ptsL*), a putative transcriptional regulator (*algB*) and genes involved in the biosynthesis of purine and pyrimidine nucleotides (*guaB*, *purA*, *pyrF*, *pyrK_2*, *purD*, *purH*, *purL*, *purQ*, *purC*) (Fig. 2). Notably, the *purD, purH,* and *purL* genes were found to exhibit higher expression upon growth in human serum in the RNA-seq analysis (Fig. 1). Nine genes were identified as negatively contributing to growth in serum, i.e. the transposon mutants in these genes were significantly enriched upon growth in serum. The effects of these mutations were relatively limited, compared to the major effects observed in the transposon mutants discussed above (Supplementary Table 6), but it is notable that five (*clsA_1*, *ddcP, ldt$_{fm}$, mgs*, and *lytA_2*) of these genes have roles in cell wall and cytoplasmic membrane biosynthesis.

E. faecium genes involved in nucleotide biosynthesis or carbohydrate metabolism contribute to growth in human serum

We developed a PCR-based method (Supplementary Fig. 3) to selectively isolate five transposon mutants (in the purine metabolism genes *purD* and *purH*, the pyrimidine metabolism genes *pyrF* and *pyrK_2* and the PTS gene *manY_2*) from the transposon library. Growth in rich medium of these transposon insertion mutants was equal to the parental strain. However, all mutants were significantly impaired in their growth in human serum (Fig. 3A), confirming the results of the Tn-seq experiments.

E. faecium pyrK_2 and *manY_2* contribute to intravenous infection of zebrafish

Next, we investigated whether the transposon insertion mutants in the *manY_2* and *pyrK_2* genes were attenuated *in vivo* (Fig. 3B). The mutants in these genes were selected because they represent the mutants in nucleotide and carbohydrate metabolism genes that were previously shown to contribute to the growth of *E. faecium* in human serum. As a model for intravenous infection, we used a recently described model in which *E. faecium* was injected into the circulation of zebrafish embryos to mimic systemic infections (43). We showed that both the *manY_2* and the *pyrK_2* mutant were significantly less virulent than the parental strain. The overall survival at 92 hours post infection (hpi) of zebrafish embryos infected with WT strain was 53%, compared to 88% and 83% respectively, for zebrafish embryos that were infected with the transposon insertion mutants in *manY_2* and *pyrK_2*.

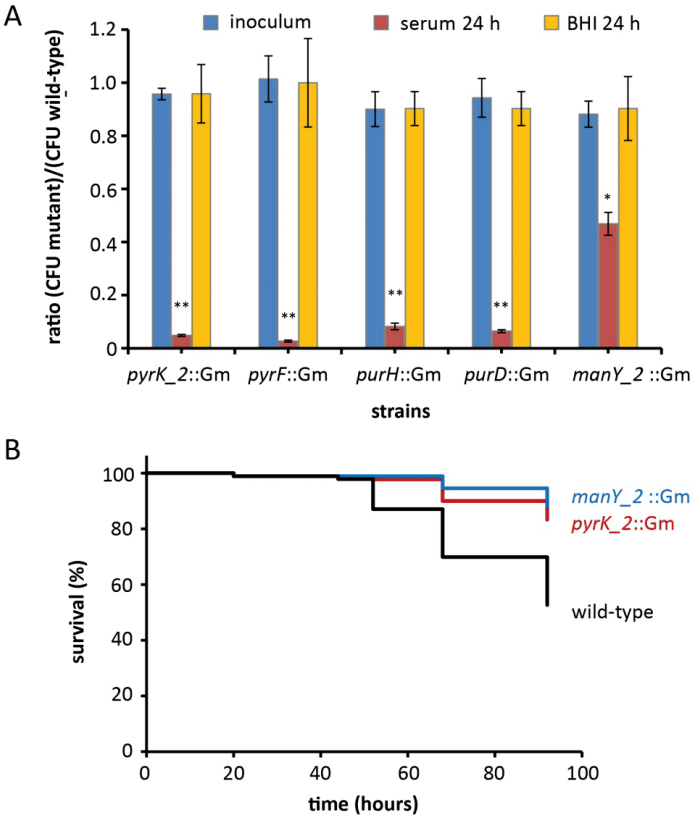

Fig. 3. *E. faecium* transposon mutants with a growth defect in human serum and an attenuated phenotype in a zebrafish model. (A) Ratios of the viable counts of five mutants compared to wild-type *E. faecium* before (blue bars) and after 24 h of growth in human serum (red bars) or BHI (yellow bars). The viable counts of wild-type *E. faecium* E745 were (3.52 ± 0.07) x 10^5/ml in the inocula, (2.92 ± 0.14) x 10^8/ml after 24 h of growth in serum and (1.20 ± 0.20) x 10^9/ml after 24 h of growth in BHI, respectively. Error bars represent the standard deviation of the mean of three independent experiments. Asterisks represent significant difference (*: $p < 10^{-3}$,**: $p < 10^{-5}$ as determined by a two-tailed Student's *t*-test) between the mutant strains and wild-type. (B) Kaplan-Meier survival curves of zebrafish embryos upon infection with *E. faecium*. The virulence of *E. faecium* mutants upon intravenous infection of zebrafish embryos was determined upon injection with 1.2x10^4 cfu of the *manY_2*::Gm and *pyrK*::Gm transposon mutants and wild-type *E. faecium* E745. The experiment was performed three times and the mutants were significantly different ($p<0.01$) from the wild-type in each experiment as determined by the Log-rank (Mantel-Cox) test with Bonferroni correction for multiple comparisons. This figure represents the combined results of the three replicates for *E. faecium* E745 ($n = 93$ zebrafish embryos), *manY_2*::Gm ($n = 92$) and *pyrK*::Gm ($n = 90$).

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The vancomycin-resistant *E. faecium* strain E745 was used throughout this study. This strain was isolated from feces of a hospitalized patient, during a VRE outbreak in a Dutch hospital (24, 25). Unless otherwise mentioned, *E. faecium* was grown in brain heart infusion broth (BHI; Oxoid) at 37°C. The *E. coli* strains DH5*α* (Invitrogen) was grown in Luria-Bertani (LB) medium. When necessary, antibiotics were used at the following concentrations: chloramphenicol 4 μg ml^{-1} for *E. faecium* and 10 μg ml^{-1} for *E. coli*, and gentamicin 300 μg ml^{-1} for *E. faecium* and 25 μg ml^{-1} for *E. coli*. All antibiotics were obtained from Sigma-Aldrich. Growth was determined by measuring the optical density at 660 nm (OD_{660}).

Genome sequencing, assembly and bioinformatic analysis

E. faecium E745 was sequenced using a combination of Illumina HiSeq 100 bp paired-end sequencing, long-read sequencing using the Pacific Biosciences RS II SMRT technology and the MinION system with R7 flowcell chemistry (Oxford Nanopore Technologies). Corrected PacBio reads were assembled using the Celera assembler (version 8.1) (26) and assembled contigs were then corrected by aligning Illumina reads using BWA (version 0.7.9a), with default parameters for index creation and BWA-MEM algorithm with *-M* option for the alignment (27). This approach resulted in 15 contigs, including one contig covering the entire 2.77 Mbp chromosome. After discarding contigs with low-coverage, the remaining contigs constituted 5 circular plasmid sequences and 5 non-overlapping contigs. These 5 contigs were aligned against the NCBI Genbank database and all were found to be part of the *E. faecium* plasmid pMG1 (28). Based on this alignment the presumed order of contigs was determined and confirmed by gap-spanning PCRs and sequencing of the products. A single gap between two contigs, could not be closed by PCR. Thus, we assembled Illumina reads together with MinION 2D reads using the SPAdes assembler (version 3.0) (29), which produced a contig that closed the gap, resulting in a complete assembly of this plasmid. Sequence coverage of chromosomal and plasmid sequences was determined with SAMtools (version 0.1.18) using short read alignments to the assembly, which were generated using BWA (version 0.7.9a). SAMtools was also used to identify possible base-calling and assembly errors, by aligning short reads to assembled contigs. A base was corrected using the consensus of aligned reads (30). The corrected sequences were annotated using Prokka (version 1.10) (31). The annotated genome of *E. faecium* E745 is available from NCBI Genbank database under accession numbers CP014529 – CP014535.

Transcriptome profiling

Approximately 3 x 10^7 cfu of *E. faecium* E745 were inoculated into 14 ml of BHI broth and heat-inactivated serum, and grown at 37°C until log phase. Cultures were centrifuged at room temperature (15 s; 21.380 *g*), and pellets were flash frozen in liquid N_2 prior to RNA extraction, which was performed as described previously (19). The ScriptSeq Complete Kit (Bacteria) (Epicentre Biotechnologies, WI) was used for rRNA removal and strand-specific library construction. Briefly, rRNA was removed from 2.5 µg of total RNA. To generate strand specific RNA-seq data, approximately 100 ng of rRNA-depleted RNA was fragmented and reverse transcribed using random primers containing a 5' tagging sequence, followed by 3' end tagging with a terminal-tagging oligo to yield di-tagged, single-stranded cDNA. Following magnetic-bead based purification, the di-tagged cDNA was amplified by PCR (15 cycles) using ScriptSeq Index PCR Primers (Epicentre Biotechnologies, WI). Amplified RNA-seq libraries were purified using AMPure XP System (Beckman Coulter) and sequenced by a 100 bp paired end reads sequencing run using the Illumina HiSeq 2500 platform (University of Edinburgh, United Kingdom). Data analysis was performed using Rockhopper (32) using the default settings for strand specific analysis.

Confirmation of RNA-seq data by quantitative real-time RT-PCR (qRT-PCR)

Total RNA isolated as described previously was used to confirm the transcriptome analysis by qRT-PCR. cDNA was synthesized as described above and qRT-PCR on these cDNAs was performed using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Breda, The Netherlands) and a StepOnePlus instrument (Life Technologies). The expression of *tufA* was used as a housekeeping control. Ct values were calculated using the StepOne analysis software v2.2. Transcript levels, relative to *tufA,* of the assayed genes were calculated using REST 2009 V2.0.13 (Qiagen, Venlo, The Netherlands). This experiment was performed with three biological replicates.

Generation of *mariner* transposon mutant library in *E. faecium*

To create a transposon mutant library in *E. faecium* E745 suitable for Tn-seq, the *mariner* transposon cassette (carrying a gentamicin resistance gene) in the transposon delivery plasmid pZXL5 (19) was adapted as follows. The transposon from pZXL5 was amplified by PCR using the set of primers: pZXL5_MmeI_SacII_Fw and pZXL5_MmeI_SacII_Rv (primer sequences listed in Supplementary table 7). These primers introduced MmeI restriction sites in the inverted repeats on both

sides of the transposon. The modified transposon delivery vector, termed pGPA1, was generated by the digestion of pZXL5 with SacII, followed by the insertion of the SacII-digested *mariner* transposon that contained MmeI restriction sites at its extreme ends. pGPA1 was electroporated into *E. faecium* E745 and the transposon mutant library was generated by selecting for gentamicin-resistant transposon mutants as described previously (19).

Tn-seq analysis of conditionally essential genes in *E. faecium* E745

The transposon mutant library created in E745 was prepared for Tn-seq analysis, similar to previously described procedures (33). To identify genes that are essential for the viability of *E. faecium* in BHI, we used ten experimental replicates of the mutant library. Aliquots (20 µl) of the transposon mutant library, containing approximately 10^7 cfu, were used to inoculate 20 ml BHI broth and grown overnight at 37°C. Subsequently, 1 ml aliquots of the cultures were spun down (15 s, 21.380 *g*) and used for the extraction of genomic DNA (Wizard genomic DNA purification kit, Promega Benelux). 2 µg of the extracted DNA was digested for 4 hr at 37°C using 10U MmeI (New England Biolabs) and immediately dephosphorylated with 1U of calf intestine alkaline phosphatase (Invitrogen) during 30 min at 50°C. DNA was isolated using phenol-chloroform extraction and subsequently precipitated using ethanol. The DNA pellets were then dissolved in 20 µl water. The samples were barcoded and prepared for Tn-seq sequencing as described previously (33). The sequence reads from all ten experimental replicates were mapped to the genome, and the mapped read-counts were then tallied for the analysis of the essentiality of the genes in the *E. faecium* E745 genome (further described below).

To identify genes that are required for growth in human serum, 20 µl aliquots of the frozen mutant library in E745 were inoculated in BHI broth and grown overnight as described above. Subsequently, bacterial cells were washed with physiological saline solution. Approximately $3x10^7$ cfu were inoculated into 14 ml BHI broth, and approximately $3x10^6$ cfu were inoculated into 14 ml human serum obtained from Sigma (Cat. No. H4522; Sterile filtered type-AB human serum) or heat-inactivated human serum (the same, after incubation for 30 min at 56°C). The different inoculum-sized were used in order for a similar number of divisions to occur during the experiment. Cells were incubated at 37°C for 24 hours without shaking and then further processed for Tn-seq (33). This experiment was performed in triplicate.

Tn-seq samples were sequenced (50 nt, single-end) on one lane of a Illumina Hiseq 2500 (Baseclear, Leiden, the Netherlands and Sequencing facility University Medical Center, Utrecht, The Netherlands), generating an average of 15 million high quality reads per sample.

Tn-seq data analysis

Raw Illumina sequence reads from Illumina sequencing were split, based on their barcode, using the Galaxy platform (34), and 16-nucleotide fragments of each read that corresponded to E745 sequences, were mapped to the E745 genome using Bowtie 2 (35). The results of the alignment were sorted and counted by IGV (36) using a 25-nucleotide window size and then summed over the gene. Read mapping to the final 10% of a gene were discarded as these insertions may not inactivate gene function. Read counts per gene were then normalized to the total number of reads that mapped to the genome in each replicate, by calculating the normalized read-count RPKM (Reads Per Kilobases per Million input reads) via the following formula: RPKM = (number of reads mapped to a gene x 10^6) / (total mapped input reads in the sample x gene length in kbp). Statistical analysis of the RPKM-values between the experimental conditions was performed using Cyber-T (37). Genes were determined to be significantly contributing to growth in human serum when the Benjamini-Hochberg corrected *P*-value was ‹0.05 and the difference in abundance of the transposon mutant during growth in BHI and serum was ›2. To determine the essentiality of *E. faecium* genes during growth in the rich medium BHI, the normalized read-counts of the ten replicates in BHI were used as data input for the EL-ARTIST analysis, as described in the user manual of the ARTIST pipeline (38).

