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# Adaptations of hospital-acquired *Enterococcus faecium*

## Aanpassingen van in het ziekenhuis verworven *Enterococcus faecium*

(met een samenvatting in het Nederlands)

**Proefschrift**

ter verkrijging van de graad van doctor aan de
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**Vincent de Maat**

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Prof. dr. R.J.L. Willems
Prof. dr. W. van Schaik

**“The journey. Not the destination matters.”**

**- T.S. Eliot**

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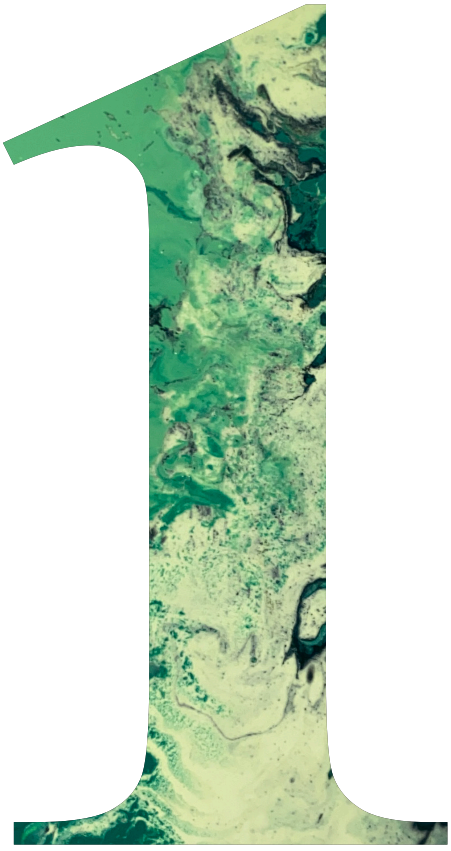

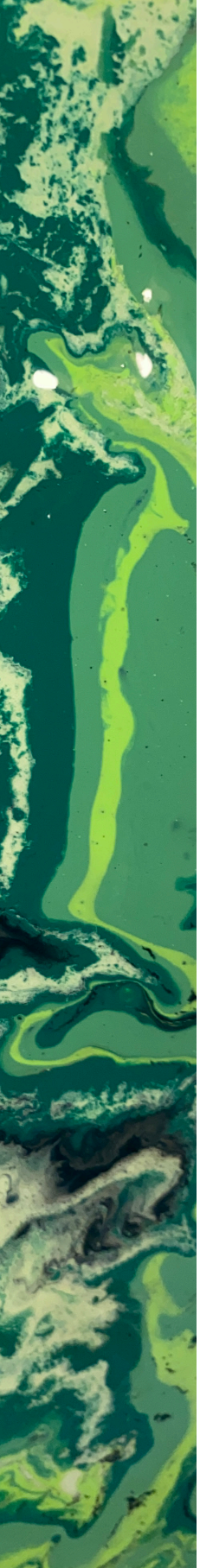

# Chapter 1

## General introduction

Bacteria are everywhere around us and most of these live alongside us without causing harm. This is, to a large extent, also the case for the genus *Enterococcus* which is a Gram-positive commensal in the gut of many mammals, insects and aquatic species (1–3) However, two species, *Enterococcus faecalis* and *Enterococcus faecium*, have become an increasingly important cause of hospital-associated infections. Since the 1970s these bacteria have emerged as opportunistic pathogens with *E. faecalis* causing approximately 60% of all enterococcal infections and *E. faecium* being responsible for the remainder (4). While both species have the capacity to cause nosocomial infections, there is a clear difference between *E. faecalis* and *E. faecium* in the distribution of adaptive genetic elements between hospital and commensal strains. In *E. faecium*, a clear divide can be found between clones that are associated with healthy humans, animals and hospitalized patients (5–7). This is, however, not the case for *E. faecalis,* where strains causing clinical infections are not exclusively found in hospitals but are also present in healthy individuals and animals (2, 6)**.** The adaptation strategies of these two species are thus considerably different with *E. faecalis* having a broadly generalist lifestyle while *E. faecium* exhibits a degree of host specialisation. *E. faecium* and *E. faecalis* are also distinct in their ability to acquire antibiotic resistance determinants. In *E. faecium* resistance to ampicillin, aminoglycosides, and fluoroquinolones is common, particularly in clinical isolates, while resistance to the glycopeptide vancomycin is also increasingly detected. In *E. faecalis* resistance to aminoglycosides is also common, but resistance to ampicillin and vancomycin is rarer than in *E. faecium* (2, 8–10). In **Figure 1** an overview of prevalence of aminopenicillin and vancomycin resistance throughout Europe in 2008 and 2018 is shown. This figure highlights the difference in antibiotic resistance acquisition between *E. faecalis* and *E. faecium*, where the latter species does not only have a higher incidence of resistance across the continent but is also more widely distributed throughout Europe. Acquiring resistance to antibiotics is not the only adaptation in clinical strains of *E. faecium* that aid in their success as hospital-adapted pathogens. Other relevant adaptations include the ability to survive in the bloodstream or wounds, the formation of biofilms, tolerance to disinfection and an ability to efficiently transfer from patient to patient, which will be further discussed below (5, 11–15).

## Population structure and genetic adaptations of clinical *E. faecium* strains

Advancements in genome sequencing have allowed extensive phylogenetic studies on *E. faecium* isolates from healthy humans, animals and patients. These studies identified three major clades, in which Clade A1 predominantly contains isolates from hospitalized patients, Clade A2 mainly has animal isolates and is closely related

to Clade A1. Clade B, is predominantly occupied by commensals from healthy humans (7, 16)Originally it was thought that nosocomial strains were derived from animal isolates, however recent studies show that there is limited sharing of genes between life stock and nosocomial strains (7, 17, 18).

Additionally there are indications that Clade A2 is not a single clade, as was previously thought, but consists of specialized clusters of strains that can be grouped on the basis of their animal host (7, 18). Strains from clade A1 have acquired a variety of adaptations that aid them in their success as a hospital-adapted opportunistic pathogen. These adaptations include mechanisms that aid to invade the body, to evade the immune system and to adapt to the relatively low nutrient levels found in blood (19–21).

The ability to form biofilms on abiotic surfaces is also recognized as an important virulence property of enterococci (22–24). Enterococci have been associated with biofilms in endocarditis, urinary tract infections, root canal infections, and ocular infections (24–27) and additionally in device-related infections such as artificial hip prostheses, prosthetic heart valves, intrauterine devices, and catheters (24, 28–30). A biofilm is a tightly connected growth of microbial cells attached to a surface. The biofilm is enclosed in a matrix of primarily polysaccharide material giving the community rigidity and providing it with the capacity to adhere to surfaces. This stable and enclosed community of microbial cells is an optimal environment to exchange genetic material between bacteria and increase resistance to environmental stress, antibiotics, and the host immune response (24, 31). While biofilm formation is a common trait for enterococci, the integrative and conjugative element ICE*Efm1*, which contains the *esp* operon, is predominantly found in strains belonging to clade A1 and that are associated with infections. Esp is a surface protein associated with adhesion and biofilm formation (12, 32). Deletion mutants in *esp* have a significantly decreased adhesion to polystyrene and biofilm formation (12, 32). Strains with ICE*Efm1* will thus form biofilms more readily, which will enhance virulence of *E. faecium* as evidenced by the contribution of Esp in an animal model of urinary tract infection (33) and endocarditis (12).

Although the exact mechanistic underpinnings are not always fully explored, studies have revealed that *E. faecium* genes associated with increased risk of bacteremia are more prevalent in clade A1(12, 34). Among these are *E. faecium* genes encoding TIR- (Toll/interleukin-1 receptor-) domain-containing proteins which interact with human Toll-like receptors (35–37). Toll-like receptors are key components of the innate immune response as they recognize highly conserved microbial structures,

termed pathogen-associated molecular patterns (PAMPs) (38, 39). Bacterial TIR-domain-containing proteins actively block detection of PAMPs by Toll-like receptors or even initiate degradation of the receptors thus preventing or limiting an adequate immune response (35, 37, 40).

## *E. faecium* and the hospital environment

To be successful as a nosocomial pathogen, *E. faecium* does not only require adaptations to colonize and invade patients, it also needs to adapt to the unique environment that is present in hospitals. In general all gut microbes will need to be able to survive outside their host, in an environment that may be inhospitable to them, to transfer from host to host (1, 5). *E. faecium* may find itself in the environment for considerable periods of time before it can enter a new host through the fecal-oral route. For this reason, *E. faecium* possesses, as a non-spore-forming bacterium, a remarkable ability to survive for prolonged periods of time outside a host. Several studies have been performed to determine the potential of nosocomial pathogens to survive outside their host in the environment (15, 41–44). Hospitals form a particularly challenging environment for bacteria, as staff aims to minimize microbial load on surfaces to reduce the risk of patient-to-patient spread. Floors and surfaces are cleaned often, beds are cleaned between patients, and doctors and nurses implement hand hygiene frequently, thus posing additional challenges for bacteria in the hospital environment. Several bacterial species, including coagulase-negative staphylococci (CNS), *Staphylococcus aureus* and enterococci survive for one week to more than 90 days on an inanimate surface (41).

Several studies have investigated why enterococci survive well in the environment, revealing that the coevolution of enterococci with mammals has shaped the genus to be highly adaptable and resilient to starvation, desiccation, low temperatures, and a wide range of pH (46, 47), combined with the ability to utilize numerous carbon sources (1). While these adaptations are also present in other bacteria, the combination of these traits in *Enterococcus* results in a superior ability to survive outside he host. *E. faecium* can survive 3- to 5-times longer on inanimate objects compared to other Gram-positive nosocomial pathogens, such as *Enterococcus faecalis*, *Staphylococcus aureus*, and *Streptococcus* (41, 44, 45, 48–50). Research into the mechanisms by which bacteria manage to survive these nutrient-limited conditions has so far mostly focused on *Escherichia coli* and has only recently been extended to other species (47, 51–53). Despite the limited knowledge on starvation and environmental survival, there are a handful of genes and molecular systems known that play a vital role in survival or recovery from stationary phase (54–56). One such molecular mechanism is the stringent response under control of

the signal molecule (p)ppGpp (53, 57, 58). This highly conserved mechanism can be found in most Gram-positive and Gram-negative bacteria in which the levels of (p)ppGpp regulates bacterial response to starvation, heat shock, oxidative stresses and desiccation (53, 57, 58). While the stringent response is conserved, the regulatory pathways leading to the accumulation of (p)ppGpp and the identity and functions of genes regulated through the stringent response may differ among bacterial species.

## Alcohol and disinfectant tolerance

While most bacteria that inhabit the human body have a symbiotic or commensal relationship with their host, a relatively small number of species can cause infections, particularly in immunocompromised individuals. Particularly, *E. faecium* presents an significant challenge since it tends to densely colonize the large intestine of patients on an antibiotic regime (59, 60). This contributes to the presence of high levels of *E. faecium* on the skin and in the immediate environment of the patient, which increases the chance of an infection at a catheter, intravenous drip or operation wound sites (61, 62). To minimize the risk of infections by opportunistic pathogens like *E. faecium*, there are strict hygiene rules in hospitals to reduce the transmission of nosocomial pathogens, including vancomycin-resistant enterococci. The immediate environment of a patient is frequently cleaned to prevent spread of pathogens and healthcare workers are required to clean their hands in between treating patients to minimize transfer of pathogens throughout a ward (63–65). Several studies have demonstrated that disinfection of catheter sites with antiseptic agents such as chlorhexidine can reduce risk of hospital-acquired bloodstream infections by multi-drug resistant bacteria like *E. faecium* and *S. aureus* (61, 66, 67). Some studies show that bathing high-risk patients with chlorhexidine-containing solutions can reduce VRE contamination of the patient's skin by 2.5 logs compared to regular water and soap washing. In addition VRE contamination on surfaces and healthcare workers were reduced by 30% and 40%, respectively (68). These findings have been confirmed by several studies (62, 69, 70) but are disputed by others (71). In addition there is evidence that use of cationic biocides, like chlorhexidine, can contribute to cross-resistance in *E. faecium* to antibiotics including vancomycin, ampicillin, cefotaxime, ceftazidime, sulfamethoxazole and bacitracin (72–74). Additionally *E. faecium* is able to develop intrinsic resistance to chlorhexidine which further limits the effectiveness of preventative treatment of chlorhexidine-based body washes (74, 75).

An additional line of defense against the spread of *E. faecium* in the hospital is the used of disinfectants containing alcohols. Most hospitals have implemented alcohol-based hand rubs (ABHR) for healthcare workers to easily, effectively and quickly clean their hands in between patients. Since 2009, the composition and

minimal efficacy of ABHR have been formalized by the World Health Organization, which has contributed to the widespread use of ABHR and resulted in a decline in prevalence of several nosocomial pathogens, including methicillin-resistant *Staphylococcus aureus* (65, 76). ABHR consist of at least one alcohol, most often 2-propanol, 1-propanol and ethanol, and additional anti-microbial agents, such as mecetronium ethyl sulphate, to further increase their efficacy (64, 65). Despite the use of ABHR, *Enterococcus* outbreaks are still common and have led to research into the emergence of alcohols tolerance in clade A *E. faecium* strains (77). Tolerance to alcohols is known to exist in other bacteria, including in *Acinetobacter baumanii* and some lactobacilli, but the exact mechanisms by which these organisms achieve tolerance is unknown (78, 79). While clinical *E. faecium* strains have been reported to be tolerant to alcohols, which has been hypothesized to contribute to their ability to spread through hospitals (77), more mechanistic studies are required to fully understand the extent of alcohol tolerance in *E. faecium* and the molecular mechanisms that are contributing to this trait.

## Functional genomics in *E. faecium*

To understand microbial behavior, it is of importance to generate mutants to characterize the functional role of genes in any relevant phenotype. The genetic tools for the genetic manipulation of *E. faecium* are currently limited, with clinical isolates being notoriously difficult to study due to the high prevalence of genes conferring resistance to antibiotics that are routinely used in the laboratory, including erythromycin, ampicillin and chloramphenicol (2, 8, 21). In addition, most genetic modification strategies rely on plasmids to carry genomic modifications into the bacterium. Clinical strains of *E. faecium* often carry numerous plasmids which can cause issues due to plasmid incompatibility during the generation of mutants (16). An important extension of the genetic toolbox of *E. faecium* was provided by the development of microarray-based transposon mapping (M-TraM (80)) which allowed for the unbiased screening of all non-essential genes under various biological relevant conditions. M-TraM utilized the random insertion of *mariner* transposons to generate a mutant library. The relative abundance of transposons was detected by microarray-based hybridizations, resulting in an overview of all transposon locations and the identification of conditionally essential genes. While M-TraM was used to identify genes with a role in resistance to ampicillin (80), bile salts (81) and chlorhexidine (74), the technique had several limitations including being labour-intensive and a potential bias for microarray hybridization with shorter fragments which could impact the detection of transposons and the accurate quantification of their abundance. Most of these drawbacks are due to the digestion of the genome with AluI in M-TraM, which results in various fragment lengths.

Once genes of interest have been identified it is common practice to generate deletion mutants in those genes to confirm and characterize their function within *E. faecium*. The current method relies solely on homologous recombination to replace genes with an antibiotic resistance cassette, which can later be removed via the *Cre-lox* system (80). This method is not particularly efficient, requiring screening of hundreds of colonies screened by PCR before a successful deletion mutant is generated. Additionally, there is currently no method to generate insertion mutants or targeted point mutations without causing scars in the genome. There is thus considerable room for improvement in the genetic toolbox for the study of clinical *E. faecium* isolates.

# OUTLINE OF THIS THESIS

This thesis will focus on identifying and characterizing new genetic elements which allow clinical strains of *E. faecium* to adapt to a nosocomial lifestyle. For this, we aimed to develop new techniques to perform large scale genomic screenings and improve targeted mutagenesis in *E. faecium*.

In **Chapter 2** we outline a new genomic toolkit to facilitate the generation of targeted mutants in clinical *E. faecium* strains. By implementing CRISPR-Cas9 as a counter-selective strategy against wild-type cells, we reduce the screening required to isolate mutants and prevent the need of integration of genetic markers in the genome, while also allowing for gene insertions and point mutations to be made.

In **Chapter 3** we implement transposon insertion sequencing (Tn-seq) to identify genes that are vital for growth in human serum in the clinical *E. faecium* strain E745. Tn-seq, combined with transcriptome analysis by RNA-seq, revealed the importance of carbon uptake, transcriptional regulation and the biosynthesis of purine and pyrimidine nucleotides for growth in human serum.

The aim of **Chapter 4** is to increase our understanding of the starvation response of *E. faecium* E745. The strain was exposed to nutrient limiting conditions for 7 days(?), after which Tn-seq was performed to uncover genes that impact survival in the absence of nutrients. In total we identified 18 unique genes covering multiple functional groups including, metabolism, DNA repair, general stress response and cell wall homeostasis. We confirmed the role of the *usp* gene, which encodes a universal stress protein, in survival during starvation.

In **Chapter 5**, we used Tn-seq to identify genes involved in tolerance to 2-propanol. We first examined strains from different isolation years and sources and show they have varying levels of tolerance to 2-propanol. The clinical strain *E. faecium* E8202 was among the most tolerant strains and Tn-seq was performed to identify genes involved in alcohol tolerance. We identified three genes that were conditionally essential for alcohol tolerance, one of which, *spxA* (encoding a transcription regulator) was experimentally confirmed to contribute to 2-propanol tolerance.

The implications of the studies described in this thesis are discussed in **Chapter 6**.

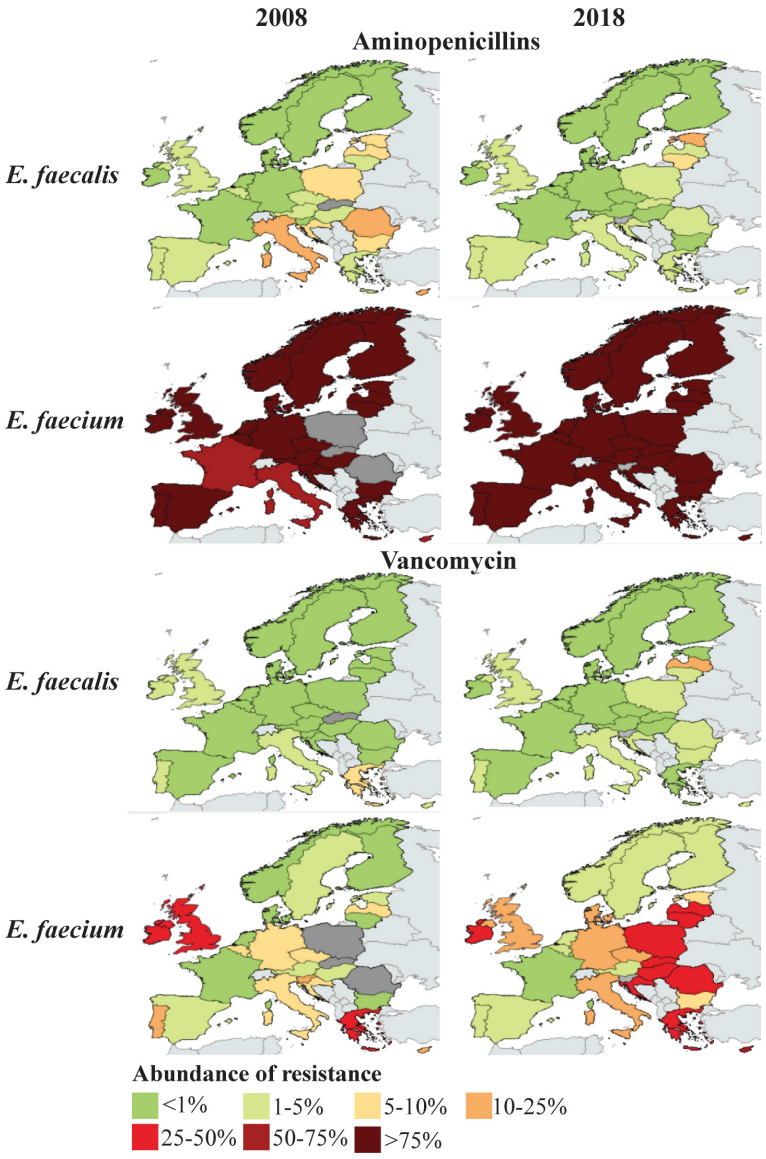


**Figure 1: Overview of aminopenicillin and vancomycin resistance in *E.faecalis* and *E. faecium* in 2008 and 2018 throughout Europe.**

Image is adapted from the ECDC Surveillance Atlas – Antimicrobial Resistance. Data are based on antimicrobial resistance data from invasive isolates reported to the European Antimicrobial Resistance Surveillance Network by 30 EU/EEA countries. (*https://atlas.ecdc.europa.eu/public/index.aspx?Dataset=27&HealthTopic=4*)

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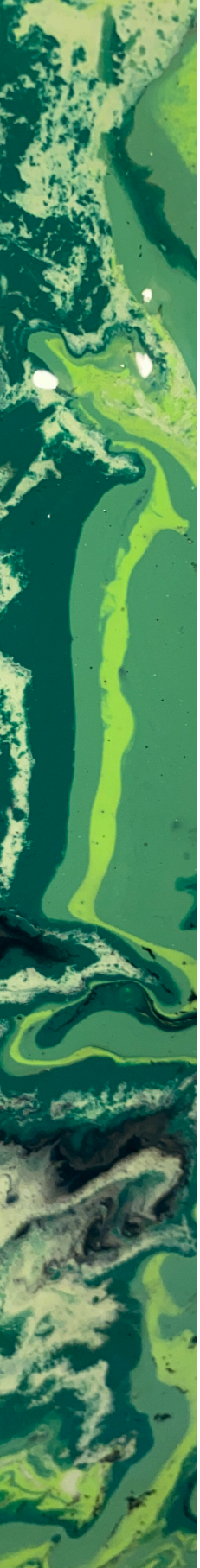

# Chapter 2

## CRISPR-Cas9-mediated genome editing in vancomycin-resistant *Enterococcus faecium*

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# ABSTRACT

The Gram-positive bacterium *Enterococcus faecium* is becoming increasingly prevalent as a cause of hospital-acquired, antibiotic-resistant infections. A fundamental part of research into *E. faecium* biology relies on the ability to generate targeted mutants but this process is currently labour-intensive and time-consuming, taking 4 to 5 weeks per mutant. In this report, we describe a method relying on the high recombination rates of *E. faecium* and the application of the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-Cas9 genome editing tool to more efficiently generate targeted mutants in the *E. faecium* chromosome. Using this tool and the multi-drug resistant clinical *E. faecium* strain E745, we generated a deletion mutant in the *lacL* gene, which encodes the large subunit of the *E. faecium* β-galactosidase. Blue/white screening using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) could be used to distinguish between the wild-type and *lacL* deletion mutant. We also inserted two copies of *gfp* into the intrinsic *E. faecium* macrolide resistance gene *msrC* to generate stable green fluorescent cells. We conclude that CRISPR-Cas9 can be used to generate targeted genome modifications in *E. faecium* in 3 weeks, with limited hands-on time. This method can potentially be implemented in other Gram-positive bacteria with high intrinsic recombination rates.



**Keywords:** *Enterococcus faecium*; genome editing; CRISPR-Cas9; molecular biology; mutants; counterselection

# INTRODUCTION

Antibiotic resistance is currently recognised as a global threat to human health (Ferri *et al.* 2017). Enterococci are among the most problematic multi-drug resistant bacteria causing infections among hospitalised patients, contributing to 10 000– 25 000 deaths per year in the USA alone (McKinnell *et al.* 2012). Clinically, the two most important enterococcal species are *Enterococcus faecalis* and *Enterococcus faecium*. While historically *E. faecalis* has been the most prominent enterococcal pathogen, since the 1990s *E. faecium* has rapidly emerged as a nosocomial pathogen of major importance. Infections caused by *E. faecium* are generally more difficult to treat as vancomycin resistance is more widespread in *E. faecium* than in *E. faecalis* (Gilmore, Lebreton and Schaik 2013; García Solache and Rice 2019). Until we understand the molecular underpinnings that contribute to the transfer of antibiotic-resistant genes and pathogenicity, we will be hampered in our ability to develop treatment strategies. To drive functional studies, efficient g enome editing tools are essential, which are currently lacking. Current methods to generate targeted mutations in *E. faecium* mostly rely on allelic exchange between the chromosome and a temperature-sensitive vector which contains an antibiotic resistance cassette and sequences that flank the target site on the *E. faecium* genome (Maguin *et al.* 1996; Nallapareddy, Singh and Murray 2006; Zhang *et al.* 2012). The antibiotic cassette can be removed using the Cre- *lox* system, but a single *lox* site remains as a scar (Zhang *et al.* 2012). These protocols are time-consuming, taking upwards of 4 to 5 weeks. The process involves several days of sub-culturing and selection of colonies on media with different antibiotics, to screen for a double cross-over event and then removal of the resistance marker by Cre-*lox*. In addition, extensive screening by colony PCR is needed to eliminate false positives and retrieve the desired double cross-over mutant. The process to generate targeted mutants in *E. faecium* was improved by the use of counter-selection system against single cross-over mutants by the use of *pheS*∗, a mutated allele of the *E. faecalis* phenylalanyl tRNA synthetase α-subunit that confers susceptibility to p-chloro-phenylalanine in enterococci (Kristich, Chandler and Dunny 2007; Thurlow, Thomas and Hancock 2009; Somarajan *et al.* 2014; Bhardwaj, Ziegler and Palmer 2016).

To further expand the genetic toolbox for multi-drug resistant *E. faecium*, we explored the use of clustered regularly interspaced palindromic repeats (CRISPR) and its associated Cas9 protein to generate mutants in *E. faecium*. The Cas9 nuclease introduces double-strand breaks in DNA that is targeted by a CRISPR and, together with other CRISPR-asssociated proteins, serves as a defence against invading bacteriophages and plasmids in prokaryotes (Brouns *et al.* 2008; Marraffini and

Sontheimer 2008). The combination of CRISPR and Cas9 has been successfully used for genome editing in eukaryotes where CRISPR-Cas9 drives the generation of mutants by inducing double-strand DNA breaks, which are then repaired by non-homologous end-joining (NHEJ) (Cong *et al.* 2013). While some bacteria have NHEJ systems, there is no evidence for their presence in *Enterococcus* and other Lactobacillales and thus *E. faecium* can only escape the lethal effect of CRISPR-Cas9 targeting a chromosomal site by utilising homologous recombination (HR) (Bowater and Doherty 2006). One approach to use CRISPR-Cas to identify recombinant genotypes is to introduce a vector that contains DNA identical to the flanking sequence of the target region while the cell produces Cas9 and a CRISPR-array homologous to the target sequence. Most surviving cells will have undergone a HR event thereby escaping CRISPR-Cas-mediated killing (Jiang *et al.* 2013; Wang et al. 2015, 2018). Genome editing approaches using HR and CRISPR-Cas9 have been used for numerous bacterial species, including Gram-positive lactic acid bacteria (Mougiakos *et al.* 2016; Leenay *et al.* 2019). *Enterococcus faecium* has a high intrinsic recombination rate and readily integrates novel exogenous DNA in its genome (de Been *et al.* 2013), making it particularly suited for the implementation of CRISPR-Cas9 as a counterselection strategy during the generation of targeted mutants.

In this study, we aimed to develop a CRISPR-Cas9 based genome editing approach for *Enterococcus faecium*. We adapted a CRISPR-Cas9-based genome editing approach previously developed for the lactic acid bacterium *Lactobacillus reuteri* (Oh and Van Pijkeren 2014), relying on the high intrinsic recombination rate of *E. faecium* for allelic exchange combined with CRISPR-Cas9 to counterselect against wild-type cells.

