Identification of genes involved in acid tolerance, antimicrobial resistance and virulence of *Enterococcus faecium*

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Declaration

I hereby certify that the work embodied in this thesis is the result of my own investigation, except where otherwise stated.

Abstract

Enterococcus faecium is an opportunistic human nosocomial pathogen that has developed resistance to many existing antimicrobial therapies. Treatment of enterococcal infections is becoming increasingly challenging and there are limited therapeutic options against multidrug-resistant enterococci. The work described in this thesis aimed to identify genes involved in resistance to host stresses and virulence as these could potentially be future therapeutic targets. An *E. faecium* transposon mutant library was screened for altered resistance to lysozyme and to nisin as a model antimicrobial peptide. This approach led to the identification of several genes that contribute to lysozyme and nisin resistance. A number of mutants that were sensitive to nisin had a transposon insertion in a gene predicted to encode a tyrosine decarboxylase. It was shown that the tyrosine decarboxylase plays a role in acid tolerance and mediates virulence of *E. faecium* in a *Galleria mellonella* larvae infection model. A targeted genetic approach was used to examine the role of an *E. faecium* serine threonine protein kinase (Stk1) in antimicrobials resistance, host cell stresses and virulence. Disruption of the *stk1* gene led to higher sensitivity to antibiotics that target the penicillin-binding proteins and bile salts compared to the wild type. Providing the *stk1* gene from either *E. faecium* or *ireK* of *E. faecalis in trans* restored the resistance of the *E. faecium stk1* mutant to ceftriaxone. The *stk1* gene also contributed to *E. faecium* virulence in the *G. mellonella* infection model. Through bioinformatic analysis a putative ATP-binding-cassette (ABC) transporter system with the potential to be involved in antimicrobial peptide resistance was identified. Markerless deletion of the gene encoding the permease component of the ABC transporter reduced *E. faecium* resistance to the antimicrobial peptide nisin and altered the net charge of the bacterial cell surface. Genes involved in acid tolerance, antimicrobial resistance and virulence of *E. faecium* were identified in this study.

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Abbreviations

˚C	Degrees Celsius
BHI	Brain heart infusion
bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
g	Gravitational force
h	Hour
kb	Kilobase
LB	Luria-Bertani
µg	Microgram
µL	Microlitre
µm	Micrometre
mL	Millimetre
µM	Micromolar
mM	Millimolar
min	Minute
nm	Nanometre
rpm	Revolutions per minute
kV	Kilovolts
µF	Microfarad
Ω	Ohms

Chapter 1

General Introduction

1 General Introduction

1.1 *Enterococcus faecium*

1.2 Enterococci

Enterococci are gram-positive bacteria which are facultative anaerobes. These bacteria occur ubiquitously in soil, on plants and foods. Enterococci are able to grow at temperatures between 10˚C and 45˚C, can survive for at least 30 minutes at 60˚C and can grow at pH 9.6 and in the presence of 6.5 % (w/v) sodium chloride (Sherman, 1937). They appeared as gram-positive cocci and are negative in the standard catalase test. Sometimes they produce pseudocatalase if grown on blood agar. However, the reaction is usually weak (Devriese et al., 1993). They appear positive in the bile-esculin medium test since they are able to hydrolyze esculin in the presence of 40 % bile salts. Most enterococci are positive in the production of pyrrolidonyl arylamidase and leucine aminopeptidase. Almost all strains are homofermentative. The end-product is mostly lactic acid and gas is not produced. Most of the species produce a cell wall-associated glycerol teichoic acid antigen which in past history resulted them being classified as Lancefield streptococcal group D (Devriese et al., 1993).

The ability to survive in such a broad range of environments and their presence as normal microflora in the gastrointestinal tract and faeces of vertebrates makes them a useful faecal contamination indicator (Stiles and Holzapfel, 1997) but also means that they can easily spread in the environment. The genus Enterococcus consists of non-spore-forming, ovoid bacteria that are arranged in single cells, pairs, or chains (Naser et al., 2005) and comprise at least 40 published species (www.bacterio.cict.fr/e/enterococcus.html). Enterococci are also a potential threat to humans as they can cause infections of the urinary tract, bloodstream, endocardium, abdomen, biliary tract and in burn wounds (Jett et al.,

1994). Enterococci are the most frequently reported pathogens in nosocomial surgical-site infections and the third most common pathogens in nosocomial primary bloodstream infections (BSIs) after coagulase-negative Staphylococci and *Staphylococcus aureus* (Richards et al., 2000). Two main enterococcal species have been identified to be responsible for the majority of human infections are *E. faecalis* and *E. faecium*. Both of these species account for more than 90 % of clinical isolates. While other enterococcal species such as *E. gallinarum*, *E. casseliflavus*, *E. durans*, *E. avium* and *E. raffinosis* are isolated much less frequently and account for less than 5 % of clinical isolates (Gordon et al., 1992).

E. faecalis and *E. faecium* are the most common isolates in the human gastrointestinal tract (Fisher and Phillips, 2009). Although for years enterococci were considered to be harmless inhabitants of the gut flora, they are now among the leading causes of nosocomial infections (Leavis et al., 2007; Willems et al., 2005; Top et al., 2008).

E. faecium strains hydrolyze arginine, form acid in mannitol and arabinose broth but not in sorbose broth. They are negative in the motility test and do not form acid from methyl-α-D-glucopyranoside. Occasionally, they appear mannitol-negative (Devriese et al., 1993).

The *E. faecium* strain E1162 that was used in this study was originally isolated from a bloodstream infection in France. This clinical isolate is belonged to ST17 and the G+C content in this strain is 38 % (van Schaik et al., 2010).

1.3 Infections caused by Enterococci

Enterococci are commensal organisms in the human gastrointestinal tract (Fisher and Phillips, 2009). Although they are potentially pathogenic, enterococci have been used as

probiotics in humans and domesticated animals. Infective endocarditis was the first documented case of an enterococcal infection (Maccallum and Hastings, 1899). Subsequent years showed that enterococci caused urinary tract infections, pelvic infections and neonatal infections (Arias and Murray, 2012). Enterococci are the second most common organisms recovered from skin and soft-tissue infections (Arias and Murray, 2008). These bacteria cause nosocomial infections of surgical wounds, urinary tract, blood-stream and other sites (Arias and Murray, 2012; Gilmore et al., 2013)

The possible routes of enterococcal infection are depicted in Figure 1.1. From the gastrointestinal tract, enterococci can move across the intestinal lining in the immunocompromised patients and pass through the liver. From here, the enterococci can access the bloodstream causing bacteremia, the heart where it can cause infective endocarditis. Patients could potentially be infected by faecal contamination from the environment or other patients. Enterococci can also access the patient's skin and mucosal surfaces from the urinary tract or intravenous catheters.

In late 1980s, enterococci had emerged as one of the most important nosocomial pathogens due to outbreaks of vancomycin resistant enterococci (VRE) (Bensoussan et al., 1998). Among the enterococci, *E. faecalis* and *E. faecium* are responsible for most enterococcal infections (Huycke et al., 1998). It has been reported that *E. faecium* infections are more difficult to treat than *E. faecalis*. Only 5 % of *E. faecalis* isolates are vancomycin resistant while more than 80 % *of E. faecium* are vancomycin resistant (Hidron et al., 2008).

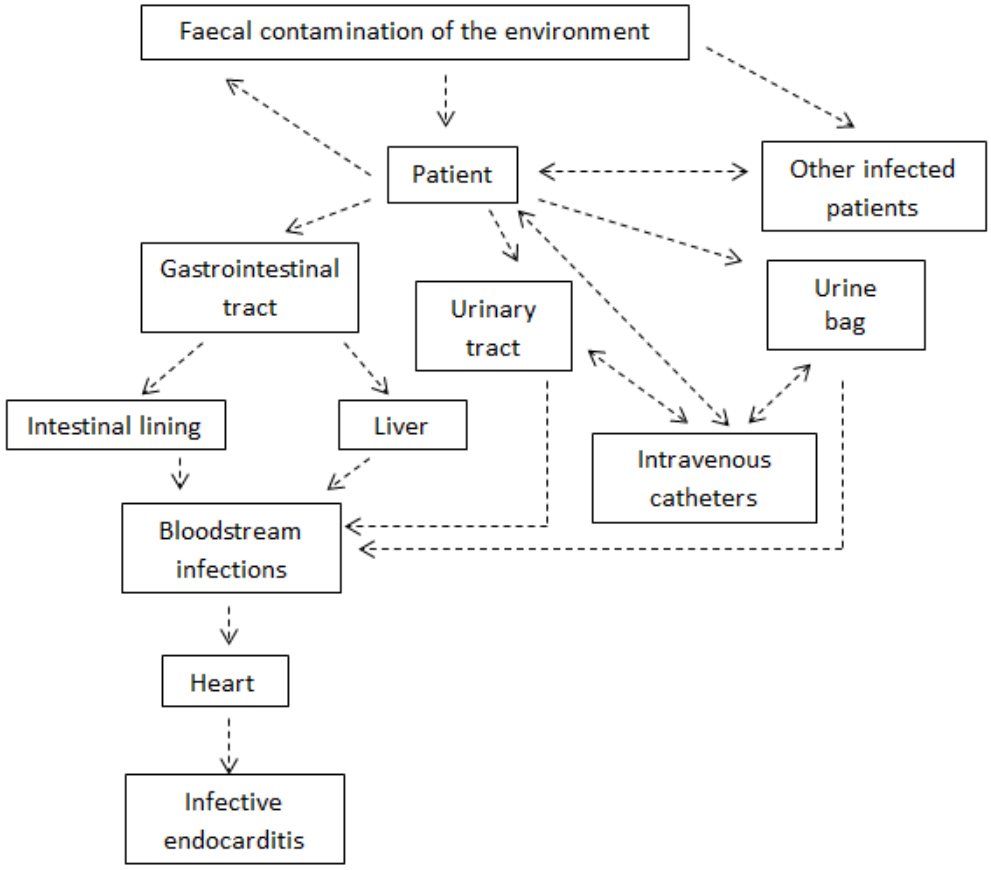


Figure 1.1 Possible routes of enterococcal infection and spread

1.4 Emergence of Clonal Complex 17 *Enterococcus faecium*

A study of the evolutionary genetics, population structure and geographic distribution of vancomycin resistant and vancomycin-susceptible *E. faecium* isolates recovered from human, nonhuman sources as well as community and hospital reservoirs in several continents has revealed an epidemic lineage clonal complex 17 (CC-17) (Willems et al., 2005). The majority of isolates clonal complex CC-17 is characterized by the presence of the enterococcal surface protein (Esp), resistance to quinolones and ampicillin (Willems et al., 2005). It has been hypothesized that the abundance of the insertion sequence IS*16* element contributed to the adaptation of this bacterium to the hospital

environment. Many strains of *E. faecium* are multidrug resistant and can potentially transfer this phenotype to other bacteria via lateral gene transfer (Leavis et al., 2007).

Another study revealed that infections caused by ampicillin-resistant *E. faecium* (AREfm) in a European hospital increased 16 fold within 11 years (from 2 % in 1994 to 32 % in 2005) (Top et al., 2007). In that survey, 35 % of patients with invasive AREfm infections died and most of the identified AREfm isolates (97 %) belonged to clonal complex 17 (CC-17). The same researchers observed the emergence of the CC-17 in the Netherlands (Top et al., 2008). Enterococcal bloodstream infections caused by AREfm also increased 5 fold during the same study period (from 4 % in 1994 to 20 % in 2005) and majority of the AREfm isolates belonged to CC-17 (Top et al., 2008). The emergence of the clonal complex CC-17 was also observed in Sweden. All the outbreak strains were Esp-positive and belonged to CC-17 (Billstrom et al., 2009). Another outbreak of the clonal complex CC-17 was reported in the northern Spain. All of *E. faecium* isolates were Tn*5382* positive which are linked to ampicillin resistance (Valdezate et al., 2009).

Although earlier findings suggested that all CC-17 isolates were resistant to ampicillin, a later study showed that not all isolates within this clonal complex are ampicillin-resistant. It has been reported that *fms* (putative pili or adhesions) genes are more accurate markers to identify the CC17 genogroup (Galloway-Pena et al., 2009).

Genotyping of enterococcal isolates has also been conducted in South America between 2006 to 2008 (Panesso et al., 2010). These studies found that almost all VREfm isolates have the *pilA*, hyl_{Efm} and *van*(A) genes. Another study was conducted on the global spread of clonal complex CC-17 from 1986 to 2009 (Freitas et al., 2010). The *E. faecium* strains were isolated from 23 countries and several continents (Europe, North America, South America, Australia, Asia, and Africa). Most of the *E. faecium* isolates had a large

plasmid (>150 kb) and they also hypothesized that the presence of the large plasmid might enhance the antibiotic resistance and contribute to the emergence of CC-17 populations (Freitas et al., 2010).

1.5 Emergence of antibiotic resistant enterococci

E. faecium is classified in a group of bacteria that are referred to as 'the ESKAPE bacteria'. This group of bacteria escape the lethal action of antibiotics and associated with healthcare-associated infections in both developed and the developing world. The ESKAPE bacteria consist of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species (Rice, 2008). The ESKAPE pathogens are involved in more than 40 % of infections in patients in intensive care units (Hidron et al., 2008).

Among gram-positive bacteria, antibiotic-resistant *E. faecium* and methicillin-resistant *Staphylococcus aureus* are leading bacteria of healthcare-associated infections in the 21st century (Arias and Murray, 2008). Also, enterococci can transfer their antibiotic resistance to methicillin-resistant *Staphylococcus aureus* (MRSA) leading to even more severe threat (Flannagan et al., 2003). The ability of *E. faecium* to acquire foreign DNA caused rapid accumulation of antibiotic resistance genes. Recently, researchers have shown the presence of antibiotics such as cephalosporins during antibiotic treatment could create the microbial imbalance in the large intestine thus allowed the outgrowth of multiresistant *E. faecium* (Hendrickx et al., 2015). The level of resistance against a broad range of antibiotics covering most antimicrobial classes among *E. faecium* isolates have increased over the last two to three decades (Hegstad et al., 2010).

The emergence of Vancomycin-susceptible *E. faecium* into Vancomycin-resistant *E. faecium* presumably occurs in the human bowel during antibiotic therapy (Howden et al., 2013). Vancomycin-resistant *E. faecium* (VREF) has emerged as an important worldwide cause of nosocomial infections second only to ampicillin-resistant *E. faecium* (AREfm), especially in immunocompromised patients (Top et al., 2007). In 2015, Thaker and colleagues have reported that the vancomycin-susceptible enterococci are able to convert to vancomycin-resistant enterococci during the antibiotic therapy resulting in treatment failure (Thaker et al., 2015). Amino acid substitutions in a low-affinity penicillin-binding protein (PBP5) leads to high-level ampicillin resistance in *E. faecium* (Ligozzi et al., 1996), while the modification of peptidoglycan leads to vancomycin resistance in this bacterium (Bugg et al., 1991).

The *van*(A) and *van*(B) were identified as the most frequently encountered genotypes among clinical vancomycin-resistant enterococci (VRE). Enterococci harbouring *van*(A) genes have a high-level resistance to glycopeptide antibiotics, vancomycin and teicoplanin (Arthur et al., 1993) while those carrying the *vanB*-type genes have a moderate level of resistance to vancomycin but remain susceptible to teicoplanin (Quintiliani, Jr. et al., 1993). The *van*(D)-type resistance mediates moderate-level resistance to both vancomycin and teicoplanin. The *van*(D)-operon is present on the chromosome (Casadewall and Courvalin, 1999) while the *van*(A) genotype is due to Tn*1546* carriage on plasmids and the *vanB* genotype is due to Tn*1549* on the chromosome (Werner et al., 2008). The majority of *E. faecium* clinical isolates nowadays are resistant to the mentioned antibiotics (van Schaik et al., 2010).

In *Enterococcus faecium*, it has been reported that Pbp5 is a low-affinity class B PBP that confers β-lactam resistance to this bacterium. Although closely related to the Pbp5 of

E. faecalis, *E. faecium* gene has a higher capacity to acquire mutations that give rise to high levels of ampicillin resistance under the selective pressure of this antibiotic (Rice et al., 2001). Amino acids alterations of Pbp5 in *E. faecium* have been associated with overproduction of the enzyme and lead to resistance at levels exceeding therapeutically achievable drug concentrations (Rice et al., 2001). A further analysis of the Pbp5 protein of *E. faecium* has shown that amino acids alterations in this protein contributed to ampicillin resistance (Galloway-Pena et al., 2011). Comparative genetic analysis was done on ampicillin sensitive and resistant *E. faecium* strains and it was found that all of the amino acid differences have occurred in the transpeptidase domain (C terminal) (Galloway-Pena et al., 2011). This hypothesis has recently been challenged by Belhaj and colleagues (2015) as their study revealed that the levels of ampicillin resistance is not correlated with the amino sequence polymorphism of PBP5 (Belhaj et al., 2015).

Other factor that contributes to ampicillin resistance are the transpeptidases. The β-lactam antibiotics act as 'suicide' substrates of the DD-transpeptidases that catalyze the last crosslinking step of peptidoglycan synthesis by mimicking the D-alanyl$_4$-D-alanine$_5$ extremity of peptidoglycan precursors (Mainardi et al., 2007). However, DD-transpeptidases is not the sole essential transpeptidase enzyme in *Enterococcus faecium* as a LD-transpeptidase (Ldtfm) has been found to be another transpeptidases that leads to high level resistance to ampicillin. The Ldtfm catalyses the formation of a 3 → 3 cross-link between peptidoglycan side chains instead of the classical 4 →3 crosslinks formed by DD-transpeptidase (Mainardi et al., 2007; Mainardi et al., 2000). It has also been shown that a metallo-DD-carboxypeptidase, DdcY, is an important component in the LD-transpeptidase mediated pathway of peptidoglycan cross-linking (Sacco et al., 2010)

These genetic determinants for ampicillin resistance were also identified by Zhang and colleagues (2012) by screening of a transposon mutant library using a Microarray-based Transposon Mapping (M-TraM) technique. Similar to previous studies, they identified *ddcP* that encodes for D,D-carboxypeptidase and *ddcY* that encodes for L,D-transpeptidase, Ldtfm. They also identified another two genetic determinants which were *pgt* that encodes a glycosyl transferase group 2 family protein and *lytG* that was predicted to encode an exo-glucosaminidase. Individual deletion of *ddcP* and *ldtfm* enhanced sensitivity of these isolates to lysozyme (Zhang et al., 2012). In addition to ampicillin resistance the low affinity penicillin-binding proteins Pbp5 of *E. faecium* confers imipenem resistance (El Amin et al., 2002). It was therefore concluded that *pbp5, ddcP, ldtfm and pgt* are important for ampicillin resistance in *E. faecium*. Recently, low affinity penicillin-binding protein 5 (Pbp5) and D-alanyl-D-alanine carboxypeptidase (DdcP) were identified as a vaccine candidates for interococcal infections (Romero-Saavedra et al., 2014).

Resistance towards aminoglycosides in *E. faecium* is due to a chromosomally encoded 6'-N-aminoglycoside acetyltransferase (Costa et al., 1993). Recently, another additional genetic determinant for aminoglycosides in *E. faecium* was found. The chromosomally encoded *efmM* that encodes for a metyltransferase confers resistance to the aminoglycosides kanamycin and tobramycin (Galimand et al., 2011).

The *lnu*(B) gene of *E. faecium* confers lincosaminade (lincomycin and clindamycin) resistance. It was found that this gene is located on a large plasmid (Bozdogan et al., 1999b). The *vat*(D) which formerly known as *sat*(A), mediates resistance to dalfopristin (streptogramin A) (Bozdogan et al., 1999b; Roberts et al., 1999). This gene has been detected on a plasmid that contains the *erm*(B) and *van*(A) resistance gene cluster (Bozdogan et al., 1999b). Another genetic determinant that mediates resistance to

dalfopristin, the *vat*(E) gene, which was formerly known as *sat*(G), was also identified (Soltani et al., 2000). Meanwhile, the *vgb*(A) gene confers resistance to quinupristin (streptogramin B) (Bozdogan and Leclercq, 1999a). The presence of *vat*(D) or *vgb*(A) confers partial resistance to the streptogramin combination of quinupristin and dalfopristin. Full resistance to the streptogramin combination of quinupristin and dalfopristin occurs when both of *vat*(D) or *vgb*(A) are present in *E. faecium* isolates (Bozdogan and Leclercq, 1999a).

Some *E. faecium* isolates are resistance to chloramphenicol due to the presence of a gene coding for chloramphenicol acetyl transferase which is found on a plasmid or the chromosome (Trieu-Cuot et al., 1993). In another study, a *tet*(M) gene that confers resistance to tetracycline was identified in *E. faecium* E1162 (van Schaik et al., 2010). This gene is the most common tetracycline-resistant determinant in enterococci. *E. faecium* isolates can also be resistant to tetracycline due to other genetic determinants including *tet*(L), *tet*(K), *tet*(O), *tet*(S)and *tet*(U) (Bentorcha et al., 1991). A recent study by Fiedler and colleagues (2015) reports that higher expression of both *tet*(L) and *tet*(M) confers tigecycline resistance among clinical isolates of *E. faecium* (Fiedler et al., 2015). A mutation in the ribosomal protein L6 is associated with evernimicin resistance (Aarestrup and Jensen, 2000). Mutations of *parC* (topoisomerase IV subunits) and *gyrA* (DNA gyrase subunits) are found in quinolone (ciproflaxin)-resistance *E. faecium* isolates (El Amin et al., 1999).