Isolation of mutants from the transposon mutant library pool

To recover a targeted transposon mutant from the complete mutant pool, a PCR-based screening strategy was developed (Supplementary figure 3). 40 µl of the transposon mutant library was inoculated into 40 ml of BHI broth with gentamicin and grown overnight at 37ºC with shaking (200 rpm). The overnight culture, containing approximately 10^9 cfu/ml, was then diluted to approximately 20 cfu/ml in 500 ml of BHI with gentamicin and kept on ice. Subsequently, 200 µl aliquots were transferred to wells of sterile 96 wells plates (n = 12, Corning Inc.). After overnight incubation at 37ºC without shaking, aliquots (15 µl) of each one of the 96 wells, were further pooled into a single new 96 well plate, as described in Supplementary figure 3.

PCRs were performed on the final plate in which the transposon mutants were pooled, to check for the presence of the Tn-mutants of interest, using the primer ftp_tn_both_ends_MmeI, which is complementary to the repeats flanking the transposon sequence, in combination with a gene-specific primer (Supplementary table 7). When a PCR was found to be positive in one of the wells of this plate, the location of the Tn-mutant was tracked backwards to the wells containing approximately 4 independent transposon mutants, by performing PCRs mapping

the presence of the transposon mutant in each step. Cells from the final positive well were plated onto BHI with gentamicin and colony PCR was performed to identify the desired transposon mutant.

Growth of *E. faecium* E745 and individual mutants in human serum

Wild-type E745 and the mutant strains were grown overnight at 37°C in BHI broth. Subsequently, bacterial cells were washed with physiological saline and approximately 3 x 10^5 cfu were inoculated into 1.4 ml BHI broth or heat-inactivated serum. Cells were grown in 1.5 ml tubes (Eppendorf) in triplicate for each condition and incubated at 37°C for 24 hours without shaking. Bacterial growth was determined by assessing viable counts, for which the cultures were serially diluted using physiological saline solution and plated onto BHI agar followed by overnight incubation at 37°C.

Intravenous infection of zebrafish embryos

London wild-type (LWT) inbred zebrafish embryos, provided by the aquarium staff of The Bateson Center (University of Sheffield), were used for infection experiments. The parental E745 strain and its *pyrK_2* and *manY_2* transposon mutants were grown in BHI broth until they reached an optical density at 600 nm of approximately 0.5 and were then harvested by centrifugation (5,500 *g*, 10 min). Bacteria were microinjected into the circulation of dechorionated zebrafish embryos at 30 hours post fertilization, as previously described **(39)**. Briefly, anesthetized embryos were embedded in 3% (w/v) methylcellulose and injected individually with approximately 1.2 x 10^4 cfu using microcapillary pipettes. For each strain, 29 to 32 infected embryos were observed for survival up to 90 hours post infection (hpi). This experiment was performed in triplicate.

Data availability

Sequence reads generated in this study have been made available at the European Nucleotide Archive under accession number PRJEB19025.

Discussion

E. faecium can contaminate the skin and from there colonize indwelling devices such as intravenous catheters, or it can translocate from the gastrointestinal tract in immunosuppressed patients, leading to the development of bacteremia and endocarditis. *E. faecium* infections are often difficult to treat, due to the multi-drug resistant character of the strains causing nosocomial infections (3, 4). However, the bloodstream poses challenges for the proliferation and survival of *E. faecium*, including a scarcity of nutrients.

In the present study we sequenced the complete genome of a vancomycin-resistant *E. faecium* strain, and identified *E. faecium* genes that were essential for growth in human serum. A total of 37 genes were found to be required for fitness of *E. faecium* E745 in serum, among which genes with roles in carbohydrate uptake and nucleotide biosynthesis. Previously, fitness determinants for growth in human serum have been identified through large-scale screening of mutant libraries in both a Gram-negative (*Escherichia coli*) and a Gram-positive (*Streptococcus pyogenes*) pathogen (44, 45). Notably, these studies have also identified the ability for *de novo* synthesis of purines and pyrimidines as a crucial factor for growth in serum. In addition, in diverse bacteria (including *Burkholderia cepacia, Pasteurella multocida*, *Acinetobacter baumannii*, *Salmonella enterica* serovar Typhimurium, *Bacillus anthracis*, and *Streptococcus pneumoniae*), the ability to synthesize nucleotides contributes importantly to virulence (46–51). The data presented here indicate that *de novo* biosynthesis of nucleotides is also required for *E. faecium* growth in serum and virulence. The nucleotide biosynthesis pathway of *E. faecium* may be a promising target for the development of novel antimicrobials for the treatment of *E. faecium* bloodstream infections. Indeed, compounds that target guanine riboswitches, thereby inhibiting nucleotide biosynthesis, have already shown their efficacy in a *Staphylococcus aureus* infection model (52).

Three genes (*ptsL*, *manY_2* and *manZ_3*) encoding subunits of PTSs were found to contribute to growth in serum in our Tn-seq experiments. Previously, PTSs have been associated with gut colonization (5) and endocarditis (7) in another clinical *E. faecium* strain. The PTSs identified in this study are different from these previously characterized systems, suggesting that the remarkably large number of genes encoding for PTSs in a typical *E. faecium* genome (4) provide metabolic flexibility for growth in a wide variety of environments.

It is notable that among the nine genes that exhibited increased fitness upon inactivation by transposon insertion, five genes are predicted to have a role in cell wall or cytoplasmic membrane biosynthesis. The protein encoded by *ddcP* was previously characterized as a low-molecular-weight penicillin binding protein with D-alanyl-D-alanine carboxypeptidase activity (19), while ldt_{fm} acts as a peptidoglycan L,D transpeptidase (53). The predicted α-monoglucosyldiacylglycerol synthase gene *mgs* is orthologous (73% amino acid

identity) to *bgsB* in *E. faecalis*, which is required for the biosynthesis of membrane glycolipids (54). The *clsA_1* gene is predicted to be responsible for the synthesis of cardiolipin (bisphosphatidylglycerol) and its inactivation may modulate the physical properties of the cytoplasmic membrane (55). Finally, *lytA_2* is predicted to encode an autolysin, which may be involved in the turnover of peptidoglycan in the cell wall (56). The transposon mutants in these genes were not further characterized in this study, but our findings suggest that genes involved in cell wall or cytoplasmic membrane remodeling may confer subtle fitness defects to *E. faecium* when growing in serum.

Our RNA-seq-based transcriptional profiling of *E. faecium* E745 during mid-exponential growth in serum showed pervasive changes in gene expression compared to exponential growth in rich medium. The purine metabolism genes *purL*, *purH*, *purD,* which were found to be required for growth in serum in our Tn-seq experiments, were among those that were significantly upregulated during growth in serum compared to growth in rich medium. Notably, a single prophage was expressed at higher levels during growth in serum than in rich medium. The abundance of prophage elements in the genome of *E. faecium* has been noted before (4, 57, 58). Interestingly, in the related bacterium *Enterococcus faecalis* prophages encode platelet-binding proteins (59) and may have a role in intestinal colonization (60). The contribution of *E. faecium* prophages to traits that are important for colonization and infection may provide important insights into the success of *E. faecium* as a nosocomial pathogen.

Collectively our data indicates that nucleotide biosynthesis and carbohydrate metabolism are critical metabolic pathways for the proliferation and survival of *E. faecium* in the bloodstream. The proteins encoded by the genes required for growth in human serum that were identified in this study, could serve as candidates for the development of novel anti-infectives for the treatment of bloodstream-infections by multi-drug resistant *E. faecium*.

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Author contributions

X.Z, R.J.L.W. and W.v.S. designed the study. X.Z., A.M.G.P., T.K.P., M.B., J.R.B., M.R. and S.M. performed experiments. All authors contributed to data interpretation. The manuscript was written by Z.X., A.M.G.P., M.J.M.B., R.J.L.W. and W.v.S.

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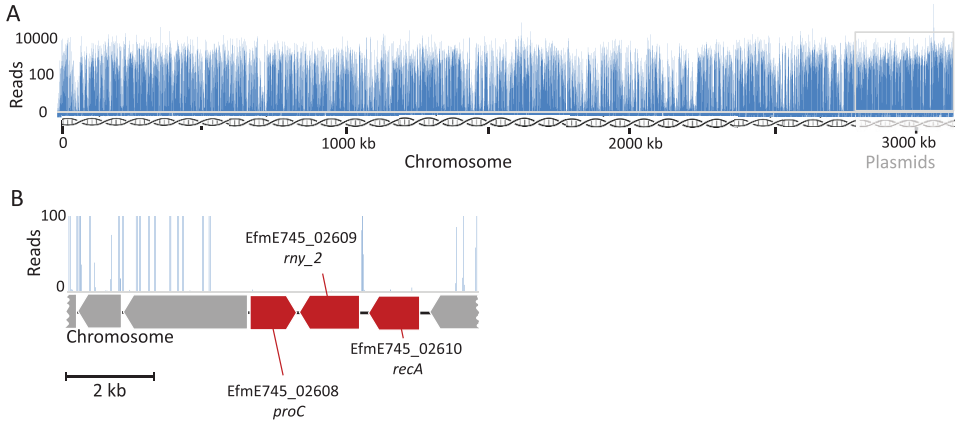
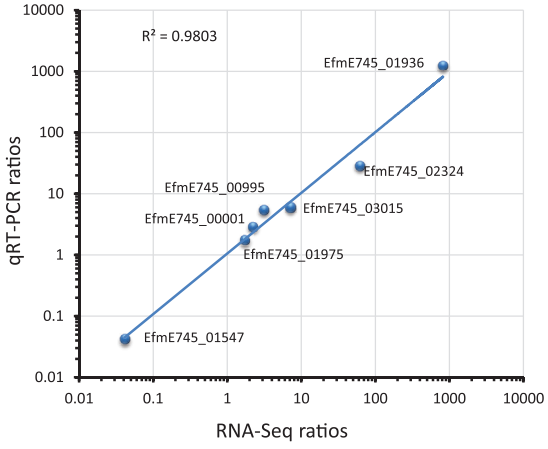
Supplementary information

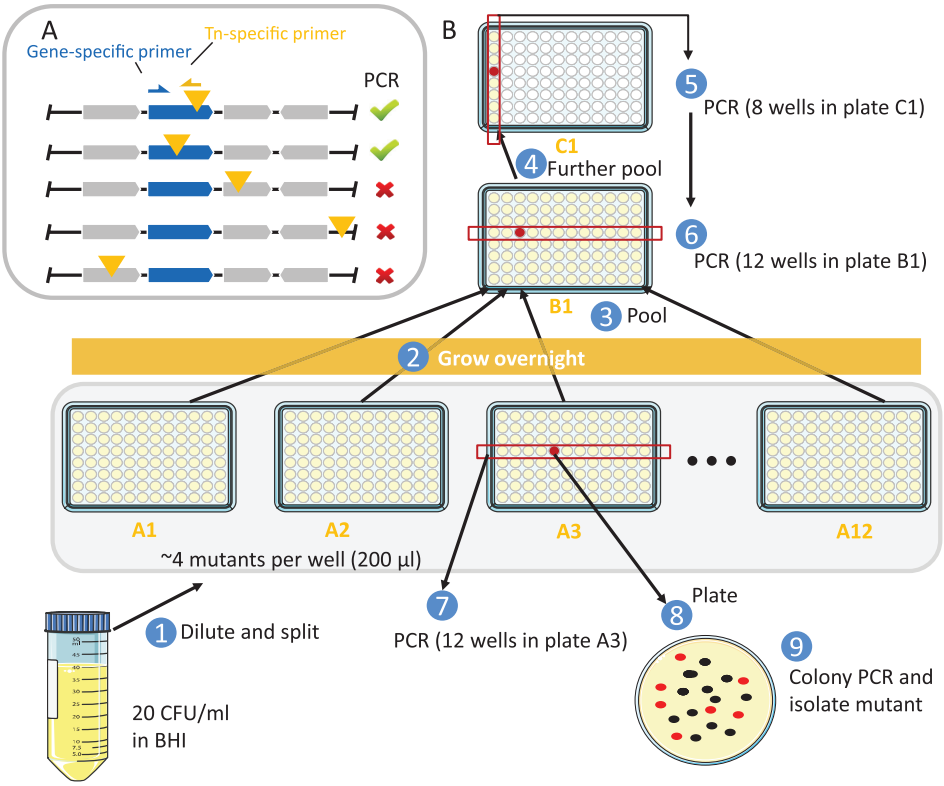
Supplementary tables not found in this chapter, can be found at:
http://biorxiv.org/content/early/2017/01/21/101329.figures-only



Supplementary Fig. 1. Characterization of the *E. faecium* E745 transposon mutant library. Panel A shows reads that were mapped to the *E. faecium* E745 chromosome and plasmids. The height of each peak represents the read abundance at a specific insertion site. An example of gene that was identified as essential on the *E. faecium* E745 chromosome is shown in panel B. In the y-axis, the number of mapped reads are shown in log scale.



Supplementary Fig. 2. qRT-PCR confirms the results of RNA-Seq experiments. Correlation of RNA-Seq and qRT-PCR expression ratios for the seven genes with various expression levels and genomic locations. The gene expression ratios obtained from both qRT-PCR and RNA-Seq were normalized by a housekeeping control gene Efm745_00056 (*tufA*). The experiments was performed with three biological replicates.



Supplementary Fig. 3. Isolation of mutants from the transposon mutant library pool. (A) Schematic representation of the PCR reaction designed to find a particular Tn-mutant within the transposon mutant library. This PCR uses a combination of a gene-specific primer (blue arrow) and a transposon specific primer primer (yellow arrow). Positive PCR products, indicated by the green check marks, should occur when the transposon (depicted as a yellow triangle) is inserted in the gene of interest (depicted in blue). If the transposons inserted in adjacent genes or intergenic regions, no PCR product can be amplified (red crosses). (B) Schematic workflow to isolate Tn-mutants from the mutant library. The transposon mutant library is split into 12 plates (A1 - A12) of 96 wells, with each 200 µl well containing an average of 4 mutants. Plates were incubated overnight (Step 2). Plate A1 was then pooled into the first column of a new 96 well plate, denominated plate B1 (Step 3) and the same was done for plates A2 to A12. Subsequently, plate B1 was pooled again into the first column of a third plate, denominated C1 (Step 4). PCR using the gene-specific primer and the transposon specific primer was performed on the 8 wells of plate C1 (Step 5). A positive PCR was suggestive of the presence of a particular transposon-mutant (depicted as a red dot). The presence of the transposon mutant was then confirmed by PCR in plate B1 (step 6) and the corresponding plate A (step 7). Once a transposon-mutant was located to a particular well in plate A, the well was plated on BHI plates containing gentamicin, and the colonies were screened for the presence of the transposon mutant by PCR (step 8).