# MATERIALS AND METHODS

## Bacterial strains, plasmids, growth conditions and oligonucleotides

The vancomycin-resistant *E. faecium* strain E745 (Zhang *et al.* 2017) was used throughout this study. This strain was isolated from a rectal swab of a hospitalized patient, during routine surveillance of a VRE outbreak in a Dutch hospital. Unless otherwise mentioned, *E. faecium* was grown in brain heart infusion broth (BHI; Oxoid) at 37°C. The *E. coli* strain EC1000 (Leen-houts *et al.* 1996) was grown in Luria-Bertani (LB) medium at 37°C with shaking at 200 rpm. *Lactobacillus lactis* MG1363 was grown in M17 broth supplemented with 0.5% w/v lactose. When required, antibiotics were used at the following concentrations: erythromycin 50 μg $ml^{-1}$ for *E. faecium* and 5 μg $ml^{-1}$ for *L. lactis* and spectinomycin 200 μg $ml^{-1}$ for

*E. faecium*, 100 µg ml$^{-1}$ for *E. coli*, and tetracycline 10 µg ml$^{-1}$ for *L. lactis*. Where indicated, plates were supplemented with 20 µg ml$^{-1}$ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The vectors pREG696 (Grady and Hayes 2003), pWS3 (Zhang *et al.* 2011) and pET-3α (Novagen) were obtained from our laboratory's culture collec-tion. pREG696-*gfp* was derived from pREG696 by inserting the *gfp* gene under the control of the promoter of the *bacA* gene (Pbac) of *E. faecalis* (Heikens, Bonten and Willems 2007) in the NotI and XhoI restriction sites of pREG696 (J. Top, personal communication). Plasmids pVPL3004 and pVPL3115 were described in previous work (Oh and van Pijkeren 2014). The sequences of the oligonucleotides used in this study are listed in Table 1.

## Isolation and transformation of plasmids

Plasmid isolation from *E. coli* was performed using the GeneJET plasmid miniprep kit (Thermo Fischer Scientific, Bleiswijk, the Netherlands) according to the manufacturer's instructions. Isolation of plasmids from *L. lactis* was as described previously (O'Sullivan and Klaenhammer 1993) with slight modifications. In short, 5 ml overnight cultures were centrifuged for 10 min at 3000 g. The cell pellet was resuspended in 250 µl THMS-buffer (30 mM Tris-HCL pH 8, 25% sucrose, 3 mM $MgCl_2$) supplemented with 2 mg ml$^{-1}$ lysozyme. The cell suspension was incubated for 10 min at 37°C after which 500 µl 1% SDS in 0.2 M NaOH was added. The tubes were mixed gently and incubated on ice for 5 min. About 375 µl ice-cold 3 M potassium acetate pH 5.5 was added and mixed by inversion, followed by incubation on ice for 5 min. Cell debris was pelleted via centrifugation at 20 000 g for 5 min, after which the supernatant was transferred to a new tube and an equal amount of isopropanol was added. After a 10-min incubation at room temperature the tubes were centrifuged at 20 000 g for 10 min to precipitate the DNA. The pellet was washed with 70% ethanol, air dried and dissolved in sterile $dH_2O$. Transformation of plasmids into *E. faecium* E745 was performed as previously described (Zhang *et al.* 2012), typically resulting in 500– 1000 transformants/µg DNA.

## Construction of the pVDM1001 CRISPR delivery vector and generation of *lacL*-deletion and *gfp*-insertion mutants

We first aimed to construct a vector that could be used for genome editing in *E. faecium* E745. This vector, termed pVDM1001, was constructed by cloning a 0.7-kbp fragment, which contains the CRISPR sequences from pVPL3115 in the XhoI and EcoRI sites of pWS3. The fragment was amplified from pVPL3115 using the primers oVDM1001–oVDM1002. The pVDM1001 vector was then implemented for the generation of a *lacL* deletion and *gfp* insertion mutant by modifying the CRISPR sequence via digestion with BsaI and annealing two oligos, oVDM1022-oVDM1023

and oVDM1024-oVDM1025, which contain a protospacer targeting *lacL* or *msrC,* respectively. This created pVDM-x*lacL* and pVDM-x*msrC*. CRISPRs were designed by identifying 30 bp sequences, that were directly followed by the protospacer adjacent motif (PAM) NGG (Jiang and Doudna 2017). The CRISPR was only used if the last 6 bp, corresponding to the seed sequence, which is crucially important for target site recognition (Jiang and Doudna 2017), did not align to another site on the *E. faecium* E745 genome with an immediately adjacent PAM site. Finally, additional nucleotides were added to create the necessary overhang for ligation into the BsaI site in pVDM1001. The nucleotide sequence of pVDM1001 has been made available on NCBI Genbank with accession number MN580666.

To create a *lacL* deletion mutant, a DNA template consisting of a 365 bp upstream region of *lacL* fused together with a 225 bp downstream region of *lacL* (Table S1) was ordered from Integrated DNA Technologies (Leuven, Belgium) and amplified using oVDM1003–oVDM1004. The amplified template was cloned into pVDM-x*lacL* after digestion with SmaI and a blunt end ligation creating pVDM-*dlacL*.

To create a *gfp* knock-in construct we amplified 773 bp upstream region of *msrC* and a 507 bp fragment overlapping with the 3' region of *msrC* using primers oVDM1012–oVDM1013 and oVDM1014–oVDM1015, respectively. Each fragment was separately cloned into pWS3 using KpnI-ApaI for the upstream fragment and SmaI-NotI for the downstream fragment, creating pWS3-*msrCup* and pWS3-*msrCdwn*, respectively. Downstream of the *msrCup* fragment a Pbac promotor was inserted. The promotor site was amplified from pREG696-*gfp* using primers oVDM1020–oVDM1021 and inserted after ApaI-EcoRI digestion creating pWS3-*msrCup*-Pbac. To pWS3-*msrCdwn* a T7 terminator was added which was amplified from pET3α using primers oVDM1028-oVDM1029 and digested with SmaI-SpeI to create pWS3-T7-*msrCdwn*. pWS3-*msrCup*-Pbac was then digested with KpnI-EcoRI and the *msrCup*-Pbac fragment was transferred to pWS3-*msrCdwn*-T7 to create pWS3-*msrC*-Pbac-T7. To compensate for the low copy number of the *gfp* integration in the chromosome, we amplified two copies of *gfp* from pREG696-*gfp* (laboratory collection) using primers with different restriction sites, oVDM1016–oVDM1026 (EcoRI-SphI) and oVDM1018–oVDM1027 (SphI-SmaI), and consequently ligated together after digestion with SphI. This construct with two *gfp* genes in tandem was inserted into pWS3-*msrC*-Pbac-T7 via EcoRI-SmaI digestion creating the complete *msrC::gfp* template. This template was amplified using oVDM1052-oVDM1053 and transferred to pVDM1001 by digestion with SmaI creating pVDM-*msrC::gfp*.

**Table 1.** List of oligonucleotides used in this study.

| Name | Sequence 5 - 3 (restriction sites are underlined) |
|---|---|
| oVDM1001 | AAAACTCGAGCCACTCACCATGGGTACTGCAG |
| oVDM1002 | AAAAGAATTCAACGTTGGCGATTCGTTGGCGATTGA |
| oVDM1003 | /5Phos/GGCGAGTCCTTTTGAAGAAAATATTGCC |
| oVDM1004 | /5Phos/AGCCATTCTTTTCCGTTTTTATTGAGCG |
| oVDM1005 | TCATTGTCGCAACAGATAGC |
| oVDM1006 | GGAACATCTGTGGTATGGCG |
| oVDM1007 | GGCCGAATTGATGACAGTTG |
| oVDM1008 | CTCTCCAGCGATTTGGTAG |
| oVDM1009 | GTAGGCAATCTGTACCACTC |
| oVDM1011 | TGCGTCCTTTGATCCGTTTC |
| oVDM1012 | CACGATGGTACCTGCGTCCTTTGATCCGTTTC |
| oVDM1013 | CATGATGGGCCCCATGTAAAACAACAATTATCG |
| oVDM1014 | CATGATACTAGTATCCGCAAACAAGGAGAAGG |
| oVDM1015 | CTAGATGCGGCCGCGTAGGCAATCTGTACCACTC |
| oVDM1016 | CATGATGAATTCAGGAGGATTAACATATGAGCAAAGGAGAAG |
| oVDM1018 | CATGATGCATGCATGAGCAAAGGAGAAG |
| oVDM1020 | CATGATGGGCCCGCTTGCATCAAAATAAAC |
| oVDM1021 | CACGATGAATTCGTAGAAAATATTTTTGAAATGCATTTC |
| oVDM1022 | AAACGATCTTCAGAGATGTCTTCTTAGTTGCTCGG |
| oVDM1023 | AAAACCGAGCAACTAAGAAGACATCTCTGAAGATC |
| oVDM1024 | AAACTTCCGCTCTGAAGTTTCTTCCAGTCTTAACG |
| oVDM1025 | AAAACGTTAAGACTGGAAGAAACTTCAGAGCGGAA |
| oVDM1026 | CACTATGCATGCTTAGTGGTGGTGGTGGTGGTGGGATC |
| oVDM1027 | CATGATCCCGGGTTAGTGGTGGTGGTGGTGGTGGGATC |
| oVDM1028 | CTAGATCCCGGGGCTGAGCAATAACTAGCATAAC |
| oVDM1029 | CACGATACTAGTCAAAAAACCCCTCAAGACC |
| oVDM1052 | /5Phos/TGCGTCCTTTGATCCGTTTC |
| oVDM1053 | /5Phos/GTAGGCAATCTGTACCACTC |
| oVDM1054 | GGGCGGTGATCACTGATGAATATA |
| oVDM1055 | ACCAATAATTCCTCAGTACCATCCAT |
| oVDM1056 | ATGACCAATTTGATTAACGG |
| oVDM1057 | CTAATTGAGAGAAGTTTCTATA |

To perform the chromosomal modifications we first transformed E745 with pVPL3004, with selection for transformants by plating on BHI with 50 μg $ml^{-1}$ erythromycin and 24 h incu-bation at 37°C. Presence of pVPL3004 in E745 was confirmed via PCR using primers oVDM1005-oVDM1006. A colony positive for pVPL3004 was grown in the presence of 50 μg $ml^{-1}$ erythromycin and made competent to receive pVDM1001 or one its derivates described above. After transformation with these vectors the transformants were selected on BHI agar with 200 μg $ml^{-1}$ spectinomycin and 70 μg $ml^{-1}$ erythromycin and incubated 48–72 h at 30°C. Successful deletion of *lacL* was confirmed by PCR with primers oVDM1007-oVDM1008. Insertion of *gfp* was confirmed by PCR with primers oVDM1009-oVDM1011.

### Curing of CRISPR and Cas9 plasmids

A colony that was positive for the desired mutation was transferred to 200 ml BHI without antibiotics and incubated overnight at 37°C at 250 rpm after which 200 µl was transferred to 200 ml pre-warmed BHI and incubated overnight at 37°C. This process was repeated a third time after which a 100 µl sample was taken and diluted 1000 times of which 25 µl was transferred and spread on a BHI agar plate. After 24 h incubation at 37°C, 50 colonies were transferred to BHI agar, BHI agar with 200 µg $ml^{-1}$ spectinomycin or BHI agar with 50 µg $ml^{-1}$ erythromycin. After incubation overnight at 37°C the plates were examined for colonies that were susceptible to both spectinomycin and erythromycin. Curing of the Cas9 delivery vector pVPL3004 and the CRISPR containing vectors derived from pVDM1001 was confirmed via colony PCR using the primer sets oVDM1054-oVDM1055 and oVDM1056-oVDM1057, respectively.

### Flow cytometric analysis of GFP fluorescence in E745

To confirm the phenotype of the *gfp* integration mutant, cultures of E745, E745::*msrC::gfp*, and E745 + pREG696-*gfp* in 3 ml BHI, supplemented with 250 µg $ml^{-1}$ spectinomycin if required, were started and incubated overnight at 37°C. The fluorescence of the cultures was then determined by flowcytometric analysis after adjusting the cultures to an $OD_{600}$ of 0.2. These were then diluted 25-fold in a 2-ml volume of PBS of which 200 µl was transferred to a round bottom 96-well plate, which was placed into a MACSQuant (Miltenyi Biotech) machine. Flow cytometric analysis was performed by measuring fluorescence at 488 nm excitation and 525 nm emission at 35.000 events in total. Bacteria were gated on single cells based on forward and side scatter. Data was further processed in FlowJo (FlowJo LLC).

## RESULTS AND DISCUSSION

### Implementation of CRISPR-Cas9-mediated genome editing in *E. faecium*

We initially attempted to combine single-stranded DNA recombineering and CRISPR-Cas genome editing in *E. faecium*, as was previously demonstrated in the lactic acid bacterium *Lactobacillus reuteri* (Oh and Van Pijkeren 2014). We were, however, unsuccessful in generating mutants in *E. faecium* using this methodology. Either not enough oligonucleotides were transformed into the cells due to the inherent low transformation efficiency in *E. faecium*, or the activity of the single-stranded DNA binding protein RecT was too low to support incorporation of the oligonucleotide into the chromosome. We then decided to adapt the *L. reuteri*

system by relying on the high intrinsic recombination rate of *E. faecium* for allelic exchange and by using CRISPR-Cas9 to counter select against wild-type cells. For this we used the vectors pVPL3004, which encodes Cas9 and pVPL3115, encoding the CRISPR array to which the protospacer target sequence can be added. To facilitate further adaptations needed for genomic modifications we transferred the CRISPR guide RNA section from pVPL3115 to the vector pWS3 to create pVDM1001. This plasmid has the benefit of having a temperature-sensitive replicon for Gram-positive bacteria and can replicate in *E. coli* EC1000, facilitating further cloning procedures.

The *E. faecium* CRISPR-mediated genome engineering plasmid thus relies on pVPL3004 and the novel vector pVDM1001 being present in the strain of interest (Fig. 1A). The general workflow is depicted in Fig. 1B**.** The plasmid pVPL3004 was first transformed into *E. faecium* E745 to allow for CRISPR-based genome modifications. We then exchanged the control protospacer in pVDM1001 for one that targets the region on the *E. faecium* chromosome that we intended to manipulate. Third, we added a HR template that contained the desired mutation. Lastly, the resulting pVDM1001-derived plasmid was transformed into E745 containing pVPL3004. Transformants were selected on BHI agar plates containing both erythromycin and spectinomycin, and were subjected to PCR to determine the recombinant genotype. As a proof-of-principle in this study, we generated a deletion mutant in *lacL* (locus tag: EfmE745 01561), the gene encoding the large sub-unit of the *E. faecium* β-galactosidase, and we integrated *gfp* in the chromosomal *msrC* gene (Singh, Malathum and Murray 2001)(locus tag: EfmE745 02638) to generate a fluorescently tagged *E. faecium* strain.

## Generation of a deletion mutant and a chromosomal integration mutant

To delete *lacL* we adapted pVDM1001 to contain a CRISPR targeting the wild type locus of *lacL* (pVDM-x*lacL*). The vector pVDM-*dlacL* contained, in addition to the CRISPR targeting *lacL*, a HR template consisting of two regions flanking *lacL*, which allowed the generation of a targeted deletion mutant. To insert *gfp* in the chromosome, we created a HR template containing flanking regions of *msrC* and two copies of *gfp* in tandem as a transcriptional fusion under control of the constitutively expressed Pbac promotor. We cloned the *gfp* HR template and a specific CRISPR targeting *msrC* into pVDM1001 to create pVDM-*msrC::gfp*.

In a representative experiment to generate the *lacL* deletion mutant, we transformed E745 + pVPL3004 with various constructs to quantify the emergence of spontaneously resistant or CRISPR escape mutants and the selective efficiency of the CRISPR. We

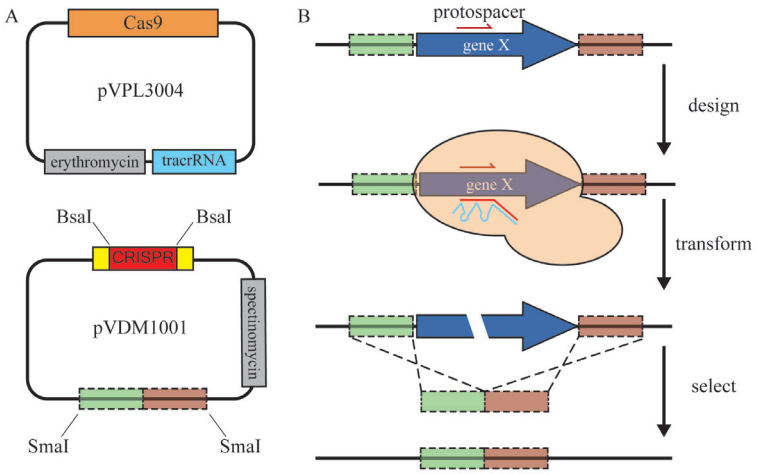


**Figure 1. Schematic overview of the CRISRP-Cas9-mediated genome editing.**
This system consists of two plasmids (panel A), pVPL3004; which contains cas9 from *S. pyogenes,* tracrRNA and an erythromycin selection marker, and pVDM1001; which contains a CRISPR targeting the desired region, the template DNA which carries the desired mutation and a spectinomycin selection marker. The general workflow for generating mutants is shown in panel B, and includes the design of the CRISPR-protospacer and repair template which are incorporated in pVDM1001. The second step is the transformation of the plasmids pVPL3004 and the relevant pVDM10001 derivative into *E. faecium*, followed by direct selection of the mutant.

transformed *E. faecium* E745 with $dH_2O$ (background), pVDM1001 (empty vector), pVDM-x*lacL* (carrying a CRISPR that targets *lacL*) and pVDM-*dlacL* (carrying both the *lacL*-targeting CRISPR and the HR template for the generation of the *lacL* deletion mutant). This resulted in 70, 250, 68 and 80 colonies, respectively, after selection on BHI agar plates containing erythromycin and spectinomycin to select for both pVPL3004 and pVDM1001 and its derivatives. The relatively high background in the water control revealed the appearance of spontaneously spectinomycin-resistant colonies. Our data also indicated that we could successfully transform pVDM1001, which lacks an *E. faecium* CRISPR-array or HR template, into *E. faecium*. The addition of a CRISPR that targets the *lacL* gene in pVDM-*xlacL* reduced colony numbers down to background levels (68 colonies versus 70 in the water control), suggesting that CRISPR-Cas9 generated lethal double-strand DNA breaks in the *E. faecium* chromosome. Transformation of pVDM-*dlacL* resulted in a slight increase in colony numbers (80 colonies), potentially indicating successful integration of the HR template. This was confirmed by PCR (Fig. 2A) and subsequent Sanger sequencing as we found that approximately 15% of screened colonies were *lacL* deletion mutants. We obtained comparable results in our attempt to integrate *gfp* in the *msrC* gene, with a background of spontaneously spectinomycin-resistant

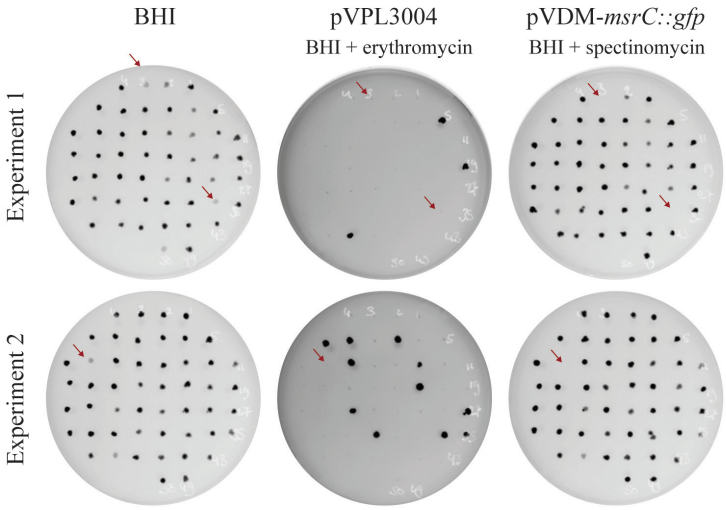


**Figure 2. Clearing efficiency of pVPL3004 and pVDM-*msrC::gfp*.**
After three days of sub-culturing to clear the plasmids, 50 colonies per mutant were transferred to BHI, BHI + 50 µg/ml erythromycin and BHI 200 µg/ml spectinomycin to screen for clones that have lost both plasmids (indicated by the red arrows). The overall clearance of pVPL3004 is 80%–90% and of pVDM-*msrC*::*gfp* is 2%–5%, resulting in at least one colony that has lost both plasmids. The results show results of two independent experiments to clear pVPL3004 and pVDM-*msrC*::*gfp* from the insertion mutant. Colonies were visualized by the ImageQuant LAS4000 imager through their production of GFP. Note that the fluorescent signal is lower in the *gfp* integration mutants than in the colonies where *gfp* is still present on a multi-copy plasmid.

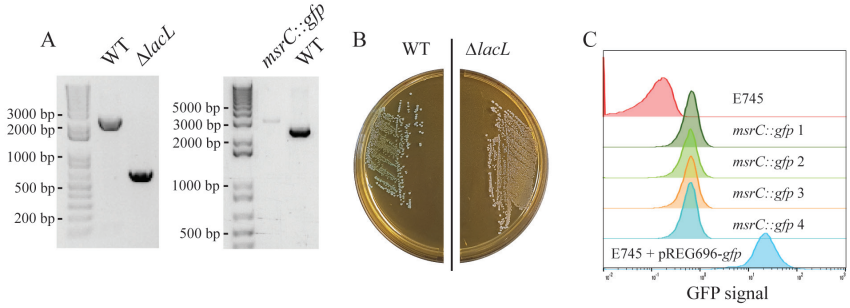


**Figure 3. Generation and phenotypes of the *dlacL* and *msrC*::*gfp* mutants.**
(A), Confirmation of *lacL* deletion and *gfp* insertion into *msrC* via PCR. Deletion of *lacL* results in a 1800 bp reduction in size of the PCR product from 2.5 kbp to 0.7 kbp, while insertion of the *gfp* construct into the *msrC* site results in a shift from 2.8 kbp to 3.2 kbp. (B), Growth of wild-type E745 and *dlacL* on BHI with 20 µg/ml X-gal. (C), Flow cytometric analysis of GFP fluorescence levels, from top to bottom, wild-type E745, four different *msrC*::*gfp* clones and, as a positive control, E745 containing pREG696-gfp.

mutants in the control experiments but a higher number of transformants upon electroporation with pVDM-*msrC*::*gfp* (data not shown). Our overall success rate in generating mutants was considerable higher in comparison to the homologous recombination-based technique we previously developed (Zhang *et al.* 2012), in which we routinely have to screen 100 or 200 colonies, after several days or even weeks of sub-culturing, before we can isolate the desired mutant that had undergone a double cross-over event.

Once we confirmed that we had successfully generated the *lacL* deletion mutant and the *msrC::gfp* insertion mutant, the CRISPR-related plasmids were cured by sub-culturing in BHI broth without antibiotics for three days, or between 20 and 25 generations. Between 50 and 100 colonies isolated from this culture were then transferred to three different BHI agar plates, i.e. BHI agar without antibiotics, BHI agar with spectinomycin and BHI agar with erythromycin to isolate colonies that had cleared both pVDL3004 and the pVDM1001-derivative. Two representative examples of experiments in which we cured the pVPL3004 and the pVDM1001-derivative are shown in Fig. 3. Curing ratios for pVPL3004 were typically around 60%–90% while pVDM1001-derived vectors was more difficult to cure as 1%–5% of colonies had lost the vector. Typically, we obtained 3 to 5 colonies in which both plasmids had cleared per 100 colonies.

## Phenotypic characterization of E745 *dlacL* and E745 *msrC::gfp*

Wild-type (WT) E745 and E745 *dlacL*, which were cleared of pVDL3004 and pVDM-*d*lacL as outlined above, were grown on BHI supplemented with the chromogenic substrate X-gal to confirm that the genomic alteration affected β-galactosidase activity. While WT colonies were light blue upon growth on medium containing X-gal, the E745::*dlacL* colonies were creamy white (Fig. 2B), indicating that they could no longer convert X-gal due to the lack of an active β-galactosidase. We determined production of GFP by flow cytometry (Fig. 2C) and we found that the GFP signal is higher in E745 *msrC::gfp* compared to WT, but considerably lower than the strain in which *gfp* is carried on a plasmid. This most likely reflects differences in copy number of the chromosomally integrated *gfp* construct versus *gfp* carried on the multi-copy pREG696 plasmid.

# CONCLUSIONS

In this proof-of-principle study we applied CRISPR-Cas9 as a counter-selection strategy to aid in the generation of targeted modifications in the chromosome of a clinical strain of *E. faecium*. Our approach for genome editing in *E. faecium* does not require specialized media and does not leave a scar in the chromosome. Mutants could be efficiently identified by PCR and the plasmids used to generate the mutants were readily cured. In comparison with our previous protocol (Zhang *et al.* 2012), processing time was reduced by up to 2 weeks and the total number of colonies that need to be screened is reduced by approximately 4-fold. It is important to note that the use of CRISPR-Cas9 allowed us to generate deletion mutants but also to insert genes into the genome, which can be useful for a number of applications. The stable insertion of fluorescent or bioluminescent tags into the genome can be of particular use during *in vivo* experiments, e.g. to track colonization and infection by *E. faecium*. We note that the CRISPR/Cas9 system described here can be improved further, e.g. by changing the selection markers to reduce the number of spontaneously resistant colonies or by the addition of phenotypic markers (e.g. genes encoding for bioluminescent or fluorescent proteins) that can facilitate screening for the loss of the plasmid. For unknown reasons, the vector is also not always lost during growth at 37°C, and this could be another target for further improvement. For any in-depth phenotypic characterization of any mutants generated with this method, or any other method involving genome manipulation, we recommend the use of whole-genome sequencing to rule out the introduction of non-target mutations. We also stress the importance of complementation of mutations upon the generation of mutants. Native CRISPR systems are relatively rare in multi-drug resistant clinical *E. faecium* strains (Palmer and Gilmore 2010; Lebreton *et al.* 2013) and there is therefore little risk of interference with the system we implemented here.

Even though E. faecium is broadly recognized as an important multi-drug resistant nosocomial pathogen, there is still a limited mechanistic understanding of its basic biology and the traits that contribute to its transition from gut commensal to opportunistic pathogen. Efficient genome editing tools for E. faecium are essential to mechanistically characterise its of resistance to antimicrobials and disinfectants and other adaptations that have contributed to E. faecium becoming a globally important nosocomial pathogen. The CRISPR-Cas9-based approach described here improves the current genetic toolbox for E. faecium and we anticipate that it will accelerate research into this species. We note that the approach we developed here for E. faecium might also be successfully implemented in other enterococci and low-GC Gram-positive bacteria with high recombination rates, including several species in the genera Lactobacillus, Streptococcus and Staphylococcus (Gonza´lez-Torres et al. 2019).

# SUPPLEMENTARY DATA

**Supplementary Table 1.** Sequence of the repair template used for the deletion of *lacL*.

| *lacL* repair template |
|---|
| GAGTCCTTTTGAAGAAAATATTGCCGCATGGATGTTTGTGTCTCCTAAACAAGATGAAGC-<br>TATTGTGTTTTTAGGGAGGATACTAGCTTCAGCTCAACCAGCGTTCCATGAAGTATATCTGAT-<br>GGGGTTAGATGATGAGGCACTTTATCAGGAACAGACCTCGAAGCGGATATTTTCGGGGGC-<br>CGAATTGATGACAGTTGGACTTTACTTCCCCGATTTTCAAGGTGATTTCCAAACAGAACTGCT-<br>TCATTTCAAAAAGTTATGAGAGAGAAGGAAAAAAGTATGAAAGCAAATATAATGATCCAAT-<br>CACAGGCAGAGAAGTGATGCGCTATGGCGGTGACTTTGACGATAAACCAAGTGACTATGAAT-<br>TCTCAGGGAATGGGATCGTTTTTGCAGATGGACAAGAAAAACCCGCCATGCAGGAGGTAA-<br>GATATTATTATGAAAAATACAGTAAATAAAAGTCATATGGATACGGAAAAAGTTGCAATCGTCT-<br>TCGGCGACTGTACATTAGGTGTCAAATCGGGGAATACGCATTATATTTTTTCTTATACAAGAGG-<br>CGGACTGGAATCGCTCAATAAAAACGGAAAAGAATGGCTA |

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***Conflicts of interest.*** None declared.