1.5.1 Bile salts resistance

Commensal and pathogenic microorganisms must resist the lethal actions of bile in order to survive and colonize the human intestinal tract (Begley et al., 2005). A gene, *bsh*, that encodes for bile salt hydrolase was initially identified in *E. faecium* as conferring resistance to bile salts *(*Wijaya et al., 2004). Later, two general stress proteins Gls24 and

GlsB were identified. These proteins are implicated in *E. faecium* bile salt resistance. The absence of either gene resulted in increased sensitivity to bile salts. The deletion of both genes exhibited additional decreased tolerance to bile salts (Choudhury et al., 2010). Individual deletions of these genes did not have a significant effect on virulence in a mouse peritonitis model. However, a double-deletion mutant was significantly attenuated in this model.

Some other additional genetic determinants for bile salt resistance were identified (Zhang et al., 2013). These resistant determinants were identified from the screening of a transposon mutant library using a Microarray-based Transposon Mapping (M-TraM) technique. A gene, *gltK*, which encodes for glutamate/aspartate transport system permease protein was identified. The deletion of this gene resulted increased sensitivity of *E. faecium* to bile salts. In that study, they also performed transcriptome analysis to identify which genes were regulated by exposure to 0.02 % bile salts. Major transcriptional changes in genes involved in carbohydrate, nucleotide and coenzyme transport and metabolism were identified. Another general stress protein GspA, was also identified as playing a role in the early response to (5-15 minutes) bile salts treatment (Zhang et al., 2013).

1.6 Enterococcal cell wall

1.6.1 Peptidoglycan

Murein or peptidoglycan is a polymer that forms a component of the bacterial cell wall (Rogers, 1970). The peptidoglycan consists of glycan chains interlinked by short peptides (Vollmer et al., 2008). The peptidoglycan layer not only protects the entire cell from the adverse effects of the environment (Silhavy et al., 2010), it also serves as a

structure for anchoring cell wall components such as cell wall proteins (Dramsi et al., 2008) and acidic polymers such as teichoic acids (Neuhaus and Baddiley, 2003).

Interspecies variation occurs either in the glycan strands or in the position of the interpeptide bridge (Schleifer and Kandler, 1972). In most gram-positive species, L-Ala is the first amino acid of the peptide stem and D-Isoglutamine is at the second position (Vollmer et al., 2008). In all species, D-Glu is the second amino acid of the peptide stem. Most alterations occurs the third position of the peptide stem (Schleifer and Kandler, 1972). Most bacilli have m-Dpm (meso-diaminopimelic acid) residue as their third residue (Shah and Dworkin, 2010). Most other gram-positive bacteria such as *E. faecalis*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Lactobacillus lactis* have L-Lys at their third position (Shah and Dworkin, 2010). In *E. faecium*, D-isoasparagynyl or D-isoaspartyl (D-iAsx) residues occur at the third position (Bellais et al., 2006).

1.6.2 Wall teichoic acids and lipothechoic acids

The cell wall of most gram-positive bacteria mainly contains peptidoglycan and additional cell wall glycopolymers (CWGs) (Neuhaus and Baddiley, 2003). The peptidoglycan provides mechanical stability to the bacterial cell and serves as a scaffold for CWGs. Two types of CWGs are present in most gram-positive bacteria which are the membrane glycolipid-anchored polymers and covalently peptidoglycan-anchored polymers. The CWG is referred to as teichoic acid (TA) if the polymer backbone contains diester-linked phosphate groups. Most TAs have negatively charged phosphate groups and additional D-alanine residues on the repeating units. The D-alanine residues normally have free positively charged amino groups (Neuhaus and Baddiley, 2003).

The peptidoglycan-anchored polymer units are attached to N-acetylmuramic acid of the peptidoglycan via a phosphodiester bond. The N-acetylmuramic acid is linked to N-acetylglusamine acid, which is connected to further sugars of the linkage unit or directly to the CWG. The covalently linked peptidoglycan-cell wall glycopolymers are referred to as wall teichoic acids (WTA) (Neuhaus and Baddiley, 2003). The composition of WTA is variable between bacterial species or even strains (Naumova et al., 2001). In general, the linkage unit consists of 1 GlcNac, 1 N-acetylmannosamine and 2 glycerolphosphate (Gro-P) residues. The WTA repeating units contain trioses, peptoses and hexoses. For example, it was found that the WTA polymer in *S. aureus* strains is formed by up to 40 repeating unit of ribitolphosphate. However, simpler WTA structures are found in *Staphylococcus epidermidis* where the glycerolphosphate (Gro-P) residues form the entire polymer (Endl et al., 1983). In some bacteria such as *Streptococcus agalactiae*, the WTA polymer is more complex. The WTA of that bacterium is composed of different types of repeating units and branched (Sutcliffe et al., 2008). In *E. faecium*, the repeating unit of WTA consisted of two residues of N-acetylgalactosamine, glycerol (Gro) and phosphate (Bychowska et al., 2011).

The lipotechoic acids (LTA) are cell wall polymers that are present on the bacterial cell membrane (Fischer, 1988). The LTA are attached to the cytoplasmic membrane by linking the polymer to glycolipids. The structure of LTA is generally less diverse than WTA. In many gram-positive bacteria such as *S. aureus*, the backbone of the LTA is formed by glycerolphosphate (Gro-P) residues (Fischer, 1994). However, some bacteria such as *Streptococcus pneumoniae* form very complex LTA. The structure of LTA in that bacterium is identical with its WTA (Fischer et al., 1993).

Both WTA and LTA have a positive charge due to the incorporation of D-alanine (Neuhaus and Baddiley, 2003). The incorporation of D-alanine into WTA and LTA is highly

conserved in low G+C gram-positive bacteria and the *dltABCD* genes which are an operon are responsible for D-alanine activation and incorporation. After biosynthesis of the TA polymers is completed, the D-alanine is incorporated. The net charge of teichoic acid is affected by D-alanine modification (Weidenmaier and Peschel, 2008). D-alanine is activated in the cytoplasm by *dltA* which links D-alanine to a D-alanine carrier protein (DltC). DltB, which is an integral membrane protein, translocates the D-alanine and DltD incorporates the D-alanine into TAs (Neuhaus and Baddiley, 2003).

Recently, a novel synthetic LTA-fragment conjugate was developed and potentially be a promising vaccine candidate for immunotherapy against *E. faecium* and other nosocomial gram-positive bacteria (Laverde et al., 2014).

1.6.3 Cell wall-anchored proteins

In general, cell wall-anchored proteins (CWAPs) have an N-terminal signal sequence peptide and a C-terminal cell wall sorting signal (CWS). The CWSs typically contain a conserved Leu-Pro-X-Thr-Gly (LPXTG) sortase substrate motif (where X denotes any amino acid) (Schneewind et al., 1993). This LPXTG motif is followed by a hydrophobic domain and positively charged amino acids. The precursor CWAP is translocated across the plasma membrane and covalently anchored to the cell wall peptidoglycan by sortase- membrane-anchored transpeptidase activity (Perry et al., 2002). Sortase cleaves between the threonine (T) and the glycine (G) of the LPXTG motif. Then, this enzyme catalyzes the formation of an amide bond between the carboxyl group of threonine residue and the amino groups of the cell wall cross bridges. Detailed examination of LPXTG motif by Guzman Prieto and colleagues (2015) showed that this motif is rich in proline residues. A transcriptome analysis of *E. faecium* E1162 revealed that a surface-exposed protein, PrpA, is the most abundant protein expressed during the exponential growth at 37°C (Guzman Prieto et al., 2015). Some

of the cell wall-anchored proteins such as a collagen-binding adhesion of *E. faecium* (Acm) are implicated in bacterial virulence (Hendrickx et al., 2007; Nallapareddy et al., 2008).

1.7 Virulence-associated factors in *E. faecium*

Overview of virulence-associated factors in *E. faecium* is depicted in Figure 1.2.

1.7.1 Esp

The enterococcal surface protein gene, *esp*, has been implicated in biofilm formation and adherence to abiotic materials contributing to the establishment of nosocomial infections via formation of biofilms on medical devices (Heikens et al., 2007). The *esp* gene of *E. faecium* has been demonstrated to be involved in urinary tract infections. Esp enhances kidney and bladder infections in mice (Leendertse et al., 2009). Later, the capacity of the same strains to adhere to human colorectal adenocarcinoma cells (Caco-2 cells) was tested and it was found that Esp does not contribute to adherence to these cells (Heikens et al., 2009). The capacity of the same strains to colonise the intestine of cefriaxone treated-mice was also tested. No differences between the colonization of wildtype and *esp* mutant were found (Heikens et al., 2009). The level of Esp expression on the cell surface varies between different strains. The expression of Esp was higher when bacteria were grown at 37°C compared to 21°C. The expression of Esp was also higher when grown under anaerobic compared to aerobic conditions (Van Wamel et al., 2007).

The Esp plays an important role in initial adherence to abiotic surfaces and in biofilm formation in *E. faecium* E1162 (Heikens et al., 2007). In that study, the cell surface expression of Esp was analyzed by flow cytometry, whole cell ELISA and electron microscopy using rabbit anti-Esp. Lack of cell surface Esp in the mutant was observed similar to Esp negative strain (E135).

Since Esp is common to clonal complex CC-17, this protein might play an important role in the evolutionary development of this clonal complex and contribute to the ecological success of this clonal complex in the hospital environment (Heikens et al., 2007). The *esp* gene is part of a cluster of six genes that have been identified as a putative pathogenicity island (PAI) in *E. faecium*. Interestingly, the PAI is present in epidemic and clinical isolates but not in all human surveillance and animal isolates. Variations in the percentage of GC content of the Open Reading Frame (ORF) present in the putative PAI implies that this island was acquired through a complex evolutionary process, involving horizontal gene transfer from a number of different bacterial ancestors (Leavis et al., 2004). This PAI is different from the *esp*-containing pathogenicity island of *E. faecalis* with the exception of the presence of the *esp* and *araC* genes. The arrangement of genes within the two PAIs are also different, *araC* is located upstream of *esp* in *E. faecium*, whilst the same gene is located downstream of *esp* in the *E. faecalis* PAI. The *araC* gene is often found on PAIs (Leavis et al., 2004; Hacker and Kaper, 2000) and probably plays a role in gene regulation since AraC is a member of a family of transcriptional activator proteins (Egan, 2002). The entire *esp* PAI can be transferred horizontally and inserts in a site-specific manner. Thus, demonstrating that entire set of genes involved in environmental persistence, colonization and virulence can be transferred and acquired by this nosocomial pathogen (van Schaik et al., 2010). It was found that *esp* PAI in E1162 is 64 kb and 3' end of the *esp* PAI encodes a 120 kDa LPXTG-type cell wall anchored protein. Analysis of *esp* containing PAI of several *esp*+ strains demonstrated that its varies in size between strains (68 kb in U0317) and (104 kb in E1679). The % G+C content of the *esp* PAI between strains are also varies (35.4 % in U0317, 36.6 % in E1679 and 35.7 % in E1162). Since the % G+C content of the *esp* PAI is lower than the average % G+C-

content over the entire genome it has been suggested that the DNA of the *esp* PAI was acquired exogenously (van Schaik et al., 2010).

More recently, the mobility of the mobilization of *esp* has been studied and it was concluded that the *esp* PAI is an integrative conjugative element (ICE) rather than a pathogenicity island (Top et al., 2011). The *esp* PAI is considered as an ICE due to the presence of *intA* that encodes integrase. The IntA is involved in the excision and formation of circular intermediates (CI) of the *esp* PAI. The *esp* PAI was found to be a self-transmissible element and name has been changed from the *esp* PAI to ICEEfm1 (Top et al., 2011).

It was found that the putative AraC type of regulator (EfmE1162_2351, which was renamed as *ebrB*, regulates the *esp* operon. The *araC* is located upstream of the *esp*. A reduction of Esp expression was observed in mutants of *ebrB*. In that study, they had also found that *ebrB* expression levels did not change over time during growth. However, an increase in *esp* expression at both RNA and protein level was observed during mid-log and late-log phase. Thus, they hypothesized that a presence of a secondary regulation system for *esp* (Top et al., 2013). A recent study by Kafil and Mobarez (2015) showed a significant correlation between the resistance of enterococci to antibiotics and the presence of *esp* (Kafil & Mobarez, 2015).

1.7.2 Hyl$_{Efm}$

The *hyl*$_{Efm}$ gene which encodes for a putative hyaluronidase is also primarily found in epidemic vancomycin-resistant *E. faecium* isolates of clinical origin (Rice et al., 2003). The *hyl*$_{Efm}$ was associated with a large plasmid (more than 145 kb). It has been shown that this gene enhances the ability of this bacterium to colonize the gastrointestinal tract (Arias et al., 2009). This gene has also been observed to be responsible for the increased virulence of *E.*

faecium in a mouse peritonitis model. The *van*(A) gene cluster and gentamicin resistance determinants are co-transferred and genetically linked to the hyl_{Efm} gene (Arias et al., 2009; Rice et al., 2003).

It was found later that hyl_{Efm} does not have hyluroidase activity but codes for a putative glycosyl hydrolase of unknown function (Panesso et al., 2011). In that study, deletions in genes adjacent to hyl_{Efm} were also created. It was observed that four genes of the hyl_{Efm} region do not mediate virulence conferred in murine peritonitis.

1.7.3 SagA

Among the putative virulence factors which have been identified in *E. faecium* is the *sagA* gene which may be involved in cell wall metabolism. This gene is located in a cluster of genes encoding cell wall metabolism proteins (MreCD) and encodes a protein which has sequence similarity to other cell wall metabolism-related proteins in other bacteria such as SagA of *S. mutans*, P60 and P45 of *L. monocytogenes*. The SagA protein is also able to bind various human extracellular matrix proteins including fibrinogen, collagen type I, collagen type IV, fibronectin and laminin (Teng et al., 2003).

It has been shown that SagA could be a promising vaccine target to treat bacteraemia caused by VRE (Kropec et al., 2011). SagA induces opsonic antibiodies in rabbits immunized with purified SagA protein. A significant reduction in *E. faecium* colony counts in the blood of mice infected with the strain from which SagA was derived, was observed with immune rabbit serum compared to preimmune serum. A reduced colony counts in the same model was also observed using heterologous vancomycin-resistant enterococci (VRE) strain (Kropec et al., 2011).

1.7.4 Acm

The cell wall-anchored collagen adhesin, Acm, has also has been extensively studied in *E. faecium*. Acm is detected predominantly in clinically derived isolates particularly in CC-17 (Nallapareddy et al., 2008). A significant correlation between Acm and the clinical origin of *E. faecium* isolates has also been found. Acm bounds to collagen type IV but to a lesser degree than type I collagen (Sillanpaa et al., 2008).

It was shown that this gene plays an important role in *E. faecium* pathogenesis in a rat model of endocarditis but not in peritonitis (Nallapareddy et al., 2008). In addition to collagen, capability of Acm to bind fibronectin (Fn) and laminin (Ln) may have contributed to the emergence and adaptation of the endocarditis-enriched nosocomial genogroup CC-17 (Zhao et al., 2009). A recent study by Somarajan and colleagues (2015) revealed that apart from this protein, a fibronectin-binding protein, Fnm, also binds to fibronectin and plays important role in endocarditis (Somarajan et al., 2015).

Recently, it was found that AtlAEfm is the major autolysin of *E. faecium* and it is implicated in Acm localization. AtlAEfm facilitates the binding to collagen types I and IV. AtlAEfm is also important for cell separation, cell adhesion and biofilm formation of this bacterium (Paganelli et al., 2013).

1.7.5 Scm

A genome-based bioinformatics study on the draft genome of *E. faecium* endocarditis-derived strain TX*0016* was done (Sillanpaa et al., 2008). Based on the analysis, these authors identified a number of genes potentially coding for microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) in *E. faecium*. Among the 15 anchored *E. faecium* surface proteins (Fms) identified Fms10, which was later known as

Scm is found to encode a second collagen. Unlike Acm that shows affinity for collagen types I and IV, the Scm showed affinity for collagen type V. Since collagen type V is present in the intestinal submucosa, they hypothesized that Scm might involved in colonization and persistence in the intestinal tract (Sillanpaa et al., 2008).

1.7.6 SgrA

Five genes encoding surface-exposed LPXTG cell wall-anchored proteins (CWAPs) distinctively enriched in clonal complex 17 (CC-17) *E. faecium* were identified (Hendrickx et al., 2007). In addition of Esp and Acm, an LPXTG surface protein, SgrA, which has a serine-rich repeat region and it was speculated that this protein could adhere to fibrinogen (Hendrickx et al., 2007; Hendrickx et al., 2009). The expression of SgrA was detected in all phases of growth. A significant reduction in binding of an *E. faecium sgrA* mutant to the components of the basal lamina, fibrinogen and ultrapure laminin was observed. The *sgrA* mutant cells also displayed a significantly lower surface hydrophobicity compared to the wildtype. The *sgrA* mutant displayed decreased biofilm formation on an abiotic material which was a polystyrene surface (Hendrickx et al., 2009).

1.7.7 EcbA

Initially, the *E. faecium* collagen binding protein A, EcbA was known as LPXTG surface protein (Hendrickx et al., 2007) and that the gene for this protein was also enriched in clonal complex (CC-17). EcbA is one of the *E. faecium* MSCRAMMs that binds to collagen type V (Hendrickx et al., 2009). This protein is encoded by *ecbA* which is present on the chromosome. In that study, they also found other EcbA-like proteins that share 90 % amino acid identity to the EcbA. EcbA bounds to fibrinogen and this protein is only expressed in exponential and late exponential phase (Hendrickx et al., 2009).

1.7.8 Pili

Apart from *scm* and *acm*, other gene, *pilA* was identified. The *E. faecium ebpABC* (*ebpABCfm*), is transcribed as a single operon, polymerises into a major pilus subunit protein, EbpCfm (Sillanpaa et al., 2008). EbpCfm is now referred to as PilB and this protein is presence on the surface of *E. faecium* (Hendrickx et al., 2008; Sillanpaa et al., 2008). Further study by them showed the importance of the polymerised pilus material in the interaction of and adherence between individual cells which could mediate the formation of biofilm matrix (Sillanpaa et al., 2010).

Hendrickx and colleagues also described another pilus which is PilA (previously known as Fms21) (Hendrickx et al., 2008). The surface expression of PilA and PilB pili was regulated in a growth-phase and temperature-dependent manner. In that study, it was found that both types of pili were expressed from two different gene clusters, pilin gene cluster PGC-1 and PGC-3. It was found that both PGCs had genes that encode class C sortase genes. PGC-1 which encodes PilA pili has an additional sortase gene, which is a class A sortase gene. PGC-3 encoding PilB pili is similar to the *E. faecalis ebpABC* cluster (Hendrickx et al., 2008).

Both of the pili were expressed during early exponential phase (Hendrickx et al., 2008). Polymerisation of PilA into PilA multimers occurs only when cells were grown on solid media but not in broth culture. Hence, it was suggested that the polymerization of PilA is induced when the cells are in a contact with a surface. The PilB type pili were expressed throughout all phases of growth. The PilB type pili were polymerized and migrated towards the poles of cells during the exponential phase. The polymerized PilB type pili were expressed at the polls of the cells during the stationary phase. Polymerization of both pili

was observed when cells were grown at 37°C but not 21°C. Putative pili subunit genes of PilA and PilB are enriched among hospital-acquired *E. faecium* isolates (Hendrickx et al., 2008).

The *ebpABC*$_{fm}$ is transcribed as an operon and deletion of the *ebpABC*$_{fm}$ operon eliminated the *EbpC*$_{fm}$ (PilB) expression on the cell surface of *E. faecium* (Sillanpaa et al., 2010). It was shown that biofilm formation of *ebpABC*$_{fm}$ mutant in TX82 strain which is lacking *esp* is comparable with an *esp* mutant of E1162. This suggested that several genetic determinants can contribute differently to biofilm formation in different strains. The *ebpABC*$_{fm}$ operon plays a role in virulence in a murine model of urinary tract infection (Sillanpaa et al., 2010).

Further analysis of the *pilA* (fms21) locus showed that this locus is resided on a plasmid since a *repA* gene was identified in the neighbourhood of the locus. In fact, the *pilA* (fms21) locus and *hyl*$_{Efm}$ are co-localized on the same plasmid (Kim et al., 2010).