Supplementary Table 1: Genome sequencing information of *E. faecium* E745

Plasmid name	Size (bp)	Sequence coverage*	Accession numbers
Chromosome	2.765.010	85,74	CP014529
pE745-1	223.688	99,69	CP014530
pE745-2	32.423	340,23	CP014531
pE745-3	9.310	396,93	CP014532
pE745-4	17.254	81,08	CP014533
pE745-5	55.167	166,16	CP014534
pE745-6	65.558	176,04	CP014535

* Sequence coverage of chromosomal and plasmid sequences was determined with SAMtools (version 0.1.18) using short read alignments to the assembly, which were generated using BWA (version 0.7.9a).

Supplementary Table 7. Oligonucleotides used in this study.

Primer	Sequence (5' – 3')
pZXL5_MmeI_SacII_Fw	TCCCCGCGGTAACAGGTTGGATGATAAGTCCCCGGTCT
pZXL5_MmeI_SacII_Rv	AGACCGGGGACTTATCATCCAACCTGTTACCGCGGGGA
ftp_tn_both_ends_MmeI	CGGGGACTTATCATCCAACC
ftp_tn_pyrF	TGCAGACAGTGGATGATGTTCT
ftp_tn_pyrK	AAGATCGGGGAAATGCTTCT
ftp_tn_purH	TCAACAGGAGGAACAAAGCA
ftp_tn_purD	TACTGGCAAACGAACGTCAG
ftp_tn_ptsE	GGCATGTACATTGATCGGTTT
qPCR_Ctrl_tuf_F	TACACGCCACTACGCTCAC
qPCR_Ctrl_tuf_R	AGCTCCGTCCATTTGAGCAG
qPCR_EfmE745_02324_F	AAGTTGGGCAAGGAAAGAAA
qPCR_EfmE745_02324_R	AGCTTCTCTCGTCGTCCTGT
qPCR_EfmE745_00001_F	CAATCCTGCAGATCCAAGAA
qPCR_EfmE745_00001_R	ACGTGCCAAATACATAGCGA
qPCR_EfmE745_00995_F	GCGAATCAATTGAAAGCAGA
qPCR_EfmE745_00995_R	TTTCGAACGATGAACTGTCC
qPCR_EfmE745_01975_F	TCTTTGACAACTCCAGGGAA
qPCR_EfmE745_01975_R	AAACGGCTTTCCAAATGATG
qPCR_EfmE745_03015_F	GTACACATGCGGACTTGGAT
qPCR_EfmE745_03015_R	TTAGCTTCCTCTTCTATTTGATCG
qPCR_EfmE745_01936_F	ATCATTGGACCGGTATTGCT
qPCR_EfmE745_01936_R	TTACTCCAGCTTCAAAGGCA
qPCR_EfmE745_01547_F	ACGAAAGATCCAATCGAAGC
qPCR_EfmE745_01547_R	TTTCAACTTCTGGCAGCAAC

Supplementary Table 5. Tn-seq data: comparison of heat-inactivated and native serum

Synonym	Chromosome/ Plasmid	Name	Product	fold change HSerum/Serum Tn-seq[a]	*q* HSerum/ Serum Tn-seq[b]
EfmE745_03040	Plasmid	-	hypothetical protein	17,16	0,01
EfmE745_03130	Plasmid	-	hypothetical protein	10,79	0,01
EfmE745_01216	Chromosome	-	hypothetical protein	4,13	0,01
EfmE745_02575	Chromosome	-	hypothetical protein	-1,88	0,01
EfmE745_02308	Chromosome	-	hypothetical protein	-4,73	0,01
EfmE745_03071	Plasmid	-	hypothetical protein	-Inf	0,02
EfmE745_01776	Chromosome	lsrC_1	Autoinducer 2 import system permease protein LsrC	3,82	0,02
EfmE745_01187	Chromosome	ddl_2	D-alanine--D-alanine ligase	3,00	0,02
EfmE745_02714	Chromosome	rpmF_2	50S ribosomal protein L32	-2,23	0,02
EfmE745_03183	Plasmid	-	hypothetical protein	13,84	0,03
EfmE745_01658	Chromosome	-	Helix-turn-helix domain protein	7,86	0,03
EfmE745_00241	Chromosome	-	hypothetical protein	3,32	0,03
EfmE745_03156	Plasmid	-	hypothetical protein	2,38	0,03
EfmE745_02693	Chromosome	-	hypothetical protein	-21,46	0,04
EfmE745_02525	Chromosome	-	putative ABC transporter ATP-binding protein	-3,14	0,04
EfmE745_01900	Chromosome	-	hypothetical protein	48,17	0,04
EfmE745_01676	Chromosome	-	hypothetical protein	4,76	0,04
EfmE745_01402	Chromosome	-	hypothetical protein	3,48	0,04
EfmE745_02057	Chromosome	fur	Ferric uptake regulation protein	2,74	0,04
EfmE745_00371	Chromosome	-	Transcriptional regulator PadR-like family protein	2,58	0,05
EfmE745_00731	Chromosome	licA_1	Lichenan-specific phosphotransferase enzyme IIA component	17,97	0,05

[a] Relative abundance of transposon mutants in the indicated genes in heat-inactivated serum (HSerum) versus native serum. A negative sign indicates that transposon mutants were less abundant upon growth in HSerum than in native serum.

[b] Benjamini-Hochberg corrected P-values (*q*) of transposon insertions in the indicated genes upon growth in growth in heat-inactivated serum (HSerum) versus native serum.

[c] RPKM (Reads Per Kilobase per Million mapped reads) for each individual experiment for the indicated gene.

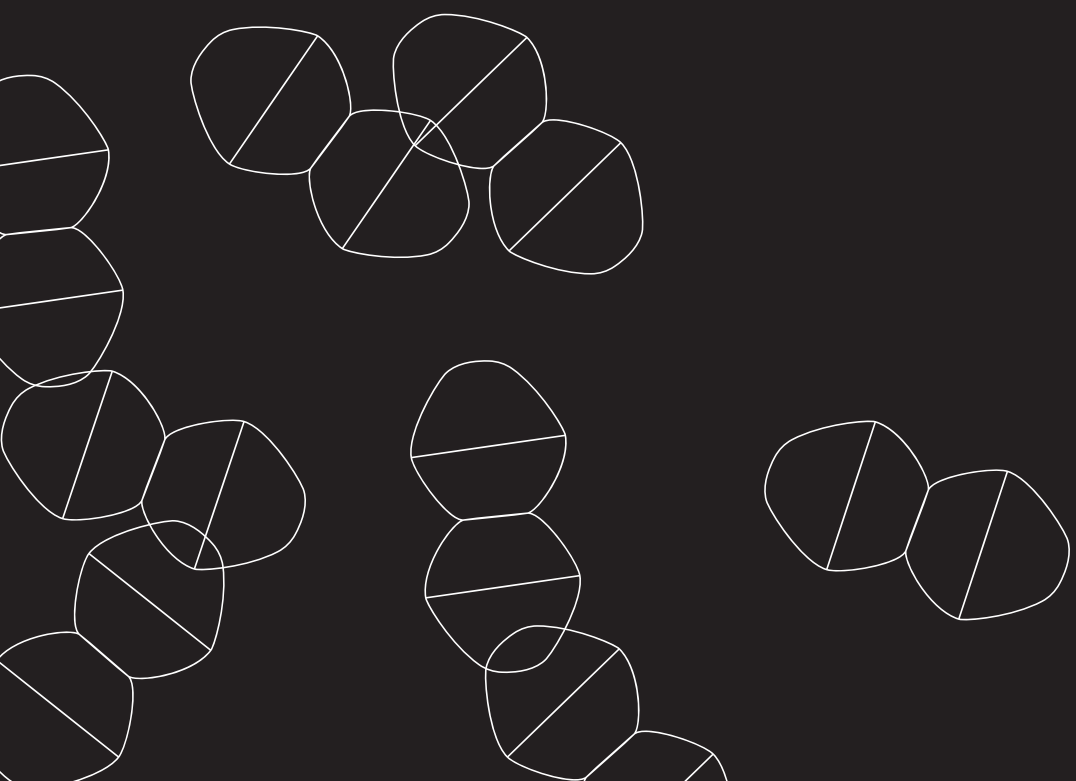
RPKM HSerum1 Tn-seq[c]	RPKM HSerum2 Tn-seq[c]	RPKM HSerum3 Tn-seq[c]	RPKM Serum1 Tn-seq[c]	RPKM Serum2 Tn-seq[c]	RPKM Serum3 Tn-seq[c]
45,59	0,00	0,00	253,97	264,20	264,20
116,73	0,00	58,41	444,34	723,14	723,14
790,00	779,53	586,28	1750,60	3579,55	3579,55
11706,58	12917,45	12937,59	6546,70	6698,41	6698,41
1879,32	1939,09	2595,19	794,46	280,73	280,73
437,17	283,84	221,11	0,00	0,00	0,00
105,26	144,84	338,94	718,31	765,26	765,26
686,80	439,87	574,12	1933,12	1583,49	1583,49
8632,23	10819,93	9901,04	6415,06	3375,12	3375,12
0,00	0,00	78,35	171,47	456,45	456,45
1916,22	424,30	2513,27	2193,52	17972,95	17972,95
127,30	225,97	299,48	506,01	830,43	830,43
4166,22	2569,00	2244,37	5776,92	7795,57	7795,57
135,37	373,52	406,82	42,67	0,00	0,00
1434,32	1171,47	1700,74	639,21	365,34	365,34
0,00	0,00	13,78	63,95	300,02	300,02
91,66	0,00	180,30	356,65	468,70	468,70
246,23	100,96	261,09	376,88	870,34	870,34
551,86	612,65	1067,71	2448,41	1833,32	1833,32
2658,69	2569,52	2724,08	4025,18	8264,57	8264,57
92,20	0,00	113,83	94,61	1803,49	1803,49

Supplementary Table 6. E745 genes that significantly (q<0.05 and fold-change <-2 or >2) contribute to growth in human serum, as identified by Tn-seq.

Synonym	Chromosome/Plasmid	Name
EfmE745_00013	Chromosome	purA
EfmE745_01785	Chromosome	pyrF
EfmE745_01958	Chromosome	-
EfmE745_00690	Chromosome	manY_2
EfmE745_01942	Chromosome	purC
EfmE745_00691	Chromosome	manZ_3
EfmE745_00687	Chromosome	algB
EfmE745_02204	Chromosome	ptsI
EfmE745_01788	Chromosome	pyrK_2
EfmE745_02782	Chromosome	guaB
EfmE745_01934	Chromosome	purD
EfmE745_01940	Chromosome	purQ
EfmE745_01082	Chromosome	rpoN1
EfmE745_00161	Chromosome	artM_1
EfmE745_01939	Chromosome	purL
EfmE745_01935	Chromosome	purH
EfmE745_01741	Chromosome	hmpT
EfmE745_02715	Chromosome	rpsN2_2
EfmE745_01776	Chromosome	lsrC_1
EfmE745_03220	Plasmid	-
EfmE745_03139	Plasmid	-
EfmE745_00139	Chromosome	bioY2
EfmE745_03147	Plasmid	-
EfmE745_03131	Plasmid	-
EfmE745_03137	Plasmid	hypBA2
EfmE745_03101	Plasmid	-
EfmE745_03141	Plasmid	-
EfmE745_01243	Chromosome	lytA_2
EfmE745_03219	Plasmid	-
EfmE745_02302	Chromosome	-
EfmE745_01129	Chromosome	clsA_1
EfmE745_00881	Chromosome	-
EfmE745_02575	Chromosome	ldt_{fm}
EfmE745_01213	Chromosome	ddcP
EfmE745_01959	Chromosome	afr_2
EfmE745_02202	Chromosome	mgs

[a] Relative abundance of transposon mutants in the indicated genes in BHI versus heat-inactivated serum. A negative sign indicates that transposon mutants were more abundant upon growth in heat-inactivated serum than in BHI.
[b] Benjamini-Hochberg corrected P-values (*q*) of transposon insertions in the indicated genes upon growth in BHI versus growth in heat-inactivated serum.

Product	fold change BHI/ HSerum Tn-seq[a]	*q* BHI/ HSerum_Tn-seq[b]
Adenylosuccinate synthetase	›100	3,04E-05
Orotidine 5'-phosphate decarboxylase	›100	2,27E-03
hypothetical protein	›100	9,63E-03
Mannose permease IIC component	77,76	1,71E-10
Phosphoribosylaminoimidazole-succinocarboxamide synthase	54,05	0,01
Mannose permease IID component	37,70	9,16E-11
Alginate biosynthesis transcriptional regulatory protein AlgB	32,74	1,33E-06
Phosphoenolpyruvate-protein phosphotransferase	21,48	1,35E-03
Dihydroorotate dehydrogenase B (NAD(+)), electron transfer subunit	15,00	1,92E-03
Inosine-5'-monophosphate dehydrogenase	14,56	8,04E-05
Phosphoribosylamine--glycine ligase	12,86	3,92E-03
Phosphoribosylformylglycinamidine synthase 1	10,03	0,01
RNA polymerase sigma-54 factor 1	8,40	0,02
Arginine transport ATP-binding protein ArtM	7,42	0,02
Phosphoribosylformylglycinamidine synthase 2	7,19	0,04
Bifunctional purine biosynthesis protein PurH	6,17	4,52E-03
Thiamine precursor transporter HmpT	4,23	0,04
Alternate 30S ribosomal protein S14	3,92	0,01
Autoinducer 2 import system permease protein LsrC	3,66	0,02
hypothetical protein	3,53	8,19E-03
hypothetical protein	3,49	1,67E-03
Biotin transporter BioY2	2,91	0,04
TraM recognition site of TraD and TraG	2,55	0,02
Sortase family protein	2,23	0,04
Beta-L-arabinobiosidase precursor	2,16	0,00
hypothetical protein	2,10	0,02
hypothetical protein	2,10	0,01
Autolysin	-2,02	3,93E-04
hypothetical protein	-2,61	0,04
hypothetical protein	-2,91	0,03
Major cardiolipin synthase ClsA	-6,55	4,82E-04
Acetyltransferase (GNAT) family protein	-6,79	0,04
beta-lactam-insensitive peptidoglycan transpeptidase	-7,48	7,05E-09
D-alanyl-D-alanine carboxypeptidase DacA precursor	-9,10	2,10E-08
1,5-anhydro-D-fructose reductase	-10,73	1,35E-03
Alpha-monoglucosyldiacylglycerol synthase	-16,37	1,51E-04



5

Contribution of the ribosomal protein RpsJ in reduced susceptibility to tigecycline in *Enterococcus faecium*

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Adapted from:

Deletions in a ribosomal protein-coding gene are associated with tigecycline resistance in *Enterococcus faecium*.