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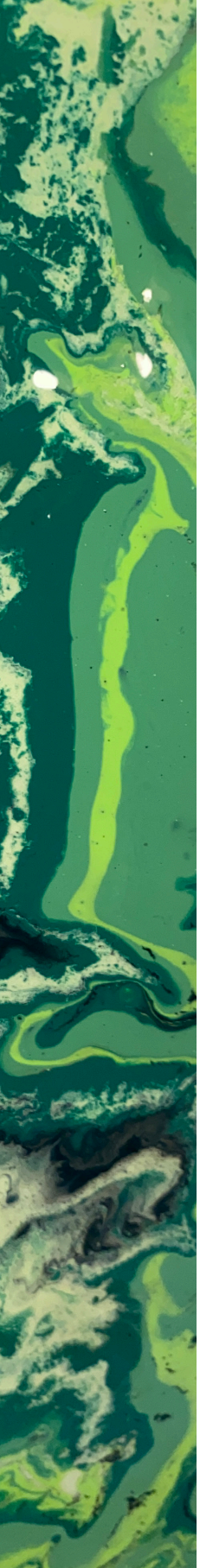

# Chapter 3

## RNA-seq and Tn-seq reveal fitness determinants of vancomycin-resistant *Enterococcus faecium* during growth in human serum

Xinglin Zhang, Vincent de Maat, Ana M. Guzmán Prieto, Tomasz K. Prajsnar, Jumamurat R. Bayjanov, Mark de Been, Malbert R.C. Rogers, Marc J.M. Bonten, Stéphane Mesnage, Rob J.L. Willems, Willem van Schaik

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# ABSTRACT

## Background

The Gram-positive bacterium *Enterococcus faecium* is a commensal of the human gastrointestinal tract and a frequent cause of bloodstream infections in hospitalized patients. The mechanisms by which *E. faecium* can survive and grow in blood during an infection have not yet been characterized. Here, we identify genes that contribute to growth of *E. faecium* in human serum through transcriptome profiling (RNA-seq) and a high-throughput transposon mutant library sequencing approach (Tn-seq).

## Results

We first sequenced the genome of *E. faecium* E745, a vancomycin-resistant clinical isolate, using a combination of short- and long read sequencing, revealing a 2,765,010 nt chromosome and 6 plasmids, with sizes ranging between 9.3 kbp and 223.7 kbp. We then compared the transcriptome of *E. faecium* E745 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 in these two conditions. A gene cluster with a role in purine biosynthesis was among the most upregulated genes in *E. faecium* E745 upon growth in serum. The *E. faecium* E745 transposon mutant library was then used to identify genes that were specifically required for growth of *E. faecium* in serum. Genes involved in *de novo* 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 *E. faecium* 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 were tested for their virulence in an intravenous zebrafish infection model and exhibited significantly attenuated virulence compared to *E. faecium* E745.

## Conclusions

Genes involved in carbohydrate metabolism and nucleotide biosynthesis of *E. faecium* are essential for growth in human serum and contribute to the pathogenesis of this organism. These genes may serve as targets for the development of novel anti-infectives for the treatment of *E. faecium* bloodstream infections.



## Keywords:

*Enterococcus faecium*, transcriptome, transposon mutant library screening, nucleotide biosynthesis, carbohydrate metabolism, virulence, zebrafish

# BACKGROUND

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]. Clade A-1 *E. faecium* strains are rarely found in healthy individuals but can colonize the gut of immunosuppressed, hospitalized patients to high-levels. These strains can then cause infections by direct translocation from the gut into the bloodstream [10–12]. In addition, due to faecal contamination of the skin in hospitalized patients, the use of intravenous catheters is another risk factor for the introduction of *E. faecium* into the bloodstream [3,13,14]. 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 [15–17]. 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 [18].

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 [19,20]. 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 [21], bile [22] and disinfectants [23]. 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 [24]

and TraDIS [25], which are based on high-throughput sequencing of the junctions of the transposon insertion sites and genomic DNA, have been developed [26].

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 two *E. faecium* genes that contribute to growth in serum and in virulence, in a zebrafish model of infection. 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. *E. faecium* E745 was isolated from a rectal swab of a hospitalized patient as part of routine surveillance during an outbreak of VRE in the nephrology ward of a Dutch hospital in 2000 [27,28]. 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 closed chromosomal sequence of 2,765,010 nt and 6 complete plasmids sequences, with sizes ranging between 9.3 kbp and 223.7 kbp (Additional file 1). Taken together, the chromosome and plasmids have 3,095 predicted coding sequences. Phylogenetic analysis of the core genome of E745 and a set of 72 genomes representing global *E. faecium* diversity [4], showed that *E. faecium* E745 is a clade A-1 strain (Fig. 1). The E745 chromosome contains a pathogenicity island with the *esp* gene, which encodes a 207-kDa surface protein that is involved in biofilm formation and infection [6,29,30]. The vancomycin resistance genes of *E. faecium* E745 are of the *vanA* type [31] and are carried on the 32.4-kbp plasmid 2. Additional antibiotic resistance genes in the *E. faecium* E745 genome are the trimethoprim resistance gene *dfrG* [32], which is located on the plasmid 6 and the chromosomally encoded macrolide resistance gene *msrC* [33].

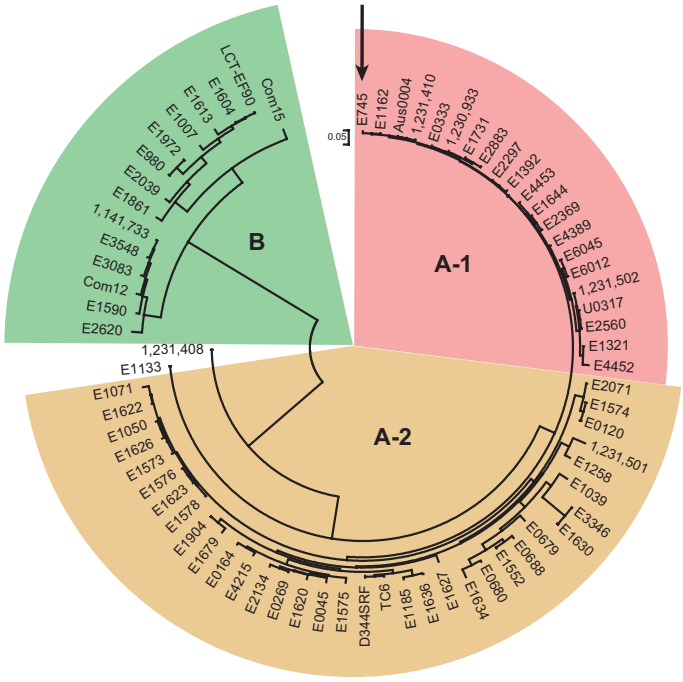


**Figure 1. Maximum likelihood phylogenetic tree of *E. faecium*.**
The phylogenetic tree was based on a core genome alignment of 1,545,750 positions that was generated by ParSNP [62]. The tree includes the *E. faecium* E745 genome sequence generated in this study and the 72 *E. faecium* whole genome sequences described in Lebreton *et al*. [4]. The tree was visualized and mid-point rooted using MEGA 7.0.26 [63]. The different *E. faecium* clades are indicated. The position of *E. faecium* E745 in the phylogenetic tree is highlighted by an arrow.

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

After confirming that serum can support the growth of *E. faecium* E745, though at a lower growth rate than the rich medium BHI (Additional data 2), the transcriptional profile of E745 was determined by RNA-seq during exponential growth in 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. 2). A total of 3217 transcription units were identified, including 651 predicted multi-gene operons, of which the largest contains 22 genes (Fig. 2A and Additional file 3).

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 (Additional file 4). 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. 2B). In addition, we found a 58.4 kbp prophage-like gene cluster that exhibited higher expression in E745 during growth in serum (Fig. 2C). Analysis of this phage sequence against genome sequences deposited at NCBI Genbank revealed that an essentially identical element (with 99% nucleotide identity) could be identified in *E. faecium* ATCC 700221 but not in other genome sequences of *E. faecium* or other bacteria.

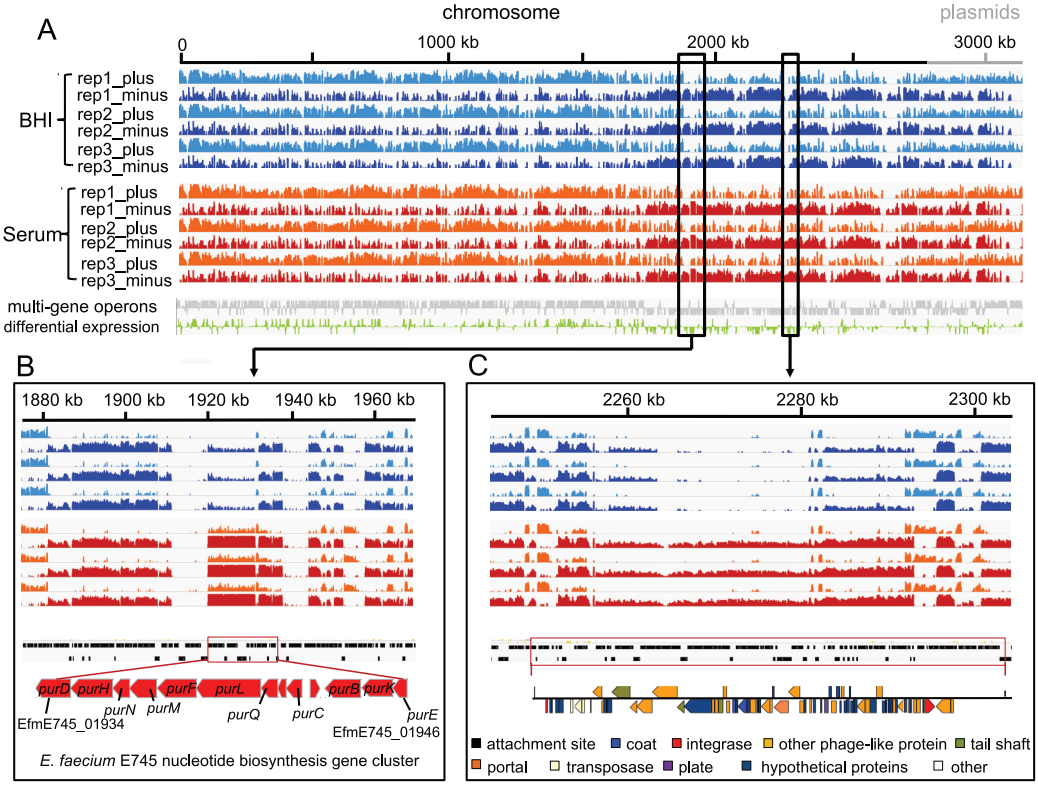


**Figure 2. Coverage plots of RNA-seq data aligning to chromosome and plasmid DNA.**
The y-axis of each track indicates reads coverage and is represented on a log scale, ranging from 0 to 10000. The x-axis represents the genomic location. Light blue (BHI) or orange (serum) tracks correspond to sequencing 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.

To confirm the RNA-seq analysis, we independently determined expression levels of eight genes during growth in serum versus growth in BHI by qPCR (Additional file 5). RNA-seq and qPCR data were highly concordant ($r^2 = 0.98$).

## *E. faecium* E745 genes required for growth in human serum

A *mariner*-based transposon mutant library was generated in *E. faecium* E745 and Tn-seq [24] was performed on ten replicate transposon mutant libraries (after overnight growth 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 25-nt windows, 49,984 (45%) 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 (Additional file 6).

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 was heat-treated to inactivate the complement system [34]. Minor differences were observed among conditionally essential genes between the experiments performed in native human serum or heat-inactivated human serum (Additional file 7) 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 [35,36].

We identified 37 genes that significantly contributed to growth of E745 in human serum (Fig. 3 and Additional file 8): 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 multifactorial process. The genes that conferred the most pronounced effect on growth of *E. faecium* in serum included genes that are 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. 3). Notably, the *purD, purH,* and *purL* genes were found to exhibit higher expression upon growth in human serum in the RNA-seq analysis (Fig. 2). 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 (Additional file 8), compared

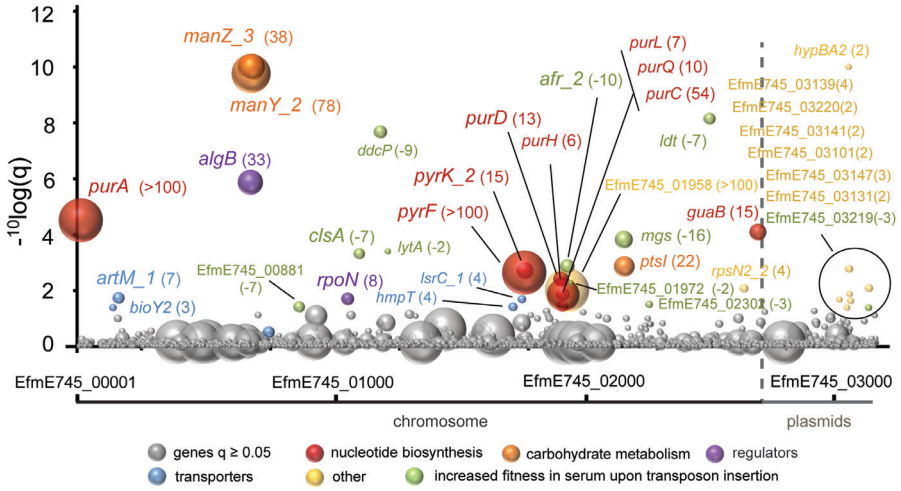


**Figure 3. Tn-seq analysis to identify *E. faecium* genes required for growth in human serum.** Bubbles represent genes, and bubble size corresponds to the fold-changes (for visual reasons, a 100-fold change in transposon mutant abundance is set as a maximum) derived from the read-count ratio of libraries 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 in parentheses. Negative values indicate that mutants in these genes outgrow other mutants in serum, suggesting that these mutants, compared to the wild-type strain *E. faecium* E745, have a higher fitness in serum.

to the major effects observed in the transposon mutants discussed above, but it is notable that five (*clsA_1*, *ddcP*, $ldt_{fm}$, *mgs*, and *lytA_2*) of these genes have predicted roles in cell wall and cytoplasmic membrane biosynthesis.

We developed a PCR-based method (Additional file 9) to selectively isolate five transposon mutants (in the purine metabolism genes *purD* and *purH*, the pyrimidine metabolism genes *pyrF* and *pyrK_2* and the phosphotransferase system (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. 4A), confirming the results of the Tn-seq experiments.

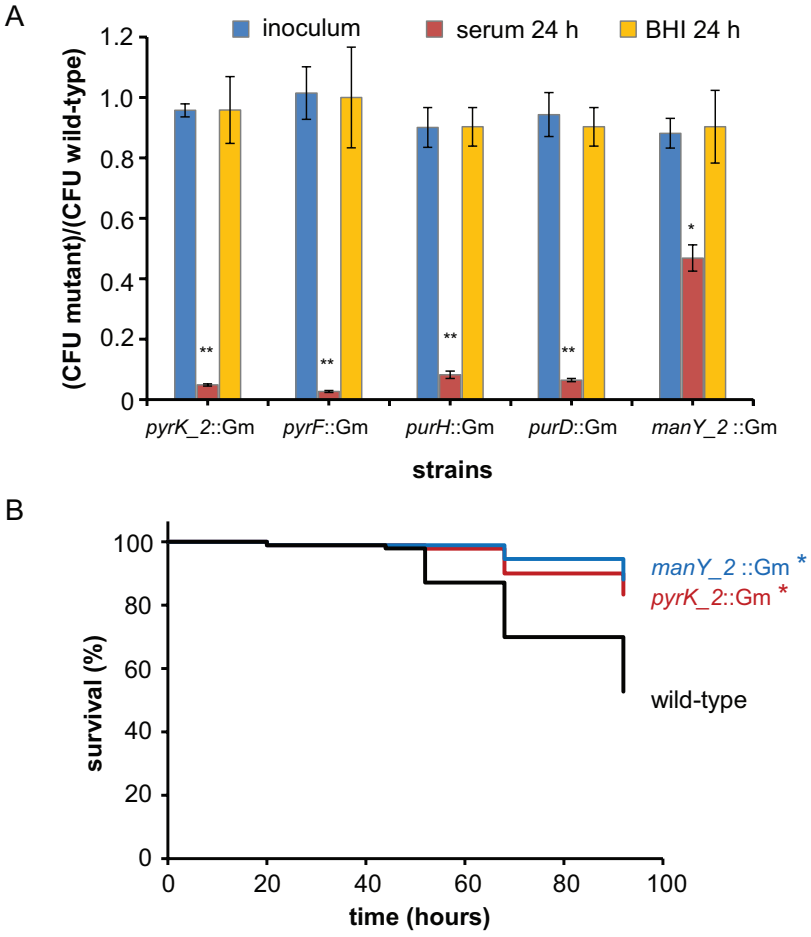


**Figure 4. *E. faecium* transposon mutants with a growth defect in human serum and an attenuated phenotype in a zebrafish infection model.**

(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 differences (***: $p < 0.001$, ****: $p < 0.0001$) 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*. Infection was initiated by the injection of 1.2x$10^4$ CFUs of the *manY_2*::Gm and *pyrK*::Gm transposon mutants and the wild-type *E. faecium* E745 into the circulation of zebrafish embryos 30 hours post fertilisation. 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).

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

Finally, we investigated whether the transposon insertion mutants in the *manY_2* and *pyrK_2* genes were attenuated *in vivo* (Fig. 4B). 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 [37]. We showed that both the *manY_2* and the *pyrK_2* mutant were significantly less virulent than the parental strain. At 92 hours post infection, survival of zebrafish embryos infected with the WT strain was 53%, as compared to 88% and 83% for zebrafish embryos that were infected with the transposon insertion mutants in *manY_2* and *pyrK_2*, respectively.

## 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, including genes with roles in carbohydrate uptake and nucleotide biosynthesis, were found to be required for fitness of *E. faecium* E745 in serum. 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 [38,39]. 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 pathogenic bacteria (including *Burkholderia cepacia, Pasteurella multocida*, *Acinetobacter baumannii*, *Salmonella enterica* serovar Typhimurium, *Bacillus anthracis*, and *Streptococcus pneumoniae*), nucleotide biosynthesis contributes importantly to virulence [40–45]. The ability to synthesize nucleotides *de novo* thus appears to be an essential trait for the success of a pathogen that spreads through

the bloodstream [38]. 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 [46].

Three genes, *ptsL*, *manY_2* and *manZ_3*, encoding subunits of *E. faecium* PTSs were found to contribute to growth in serum in our Tn-seq experiments. The *ptsL* gene is predicted to encode an enzyme that confers a phosphate group from phosphoenolpyruvate to Enzyme I of PTS, while *manY_2* and *manZ_3* are predicted to form the IIA and IIBC components of a permease system that is homologous (64% and 69% amino acid identity, respectively) to the PtnAB PTS permease of *Lactococcus lactis* MG1363. PtnAB is one of the glucose uptake systems of *L. lactis* [47] and the *E. faecium* homolog may have a similar function, which could explain its essential role during growth in serum, as glucose is the only carbohydrate that occurs in the free state in appreciable amounts in serum [18].

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 [21], while $ldt_{fm}$ acts as a peptidoglycan L,D transpeptidase [48]. 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 [49]. 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 [50]. Finally, *lytA_2* is predicted to encode an autolysin, which may be involved in the turnover of peptidoglycan in the cell wall [51]. The transposon mutants in these genes were not further characterized in this study, but our findings suggest that non-essential pathways of cell wall or cytoplasmic membrane remodelling may confer subtle fitness defects to *E. faecium* when growing in a nutrient-poor environment, like 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 large number of differentially

expressed genes may not all reflect the different growth conditions (serum and BHI) *per se*, but could also be influenced by the difference in growth rate during mid-exponential growth in serum and BHI (Additional file 2). 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 [4,52,53]. Interestingly, in the related bacterium *Enterococcus faecalis* prophages encode platelet-binding proteins [54] and may have a role in intestinal colonization [55]. 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.

# CONCLUSIONS

Our data indicate 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*.

# METHODS

## Bacterial strains, plasmids, growth conditions, and oligonucleotides

The vancomycin-resistant *E. faecium* strain E745 was used throughout this study. This strain was isolated from a rectal swab of a hospitalized patient, during routine surveillance of a VRE outbreak in a Dutch hospital [27,28]. Unless otherwise mentioned, *E. faecium* was grown in brain heart infusion broth (BHI; Oxoid) at 37°C. The *E. coli* strains DH5$\alpha$ (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 ($OD_{660}$). The sequences of all oligonucleotides used in this study are listed in Additional file 10.

## 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) [56] and assembled contigs were then corrected by aligning Illumina reads using BWA (version 0.7.9a), with default parameters for index creation and the BWA-MEM algorithm with the *-M* option for the alignment [57]. 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 [58]. 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) [59], 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 [60]. The corrected sequences were annotated using Prokka (version 1.10) [61]. A maximum likelihood phylogenetic tree based on the core genome of *E. faecium* E745 and an additional 72 *E. faecium* strains representing the global diversity of the species [4], was generated using ParSNP [62] with settings -c (forcing inclusion of all genome sequences) and -x (enabling recombination detection and filtering). The resulting phylogenetic tree was visualized using MEGA 7.0.26 [63]. Antibiotic resistance genes in the assembled genome sequence of *E. faecium* E745 were identified using ResFinder [64]. The annotated genome of *E. faecium* E745 is available from NCBI Genbank database under accession numbers CP014529 – CP014535.

## RNA-seq

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 exponential 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 [21]. 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 [65] using the default settings for strand specific analysis.

## Validation of RNA-seq results 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 [21] 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. 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 [21].

## 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 [66]. 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 [66]. 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 [66]. 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 [67], and 16-nucleotide fragments of each read that corresponded to E745 sequences, were mapped to the E745 genome using Bowtie 2 [68]. The results of the alignment were sorted and counted by IGV [69]

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 Kilobase 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 [70]. 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.

## 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 (Additional data file 9). 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 Additional file 9.

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. 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 [71]. Briefly, anesthetized embryos were embedded in 3% (w/v) methylcellulose and injected individually with approximately $1.2 \times 10^4$ 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.

# ABBREVIATIONS

| | |
|---|---|
| BHI | Brain Heart Infusion broth |
| cDNA | complementary DNA |
| cfu | colony forming units |
| hpi | hours post-infection |
| kbp | kilo-base pair |
| LB | Luria-Bertani |
| LWT | London wild-type |
| NCBI | National Center for Biotechnology Information |
| nt | nucleotides |
| PTS | phosphotransferase system |
| qPCR | quantitative polymerase chain reaction |
| RNA-seq | RNA sequencing |
| RPKM | reads per kilobase per million input reads |
| rRNA | ribosomal RNA |
| Tn-seq | transposon sequencing |
| TraDIS | transposon directed insertion sequencing |
| U | unit(s) |

# DECLARATIONS

## Ethics approval and consent to participate

Strain E745 was isolated as part of routine diagnostic procedures during a VRE outbreak. This aspect of the study did not require consent or ethical approval by an institutional review board. Zebrafish work was performed according to guidelines and legislation set out in United Kingdom law under the Animals (Scientific Procedures) Act 1986. Ethical approval was given by the University of Sheffield Local Ethical Review Panel. All zebrafish experiments in this study were performed on larvae before the free feeding stage (5.2 days post fertilization) and consequently did not fall under the animal experimentation law according to the Act.

## Consent to publish

Not applicable

## Availability of data and materials

Sequence reads generated in this study have been made available at the European Nucleotide Archive under accession number PRJEB19025. The core genome alignment and Newick-formatted tree file that were generated for Fig. 1, are available in the Figshare repository (https://doi.org/10.6084/m9.figshare.5545327.v1). Strains and vectors can be requested by contacting the corresponding author.

## Competing interests

The authors declare that they have no competing interests

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## Authors' contributions

XZ, VdM, AMGP, and TKP performed experiments. JRB, MdB and MRCR contributed bioinformatic analyses. XZ, MJMB, SM, RW and WvS designed the study. XZ, AMGP, JRB and WvS drafted the manuscript. All authors read and approved the final manuscript.

## Acknowledgements

Not applicable.

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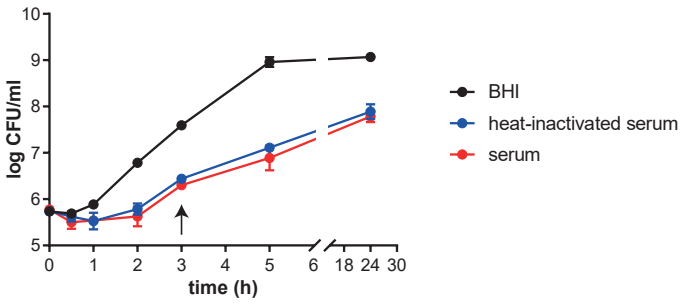
# ADDITIONAL FILES

**Additional file 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).



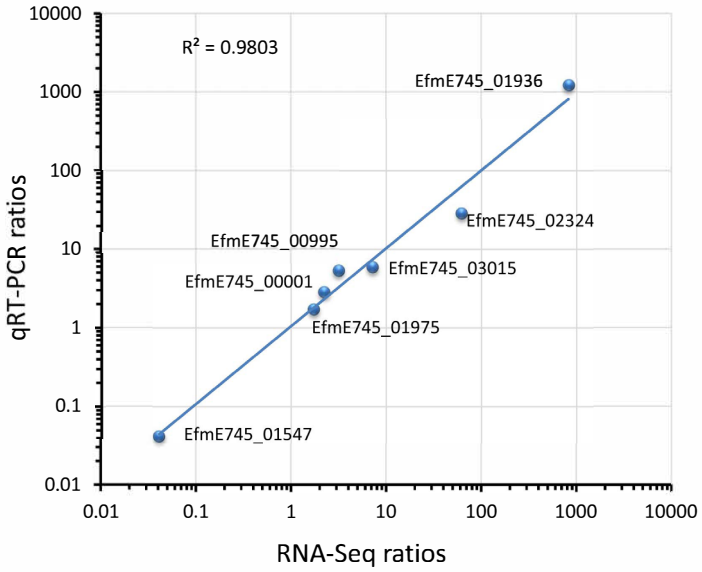

**Additional file 2.**Growth curves of E. faecium E745 in Brain Heart Infusion (BHI) broth, heat-inactivated and native serum at 37°C. The results are the average of three independent experiments for BHI, and two independent experiments for the sera. Error bars indicate standard deviations. The arrow corresponds to the time of sampling for the RNA-seq experiments.