Analysis of PGC-1 which encodes PilA pili showed that this locus codes for two additional pili, which are *pilE* and *pilF* (Hendrickx et al., 2010). The *pilA* locus is present in the polyclonal hospital-acquired genetic lineage but not in non-hospital-acquired strains of *E. faecium*. The expression of PilA, PilE and PilF pili is higher at 21°C than 37°C. PilF is associated with polymerization of PilA type pili (Hendrickx et al., 2010).

1.7.9 AsrR

An oxidative stress sensor and response regulator, AsrR (antibiotic and stress response regulator) was identified in *E. faecium* and reported to sense hydrogen peroxide. This gene is conserved among all *E. faecium* isolates (Lebreton et al., 2012). Homologs of *asrR* are not present in *E. faecalis* but are present in *Enterococcus casseliflavus* and *Enterococcus gallinarum*. Deletion of *asrR* increased the resistance of *E. faecium* to cationic

antimicrobial peptides and vancomycin. Pbp5 expression increases in the *asrR* mutant resulting in increased resistance to β-lactam antibiotics. Autolysis in the *asrR* mutant was slower than the parental strain and increased the transfer frequency of conjugative transposon Tn*916*. Two major adhesions, *acm* and *ecbA* are overexpressed in the *asrR* mutant. Deletion of *asrR* also increased biofilm formation and adherence to human intestinal cells. The *asrR* mutant showed increased susceptibility to oxidative stress *in vitro* and in murine macrophages. However, the deletion of *asrR* resulted an increased host-persistence in both *Galleria mellonella* and mouse systemic infection models. This data suggests that *asrR* regulates the antimicrobial resistance and pathogenicity in *E. faecium* (Lebreton et al., 2012).

1.7.10 GelE

Two secreted proteases a gelatinase and a serine protease were initially identified in *E. faecalis* (Gutschik et al., 1979). These enzymes, which are responsible for proteolysis are also known as gelatinase since they are able to liquefy gelatine. Gelatinase, encoded by the chromosomal *gelE*, is an extracellular zinc endopeptidase that hydrolyzes collagen, gelatin, and small peptides (Gutschik et al., 1979). *gelE* has been shown to exacerbate endocarditis in an animal model (Su et al., 1991).

gelE was detected in one clinical strain of *E. faecium*; however, gelatinase activity was lost during subculture from the original stock (Eaton and Gasson, 2001). Recently, the presence of *gelE* was reported in one clinical strain of *E. faecium* isolated from patients suffering from a Crohn's disease (Golinska et al., 2013).

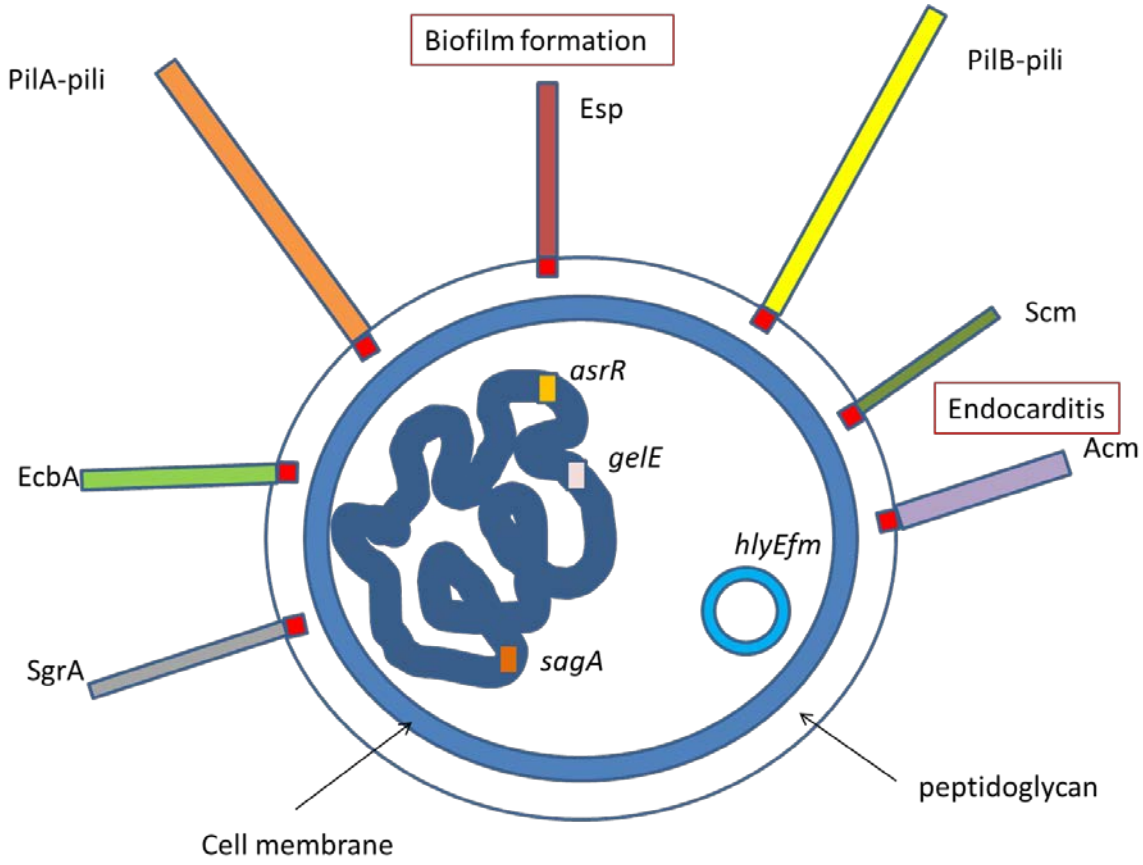


Figure 1.2 Overview of virulence-associated factors in *E. faecium*. Surface proteins are covalently attached via the LPXTG motif (red square) to the cell wall peptidoglycan. Different colours represent different proteins.

1.8 Aim of this project

E. faecium is an opportunistic human nosocomial pathogen that has developed resistance to many existing antimicrobial therapies. Treatment of enterococcal infections is becoming increasingly challenging and there are limited therapeutic options against multidrug-resistant enterococci. The work described in this thesis aimed to identify genes involved in acid tolerance, antimicrobial resistance and virulence of *E. faecium*.

The aim of this project is to evaluate methods for creating random mutations in *E.faecium* E1162 by using mariner-based transposon delivery vector (pJAWTRASH2). An *E. faecium* transposon mutant library was screened for altered resistance to lysozyme and to

nisin as a model antimicrobial peptide. This approach led to the identification of several genes that contribute to lysozyme and nisin resistance.

A number of mutants that were sensitive to nisin had a transposon insertion in a gene predicted to encode a tyrosine decarboxylase. It was shown that the tyrosine decarboxylase plays a role in acid tolerance and mediates virulence of *E. faecium* in a *Galleria mellonella* larvae infection model.

This project also examined the role of *E. faecium* serine threonine protein kinase (Stk1) in antimicrobial resistance, host cell stresses response and virulence. It was anticipated that the data obtained from this study would improve our understanding of the role of Stk1 in *E. faecium* E1162.

Finally, it was hypothesized that a permease component of the ABC transporter putative ATP-binding-cassette (ABC) transporter system involved in antimicrobial peptide resistance. To examine this, the markerless deletion of the gene encoding the permease component of the ABC transporter mutant was characterized. The aim was to improve our understanding of Antimicrobial peptide resistance mechanisms in *E. faecium* E1162.

Chapter 2

Materials and Methods

2 Materials and methods

This chapter describes the materials and methods that are used throughout the project. The specific materials and methods are outlined at the beginning of the relevant chapter.

2.1 Bacterial strains

The bacterial strains used in this study are listed in Table 2.1. All bacterial stocks were maintained in 20 % (v/v) glycerol in BHI at -80°C.

Table 2.1 Bacterial strains used in this study

Strain	Relevant characteristics	Reference or source
E. faecium		
E1162	Clinical blood isolate; CC17; Amp^r Van^s Chl^s Gen^s Ery^s Esp	Willems et al., 2005
E. faecium		
201c	E1162 Δ*stk1* Amp^r Van^s Chl^r Gen^s Ery^s Esp	Sean Nair. (unpublished)
E. faecium		
47N	E1162 ΔEfmE1162_0264 Amp^r Van^s Gen^s Ery^s Esp	This study
pHFH12	repaired mutant 47N	This study
E. faecalis		
OG1RF	Ery^s Chl^s	Kristich et al., 2007
E. coli		
DH5α	*E.coli* host strain for routine cloning	Invitrogen
EC1000	*E. coli* cloning host; MC1000 containing pWV01 *repA* on chromosome	Leenhouts et al., 1996

r: resistant, s: sensitive, Amp: Ampicillin, Van: Vancomycin, Chl: Chloramphenicol, Gen: Gentamicin, Ery: Erythromycin, Esp: Enterococcal surface protein, *repA*: Regulatory protein RepA

2.2 Plasmids

Plasmids used in this study are listed in Table 2.2

Table 2.2 Plasmids used in this study

Plasmid	Relevant characteristics	Reference or source
pCJK96	Amp^r Van^s Chl^r Gen^s Ery^r Esp	Kristich et al., 2007
pEA201	pCJK96:*stk1* from *E. faecium*; Ery^r	Elaine Allan (unpublished)
pJAWTRASH2	pCM38/39; T7 promoter; Ery^r	Wright et. al., (unpublished data)
pCR 2.1	Amp^r	Invitrogen
pHFH1	pCJK96:chloramphenicol acetyl transferase gene from pSK236; Ery^r	This study
pSK236	Amp^r	This study
pHFH9	pSK236;Amp^r Ery^r	This study
pHFH10	pHFH9 containing *stk1* with RBS	This study
pHFH11	pHFH9 containing *ireK* with RBS	This study
pHFH4	pSK236; kanamycin promoter; Amp^r Clm^r	This study
pHFH12	pHFH4 containing EfmE1162_0264 with RBS	This study

r: resistant, s: sensitive, Amp: Ampicillin, Ery: Erythromycin, Van: Vancomycin, Gen: Gentamicin, Chl: Chloramphenicol, Esp: Enterococcal surface protein, RBS: Ribosomal binding site

2.3 Media

All media is shown below (Table 2.3)

Table 2.3 Media used in this study

Media	Components
Brain Heart Infusion	As supplied by Oxoid (CM1135)
Tryptic Soy broth	As supplied by Sigma (T8907)
Luria Bertani broth	As supplied by Sigma (L3022)
Todd–Hewitt broth	As supplied by Oxoid (CM0189)
MM9YE	33.9 g/L disodium phosphate 15 g/L monopotassium phosphate 2.5 g/L sodium chloride 5 g/L ammonium chloride 2.5 g/L Yeast extract 2 g/L ribose
SOC medium	20 g/L tryptone 5 g/L yeast extract 0.5 g/L NaCl 2.5 M KCl 20 M glucose
Solid media	1.5 % agar

2.4 Growth conditions

Bacteria were grown at 37˚C unless stated otherwise. *Escherichia coli* DH5α containing plasmids were grown in LB broth and on LB agar. Enterococcal strains were grown either in Tryptic Soy Broth (TSB), Todd–Hewitt broth (THB) or Brain Heart Infusion

(BHI) media. Enterococci containing plasmids pHFH9, pHFH10 and pHFH11 were grown in broth or medium supplemented with 40 µg/mL erythromycin. Enterococci containing plasmids pHFH4 and pHFH12 were grown in broth or medium supplemented with 20 µg/mL chloramphenicol. All bacterial cultures were incubated under shaking condition (200 rpm).

2.5 Antibiotics

Antibiotics were obtained from Sigma-Aldrich (Poole, UK) and were used at concentration of 20 µg/mL chloramphenicol, 40 µg/mL erythromycin and 100 µg/mL ampicillin, unless stated otherwise.

2.6 Molecular biology techniques

2.6.1 Plasmid DNA purification

All procedures for DNA extraction were performed according to the manufacturer's instructions for spin column plasmid DNA miniprep kits (NBS Biologicals).

2.6.2 Genomic DNA purification

All procedures for DNA extraction were carried out according to manufacturer's instruction (QIA amp®DNA Mini Kit), with the exception that in the step after the bacterial pellet was suspended by vigorous vortexing in 170 µL TE buffer and 20 µL 50 mg/mL lysozyme were also added. The subsequent procedures were done according to manufacturer's instruction.

2.6.3 Agarose gel electrophoresis

DNA was analysed via gel electrophoresis. Powdered agarose (1 %) was dissolved in 1 x TAE buffer (40 mM Tris, 40 mM acetate, and 1 mM EDTA, pH 8.3) to a final concentration of 0.8-1.5 % (w/v) and 3 µL of 10 mg/mL ethidium bromide was added. The DNA samples

were mixed with 6 x loading dye (200 mM EDTA, pH 8, 50 % (v/v) glycerol, 0.1 % (w/v) bromophenol blue) and run in 1 x TAE at a constant voltage of 80-120 V for an appropriate time. The 1 kb plus DNA ladder (Invitrogen) was used to determine the size of DNA fragments. The DNA bands were visualized under UV-light using Alpha Imager (Alpha Innotech).

2.6.4 Standard PCR protocol

PCR amplification was performed using Biometra T3000 Thermocycler with the following standard conditions: initial denaturation step at 95°C for 4 min, 30 PCR cycles (94°C, 1 min; 53-60°C, 1 min (dependent on primer annealing temperature); 72°C, 1-4 min (dependent on expected amplicon size, usually 1 min for each 1 kb); final extension of 4 min at 72°C and cooled at 4°C until required. All of the temperature and times are variable. The melting temperatures (Tm) of the primers were calculated by the following equation: Tm=67.5 + [0.34 x % (G+C)]-(395/n), where n is the length of the primer. The annealing temperature was derived as 5°C below the Tm of the primer with the lowest Tm. The PCR reaction mixture contained 5 µL of 10 x *Taq* polymerase buffer (NEB, Hertfordshire, UK), 1 µL of 10 pmol/ µL of each forward and reverse primers (Sigma-Aldrich, Poole, UK) (Table 2.4), 1 µL of 10 mM dNTP mix (NEB, Hertfordshire, UK), 0.5 U/µL of *Taq* polymerase (NEB, Hertfordshire, UK) and 1 µL of DNA template (0.2 ng/µL). The total volume of PCR mixture was made up to 50 µL using distilled water. Products were analysed by electrophoresis on a 1 % agarose gel.

Table 2.4 Oligonucleotides used in this study

Oligonucleotides	Sequence
Linker I-Taq$^{\alpha}$I	5' TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACAT 3'
Linker II-Taq$^{\alpha}$I	5' CGATGTCCCCGTACATCGTTAGAACTACTCGTACCATCCACAT 3'
Linker I- AseI	5' TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACAAT 3'
Linker II- AseI	5' TAATTGTCCCCGTACATCGTTAGAACTACTCGTACCATCCACAT 3'
Marq255	5' CAGTACAATCTGCTCTGATGCCGCATAGTT 3'
Marq256	5' TAGTTAAGCCAGCCCCGACACCCGCCAACA 3'
Y linker primer	5' CTGCTCGAATTCAAGCTTCT 3'
Transposon primer R	5' GGAATCATTTGAAGGTTGGTA 3'
catF	5' GTGCATGCAATTTAGGAGGCATATCAAATG 3'
cattermR	5' GTTCTAGA AGTGACATTAGAAAACCGAC 3'
MabcUF (A)	5' AAGCGGCCGC ATGCAGGATAAAT AGAAAATACC 3'
MabcUR (B)	5' ATGAACAAATGGCGTTCTAATCCAAAATTGAAT 3'
MabcDR(D)	5' AAGAATTCATGAAAAATGAAAGAGGAGG 3'
MabcpUF(I)	5' AAGCGGCCGCGATGTGACACTGTCACACTC 3'
MabcpUR(J)	5' ATGGAAATCAATGAACAAAATTTTGGCGAA 3'
MabcpDF(K)	5' TTTTGTTCATTGATTTCCATAAAGCATAGTCAATCTCCCC 3'
MabcpDR(L)	5' AAGAATTCATGAACAAAATAGTCGAAGTACAG 3'
abcF	5 'TTATCTAGAAAGTAATAGACATGCAGGCG 3'
abcR	5' ATTGCATGCAATAAAGAGGTTGTGACAAA 3'
EA 126F	5' GACAACATGCAGTATCTGGTG 3'
EA 126R	5' CTTCGCGTTTTTGCTTGACTG 3'
EA 127F	5' TGATTTAGACAATTGGAAGAG 3'
EA 127R	5' CAGTCATTAGGCCTATCTGAC 3'
EA 128F	5' TCACGCAGACGAATACGATGC 3'
EA 128R	5' TAGCTAATAGGATCAGCCACC 3'
EFMSTPKF	5' TTATCTAGAGGATAATATCACCGTTCTTC 3'
EFMSTPKR	5' ATTGCATG AGGAAAGTAGGTGTTATTCC3'
EFCSTPKF	5' TTATCTAGATACCGTGTTAGTGATACACT 3'
EFCSTPKR	5' ATTGCATGCTTCTTCTTATTGCTGAGAAA 3'

2.6.5 Colony PCR

The protocol for colony PCR is the same as that for standard PCR, with the difference that a single bacterial colony is used as the DNA template instead of purified genomic DNA. One colony was selected from a selection plate using a sterile tip. The tip was touched to a separate agar plate (so that a stock of the colony was retained) and then dipped into the PCR reaction and stirred gently. PCR amplification was then carried out as described above.

2.6.6 Lyse and go reaction

One colony was selected from a selection plate using a sterile tip and dispensed into 5 µL of distilled water. Ten microlitre of Lyse and Go buffer (Thermo Scientific) was dispensed into the cell suspension. The tube was then placed into the Biometra T3000 Thermocycler, followed by incubation at 65°C at 30 seconds, 8°C at 30 seconds, 65°C at 90 seconds, 97°C at 180 seconds, 8°C at 60 seconds, 65°C at 180 seconds, 97°C at 60 seconds, 65°C at 60 seconds and 80°C at 300 seconds. For PCR amplification reactions, the bacterial suspension was then spun at 11952 x g (10000 rpm) a table-top microcentrifuge for 1 minute. The supernatant (4 µL) was used as DNA material in PCR reaction.

2.6.7 DNA purification from gel

After agarose gel electrophoresis was performed, DNA fragments were visualized on a T2201 UV Transilluminator (Sigma Chemical Company) at 302 nm. The DNA fragment was excised from the agarose gel with a clean and sharp scalpel. The gel slice was weighed in a microcentrifuge tube and purified using the Spin Column Gel Extraction Kit (NBS Biologicals) following the manufacturer's instructions.

2.6.8 Restriction endonuclease reactions

DNA was digested using restriction enzymes (NEB, Hertfordshire, UK) according to the manufacturer's instructions. All restriction endonuclease reactions were carried out at 37°C unless stated otherwise.

2.6.9 Ligation

Ligation of DNA was carried out using T4 DNA ligase (NEB, Hertfordshire, UK) according to the manufacturer's instructions. Ligation was carried out using a molar ratio of 1:3 vector to insert. The ligation mixture was incubated overnight at 12°C with T4 DNA ligase.

2.6.10 Dephosphorylation reactions

Dephosphorylation was carried out remove the 5'-phosphate groups from each end of the linearised vector to minimize the self-ligation of the vector during cloning. The linearised vector (1 µg) was incubated with 0.01 U/pmol of Calf Intestinal Alkaline Phosphatase (CIAP) and 5 µL of CIAP 10 x reaction for 30 min at 37°C. Dephosphorylation mixture was set up in 50 µL reaction. After 30 min incubation, an equivalent amount of CIAP previously used was added to the reaction mixture and incubation continued for an additional 30 min. Dephosphorylation was stopped with the addition of 0.5 mM ethylenediaminetetraacetic acid (EDTA) and incubated at 75°C for 10 min. Subsequently, the dephosphorylated vector was purified using Spin Column PCR Purification Kit (NBS Biologicals) following the manufacturer's instructions.

2.6.11 5'-Phosphorylation of DNA fragment

Phosphorylation was carried out to add the 5'-phosphate group to the DNA fragment. The following components were added to a microcentrifuge tube; 300 pmol

oligonucleotides, 5 µL 10 x T4 Polynucleotide Kinase Reaction Buffer, 1 µL T4 Polynucleotide Kinase 10 U/µL, 5 µL 10 mM ATP and distilled water to a final volume of 50 µL. The reaction mixture was incubated at 37°C for 30 min. The T4 Polynucleotide Kinase was activated at 65°C for 20 min.

2.7 DNA Sequencing

DNA sequencing of purified plasmids and PCR products was performed by DNA Sequencing Service (Source BioScience, London, UK). The concentrations used for DNA sequencing were 100 ng/µL for plasmids and 1 ng/µL per 100 bp for PCR products. The concentration of DNA was determined either by agarose gel electrophoresis or the NanoDrop^{TM} 1000 Spectrophotometer (Thermo Scientific, Surrey, UK).