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Abstract

Enterococcus faecium is an emerging nosocomial pathogen associated with antibiotic therapy in the hospital environment. Whole-genome sequences were determined for three pairs of related, consecutively collected *E. faecium* clinical isolates to determine putative mechanisms of resistance to tigecycline. The first isolates (1S, 2S and 3S) in each of the three pairs were sensitive to tigecycline [minimum inhibitory concentration (MIC) of 0.125 mg/L for 1S and 3S and 0.047 mg/L for 2S]. Following tigecycline therapy, the second isolate in each pair demonstrated increased resistance to tigecycline. Two isolates (1R and 2R) were resistant (MIC of 8 mg/L and 4 mg/L respectively) and one isolate (3I) demonstrated reduced susceptibility (MIC of 0.5 mg/L). Mutations distinguishing each pair of sensitive and resistant isolates were determined through alignment to a reference genome and variant detection. In addition, a *de novo* assembly of each isolate genome was constructed to confirm mutations. A total of 16 mutations in eleven coding sequences were determined. Mutations in the *rpsJ* gene, which encodes a structural protein forming part of the 30S ribosomal subunit, were detected in each of the pairs. Mutations were in regions proximal to the predicted tigecycline-binding site. Predicted amino acid substitutions in this region were detected in 1R and 3I. The resistant strains were additionally associated with deletions of 15 nucleotides (2R) and 3 nucleotides (1R) in *rpsJ*. Introduction of the *rpsJ* gene from strain 2R into *E. faecium* E1162, a tigecycline-sensitive (MIC 0.047 mg/L) clinical isolate, led to a 3-fold increase of the MIC (0.125 mg/L). This study confirms that amino acid substitutions and deletions in *rpsJ,* contribute towards reduced susceptibility to tigecycline in *E. faecium*. However, mechanisms other than *rpsJ* mutations are likely required to reach tigecycline MICs of ≥4 mg/L.

Introduction

Enterococcus faecium is a nosocomial pathogen associated with antibiotic therapy in the hospital environment that is increasing in prevalence (1, 2). Compared with *Enterococcus faecalis*, which remains the most common *Enterococcus* species in clinical infections, *E. faecium* is intrinsically resistant to a greater number of antibiotics and can efficiently acquire additional antibiotic resistance mechanisms necessitating the use of alternative antibiotics for therapy. Vancomycin resistance is a common and pressing clinical problem in *Enterococcus*. This results from transposon-mediated insertion of genes clusters, e.g *vanA* and *vanB*, coding for two-component systems and enzymes able to alter terminal amino acids of peptidoglycan precursors, leading to vancomycin resistance (3). Tigecycline, a derivative of minocycline, has bacteriostatic activity through binding to the 30S subunit of the bacterial ribosome and inhibiting protein synthesis (2, 4). It provides broad-spectrum activity against multiresistant pathogens such as meticillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and Enterobacteriaceae with extended-spectrum β-lactamases (ESBLs) or carbapenemases. Tigecycline resistance has been reported (4), mainly in Gram-negative pathogens, e.g. *Acinetobacter baumannii* and *Klebsiella pneumoniae* (5, 6), although rarely also in Gram-positive pathogens such as *E. faecium, E. faecalis* and MRSA (7–11). Tigecycline resistance is most commonly associated with overexpression of efflux pumps, e.g. MarA-mediated overexpression of AcrAB in *Escherichia coli* (4, 12).

Whole-genome sequencing (WGS) is a powerful technique that can be used to pinpoint mutations that arise in clinical isolates which have been exposed to antibiotics during treatment. This technique has allowed detailed analysis of pairs of consecutively isolated tigecycline-susceptible and -resistant isolates of Gram-negative pathogens to be undertaken (6, 13). In this study, WGS was used to investigate the nature of potential mechanism(s) associated with tigecycline resistance and reduced susceptibility in three clinical, vancomycin-resistant *E. faecium* isolates.

Results and discussion

The antibiotic susceptibility profiles revealed increased resistance to tigecycline in each of the three post-therapy isolates (Table 1). Each of the pairs showed different mutations in *rpsJ*, which encodes the S10 structural protein in the small 30S ribosomal subunit.

Table S1: Antibiotic susceptibilities of *Enterococcus faecium* isolates.

Isolate		MIC (mg/L) [interpretation[a]]					
	MLST	**Ampicillin**	**Vancomycin**	**Teicoplanin**	**Linezolid**	**Daptomycin[b]**	**Tigecycline**
1S	280	›8 [R]	›32 [R]	›32 [R]	2 [S]	2	0.125 [S]
1R	280	›8 [R]	›32 [R]	›32 [R]	›8 [R]	2	8 [R]
2S	203	›8 [R]	›32 [R]	16 [R]	2 [S]	4	0.047 [S]
2R	203	›8 [R]	›32 [R]	8 [R]	1 [S]	4	4 [R]
3S	–	›8 [R]	›32 [R]	›32 [R]	4 [S]	1	0.125 [S]
3I	–	›8 [R]	›32 [R]	›32 [R]	2 [S]	2	0.5 [I]
E1162	17	-		-	-	-	0.047 [S]
M0346	-	-	-	-	-	-	0.032 [S]
M0347	-	-	-	-	-	-	0.125 [S]

MLST, multilocus sequence typing; MIC, minimum inhibitory concentration; R, resistant; S, susceptible.
[a] According to British Society for Antimicrobial Chemotherapy (BSAC) guidelines [14].
[b] No BSAC MIC breakpoint is available for this antibiotic for *E. faecium*.

Comparison of genomes for pair 1 showed a codon deletion (ATC) and a non-synonymous transition (T›C) mutation. Pair 2 had a deletion of five codons (ATCCGTGCGACTCAT). Pair 3 had two non- synonymous transversion (C›A and A›G) mutations (Fig. 1). All detected mutations were found in close proximity of each other, corresponding to amino acid positions 52–60 of *E. coli* K-12 RpsJ. The introduction of the mutated *rpsJ* allele from the tigecycline-resistant strain 2R, containing the deletion of five codons, on a vector in *E. faecium* E1162, led to a 3-fold increase in the MIC for tigecycline (from 0.047 mg/L to 0.125 mg/L). The introduction of the mutated *rpsJ* allele alone was apparently not sufficient, when placed in the E1162 genetic background, to recapitulate the level of resistance to tigecycline observed in strain 2R, possibly due to the presence of the intact chromosomal copy of *rpsJ* in E1162. Attempts to transform the construct containing the mutated *rpsJ* allele into the tigecycline-susceptible parental strain 2S were unsuccessful, due to the instability of the vector in this background.

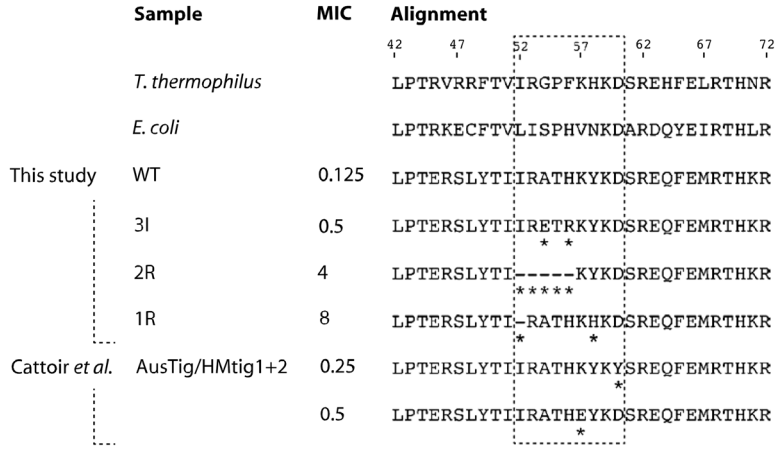

Fig. 1. Mutations identified in the *rpsJ* gene potentially implicated in tigecycline resistance. The relevant amino acid sequence of the early wild-type (WT) isolates in comparison with the mutated isolates identified both in the intermediate (3I) and resistant (1R and 2R) strains from the current study and those from Cattoir *et al*. (10). Sequences from *Escherichia coli* K-12 and *Thermus thermophilus* are shown for context. Codons are numbered according to annotations in *E. coli* K-12. The polymorphic region is indicated with a dotted box, and mutations are indicated by asterisks.

In Gram-negatives, tigecycline resistance may result from changes to the regulation of efflux pumps, with other reported mechanisms including enzymatic degradation (4). In *E. faecium*, the overexpression of the major facilitator superfamily efflux pump TetL and the ribosomal protection protein TetM, have been proposed to contribute to resistance to tigecycline (11). TetM is encoded by the E1162 genome (17). In the current study, we did not find evidence for mutations leading to overexpression of *tetL* and *tetM* (Supplementary Table S1) in any of the resistant isolates. Analysis of gene content demonstrated that neither isolates 1R or 2R were found to contain any additional coding sequences compared with their respective sensitive counterparts. Isolate 3I, however, was found to contain an additional 73 coding sequences, the majority of which were located within a 48-kb region of the genome compared with the reference genome Aus0004 (18). We found no evidence for the presence of *tetA* or *tetR* or additional efflux pump-related sequences in this region. There was no commonality between lost or gained sequences between isolate pairs, making it unlikely that changes in gene content between isolates accounted for the phenotypic changes observed.

A mutation at codon 57 in *rpsJ* was previously described in a tigecycline-resistant *K. pneumoniae* (13). It was hypothesized that this mutation, which is in close proximity to the tigecycline-binding site, could disturb the ribosomal structure, in turn affecting the strong affinity of tigecycline to the ribosome. Our

analysis indicates that amino acid substitutions and deletions in *rpsJ* contribute to increased tigecycline MICs in *E. faecium*. The role of mutations in *rpsJ* in tigecycline has recently also been recognized in *S. aureus, A. baumannii and E. coli* (19). Notably, Cattoir *et al*. also suggested that intermediate resistance (MICs of 0.25–0.5 mg/L) in *E. faecium* is associated with a single nucleotide substitution in *rpsJ* resulting in a non-synonymous change in the conserved KYKD (to EYKD or KYKY) motif at positions 57–60 (10). This is important because the mutations are located in the same region of *rpsJ* as the mutations detected here. However, because we were not able to recapitulate the tigecycline-resistant phenotype by providing the mutated allele of strain 2R *in trans* in E1162, we conclude that mutations in *rpsJ* may be insufficient to produce a tigecycline-resistant phenotype.

According to an X-ray crystal structure of the *Thermus thermophilus* ribosome, the tigecycline-binding site, which is located in the small ribosomal subunit, is composed almost exclusively of ribosomal RNA. All of the mutations that increase resistance to tigecycline occur in a relatively poorly conserved loop of S10, which appears to be involved in maintaining the structure of the tigecycline-binding site (20). All of the mutations that potentially increase resistance substantially (1R and 2R) result in the deletion of one or multiple amino acids from this loop and would likely significantly distort the tigecycline-binding site. Isolate 1R contains a missense mutation resulting in a tyrosine to histidine substitution at position 58 (Supplementary Fig. S1). Interestingly, *T. thermophilus* naturally contains a histidine at this position (20), suggesting that the substitution could compensate for a destabilization caused by the deletion of isoleucine-52.

Two isolates (2R and 3I) had mutations in a coding sequence HMPREF0351-12599, annotated as a hypothetical protein. Protein domain searches of this product identified a YacP-like NYN (N4BP1, YacP-like nuclease) domain. YacP proteins are predicted to interact in a processome complex that catalyses the maturation of rRNA and tRNA, suggesting this mutation may affect the structure of the small subunit of the ribosome (21).

This study highlights the importance of screening for short insertions and deletions (indels) as well as single nucleotide polymorphisms when performing mutant analysis. Indels are often not specifically looked for during routine bioinformatics analysis owing to historical difficulties in mapping reads containing indels when using Illumina short-read sequencing, and high indel rates associated with sequencing platforms such as 454, Ion Torrent and Pacific Biosciences (22, 23). These mutations will be important when constructing databases to perform in silico antibiotic resistance determination directly from whole-genome data.

In conclusion, we have shown that tigecycline resistance in *E. faecium* can arise through several alternative evolutionary paths in *rpsJ* following tigecycline therapy and that in-frame deletions in *rpsJ* contribute to resistance. However, additional, so far uncharacterized, mechanisms are likely also involved.

Materials and methods

Bacterial isolates

Two pairs of isolates (1S and 1R, and 3S and 3I) were obtained from patients in a hospital in the West Midlands, UK. Both patients were previous liver transplant recipients and had been diagnosed with hepatic artery thrombosis and intrahepatic collections. A tigecycline-resistant VRE (1R) was isolated from the first patient following a total of 38 days of treatment with tigecycline. The VRE isolate with reduced susceptibility to tigecycline (3I) was isolated following two courses of tigecycline, totaling 43 days of treatment. A third pair consisted of two isolates (2S and 2R), which despite originating from different patients belonged to a persistent endemic clone [determined by pulsed-field gel electrophoresis (PFGE)], isolated in a hospital in Scotland. Isolate 2S was cultured from urine from a renal patient, whereas isolate 2R was grown from a rectal VRE screening sample in a patient with complex intra-abdominal infection who had received tigecycline for only 3 days.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by agar dilution or Etest (AB bioMérieux, Solna, Sweden) on Iso-Sensitest agar (Oxoid Ltd., Basingstoke, UK) and were interpreted according to British Society for Antimicrobial Chemotherapy (BSAC) guidelines (14).

Whole-genome sequencing

DNA was extracted using an Ultraclean® Microbial DNA Kit (Cambio Ltd., Cambridge, UK) for 1S, 1R, 3S and 3I according to the manufacturer's instructions and was quantified using the dsDNA HS Assay on a Qubit® fluorometer (Thermo Fisher Scientific, Ren- frew, UK). Using 1 ng of input DNA, sequencing libraries were then generated using a Nextera XT Sample Preparation Kit (Illumina, Great Chesterford, UK) according to the manufacturer's recommendations. Sequencing was performed on a MiSeq™ Sequencing System (Illumina) using v2 reagents and generated 300 base (forward) and 200 base (reverse) paired-end reads. For isolates 2S and 2R, DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI) and was sequenced on a HiSeqTM 2000 instrument (Illumina) using 90 base paired-end reads at BGI (Shenzhen, China).