**Additional file 3.** Operons identified by RNA-seq in *E. faecium* E745

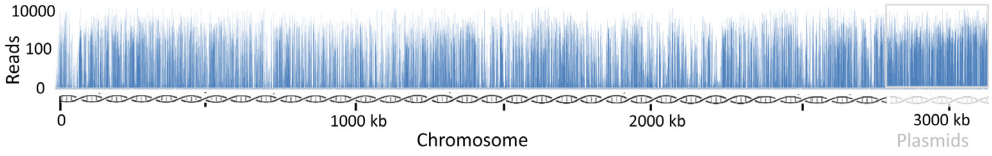
| Start | Stop | Strand | Number of Genes | Genes |
|---|---|---|---|---|
| 2826 | 8679 | + | 4 | EfmE745_00003, recF, gyrB, gyrA |
| 8915 | 10038 | + | 3 | rpsF, ssb_1, rpsR |
| 10181 | 12612 | + | 2 | nrnA_1, rplI |
| 23864 | 24674 | + | 2 | EfmE745_00020, EfmE745_00021 |
| 30527 | 30977 | + | 2 | EfmE745_00026, EfmE745_00027 |
| 31802 | 33282 | - | 2 | yvdM_1, lacR_1 |
| 33519 | 38592 | + | 4 | lacG_1, lacF_1, lacE_1, ganB |
| 39083 | 41630 | - | 2 | guaA_1, coaA |
| 43896 | 46324 | + | 3 | pdp, deoC2, cdd |
| 47684 | 54159 | + | 6 | mglA, EfmE745_00045, EfmE745_00046, deoB, punA, deoD |
| 57031 | 60179 | + | 3 | rpsL, rpsG, fus |
| 61944 | 69536 | + | 17 | rpsJ, rplC, rplD, rplW, rplB, rpsS, rplV, rpsC, rplP, rpmC, rpsQ, rplN, rplX, rplE, rpsZ, rpsH, rplF |
| 69697 | 73256 | + | 6 | rplR, rpsE, rpmD, rplO, secY, adk |
| 73447 | 76040 | + | 6 | infA, rpmJ, rpsM, rpsK, rpoA, rplQ |
| 76312 | 78783 | + | 3 | ecfA1, ecfA2, ecfT |
| 85086 | 85969 | - | 2 | EfmE745_00095, EfmE745_00096 |
| 86072 | 87708 | + | 2 | EfmE745_00097, EfmE745_00098 |
| 93409 | 96495 | + | 2 | recG, plsX |
| 98747 | 104406 | + | 5 | oppD_1, oppF_1, gsiC, oppC, appA |
| 104754 | 110869 | + | 4 | rnc, smc_1, yidA_1, ftsY |
| 114012 | 117642 | + | 4 | feuB_1, hmuU, fhuC, yclQ |
| 121216 | 122481 | + | 2 | EfmE745_00126, EfmE745_00127 |
| 128759 | 132444 | + | 5 | lrgA, yohK, EfmE745_00135, EfmE745_00136, fabG_1 |
| 134978 | 137267 | + | 3 | nirC, EfmE745_00142, EfmE745_00143 |
| 137675 | 144200 | + | 5 | EfmE745_00144, EfmE745_00145, mutS_1, mutL, maf |
| 147902 | 150659 | + | 4 | ruvA, ruvB, dhaS, yfkJ |
| 156564 | 159139 | - | 3 | artM_1, glnP_1, EfmE745_00163 |
| 159949 | 162033 | + | 3 | cobB, maa, nadR |
| 173508 | 174932 | + | 2 | rsuA, yfnB_1 |
| 176159 | 178679 | + | 4 | EfmE745_00182, EfmE745_00183, ydjZ, EfmE745_00185 |
| 179003 | 180827 | + | 2 | EfmE745_00186, EfmE745_00187 |
| 183017 | 185598 | + | 2 | ddl_1, murF |
| 187561 | 189421 | + | 3 | acpS, alr, ndoA |
| 197235 | 197764 | + | 2 | EfmE745_00199, EfmE745_00200 |
| 198995 | 202397 | - | 4 | opuCD, opuCC, opuCB, opuCA_1 |
| 204240 | 206879 | + | 2 | yoaB, EfmE745_00209 |
| 207962 | 209584 | + | 2 | tgt, EfmE745_00212 |
| 215741 | 220859 | + | 5 | rhaB, rhaA, rhaD, rhaM, fucO |
| 221844 | 225182 | + | 5 | kdgA, kdgK, kdul1, hdhA, gno |
| 225431 | 229504 | - | 4 | fni, thrB_1, thrB_2, galK_1 |
| 229826 | 231469 | + | 2 | EfmE745_00232, corA_1 |
| 232423 | 233631 | + | 3 | infC, rpmI, rplT |
| 235268 | 238061 | + | 2 | apc1, EfmE745_00241 |
| 239194 | 240727 | - | 3 | EfmE745_00243, ybgC, nnrD |
| 246208 | 247025 | + | 2 | ytpP, EfmE745_00253 |
| 250588 | 251750 | - | 2 | EfmE745_00257, EfmE745_00258 |
| 253259 | 255108 | + | 2 | EfmE745_00260, melR |
| 255205 | 258629 | + | 2 | rafA_1, ymfD_1 |
| 260531 | 261708 | + | 2 | EfmE745_00265, EfmE745_00266 |
| 261897 | 262531 | + | 2 | EfmE745_00267, EfmE745_00268 |
| 265086 | 265668 | + | 2 | EfmE745_00271, EfmE745_00272 |
| 270275 | 273544 | + | 3 | EfmE745_00276, EfmE745_00277, pinR |
| 275124 | 276370 | + | 2 | EfmE745_00280, EfmE745_00281 |
| 276603 | 279683 | + | 2 | EfmE745_00282, nifA_1 |
| 279755 | 284258 | + | 6 | sorB_1, agaC_1, manZ_1, EfmE745_00287, EfmE745_00288, gmuE_1 |
| 284821 | 286813 | + | 3 | EfmE745_00291, EfmE745_00292, EfmE745_00293 |
| 290396 | 292719 | + | 3 | licR_1, cmtB_1, ulaB_1 |
| 293789 | 295503 | + | 3 | hosA, paiA, EfmE745_00303 |
| 295703 | 296315 | + | 2 | EfmE745_00304, EfmE745_00305 |
| 299945 | 302164 | + | 2 | malL_1, EfmE745_00309 |
| 302265 | 304063 | + | 2 | EfmE745_00310, hchA |
| 304406 | 307151 | + | 2 | npr_1, EfmE745_00313 |
| 307287 | 311337 | + | 3 | glpK, glpO, glpF |
| 314859 | 315447 | - | 2 | EfmE745_00319, csoR |
| 315617 | 319805 | + | 4 | moeZ, cdr_1, helD_1, EfmE745_00324 |
| 323340 | 325353 | + | 3 | dhaM, dhaK, dhaL |
| 327184 | 329427 | - | 2 | EfmE745_00334, ecsA_1 |
| 338754 | 340122 | + | 2 | nhaC_1, nhaC_2 |

**Additional file 4**. E745 genes that exhibited significant (q<0.001 and fold-change>2) differential expression in human serum, as determined by RNA-Seq.

| Synonym | Chromosome/Plasmid | Name | Product | Fold HSerum/BHI RNA-Seq[a] | q HSerum/BHI RNA-Seq[a] |
|---|---|---|---|---|---|
| EfmE745_01939 | Chromosome | purL | Phosphoribosylformylglycinamidine synthase 2 | 422.56 | 0 |
| EfmE745_01937 | Chromosome | purM | Phosphoribosylformylglycinamidine cyclo-ligase | 322.70 | 0 |
| EfmE745_01935 | Chromosome | purH | Bifunctional purine biosynthesis protein PurH | 287.82 | 0 |
| EfmE745_01934 | Chromosome | purD | Phosphoribosylamine--glycine ligase | 266.60 | 0 |
| EfmE745_01936 | Chromosome | purN | Phosphoribosylglycinamide formyltransferase | 260.33 | 0 |
| EfmE745_01881 | Chromosome | - | hypothetical protein | 202.95 | 0 |
| EfmE745_01665 | Chromosome | gspA_2 | General stress protein A | 197.50 | 0 |
| EfmE745_01664 | Chromosome | gspA_1 | General stress protein A | 196.00 | 0 |
| EfmE745_01667 | Chromosome | corA_2 | Magnesium transport protein CorA | 175.33 | 0 |
| EfmE745_00732 | Chromosome | chbC | N,N'-diacetylchitobiose permease IIC component | 173.50 | 0 |
| EfmE745_00882 | Chromosome | dppE | Dipeptide-binding protein DppE precursor | 144.79 | 0 |
| EfmE745_00884 | Chromosome | oppB | Oligopeptide transport system permease protein OppB | 144.38 | 0 |
| EfmE745_02018 | Chromosome | folT_2 | Folate transporter FolT | 141.57 | 0 |
| EfmE745_01668 | Chromosome | yghA | putative oxidoreductase YghA | 141.09 | 0 |
| EfmE745_02293 | Chromosome | - | hypothetical protein | 131.00 | 0 |
| EfmE745_02508 | Chromosome | licB_3 | Lichenan-specific phosphotransferase enzyme IIB component | 104.00 | 0 |
| EfmE745_00885 | Chromosome | dppC | Dipeptide transport system permease protein DppC | 97.40 | 0 |
| predicted RNA | | - | antisense: EfmE745_02338 | 93.50 | 0 |
| predicted RNA | | - | - | 89.54 | 0 |
| EfmE745_00888 | Chromosome | - | Helix-turn-helix domain protein | 80.00 | 0 |
| EfmE745_00887 | Chromosome | oppF_2 | Oligopeptide transport ATP-binding protein OppF | 79.50 | 0 |
| EfmE745_00118 | Chromosome | feuB_1 | Iron-uptake system permease protein FeuB | 77.20 | 0 |
| EfmE745_00886 | Chromosome | oppD_2 | Oligopeptide transport ATP-binding protein OppD | 76.35 | 0 |
| EfmE745_02339 | Chromosome | - | hypothetical protein | 70.50 | 0 |
| EfmE745_00121 | Chromosome | yclQ | putative ABC transporter solute-binding protein YclQ precursor | 60.82 | 0 |
| EfmE745_00119 | Chromosome | hmuU | Hemin transport system permease protein HmuU | 58.31 | 0 |
| predicted RNA | | - | - | 57.92 | 0 |
| EfmE745_00139 | Chromosome | bioY2 | Biotin transporter BioY2 | 57.31 | 0 |
| predicted RNA | | - | antisense: EfmE745_02336 | 54.18 | 0 |
| predicted RNA | | - | - | 50.85 | 0 |
| EfmE745_01933 | Chromosome | ydhF | Oxidoreductase YdhF | 49.82 | 0 |
| EfmE745_00880 | Chromosome | gldA_1 | Glycerol dehydrogenase | 49.80 | 0 |
| EfmE745_00730 | Chromosome | licB_1 | Lichenan-specific phosphotransferase enzyme IIB component | 48.80 | 0 |
| EfmE745_02337 | Chromosome | - | hypothetical protein | 48.45 | 0 |
| EfmE745_00897 | Chromosome | niaX | Niacin transporter NiaX | 48.35 | 0 |
| EfmE745_00120 | Chromosome | fhuC | Iron(3+)-hydroxamate import ATP-binding protein FhuC | 44.54 | 0 |
| EfmE745_01745 | Chromosome | ysnF | Stress response protein YsnF | 44.15 | 0 |
| predicted RNA | | - | - | 43.76 | 0 |
| EfmE745_00731 | Chromosome | licA_1 | Lichenan-specific phosphotransferase enzyme IIA component | 43.61 | 0 |
| predicted RNA | | - | - | 42.89 | 0 |
| EfmE745_01544 | Chromosome | - | hypothetical protein | 41.50 | 0 |
| EfmE745_00767 | Chromosome | - | hypothetical protein | 40.00 | 0 |
| predicted RNA | | - | - | 39.24 | 0 |
| EfmE745_02942 | Plasmid 1 | ohrB_2 | Organic hydroperoxide resistance protein OhrB | 36.75 | 0 |
| EfmE745_01946 | Chromosome | purE | N5-carboxyaminoimidazole ribonucleotide mutase | 36.00 | 0 |
| EfmE745_02296 | Chromosome | - | hypothetical protein | 35.67 | 0 |
| EfmE745_02329 | Chromosome | - | hypothetical protein | 34.67 | 0 |
| EfmE745_00214 | Chromosome | adhE | Aldehyde-alcohol dehydrogenase | 31.67 | 0 |
| EfmE745_02972 | Plasmid 1 | manX_9 | PTS system mannose-specific EIIAB component | 31.33 | 0 |
| EfmE745_02017 | Chromosome | - | Glyoxalase-like domain protein | 31.11 | 0 |
| EfmE745_00487 | Chromosome | vioD | Capreomycidine synthase | 30.88 | 0 |
| EfmE745_00619 | Chromosome | manX_1 | PTS system mannose-specific EIIAB component | 30.87 | 0 |
| EfmE745_02335 | Chromosome | - | Phage antirepressor protein KilAC domain protein | 30.32 | 0 |
| EfmE745_01662 | Chromosome | - | Isochorismatase family protein | 30.10 | 0 |
| EfmE745_02332 | Chromosome | - | hypothetical protein | 29.95 | 0 |
| EfmE745_02331 | Chromosome | - | hypothetical protein | 29.17 | 0 |
| EfmE745_01760 | Chromosome | ulaC | Ascorbate-specific phosphotransferase enzyme IIA component | 26.83 | 0 |
| EfmE745_02334 | Chromosome | - | hypothetical protein | 26.41 | 0 |
| EfmE745_00486 | Chromosome | arcD | Arginine/ornithine antiporter | 26.33 | 0 |
| EfmE745_00096 | Chromosome | - | hypothetical protein | 26.29 | 0 |
| EfmE745_02333 | Chromosome | - | hypothetical protein | 26.13 | 0 |
| EfmE745_00620 | Chromosome | - | putative phosphotransferase enzyme IIB component | 25.09 | 0 |
| EfmE745_01620 | Chromosome | - | hypothetical protein | 24.38 | 0 |
| EfmE745_02167 | Chromosome | pbuO | Guanine/hypoxanthine permease PbuO | 23.73 | 0 |
| EfmE745_01996 | Chromosome | - | hypothetical protein | 22.75 | 0 |
| EfmE745_02325 | Chromosome | - | NUMOD4 motif protein | 22.13 | 0 |
| EfmE745_02941 | Plasmid 1 | - | hypothetical protein | 21.25 | 0 |
| EfmE745_02323 | Chromosome | - | hypothetical protein | 21.14 | 0 |
| EfmE745_01663 | Chromosome | yidA_3 | Sugar phosphatase YidA | 21.00 | 0 |
| EfmE745_02940 | Plasmid 1 | hsdS | Type-1 restriction enzyme EcoKI specificity protein | 20.24 | 0 |
| EfmE745_02324 | Chromosome | - | hypothetical protein | 19.77 | 0 |
| EfmE745_00746 | Chromosome | - | Phage antirepressor protein KilAC domain protein | 19.45 | 0 |
| EfmE745_01350 | Chromosome | - | hypothetical protein | 19.36 | 0 |
| EfmE745_00447 | Chromosome | yecD_2 | Isochorismatase family protein YecD | 19.11 | 0 |
| EfmE745_02330 | Chromosome | - | hypothetical protein | 18.68 | 0 |
| EfmE745_02207 | Chromosome | - | hypothetical protein | 18.56 | 0 |
| EfmE745_02971 | Plasmid 1 | manY_3 | Mannose permease IIC component | 18.50 | 0 |
| EfmE745_00747 | Chromosome | - | hypothetical protein | 18.22 | 0 |
| EfmE745_00569 | Chromosome | pheP | Phenylalanine-specific permease | 18.00 | 0 |
| EfmE745_01670 | Chromosome | - | hypothetical protein | 17.98 | 0 |
| EfmE745_00446 | Chromosome | merA | Mercuric reductase | 17.90 | 0 |
| EfmE745_01995 | Chromosome | - | esterase | 17.33 | 0 |
| EfmE745_01671 | Chromosome | - | hypothetical protein | 17.00 | 0 |
| EfmE745_02973 | Plasmid 1 | manX_10 | PTS system mannose-specific EIIAB component | 16.80 | 0 |
| predicted RNA | | - | - | 16.52 | 0 |
| EfmE745_00456 | Chromosome | ydbD | putative manganese catalase | 16.17 | 0 |
| EfmE745_01964 | Chromosome | dcuS_1 | Sensor histidine kinase DcuS | 14.14 | 0 |
| EfmE745_00243 | Chromosome | - | hypothetical protein | 14.12 | 0 |
| EfmE745_01672 | Chromosome | - | Transglycosylase associated protein | 13.51 | 0 |
| EfmE745_01839 | Chromosome | - | hypothetical protein | 13.44 | 0 |
| predicted RNA | | - | - | 13.32 | 0 |
| EfmE745_01449 | Chromosome | yfiY | putative siderophore-binding lipoprotein YfiY precursor | 12.78 | 0 |
| EfmE745_02319 | Chromosome | - | hypothetical protein | 12.73 | 0 |
| EfmE745_00621 | Chromosome | manY_1 | Mannose permease IIC component | 12.69 | 0 |
| EfmE745_02321 | Chromosome | - | hypothetical protein | 12.64 | 0 |
| EfmE745_02328 | Chromosome | - | hypothetical protein | 12.52 | 0 |
| EfmE745_00901 | Chromosome | folT_1 | Folate transporter FolT | 12.33 | 0 |
| EfmE745_00751 | Chromosome | - | ORF6C domain protein | 12.33 | 0 |




**Additional file 5. qRT-PCR validation 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.




**Additional file 6.** Characterization of the E. faecium E745 transposon mutant library, showing the number of 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. On the y-axis, the number of mapped reads are shown in log scale.

**Additional file 7.** 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] | 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] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| EfmE745_00241 | Chromosome | - | hypothetical protein | 3.32 | 0.03 | 127.30 | 225.97 | 299.48 | 506.01 | 830.43 | 830.43 |
| EfmE745_00371 | Chromosome | - | Transcriptional regulator PadR-like family protein | 2.58 | 0.05 | 2658.69 | 2569.52 | 2724.08 | 4025.18 | 8264.57 | 8264.57 |
| EfmE745_00731 | Chromosome | licA_1 | Lichenan-specific phosphotransferase enzyme IIA component | 17.97 | 0.05 | 92.20 | 0.00 | 113.83 | 94.61 | 1803.49 | 1803.49 |
| EfmE745_01187 | Chromosome | ddl_2 | D-alanine--D-alanine ligase | 3.00 | 0.02 | 686.80 | 439.87 | 574.12 | 1933.12 | 1583.49 | 1583.49 |
| EfmE745_01216 | Chromosome | - | hypothetical protein | 4.13 | 0.01 | 790.00 | 779.53 | 586.28 | 1750.60 | 3579.55 | 3579.55 |
| EfmE745_01402 | Chromosome | - | hypothetical protein | 3.48 | 0.04 | 246.23 | 100.96 | 261.09 | 376.88 | 870.34 | 870.34 |
| EfmE745_01658 | Chromosome | - | Helix-turn-helix domain protein | 7.86 | 0.03 | 1916.22 | 424.30 | 2513.27 | 2193.52 | 17972.95 | 17972.95 |
| EfmE745_01676 | Chromosome | - | hypothetical protein | 4.76 | 0.04 | 91.66 | 0.00 | 180.30 | 356.65 | 468.70 | 468.70 |
| EfmE745_01776 | Chromosome | lsrC_1 | Autoinducer 2 import system permease protein LsrC | 3.82 | 0.02 | 105.26 | 144.84 | 338.94 | 718.31 | 765.26 | 765.26 |
| EfmE745_01900 | Chromosome | - | hypothetical protein | 48.17 | 0.04 | 0.00 | 0.00 | 13.78 | 63.95 | 300.02 | 300.02 |
| EfmE745_02057 | Chromosome | fur | Ferric uptake regulation protein | 2.74 | 0.04 | 551.86 | 612.65 | 1067.71 | 2448.41 | 1833.32 | 1833.32 |
| EfmE745_02308 | Chromosome | - | hypothetical protein | -4.73 | 0.01 | 1879.32 | 1939.09 | 2595.19 | 794.46 | 280.73 | 280.73 |
| EfmE745_02525 | Chromosome | - | putative ABC transporter ATP-binding protein | -3.14 | 0.04 | 1434.32 | 1171.47 | 1700.74 | 639.21 | 365.34 | 365.34 |
| EfmE745_02575 | Chromosome | - | hypothetical protein | -1.88 | 0.01 | 11706.58 | 12917.45 | 12937.59 | 6546.70 | 6698.41 | 6698.41 |
| EfmE745_02693 | Chromosome | - | hypothetical protein | -21.46 | 0.04 | 135.37 | 373.52 | 406.82 | 42.67 | 0.00 | 0.00 |
| EfmE745_02714 | Chromosome | rpmF_2 | 50S ribosomal protein L32 | -2.23 | 0.02 | 8632.23 | 10819.93 | 9901.04 | 6415.06 | 3375.12 | 3375.12 |
| EfmE745_03040 | Plasmid 2 | - | hypothetical protein | 17.16 | 0.01 | 45.59 | 0.00 | 0.00 | 253.97 | 264.20 | 264.20 |
| EfmE745_03071 | Plasmid 3 | - | hypothetical protein | -Inf | 0.02 | 437.17 | 283.84 | 221.11 | 0.00 | 0.00 | 0.00 |
| EfmE745_03130 | Plasmid 5 | - | hypothetical protein | 10.79 | 0.01 | 116.73 | 0.00 | 58.41 | 444.34 | 723.14 | 723.14 |
| EfmE745_03156 | Plasmid 5 | - | hypothetical protein | 2.38 | 0.03 | 4166.22 | 2569.00 | 2244.37 | 5776.92 | 7795.57 | 7795.57 |
| EfmE745_03183 | Plasmid 6 | - | hypothetical protein | 13.84 | 0.03 | 0.00 | 0.00 | 78.35 | 171.47 | 456.45 | 456.45 |

[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.

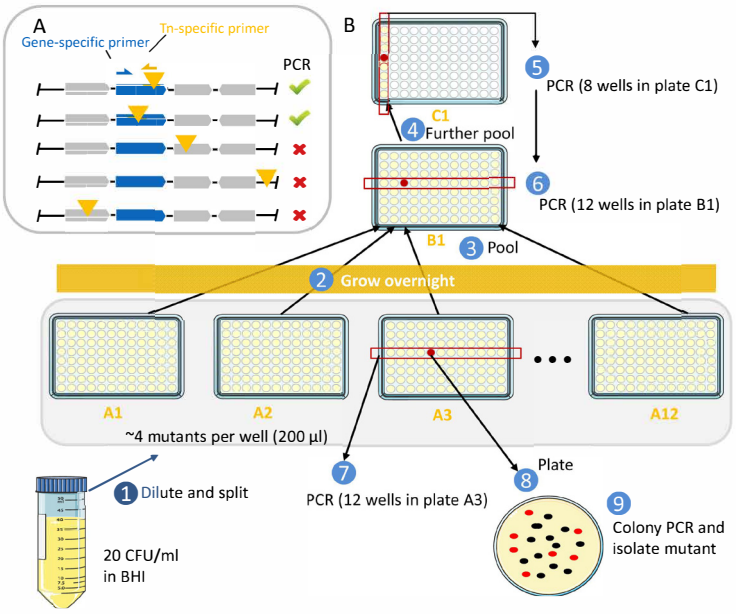
[c] RPKM (Reads Per Kilobase per Million mapped reads) for each individual experiment for the indicated gene.

**Additional file 8.** 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 | Product | fold change BHI/HSerum Tn-seq[a] | *q* BHI/HSerum_ Tn-seq[b] |
|---|---|---|---|---|---|
| EfmE745_00013 | Chromosome | purA | Adenylosuccinate synthetase | >100 | 3.04E-05 |
| EfmE745_01785 | Chromosome | pyrF | Orotidine 5'-phosphate decarboxylase | >100 | 2.27E-03 |
| EfmE745_01958 | Chromosome | - | hypothetical protein | >100 | 9.63E-03 |
| EfmE745_00690 | Chromosome | manY_2 | Mannose permease IIC component | 77.76 | 1.71E-10 |
| EfmE745_01942 | Chromosome | purC | Phosphoribosylaminoimidazole-succinocarboxamide synthase | 54.05 | 0.01 |
| EfmE745_00691 | Chromosome | manZ_3 | Mannose permease IID component | 37.70 | 9.16E-11 |
| EfmE745_00687 | Chromosome | algB | Alginate biosynthesis transcriptional regulatory protein AlgB | 32.74 | 1.33E-06 |
| EfmE745_02204 | Chromosome | ptsI | Phosphoenolpyruvate-protein phosphotransferase | 21.48 | 1.35E-03 |
| EfmE745_01788 | Chromosome | pyrK_2 | Dihydroorotate dehydrogenase B (NAD(+)), electron transfer subunit | 15.00 | 1.92E-03 |
| EfmE745_02782 | Chromosome | guaB | Inosine-5'-monophosphate dehydrogenase | 14.56 | 8.04E-05 |
| EfmE745_01934 | Chromosome | purD | Phosphoribosylamine--glycine ligase | 12.86 | 3.92E-03 |
| EfmE745_01940 | Chromosome | purQ | Phosphoribosylformylglycinamidine synthase 1 | 10.03 | 0.01 |
| EfmE745_01082 | Chromosome | rpoN1 | RNA polymerase sigma-54 factor 1 | 8.40 | 0.02 |
| EfmE745_00161 | Chromosome | artM_1 | Arginine transport ATP-binding protein ArtM | 7.42 | 0.02 |
| EfmE745_01939 | Chromosome | purL | Phosphoribosylformylglycinamidine synthase 2 | 7.19 | 0.04 |
| EfmE745_01935 | Chromosome | purH | Bifunctional purine biosynthesis protein PurH | 6.17 | 4.52E-03 |
| EfmE745_01741 | Chromosome | hmpT | Thiamine precursor transporter HmpT | 4.23 | 0.04 |
| EfmE745_02715 | Chromosome | rpsN2_2 | Alternate 30S ribosomal protein S14 | 3.92 | 0.01 |
| EfmE745_01776 | Chromosome | lsrC_1 | Autoinducer 2 import system permease protein LsrC | 3.66 | 0.02 |
| EfmE745_03220 | Plasmid 6 | - | hypothetical protein | 3.53 | 8.19E-03 |
| EfmE745_03139 | Plasmid 5 | - | hypothetical protein | 3.49 | 1.67E-03 |
| EfmE745_00139 | Chromosome | bioY2 | Biotin transporter BioY2 | 2.91 | 0.04 |
| EfmE745_03147 | Plasmid 5 | - | TraM recognition site of TraD and TraG | 2.55 | 0.02 |
| EfmE745_03131 | Plasmid 5 | - | Sortase family protein | 2.23 | 0.04 |
| EfmE745_03137 | Plasmid 5 | hypBA2 | Beta-L-arabinobiosidase precursor | 2.16 | 0.00 |
| EfmE745_03101 | Plasmid 5 | - | hypothetical protein | 2.10 | 0.02 |
| EfmE745_03141 | Plasmid 5 | - | hypothetical protein | 2.10 | 0.01 |
| EfmE745_01243 | Chromosome | lytA_2 | Autolysin | -2.02 | 3.93E-04 |
| EfmE745_03219 | Plasmid 6 | - | hypothetical protein | -2.61 | 0.04 |
| EfmE745_02302 | Chromosome | - | hypothetical protein | -2.91 | 0.03 |
| EfmE745_01129 | Chromosome | clsA_1 | Major cardiolipin synthase ClsA | -6.55 | 4.82E-04 |
| EfmE745_00881 | Chromosome | - | Acetyltransferase (GNAT) family protein | -6.79 | 0.04 |
| EfmE745_02575 | Chromosome | $ldt_{fm}$ | beta-lactam-insensitive peptidoglycan transpeptidase | -7.48 | 7.05E-09 |
| EfmE745_01213 | Chromosome | ddcP | D-alanyl-D-alanine carboxypeptidase DacA precursor | -9.10 | 2.10E-08 |
| EfmE745_01959 | Chromosome | afr_2 | 1,5-anhydro-D-fructose reductase | -10.73 | 1.35E-03 |
| EfmE745_02202 | Chromosome | mgs | Alpha-monoglucosyldiacylglycerol synthase | -16.37 | 1.51E-04 |

[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.

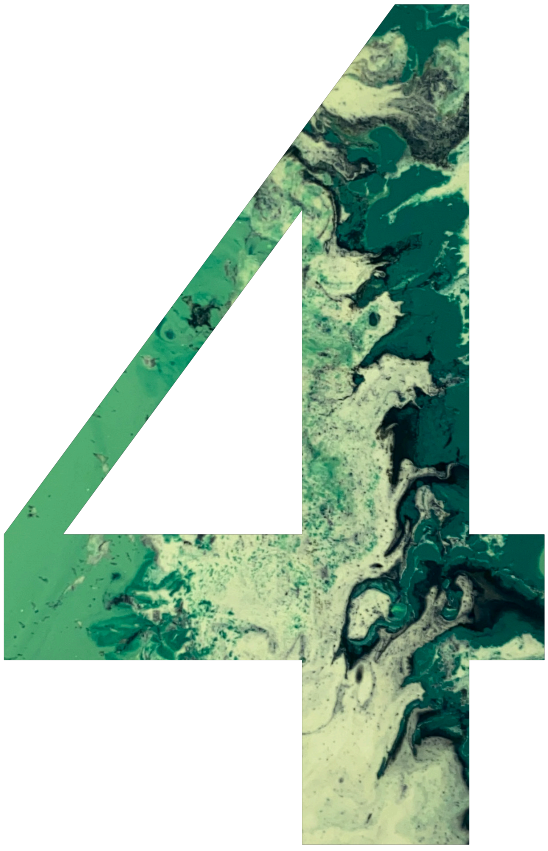
[b] Benjamini-Hochberg corrected P-values (*q* ) of transposon insertions in the indicated genes upon growth in BHI versus growth in heat-inactivated serum.