2.8 The pHOU1 counter selection system

The pHOU1 counter selection system has previously been used in *E. faecium* E1162 to make strains with deletions in genes (Panesso et al., 2011). The targeted gene was then ligated with a fragment released from pCR 2.1 after digestion with the same enzymes. The recombinant plasmid was transformed into *E.coli* EC1000 and the transformants were selected by growth on Luria-Bertani agar containing gentamicin (25 µg/mL). The plasmid can replicate in this *E. coli* strain because it produces the plasmid replication protein RepA. Subsequently, the plasmid was purified and introduced into *E. faecium* E1162 by electroporation and plated onto BHI agar plates supplemented with gentamicin (200 µg/mL) for selection of single cross-over integrants. Colonies were picked, resuspended in 50 µL of 0.85 % NaCl and plated on MM9YEG media supplemented with 7 mM phenylalanine. The *pheS* counter-selection system based on a vector which carries a *pheS** allele that has a mutation which results in an amino acid change at position 294 from Gly to Asp in this

protein (Kast et al., 1991). The *pheS* gene codes for the phenylalanine-t-RNA synthetase α subunit and a change from Gly294 to Asp294 results in a protein with a relaxed substrate specificity that tolerates incorporation of the toxic phenylalanine analogue p-chloro-phenylalanine. Thus, bacteria harboring a plasmid coding for the pheS* would be killed if grown on media-containing p-chloro-phenylalanine. The plates were incubated for 48 hours at 37°C. In order to confirm that the colonies were excisants, the corresponding colonies were grown on MM9YEG media supplemented with phenylalanine, the colonies were replica plated on BHI in the presence and absence of gentamicin. Colonies that were sensitive to gentamicin and erythromycin were selected.

2.9 Preparation of competent cells

2.9.1 Preparation of electro-competent *E. faecium* and *E. faecalis*

A colony of *E. faecium* or *E. faecalis* was grown in 10 mL of Todd-Hewitt broth (THB) for 8 hours with shaking at 37˚C. The culture was then centrifuged at 5000 x g for 10 min and then resuspended in 10 mL of fresh THB. This 10 mL was then added into 1 L THB and incubated at 37˚C without shaking. After 16 hrs the cells were recovered by centrifugation at 5000 x g for 15 min at 4˚C using a SLA-3000 rotor in a Sorval® RC-5B centrifuge. The pellet was resuspended in 1 L 10 % glycerol. In the following steps, the pellet was centifuged at 857 x g for 15 min and resuspended in 500 mL 10 % glycerol, 200 mL 10 % glycerol, 20 mL 10 % glycerol and 2 mL 10 % glycerol, respectively. The competent cells were aliquoted into 100 µL and stored at -80˚C.

2.9.2 Preparation of competent cells of *E. coli* EC1000

A 10 µL glycerol stock of an *E. coli* EC1000 containing no plasmids was allowed to thaw in ice and added to 40 mL of liquid SOC medium. This culture was incubated at 37°C

with shaking at 200 rpm for 2-3 hours until an OD_{600} of 0.4 was reached. The cells were pelleted by centrifugation at 12164 x g (8000 rpm) for 1 min at 4°C, then resuspended in one-half volume (20 mL) of sterile cold 100 mM $CaCl_2$ solution, and incubated on ice for 25 min. After another centrifugation step as above, the resulting cell pellet was resuspended in one-tenth volume (4 mL) of sterile cold 100 mM $CaCl_2$ solution to yield the final competent cell suspension. The competent cells (1.6 mL) were transferred into a tube and 0.4 mL of sterile 100 % glycerol was added to give a final concentration of 20 % glycerol. The competent cells were aliquoted into 100 µL and stored at -80˚C.

2.10 Transformation

2.10.1 Transformation of *E. faecium* and *E. faecalis*

Transformation was done using the electroporation technique. Electroporations were performed using Gene Pulser II from Bio-Rad. One hundred µL of *E. faecium* competent cells were added into a chilled 0.2 cm electroporation cuvette. One µg of the purified plasmid DNA was added to the competent cells and mixed by swirling using pipette tips. The mixture was then pulsed immediately using 2.5 kV, 25 µF capacitance and 400 Ω resistance. Nine hundred µL of Todd-Hewitt broth was added to the mixture and the cells were incubated horizontally at room temperature (25˚C) with shaking at 200 rpm for an hour and then at 28˚C for an additional hour. After incubation, 100 µL of the mixture was plated out on Tryptone Soy Agar (TSA) containing appropriate antibiotics and incubated at 28˚C for 24-96 hours. Random colonies were selected for further investigations.

2.10.2 Transformation of *E. coli*

E. coli transformation was carried out using competent cells *E. coli* EC1000 or Subcloning Efficiency ^{TM} DH5α^{TM} Chemically Competent Cells (Invitrogen) according to the

manufacturer's instruction. Three microlitres of ligation reaction was added to a 50 µL aliquot of cells and mixed gently by tapping the tube.

2.11 Blue/white colour screening

Transformants containing recombinant plasmids were selected for on LB agar containing 100 µg/mL ampicillin, 40 µg/mL 5-bromo-4-chloro-indoyl-β-D-galactopyranoside (X-Gal). Plates were incubated overnight at 37°C. White colonies, which indicate the presence of recombinant plasmids were selected for further investigation.

2.12 Determination of bacterial sensitivity to antibiotics

Minimum inhibitory concentrations for antibiotics were determined in aerobic liquid cultures by using a microtiter plate serial dilution method. Two-fold dilution series beginning at 1024 µg/mL was prepared in 96-well microtiter plates. Bacteria from stationary-phase cultures were diluted to the OD_{600} of 0.5 in TSB growth medium were inoculated into each well to a concentration of ≈ 10^5 cfu/mL (50 µL). Microtiter plates were incubated at 37°C for 48 hours. The lowest concentration of antibiotic that prevented growth was recorded as the MIC. Antibiotic concentrations ranged from 1024 to 0.25 µg/mL. Bile salts concentrations ranged from 0.01 % to 10.00 %.

2.13 Virulence test of *E. faecium* strains using *Galleria mellonella* wax worms model

The *Galleria mellonella* wax worms were purchased from Cornish Crispa, Co. (UK) and stored at 12˚C prior use. All *E. faecium* strains were grown in BHI medium at 37˚C for 16 hours with shaking at 200 rpm. Bacterial cultures (2 mL) were normalized in the same medium to OD_{600} 2.0 and cells were washed twice in PBS. For each washing step, cells were centrifuged at 5000 rpm for 10 minutes. To get ≈5 x 10^8 cells/mL, cells that were collected

by centrifugation were resuspended with 4 mL PBS. For each strain, 10 *G. mellonella* wax worms of about the same size (body weight, 200 to 289 mg) were infected. Ten µL of the cells suspension was injected into the second hindmost left proleg of each *G. mellonella* wax worms using VanishPoint® syringe. PBS (10 µL) was injected into the wax worms as a negative control. After injection, the wax worms were incubated at 37°C in petri dishes (in darkness). The number of live wax worms was scored every 24 hours after infection. Wax worms were considered dead when they displayed no movement in response to touch and had turned black. The experiment was repeated three times on three different days, and the survival percentages of wax worms inoculated with different strains were analysed using Kaplan-Meier curves. The log rank test was used to analyze different Kaplan-Meier curves. Result of the virulence test is shown in Appendix A.

2.14 Cytochrome c binding assay

To measure changes in bacterial surface charge, a cytochrome c binding assay was performed as described by Matsuo et al. (2011), with slight modifications. Bacterial cells at the appropriate growth phase were harvested by centrifugation at 5,000 rpm for 10 minutes and washed twice with 20 mM MOPS buffer, pH 7. The cells were resuspended in the same buffer to an optical density of 0.5 at 600 nm. Cell suspensions were centrifuged at 5,000 rpm for 10 minutes. The cells suspensions were incubated with 0.25 mg/mL cytochrome c for 10 minutes in the same buffer and then centrifuged. The amount of cytochrome c binding was determined by measuring the optical density at 530 nm and substracted them from the initial optical density of cytochrome c solution before the bacterial exposure. The absorbance value obtained compared with that without bacterial cells was calculated as the absorption ratio, reflecting the bacterial surface charge.

Calculations of cytochrome c bound to the cells: Cytochrome c tested- (Final optical density / Initial optical density x Cytochrome c μg/mL).

2.15 Statistical analysis

All results were represented by a minimum of three independent replicates. Mean and standard deviations were analyzed with the Mann-Whitney U test.

Chapter 3

Identification of genes involved in antimicrobial peptide resistance by utilizing transposon mutagenesis and the role of tyrosine decarboxylase in *E. faecium*

3 Identification of genes involved in antimicrobial peptide resistance by utilizing transposon mutagenesis and the role of tyrosine decarboxylase in *E. faecium*

3.1 Antimicrobial peptides

It is known that antimicrobial peptides (AMPs) play an important role in the innate immune system. AMPs have been identified on the skin and mucosae (Wiesner and Vilcinskas, 2010). AMPs including defensins are also produced by several blood cell types such as neutrophils, eosinophils and platelets (Risso, 2000; Cox et al., 2011; Malik and Batra, 2012). Mammalian defensins are classified into α, β and θ categories based on their size and pattern of disulphide bonding (Cole et al., 2004). α defensins are found in neutrophils, epithelial cells, certain macrophage populations and Paneth cells (specialized cells in the epithelium of the small intestine) (Yang et al., 2002; Cole et al., 2004; Bevins and Salzman, 2011). Paneth cells play a role in intestinal host defence and homeostasis since the dysfunction of these cells leds to higher susceptibility to chronic inflammatory bowel disease (Bevins and Salzman, 2011). β defensins are produced by epithelial cells lining various organs such as the epidermis and genitourinary tract (Yang et al., 2002). Theta defensins are only identified in other primate phagocytes but not in human (Cole et al., 2004). Although theta-defensin genes are present in human genomes, a premature termination codon blocks translation thus they are not expressed as peptides (Venkataraman et al., 2009). Besides defensins, other AMPs such as cathelicidins LL-37 are found in epithelial cells of testis, respiratory tract, gastrointestinal, in skin and leukocytes (Durr et al., 2002).

In order to investigate the genes which play a role in resistance to human AMPs resistance in *E. faecium*, lysozyme and nisin were used in this study since they are cheap alternatives to human antimicrobial peptides.

3.2 Lysozyme and nisin

Generation of antimicrobial substances including antimicrobial peptides (such as defensins and cathelicidins LL-37) and large antimicrobial proteins (such as lysozyme and lactoferrin) are some mechanisms of innate defense (Yang et al., 2002).

Bacteriocins are AMPs that are ribosomally produced by bacteria to inhibit the growth of strains or species closely related to the producer strains (Cotter et al., 2005). Bacteriocins are cationic, membrane permeabilizing and amphiphilic or hydrophobic (Nissen-Meyer et al., 2009). Nisin is an antimicrobial peptide which is produced by *Lactococcus lactis* and belongs to the Class I bacteriocins called lantibiotics. Nisin is a cationic, hydrophobic peptide of 34-amino acid with an approximate mass of 3.4 kDa. Nisin is widely used as a food preservative (Cheigh and Pyun, 2005). Nisin is a pore-forming lantibiotics that targets lipid II, a membrane-bound cell wall precursor, as a docking molecule. The N-terminus of nisin binds to the pyrophosphate of lipid II and permeabilises the plasma membrane, resulting in efflux of intracellular molecules and eventually bacterial cell death (Breukink and de Kruijff, 2006).

Lysozyme is an antimicrobial protein that was used in this study. Lysozyme has enzymatic activity, degrading peptidoglycan but also has antimicrobial activity with a similar mechanism to AMPs (Benkerrroum, 2008). Lysozyme is lipophilic, cationic and its hydrophobic nature can interfere with the plasma membrane. Characteristics of lysozyme are similar to other cationic antimicrobial peptides (Jenssen et al., 2006).

3.3 Transposon mutagenesis

Transposon mutagenesis is a valuable genetic tool for identifying genetic determinants which enable a bacterium to survive in a broad range of environments.

Transposons can be used to generate libraries of TA-specific insertion mutants. These libraries can then be screened to identify mutants which have a defective phenotype in diverse aspects of metabolism, physiology, motility and biofilm formation (Picardeau et al., 2010).

The *Himar1* mariner transposon, which was the transposon of choice in this study, was originally isolated from the horn fly, *Haematobia irritans*. This transposon is a good choice for generating libraries of random insertion mutants since the mariner family of transposable elements are widespread in nature, ranging from bacteria to humans (Plasterk et al., 1999). Therefore, mariner is an efficient transposon for the random chromosomal integration of genes and insertional mutagenesis (Hoffmann et al., 2012). For efficient transposition, the *Himar1* mariner only requires its own transposase for transposition (Lampe et al., 1996). The *Himar1* mariner recognises the dinucleotide TA sequence for insertion (Lampe et al., 1996), which makes it an ideal choice for transposition to generate random libraries in low G+C content bacteria like *Enterococcus faecium* E1162 (~38 % of guanine-cytosine content) .

Some other transposons such as Tn*917* are less random and integrate at several hot spot regions making them less ideal for the construction of random libraries (Garsin et al., 2004). Unlike Tn*917*, the transposition and the transposon orientation of *Himar1* mariner is more random (Cao et al., 2007). The efficiency of transposition of *Himar1* mariner-based transposons is more than 20-fold higher and yields about 10-fold-more mutants compared to Tn*917*-based transposon delivery systems (Cao et al., 2007).

A mariner based transposon mutagenesis system was used in the same genus, *Enterococcus faecalis* to identify genes that contributed to biofilm formation (Kristich et al., 2008). Although some of the genes identified in that study such as the *ebp* locus, encoding a

protein termed the endocarditis and biofilm pilus subunit had previously been identified involved in other studies, this technique also identified genes that were not previously known to have a role in biofilm formation (Kristich et al., 2008).

Generally, *in vivo* transposition utilizes a plasmid that contains a mariner-based transposon and a resistance gene (for positive selection) that is flanked by *Himar1* inverted repeats. These plasmids also generally code for a transposase under the control of a strong promoter, which allows high expression of the transposase (Picardeau et al., 2010). The work presented in this thesis used a modified version of a mariner-based transposon delivery vector constructed for *Listeria monocytogenes* (Cao et al., 2007). In that study, the efficiency of transposition with two mariner-based transposon delivery vectors (pMC38 and pMC39) was evaluated. In plasmid pMC38, the gene for the *Himar1* transposase was under the control of the promoter of the *mgrA* gene (P_{mgrA}) encoding a homolog of DNA-binding protein from starved cells (Dps) (Almiron et al., 1992), while in plasmid pMC39, the transposase gene was under the control of the promoter of *kat*(A) gene (P_{katA}) encoding catalase (Bol and Yasbin, 1994). The authors found that 16 % of the isolated mutants had two-to-three transposon insertions (Cao et al., 2007).

To overcome this problem, plasmid pJAWTRASH2 was used as a transposon delivery vector for *in vivo* transposition in *E. faecium* E1162 (Figure 3.1). The plasmid pJAWTRASH2 was modified from plasmid pMC38 (Cao et al., 2007). In pJAWTRASH2, Wright et al. (unpublished data) replaced the P_{mrgA} 'constitutive' promoter that controls expression of the *Himar1* transposase gene in pMC38 with an inducible $P_{xyl/tetO}$ promoter. The $P_{xyl/tetO}$ promoter can be induced by tetracycline or derivatives, including anhydrotetracycline. Therefore, using this promoter system it is possible to control the expression of *Himar1* transposase to a desired level which would mean there was less likelihood of individual

mutants having multiple insertions of the transposon. In this vector, a T7 promoter was inserted between the inverted terminal repeats located upstream of the *erm*(C) gene to enable the generation of *in vitro* transcribed RNA 'runoffs' using T7 RNA polymerase for use in microarray-based transposon insertion site identification methods such as Transposon-Mediated Differential Hybridisation (TMDH) (Chaudhuri et al., 2009). This feature was not utilised in this study. The vector (Figure 3.1) comprises the *Escherichia coli* p15A low-copy-number replication origin, the RP4 ori for conjugative transfer, the pE194ts gram-positive temperature-sensitive replication origin, a gram-negative chloramphenicol resistance gene (*cat*), a gram-positive constitutive erythromycin resistance gene (*erm*(C)) flanked by the 29-bp ITR of the Himar*1 mariner*, a gram-positive kanamycin resistance gene (*kan*) (as a screening marker for loss of the plasmid, and the *Himar1* mariner transposase gene (*tpase*).

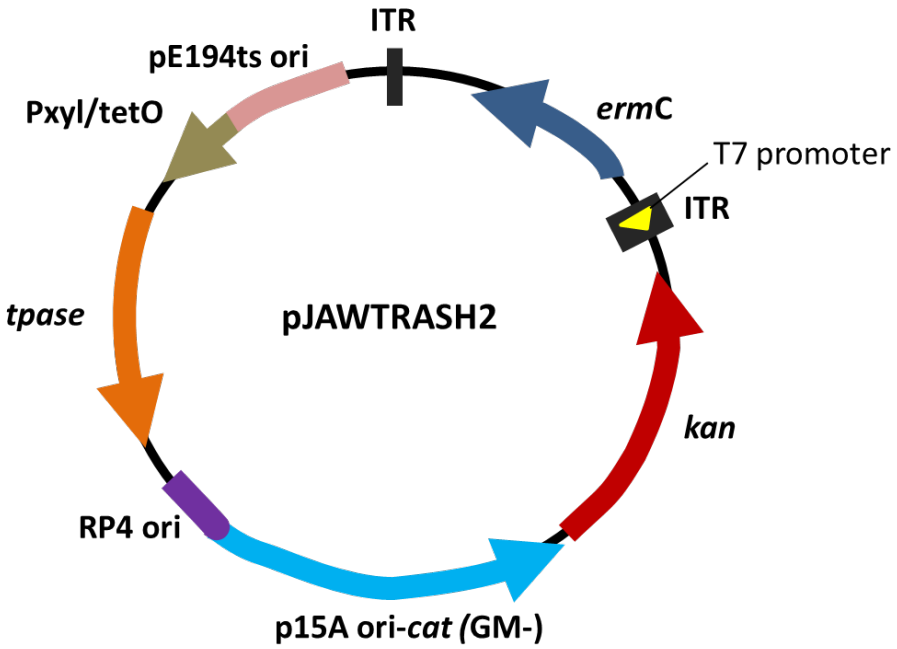


Figure 3.1 Map of the mariner-based transposon delivery vector pJAWTRASH2, modified from Cao et al. (2007).

The work described in this chapter utilised a library of *E. faecium* mariner transposon mutants which were screened for vitality in the presence of lysozyme (50 μg/mL) and increased sensitivity to nisin (256 μg/mL). Methods of screening the *E. faecium* mariner transposon mutants are mentioned in section 3.4.2 and 3.4.3.1. It was anticipated that the data obtained from this study would improve our understanding of AMPs resistance in *E. faecium*. Thus, the genes identified in this work may be useful as targets for the development of new antimicrobials against *E. faecium* infections.

3.4 Results

3.4.1 Identification of the disrupted genes in mutants that had altered sensitivity to lysozyme and nisin

In order to identify the disrupted genes in *E. faecium* mariner transposon mutants which had altered sensitivity to lysozyme and nisin compared to *E. faecium* E1162, a PCR based method for the specific amplification of transposon-flanking sequences was used (Kwon and Ricke, 2000) (Figure 3.2).

Y-linker PCR (Kwon and Ricke, 2000) was used to determine the gene that was disrupted by the transposon. The genomic DNA isolated from transposon mutants was digested with a 4 bp-recognition site restriction enzyme, Ase or TaqI. The digested DNA was ligated with a Y-linker. In the Y-linker PCR reaction, the transposon specific primer anneals to the DNA fragments containing transposon sequences. Both of the primers (Transposon specific primer and Y-linker primer) can selectively amplify DNA fragments containing transposon-specific sequences. See Table 2.4 for primers. Gel electrophoresis was done to view the amplification product. The gel image of this amplification is shown in Figure 3.3.