Overexpression of *rpsJ* alleles in *E. faecium* E1162

The *rpsJ* gene and an approximately 500 bp fragment upstream of *rpsJ* was amplified using Accuprime High Fidelity Taq Polymerase (Life Technologies, Bleiswijk, The Netherlands), from genomic DNA isolated from strains S2 and R2 (Wizard genomic DNA purification kit, Promega Benelux), using the primers rpsJ-F_SalI (5'-CCGTTGTCGACATTTATTGCAGGATCAGGAAAAAAG-3') and rpsJ-R-BamHI (5'-CCGTTGGATCCTTATAATTTGATTTCGATGTTTAC-3'), which introduce a SalI and a BamHI restriction site at the ends of the amplicon. The resulting products were cloned into pEF25 (15). The absence of mutations in the generated constructs was confirmed by sequencing. Finally, the constructs were electrotransformed (16) into *E. faecium* E1162, a drug-resistant clinical strain isolated from blood (17), generating the strains M0346 (containing a vector carrying the *rpsJ* allele from S2) and M0347 (containing a vector carrying the *rpsJ* allele from R2). These strains were cultured in the presence of 300 μg ml^{-1} spectinomycin to select for the presence of the *rpsJ*-containing vectors.

Genome analysis

Raw reads were adapter and quality trimmed using Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic). Reads were aligned against the *E. faecium* DO reference genome (accession no. NC 017960.1) using BWA-MEM (https://github. com/lh3/bwa), variants were called using VarScan2 with an allele frequency threshold of 80%, and the effect on coding sequences was predicted using SnpEff (http://snpeff.sourceforge.net). In addition, to confirm that complex mutations in *rpsJ* did not result from mapping artefacts, a *de novo* assembly was performed on each sample using SPAdes 2.5.0 (http://bioinf.spbau.ru/spades) with default parameters. The location of the *rpsJ* sequence in each draft assembly was determined by BLASTX, and matching sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/ clustalw2). To look for changes in gene content between pairs of isolates, the draft genome assemblies for each isolate were compared using the LS-BSR pipeline (https://github.com/jasonsahl/LS-BSR). The python script compare BSR.py from the same package was used to compare the resistant or intermediate isolate against the sensitive isolate for each pair. Multilocus sequence typing (MLST) sequence types were determined in silico from the *de novo* assemblies using the python script mlst (https://github.com/Victorian-Bioinformatics-Consortium/mlst). The nucleotide sequences of the *E. faecium* isolates described in this work have been deposited at the European Nucleotide Archive under the study accession no. PRJEB7380 (http://www.ebi.ac.uk/ena/data/view/PRJEB7380).

Acknowledgments

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Supplementary information

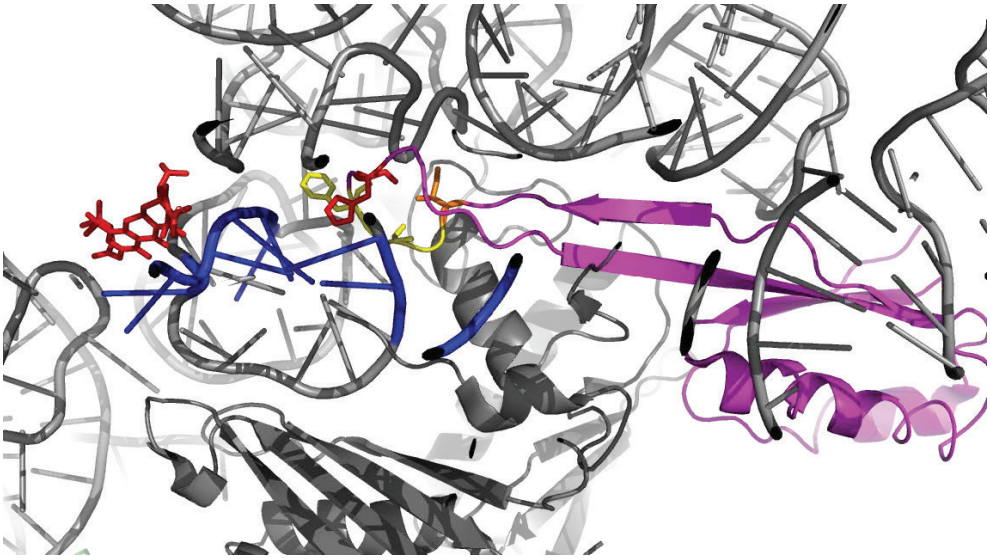
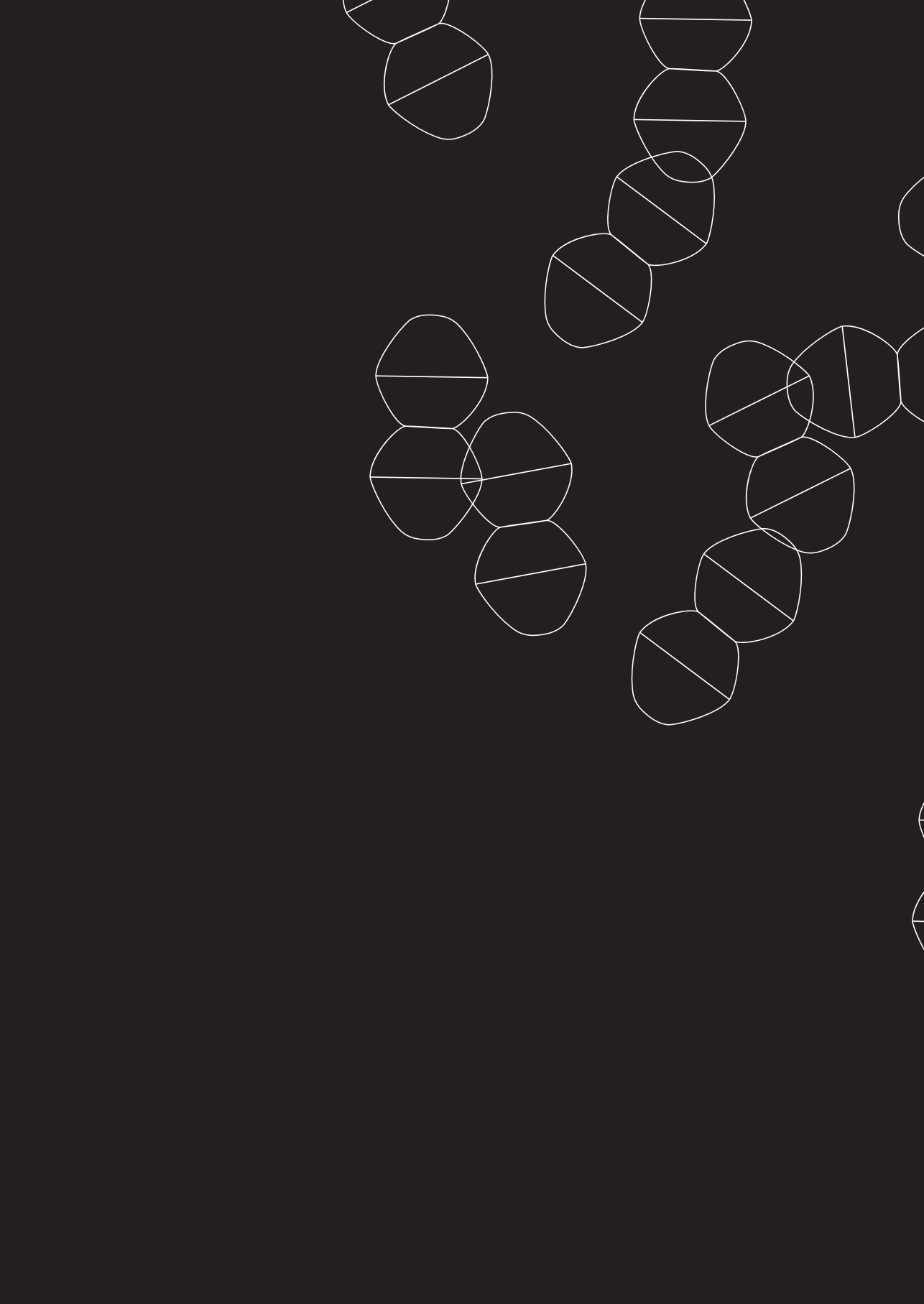


Fig. S1. PyMol figure demonstrating tigecycline (red) bound to the ribosome from *Thermus thermophilus* in cartoon format. The blue shaded area is the ribosomal RNA while the ribosomal S10 protein is depicted in magenta. Also shown are the 2R deletion (yellow), 3I substitutions overlapping 2R deletion (sticks), 1R substitution (red) and residue overlapping the 2R deletion (orange). PDB file used: 4g5t.

Table S1. (page 147) Table of all variants detected between sensitive resistant pairs of isolates. G2576T mutation which confers resistance to linezolid in 1R at ‹80% allele frequency threshold was also included. The specific position is not known as it occurs in a multi-copy gene and the AA change is absent as it occurs in a sequence that codes for ribosomal RNA.

Position	Mutation type	AA change	Locus tag	Product	1S	1R
62187	Missense	Tyr99His	rpsJ	Ribosomal subunit protein S10		295T>C
62165	Inframe deletion	Ile92del	rpsJ	Ribosomal subunit protein S10		274_276delATC
1746315	Missense	Gly311Arg	HMPREF0351_11776	Cell wall surface anchor family protein		931G>A
	G2576T		HMPREF0351_r10011	23S ribosomal RNA		C>T
					2S	**2R**
62168	Inframe deletion	Ile93_His97del	rpsJ	Ribosomal subunit protein S10		277_291delATCGTG CGACTCAT
389535	Missense	Gln170Lys	HMPREF0351_10405	Hypothetical		508C>A
928627	Synonymous	Ala89Ala	HMPREF0351_10953	Hypothetical	267T>C	
1294253	Missense	Ile148Thr	apt	Adenosine phosphoribosyl transferase		443T>C
1655982	Synonymous	Tyr400Tyr	polC	DNA---directed DNA polymerase III alpha subunit		1200C>T
1734017	Missense	Pro535Ser	alsS	Acetolactate synthase		1603C>T
1916087	Frameshift	Phe124fs	capC	Capsular polysaccharide biosynthesis protein	372delT	
2215747	Missense	Gly126Asp	oppA	Oligopeptide ABC superfamily ATP binding cassette transporter		377G>A
2265288	Missense	Val250Leu	lmrA	Multidrug resistance ABC superfamily ATP binding cassette transporter	748G>T	
2581781	Missense	Gln166Pro	HMPREF0351_12599	Hypothetical		497A>C
					3S	**3I**
62176	Missense	Ala95Glu	rpsJ	Ribosomal subunit protein S10		284C>A
62182	Missense	His97Arg	rpsJ	Ribosomal subunit protein S10		290A>G
2581877	Frameshift	Thr134fs	HMPREF0351_12599	Hypothetical		400delA



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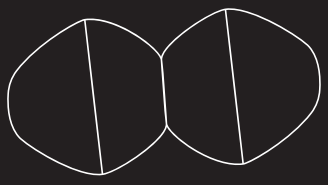
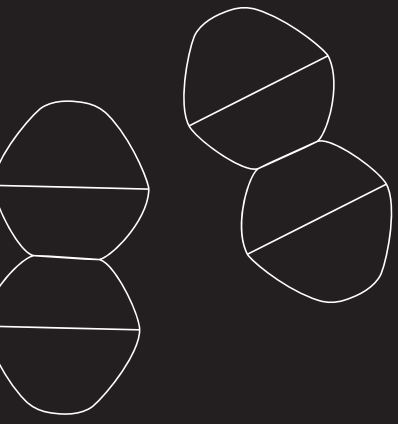
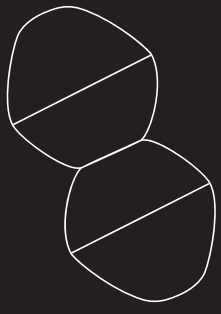
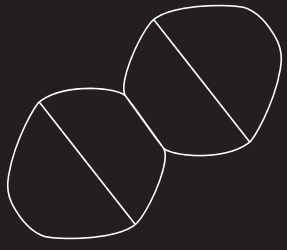
Summarizing discussion









The transition of *Enterococcus faecium* from gastrointestinal commensal into multidrug resistance pathogen has been a process driven by multiple factors that we have only recently started to understand (Arias and Murray, 2012). The intrinsic resistance of *E. faecium* to antibiotics like aminoglycosides and the rapid development of resistance to other antibiotic classes, such as fluoroquinolones and glycopeptides, including vancomycin (National Nosocomial Infections Surveillance System, 2004; Uttley et al., 1988), began to complicate treatment of infections caused by multi-drug resistant *E. faecium* in the nosocomial environment since the 1980s. With the emergence of vancomycin resistant enterococci (VRE), new antibiotics like tigecycline, linezolid and daptomycin were developed and introduced for clinical use (Rose and Rybak, 2006). However very rapidly, resistance to these antibiotics was also reported (Aksoy and Unal, 2008; Montero et al., 2008; Niebel et al., 2015; Scheetz et al., 2008). In an effort to understand and contain the rise and dissemination of vancomycin-resistant *E. faecium* (VREF), different molecular tools have been developed and used for epidemiological studies (Morrison et al., 1999). Nevertheless, with time, those tools proved to be insufficient to accurately reproduce genetic relatedness of isolates. Moreover, the global rise of VRE could be only partially explained by clonal spread of strains and it became clear that horizontal acquisition of vancomycin resistance genes from commensal microbiota contributed to *de novo* emergence of VRE (Howden et al., 2013).

Population structure of *E. faecium*.

The advent of whole genome sequencing (WGS) allowed more accurate epidemiological studies and phylogenetic analyses. The *E. faecium* population has been divided into three sub-populations denominated clade A-1, representing the majority of clinical isolates, clade A-2 representing most animal strains and clade B, which is associated with strains from healthy humans (Galloway-Peña et al., 2012; Lebreton et al., 2013). However, using core genome MLST (cgMLST) (de Been et al., 2015), we showed that animal isolates of clade A-2 group into several diverse clusters rather than an individual one, suggesting that clade A-2 strains may not have one common evolutionary ancestor, as previously postulated (Lebreton et al., 2013). The evolutionary trajectory of *E. faecium*, which has led to this clear clade structures, suggests that *E. faecium* is colonizing rugged fitness landscapes, in which characters of particular clones in clade A and B impose strong fitness differences. Hospital-adapted linages in clade A-1 carry, and easily exchange among them, mobile genetic elements containing ecologically important genomic traits not found in clade A-2 or clade B, leading to significantly larger genome sizes (Lebreton et al., 2013). These genomic traits in clade A-1 code for virulence and antibiotic resistance factors, which allow these strains, to be

successful in colonizing and infecting hospitalized patients (Hegstad et al., 2010; Leavis et al., 2003; Palmer et al., 2010; van Schaik et al., 2010). This mechanism, which is termed divergence hitchhiking (DH) (Via, 2012), explains how differences in gene exchange between diverging populations is reduced over large genomic regions, as a collateral effect of strong divergent selection on genes involved in local adaptation.