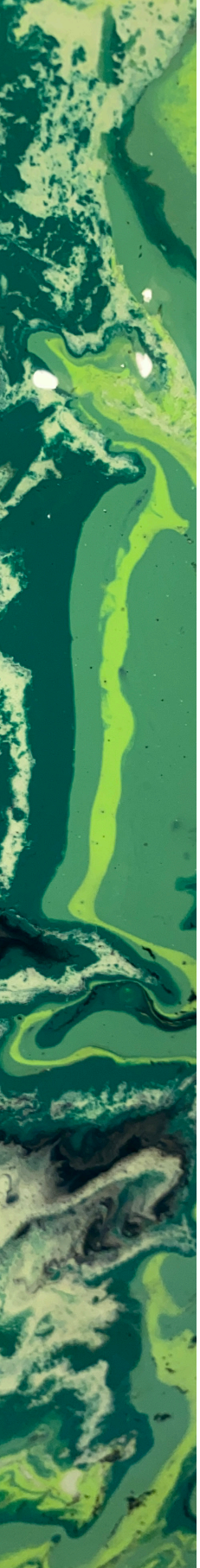



**Additional file 9. Isolation of mutants from the transposon mutant library pool.** (A) Schematic representation of the PCR reaction designed to find a particular Tn-mutant within the transposon mutant library. This PCR uses a combination of a gene-specific primer (blue arrow) and a transposon specific 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).

**Additional file 10.** 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 |





# Chapter 4

# Conditionally essential genes for survival during starvation in *Enterococcus faecium* E745

Vincent de Maat, Sergio Arredondo-Alonso, Rob J.L. Willems, Willem van Schaik

Published in BMC Genomics 2020

# ABSTRACT

**Background:** The nosocomial pathogen *Enterococcus faecium* can survive for prolonged periods of time on surfaces in the absence of nutrients. This trait is thought to contribute to the ability of *E. faecium* to spread amongpatients in hospitals. There is currently a lack of data on the mechanisms that are responsible for the ability of *E.* faecium to survive in the absence of nutrients.

**Results:** We performed a high-throughput transposon mutant library screening (Tn-seq) to identify genes that have a role in long-term survival during incubation in phosphate-buffered saline (PBS) at 20 °C. A total of 24 genes were identified by Tn-seq to contribute to survival in PBS, with functions associated with the general stress response, DNA repair, metabolism, and membrane homeostasis. The gene which was quantitatively most important for survival in PBS was usp (locus tag: EfmE745_02439), which is predicted to encode a 17.4 kDa universal stress protein. After generating a targeted deletion mutant in usp, we were able to confirm that usp significantly contributes to survival in PBS and this defect was restored by in trans complementation. The usp gene is present in 99% of a set of 1644 E. faecium genomes that collectively span the diversity of the species.

**Conclusions:** We postulate that *usp* is a key determinant for the remarkable environmental robustness of *E. faecium*. Further mechanistic studies into *usp* and other genes identified in this study may shed further light on the mechanisms by which *E. faecium* can survive in the absence of nutrients for prolonged periods of time.

# BACKGROUND

*Enterococcus faecium* is a commensal of the human gut,but has emerged over the last few decades as an opportunistic pathogen which causes infections in hospitalized patients. *E. faecium* infections are often difficult to treatdue to the high prevalence of resistance to antibiotics, including virtually all cephalosporins, aminoglycosides, clindamycin, and trimethoprim-sulfamethoxazole [1]. Additionally, resistance to the glycopeptide vancomycin is increasingly widespread in *E. faecium* strains, further complicating the treatment of infections. While the accumulation of antibiotic resistance determinants is a major contributor to *E. faecium*'s emergence as an important nosocomial pathogen, other adaptations, like the ability to form biofilms [2–4] and interact with host extracellular matrix and serum components [5], are also widespread in clinical isolates. Genomic analyses have revealed that the majority of recent clinical isolates of *E. faecium* belong to a defined sub-population which was termed clade A1, with strains from other niches, including animals and healthy humans mostly belonging to other clades in the *E. faecium* population [6–8].

*E. faecium* also has the ability to persist for long periods of time on synthetic surfaces like table tops, handrails, doorknobs and other medical surfaces [9–11]. The ability of *E. faecium* to spread via fomites is thus proposed to play a critical role in the inter-patient spread of *E. faecium* in hospital settings [12–14]. Many nosocomial pathogens are thought to spread via environmental contamination, but *E. faecium* can survive 3to 5-times longer on inanimate objects compared to other Gram-positive nosocomial pathogens, such as *Enterococcus faecalis*, *Staphylococcus aureus*, and streptococci [15–17].

In natural environments, bacteria most often exist in a physiological state that is similar to the stationary phase of growth and are adapted to survive harsh conditions [18, 19]. While survival strategies like sporulation are limited to a subset of bacterial species, most bacteria possess the ability to survive in the complete absence of nutrients (e.g. in water or buffers) for weeks, months and in some cases even years [20, 21]. Research into the mechanisms by which bacteria manage to survive these nutrient-limited conditions is currently relatively scarce. Most stress responses upon nutrient deprivation involve the expression or activity of DNA repair systems, transcriptional regulation, metabolic pathways, and the biogenesis of cell walls and membranes [21, 22].

While survival in the absence of nutrients appears to be an important factor in the spread of *E. faecium* as a nosocomial pathogen, we currently lack an understanding of the genes involved in these processes. For this study, we have elucidated the *E.*

*faecium* genes involved in survival upon starvation by high-throughput screening of a *mariner* transposon mutant library (Tn-seq) of a clinical

*E. faecium* strain upon incubation in a buffer without carbonor nitrogen-sources at room temperature for seven days. With this study, we generated the first insightsinto the mechanisms by which *E. faecium* can survive inthe absence of nutrients.

# RESULTS

## Effect of nutrients and temperature during prolongedincubation

The vancomycin-resistant, clade A1 strain *E. faecium* E745 was incubated at either 37 °C or 20 °C in the rich medium Brain Heart Infusion broth (BHI) or in phosphate-buffered saline (PBS) for up to 10 days (Fig. 1). By determining viable counts, we showed that cells in BHI were able to survive for a prolonged period of time. Indeed, the stationary phase cultures incubated in BHI at 20 °C, showed no reduction in viable counts duringthe course of the experiments, while at 37 °C the cultures were only losing their viability (0.9-$\log_{10}$ reduction)after more than 7 days. During incubation in the nutrient-free buffer PBS, significant reductions in viable counts were observed after 2 days of incubation at 37 °C and after 6 days at 20 °C, but a sub-population of cells remained viable until the end of the experiment.

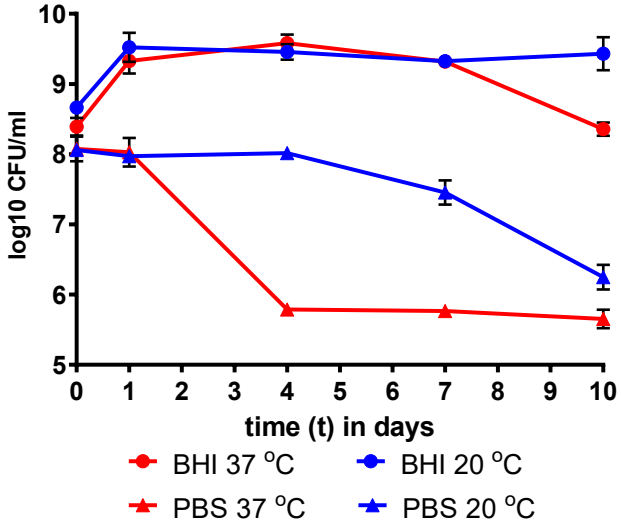



**Figure 1: Survival of E. faecium E745 during starvation.** *E. faecium* strain E745 was incubated for up to 10 days in BHI (circles) or PBS (triangles) at either 37°C (red) or 20°C (blue).

## *E. faecium* E745 genes required for survival duringstarvation

To investigate which genes were required for survival in PBS at 20 °C, we incubated a transposon mutant library of E745 in PBS for 7 days at 20 °C. The relative abundance of transposon mutants in 1631 chromosomal and plasmid genes of *E. faecium* E745 at day 7 was compared to the abundance of mutants present at the start of the experiment (Fig. 2). The abundance of transposon mutants in 24 genes was significantly reduced (BenjaminiHochberg [BH] corrected *P*-value of < 0.05) by 10-fold or more, indicating that these genes contribute importantly to the survival of *E. faecium* in PBS (Table 1). We did not find any mutations that led to a higher abundance after incubation in PBS. The predicted functions of the 24 genes contributing to survival in PBS cover four groups, i.e. general stress response, DNA repair, metabolism, and membrane homeostasis. Transposon mutants in four genes had a greatly reduced (more than a 1000-fold) abundance upon 7-day incubation in PBS, indicating that these genes have a particularly large impact on survival in nutrient-limited conditions.

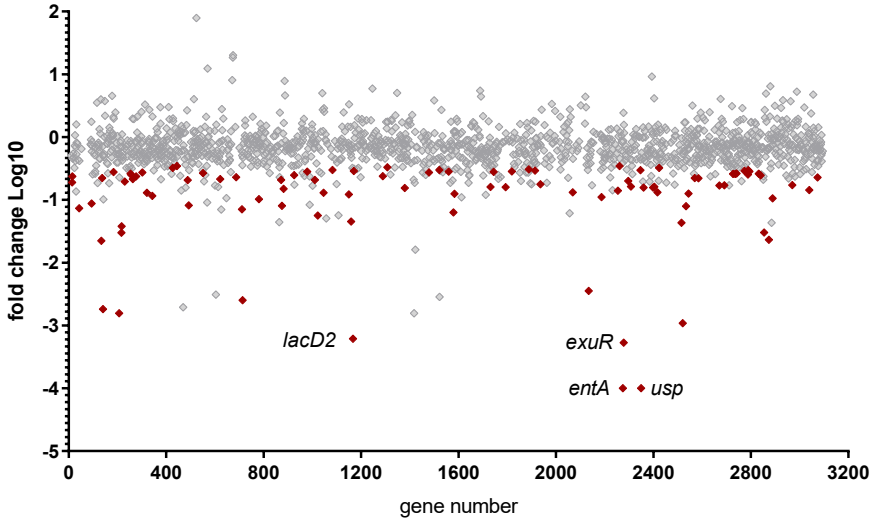



**Figure 2: Tn-seq analysis to identify genes with a role in survival during starvation.** Each diamond represents a gene represented in the transposon library. The Y-axis indicates the relative abundance of each gene after incubation in PBS at 20°C for 7 days compared to the abundance of each gene at the start of the experiment. A positive value indicates an enrichment of mutants during incubation in PBS while a negative value indicates genes that contribute to survival in PBS, with red diamonds representing genes that passed the threshold for statistical significance. The highlighted dots represent genes that are discussed in the text.

**Table 1.** Genes with a role in survival during starvation as determined by Tn-seq. Genes of which the transposon mutants were reduced by more than − 10-fold upon incubation in PBS for 7 days, using a BH-adjusted P-value of < 0.05 as cut-off for statistical significance

| Locus Tag | Name | Function | Fold Change | P-value (BH adjusted) |
|---|---|---|---|---|
| EfmE745_02439 | usp | Putative universal stress protein | <−2.5 × 105 | 0.023 |
| EfmE745_02361 | entA | Bacteriocin EntA | <−9.2 × 104 | 0.020 |
| EfmE745_02364 | exuR | DNA-binding transcriptional repressor | −1.9 × 103 | 0.034 |
| EfmE745_01205 | lacD2 | Tagatose 1,6-diphosphate aldolase 2 | −1.6 × 103 | 0.018 |
| EfmE745_02646 | sacY | Levansucrase and sucrase synthesis operon antiterminator | – 922.6 | 0.020 |
| EfmE745_00210 | – | hypothetical protein | – 639.1 | 0.042 |
| EfmE745_00142 | – | DNA alkylation repair enzyme | – 548.8 | 0.034 |
| EfmE745_00731 | licA_1 | Lichenan-specific phosphotransferase enzyme IIA component | – 396.6 | 0.039 |
| EfmE745_02217 | gbpA | GlcNAc-binding protein A precursor | −280.7 | 0.043 |
| EfmE745_00135 | – | Mga helix-turn-helix domain protein | −44.8 | 0.034 |
| EfmE745_03020 | – | hypothetical protein | −43.1 | 0.010 |
| EfmE745_00219 | rhaD | Rhamnulose-1-phosphate aldolase | −33.3 | 0.028 |
| EfmE745_02998 | – | hypothetical protein | −33.1 | 0.010 |
| EfmE745_00220 | rhaM | L-rhamnose mutarotase | −26.4 | 0.034 |
| EfmE745_02641 | – | putative HTH-type transcriptional regulator | −23.1 | 0.034 |
| EfmE745_01197 | – | hypothetical protein | −22.1 | 0.023 |
| EfmE745_01055 | – | ABC-2 family transporter protein | −17.8 | 0.040 |
| EfmE745_01627 | – | PTS system glucitol/sorbitol-specific transporter subunit IIA | −15.9 | 0.016 |
| EfmE745_00728 | cshA | DEAD-box ATP-dependent RNA helicase | −14.1 | 0.038 |
| EfmE745_00043 | tmpC | Membrane lipoprotein TmpC precursor | −13.6 | 0.016 |
| EfmE745_02660 | drrA | Daunorubicin/doxorubicin resistance ATP-binding protein | −12.6 | 0.046 |
| EfmE745_00895 | rnmV | Ribonuclease M5 | −12.4 | 0.038 |
| EfmE745_00502 | metP | Methionine import system permease protein | −12.3 | 0.033 |
| EfmE745_00095 | – | Arsenical resistance operon trans-acting repressor ArsD | −11.4 | 0.029 |

The *usp* gene (locus-tag: EfmE745_02439) was identified as the most important gene involved in survival in PBS. Inspection of the *mariner* transposon insertion sites in *usp* revealed no unusual patterns in the abundance or placement of the transposon insertion sites, supporting the validity of this hit (Fig. 3a). The *usp* gene encodes a 17.4 kDa putative universal stress protein with no clear function, although homologs are widespread among all domains of life [23, 24]. The second-highest hit in the Tn-seq analyses was found to be in the gene that encodes the previously characterised enterococcal bacteriocin EntA(locus tag: EfmE745_02361) [25, 26]. Like for *usp*, transposon insertions in *entA* were no longer detected in the library at day 7. Bacteriocins are small secreted proteins or peptides produced by bacteria to reduce competition of unrelated bacterial strains. A unique feature of bacteriocins is that they are always co-expressed with their associated immunity gene to avoid auto-inhibition [27]. When we inspected the distribution of transposon insertions in *entA*, we noted that there was only one insertion site in *entA* which is located close to the 3′ end of the gene (Fig. 3b). As the presumptive bacteriocin immunity gene was found to be overlapping with *entA* and was also disrupted by the transposon insertion, there is a distinct possibility that this transposon mutant is killed due to the production of the EntA bacteriocin during nutrient deprivation, by the other mutants in the population in which these genes were not disrupted. For this reason, we did not include *entA* in further functional analyses (described below). The transposon mutants in EfmE745_02364 (*exuR*) and EfmE745_01205 (*lacD2*) (Fig. 3c and d) were also highly reduced upon incubation in PBS, and transposon insertion sites in these genes showed no unusual patterns.

Next we studied the presence of all 24 genes identified by Tn-seq in the whole genome sequences of a collection of 1644 *E. faecium* strains isolated from healthy humans, patients, pets, pigs, and poultry (Fig. 4), previously described in [8]. We found that *usp* and *exuR* are present in 99, and 100% respectively of the isolates tested and these genes can be thus considered part of *E. faecium* core genome. The *lacD2* gene was present in 53% of genome sequences in this collection. Four genes, including *entA*, were found to be significantly enriched ($\chi^2$ test, $P < 0.05$ with Benjamini-Hochberg-correction for multiple testing)in clade A1 strains.

To further test the importance of these genes we have attempted to create gene deletion mutants in *usp*, *exuR* and *lacD2*, but only managed to construct deletions in *usp* and *exuR*. These mutants were tested for their ability to survive in PBS for a 7-day period.

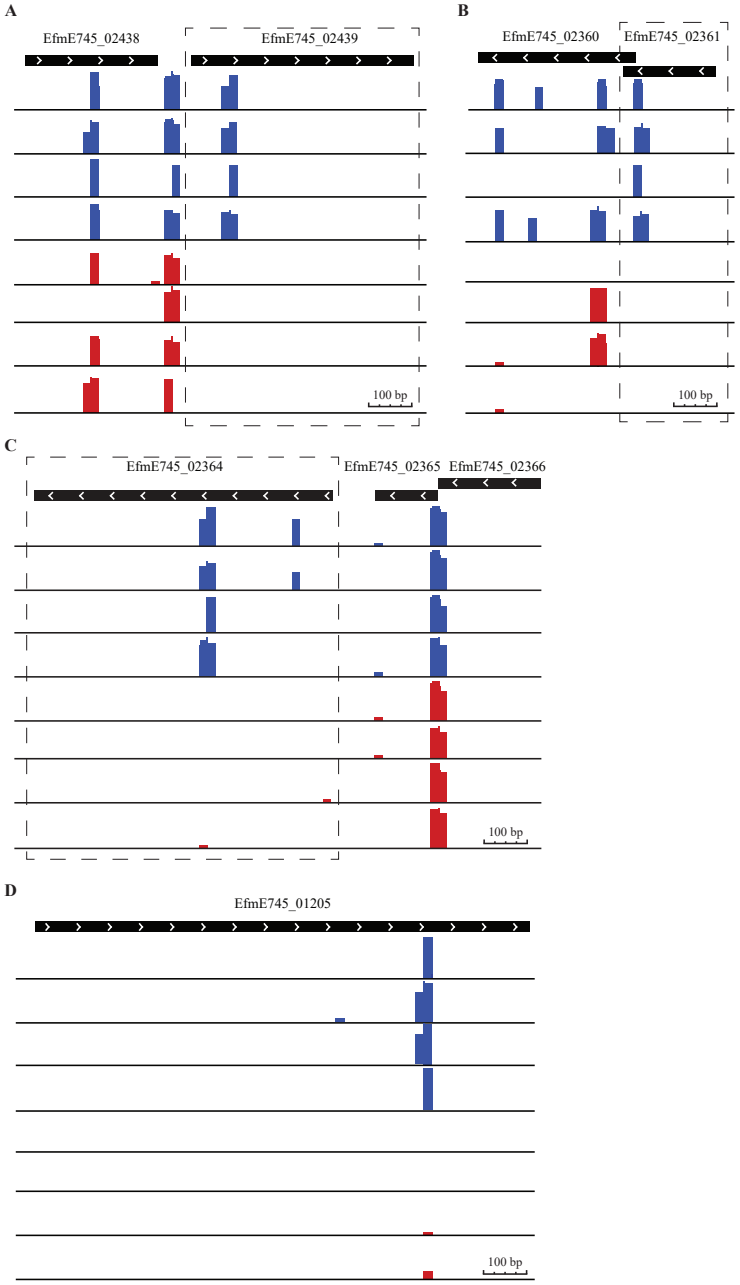



**Figure 3: Visual representation of the transposon insertion sites in *usp, entA, exuR* and *LacD2.*** Genes and gene direction are depicted by the black bars with arrows. Transposon insertion abundance is shown by the bars below the genes on a $Log_{10}$ scale. Blue and red bars denote the abundance of transposon insertion mutants at day 0 and at day 7, respectively. Abundance of transposon insertion are shown for EfmE745_02439 (*usp*), EfmE745_02361 (*entA*), EfmE745_02364 (*exuR*) and EfmE745_01205 (*lacD2*) in panel A, B, C and panel D, respectively.

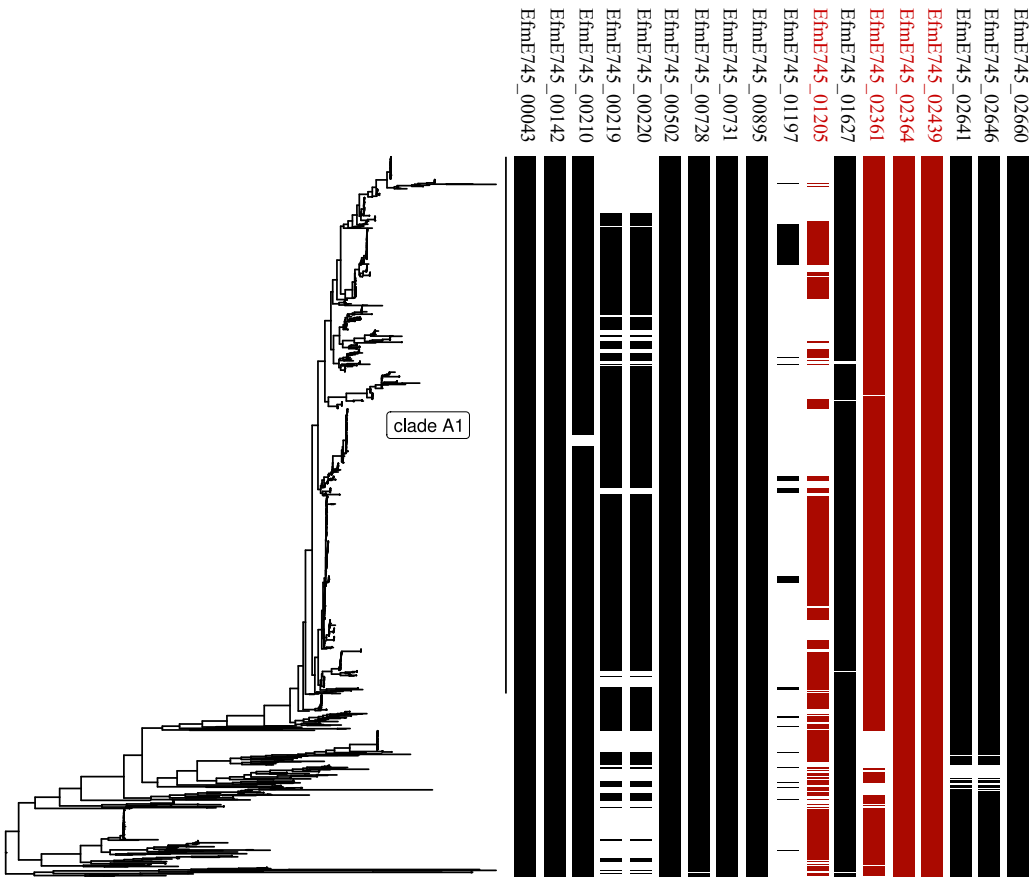



**Figure 4: Presence of genes involved in long-term survival under nutrient-limiting conditions in *E. faecium*.** Whole genome sequence based phylogenetic tree of 1644 *E. faecium* strains representing the global *E. faecium* population. The presence of the different E745 genes was plotted along the phylogeny using the R package ggtree. Highlighted in red are EfmE745_01205 (*lacD2*), EfmE745_02361 (*entA*), EfmE745_02364 (*exuR*) and EfmE745_02439 (*usp*).

## The universal stress protein Usp, but not ExuR, contributesto survival of *E. faecium* during prolonged starvation

To determine whether *usp* has an impact on the survival of E745 we created the deletion mutant E745-Δ*usp* and complemented the deletion by supplying the *usp* gene *in trans* on a plasmid (strain E745-Δ*usp*::*usp*-C). We incubated E745, E745-Δ*usp,* E745-Δ*usp*::*usp*-C, and E745-Δ*usp*:: pEF25 (the *usp* deletion mutant transformed with the empty vector used for *in trans* complementation) in PBS at 20 °C and determined viability of the cell suspension over a 7-day period (Fig. 5a). The *E. faecium*

E745 strain showed a similar survival response as seen in Fig. 1. However, the E745-Δ*usp* and E745-Δ*usp*::pEF25 cell suspension lost theirviability quicker than E745, with a statistically significant ($P < 0.05$; one way ANOVA) 1-log lower viable count after 7 days than the wild-type. The complemented strain, E745- Δ*usp*::*usp*-C, responded similar to E745 with no statistically significant difference detected at any time point. These results confirm the role of *usp* in survival of *E. faecium* E745 in the absence of nutrients. E745-Δ*exuR* was foundto survive equally well as the wild-type strain during starvation (Fig. 5b), suggesting that *exuR* does not havea role in survival during starvation.

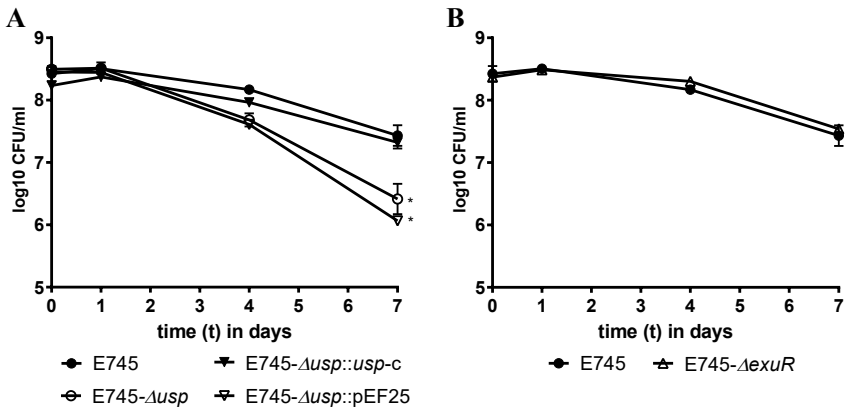



**Figure 5: Phenotypic analysis of usp and exuR deletion mutants.** The data represent the data of three biologically independent experiments, with error bars showing standard deviation. All strains were incubated in PBS at 20°C for up to 7 days. * indicates a statistical difference (P<0.05) between survival of *E. faecium* E745 and *Δusp* and *Δusp*::pEF25 at day 7, as determined by one way ANOVA.

# DISCUSSION

Most bacteria have a 'feast and famine' lifestyle where long periods of severe nutrient limitation are interrupted by short bursts of nutrient availability [21]. For *E. faecium*, we propose that this cycle is particularly relevant for the spread of this opportunistic pathogen in hospitals, where it can survive on inanimate objects until it is transferred to a human host. Indeed, recent work has suggested that this is a trait that characterizes the genus *Enterococcus* and has been selected for over hundreds of

millions of years of microbial evolution [28]. The finding that the genes most likely to contributeto the survival of *E. faecium* during starvation are part ofthe core genome of this species, support the crucial roleof starvation tolerance in the evolution of *E. faecium*. While *E. faecium* has a low intrinsic virulence, it has become a significant threat to immunocompromised patients in hospitals due to its ability to rapidly acquire genes involved in antibiotic resistance and other traits that contribute to its ability to successfully colonize patients [29]. The mechanisms by which it can survive and spread in the hospital environment have so far received less attention.

In this study we investigated the potential of vancomycin-resistant *Enterococcus faecium* to survive in the absence of nutrients. This trait may contribute to the ability of *E. faecium* to survive on inanimate objects in hospitals and thus can impact its success as a nosocomial pathogen. We noted that in the complete absence of nutrients, during incubation in PBS, *E. faecium* cultures exhibit a small loss in viability over a period of 7 days at 20 °C, while at 37 °C a significant loss of viability has occurred after four days, although a subpopulation of cells remains viable, which could represent persister cells [30]. We note that the *E. faecium* cells may be entering a 'viable but non-culturable' state after prolonged incubation in our experiments [31]. The faster decrease in viability at 37 °C, compared to 20 °C, may be due to increased enzymatic activities and higher metabolic turnover at the higher temperature [32].

To obtain a better understanding into the genes that are involved in the response upon prolonged starvation,we performed Tn-seq on a transposon mutant library that was starved for nutrients for 7 days at 20 °C. This analysis revealed a variety of genes that were potentially involved in retaining viability. Genes could be assigned to four major functional groups, i.e. DNA repair, metabolism, cell membrane and cell wall homeostasis. DNA repair, or lack thereof, can contribute to survival during starvation as reactive oxygen species and other DNA damaging molecules accumulate [33]. Metabolic adaptations during starvation are essential as shifts in metabolism, including changes in transport systems, are required for optimal acquisition of nutrients and the excretion of metabolic waste products [34, 35]. Changes to the cytoplasmic membrane and cell wall have also been described to contribute to the ability of cells to withstand harsh conditions, including starvation [28]. The final category of genes we identified in our Tn-seq analysis have roles in the general stress response, i.e. the cell's extended toolbox for responding to adverse conditions [23, 24]. Our Tn-seq data suggest that most genes involved in survival during nutrient deprivation are partof the core genome of *E. faecium*. However, four genes are enriched in clade A1 strains which could suggest that these genes may play a role in the emergence ofstrains from this clade as nosocomial pathogens.