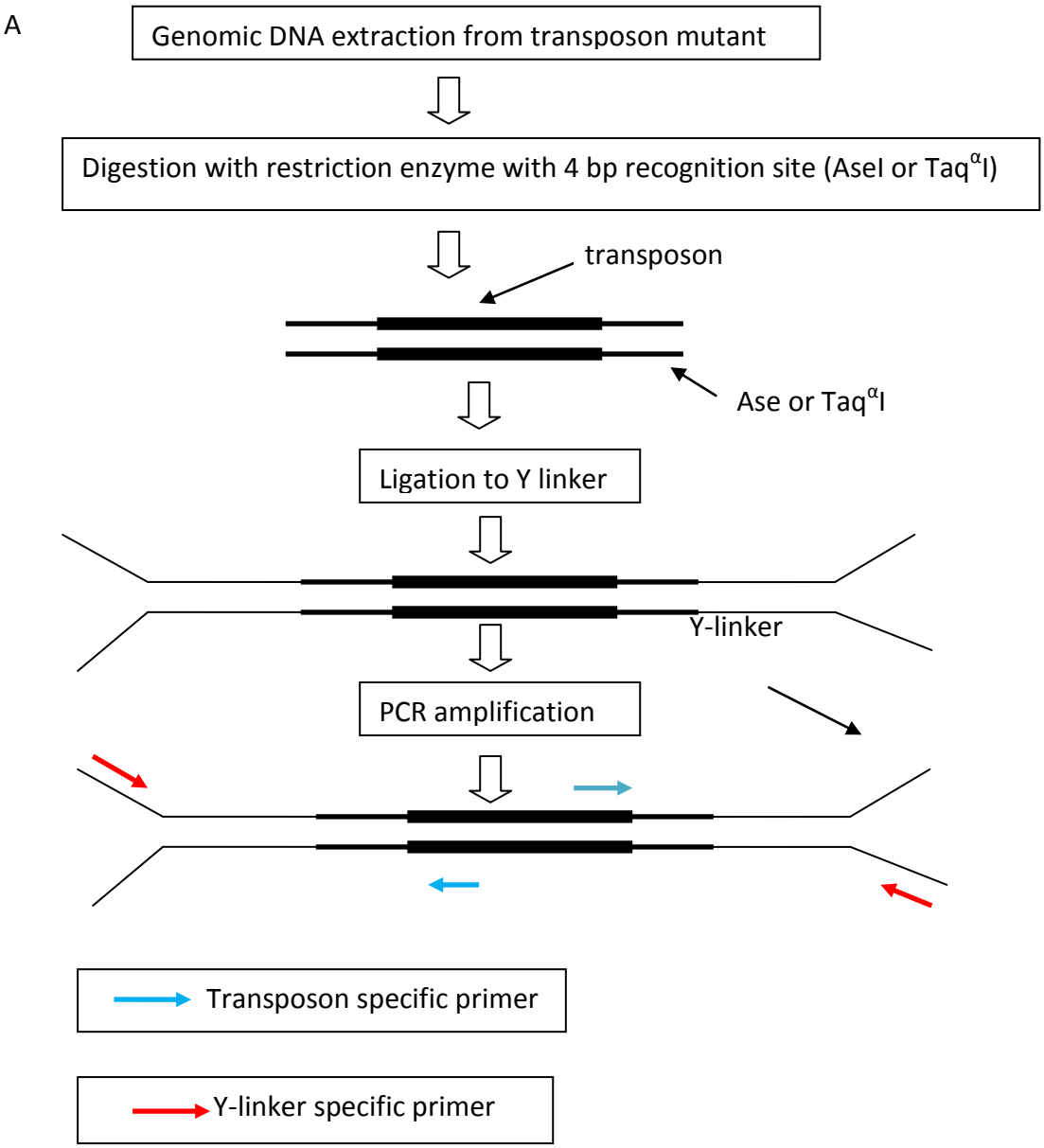


Figure 3.2 Schematic diagram of amplification of the Y linker and transposon flanking sequences. Blue arrows: Transposon specific primer and Red arrows: Y-linker specific primer

The amplification product was excised from agarose gel, purified and the nucleotide sequence was determined. The sequencing result obtained was then compared to sequences in GenBank using the Nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTN), which performed sequence-similarity searches using a nucleotide query. More

specific searches were performed against the data from the whole-genome shotgun-contigs (wgs) of *Enterococcus faecium* strain E1162 (ABQJ00000000).

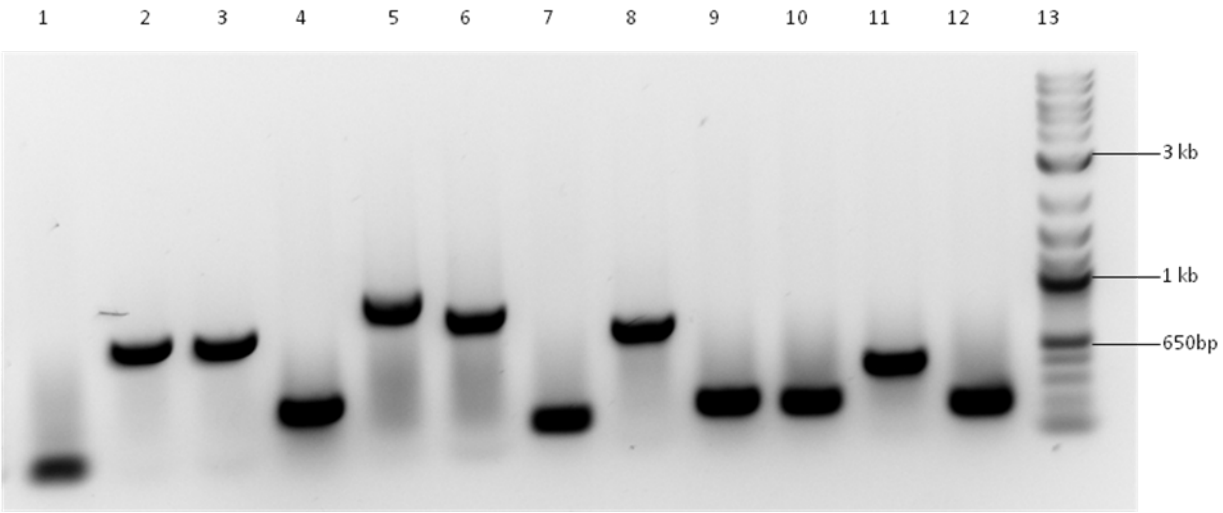


Figure 3.3 Image of the electrophoresis gel of Y-linker PCR products from mutants which had a defective phenotype different to the wildtype. Lane 1: Y-linker PCR products from *E. faecium* E1162; Lane 2-12: Y-linker PCR products from mutants which had a defective phenotype different to the wildtype using Y-linker PCR and Lane 13: 1 kb DNA ladder (Invitrogen).

3.4.2 Identification of *E. faecium* genes which are involved in lysozyme resistance

We hypothesized that using a transposon mutant library we might be able to easily identify genes which play a role in mediating lysozyme resistance of *E. faecium* by selecting for transposon mutants with increased resistance. In this study a transposon mutant library was generated by using a modified mariner transposon vector pJAWTRASH2 (the transposon library was obtained from Sean Nair). The transposon mutant library (100 µL) containing 7 x 10^5 cells/mL bacteria (contains 2.9 x 10^5 cells/mL of transposon mutants) was incubated overnight in 10 mL TSB containing a final concentration of 50 µg/mL lysozyme. The overnight culture was spread on TSA. Colonies which survived and grew on the TSA

were selected. Only one colony was recovered and named AGSA1. The gene into which the transposon had inserted was identified using a polymerase chain reaction-based method for specific amplification of transposon-flanking sequences as described in section (Figure 3.1).

The Y linker primer PCR product had a size of 700 bp. The transposon was found to be inserted at 200 bp within a gene coding for a putative ORF EFF34598 (EfmE1162_1531) encoding a protein of 93 amino acids. EFF34598 (EfmE1162_1531) is annotated as conserved hypothetical protein of unknown function. An ORF EFF34599 (EfmE1162_1532) encoding RepB and ORF EFF34600 (EfmE1162_1533) encoding RepA are located upstream of this gene. Three conserved hypothetical proteins (ORF EFF34597, ORF EFF34596 and ORF EFF34595) are located downstream of this gene (Figure 3.4).

Figure 3.4 Genetic organization of the region upstream and downstream of EfmE1162_1531 which annotated as conserved hypothetical protein of unknown function. EfmE1162_1528 = Conserved hypothetical protein; EfmE1162_1529 = Conserved hypothetical protein; EfmE1162_1530 = Conserved hypothetical protein; EfmE1162_1531 = Conserved hypothetical protein; EfmE1162_1532 = RepB and EfmE1162_1533 = Replication protein A

3.4.2.1 Growth of *E. faecium* E1162 and AGSA1 in the presence of lysozyme

In order to further characterize the lysozyme resistance of the *E. faecium* mutant AGSA1, the effect of lysozyme on growth was examined in more detail. In this bacterial cell lysis assay, a final concentration of 50 µg/mL lysozyme was added to bacteria in the exponential growth phase, and its effect on cell density was monitored spectrophotometrically. The disruption of the conserved hypothetical protein gene by the transposon in AGSA1 confers resistance to lysozyme. The optical density of mutant AGSA1 culture in the presence of lysozyme declined slowly after 24 hours incubation (1.14 %) and

declined further (19.2 %) after 48 hours incubation. On the other hand the optical density of the wild type in the presence of lysozyme decreased more rapidly (26.1 % in 24 hours) and lysed further (69.6 %) after 48 hours incubation (Figure 3.5).

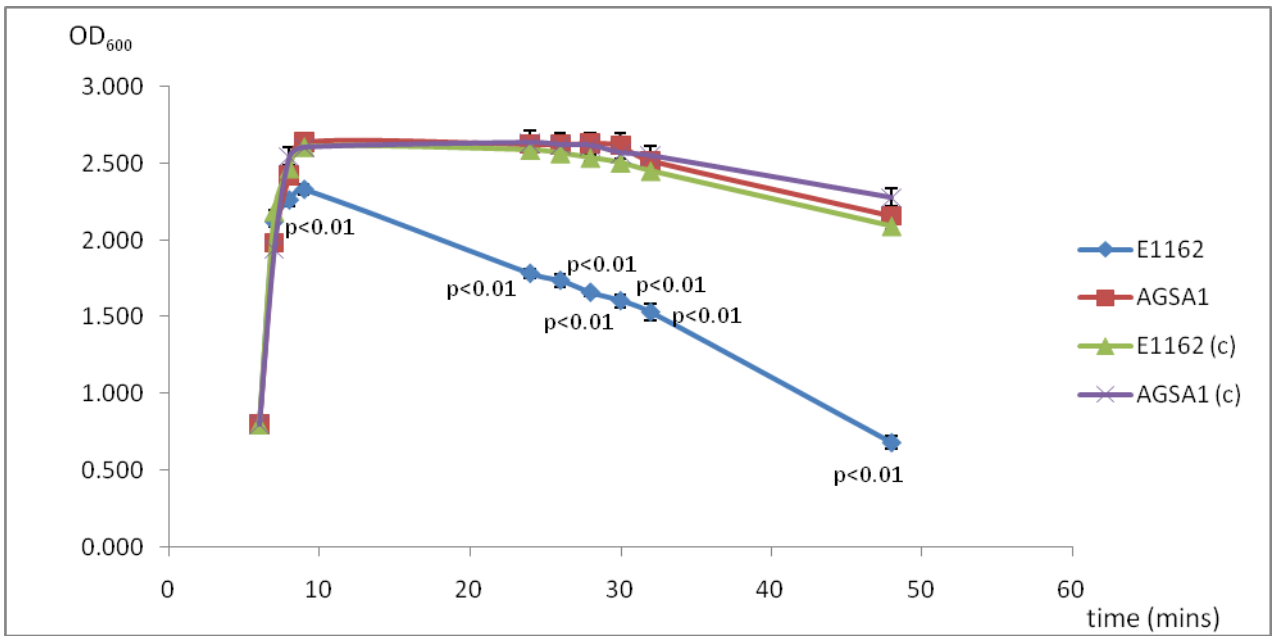


Figure 3.5 The cell lysis of the *E. faecium* E1162 and mutant AGSA1 at a final concentration of 50 µg/mL lysozyme in tryptic soy broth (TSB). Purple: AGSA1 in TSB; Red: AGSA1 in TSB +50 µg/mL lysozyme; Green: E1162 in TSB; Blue: E1162 in TSB +50 µg/mL lysozyme; (c): strain grown in TSB (as a control). Data presented are the mean of three independent experiments with error bars showing the standard deviations. The significance of any differences between E1162 and mutant AGSA1 in TSB+50 µg/mL lysozyme were analysed using Mann-Whitney U test.

3.4.3 Identification of *E. faecium* genes which are involved in nisin resistance

3.4.3.1 Nisin sensitivity of *E. faecium* E1162

In order to determine the concentration of nisin to be used in screening the transposon library (the transposon library was obtained from Sean Nair), *E. faecium* E1162 was grown on TSA supplemented with different nisin concentrations. After 24 hours incubation, it was observed that *E. faecium* E1162 was sensitive to 512 µg/mL nisin. The

transposon library was screened on Tryptic Soy Agar (TSA) supplemented with 256 µg/mL nisin.

3.4.3.2 Identification of mutants with increased sensitivity to nisin

To identify genes which play a role in nisin resistance, a serial dilution of the transposon mutant library of *E. faecium* E1162 were spread on TSA plates and incubated at 37°C so as to achieve a growth of approximately 50 colonies per plate. The next day colonies were replica plated on TSA plates containing 256 µg/mL nisin. Those colonies which grew on TSA plates but not the TSA containing nisin were putative nisin sensitive mutants.

Out of 7562 colonies, twelve nisin sensitive mutants were identified (Table 3.1). The gene into which the transposon had inserted was identified using a polymerase chain reaction- based method for specific amplification of transposon-flanking sequences (Kwon and Ricke, 2000). Although eight isolates were determined to have transposon insertions in a gene predicted to code for a tyrosine decarboxylase, only five decarboxylase mutants were selected and tested for nisin sensitivity.

Isolate 83N was determined to have a transposon inserted at 416 bp, within an ORF EFF33504 (EfmE1162_1535) encoding a predicted protein of 815 amino acids, annotated in the *E. faecium* E1162 genome sequence as a conjugative transposon protein (Figure 3.6).

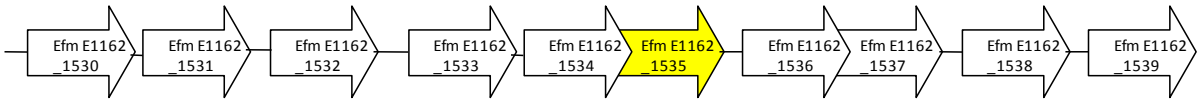


Figure 3.6 Genetic organization of the region upstream and downstream of EfmE1162_1535 which annotated as a conjugative transposon protein. EfmE1162_1530 = conserved hypothetical protein; EfmE1162_1531 = conserved hypothetical protein; EfmE1162_1532 = RepB; EfmE1162_1533 = replication Protein A; EfmE1162_1534 = conjugative transposon protein; EfmE1162_1535 = conjugative transposon protein; EfmE1162_1536 = conjugative transposon membrane protein; EfmE1162_1537= lipoprotein; Nlp/P60 family; EfmE1162_1538= conjugative transposon protein and EfmE1162_1539= transposase

Isolate 95N was determined to have a transposon at 53 bp, within an ORF EFF34739 (EfmE1162_1389) encoding a predicted protein of 416 amino acids, annotated in the *E. faecium* E1162 genome sequence as transposase (Figure 3.7).

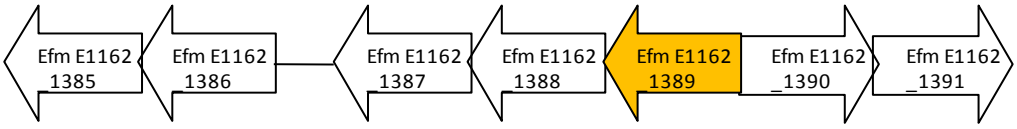


Figure 3.7 Genetic organization of the region upstream and downstream of EfmE1162_1389 which annotated as transposase. EfmE1162_1385 = hypothetical protein; EfmE1162_1386 = heat shock protein; EfmE1162_1387 = hypothetical protein; EfmE1162_1388 = hypothetical protein; EfmE1162_1389 = Transposase; EfmE1162_1390 = ascorbate-specific PTS system enzyme IIC and EfmE1162_1391 = hypothetical protein

Isolate 183N was determined to have a transposon at 348 bp, within an ORF EFF35601 (EfmE1162_0514) encoding a protein of 214 amino acids, annotated in the *E. faecium* E1162 genome sequence as Cystathionine β-synthase (CBS) domain protein (Figure 3.8).

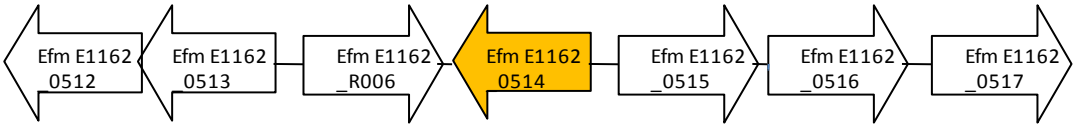


Figure 3.8 Genetic organization of the region upstream and downstream of EfmE1162_0514 which is annotated as a CBS domain protein. EfmE1162_0512 = DegV family protein; EfmE1162_0513 = hypothetical protein; EfmE1162_R006 = tRNA-Lys; EfmE1162_0514 = CBS domain protein; EfmE1162_0515 = ribosomal protein L33; EfmE1162_0516 = preprotein translocase, SecE subunit and EfmE1162_0517 = transcription termination/antitermination factor NusG

Isolate 59N was determined to have a transposon at 395 bp, within an ORF EFF33496 (EfmE1162_1543) encoding a protein of 483 amino acids, annotated in the *E. faecium* E1162 genome sequence as muramidase (Figure 3.9).

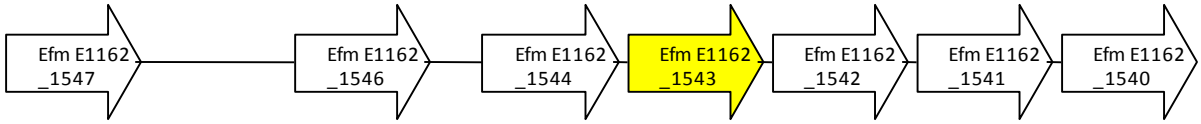


Figure 3.9 Genetic organization of the region upstream and downstream of EfmE1162_1543 which is annotated as muramidase. EfmE1162_1547 = hypothetical protein; EfmE1162_1546 = conserved hypothetical protein; EfmE1162_1544 = NADH oxidase nox; EfmE1162_1543 = Putative muramidase; EfmE1162_1542 = Conserved hypothetical protein; EfmE1162_1541 = Drug resistance transporter, Emrb/Qaca family protein; EfmE1162_1540 = Conserved hypothetical protein and EfmE1162_1539 = Transposase

Isolate 64N was determined to have a transposon at 246 bp, within an ORF EFF35914 (EfmE1162_0147) encoding a protein of 157 amino acids, annotated in the *E. faecium* E1162 genome sequence as conserved hypothetical protein of unknown function (Figure 3.10).

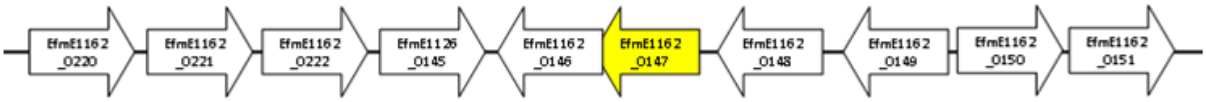


Figure 3.10 Genetic organization of the region upstream and downstream of EfmE1162_0147 which is annotated as conserved hypothetical protein of unknown function. EfmE1162_0220 = tyrosine-protein kinase YwqD; EfmE1162_0221 = putative tyrosine-protein phosphatase CapC; EfmE1162_0222 = hypothetical protein; EfmE1162_0145 = exonuclease; EfmE1162_0146 = acetyltransferase, gnat family; EfmE1162_0147 = conserved hypothetical protein; EfmE1162_0148 = phosphate acetyltransferase; EfmE1162_0149 = uracil-DNA glycosylase; EfmE1162_0150 = hydrolase, HAD superfamily, Cof family and EfmE1162_0151 = conserved hypothetical protein

Five of the identified mutants, 99N, 139N, 47N, 53N and 63N had a transposon at 630 bp, within an ORF EFF34651 encoding a protein of 625 amino acids, annotated in the *E. faecium* E1162 genome sequence as tyrosine decarboxylase.

All *E. faecium* E1162 mutants tested were two-fold more sensitive to nisin compared to the wildtype (Table 3.1). The MIC was done in BHI broth. Three independent experiments of MICs were done on separate days, each using three individual colonies as replicates.

Table 3.1 Comparison of the nisin sensitivity of the *E. faecium* E1162 and mutants

Isolates	Genes into which there was a transposon insertion	GenBank accession number	Nisin sensitivity (μg/mL)
E1162	(wildtype)	ABQJ00000000	128
99N	tyrosine decarboxylase	EFF34651	64
139N	tyrosine decarboxylase	EFF34651	64
47N	tyrosine decarboxylase	EFF34651	64
53N	tyrosine decarboxylase	EFF34651	64
63N	tyrosine decarboxylase	EFF34651	64
95N	transposase	EFF34739	64
83N	conjugative transposon protein	EFF33504	64
183N	Cystathionine beta synthase (CBS) domain protein	EFF35601	64
59N	putative muramidase	EFF33496	64
64N	conserved hypothetical protein	EFF35914	64

3.4.3.3 Growth of *E. faecium* E1162 and nisin-sensitive mutants in the presence of nisin

The *E. faecium* E1162 mutants that were sensitive to nisin were investigated further by growth in the presence of subinhibitory concentrations of nisin. Bacterial growth was monitored by measuring the optical density at 600 nm of *E. faecium* cultures growing in TSB. The *E. faecium* tyrosine decarboxylation mutant (Isolate 47N) was selected for comparison

to *E. faecium* E1162. Nisin sensitivity was assessed by adding nisin at final concentrations of a two-fold dilution series from 256 μg/mL to 1 μg/mL of nisin to see if there was a difference in the growth of the wildtype *E. faecium* E1162 and the tyrosine decarboxylation mutant in the presence of nisin. It was observed that the growth of both strains was similar in the presence of 1 μg/mL of nisin. However both strains were inhibited to some degree in the presence of 2 μg/mL of nisin. The tyrosine decarboxylase mutant (Isolate 47) showed a growth defect with a slower growth rate in early to mid-log phase (after 210 minutes) in the presence of 2 μg/mL of nisin compared to *E. faecium* E1162 (Table 3.1). Both of the strains were sensitive to the final concentration of 4 μg/mL nisin and above.