The *E. faecium* surface protein PrpA.

Among the adaptive elements that may contribute to *E. faecium*'s success as a gut commensal and nosocomial pathogen are a number of surface proteins. These are anchored to the peptidoglycan through a motif composed of leucine-proline-X-threonine/serine/alanine-glycine (Ton-That et al., 2004). These proteins can frequently recognize host extracellular matrix components (ECM) (Hendrickx et al., 2009, 2013, 2013). To successfully colonize and infect the host, *E. faecium* has to gain access to underlying tissues, which are exposed when natural barriers, like the skin, are breached by trauma, surgery or the use of small indwelling medical devices like catheters. Central lines in hospitalized patients create an access route for bacteria to reach the bloodstream, causing bacteremia and other bloodstream associated infections (Zingg et al., 2008). Compared to bloodstream infections caused by other enterococci, bacteremias caused by *E. faecium* have a poorer prognosis, particularly when they are caused by vancomycin-resistant isolates (Hayakawa et al., 2012; Munita et al., 2014). In **chapter 2** we performed a transcriptome analysis in the clinical strain *E. faecium* E1162 during growth at mammalian and environmental temperatures. We described a thermo-regulated surface-exposed protein, termed PrpA for proline-rich protein A. PrpA was detected at higher levels during growth at 37°C than at 25°C on the surface of *E. faecium* E1162 cells. We postulated that the C-terminal LPKSG motif of PrpA, may serve as an anchor of this protein to the peptidoglycan and may, simultaneously extend the proline rich repeats of the N-terminal domain externally, as described in other gram-positives (Santi et al., 2007). We showed that the functional N-terminal domain of PrpA interacts with fibrinogen and fibronectin, which are components of the ECM present in mammalian hosts (Halper and Kjaer, 2014) and also part of the coagulation cascade, in which platelets play an important role (Weisel and Litvinov, 2017). In addition to binding ECMs, the N-terminal domain of PrpA was also able to bind platelets.

PrpA was found to be produced at mammalian temperature in clinical and animal strains of clade A but not at environmental temperature or in commensal isolates that belong to clade B. The ability of PrpA to bind ECM and blood components, together with the observation of its thermo-regulated production in clade A isolates, suggests a role for PrpA in colonization and infection of the host.

A two-component system links chlorhexidine and bacitracin tolerance in *E. faecium*.

Central line-associated bloodstream infections (CLABSIs), are common in patients admitted to the Intensive Care Unit (ICU) and in patients with impaired immunity (e.g. patients who are on chemotherapy) (Steinberg et al., 2013). The combination of infection control polices and interventions, like epidemiological surveillance, outbreak investigations and aseptic insertion and maintenance of catheters with proper disinfection, is effective in reducing CLABSIs (Blot et al., 2014; Ista et al., 2016; Voor in 't Holt et al., 2017). The use of disinfectants like chlorhexidine (CHX) for daily patient bathing, diminishes the risk of VRE colonization and nosocomial infections in hospitalized patients (Kim et al., 2016; Marschall et al., 2014; Quach et al., 2014; Salzman et al., 1993). In **chapter 3** we showed that hospital-associated multi-drug resistant isolates belonging to clade A-1, are able to tolerate CHX while clade A-2 and clade B strains, are more susceptible to this disinfectant. This observation indicates that tolerance to CHX might be one of the adaptive mechanisms that has contributed to the success of particular *E. faecium* clones in the nosocomial environment (Lebreton et al., 2013; Willems et al., 2005). Exposure of multi-drug resistant isolates, like VRE, to constant prophylactic sub inhibitory concentrations of CHX, may lead to higher CHX MICs and the loss of susceptibility towards this disinfectant (Suwantarat et al., 2014). We used Microarray-based Transposon Mapping (M-TraM; (Zhang et al., 2012)), to perform a genome-wide screening of a transposon mutant library in the clinical strain *E. faecium* E1162. We identified a two-component regulatory system (2CS), termed ChtRS, that contributes to CHX tolerance in *E. faecium* E1162. In addition we showed, that a single non-synonymous nucleotide change in the response regulator component (*chtR*) found in clade A-1, but not in clade A-2 or clade B strains, is sufficient to cause the CHX-tolerant phenotype of clade A-1 isolates. This particular mutation is located in a domain that may affect dimerization of ChtR, potentially altering the DNA binding of the response regulator and consequently may change gene expression (Bachhawat et al., 2005). ChtRS in *E. faecium* is not only responsible for the regulation of, yet unidentified, effector genes, leading tolerance to CHX, but also to resistance to bacitracin, an antibiotic that may be used in the treatment of VRE colonization and infection. Our work adds to the rising concerns about the co- and cross-selection for antibiotic resistance, induced by the use of disinfectants (Bhardwaj et al., 2016; Russell, 2003).

E. faecium genes that contribute to growth in serum.

Apart from the use of catheters, bloodstream infections can result from translocation of pathogens from the intestinal tract into the bloodstream (Kamboj et al., 2014). In order to cause infections, *E. faecium* needs to overcome many challenges, like the insufficiency of basic nutrients, to be successful in the bloodstream (Krebs, 1950). However, *E. faecium* thrives in this environment and it causes approximately 40% of all enterococcal bacteremias (de Kraker et al., 2013; Sievert et al., 2013; Weiner et al., 2016). The *de novo* synthesis of purines and pyrimidines has previously been identified as an important factor for the growth of different bacteria in serum (Le Breton et al., 2013; Samant et al., 2008). Perhaps unsurprisingly, the biosynthesis of nucleotides has been proposed as an important factor involved in virulence of several bacterial pathogens (Jenkins et al., 2011; Schwager et al., 2013). In **chapter 4** we sequenced the complete genome of E745, a vancomycin-resistant *E. faecium* strain, in order to identify genes that contribute to growth of *E. faecium* in human serum. For this purpose, we used Tn-seq (van Opijnen et al., 2009), a high-throughput approach based on sequencing of the junctions of the transposon insertion sites. We identified the genes that contribute to survival and growth of *E. faecium* E745 in human serum. Among these, 37 genes were described including gene involved in carbohydrate uptake (*manZ_3, manY_2*, *ptsL*), a putative transcriptional regulator (*algB*) and genes involved in the biosynthesis of purine and pyrimidine nucleotides (*guaB*, *purA*, *pyrF*, *pyrK_2*, *purD*, *purH*, *purL*, *purQ*, *purC*). Subsequently, we used RNA-seq (Wang et al., 2009) to determine the transcriptional response of *E. faecium* E745 in rich media compared to human serum. Almost 30% of the genes in the genome were differentially expressed. The genes *purD, purH,* and *purL* with a role in purine biosynthesis, were among the genes with the highest difference in expression. Notably, these genes were also previously determined to be important for growth in serum in the Tn-seq experiments. Transposon mutants in *pyrF*, *pyrK_2*, *purD*, *purH* and *manY_2* where shown to have a growth defect in human serum. Additionally, *pyrK_2* and *manY_2*, were found to be attenuated in virulence in a zebrafish infection model. Our data supports that *de novo* biosynthesis of nucleotides is crucial for growth in serum and virulence and may be a promising pathway for the development of new anti-infectives.

Evolution of tigecycline resistance in *E. faecium*.

The rapid emergence and spread of VRE, has led to the use of combination therapies of multiple antimicrobial agents and also use of recently introduced antibiotics, like tigecycline (Miller et al., 2016). Resistance to tigecycline in enterococci has been described previously and is mediated through upregulated expression of the genes encoding the efflux pump TetL and the ribosomal

protection protein TetM (Fiedler et al., 2016). In **chapter 5** we used whole-genome sequencing to identify additional mechanisms of resistance to tigecycline, in three pairs of *E. faecium* clinical isolates. These strains were initially susceptible but acquired resistance to tigecycline during treatment with this antibiotic. Among the 16 different mutations identified, deletions and/or mutations in the *rpsJ* gene, encoding a structural protein in the 30S ribosomal subunit, were found in each one of the three resistant pairs. We provided experimental evidence that the deletion of five codons in *rpsJ*, when introduced in a tigecycline susceptible clinical isolate, led to an increase in the MIC for tigecycline, but was not sufficient to cause resistance to this antibiotic. In accordance with other studies in *E. faecium* (Beabout et al., 2015; Cattoir et al., 2015), we conclude that *rpsJ* has a role in tigecycline resistance in *E. faecium* but additional mutations may still be needed to reach MICs › 0.5 ug/ml.

Concluding remarks and future perspective

Despite its commensal nature, *Enterococcus faecium* has recently emerge a as multi-drug nosocomial pathogen, causing approximately 40% of the health care-associated infections caused by enterococci (Weiner et al., 2016). In this thesis we used a variety of functional genomic techniques, to obtain insights into the mechanisms that contribute to the global rise of *E. faecium* as a nosocomial pathogen.

The population structure of *E. faecium* is characterized by a deep phylogenetic split that separates human commensal isolates (clade B) from farm animal and hospital isolates (clade A) (Lebreton et al., 2013). The particular evolutionary trajectory of hospital adapted strains in clade A-1, has led to the acquisition of ecologically important genomic traits, like antibiotic resistance and genes linked to pathogenicity, leading to a successful subpopulation adapted to the nosocomial environment. Among the acquired antibiotic resistant determinants in clade A-1, vancomycin resistance gene clusters, particularly *vanA* and *vanB,* have contribute to the dissemination of vancomycin resistance (Courvalin, 2006). The global emergence of vancomycin resistant *E. faecium* and the relatively recent development of resistance to last-resort antibiotics like tigecycline, complicates treatment options for patients infected by this pathogen. Clearly, the widespread use of antibiotics in human and veterinary medicine are important factors that drive the current emergence of *E. faecium*. In fact, it is tempting to speculate that the deep phylogenetic split in *E. faecium* is driven by anthropogenic influences, since strains from farm animals (clade A-2) and clinical isolates (clade A-1), share a common features as they both originate from environments, where mammalian hosts are in close contact with each other and usage of antibiotics is high. It is of crucial importance to fully understand the differences in population biology of *E.*

faecium, as well as the genomic factors that favor the adaptation to and survival in diverse niches in the human host.

In hospitalized patients exposure to antimicrobial agents lead to higher densities of *E. faecium* in the intestinal tract (Donskey et al., 2000; Ubeda et al., 2010). These strains can cause difficult-to-treat infections, particularly when these infections are caused by VRE. Due to the global rise of VRE, novel antibiotic targets and the development of new antimicrobials are needed. Diverse peptidoglycan-anchored surface exposed proteins have been considered possible antimicrobial targets, since they play important roles in colonization, viability and virulence of bacteria. In other Gram-positive opportunistic pathogens targeted immunotherapy using antibodies or Fab fragments directed to bacterial exposed structures, including LPxTG-type proteins, start to show effectiveness in reducing colonization, systemic and invasive disease. The success of passive immunization is accomplished by promoting complement deposition, which supports phagocytic uptake of antibody-opsonized bacteria (Kristian et al., 2016; Rivas et al., 2004; Sause et al., 2016; Schaffer et al., 2006; Schlievert et al., 2010; Yang et al., 2016). The functional characterization of PrpA, supports a possible role of this protein in *E. faecium* pathogenesis and this surface protein may be a future target for the development of novel immunotherapy to treat or prevent *E. faecium* infections.

E. faecum can translocate from the GIT into the bloodstream or colonize the skin and indwelling medical devices via fecal contamination, leading to bloodstream infections (Kamboj et al., 2014). To thrive in serum, we showed that *E. faecium* needs genes involved in nucleotide biosynthesis. The ability to synthesize nucleotides can contribute importantly to virulence in *E. faecium*, as shown in this thesis, and in other pathogens (Le Breton et al., 2013; Samant et al., 2008). Since the time taken to develop, test and approve new therapeutic candidates, is a limiting step in the antibiotic pipeline and only few new antimicrobial compounds with activity against enterococci have been discovered with promising *in vivo* results (van Harten et al., 2017), other alternatives, including the use of nucleoside analogues, has recently been considered. These compounds have long been used as potent inhibitors of viral replication (Mitsuya et al., 1985) and as anti-proliferatives in the treatment of cancer (Plunkett et al., 1995). In short, nucleoside analogues inhibits enzymes responsible for producing the deoxynucleotides required for DNA synthesis, which in turn favors the incorporation of the nucleoside analogues into DNA, obstructing further DNA synthesis (Plunkett et al., 1995). Recently their antimicrobial potential have been extensively reviewed (Soo et al., 2016). Among nucleoside analogues, zidovudine has antibacterial activity against Gram-negatives, particularly enterobacteria (Doléans-Jordheim et al., 2011). A different analog, gemcitabine, has been shown to be effective against Gram-positive bacteria including enterococci (Jordheim et al., 2012).

In addition, while new antimicrobials are developed and approved, it is

important to use diverse infection control policies to stem the global emergence of antibiotic resistant bacteria. The use of disinfectants in clinical wards is an effective method to prevent health care–associated infections, caused by multi-drug resistant bacteria (Kim et al., 2016). However, the use of disinfectants may also lead to selection of resistant isolates (Pal et al., 2015; Russell, 2003). This topic deserves further study to fully elucidate if and how the use of disinfectants can lead to co- and cross-selection for antibiotic resistance and to develop interventions that can prevent the spread of bacteria, while minimizing the risk of co- or cross-selection for antibiotic resistance.

To better understand *E. faecium* biology and pathogenesis, the use of animal models allows the study of biofilm formation, bacteremia and endocarditis, gut colonization and antibiotic-induced dysbiosis. A recent study showed that host immune factors can restore the diversity of anaerobic commensals to promote resistance to *E. faecalis* colonization (Pham et al., 2014), opening the spectra for potential future applications in *E. faecium* biology, to improve strategies to curtail the ongoing emergence of this and other multi-drug resistant pathogens as public health threats.However, concerns remain about the relevance of animal models for human disease. An attractive alternative to animal studies is formed by miniaturized organs termed organoids, that exhibit similar functionality as the human tissue of origin (Fatehullah et al., 2016). Organoids may be used to study the mutualism between the host and the diverse microbial community of the gut microbiota. Therefore, organoids may increase our knowledge of the complex interactions between the host and the microbiota that lead to intestinal overgrowth by *E. faecium*. These systems could conceivably be used for the development of novel therapeutics and interventions targeting multi-drug resistant hospital-adapted clones of *E. faecium*.