**Table 2.** Oligonucleotides used in this study. Relevant restriction sites are underlined

| Name | Sequence (5′- 3′) |
|---|---|
| oVDM2001 | AAACACGTTCTTTCCAAACCGTTTCATCCTTTGAG |
| oVDM2002 | AAAACTCAAAGGATGAAACGGTTTGGAAAGAACGT |
| oVDM2003 | CAAACAACGGCATTAACGGAGATTACCAT |
| oVDM2004 | GTGTTTGATAAAAAACTTTGTCTACATGAC |
| oVDM2005 | CTGCATATGCTGTTTTAGGCG |
| oVDM2006 | GTGTAATTCTGTCAGAGAGC |
| oVDM2007 | AAAAAAAACCCGGGCAATTGGGGCTATCCATCGGGAAATGAATGCC |
| oVDM2008 | AAAAAAAACTCGAGTTATGACGACGGACTTCTCGC |
| oVDM2009 | GAGGGAATTCTACCGTTCGTATAGCATACATTATACGAAGTTATGA TAAACCCAGCGAACCATTTGAGG |
| oVDM2010 | CTCCGAATTCTACCGTTCGTATAATGTATGCTATACGAAGTTATTCAATCT TTATAAGTCCTTTTATAA |
| oVDM2011 | GGAGGCAGATTGCCTTGAAT |
| oVDM2012 | TCCAATAATTCGGCTCTCT |
| oVDM2013 | GACTCGTTTATGTTTTGTTCCGTC |
| oVDM2014 | GTATTTTCCAAGATCGTTCG |
| oVDM2015 | AAAAAAGTCGACACAAGAAAACGGTTCTGTTTCTGCGAGC |
| oVDM2016 | AAAAAAACCCGGGTTAAGCTTTGGTGTGGTCTTTAGCT |
| oVDM2017 | CGGAGCCACGGCGGCCGAA |
| oVDM2018 | CTGCGCGTAATCTGCTGCTTCGA |

We studied the role of the general stress protein Usp (locus tag: EfmE745_02439) which belongs to this last functional group and confirmed that it has a significant impact on survival of *E. faecium* during starvation. Universal stress proteins (USPs) are mostly studied in *E. coli* where they protect the cell against adverse conditions. However, the exact mechanism(s) by which they act are currently unknown. This could be a result of the many different subtypes found, and the complex responses they contribute to. USPs are found throughout all branches of the tree of life and are important for survival under adverse conditions in many species [23, 24]. In this study we expand these observations to *E. faecium*.

The *exuR* gene (locus-tag: EfmE745_02364) appeared to be important for survival upon nutrient starvation on the basis of our Tn-seq screening, but we were unable to confirm this finding by studying an *exuR* deletion mutant. The *E. coli* ExuR homologue represses genes involved in the metabolism of D-galacturonate and D-glucuronate [36, 37]. ExuR expression is upregulated in the absences of glucose which allows *E. coli* to use different carbon sources for its growth and survival. We speculate that ExuR in *E. faecium* is involved in rerouting carbohydrate metabolism, similar to *E. coli*. It is possible that the derepression of pathways involved in alternative carbon source utilization in the Δ*exuR* mutant might negatively affect the growth of cells during the recovery step in the glucose-rich medium BHI, which we performed to minimize a Tn-seq signal originating from dead cells in the cell suspension.

# CONCLUSIONS

In this study, we have identified 24 *E. faecium* genes that could potentially affect its survival in the absence of nutrients. Our Tn-seq data suggest that most genes involved in survival during nutrient deprivation are part of the core genome of *E. faecium*. However, four genes are enriched in clade A1 strains which could suggest that these genes may play a role in the emergence of strains from this clade as nosocomial pathogens. We have confirmed the role of the *usp* gene, but future functional characterization of other genes identified here may further increase our understanding of the mechanisms by *E. faecium* can survive outside human or animal hosts.

# METHODS

## Bacterial strains, plasmids, growth conditions, andoligonucleotides

The vancomycin-resistant, clade A1 *E. faecium* strain E745 [38] was used throughout this study. *E. faecium* E745 was isolated from a rectal swab of a patient, during routine surveillance of a VRE outbreak in a Dutch hospital, and its genome was previously sequenced to completion [38]. *E. faecium* was grown at 37 °C in brain heart infusion broth (BHI; Oxoid), unless otherwise mentioned. The *E. coli* strains EC1000 [39] and DH5α were grown in Lysogeny Broth (LB). Antibiotics were used at the following concentrations: erythromycin 50 μg $ml^{-1}$, chloramphenicol 10 μg $ml^{-1}$, spectinomycin 200 μg $ml^{-1}$ and gentamicin 300 μg $ml^{-1}$ for *E. faecium* and spectinomycin 100 μg $ml^{-1}$, chloramphenicol 4 μg $ml^{-1}$, erythromycin 100 μg $ml^{-1}$ and gentamicin 50 μg $ml^{-1}$ for *E. coli*. The transposon library in *E. faecium* E745 was previously described [38]. The vectors pWS3 [40], pVDM1001 [41], pCRE-Lox [42], pEF25 [43] and pGPA1 [38] were obtained from our laboratory's culture collection. Genomic DNA isolation was performed usingthe Wizard Genomic DNA Purification kit (Promega).

The sequences of all oligonucleotides used in this study are provided in Table 2. The sequences of synthesized DNA fragments are listed in Additional file 1.

## Isolation and transformation of plasmids

Plasmid isolation from overnight *E. coli* cultures was performed using the GeneJET plasmid miniprep kit (Thermo Fischer Scientific, Bleiswijk, The Netherlands) according to the manufacturer's instructions. Transformation of plasmids into *E. faecium* E745 was performed aspreviously described [42].

## Determination of viability during starvation

*E. faecium* E745 cultures were grown overnight in 3 ml BHI at 37 °C with shaking at 150 rpm. An aliquot of 20 µl of an overnight culture was transferred to 20 ml BHI which was again grown at 37 °C with shaking at 150 rpm until the culture reached an optical density at 600 nm ($OD_{600}$) of 0.3–0.4. Aliquots (3 ml) of these cultures were then centrifuged (3000 g, 5 min), washed once in PBS (137 mM NaCl; 2.7 mM KCl; 10 mM $Na_2HPO_4$; 2 mM $KH_2PO_4$; pH 7.4) and resuspended in 3 ml PBS and incubated at either 37 °C or 20 °C with continuous shaking at 150 rpm. Controls remained in BHI and were incubated at the same temperatures with shaking. At days 0, 1, 4, 7, and 10 samples were taken and serially diluted in PBS, after which viable counts were determined using the Miles and Misra method [44].

## Tn-seq analysis of conditionally essential genes in *E.faecium* E745 during prolonged starvation

For the identification of genes that were conditionally essential for prolonged starvation in *E. faecium*, we grew the previously constructed *mariner* transposon mutant library in *E. faecium* E745 [38] in 10-ml BHI supplemented with gentamicin overnight at 37 °C, after which 20 µl of the culture was transferred to 10 ml pre-warmed BHI supplemented with 200 µg $ml^{-1}$ gentamicin, which was incubated at 37 °C until the culture reached an $OD_{600}$ of 0.3. After pelleting the cells by centrifugation (3000 g, 5 min), washing once with PBS, and resuspension of the cells in 10 ml PBS, a 3 ml aliquot was transferred to a new tube and incubated at 20 °C for 7 days. The remaining suspension was used for the isolation of genomic DNA. At day 7, the cell suspensions were washed once with PBS and then transferred to 3 ml prewarmed BHI and incubated at 37 °C until $OD_{600}$ 0.3 to recover viable cells. The experiments with the transposon mutant library were performed with four independent replicates.

After genomic DNA extraction, Tn-seq libraries were prepared as described previously [38]. Tn-seq libraries were sequenced on one lane of a HiSeq 2500 with 50 nt single-end reads. The sequence reads of the Tn-seq experiments have been made available in the European Nucleotide Archive with accession number PRJEB37076.

## Tn-seq data analysis

Tn-seq data analysis was performed as described previously [38]. In short, Illumina sequence reads were demultiplexed, based on their barcode, using Galaxy [45], and 16-nucleotide fragments of each read, corresponding to E745 genomic sequences flanking the transposon, were mapped to the E745 genome using Bowtie 2 [46]. The number of reads per gene were determined using the Integrative Genome Viewer (IGV

[47];). Reads that mapped to the final 10% of a gene were discarded as these insertions may not inactivate gene function [48]. Read counts per gene were then normalized in each replicate by calculating the RPKM (Reads Per Kilobase per Million input reads), with subsequent statistical analysis of these values being performed with Cyber-T [49, 50]. Genes were determined to be significantly contributing to survival in PBS if the Benjamini-Hochberg corrected *P*-value was < 0.05 and the difference in abundance of a gene between day 0 and day 7 was > 10 or < − 10.

## Construction of targeted deletion mutants

The *usp* deletion mutant was created using CRISPRCas9-mediated genome editing as previously described [41]. In short, a CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) targeting *usp* was inserted into pVDM1001 by annealing oVDM2001 and oVDM2002 together and ligating this DNA fragment into BsaI-digested pVDM1001 creating pVDM-x*usp*. Next, a DNA fragment (gBlock, Integrated DNA Technologies; Leuven, Belgium) was synthesized containing the 463 bp upstream region of *usp* fused together with the 513 bp downstream region of *usp* (Additional file 1). This DNA fragment was amplified using oVDM2003 and oVDM2004 and was subsequently ligated in SmaI-digested pVDM-x*usp* creating pVDM-Δ*usp*. This plasmid was then transformed into *E. faecium* E745 which already carried pVPL3004 [51] with selection for transformants on BHI agar containing 200 µg $ml^{-1}$ spectinomycin and 50 µg $ml^{-1}$ erythromycin at 30 °C for 48 h. The deletion of *usp* was confirmed by PCR using oVDM2005 and oVDM2006. The plasmids pVPL3004 and pVDM-Δ*usp* were cured from *E. faecium* E745-Δ*usp* by sub-culturing in BHI for 72 h.

The 848-bp DNA fragment containing the promotor of *usp* and the complete *usp* gene for *in trans* complementation (Additional file 1) was synthesized, and subsequently this fragment was amplified by PCR using oVDM2015 and oVDM2016, and subsequently digested with XmaI and SalI. The digested fragment was ligated into pEF25 to form pEF25-*usp-C*. This vector was subsequently transformed into E745-Δ*usp* and transformants were selected on BHI agar supplemented with 200 µg $ml^{-1}$ spectinomycin. The presence of the vector in *E. faecium* E745-Δ*usp* was confirmed by PCR using oVDM2017 and oVDM2018 and the resulting complemented strain was named E745-Δ*usp*::*usp-C*.

We were unable to generate an *exuR* deletion mutant with the CRISPR-based genome editing approach described above and we thus used our previously described [42] allelic replacement method, with minor modifications, to generate this mutant. A DNA fragment (Integrated DNA Technologies; Leuven, Belgium) was ordered containing the 496 bp upstream region of *exuR* fused to the 515 bp

region downstream of *exuR* (Additional file 1). The DNA fragment was amplified using oVDM2007 and oVDM2008, digested with XhoI and XmaI and subsequently ligated into similarly digested pWS3 to create pVDM-*exuR*. The gentamicin resistance cassette from pGPA1 was amplified using oVDM2009 and oVDM2010, followed by digestionwith EcoRI. This DNA fragment was then ligated into similarly digested pVDM-*exuR*, resulting in pVDM*exuR*+*G*. E745 was transformed with pVDM-*exuR*+*G* and transformants were selected at 30 °C on BHI supplemented with 200 µg $ml^{-1}$ spectinomycin. The presence of pVDM-*exuR*+*G* in the transformants was confirmed by PCR using oVDM2011 and oVDM2012. To induce a double crossover event to replace *exuR* with the gentamicin resistance cassette, a colony harbouring pVDM-*exuR*+*G* was used to inoculate 200 ml BHI medium and then subcultured for 72 h at 37 °C after which the culture was plated on BHI with 300 µg $ml^{-1}$ gentamicin. At least 100 colonies were transferred to BHI agar containing either 300 µg $ml^{-1}$ gentamicin or 200 µg $ml^{-1}$ spectinomycin. Colonies that were resistant to gentamicin but not spectinomycin were checked for successful double crossover events using oVDM2013 and oVDM2014. The resulting mutant was named E745::Δ*exuR*+*G* and was consequently transformed with pCRE-*lox* to remove the gentamicin cassette from the genome, as described previously [42]. Removal of the gentamicin cassette was confirmed using PCR using oVDM2013 and oVDM2014 andcuring of pCRE-*lox* after removal of the gentamicin cassette was performed as described previously [42].

## Presence and absence of genes involved in survivalduring starvation in *E. faecium* genomes

Abricate (https://github.com/tseemann/abricate, version 0.8) was used to identify presence of the 24 *E. faecium* E745 genes that had the largest role in survival in PBS, as determined by Tn-seq, against the draft assemblies from 1644 *E. faecium* genomes that represent the global diversity of the species *E. faecium* [8]. We considered a minimum identity and coverage of 95 and 80%, respectively, to consider a gene as present in a particular draftassembly. To visualize the presence and absence of these genes in the context of the phylogeny of *E. faecium*, we used the neighbour-joining tree described in [8], based on a RAxML tree-based of 955 *E. faecium* core genes, and used the R package ggtree (version 1.14.6) to plot the presence of the different E745 genes.

# SUPPLEMENTARY INFORMATION

Supplementary information accompanies this paper at https://doi.org/10. 1186/s12864-020-06984-2.

PBS: Phosphate-buffered saline; BHI: Brain Heart Infusion broth;

BH: Benjamini-Hochberg; USPs: Universal Stress Proteins; LB: Lysogeny Broth; $OD_{600}$: Optical density at 600 nm; RPKM: Reads Per Kilobase per Million input reads; IGV: Integrative Genome Viewer; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeat

## Acknowledgements
Not applicable.

## Authors'contributions
VdM performed experiments and analysed the data. SAA contributed bioinformatic analyses. RW and WvS designed the study. VdM and WvS drafted the manuscript. All authors read and approved the final manuscript.

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## Availability of data and materials
The datasets generated in the current study are available in the European Nucleotide Archive with accession number PRJEB37076. The genome sequence of *E. faecium* E745 used in this study is available through the European Nucleotide Archive with accession number GCA_001750885.1. Requests for *E. faecium* E745 can be made by contacting the correspondingauthor.

## Ethics approval and consent to participate
Not applicable.

## Consent for publication
Not applicable.

## Competing interests

None to declare.

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# SUPPLEMENTARY FILE 1.

**usp deletion mutant**

AAAAAAACCCGGGACAAACAACGGCATTAACGGAGATTACCATCTTTTAAAGAAT-
AAAGCAATTTGCTCTTGGCAAATTGCTTTTTCTATTTGTTGCTTATTTTGGAGGATT-
TGTTCATGATTAGGATTTTAATGATTATCGCCACTTTAGTATTATTATTCGTTAGCTAT-
TATTTATTTAATAAACAGGATATCTTTTTTGTATTGATCAACAAAAATGATAAAAAT-
CAAGGATTCCTACAATTTTACGGAGCTGCATATGCTGTTTTAGGCGTAATGGGTAT-
TTTAACAGCTTTTTTCAATCAACGCTTTATTGCCTTGGTCTTTTTACTGATCGTAAT-
TATTGTTTCTGCCACCTTCAGTATCCGATTTGCAAAAAAAATTGCTGAACCAAAG-
CAGTAAATAATTCTCTTTTTCCCTAAAGTGTTCTACAATAAAAGAGTACAATAC-
GATTGACTTGAGGAGTGAATGCTTGAATTCCACGTAGACGACCTGCCAACTATCGG-
CAGGTTTTTTTGCACAAAAAACCGTGATAAAAACAAAAGACGCGGCAAGCACTCA-
TCGGTCTTTATAATGTTTAAAATAAAGGGAAAACAGTCAATGAGTTCAAGTAAAAT-
AAAAAAAAGTGAACACCAGGGCGATTAGTCGCCCTGGTGTGGTTTAAAACGACC-
TAAGCATGCCAAAGCTTATTGGTTCGTCAATGTGGAAGTTTCCGATGAATCTGATAC-
GAAAAAATATGAAGTATCGATGGCGGATACCGATTTTATCGCCAAACTAAAATTTTAT-
TTCGGGTTCGGGTACCGCGAGAAGGCAAGTGCTACATTCGGTAAATCATTCAAGAAT-
TGATGAAAGGCTACCGCTTGCCATTGCAACCACAACGATACTCCTGAACGCGATGTT-
GGTGAGATTTCCAAAGAGAAGAGCTGTTTAATGCAACGGTATTAAGCAAAT
GGCAAAAGGAAGTCATGTAGACAAAGTTTTTTATCAAACACCTCGAGTTTTTTTT

**usp complementation**

AAAAAAGTCGACACAAGAAAACGGTTCTGTTTCTGCGAGCTCTCTCATTGCTCTTTT-
TAAATTCATATGCTACTATAGAGGTGGGAAATCTGTGATGTTCGAATTGTCCTCTTTGTGT-
GTTGACCGAACATTTTTATTGATATCTTGGGACCCGTCTTGTGTCACTGCTGGTAACATG-
CCTTATCATTTGGTAGTTCGAAGCTACGACCATGGGACCCACCTGCTTGAATTCGCGGGAT-
CAATACTACAGGACAAACAACGGCATTAACGGAGATTACCATCTTTTAAAGAATAAAGCAA-
TTTGCTCTTGGCAAATTGCTTTTTCTATTTGTTGCTTATTTTGGAGGATTTGTTCATGT-
TACAACAATACAAAAAAATCATGGTTGCCGTAGATGGTTCTGATGAAGCTGAATTAGCTTT-
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TGACACACGTTCTTTCCAAACCGTTTCATCCTTTGATGGAATGCTTGCAGAACAAGCTACT-
GAAATGGCAAAGCAAACATTGGCAGATTATGAAAGCAATGCGAAGAAAGCTGGCTTGAA-
TAATGTAACCTCTGTCGTAGAATATGGCTCACCAAAACAAATTATTGCCAGAGAAAT-
CCCAGAAGATAATCAAGTAGATTTGATTATGTTAGGAGCCACAGGATTGAATGCTGTA-
GAACGTTTATTTATTGGTTCTGTTTCTGAATATGTTATTAGAAACGCAGCTTGCGATGT-
GCTTGTTGTCCGAACTGATTTAGAAAACCAATTGCCAGCTAAAGACCACACCAAAGCT-
TAACCCGGGTTTTTTT

**exuR deletion mutant**

AAAAAAAACCCGGGCAATTGGGGCTATCCATCGGGAAATGAATGCCAAGAAATGGACT-
TGGATCGCCATAGGCTATCAATGCGGTTTAGCTTATTTAGTTAGTTTTGTGATTTATCAGTT-
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GATAGGACTCGTTTATGTTTTGTTCCGTCAGCCAAAATCCAAGAACCAGTTTTATACTA-
TATTGTCATTAGAAGGAGAGGAATAGGATGGCGACATTCTTATTAAGTGTGTTAATCTTTG-
GAAGTGCAGGCGCAATTGTCTATACTCGTTTAAAATCCGGTAAGAATTGTGATGACT-
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GCAAGAGGCTATTCTCCCCTTTTTTGCTTATTTTGCTAGAGCATAGTAAGATGAGG-
GAAAGTAAAAAAAGAAAGAGGAAAGAAAATGAATTCTGTGTGAAATATTAAAAAAAT-
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ACAAGCAAATTTCTTTCATTTTTGACAAAGTGACTACATGATGTTAACATAGTGTG-
GTCACTTTTTTCAGAAAGAAAAATGAATTTACATAAAAAAGGAGAATTTGTTTGG-
TAAAAGTAGAAGTAAAACATTTAACAAAGATATTTGGTAAAAAGACACAAGCCGCTTTA-
GATATGATGAATGATCACCAGCCGAAAACGGAGATTTTAAAGAAAACCGGTGCAACGGTT-
GGTGTGTATGACGTGAATTTTGACGTAAAAGAAGGAGAAATCTTCGTGATCATGGGGCTTT-
CCGGGAGTGGGAAATCCACATTGATCCGTCTATTGAATCGTCTGATTGAGCCAACTTCGGG-
CAGTATCTATATTGATGGAGAAGATGTATCGAAGTTAAGCAAAGAAGAATTGCGAGAAGTC-
CGTCGTCATAACTCGAGTTTTTTTT

**usp in-trans complementation**

AAAAAAACCCGGGACAAACAACGGCATTAACGGAGATTACCATCTTTTAAAGAAT-
AAAGCAATTTGCTCTTGGCAAATTGCTTTTTCTATTTGTTGCTTATTTTGGAGGATT-
TGTTCATGATTAGGATTTTAATGATTATCGCCACTTTAGTATTATTATTCGTTAGCTAT-
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CAAGGATTCCTACAATTTTACGGAGCTGCATATGCTGTTTTAGGCGTAATGGGTAT-
TTTAACAGCTTTTTTCAATCAACGCTTTATTGCCTTGGTCTTTTTACTGATCGTAAT-
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CAGTAAATAATTCTCTTTTTCCCTAAAGTGTTCTACAATAAAAGAGTACAATAC-
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CAGGTTTTTTTGCACAAAAAACCGTGATAAAAACAAAAGACGCGGCAAGCACTCA-
TCGGTCTTTATAATGTTTAAAATAAAGGGAAAACAGTCAATGAGTTCAAGTAAAAT-
AAAAAAAAGTGAACACCAGGGCGATTAGTCGCCCTGGTGTGGTTTAAAACGACC-
TAAGCATGCCAAAGCTTATTGGTTCGTCAATGTGGAAGTTTCCGATGAATCTGATAC-
GAAAAAATATGAAGTATCGATGGCGGATACCGATTTTATCGCCAAACTAAAATTTTAT-
TTCGGGTTCGGGTACCGCGAGAAGGCAAGTGCTACATTCGGTAAATCATTCAAGAAT-
TGATGAAAGGCTACCGCTTGCCATTGCAACCACAACGATACTCCTGAACGCGATGTT-
GGTGAGATTTCCAAAGAGAAGAGCTGTTTAATGCAACGGTATTAAGCAAAT
GGCAAAAGGAAGTCATGTAGACAAAGTTTTTTATCAAACACCTCGAGTTTTTTTT

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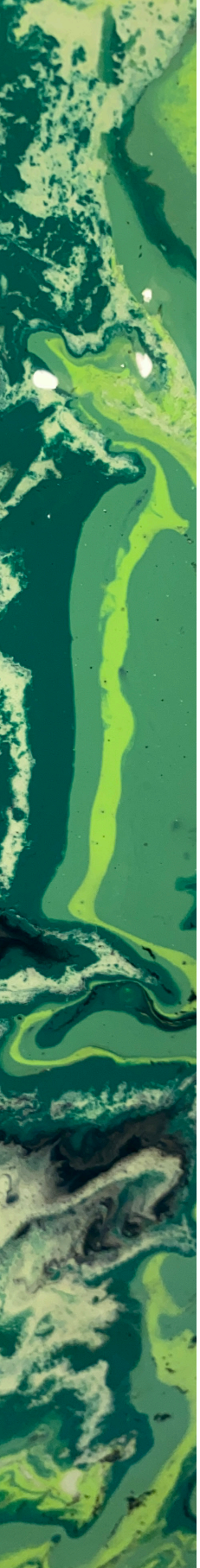

# Chapter 5

## Identification of *spxA* as an isopropanol tolerance determinant in *Enterococcus faecium* E8202

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Manuscript in preparation

# ABSTRACT

The nosocomial pathogen *Enterococcus faecium* can readily spread from patient-to-patient in hospital settings. Many hospitals have implemented the use of alcohol-based hand-rubs (ABHR) to prevent the spread *Enterococcus faecium* and other nosocomial pathogens. This practice has recently been linked to an increased tolerance of *E. faecium* towards 2-propanol, the most commonly used alcohol in ABHR. Currently there is a lack of data on the mechanisms that contribute to the ability of *E. faecium* to survive an acute exposure to 2-propanol. A high-throughput transposon mutant library screening (Tn-seq) identified three genes that contributed to survival during 2-propanol exposure, with functions associated with stress response, glycerol metabolism and peptidoglycan remodelling. The gene which was quantitatively most important for survival was *spxA* (locus tag: EQB38_RS13145), which is predicted to encode a 15.4 kDa protein of the Spx/ArsC family of transcriptional regulators. After generating a targeted insertion mutant in *spxA*, we were able to confirm that *spxA* significantly contributes to tolerance to 2-propanol. As *spxA* is present in 100% of 1644 *E. faecium* genomes that span the species' diversity, we postulate that this gene is a key determinant for the innate tolerance to alcohols of *E. faecium*. Further mechanistic studies into *spxA* and other genes identified in this study may shed further light on the mechanisms by which *E. faecium* can increase tolerance to exposure to ABHR.

# INTRODUCTION

Enterococci have emerged as major contributors to healthcare associated infections causing approximately 10% of all cases of hospital-acquired bacteraemia (1). *Enterococcus faecalis* and *Enterococcus faecium* are responsible for most enterococcal infections. In the United States of America they are the fourth and fifth leading cause of sepsis respectively (1, 2). While enterococci have relatively low virulence potential, they often cause nosocomial infections and have acquired resistance to many commonly used antibiotics, including to the glycopeptide vancomycin, greatly complicating the treatment of infected patients (3–5). The multi-drug resistant nature of *E. faecium* clinical isolates, combined with their tendency to be transmitted between patients in healthcare settings, makes it increasingly difficult to contain the dissemination of nosocomial *E. faecium* infections within and between hospitals (3–5).

Good and effective hand hygiene programs are of key importance for hospitals to control the spread of infectious diseases. While the use of water and soap is effective, it is also relatively slow and repeated washing will damage the skin. An alternative to soap are alcohol-based hand rubs (ABHR), which were standardised by the World Health Organisation (WHO) in 2009 by specifying the composition of ABHR, including alcohols, to be used to reach optimal efficacy (6). This standardisation has contributed to a decline in prevalence of several nosocomial pathogens, including methicillin-resistant *Staphylococcus aureus* (6, 7). The procedure of applying ABHR takes about 30 seconds and leads to a 3.5 $\log_{10}$ reduction in bacterial counts, which is considerably more than water and soap (8). ABHR mainly consist of a mixture of alcohols, most often including 2-propanol and 1-propanol (6). These alcohols are small molecules that are able to easily penetrate the phospholipid bi-layer of bacterial membranes, thereby disrupting membrane fluidity and polarity, and subsequently enter the cytosol. Consequently, cellular processes such as protein and DNA synthesis are also affected by alcohol exposure (9, 10)**.** Alcohol tolerance has been observed in several bacterial species but the exact mechanism by which the reported mutations and stress responses affect alcohol tolerance is not clear (9, 11).

Recently, Pidot and co-workers determined the 2-propanol tolerance of 139 *E. faecium* strains that were isolated in an Australian hospital between 1998 and 2015 (12). The authors found that more recently isolated strains have up to 2 $\log_{10}$ higher survival rates compared to strains isolated from before 2009, which coincided with the increased use of ABHR in the studied hospital. The alcohol-tolerant strains also showed increased colonisation efficiency in a mouse model for transmission after disinfection (12). Three genomic regions that positively correlated with increased tolerance were

identified but deletion mutants of these three regions did not show any reduced tolerance when exposed to high levels of 2-propanol. However, these mutants had a lower doubling time when grown in medium supplemented with 3% (v/v) 2-propanol, leading to the conclusion that alcohol tolerance in *E. faecium* is a polygenic phenotype in which multiple genes and mutations contribute to increased tolerance.

It thus remains unclear which mechanisms are involved in *E. faecium* alcohol tolerance. Therefore, a *mariner* transposon library was constructed in a 2-propanol tolerant strain to identify genes that contribute to this phenotype.