Figure 3.11 shows the effect of 2 μg/mL of nisin on the growth of *E. faecium* E1162 and *tdc* mutant in three independent experiments. Both strains were inhibited to some degree by this concentration of nisin having longer lag phase and exponential phases but eventually reaching the same optical density as bacteria not treated with nisin. The data also shows that in the presence of nisin the mutant exited lag phase slightly later than the *E. faecium* E1162 and produced lower optical densities in the early and mid-exponential phases.

The other *E. faecium* transposon mutants identified as having an increased sensitivity were also tested to see what effect 2 μg/mL of nisin had on their growth. Figure 3.12A shows the data obtained with *E. faecium* E1162 and strain 59N, which had a transposon inserted in a gene encoding for a putative muramidase (EFF33496). There was no significant difference between the growth of the *E. faecium* E1162 and strain 59N when grown in TSB without nisin. When cultured in TSB containing 2 μg/mL of nisin, strain 59N had a small but statistically significant reduced optical density compared to *E. faecium*

E1162 at 90 and 150 minutes. However the difference between the strains was not significant at the later time 270 minutes.

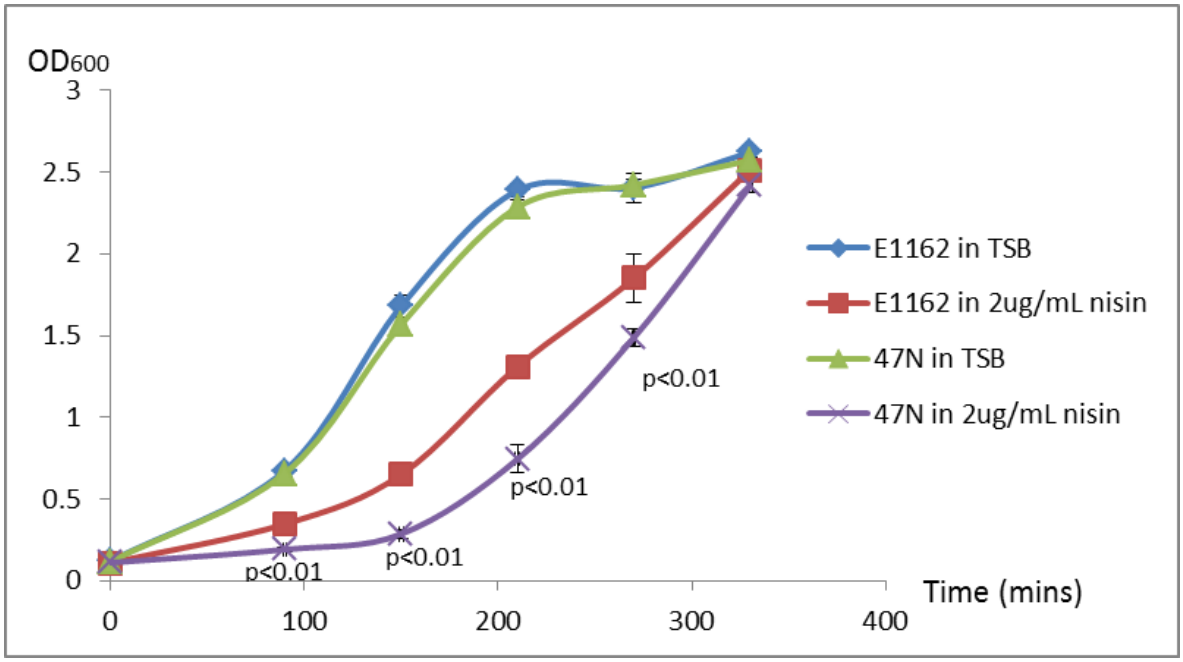


Figure 3.11 Growth of *E. faecium* E1162 and the tyrosine decarboxylation mutant 47N in the presence of 2 µg/mL nisin. Blue: E1162 in TSB; Red: E1162 in TSB+2 µg/mL nisin; Green: 47N in TSB and Purple: 47N in TSB+2 µg/mL nisin. Data presented are the mean of three independent experiments and error bars show standard deviations. Significance of the difference between E1162 and mutant 47N in TSB+2 µg/mL nisin was analysed using the Mann-Whitney U test.

Figure 3.12B shows the effect of 2 µg/mL of nisin on the growth of *E. faecium* E1162 and strain 95N, which had a transposon inserted in a gene encoding for a putative transposase (EFF34739). Similar to strain 59N, strain 95N had a small but statistically significant reduced optical density compared to *E. faecium* E1162 at 90 and 150 minutes. However, there was no difference in the growth at 210 minutes. At 270 minutes strain 95N had a small but statistically significantly reduced optical density compared to *E. faecium* E1162, however there was no difference at 330 minutes.

Figure 3.12C shows the effect of 2 µg/mL of nisin on the growth of *E. faecium* E1162 and strain 64N, which had a transposon inserted in a gene encoding for a putative

conserved hypothetical protein of unknown function (EFF35914). There was no significant difference between the growth of *E. faecium* E1162 and strain 183N when grown in TSB without nisin. When cultured in TSB containing 2 µg/mL of nisin, the mutant exited lag phase later than *E. faecium* E1162 and produced lower optical densities in the early and mid-exponential phases.

Figure 3.12D shows the effect of 2 µg/mL of nisin on the growth of *E. faecium* E1162 and strain 183N, which had a transposon inserted in a gene encoding for a putative CBS domain protein (EFF35601). The data shows that in the presence of nisin, strain 183N had a statistically significant reduced optical density compared to *E. faecium* E1162 at 90 minutes. However, the difference was less apparent at 150 minutes. The data also shows that in the presence of nisin the mutant exited lag phase very slightly later than the *E. faecium* E1162 and produced lower optical densities in the late-exponential phases, however there was no difference at later time point (330 minutes).

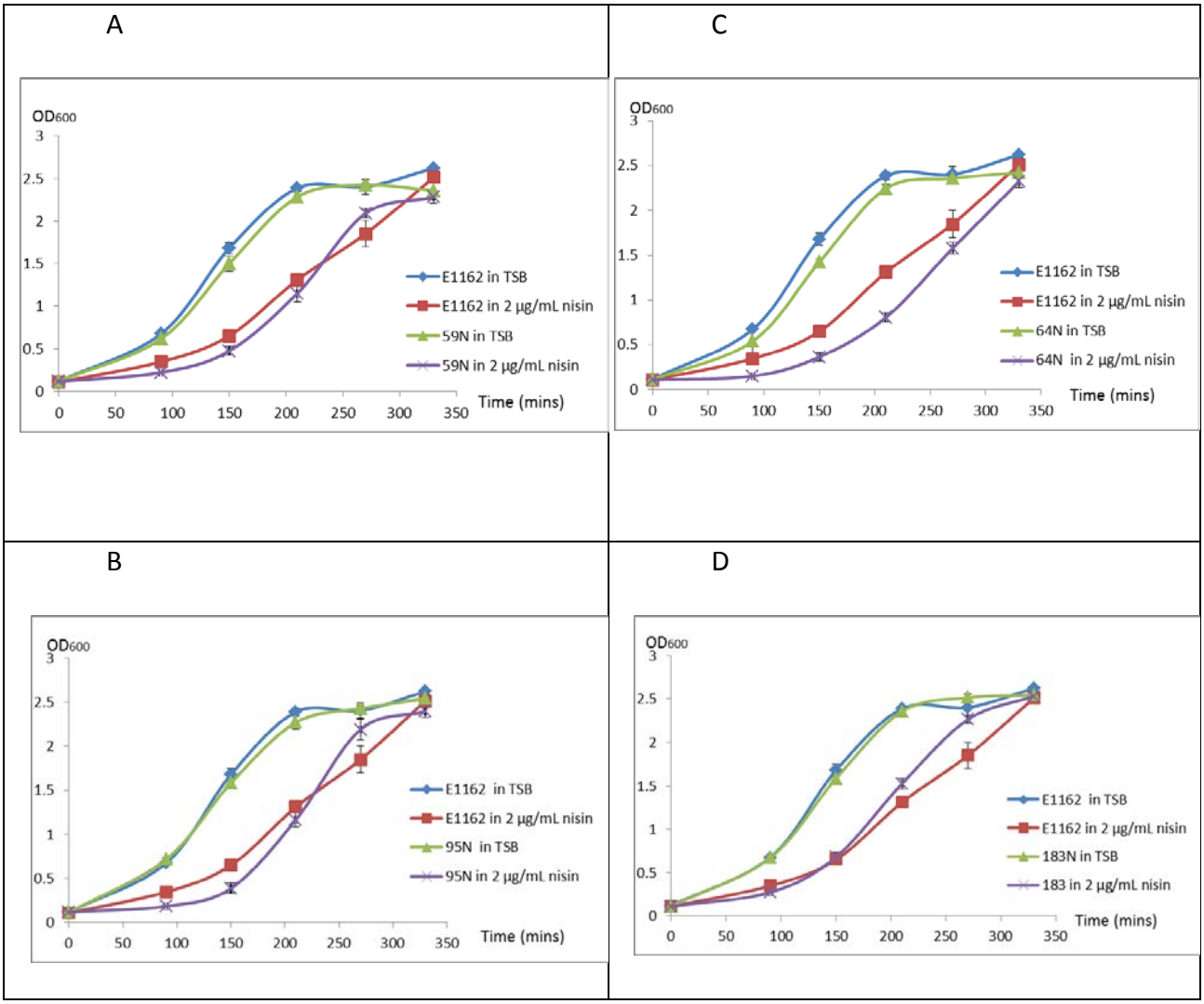


Figure 3.12 Growth of *E. faecium* E1162 and and nisin-sensitive mutants in the presence of 2 µg/mL nisin. Blue: E1162 in TSB; Red: E1162 in TSB+2 µg/mL nisin; Green: nisin-sensitive mutants in TSB and Purple: nisin-sensitive mutants in TSB+2 µg/mL nisin. Data presented are the mean of three independent experiments and error bars show standard deviations. Significance of the difference between E1162 and nisin-sensitive mutants in TSB+2 µg/mL nisin were analysed using the Mann-Whitney U test.

Figure 3.13 shows the effect of 2 µg/mL of nisin on the growth of *E. faecium* E1162 and strain 83N, which had a transposon inserted in a gene encoding for a putative conjugative transposon protein (EFF33504). There was no significant difference between the growth of *E. faecium* E1162 and strain 83N when grown in TSB without nisin. When cultured in TSB containing 2 µg/mL of nisin, the mutant exited lag phase later than *E. faecium* E1162 and produced lower optical densities in the early and mid-exponential phases.

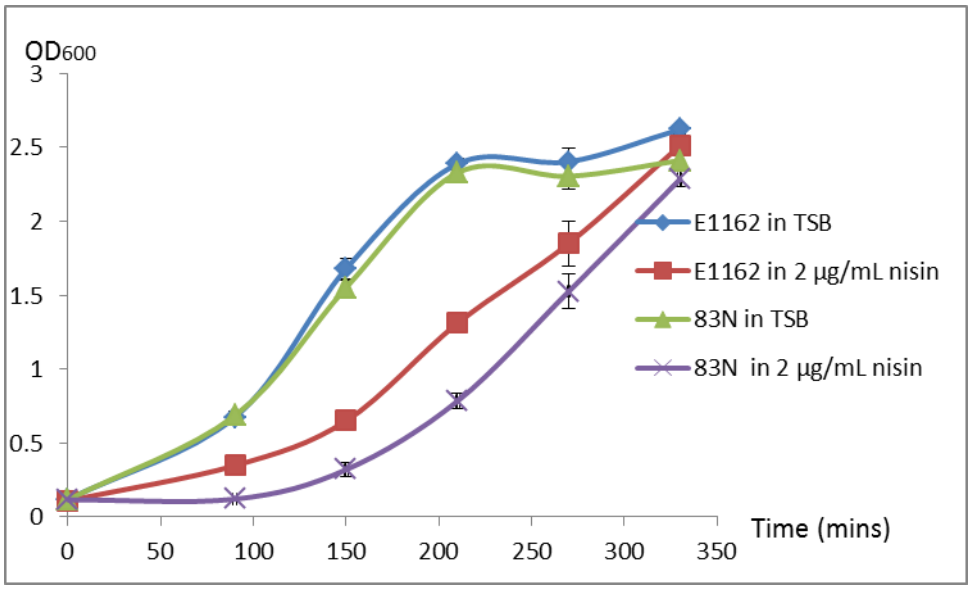


Figure 3.13 Growth of E. faecium E1162 and mutant 83N in the presence of 2 µg/mL nisin. Blue: E1162 in TSB; Red: E1162 in TSB+2 µg/mL nisin; Green: 83N in TSB and Purple: 83N in TSB+2 µg/mL nisin. Data presented are the mean of three independent experiments and error bars show standard deviations. Significance of the difference between E1162 and mutant 83N in TSB+2 µg/mL nisin was analysed using the Mann-Whitney U test.

3.4.3.4 *Galleria mellonella* virulence model

In order to determine whether the *E. faecium* mutants that were identified as being more sensitive to nisin also had reduced virulence, a *Galleria mellonella* wax worm model was used as a host for examining *E. faecium* infections (As mentioned in section 2.13). The *E. faecium* mutant with increased resistance to lysozyme was also examined in this model.

3.4.3.4.1 Comparison of the capacity of *E. faecium* E1162 and the lysozyme resistant mutant AGSA1 to kill wax worm

In order to evaluate the role of the putative conserved hypothetical protein EFF34598 in virulence, the *E. faecium* E1162 and the mutant AGSA1, which has a transposon insertion in a predicted gene coding for EFF34598 were examined in a *G. mellonella* model

(Figure 3.14). Both strains killed similar numbers of wax worms at 24 hours. After that time point the mutant killed lower numbers of wax worms than the *E. faecium* E1162. However, the difference in killing between the *E. faecium* E1162 and mutant AGSA1 was not statistically significant ($p>0.05$). This suggest that the gene coding for a conserved hypothetical protein disrupted in AGSA1 may not be involved in virulence or that the lysozyme of *G. mellonella* does not play a major role in protecting the wax worms from *E. faecium* infections.

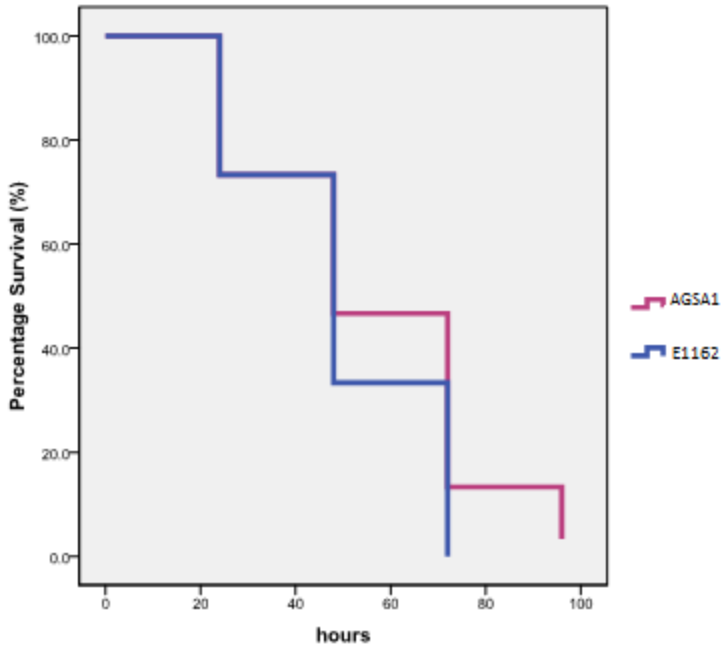


Figure 3.14 Kaplan-Meier survival curves of *Galleria mellonella* wax worms infected with *E. faecium* strains. Blue: *E. faecium* E1162 and Pink: *E. faecium* strain AGSA1 which has increased resistance to lysozyme. Data presented are the percentages of 30 wax worms which were tested in groups of 10 on three different occasions. Significance of the difference between E1162 and mutant AGSA1 were analyzed with the log rank test.

3.4.3.4.2 Comparison of the capacity of *E. faecium* E1162 and the nisin sensitive mutants to kill wax worms

The hypothesis behind screening for mutants that were more sensitive to the antimicrobial peptide nisin was that by doing so we might identify genes that are important for resistance to other classes of AMPs such as those of animals. If this were the case then

such mutants may also have reduced virulence in the wax worm model which produces antimicrobial peptides to protect against infection (Cook and McArthur, 2013).

Figure 3.15 shows that strain 59N which contained a transposon insertion in a gene coding for a putative muramidase (EFF33496), was significantly attenuated in its capacity to kill wax worms compared to the *E. faecium* E1162 ($p<0.01$). These results demonstrate that the gene coding for the putative muramidase contributes to *E. faecium* virulence in the *G. mellonella* model.

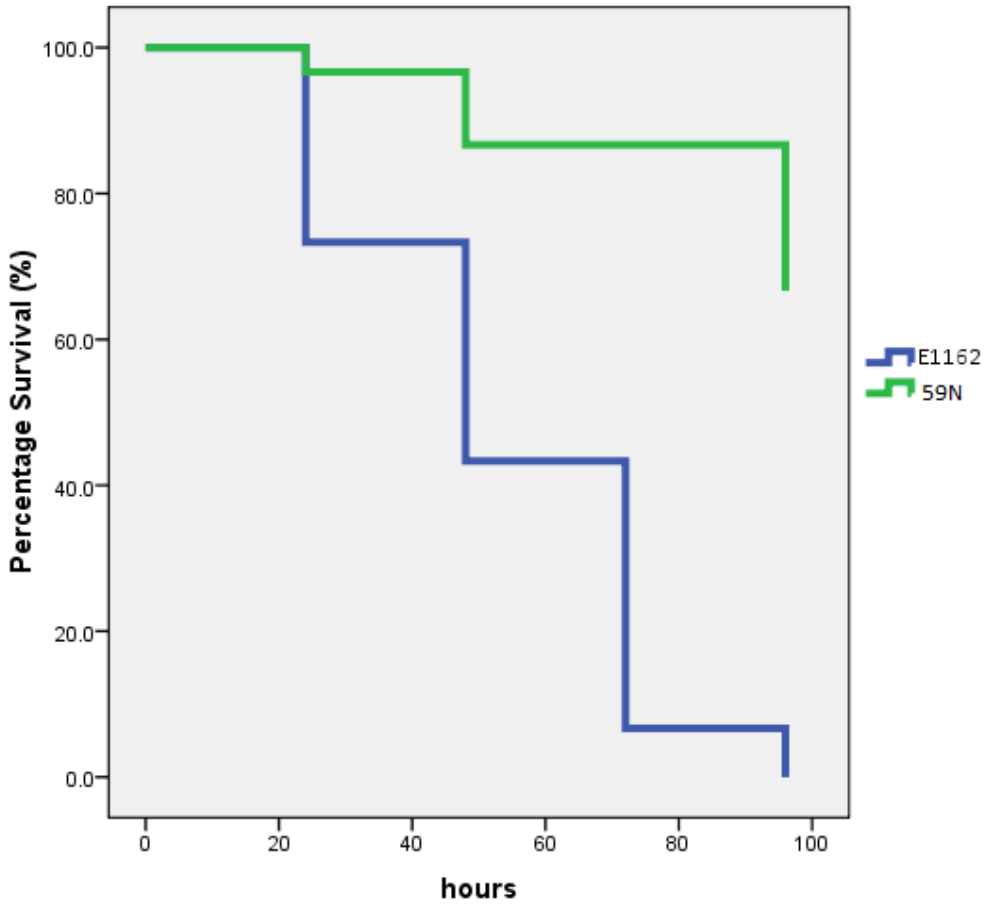


Figure 3.15 Kaplan-Meier survival curves of *Galleria mellonella* wax worms infected with *E. faecium* strains. Blue: *E. faecium* E1162 and Green: *E. faecium* strain 59N which has a transposon insertion in a gene coding for a putative muramidase. Data presented are the percentages of 30 wax worms which were tested in groups of 10 on three different occasions. Significance of the difference between E1162 and mutant 59N were analyzed with the log rank test.