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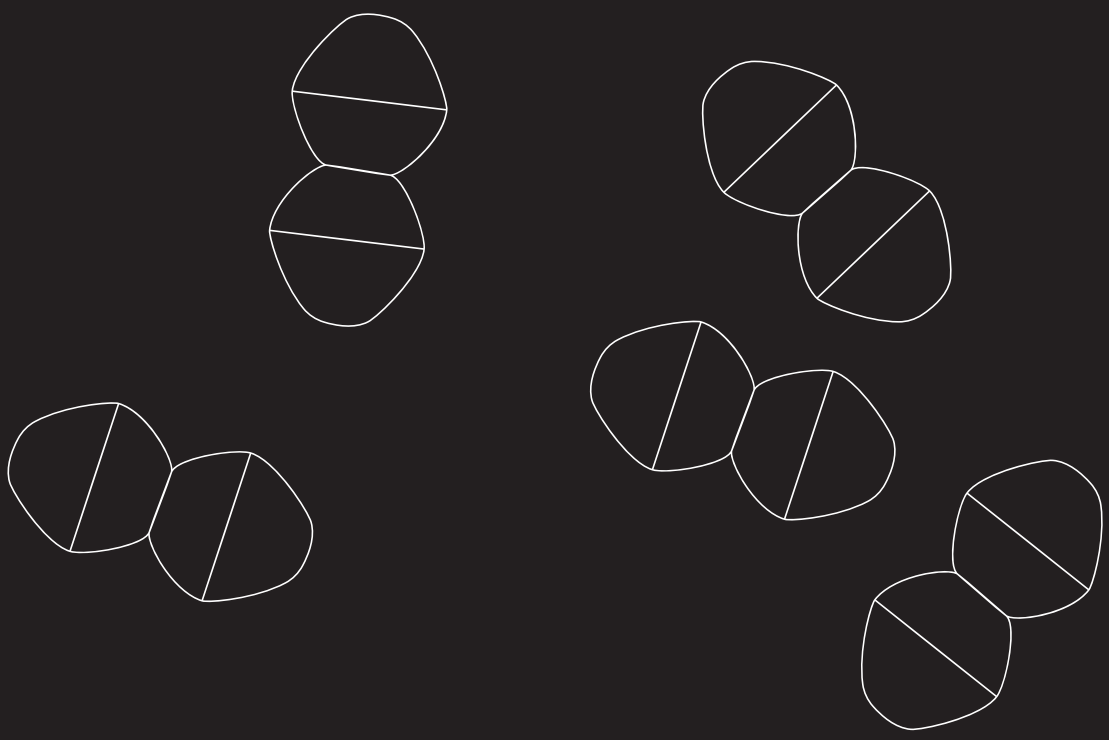
Summary



Enterococcus faecium is a Gram-positive bacteria and a common commensal of the gastrointestinal tract. However, *E. faecium* can cause a large number of hospital-acquired infections in humans and has rapidly acquired resistance to several classes of antibiotics. The ubiquitous nature of *E. faecium,* the ability to prosper in complex microbial communities, the great flexibility of its genome and the global use of antibiotics, are important factors driving *E. faecium*'s current emergence as a nosocomial pathogen around the world. The rise of vancomycin resistant enterococci (VRE) particularly complicates treatment of infections caused by enterococci in hospitalized patients. Unfortunately, up to date, only few new antibiotics have been introduced for clinical use to treat VRE infections.

It is of essence to understand the epidemiology and phylogenetics of *E. faecium* to help contain its current global rise as a nosocomial pathogen. To this aim, diverse molecular tools have been developed since the year 2000. The advent of whole genome sequencing has allowed more accurate studies on the population structure of *E. faecium*. The species *E. faecium* can be divided into three distinct sub-populations that each include the majority of clinical isolates, strains from healthy humans and animal isolates. The acquisition of adaptive elements, such as mobile genetic elements carrying antimicrobial resistance and virulence determinants, contributes to *E. faecium*'s success as gut commensal and nosocomial pathogen. For some time, the lack of suitable methods for genomic studies in *E. faecium* hindered the functional characterization of genetic determinants that are relevant to important *E. faecium* traits. Fortunately, these barriers have largely been removed over the last few years. In this thesis, we continue to expand the use of high-throughput approaches to characterize the genetic determinants of *E. faecium* that can explain its success as a nosocomial pathogen.

To successfully colonize and infect the host, *E. faecium* has to gain access to underlying tissues, which are exposed when natural barriers, like the skin, are breached by trauma, surgery or the use of small indwelling medical devices like catheters. In *E. faecium,* surface proteins anchored to the peptidoglycan can recognize host extracellular matrix components (ECM). In **chapter 2** we performed comparative transcriptome profiling of an ampicillin-resistant *E. faecium* isolate grown at mammalian (37°C) and room temperatures and identified a thermo-regulated surface-exposed protein, termed PrpA. We showed that its functional N-terminal domain interacts with fibrinogen and fibronectin, which are components of the ECM present in mammalian hosts. The same domain was also able to bind to platelets. This thermo-regulated surface-exposed protein was found to be produced at 37°C in clinical and animal strains but not at room temperature or in commensal isolates.

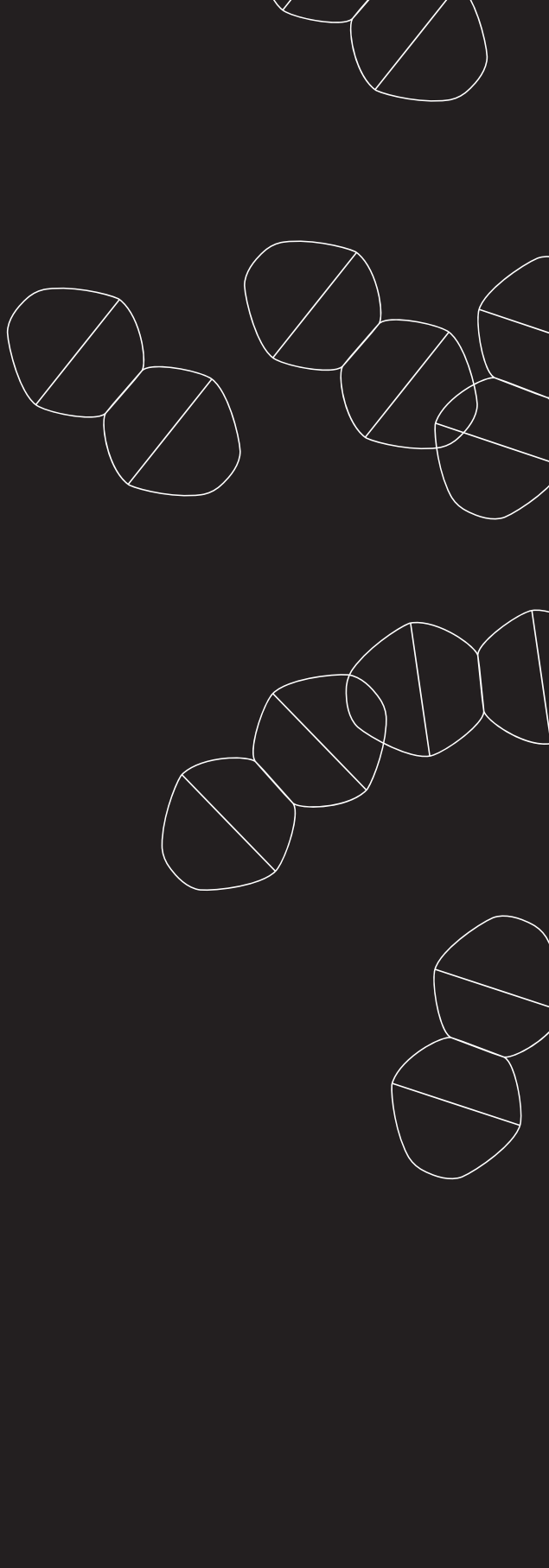
Central lines in hospitalized patients create an access route for bacteria to reach the bloodstream, causing bacteremia and other central line-associated

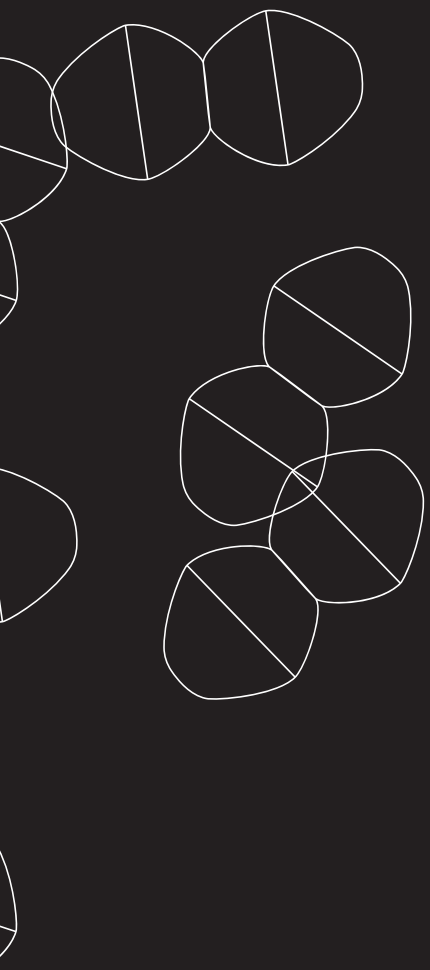
bloodstream infections (CLABSIs), which are common in critically ill patients. The use of disinfectants for daily patient bathing, is effective in reducing CLABSIs, particularly in reducing the risk of VRE infections. **Chapter 3** describes a two-component system, identified through a microarray-based transposon mutant screening method, implicated in tolerance to the disinfectant chlorhexidine. In addition, we showed that this putative two-component system is also implicated in resistance to the antibiotic bacitracin, which may be used in the treatment of VRE colonization and infection.

Bloodstream infections can result from translocation of pathogens from the intestinal tract into the bloodstream or by fecal contamination of the skin, followed by a breach in this barrier, e.g. when a catheter is placed. In either case, *E. faecium* needs to overcome the insufficiency of basic nutrients in the bloodstream, to be successful in such niche. In **chapter 4** we sequenced the genome of the vancomycin-resistant clinical *E. faecium* isolate E745 and implemented a high-throughput transposon mutant sequencing approach, to perform a sequencing-based genome-wide functional profiling of this strain. We identified the genes that contribute to survival and growth of *E. faecium* E745 in human serum, showing that genes involved in carbohydrate uptake and genes involved in the biosynthesis of purine and pyrimidine nucleotides, had a role in this phenotype. Transposon mutants in selected genes were isolated from the mutant library and were shown to have a growth defect in human serum and found to be attenuated in virulence in a zebrafish infection model.

The rise of VRE and the difficulties that are posed by the treatment of infections caused by these bacteria, highlight the need for the development of new antibiotics. Meanwhile, the implementation of different antimicrobial therapies using relatively novel broad-spectrum antibiotics and combinations of antibiotics needs to be explored. Unfortunately, resistance remains a concern with these therapeutic approaches. In **chapter 5**, we describe a novel resistance mechanism against the recently introduced antibiotic tigecycline. Resistance was caused by mutations in the *rpsJ* gene which encodes a ribosomal protein. This mechanism was identified using a combination of comparative genomic analysis of paired susceptible and resistant isolates, and functional genetic complementation.

The widespread use of antibiotic in human and veterinary medicine is a crucial factor in the emergence of succesful *E. faecium* clones that have adapted to live in the hospital. However, other traits, like the ability of the bacteria to colonize the intestinal tract and cause infections, are also important to explain the transition of *E. faecium* from gut commensal to nosocomial pathogen. The research described in this thesis contributes to our understanding of the underlying mechanisms that have made *E. faecium* an important nosocomial pathogen and may contribute to the development of new therapies aimed against this bacterium.





Samenvatting

Enterococcus faecium is een Gram-positieve bacterie die als commensaal van het maag-darm kanaal veelvuldig voorkomt. *E. faecium* veroorzaakt echter ook een groot aantal infecties bij patiënten in het ziekenhuis en heeft snel resistentie ontwikkeld tegen verschillende klasses antibiotica. Omdat *E. faecium* wijdverspreid is, kan uitgroeien in complexe microbiële ecosystemen, relatief eenvoudig horizontaal overgedragen DNA kan integreren in het eigen genoom en door het gebruik van antibiotica, is deze bacterie een steeds belangrijkere ziekenhuis-pathogeen geworden over de gehele wereld. De toename van vancomycine-resistente enterokokken (VRE) in het bijzonder, is een belangrijke complicerende factor in de behandeling van enterokokken infecties in het ziekenhuis. Helaas zijn er, tot op heden, slechts enkele antibiotica beschikbaar gekomen die gebruikt kunnen worden voor de behandeling van VRE infecties.

Het is cruciaal om de epidemiologie en phylogenie van *E. faecium* te begrijpen omdat dit kan bijdragen aan het beteugelen van de wereldwijde toename van *E. faecium* als een ziekenhuis-pathogeen. Met dit doel, zijn sinds het jaar 2000 verschillende moleculaire methodes ontwikkeld. De opkomst van nieuwe methoden om de basenvolgorde van genomen te bepalen (*whole genome sequencing*), heeft belangrijk bijgedragen aan accurate studies over de populatiestructuur van *E. faecium*. De soort *E. faecium* can opgedeeld worden in drie sub-populaties die ieder de meerderheid van klinische isolaten, stammen van gezonde mensen en dierisolaten bevatten. Het verkrijgen van adaptieve elementen, zoals mobiele genetische elementen met antibioticum resistentie en virulentie determinanten, draagt bij aan het succes van *E. faecium* als darmcommensaal en opportunistische pathogeen. Het gebrek aan methodes voor de manipulatie van het genoom van *E. faecium* is lang een hindernis geweest voor de functionele karakterisatie van genen die van belang zijn voor het succes van *E. faecium* als darmcommensaal en als ziekenhuisbacterie. Gelukkig zijn deze barrières grotendeels weggenomen gedurende de laatste jaren. In dit proefschrift hebben we de efficiënte methodes, gebaseerd op *functional genomics*, verder ontwikkeld om genen te karakteriseren die bijdragen aan het succes van *E. faecium* als ziekenhuisbacterie.