# MATERIALS AND METHODS

## Bacterial strains, plasmids, growth conditions, and oligonucleotides

The origins of all *Enterococcus faecium* strains used in this study are listed in **table 1**. Unless otherwise mentioned, *E. faecium* was grown in brain heart infusion broth (BHI; Oxoid) at 37°C. The *E. coli* strains EC1000 and DH5α were grown in Luria-Bertani (LB) medium. When necessary, antibiotics were used at the following concentrations: chloramphenicol 10 μg $ml^{-1}$, spectinomycin 200 μg $ml^{-1}$ and gentamicin 300 μg $ml^{-1}$ for *E. faecium* and spectinomycin 100 μg $ml^{-1}$, chloramphenicol 4 μg $ml^{-1}$ and gentamicin 50 μg $ml^{-1}$ for *E. coli*. The vectors pWS3 and pGPA1 were obtained from our laboratory's culture collection. (13) **(**14)

The sequences of all oligonucleotides used in this study are shown in supplementary table 1**.**

## Isolation and transformation of plasmids

Plasmids isolation from overnight *E. coli* cultures was done using the GeneJET plasmid Miniprep kit (Thermo Fischer Scientific, Bleiswijk, The Netherlands) according to the manusfacturer's instructions. Transformation of plasmids into *E. faecium* E8202 was performed as previously described (14). Genomic DNA isolation was performed using the Wizard Genomic DNA Purification kit (Promega).

## Generation of transposon mutant library in *E. faecium* E8202

The transposon library was constructed as previously described (15)we identify genes that contribute to growth of E. faecium in human serum through transcriptome profiling (RNA-seq. In short, pGPA1 was electrotransformed into *E. faecium* E8202, and presence of the plasmid in gentamicin- and chloramphenicol-resistant colonies was confirmed via PCR (primers 6 and 7). The transposase was induced by

the addition of 25 ng $ml^{-1}$ nisin to the culture after which selection for transposon mutants and loss of pGPA1 was induced by sub-culturing without antibiotic pressure at 37°C, at which temperature pGPA1 cannot replicate, for up to 3 days. Curing of pGPA1 was confirmed by plating cultures on BHI agar supplemented with either chloramphenicol or gentamicin.

## Alcohol tolerance assay

*E. faecium* E8202 cultures were grown overnight in 3 ml BHI at 37°C with shaking at 150 rpm. The optical density at 600 nm ($OD_{600}$) was measured and the culture was diluted to $OD_{600}$ = 0.5 with phosphate-buffered saline (PBS; NaCl 137 mM;

**Table 1:** strains used in this study

| Strain | Country of isolation | Isolation year | Source | reference |
|---|---|---|---|---|
| Aus0004 | Australia | 1998 | Patient blood | (31) |
| E688 | Spain | 1995 | Pig | (4) |
| E745 | Netherlands | 2000 | Patient faeces | (32) |
| E980 | Netherlands | 1998 | Healty human faeces | (33) |
| E1007 | Netherlands | 1998 | Healty human faeces | (4) |
| E1071 | Netherlands | 2000 | Patient faeces | (33) |
| E1133 | USA | 2001 | Patient faeces | (4) |
| E1162 | France | 1997 | Patient blood | (33) |
| E1590 | Ireland | 2001 | Healty human faeces | (4) |
| E1627 | Netherlands | 1979 | Patient faeces | (4) |
| E1794 | USA | 1991 | Patient | (34) |
| E1861 | Spain | 2001 | Patient faeces | (4) |
| E2071 | Denmark | 2001 | Poultry | (4) |
| E8200 | Netherlands | 2015 | Patient faeces | (22) |
| E8202 | Netherlands | 2015 | Patient faeces | (22) |
| E8303 | Netherlands | 2015 | Patient faeces | (22) |
| E8425 | Netherlands | 2015 | Patient faeces | (22) |
| E9006 | Netherlands | 2015 | Patient faeces | (22) |

2.7 mM KCl; 10 mM $Na_2HPO_4$; 2 mM $KH_2PO_4$; pH 7.4). Subsequently, 1 ml of the diluted overnight culture was transferred to a 2 ml Eppendorf tube and either 1 ml PBS or 1 ml PBS with 46% (v/v) 2-propanol was added and mixed extensively by vortexing. After 5 minutes incubation at room temperature, the tubes were again mixed extensively by vortexing after which a 20 µl sample was transferred to 180 µl PBS and further serially diluted, after which viable counts were determined using the Miles and Misra method (16).

## Tn-seq analysis of conditionally essential genes in *E. faecium* E8202 during exposure to 2-propanol

For the identification of genes that were conditionally essential for 2-propanol tolerance in *E. faecium*, we cultured the transposon library in BHI supplemented with 200 µg $ml^{-1}$ gentamicin and then exposed each culture separately to 0, 20% (v/v) and 23% (v/v) 2-propanol. After exposure the 2-propanol-exposed cultures were transferred to 18 ml fresh BHI and incubated at 37°C with shaking at 150 rpm until $OD_{600}$ = 0.3. Genomic DNA was isolated from this recovered culture using the Wizard Genomic DNA Purification kit (Promega). The experiments with the transposon mutant library were performed using four independent replicates.

Genomic DNA was prepared for Tn-seq analysis as described previously (17). In short, 2 µg genomic DNA was digested for 4 h at 37°C using 10 U MmeI (New England Biolabs) and then dephosphorylated with 1 U of calf intestine alkaline phosphatase (Invitrogen) during 30 min at 50°C. DNA was then isolated using phenol-chloroform extraction and subsequently precipitated using ethanol. The DNA pellets were dissolved in 20 µl water. The samples were further processed, including the addition of barcodes and prepared for Illumina sequencing as described previously (17). The sequence reads have been made available on the European Nucleotide Archive with accession number PRJEB37086.

## Tn-seq data analysis

Illumina sequence reads were deconvoluted, based on their barcode, using the Galaxy platform (18), and 16-nucleotide fragments of each read that corresponded to E8202 sequences, were mapped to the E8202 genome using Bowtie 2 (19). The results of the alignment were sorted and counted by IGV (20) using a 25-nucleotide window size and then summed over the gene. Reads 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 Kilobase per Million input reads) via the following formula: RPKM = (number of reads

mapped to a gene × $10^6$) / (total mapped input reads in the sample × gene length in kbp). Statistical analysis of the RPKM-values between the experimental conditions was performed using Cyber-T (21). Genes were determined to be significantly contributing to growth in human serum when the Benjamini-Hochberg corrected P-value was <0.1 and the difference in abundance of the transposon mutants between unexposed cultures and cultures exposed to either 20% or 23% (v/v) 2-propanol. In supplementary **Figure S1** is an overview of the average transposon insertions in the E8202 transposon library.

## Presence and absence of genes identified by Tn-seq in 1644 *E. faecium* genomes

Abricate (version 0.8) was used to perform a search of the three *E. faecium* E8202 genes that had the largest role in survival upon exposure to 2-propanol against the draft assemblies from 1,644 *E. faecium* genomes that represent the global diversity of the species *E. faecium* (22). We used a minimum threshold identity and coverage of 95% and 80%, respectively, to consider a gene as present in a particular draft assembly. To visualize the presence and absence of these genes in the context of the phylogeny of *E. faecium*, we used the neighbor-joining tree described at (22), based on a RAxML tree-based of 955 *E. faecium* core genes, and used the R package ggtree (version 1.14.6) to plot the presence of the different E8202 genes.

## Generation of the *spxA*::pWS3 insertion mutant

A 285 bp section of *spxA* was amplified by PCR using Phusion high-fidelity DNA polymerase (Thermo-Fisher) from *E. faecium* E8202 genomic DNA using primers 1 and 2. The amplified fragment was digested by SacII and XhoI and ligated into the thermosensitive plasmid pWS3 digested with the same enzymes, resulting in pWS3-*spxA*. Insertion of the *spxA*-fragment into pWS3 was confirmed by PCR using primers 3 and 4 subsequent Sanger sequencing of the amplicon. pWS3-*spxa* was electrotransformed in E8202, followed by recovery in BHI supplemented with 0.5M sucrose for 2 h at the permissive temperature of 30°C, after which the cells were plated on BHI plates supplemented with 200 mg $ml^{-1}$ spectinomycin at 30°C to select for transformants. Spectinomycin-resistant colonies were picked and grown overnight in 200 ml of BHI broth supplemented with 200 mg $ml^{-1}$ spectinomycin at an elevated temperature (37°C) to select for plasmid integration. The cells were then plated on BHI agar plates with spectinomycin at 37°C. Single-cross-over integration of pWS3 into *spxA* was verified by PCR with primers 4 – 5 and additionally verified via Sanger sequencing.

# RESULTS

## Alcohol tolerance across isolates from different sources and time periods

Pidot *et al.* (12) previously showed that alcohol tolerance has steadily increased over the past 15 years in *E. faecium* strains isolated from an Australian hospital. In order to identify genes essential in alcohol tolerance we first analysed alcohol tolerance in a limited set of isolates, including non-hospital and animals isolates from different time periods. This revealed that alcohol tolerance differs considerably from strain to strain regardless of isolation source and date (**Figure 1**). Among recently isolated nosocomial strains, only E8200 was significantly more tolerant when compared to older hospital strains. Unfortunately we were unable to generate a mariner transposon library in this strain and therefore decided to continue with the second-most tolerant strain, E8202.

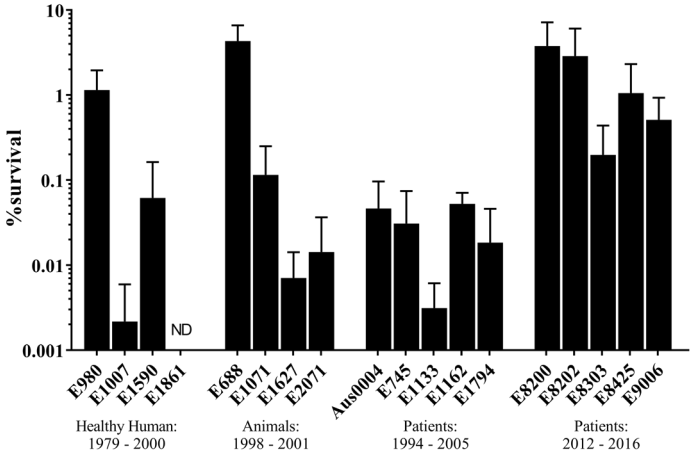


**Figure 1: Tolerance to 2-propanol of *E. faecium* isolates.** Tolerance to 2-propanol was determined by exposing each strain to 23% (v/v) 2-propanol for 5 minutes and determining survival. *E. faecium* strains were collected from different isolation sources including healthy humans (human commensal), animals, and hospitalized patients from two different decades. (ND = not detected/below detection limit)

## Tn-seq analysis for genetic elements involved in alcohol tolerance in E8202

To identify genes implicated in alcohol tolerance we constructed a mariner transposon library in the 2-propanol-tolerant strain E8202. The relative abundance of transposon mutants in 2682 chromosomal and plasmid genes of *E. faecium* E8202 exposed to 23% v/v 2-propanol was compared to the abundance of mutants present in the control samples to which an equal volume of PBS was added **(Figure 2A)**. The abundance of transposon mutants of 237 genes was significantly impacted

(Benjamini-Hochberg (BH) corrected P-value of <0.1) by 2-fold or more. We hypothesised that the relatively high number of genes identified here, may be due to the relatively large part of the population of transposon mutants losing viability in a stochastic fashion during exposure to 23% 2-propanol. We therefore also exposed the transposon library to 20% (v/v) 2-propanol (**Figure 2B)** and this resulted in only 3 genes with a significantly reduced abundance (BH-adjusted P-value <0.1, fold change 2-fold or more), indicating that these genes potentially contributed to the survival of *E. faecium* when exposed to high concentrations of 2-propanol (**Table 2)**. Transposon insertion mutants of two genes were only moderately selected against during 2-propanol exposure and these encoded an alpha-glycerophosphate oxidase (locus tag: EQB38_RS01810, *glpO*), which is involved in glycerol metabolism, and a serine-type D-Ala-D-Ala carboxypeptidase (locus tag: EQB38_RS06180), which is a member of the penicillin binding proteins which are involved in peptidoglycan

**Table 2:** Genes with a role in 20% (v/v) 2-propanol tolerance as identified by Tn-seq.

| Locus tag | Name | Description | Fold change | P-value (BH-adjusted) |
|---|---|---|---|---|
| EQB38_RS01810 | glpO | alpha-glycerophosphate oxidase | -2.18 | 0.076 |
| EQB38_RS06180 | - | serine-type D-Ala-D-Ala carboxypeptidase | - 2.26 | 0.047 |
| EQB38_RS13145 | spxA | transcriptional regulator Spx | -11.11 | 0.076 |

synthesis and remodelling. The third gene to be identified after Tn-seq analysis upon exposure to 20% 2-propanol encodes the transcriptional regulator Spx (locus tag: EQB38_RS13145, *spxA*). Both *glpO* and *spxA* were identified in the Tn-seq screening upon exposure to both 20% and 23% 2-propanol. Mutants in *spxA* were particularly strongly reduced in our Tn-seq screening. Further inspection of the transposon abundance showed numerous insertion sites within *spxA* which were all reduced after exposure (**Figure 3).** The *spxA* gene encodes a 15.4 kDa protein of the Spx/ArsC family of transcriptional regulators and is conserved among low-GC Gram-positive bacteria like *Bacillus subtilis* and *Lactococcus lactis*, in which these homologues are involved in the oxidative stress response, competence development and stress tolerance (23–25). We studied the presence of the three genes identified by Tn-seq in whole genome sequences of a collection of 1646 *E. faecium* strains(26) isolated from healthy humans, patients, pets, pigs, and poultry **(Figure 4)**. We found that *glpO* is present in 99% of the isolates tested while EQB38_RS06180 and *spxA* are present in 100% of the isolates tested and thus all can be considered part of *E. faecium* core genome. We decided to further study the transcriptional regulator *spxA* and its impact on alcohol tolerance in *E. faecium*.

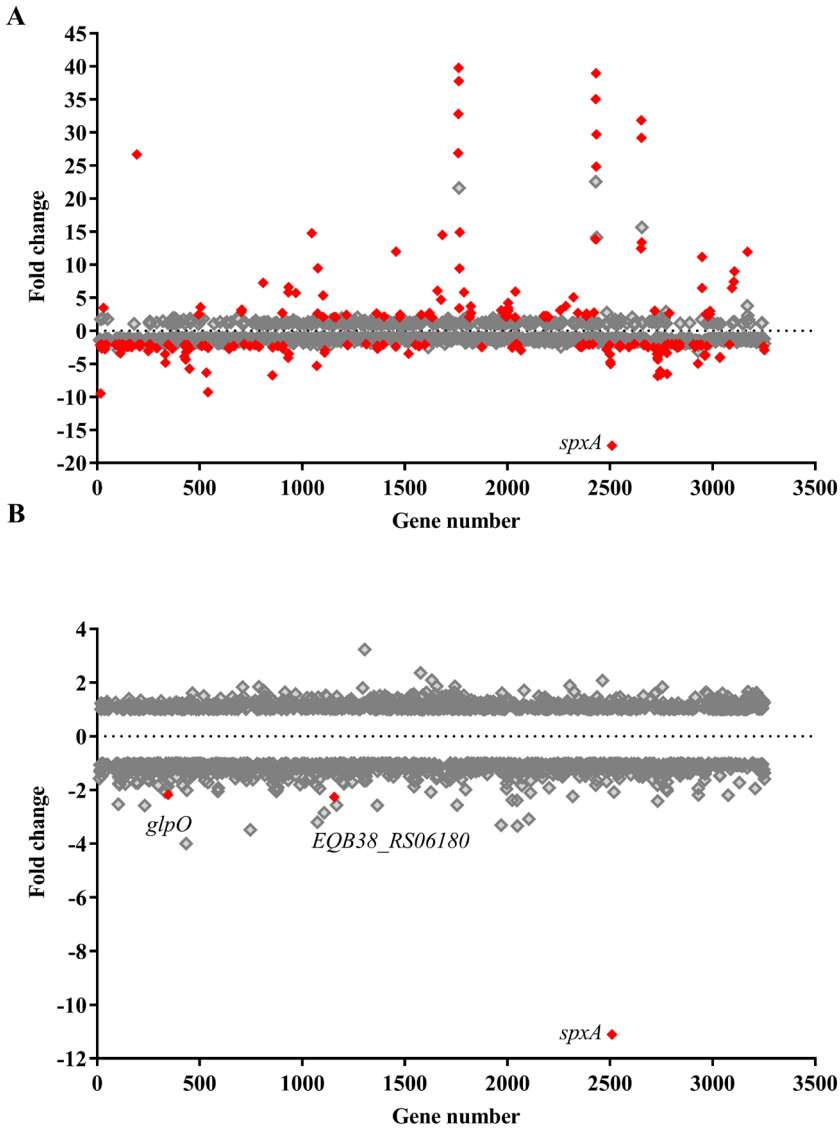


**Figure 2: Tn-seq analysis to identify genes with a role in 2-propanol tolerance.** Each diamond represents a gene in the transposon library. The Y-axis indicates the relative abundance of transposons in each gene after a 5 minute incubation with 23% (A) or 20% (B) (v/v) 2-propanol compared to the abundance of insertions in each gene at the start of the experiment. A positive value indicates an enrichment of mutants during incubation in 2-propanol while a negative value indicates genes that contribute to survival in 2-propanol, with red diamonds representing genes that have a Benjamini-Hochberg adjusted p-value of <0.1 and a fold change of > 2 or < -2.

## The transcriptional regulator SpxA contributes to tolerance to 2-propanol in *E. faecium* E8202

To determine whether *spxA* has an impact on the survival of E8202 when exposed to 2-propanol we created a disruption mutant in *spxA* (E8202-spxA::pWS3). We exposed E8202 and E8202-spxA::pWS3 to 23% v/v 2-propanol for 5 minutes to determine their individual tolerance **(Figure 5)**. E8202 has a similar response as previously observed with a 1.8 $\log_{10}$ reduction in viable counts. E8202-spxA::pWS3 was considerably more susceptible to 2-propanol exposure, with a reduction in viable counts by 4 $\log_{10}$ CFU/ml. E8202-spxA::pWS3 was recovered on BHI agar with and without supplementation of spectinomycin to confirm that any reduction in CFU was not due to excision of the plasmid that had integrated in *spxA*. Survival was identical on both media, indicating that reversion of the insertion mutant to the wild-type genotype did not have an appreciable impact on the outcome of 2-propanol tolerance assays. These results thus confirm the role of *spxA* in tolerance of E8202 to 2-propanol. We lacked the time to perform *in trans* complementation of the mutant.

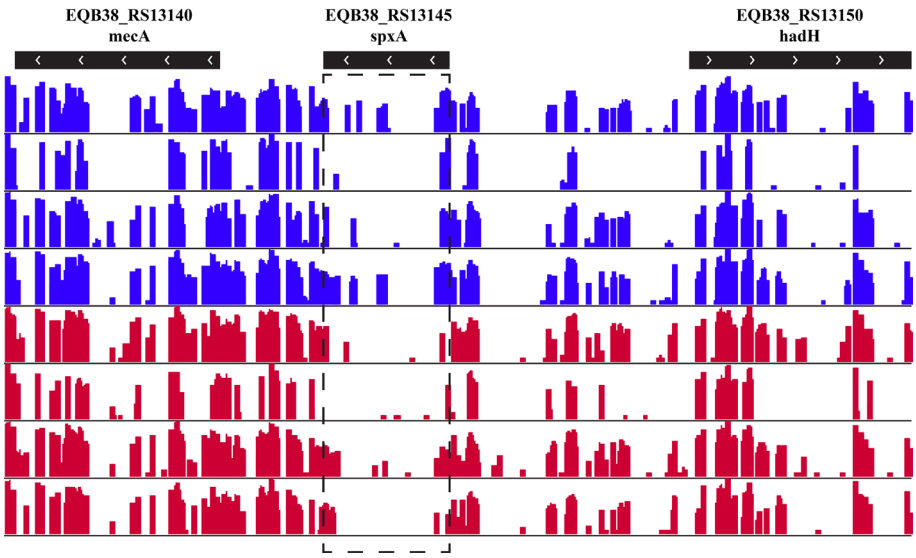


**Figure 3: Visual representation of the transposon inserts in EQB38_RS13145 and its surrounding genes.** Genes and gene direction are depicted by the black bars with arrows. Transposon insertion abundance is shown by the bars below the genes on a Log10 scale. Abundance of transposon insertions in the negative control (blue) and transposon abundance upon exposure to 20% 2-propanol for 5 minutes (red) is shown. Transposons within EQB38_RS13145 are outlined with a dashed line.

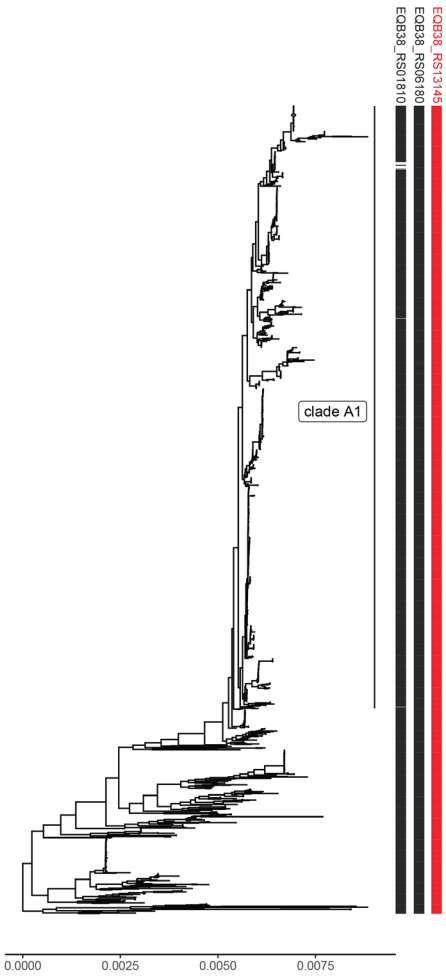


**Figure 4: Presence of genes involved in 2-propanol tolerance in the *E. faecium* population.** Whole genome sequence based phylogenetic tree of 1644 *E. faecium* strains representing the global *E. faecium* population. The presence of the different E8202 genes was plotted along the phylogeny using the R package ggtree. The *spxA* gene is highlighted in red.

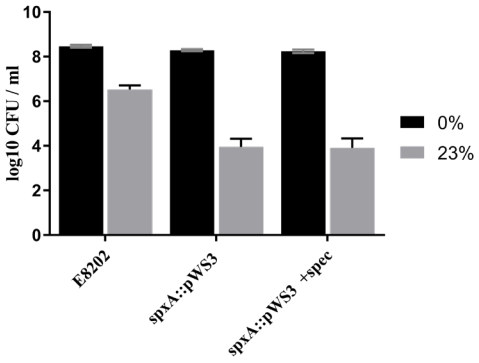


**Figure 5: Phenotype of the *spxA*::pWS3 mutant.** Strains were exposed for 5 minutes to either 0% (black bars) or 23% (grey bars) 2-propanol at room temperature. The *spxA*::pWS3 + spec samples were recovered on BHI agar plate supplemented with 200 µg $ml^{-1}$ spectinomycin while the other two were recovered on BHI agar without antibiotic supplementation. The graph depicts the average data of four biological replicates with error bars depicting standard deviations.

# DISCUSSION

In 2009 the WHO formalised the use of ABHR and set minimum requirements for their efficacy. After this standardization and widespread implementation of ABHR many hospitals saw a noticeable decrease of hospital-associated bacterial infections (6). A recent study in an Australian hospital revealed a trend of increasing tolerance to alcohols in hospital-associated *E. faecium* strains isolated after 2009 (12). Furthermore they showed that these more tolerant strains have a better chance of colonizing mice in an infection model, supporting the notion that this increase in tolerance allows for more efficient transmission to new hosts (12).

In our study we investigated strains from clinical and non-clinical sources for increased alcohol tolerance and aimed to identify genetic elements that contribute to this increased tolerance. We observed that two strains, E980 and E688, which were isolated from healthy humans and animals prior to 2012, were 2-propanol-tolerant to the same level as recently isolated hospital strains. This indicates that tolerance to alcohols is a phenotype that is unlikely to be limited to *E. faecium* hospital isolates and suggests that the increase of tolerance is not due to newly acquired genetic elements but due to selection of strains with an intrinsic 2-propanol tolerance. To obtain a better understanding into the genes that contribute to 2-propanol tolerance, we performed Tn-seq on a transposon mutant library that was exposed to 23% and 20% 2-propanol for 5 minutes. This analysis revealed a variety of genes that were potentially involved in retaining viability, with the *spxA* gene being most prominent. In other Gram-positive bacteria, SpxA is an important transcriptional regulator that is induced by oxidative stress and has an impact on the oxidative stress response and competence (27–29). It is as yet unclear whether alcohols cause oxidative stress directly or if it is a side effect of its disrupting effect on membranes, DNA and protein synthesis (30). Our data shows that *spxA* is important to survive exposure to 2-propanol in *E. faecium* E8202. Because *spxA* is present in all *E. faecium* strains, including in alcohol-sensitive strains, this gene is not the sole factor that determines whether a strain is tolerant or sensitive to alcohols. It is possible that upstream regulators of *spxA* and genes that are regulated by *spxA* could be responsible for the remarkable differences in alcohol tolerance among *E. faecium* strains. Further research is thus required to better understand which genetic elements are important to impact alcohol tolerance in this opportunistic pathogen.

# SUPPLEMENTARY FIGURES

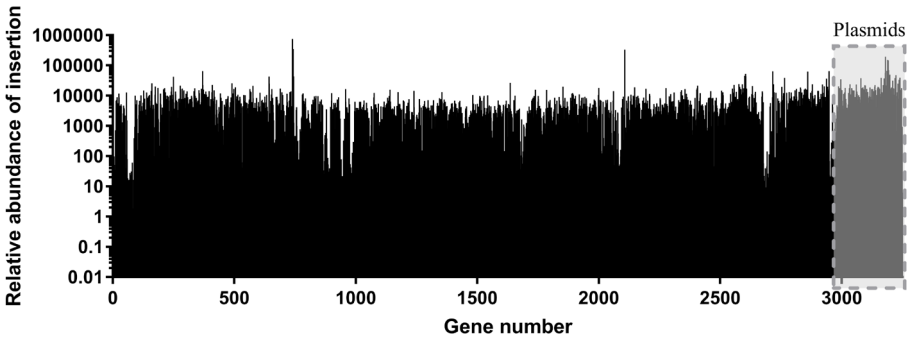


**Figure S1: Overview of relative abundance of transposon insertions throughout the genome of E8202.** Each bar represents the average insertion abundance per gene over four replicates.

**Table S1:** Oligonucleotide sequences[a]

| Name | Sequence 5' - 3' |
|---|---|
| Primer 1 - XhoI | AAAAAAAACTCGAGGTTGTACTTCCTGCAGAAAGGCTC |
| Primer 2 - SacII | AAAAAAAACCGCGGCCAACTTGCAGACGTTTATCA |
| Primer 3 | TGTGCTGCAAGGCGATTAAGTTGG |
| Primer 4 | AAAGCGTCCCTACTACTGACAGCTTC |
| Primer 5 | AGTACACGGATCGTATTTTCATTAATATGTTC |

[a] **Restriction sites are underlined.**

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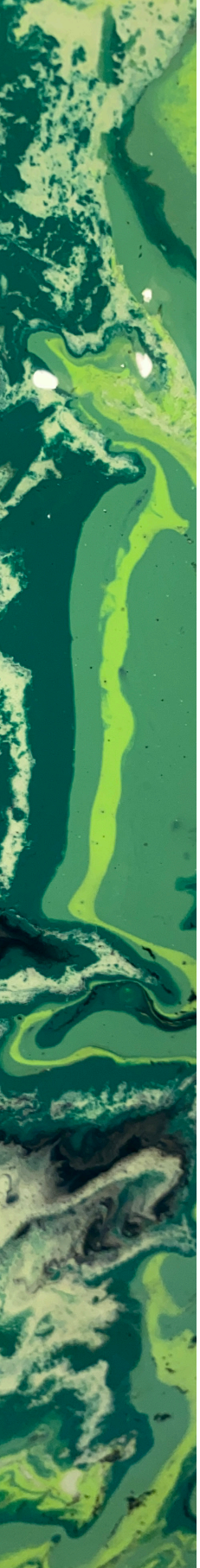

# Chapter 6

## Discussion

# SUMMARIZING DISCUSSION AND FUTURE PERSPECTIVES

*Enterococcus faecium* is a commensal gut bacterium that does not cause harm to an immunocompetent host. Yet in immunocompromised patients it may become a problematic nosocomial pathogen by acquiring genes conferring resistance to antimicrobial compounds (1–3). Moreover, *E. faecium* is adapted to a lifestyle outside its hosts which allows it to persist in hospital wards which increases the chance of reoccurring infections (2, 4). There is an urgent need for insight into the mechanisms by which these multidrug-resistant strains adapted to a nosocomial lifestyle (5–8).