Figure 3.16A shows that strain 64N which contained a transposon insertion in a gene coding for a conserved hypothetical protein (EFF35914), was significantly attenuated in its capacity to kill wax worms compared to the *E. faecium* E1162 ($p<0.01$). These results demonstrate that the gene coding for conserved hypothetical protein contributes to *E. faecium* virulence in the *G. mellonella* model.

Figure 3.16B shows that strain 95N which contained a transposon insertion in a gene coding for transposase (EFF34739), was almost similar to the wildtype *E. faecium* E1162 ($p>0.05$) in its capacity to kill wax worms. These results demonstrate that the gene coding for transposase does not contribute to *E. faecium* virulence in the *G. mellonella* model.

Figure 3.16C shows that strain 183N which contained a transposon insertion in a gene coding for a putative CBS domain protein (EFF35601), was significantly attenuated in its capacity to kill wax worms compared to the *E. faecium* E1162 ($p<0.05$). These results demonstrate that the gene coding for a putative CBS domain protein contributes to *E. faecium* virulence in the *G. mellonella* model.

Figure 3.16D shows that strain 47N which contained a transposon insertion in a gene coding for a putative tyrosine decarboxylase (EFF34651), was significantly attenuated in its capacity to kill wax worms compared to the *E. faecium* E1162 ($p<0.01$). These results demonstrate that the gene coding for a putative tyrosine decarboxylase contributes to *E. faecium* virulence in the *G. mellonella* model.

Figure 3.16E shows that strain 83N which contained a transposon insertion in a gene coding for a putative a putative conjugative transposon protein (EFF33504), was significantly attenuated in its capacity to kill wax worms compared to the *E. faecium* E1162 ($p<0.05$). These results demonstrate that the gene coding for a putative conjugative transposon protein contributes to *E. faecium* virulence in the *G. mellonella* model.

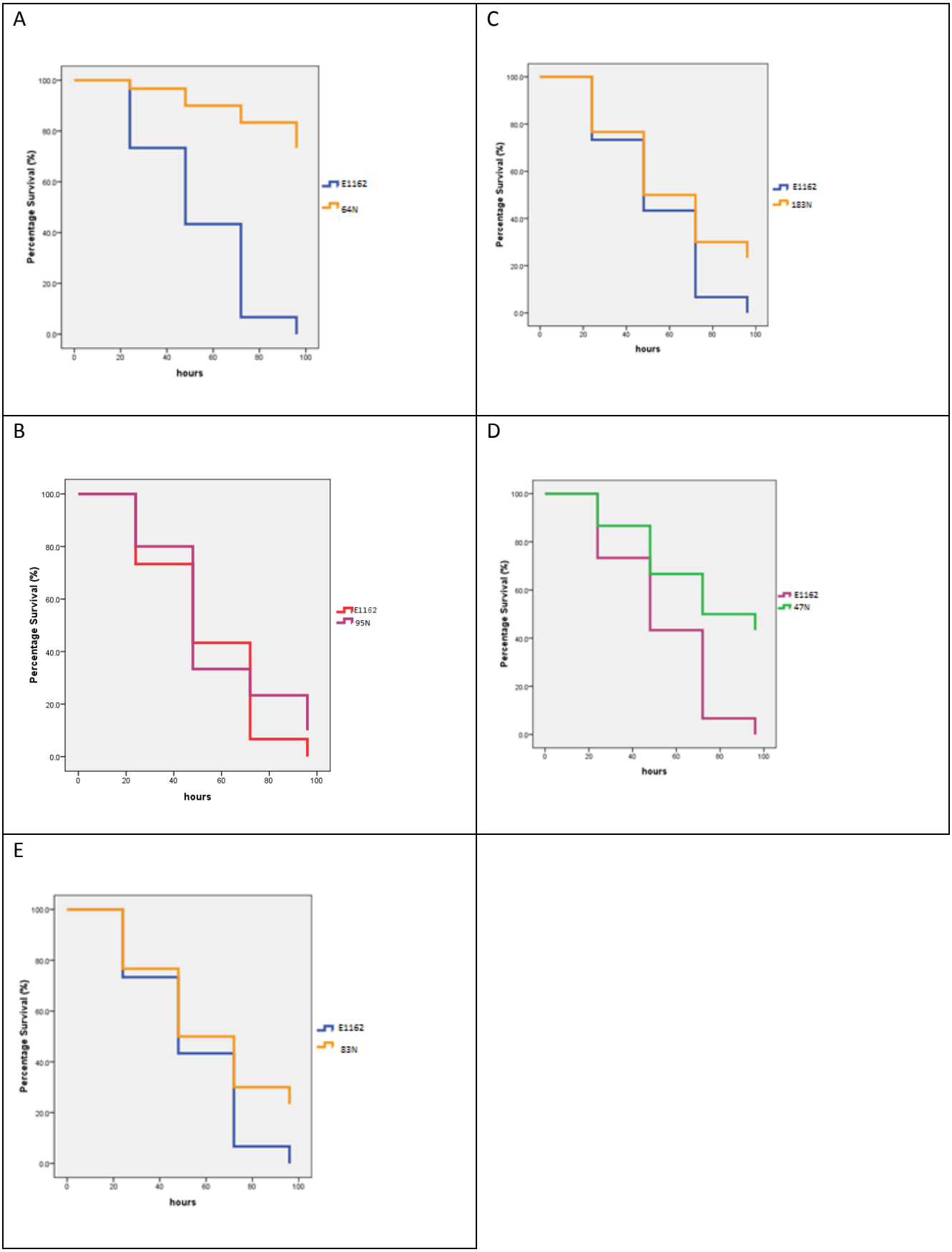


Figure 3.16 Kaplan-Meier survival curves of *Galleria mellonella* wax worms inoculated with *E. faecium* strains. A: Blue: *E. faecium* E1162; Orange: *E. faecium* strain 64N which has a transposon insertion in a gene coding for a putative a conserved hypothetical protein; B:

Red: *E. faecium* E1162; Purple: *E. faecium* strain 95N which has a transposon insertion in a gene coding for a putative transposase; C: Blue: *E. faecium* E1162; Orange: *E. faecium* strain 183N which has a transposon insertion in a gene coding for a putative CBS domain protein; D: Purple: *E. faecium* E1162; Green: *E. faecium* strain 47N which has a transposon insertion in a gene coding for a putative tyrosine decarboxylase and E: Blue: *E. faecium* strain 83N which has a transposon insertion in a gene coding for a putative conjugative protein. Data presented are the percentages of 30 worms which were tested in groups of 10 on three different occasions. Significance of the difference between E1162 and mutants were analyzed with the log rank test.

3.4.4 Further characterization of the tyrosine decarboxylase mutant of *E. faecium* E1162

Since 8 nisin sensitive transposon mutants of *E. faecium* had insertions in a gene coding for a tyrosine decarboxylase and because this gene was also involved in virulence in the *Galleria mellonella* infection model, one of these mutants (strain 47N) was characterised further.

3.4.4.1 Genetic organization of the tyrosine decarboxylase cluster in *E. faecium* E1162

Bioinformatic analysis of the genome sequence of *E. faecium* E1162 revealed that the gene coding for the putative tyrosine decarboxylase (EFF34651) is the second gene in a putative operon that consists of 4 genes (Figure 3.17) encoding a predicted tyrosyl-tRNA synthetase gene, *tyrS*, tyrosine permease, *tyrP* and Na+/H+ antiporter, *nhaC*.

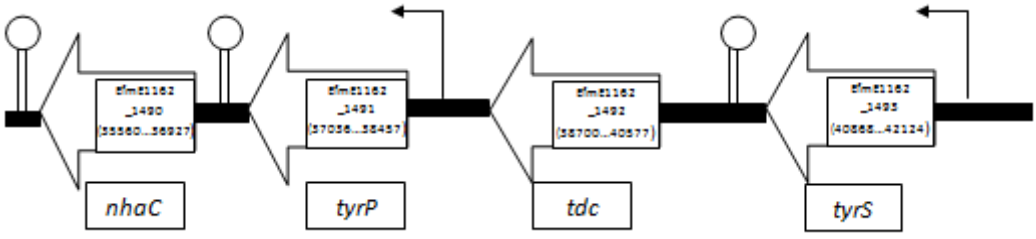


Figure 3.17 Genetic organization of the tyrosine decarboxylase gene cluster of *E. faecium* E1162.

Analysing the sequence data using BPROM and Prokaryote Promoter prediction software, it was predicted that a promoter is present upstream of the tyrosyl tRNA synthetase, *tyrS* and tyrosine permease, *tyrP* genes (indicated by the arrows in Figure 3.17). The presence of terminators were predicted at the end of tyrosyl tRNA synthetase, *tyrS*, the Na+/H+ antiporter, *nhaC* and the tyrosine permease, *tyrP* genes (indicated by the loop in Figure 3.17).

3.4.4.2 Investigation of the role of tyrosine decarboxylase in acid tolerance

We tested the ability of *E. faecium* E1162 and the tyrosine decarboxylase mutant strain 47 to convert tyrosine to tyramine (Figure 3.18). This was assayed using LB medium containing 5 mM tyrosine and bromocresol purple which detected the consumption of H^+ in the reaction and the subsequent pH change from below pH 5.2 (yellow) to neutral pH 7 (purple).

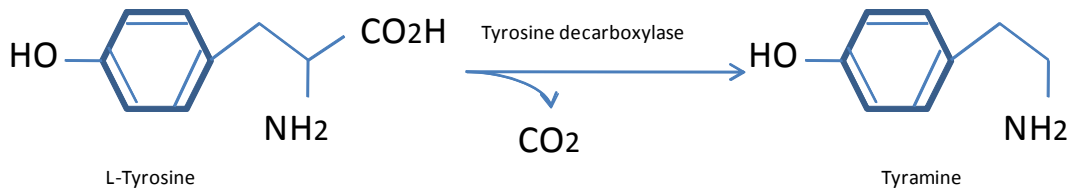


Figure 3.18 Conversion of L-tyrosine to tyramine by tyrosine decarboxylase

It was observed that when strain 47N, which is disrupted in the putative *tdc* gene, was grown in the presence of tyrosine, there was no change in the colour of the bromocresol purple indicating that strain 47 failed to use the substrate. On the other hand, the *E. faecium* E1162 strain produce a change in colour from yellow to purple in the presence of tyrosine, indicating that this strain could utilize the substrate.

Tyrosine decarboxylase has been shown to be involved in acid tolerance of *E. faecium* E17 (Pereira et al., 2009). We examined the role of tyrosine in the acid tolerance of *E. faecium* E1162 by measuring bacterial survival at low pH. Experiments were performed in 1 mM potassium phosphate buffer (KH_2PO_4) at pH 3.0 and pH 7.0 (control) with or without 5 mM tyrosine (Figure 3.19). The percentage survival of bacteria in each treatment condition was calculated by dividing the number of colony forming units per milliliter (CFU/mL) after 3 hours incubation (t=3) by the number of CFU/mL at time 0 hrs multiplied by 100. The survival of *E. faecium* E1162 cells was higher at pH 7.0 (98.9 %) than at pH 3.0 (43 %). The survival of *E. faecium* E1162 was significantly higher in the presence of tyrosine (77.9 %) at pH 3.0 ($p<0.01$). In the case of strain 47N, 56.7 % of cells survived in the presence of tyrosine while 46.2 % survived without tyrosine at pH 3.0. At neutral pH (pH 7.0), the difference between *E. faecium* E1162 and strain 47N was not significant.

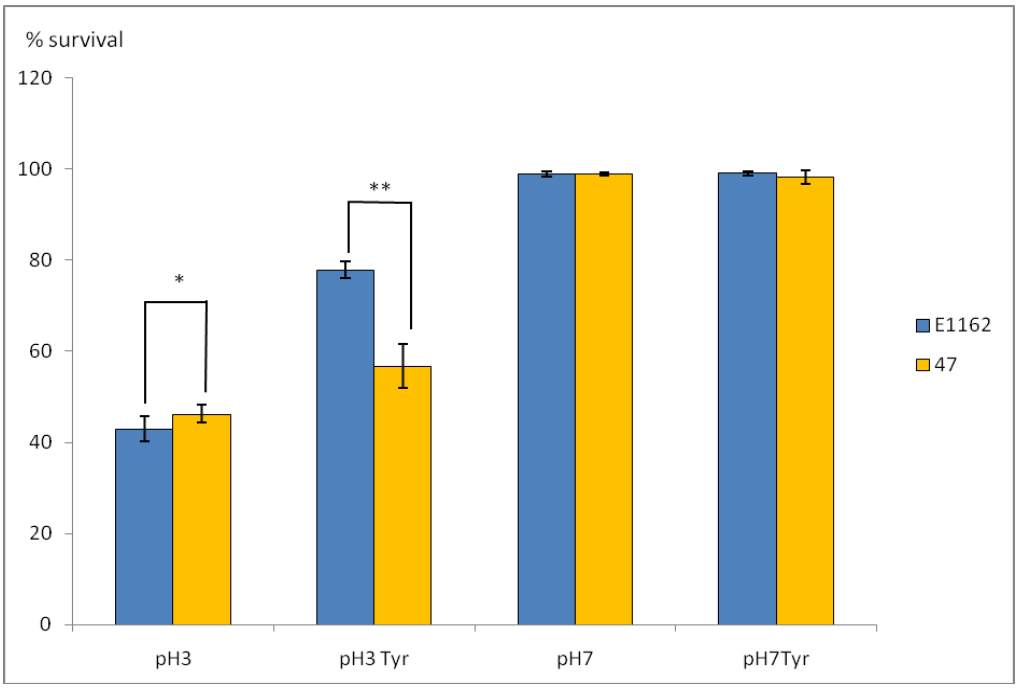


Figure 3.19 Percent survival after 3 hours incubation in 1 mM potassium phosphate buffer (KH_2PO_4). Blue: *E. faecium* E1162 and Orange: strain 47. Data presented are the mean of three independent experiments with error bars showing the standard deviations. Significance of the difference between E1162 and mutant 47N were analyzed using the Mann-Whitney U test, *: $p<0.05$, **$p<0.01$

3.4.4.3 Allelic exchange to repair the *tdc* mutant

It was possible that, all or some of, the defective phenotypes that we observed in strain 47N were not due to the insertion of the transposon into the *tdc* gene, but due to point mutations having arisen elsewhere or due to polar effects of the transposon insertion. One way to rule out this possibility would be to complement strain 47N with a copy of the *tdc* gene *in cis*. The *tdc* gene was repaired using a pheS counter-selection system (Panesso et al., 2011). Figure 3.20 shows the process of construction of strain GHFH12 which is a repaired 47N mutant using pheS counter-selection (as described in section 2.8). Primers tdcUF and tdcDR were used to amplify a 3976 bp region which consisted of the *tdc* gene and 741 bp upstream and 1354 bp downstream of the gene. This PCR product was subsequently ligated in pHOU1 and was verified by sequencing (see section 2.8 for methods). The construct was introduced in strain 47N to repair the *tdc* gene by homologous recombination as described in section 2.8. To confirm the integration of *tdc* into the chromosome, PCR was used to identify clones in which the *tdc* gene had been repaired and the length of PCR product was the same as in the wildtype *E. faecium* E1162 (Figure 3.21).

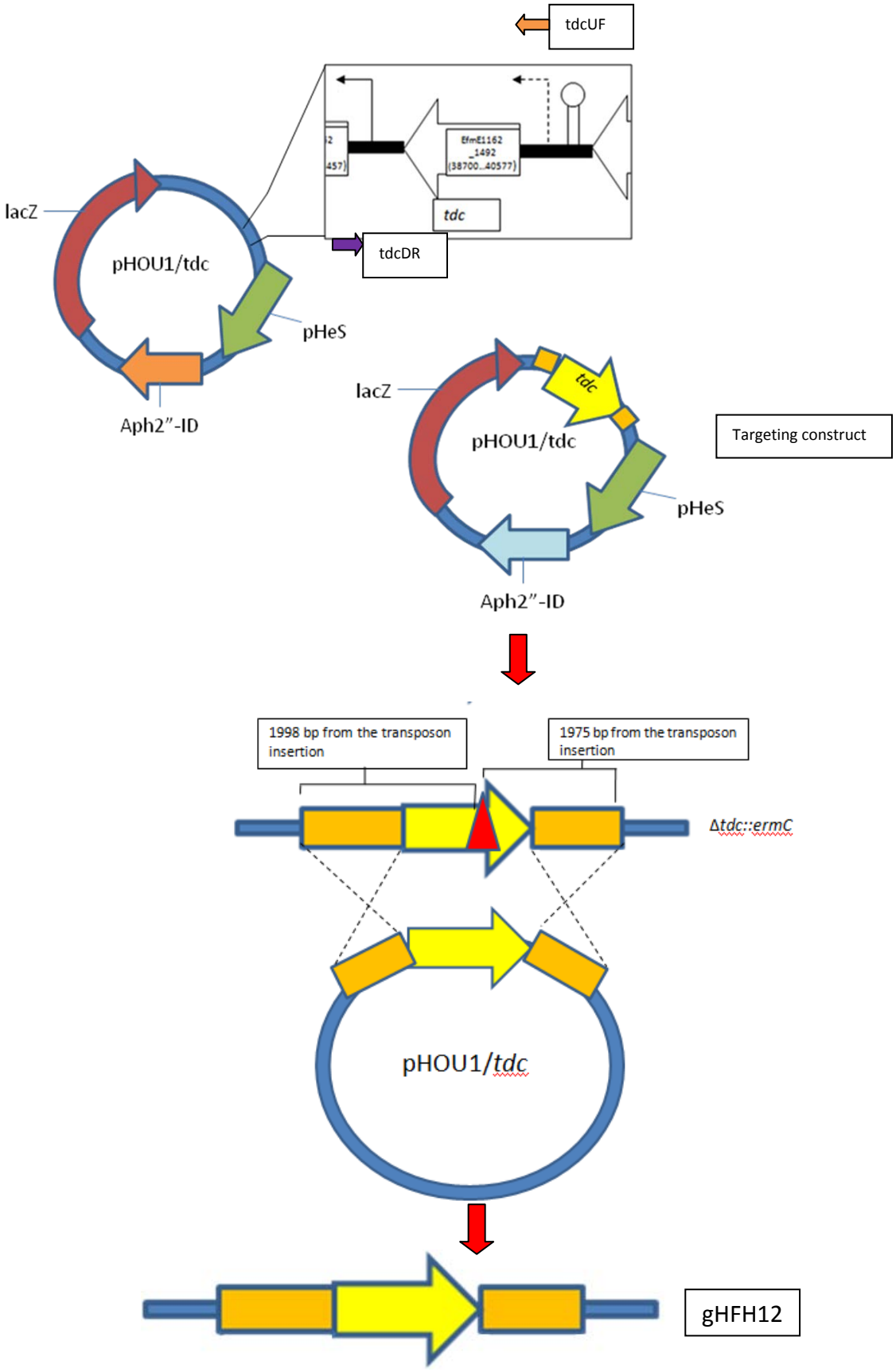


Figure 3.20 Construction of the repaired mutant 47N (GHFH12) was done using a pheS counter-selection system

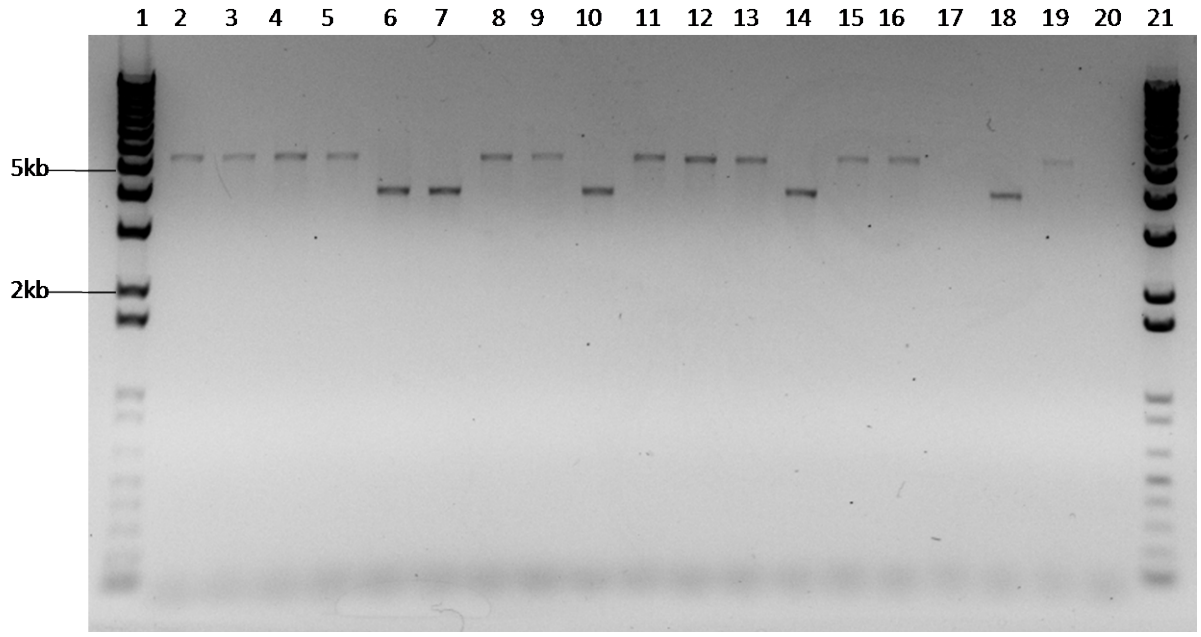


Figure 3.21 Image ofnelectrophoresis gel of PCR products for the *tdc* gene using primers, tdcUF and tdcDR. Lane 1 and 21: 1 kb plus DNA ladder (Invitrogen). Lanes 2-5, 8-9, 11-13, 15-6: PCR products from *E. faecium* strain 47N (Δ*tdc:*); Lanes 6-7, 10 and 14: PCR products from the clones with repaired *tdc* genes; Lane 18: *E. faecium* E1162; Lane 19: *E. faecium* strain 47N (Δ*tdc*); Lane 20: Negative control

3.4.4.3.1 Evaluation of the effect of the tyrosine decarboxylase gene repair in *E. faecium* strain 47N on acid tolerance

To confirm a role for the tyrosine decarboxylase gene of *E. faecium* in acid tolerance, the survival of one of the repaired strains GHFH12, was compared to that of the *tdc* mutant 47N, and the wild type strain E1162 upon exposure to 1 mM potassium phosphate buffer, pH 3.0 at 37°C for 3 hours in the presence or absence of tyrosine (Figure 3.22). At pH 3 in the absence of tyrosine, survival of the wild type, the *tdc* mutant strain 47N and the repaired strain GHFH12 were not significantly different. However, in the presence of tyrosine, survival of the wild type, the *tdc* mutant strain 47N and the repaired strain GHFH12 was 76.4 %, 48.3 % and 74.2 %, respectively. There was no significant difference between survival of the wild type strain E1162 and the *tdc* repaired strain GHFH12 at pH3.0 in the

presence of tyrosine. However both strains had higher survival rates under these conditions compared to the *tdc* mutant. These results demonstrate that the reduced acid tolerance phenotype of the *tdc* mutant strain 47N is due to the insertion of a transposon into the *tdc* gene and not other random mutations in the genome of *E. faecium* E1162.