Om de gastheer succesvol te koloniseren en te infecteren moet *E. faecium* toegang verkrijgen tot onderliggende weefsels, die bloot komen te liggen als natuurlijk barrières, zoals de huid, zijn beschadigd door trauma, chirurgie of het gebruik van medische hulpmiddelen, zoals intraveneuze katheters. In *E. faecium* kunnen oppervlakte-eiwitten die verankerd zijn aan het peptidoglycaan, componenten van de extracellulaire matrix herkennen. In **hoofdstuk 2** beschrijven we een transcriptome studie die is uitgevoerd met een ampicilline-resistente *E. faecium* stam tijdens groei bij kamer-temperatuur en de temperatuur van een zoogdier (37°C). Hierdoor identificeerden we een temperatuur-gereguleerd oppervlakte-eiwit, dat we PrpA hebben genoemd. We hebben laten zien dat het functionele N-terminale domein van dit eiwit interacties aangaat met

fibrinogeen en fibronectine. Deze eiwitten zijn belangrijke onderdelen zijn van de extracellulaire matrix van zoogdieren. Hetzelfde domein was ook in staat om aan bloedplaatjes te binden. Dit temperatuur-gereguleerde oppervlakte-eiwit werd door klinische en dier-stammen gemaakt bij 37°C maar niet bij kamertemperatuur of door commensale *E. faecium* isolaten.

Centrale lijnen in ziekenhuis-patiënten creeëren een toegangsroute voor bacteriën om de bloedbaan te bereiken, waar zij bacteremie en andere centrale lijn geassocieerde bloedbaaninfecties (CLABSIs) kunnen veroorzaken, in het bijzonder in ernstige zieke patiënten. Het gebruik van disinfectantia voor het dagelijks baden van patiënten is een effectieve manier om CLABSIs te reduceren, in het bijzonder de infecties die worden veroorzaakt door VRE. **Hoofdstuk 3** beschrijft een twee-componenten systeem dat is betrokken bij de tolerantie tegen het disinfecterende middel chlorhexidine. Bovendien laten we zien dat dit twee-componenten systeem ook een rol heeft in de tolerantie tegen het antibioticum bacitracine, dat mogelijk gebruikt kan worden voor de behandeling van *E. faecium* infecties.

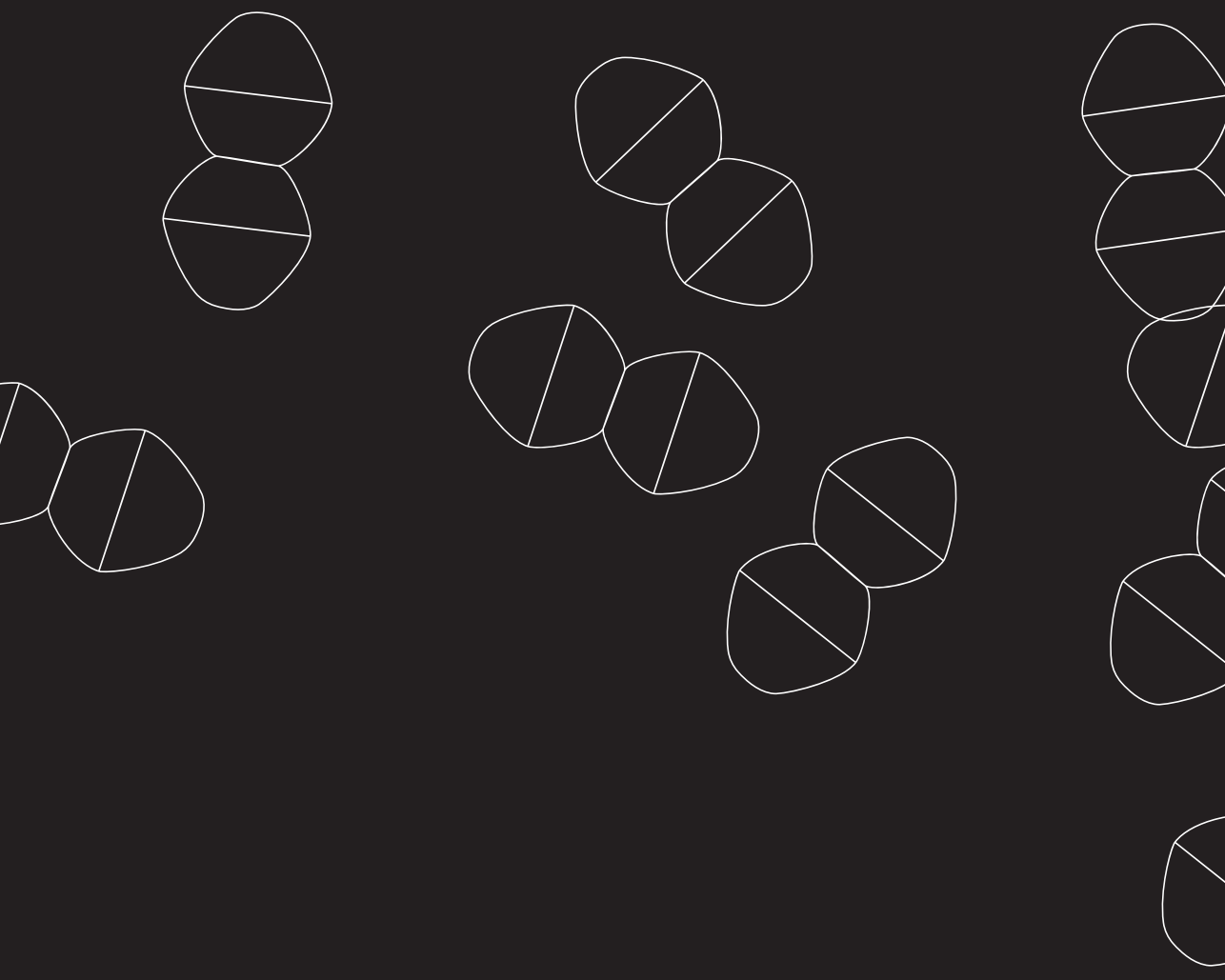
Bloedbaaninfecties kunnen ontstaan als gevolg van de translocatie van pathogene bacteriën van de darmen naar de bloedbaan of door fecale besmetting van de huid, gevolgd door een verstoring van deze barrière, bijvoorbeeld wanneer een catheter wordt geplaatst. In beide gevallen moet *E. faecium* het tekort aan voedingsstoffen in de bloedbaan kunnen overwinnen om succesvol te zijn in deze niche. In **hoofdstuk 4**, hebben we het genoom van het vancomycine-resistent klinisch *E. faecium* isolaat E745 gesequenst en pasten we een efficiënte methode toe om transposon-mutanten banken te analyseren, wat het mogelijk maakte om genoom-wijde functionele experimenten met deze *E. faecium* stam uit te voeren. We identificeerden de genen die bijdragen aan overleving en groei van *E. faecium* E745 in menselijk serum en laten zien dat genen met een rol in suikertransport en de biosynthese van purine en pyrimidine nucleotiden bijdragen aan dit fenotype. Transposon mutanten in een aantal van deze genen werden geïsoleerd uit de transposon-mutanten bank en er werd vastgesteld dat deze mutanten een groeidefect hadden in menselijke serum en minder virulent waren in een zebravismodel voor infectie.

De toename van VRE en de problemen die veroorzaakt worden door infecties met deze bacteriën, onderstreept het belang van de ontwikkeling van nieuwe antibiotica. Ondertussen is het een optie om verschillende antimicrobiële therapiën met relatief nieuwe, breed-spectrum antibiotica en combinaties van antibiotica te onderzoeken. Helaas vormt ook hier resistentie een probleem. In **hoofdstuk 5**, beschrijven wij een nieuw resistentiemechanisme tegen het recent geïntroduceerde antibioticum tigecycline. Resistentie werd veroorzaakt door mutaties in het *rpsJ* gen dat codeert voor een ribosomaal eiwit. Dit mechanisme werd geïdentificeerd met behulp van een combinatie van vergelijkende genoom

analyses van gepaarde gevoelige en resistente stammen, en functionele genetische complementatie.

Het wijdverspreide gebruik van antibiotica in de humane geneeskunde en diergeneeskunde is een cruciale factor die bijdraagt aan het ontstaan van succesvolle *E. faecium* klonen die zich hebben aangepast aan het leven in het ziekenhuis. Andere eigenschappen, zoals het vermogen om de darm te koloniseren en infecties te veroorzaken, zijn echter ook belangrijk om de overgang van *E. faecium* van darmcommensaal tot ziekenhuispathogeen te kunnen verklaren. Het onderzoek dat beschreven is in dit proefschrift draagt bij aan het begrip van de onderliggende mechanismes die *E. faecium* tot een belangrijke ziekenhuisbacterie hebben gemaakt en kunnen bijdragen aan de ontwikkeling van nieuwe therapiën gericht tegen deze bacterie.

Acknowledgements/ Agradecimientos



"Don't cry because it's over. Smile because it happened." Q.A….and… so finishes a long road full of uncountable memories and wonderful people, all which have impacted my academic and personal life on so many levels and from whom I stole a bit of their history and hopefully they also took a bit of mine.

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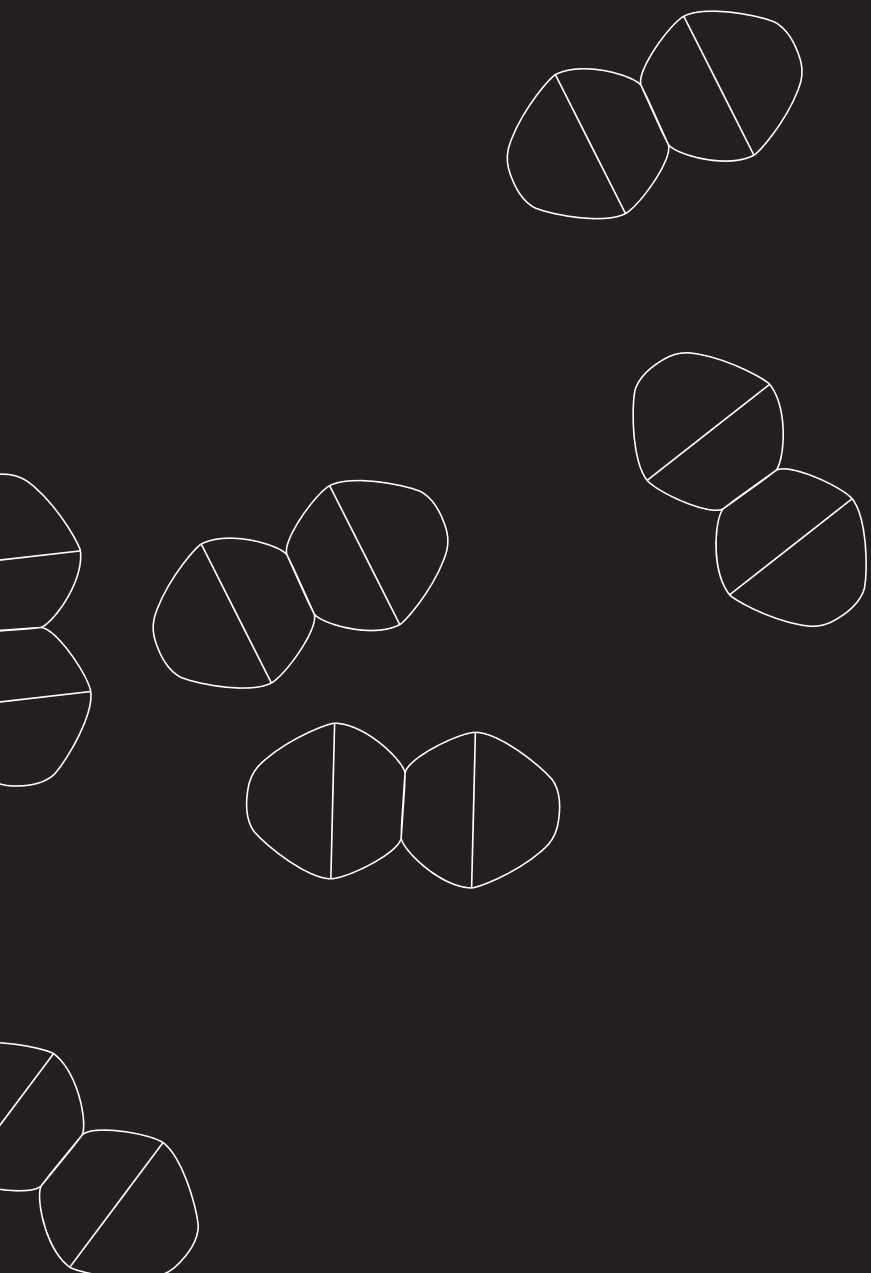

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About the author

Ana María Guzmán Prieto was born on the 14th of September, 1982 in Santiago de Cali, Valle del Cauca, Colombia. She completed her secondary education at Liceo Benalcazar (Santiago de Cali, Colombia) in 1999. Upon graduation from secondary school, she was awarded an American Field Service (AFS) Intercultural Programs scholarship and became a foreign exchange student at Marshal Senior High School in Marshal, Minnesota, United States of America (USA), where she completed her senior year in 2000. After her academic experience in the USA, she started her bachelor's degree at Universidad del Valle, Colombia, where half of her studies were funded with a scholarship awarded by Universidad del Valle, to top students. She received her bachelor's degree in Clinical Microbiology in 2006. Following, she was employed as a research assistant at the International Center for Medical Research and Training (CIDEIM), located in her hometown. For two years, she worked on the antimicrobial resistance line, which was dedicated to the detection of mechanisms of resistance in Gram negative bacteria, isolated from tertiary care hospitals in Colombia. This research particularly focused on studying carbapenem resistance. In 2009, she was awarded the Utrecht Excellence Scholarship to fully fund her Molecular and Cellular Life Sciences master's program at Utrecht University, The Netherlands. During this program, she performed her minor internship at the Hubrecht Institute, under the supervision or Dr. Katherine Rabouille, investigating the role of GRASP65 in the transport of junctional proteins in mammalian epithelial cells (MDCK). She performed the major research project of her master's studies under the supervision of Prof. dr. Fulvio Reggiori in the department of Cell Biology at the University Medical Center Utrecht (UMCU), where she studied the molecular basis of the interaction between Atg2, Atg18 and Atg9 autophagy proteins in yeast. Upon completion of her master's internships, she wrote her master thesis entitled "The molecular mechanism of *Coxiella burnetii* autophagy-mediated infection". She received her Master of Science degree in 2011. Also in 2011, she started her PhD project in the department of Medical Microbiology at the UMCU in the group of Prof. dr. Marc Bonten, under the supervision of Prof. dr. ir. Willem van Schaik and Prof. dr Rob Willems. The results of her PhD research are reported in this thesis and have been published in various international scientific journals.

Ana María has moved to Santa Barbara, California, USA to continue pursuing her dreams and career.