Advances in whole genome sequencing have allowed I) the identification of clones that are associated with nosocomial infections new strains, II) the study of plasmid dynamics, III) the identification of genomic islands involved in gut colonization and virulence, and IV) the quantification of the spread of novel antibiotic resistance mechanisms in clinical *E. faecium* strains (9–11). In addition, genetic tools have been developed to study, compare and manipulate the genomic content of multi-drug resistant *E. faecium* (11–13). However, the genetic study of *E. faecium* is still complex, primarily due to its diversity of naturally occurring and acquired antibiotic resistance mechanisms, thereby hampering the identification of the mechanisms by which *E. faecium* adapts to life as a nosocomial pathogen (3, 9, 14).

In this thesis, we have addressed these limitations by developing novel techniques and applied them to clinical multi-drug resistant strains of *E. faecium* (15, 16). These techniques enabled large-scale genomic screenings and improved targeted mutagenesis. The combination of these techniques allowed for the identification and characterization of novel genetic elements that had a role in processes that contribute to the success of *E. faecium* as a nosocomial pathogen.

## CRISPR-Cas9-based genome manipulation of Enterococcus faecium

Traditional methods for genomic modification rely solely on homologous recombination and insertion/excision of antibiotic resistance markers in the DNA of *E. faecium*. While effective, this approach is labour-intensive and time-consuming. Additionally, the essential step of selecting transformants and mutants in this approach is hampered by the presence of naturally occurring antibiotic resistance in clinical strains. Application of the CRISPR-Cas9 DNA editing technique should allow for the genomic alterations without the need for inserting an antibiotic resistance marker into the genome. This method should also act as a powerful counter-selective pressure to enhance the successful selection of the desired mutants.

In **Chapter 2**, we successfully demonstrate the implementation of CRISPR-Cas9 based genome editing in the clinical vancomycin resistant clinical strain (E745) of *E. faecium* (15). Hereby, a clean gene deletion mutant was generated without genomic scarring while only requiring half the time compared to traditional methods. These data underline the potential power of CRISPR-Cas9 in the study of clinical strains of *E. faecium* to identify and characterise elements involved in its adaption to a nosocomial life style.

Currently, our CRISPR-Cas9 based approach consists of two separate plasmids, one carrying the Cas9 gene and the second the CRISPR and the homologous recombination template. As such, two unique selection markers are required which may still be problematic for highly multidrug-resistant strains. Ideally, a single plasmid would be developed containing all the CRISPR-Cas9 elements as has been described for the use in yeast and *Clostridium* (17–19). Additionally, this plasmid should contain a single universal selection marker to further streamline its application independent of the multidrug-resistance nature of *E. faecium*. Such a universal selection marker could be an auxotroph-selection system, which have already successfully been applied in numerous (industrial) strains (20–22). While this approach would overcome a major hurdle, it requires the presence of large functional elements, which could potentially lead to a significant increase in plasmid size and thereby decrease transformation efficiency. There is still scope for further optimisation of gene deletion methodologies and transformation protocols of *E. faecium*. Indeed, the recent description of a novel protocol combining CRISPR-Cas9 and RecT-mediated recombineering appears to further facilitate genome editing in clinical *E. faecium* strains. https://www.biorxiv.org/content/10.1101/2020.09.01.278044v2.full

## Analysis of *Enterococcus faecium* transposon mutant libraries

Where, commensal *E. faecium* is restricted to the large intestines, clinical strains have shown the ability to survive in the bloodstream. Patients can become infected via either contaminated indwelling medical devices or by the acquired ability of clinical *E. faecium* strains to cross the intestinal barrier (5, 8, 23).

To survive and grow in the bloodstream, *E. faecium* needs to utilize different nutrients and evade various components of the immune system to ensure its growth (2, 24, 25). Additional adaptations that contribute to its success as a nosocomial pathogen include the ability to survive starvation on inanimate surfaces and to withstand the use of cleaning alcohols in clinical wards (4, 26). Identification of genetic elements responsible for these adaptations would be of value in the development of novel intervention strategies against *E. faecium*.

Ideally, to identify the responsible genetic elements, all ~2600 genes in a particular *E. faecium* would be disrupted by a transposon or deleted (as described in **chapter 2**). While this approach may be achievable, as has been shown in various pathogens like *E. coli* (27)*, Pseudomonas aeruginosa* (28) and *Staphylococcus aureus* (29), it would require a significant effort and thus poses a huge logistical challenge. An effective alternative is to implement targeted-sequencing of randomly inserted *mariner* transposons (Tn-Seq) in the genome (including naturally occurring plasmids) of clinical *E. faecium* strains (30, 31). An example of the broad application of this approach is highlighted in an article by Gilmore *et al* (31), who set out to genes involved in fitness and in intrinsic resistance to 10 different antibiotics in *E. faecalis*. Using this technique they created a detailed overview of genes and pathways involved, which could serve as a basis to design a targeted and more detailed investigation in the future. We decided to implement Tn-seq to identify important genetic systems in *E. faecium* which contribute to its survival in and outside the patient.

Using Tn-seq we were able to generate transposon mutant libraries in two different clinical strains, with both libraries containing functional knock-outs of non-essential genes (approximately 80-90% of all genes in the genome). The library based upon the E745 strain, which is resistant to antibiotic vancomycin, was used to investigate the systems responsible for its ability to survive in human serum (**Chapter 3**). Moreover, this library was also applied to identify important pathways involved in the survival under nutrition deprivation (**Chapter 4**). The second library was generated in the clinical strain of *E. faecium* E8202, which shows an increased tolerance against cleaning alcohols most commonly used alcohol based hand rubs (32) (**Chapter 5**). Outcomes of these studies are discussed below in greater details.

It has to be noted that, while transposon mutant library screening is a powerful technique, it has limitations. First, it can only identify the role of non-essential genes, since any disruption of essential genes results in the loss of the transposon mutant from the library. Involvement of essential genes could be better studied via inducible transposon insertion by TRADIS-XPress (33) or via gene knockdown approaches, like those described for CRISPRi (34). Secondly, transposon library screening cannot detect genes in pathways for which functional redundancy exists. Finally, insertion of a transposon in an operon could cause pleiotropic effects due to the loss of transcription of downstream genes, requiring the use of TRADIS-XPress or follow-up investigation to identify the responsible element via CRISPR-Cas9 as discussed earlier (**Chapter 2**).

## Determinants of growth of *Enterococcus faecium* in human serum

In **Chapter 3**, by implementing Tn-seq the importance of nucleotide biosynthesis for the growth of *E. faecium* in human serum was revealed. Several other genetic elements involved in growth in human serum were also identified, although their effect on growth was limited compared to the nucleotide biosynthesis pathways. The importance of nucleotide synthesis for growth in serum has been described in other Gram-positive and Gram-negative bacteria including *Streptococcus pyogenes*, *Escherichia coli*, *Acinetobacter baumannii*, and *Salmonella enterica* (16, 35–38). Moreover, we found that the mannose phosphotransferase system responsible for carbohydrate uptake is also essential for growth in serum. These observations were further supported by RNA-seq in which changes in expression profiles are in line with the genes identified in the transposon library, highlighting their potential importance in human bloodstream infections. Pathways related to carbohydrate utilization and nucleotide synthesis are commonly associated with adaptations for survival in human serum (39, 40). Therefore, these pathways are thought to be excellent targets for the treatment and prevention of bloodstream infections with multidrug-resistant *Enterococci*. To this aim, either blocking or limiting nucleotide biosynthesis or uptake of essential carbohydrates should be sufficient to limit the survival and growth of *E. faecium* in human serum. This would provide a much needed alterative approach to treat these multi-drug resistant strains (35, 41).

## Survival of *Enterococcus faecium* in the absence of nutrients

The ability of *E. faecium* to survive on inanimate surfaces such as table tops, doorknobs and handrails is often overlooked as an important contributing factor to its success as nosocomial pathogen (42–44). In **Chapter 4**, we identified 24 genes that have a significant impact *E. faecium* (E745) on survival during nutrient starvation, triggered by incubation in phosphate buffered saline. These 24 genes are associated with general stress response, DNA repair, metabolism, and membrane homeostasis. The most notably effecting transposons insertions were located in the bacteriocin *entA* and that of the universal stress protein *usp*. The relevant transposon mutants completely disappear from the library upon incubation in PBS at room temperature. The identification of the *entA* bacteriocin gene was surprising as bacteriocins are thought to be involved in reducing growth of competing strains in a niche in which nutrients are scarce (45). Follow-up study showed that the transposon insertion also disrupted the production of the association immunity gene against the EntA protein. During incubation in PBS, the EntA transposon mutant may thus have been killed by the bacteriocins that were produced by neighbouring cells, rather than its inability to survive this nutrient-deprived state.

Involvement of the universal stress protein *usp* was verified via a single-deletion mutant (generated by CRISPR-Cas) to have a significant impact on survival during nutrient deprivation. The *usp* gene family is well studied in *E. coli* and is known to play a role in their survival and general stress response (46–48). While the exact function of *usp* genes is unknown, they are found to be of great importance during the stationary phase, starvation from carbon and other nutrients, exposure to heat, oxidants, metals, antibiotics and ethanol, and various other environmental stresses (46–48). To identify their roles, targeted mutagenesis coupled with transcriptomics will lead to a better understanding of their function and identification of potential co-factors. Finally, it remains to be determined whether the identified *usp* gene is also involved in survival on solid surfaces such as glass, ceramics or metals, as would be relevant during transmission in clinical wards.

During the screening, the transcriptional regulator *exuR* was also identified to be of influence during nutrient starvation. Nonetheless, the single-deletion mutant did not confirm this observation. We speculate that the insertion of the transposon caused a disruption in the expression of genes surrounding *exuR*. A follow-up study containing deletion mutants in one or more of these surrounding genes could reveal the gene responsible for the reduction in survival. The remaining genes that were identified in this starvation experiment can also be investigated to obtain a comprehensive overview of their impact on the survival response. A study by LeBreton *et al* (24) revealed that there is a distinct difference in desiccation and starvation resistance between *E. faecalis* and *E. faecium* strains. In general, *E. faecalis* is among the most resistant to desiccation of the enterococcal species while *E. faecium* is more proficient at handling starvation stresses (24). Nevertheless, all enterococcal species are highly tolerant to both forms of environmental stress which lays at the basis of their successful transition from commensal to hospital-acquired pathogen.

## Tolerance of *Enterococcus faecium* against alcohol-based hand rubs

In clinical wards, colonization of surfaces by multidrug-resistant bacteria and spread from patient to patient is actively targeted. For this reason, extensive hand washing by nurses and other hospital staff is performed. Most hospitals have implemented alcohol-based hand-rubs (AHBR) to make this process easier, quicker and more effective. Worryingly, an improved tolerance towards alcohols in bacteria, including *E. faecium*, has been reported among clinical isolates (49–51).

In **Chapter 6** we identified genes that contribute to tolerance of *E. faecium* E8202 to isopropanol, as it is the alcohol that is mainly used in ABHR. A 5-minute exposure to 23% v/v isopropanol revealed a clear distinction in susceptibility between strains

prior or after the broad implementation of ABHR in hospitals, as advised in 2009 by WHO (32). Compared to strains prior 2009, the new strains showed up to a 2 $^{10}$log-fold reduction is susceptibility towards isopropanol (26). Interestingly, this observation was not only limited to clinical strains, but also occasionally observed in strains isolated from animals or healthy individuals. This suggests that alcohol tolerance, or at least tolerance to isopropanol, is intrinsic to some strains of *E. faecium* (26).

When the transposon mutant library in the isopropanol-tolerant *E. faecium* E8202 strain was tested an overall significant decrease in viability of the entire library was observed in response to 23% v/v isopropanol. These data shows that this concentration may introduce a high level of stochasticity in survival of individual cells and may thus introduce noise in the Tn-seq experiment. Using a lower concentration of isopropanol (20% v/v), three genes were found to be significantly contributing to isopropanol tolerance. The transposon mutants in gene *spxA* showed a >10 fold reduction in both the 20% v/v and 23% v/v isopropanol challenge. The protein encoded by *spxA* is part of a family of transcriptional regulators commonly found in low-GC Gram-positive bacteria and is involved in the oxidative stress response, competence development and overall stress tolerance (52–54). Nonetheless, the exact manner in which *spxA* influences these pathways is unknown. By studying 1646 *E. faecium* strains from healthy humans, patients, pets, pigs, and poultry we discovered that *spxA* is present in all strains, making this gene part of the *E. faecium* core genome. This is unexpected since not all strains are equally tolerant to isopropanol. Indeed, a single cross-over, disruption mutant in *spxA*, did not completely reduce the capacity of E8202 to survive upon isopropanol exposure. As such, the presence of the *spxA* gene is insufficient to explain our observations, but might be explained by variability and available repertoire of the promoters that are targeted by SpxA in individual *E. faecium* strains.

# CONCLUSION

*Enterococcus faecium* is a bacterium with a high potential to adapt to novel challenging environments. Its ability to evolve and acquire resistance to multiple antibiotics, as well as its capacity to survive under adverse conditions make it a major threat to patient care. The work presented in this thesis reveals novel insights into essential genetic elements that have contributed to the emergence of *E. faecium* as an opportunistic pathogen. Interestingly, many of the genes identified during starvation or isopropanol exposure were present in more than 80% of the 1646 strains that span the diversity of the species. This suggests that these genes are part of the *E. faecium* core genome and could explain its unique ability to survive under these adverse conditions, leading to its rapid emergence as an important nosocomial pathogen. *E. faecium* may be uniquely adapted to fill novel niches in modern healthcare systems, with the infection of patients being an unfortunate coincidence.

The work presented here was achieved by combining both the *mariner* transposon mutant library and CRISPR-Cas9 approach in elucidating molecular mechanisms in *E. faecium*. We show the value of modern functional genomic approaches in the study of these multidrug-resistant opportunistic pathogens, and anticipate that these will help to gain further insights into these unique and challenging bacteria while opening up avenues for novel preventative interventions or treatment methods.

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# NEDERLANDSE SAMENVATTING

## Introductie.

Bacteriën zijn overal in de natuur te vinden en zijn een belangrijk onderdeel van elk ecosysteem. Bacteriën werken samen met andere organismen om zo de delicate balans van de natuur te behouden. Ook op en in het menselijk lichaam vind je een groot aantal bacteriën, zoals op de huid, in de neus en dikke darm, waar ze samen met de gastheer in harmonie leven zolang ze in hun specifieke niche blijven. Een mooi voorbeeld is de commensaal darmbacterie *Escherichia coli* (*E. coli*) welke veel voorkomt in onze dikke darm en daar vrijwel nooit problemen veroorzaakt. Maar wanneer *E. coli* in de blaas of urinewegen terecht komt kan het een heftige ontsteking veroorzaken die vaak met antibiotica moet worden behandeld. Het gebruik van antibiotica voor dit soort infecties heeft ertoe geleid dat antibioticaresistenties tegenwoordig veelvuldig voorkomen, wat de behandeling aanzienlijk bemoeilijkt. Een aantal van deze antibioticaresistente bacteriën hebben zich gespecialiseerd en kunnen zo overleven in gezondheidsinstellingen zoals het ziekenhuis. Dit zijn onder andere de bekende meticilline-resistente *Staphylococcus aureus* (MRSA) maar ook de vancomycin-resistente *Enterococcus faecium* (VRE). Dit proefschrift focusseert zich op *E. Faecium* en hoe het zich heeft aangepast om te overleven in menige gezondheidsinstelling.

## Doel van dit proefschrift.

Naast het verkrijgen van antibioticaresistente, moet *E. faecium kunnen omgaan met schoonmaakalcoholen en kunnen overleven in voedingstofarme omstandigheden.* Veel onderzoek is al gedaan naar de mechanismes waarmee *E. faecium* nieuwe resistenties opneemt. Hieruit blijkt dat *E. faecium* zeer goed is in het verkrijgen van nieuwe stukken DNA van andere bacteriën om zo zijn eigen overlevingskansen te vergroten. In dit proefschrift bekijken wij hoe *E. faecium* zich aanpast om te overleven met gebrek aan voedingstoffen of in het bloed, wat een compleet nieuwe omgeving is voor deze bacterie. Verder hebben wij gekeken welke mechanismes van *E. faecium* belangrijk zijn om om te kunnen gaan met schoonmaakalcoholen. Om dit onderzoek te bewerkstelligen hebben we nieuwe technieken ontwikkeld om te bepalen welke genen van *E. faecium* belangrijk zijn om te kunnen overleven onder verschillende omstandigheden. Met de informatie van onze studies, kunnen hopelijk nieuwe behandelmethodes worden ontwikkeld om de verspreiding van vancomycin-resistente *E. faecium* in gezondheidsinstellingen tegen te gaan.

## Een nieuwe methode om genen uit te schakelen van *E. faecium*.

Het aanpassen van de genen in *E. faecium* is een essentiële methode om mechanismes te kunnen ontdekken waarmee deze bacterie zich kan aanpassen aan verschillende omstandigheden. Om dit werk goed uit te kunnen voeren moeten de aangepaste *E. faecium* kunnen worden onderscheiden van de normale stam. Dit gebeurt door antibioticaresistentie in de aangepaste stammen aan te brengen. Helaas hebben veel klinische stammen van *E. faecium* al veel van deze antibioticaresistenties waardoor deze technieken lastig toe te passen zijn. Om dit probleem te overkomen hebben wij in **Hoofdstuk 2** gebruik gemaakt van de nieuwe techniek "CRISPR-Cas9". Met CRISPR-Cas9 kan heel gericht een knip aangebracht worden in het DNA van *E. faecium*. Deze knip kan *E. faecium* niet repareren. Daarom bieden wij een nieuw stuk DNA aan dat deels overeen komt met de regio waar de knip heeft plaatsgevonden. *E. faecium* zal dit nieuwe stuk DNA gebruiken als voorbeeld om het DNA te repareren. Door het voorbeeld-DNA aan te passen kan het reparatie proces worden beïnvloed, hierbij kunnen genen worden uitgeschakeld of aangepast. Wanneer nodig kunnen hiermee zelfs nieuwe genen worden geïntroduceerd. Deze nieuwe varianten van *E. faecium* (mutanten) kunnen vervolgens in studies worden gebruikt om te kijken hoe de aangepaste genen belangrijk zijn voor het overleven van *E. faecium.*

## Identificatie van genen die bijdragen aan de overleving *E. faecium* in de patiënt.

Als commensaal, heeft *E. faecium* zich aangepast om te kunnen overleven in de dikke darm. Verandering van omgeving, dwingt *E. faecium zich aan te passen om te kunnen overleven.* De varianten van *E. faecium* die voorkomen in het ziekenhuis en met regelmaat infecties veroorzaken waarbij ze in de bloedbaan terechtkomen moeten dus grote aanpassingen maken om hierin te overleven. In **Hoofdstuk 3** bestuderen we via een nieuwe techniek genaamd Tn-seq hoe *E. faecium* zich aanpast om te kunnen overleven in menselijk bloed. Met deze techniek kunnen in één keer alle niet essentiële genen getest worden onder verschillende omstandigheden. Via deze methode hebben we genen geïdentificeerd die betrokken zijn bij koolhydraatmetabolisme en de aanmaak van DNA. Deze genen zijn essentieel voor goede overleving in bloed.

## Identificatie van genen die bijdragen aan de overleving van *E. faecium* buiten de patiënt.

*Enterococcus faecium* is een gevaarlijke bacterie omdat het gemakkelijk van patiënt naar patiënt verspreidt en daarnaast lang op een afdeling kan overleven buiten de patiënt om. Dit kan leiden tot grote uitbraken die grote groepen met patiënten aandoen. Hiervoor zal *E. faecium* het lichaam van de gastheer moet verlaten en langdurig op oppervlaktes buiten het lichaam moeten overleven. Dit is een zeer grote

overgang ten opzichte van de darm, waarvoor *E. faecium* zich zal moeten aanpassen op het gebied van temperatuur, voedseltekort, uitdroging en het weerstaan van schoonmaakmiddelen. In **Hoofdstuk 4** bestuderen we welke genen van *E. faecium* betrokken zijn bij overleving op kamertemperatuur en gedurende voedseltekort. In **Hoofdstuk 5** bestuderen we welke genen betrokken zijn om de blootstelling aan schoonmaakalcoholen te kunnen overleven. In beide hoofdstukken maken we gebruik van de Tn-Seq techniek om de essentiële genen te identificeren.

In **Hoofdstuk 4** identificeren we 24 unieke genen welke een aanzienlijke invloed hebben op de overleving op kamertemperatuur gedurende voedseltekort. Deze genen zijn onder andere betrokken bij DNA reparatie, membraan homeostasis, algemene stressreactie en metabolisme. Het grootste effect werd geobserveerd toen we het gen voor *Universal stress protein* (USP) hadden uitgeschakeld. Dit gen is bekend van zijn rol in het reageren op vele vormen van stress zoals, zuurtegraad, temperatuur en uitdroging. Om te evalueren hoe belangrijk deze 24 genen zijn, hebben wij de genomen van alle 1644 *E. faecium* bekeken. Hieruit blijkt dat veel van de 24 genen aanwezig zijn in meer dan 90% van deze stammen. Voor het USP gen was dit zelfs 99%. Dit bevestigt dat deze 24 genen onderdeel maken van de basisgenen van *E. faecium* en essentieel zijn voor zijn overleving in gezondheidsinstellingen.

Om de overdracht van bacteriën zoals *E. faecium* tussen patiënten te beperken worden in ziekenhuizen alcoholische handgels gebruikt als desinfectie. Deze gelen bestaan uit een mengeling van alcoholen waaronder Isopropanol. In **Hoofdstuk 5** tonen we aan dat tolerantie tegen Isopropanol langzaam aan het oplopen is onder *E. faecium*, waardoor deze bacteriën een vergrote kans hebben om te overleven. Dit is problematisch aangezien dit de kans vergroot op overdracht van patiënt naar patiënt. In onze studie hebben wij het gen SpxA geïdentificeerd, dat een aanzienlijke invloed heeft op de Isopropanol tolerantie. Dit gen is aanwezig in alle 1644 *E. faecium* genomen. Ondanks de aanwezigheid van dit gen zijn er wel grote fluctuaties in Isopropanol tolerantie tussen de verschillende stammen. Dit suggereert dat er een andere component aanwezig is die de Isopropanol tolerantie beïnvloedt. Een mogelijke verklaring kan zijn dat SpxA de activiteit van andere genen kan reguleren. Toekomstig onderzoek zal moeten uitwijzen welke genen worden beïnvloed door SpxA en welke daarvan belangrijk zijn voor de Isopropanol tolerantie van *E. faecium*.

## Ter conclusie

*Enterococcus faecium* is een beruchte ziekenhuisbacterie die heeft bewezen zich makkelijk aan te kunnen passen om te overleven in verschillende omgevingen, zowel binnen als buiten de patiënt. Het onderzoek beschreven in dit proefschrift

heeft essentiële genen geïdentificeerd die hiervoor belangrijk zijn. Deze genen zijn aanwezig in vrijwel alle stammen, wat bevestigt dat *E. faecium* van nature al uitermate geschikt is om te overleven in ziekenhuizen. Voor dit onderzoek hebben wij twee krachtige genetische technieken geïntroduceerd waarmee inzichten in de genetica van *E. faecium* zowel verbreed als verdiept kunnen worden. Dit zal op den duur leiden tot een beter begrip van de interne werking van *E. faecium* en nieuwe aanknopingspunten geven voor een verbeterde behandeling.

# ACKNOWLEDGEMENTS

Here it finally is, the very end of my Thesis. It is however not an end in the traditional sense. It is an end that is not really and end, it is more an ending of a beginning. It is written last and located at the very end of the thesis, it is however read at the very beginning. The acknowledgements mark the end of my PhD which has been a journey for me and as one journey ends a new one begins. I did not do this journey alone, I met many people along the way and would like to thank you all for the time spend with me on this journey.

### Let us start at the beginning of the end that is not an end.

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When I was not in the lab I could be found in the relative quietness of the office which was occasionally disrupted by “het mannen-uurtje” with met **Mike, Steven, Kobus,** and **Rob** when the ladies were not present. This was an integral part of the office dynamics for which even Sinterklaas had to be involved. When I just arrived

at the department the Saint challenged us to determine the Alpha-man of the group. Short to say this was a very intense welcome to the office. Of course I cannot ignore the ladies, **Anouk, Kirsten, Samantha, Stephanie,** and **Yuxi**, without whom this office group would have been half as great. Thank you all for the pleasant times there with the occasional needed distractions between experiments.

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Dear parents **Rob** and **Dominique**, thank you two for being the great supportive parents that you are. It is with your help that I could keep on going even when it got tough for me. It helped me to always have a stable base to work from. I'm sorry for all the grey hair I caused to grow, but I have to say it looks very good on the both of you.

**Steven**, big brother, fellow PhD. It is finally here, I made it to PhD! For which you have helped me greatly in proofreading parts of my thesis and help me focus on the parts that really mattered. Thank you for offering your Scientific and Brotherly knowledge during the dark times known as the end of the PhD. Of course I cannot forget your beautiful wife **Judith**, who was kind enough to lend you to me to help me with the final writing phases. Thank you **Judith**. I wish you all the best with the completion of your very own Thesis and I am looking forward to congratulate you as fellow Doctor.

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# ABOUT THE AUTHOR

Vincent de Maat was born on November 30th 1988 in Apeldoorn, the Netherlands. In 2007, he graduated from secondary education with a VWO degree from the Heemlanden, in Houten, the Netherlands.

In early 2008 he started his Bachelor of Science degree in Life Sciences at de Hoogeschool Utrecht. For his minor he participated in Innovative Strategies for Alternative to Animal Testing where he worked on a single cell gel-electrophoresis technique to determine DNA damage after UV exposure to test efficacy of sun blocker ingredients. To finalise his bachelor he went to Portland, Oregon, in the United States for a 10 month internship at Oregon Health & Science University. Here he studied the repair mechanism of DNA-DNA crosslinking in yeast under supervision of Dr Adam Clore.

After receiving his Bachelor of Applied Science degree in 2011 he started a Masters program at Utrecht University titled Molecular and Cellular Life Sciences. For his degree Vincent did two internships, one of 6 months and one of 9 months. The 6 month internship was done at University Utrecht under guidance of Dr Jésus Arénas. Vincent studied a new cluster of genes in *N. meningitidis* which revealed to be a new contact dependent inhibition system. The second internship was done at Sanquin in Amsterdam, the Netherlands under supervision of Jaap van Buul. Here he studied the molecular forces on VE-Cadherin of endothelial cells. For this Vincent designed an artificial system to carefully regulate the pulling forces applied and detect cellular changes via western blot and fluorescence microscopy. In September 2014 Vincent received his Master of Science.

In January 2015, Vincent started his PhD degree at the department of Medical Microbiology of the UMC Utrecht under supervision of Prof. Willem van Schaik. Here he studied genetic adaptation of hospital-acquired *Enterococcus* faecium. The results have been presented at multiple (inter)national conferences, submitted for publication in peer-reviewed international scientific journals, and are collected in this thesis.

Vincent is currently working as a R&D Scientist at Kinetic Evaluation Instruments in Leusden, the Netherlands. Where he develops new assays to detect *M. tuberculosis* in human blood.

# LIST OF PUBLICATIONS

## Related to this thesis

**de Maat, V.**, Stege, P.B., Dedden, M., Hamer, M., van Pijkeren, J., Willems, R.J.L., van Schaik, W. CRISPR-Cas9-mediated genome editing in vancomycin-resi stant *Enterococcus faecium*. *FEMS Microbiology Letters*, Volume 366, Issue 22, November 2019, fnz256

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## Other publications

Arenas J., Catón, L., van den Hoeven, T., **de Maat, V.**, Herrero, J.C., Tommassen, J. (2020) The outer-membrane protein MafA of Neisseria meningitidis constitutes a novel protein secretion pathway specific for the fratricide protein MafB, Virulence, 11:1, 1701-1715,

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Arenas, J., **de Maat, V.**, Catón, L., Krekorian, M., Herrero, J. C., Ferrara, F., & Tommassen, J. (2015). Fratricide activity of MafB protein of N. meningitidis strain B16B6. BMC microbiology, 15(1), 156.