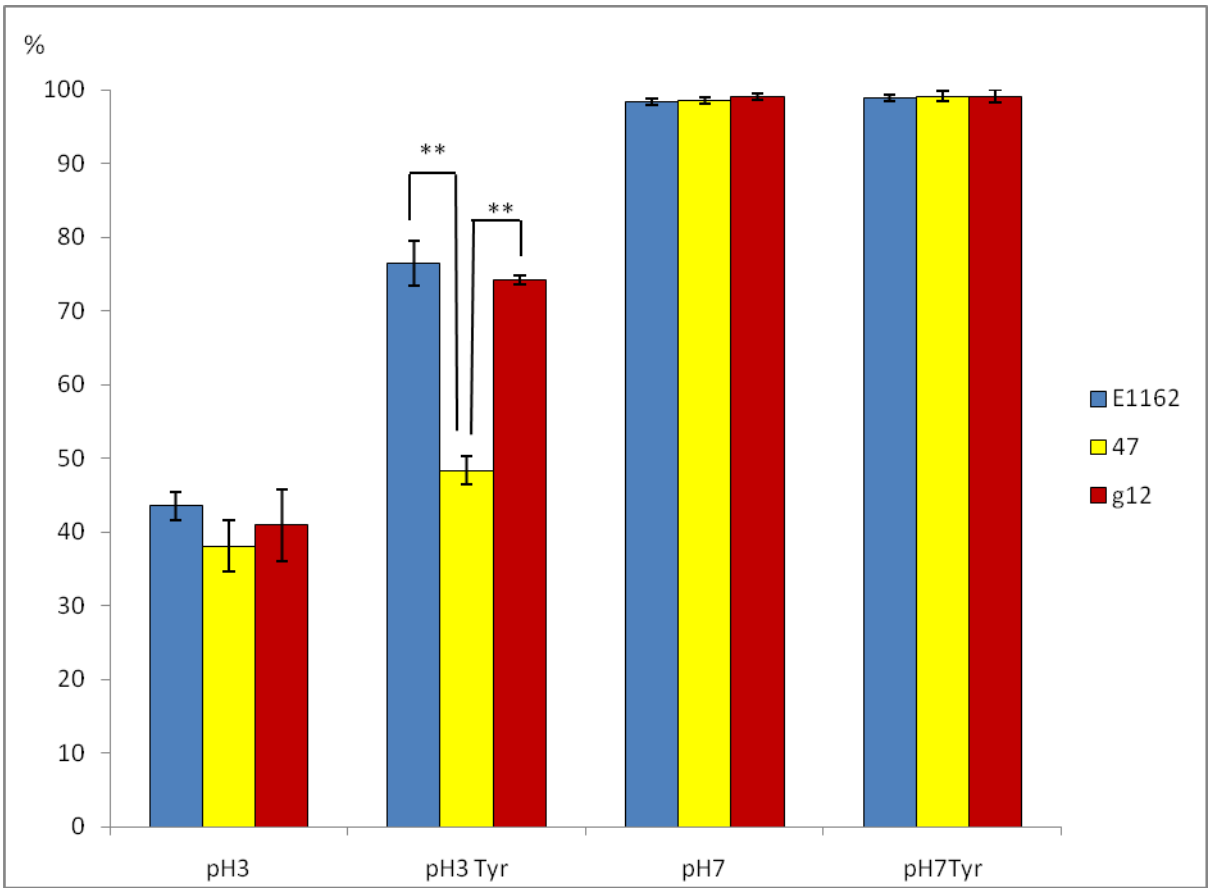


Figure 3.22 Survival of *E. faecium* E1162, the *tdc* mutant strain 47N and repaired strain GHFH12 upon exposure to 1 mM potassium phosphate buffer, pH 3.0 at 37 °C for 3 hours in the presence or absence of tyrosine. Blue: *E. faecium* E1162; Yellow: the *tdc* mutant strain 47N and Red: repaired strain GHFH12. Data presented are the mean of three independent experiments with error bars showing the standard deviations. Significance of the difference between E1162, the mutant 47N and the repaired strain GHFH12 were analyzed with the Mann-Whitney U test, **p<0.01

3.4.4.3.2 Determination of the effect of repairing the tyrosine decarboxylase gene in *E. faecium* strain 47N on nisin resistance

To confirm a role for the tyrosine decarboxylase gene of *E. faecium* in resistance to nisin, the minimum inhibitory concentration of nisin for the *tdc* repaired strain GHFH12, was compared to that of the *tdc* mutant 47N and the wildtype *E. faecium* E1162. It was observed that the MIC of nisin for the *E. faecium* E1162 and the *tdc* repaired strain was 128 µg/mL, while the MIC of nisin for the *tdc* mutant was 64 µg/mL nisin. The MIC value is the mean of three independent experiments. These results confirm that the phenotype of reduced resistance to nisin in the *tdc* disrupted strain 47N is the result of a transposon insertion in the *tdc* gene.

3.4.4.3.3 Determination of the effect of repairing the *tdc* disrupted gene in *E. faecium* strain 47N on virulence in the *G. mellonella* wax worms model

To confirm a role for the tyrosine decarboxylase gene in the virulence of *E. faecium*, in the *G. mellonella* wax worms model, the virulence of the *tdc* repaired strain, was compared to *E. faecium* E1162 and the *tdc* mutant strain 47N.

It was observed that killing of wax worms by the *tdc* mutant strain 47N was significantly ($p<0.05$) slower than that caused by the *E. faecium* E1162 or the *tdc* repaired strain, GHFH12 whereas there was no significant difference between strains E1162 and GHFH12 (Figure 3.23). This result confirmed that the phenotype of reduced virulence in the *G. mellonella* model of the *tdc* disrupted strain 47N is the result of a transposon insertion in the *tdc* gene.

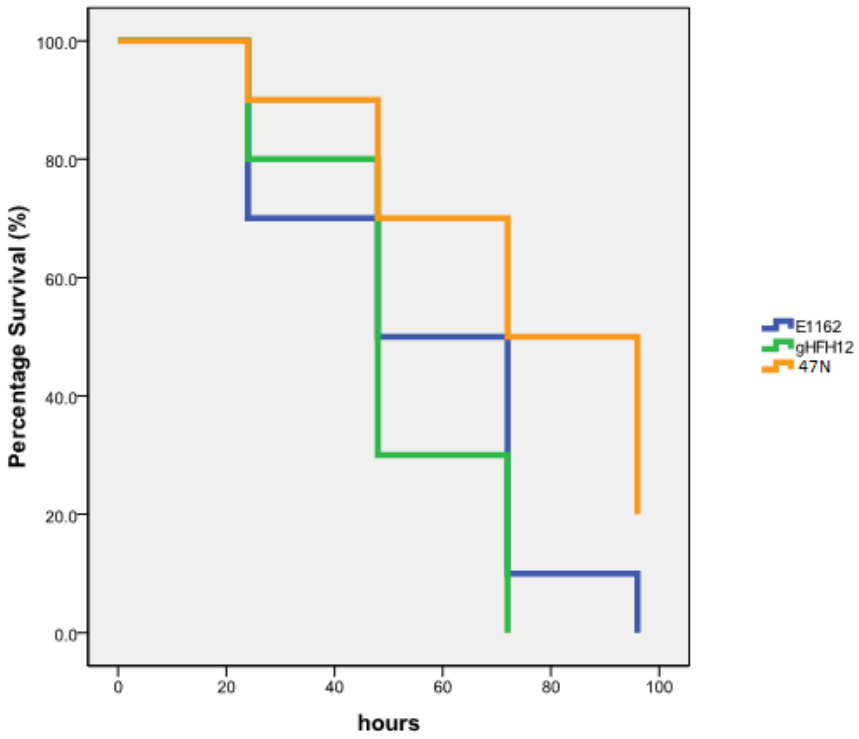


Figure 3.23 Kaplan-Meier survival curves of *Galleria mellonella* infected with *E. faecium.* Blue: *E. faecium* E1162; Green: repaired strain GHFH12 and Yellow: mutant 47N. Data presented are the percentages of 30 wax worms which were tested in groups of 10 on three different occasions.

3.5 Discussion

In the work described in this chapter a transposon mutagenesis approach was used to identify genes involved in antimicrobial peptide resistance of *E. faecium* E1162. Initially we looked for genes that are involved in lysozyme resistance. The result showed that disruption of a gene that codes for a conserved hypothetical protein (EFF34598) in strain AGSA1 confered resistance to lysozyme.

The fact that the EFF34598 is located in close proximity to putative RepA and RepB proteins suggests that this gene might reside on a plasmid. Analysis of the sequence of gene encodes for a conserved hypothetical protein and adjacent genes shows that this gene might be involved in toxin-antitoxin system. A tetracycline resistance-encoding plasmid

from *E. faecium* was reported by Li et al. (2011). That study revealed the presence of toxin-antitoxin independent plasmid mechanism and this system is widely distributed in *E. faecium* isolates. The toxin-antitoxin systems have a toxin protein and antitoxin components which play important role for this pathogen to survive in harsh environment including under exposure of antibiotics (Li et al., 2011). In other gram-positive bacteria such as *S. aureus*, toxin-antitoxin systems are involved in plasmid maintenance and in global regulation of virulence (Bukowski et al., 2013). Hence, it is also possible that the similar system is also presence in *E. faecium*.

In the present study, it was also observed that the killing of *Galleria mellonella* wax worms was insignificantly different between the wildtype and AGSA1 mutant. Hence, this implies that the conserved hypothetical protein is not involved in virulence of *E. faecium* in the *Galleria mellonella* model.

Different kinds of stresses are encountered by *E. faecium* during infections including antimicrobial peptides that are part of the innate immune system and which are also produced by bacteria to compete against each other. Using a transposon mutagenesis approach, we also identified genes involved in nisin resistance. Nisin was used in this study as a surrogate for human antimicrobial peptides since it is a relatively cheap alternative.

Strain 59N had a transposon inserted within a predicted ORF EFF33496. This is annotated in the *E. faecium* E1162 genome sequence data as coding for a muramidase. The insertion of the transposon within this gene caused a higher susceptibility to nisin. β-N-acetylmuramidase are enzymes which hydrolyze β-N-glycosidic linkages between linked N-acetylmuramic acid and N-acetylglucosamine. Given this fact it may be that the putative muramidase EFF33496 is involved in remodelling the cell wall and that this affects the ability

of nisin to interact with its target. Changes in cell wall compositions could prevent nisin interaction with lipid II (Kramer et al., 2004). Further studies on the cell wall composition of strain 59N compared to the wild type strain would need to be performed to confirm this hypothesis. Strain 59N was also significantly impaired in killing *G. mellonella* larvae compared to the *E. faecium* E1162 wild type strain. The gene coding the putative muramidase EFF33496 was located within a 150-kb pathogenicity island of *E. faecium.* Therefore the insertion of the transposon into the gene coding for EFF33496 might affect expression of one or more adjacent genes on this pathogenicity island which might account for the reduced virulence rather than it being a direct result of inactivation of EFF33496. The enterococcal surface protein gene, *esp*, is located upstream of the gene coding for EFF33496 and is in biofilm of *E. faecium* (Heikens et al., 2007), but it is not essential for cell adhesion and intestinal colonization in mice (Heikens et al., 2009). However one study has shown that Esp is involved in the *in vitro* binding of bladder and kidney epithelial cells (Leenderste et al., 2009). Further studies need to be conducted to determine if the phenotype is due to disruption of the gene coding for EFF33496.

Another mutant isolated in the nisin sensitivity screen, 64N, had a transposon integrated into a gene predicted to code for a conserved hypothetical protein (EFF35914). When compared with other protein, this conserved hypothetical protein has a putative conserved domain architectures suggesting it belongs to YjeE family, which is essential for bacterial viability (Alliali-Hassani et al., 2004). YdiB which is orthologue of YjeE in *Bacillus subtilis* plays a role in nutrient-limited conditions (Karst et al., 2009). The mutant 64N was significantly less virulent in the *G. mellonella* larvae model compared to the wild type *E. faecium* E1162. This indicates that disruption of this gene or polar effects on neighbouring genes, due to the transposon insertion, might be important in virulence of this bacterium.

Isolate 95N which was sensitive to nisin was determined to have a transposon within a gene that codes for a putative transposase. It is not clear how a transposase could be involved but it is possible the disruption caused polar effects on nearby genes in this strain. Upstream of the putative transposase is a gene that encodes a putative ascorbate-specific PTS system enzyme IIC. In other bacteria like *Listeria monocytogenes*, part of phosphotransferase system component IIC is important for a specific interaction with bacteriocins (Kjos et al., 2010). In *E. faecalis*, the ascorbate-specific transporter subunit IIC protein (EF1127) is involved in L-ascorbate metabolism (Mehmeti et al., 2013). This might indicate that *E. faecium* can utilise ascorbic acid, which might be important during urinary tract infections. It was observed that the killing of *Galleria mellonella* wax moth larvae was insignificantly different between the wildtype and 95N mutant. Hence, this implies that the conserved hypothetical protein or polar effects on neighbouring genes is not involved in virulence of *E. faecium* in the *G. mellonella* model.

Isolate 183N which was sensitive to nisin was determined to have a transposon within putative ORF EFF35601 (CBS domain protein). Cystathione β-synthase (CBS) is involved in bile resistance, which mediates bile tolerance of *Bifidobacterium breve* UCC2003 (Ruiz et al., 2012). Thus, this indicates that *E. faecium* might use a similar mechanism and utilize this gene or other neighbouring genes that might be important to survive in gastrointestinal tract. This mutant was also significantly attenuated in its capacity to kill wax worms compared to *E. faecium* E1162 ($p<0.05$). These results demonstrate that CBS domain protein or neighbouring genes contribute to *E. faecium* virulence in the *G. melonella* model.

Isolate 83N which was sensitive nisin was determined to have a transposon within a gene that codes for conjugative transposon protein (EFF33504). Conjugative transposons have capacity to carry different putative virulence genes in streptococci (Chuzeville et al.,

2012). The conjugative transposon increased the survival of *Legionella pneumophila* in mouse macrophages(Flynn & Swanson, 2014). Hence, this might indicate that *E. faecium* might use a similar mechanism. The mutant was significantly attenuated in its capacity to kill wax worms compared to to *E. faecium* E1162 ($p<0.05$). These results demonstrate that conjugative transposon protein or neighbouring genes contribute to *E. faecium* virulence in the *G. melonella* model.

Screening of the transposon mutant library of *E. faecium* for sensitivity to nisin also resulted in the isolation of 8 mutants that had a transposon inserted within ORF EFF34651 coding for a putative tyrosine decarboxylase. The gene coding for EFF34651 was present in a putative operon that had a similar genetic organisation to operons found in *Lactobacillus brevis* IOEB 9809, *Lactobacillus lactis* IPLA 655 (Bearson et al., 1997; Fernandez et al., 2004) and *E. faecium* E17 (Pereira et al., 2009). In general, two proteins are involved in amino acid decarboxylation pathways; a decarboxylase and a transporter protein. Tdc catalyzes the decarboxylation of tyrosine to tyramine. The transporter TyrP takes up tyrosine from the medium and exchanges tyramine to the outside of the cell. Tyramine has a positive charge while tyrosine has no net charge. In low pH environments, internal tyrosine is converted to external tyramine. During this decarboxylation of tyrosine, one positive charge is translocated across the membrane and one proton is removed from the membrane (Wolken et al., 2006). This contributes to the pH homeostasis and survival of this bacterium in acidic environments.

The transcription of *tyrS* in *Enterococcus durans*, which is upstream of *tdc*, has been reported to be induced by low pH conditions and its expression is enhanced in the absence of tyrosine leading the authors to suggest that, tyrosine is a strong repressor of *tyrS* (Linares et al., 2012).

The Na+/H+ antiporter, *nhaC*, which is the last gene in the *tdc* operon, might contribute in pH and Na+ homeostasis (Liew et al., 2007). This gene is not essential in the tyrosine decarboxylation pathway but might be important for maintaining pH and Na+ homeostasis during growth (Pereira et al., 2009). Some tyrosine decarboxylases are also able to catalyze phenylalanine decarboxylation but this reaction only occurs when tyrosine is depleted (Pessione et al., 2009).

As mentioned in section 3.4.4.1, from the bioinformatics analysis *tdc* seems to be part of a putative operon that may be under complex regulation. Furthermore there was more than one promoter predicted that might be able to drive the transcription of the *tdc* gene. For these reason, complementing strain 47N *in trans* with the *tdc* gene under the control of a foreign promoter or one of the two putative promoters in the operon which may control its transcription might not restore all or any of the defective phenotypes. Therefore it was decided to repair the disruption in strain 47N by allelic exchange. The *tdc* gene was repaired using the pHOU1 vector system (Kast et al., 1991), which allows for positive selection for the loss of the plasmid backbone. The repaired strain (GHFH12) was able to fully restore nisin resistance to the same level as *E. faecium* E1162. These data demonstrate that the altered phenotypes in strain 47 are due to *tdc* disruption and not due to other coincident mutations.

It was reported by Pereira and colleagues that the *tdc* gene was important for survival in acidic environment (Pereira et al., 2009). Here, a control at pH 7.0 was incorporated, which was not provided by Pereira and colleagues. Similar to the result reported in *E. faecium* E17 (Pereira et al., 2009), we found that the tyrosine decarboxylase is important in low pH environments. Similar to Pessione and colleagues (2009), our data also shows that the *tdc* gene is also able to catalyze phenylalanine decarboxylation. Our data

demonstrates that the *tdc* is also involved in mediating resistance to nisin. In the gram-positive bacterium *Listeria monocytogenes* another amino acid decarboxylase, glutamate decarboxylase mediates nisin resistance (Begley et al., 2010). In their study, the glutamate decarboxylase mutant exhibited a longer lag phase and lower growth rate compared to the wild type in the presence of 100 µg/mL nisin powder than in the absence of nisin. Nisin powder is impure (the balance of the powder containing sodium chloride and denatured milk solids) and based on my calculations, the actual nisin concentration that they used in their study was 2.5 µg/mL. A similar phenotypic defect was observed in my study, where the *tdc* mutant was more sensitive to nisin at 2 µg/mL. These results showed that tyrosine decarboxylase plays a role in mediating nisin resistance in *E. faecium*.

The glutamate decarboxylase of *Listeria monocytogenes* mediates nisin resistance by contributing to the intracellular ATP pools (Begley et al., 2010). Hence, it could be hypothesized that tyrosine decarboxylase in *E. faecium* might play a similar role. To confirm this, further analysis of intracellular ATP levels would need to be performed to detect the differences in wild type and *tdc* mutant.

To further understand the role of *tdc* in *E. faecium* pathogenesis, we evaluated the capacity of strain 47N to kill wax worms. The mutant with a disruption in the *tdc* gene displayed significantly impaired killing of *G. mellonella* wax worms compared to the wild type *E. faecium* E1162. This indicates that this gene also plays an important role in virulence and persistence of this bacterium in this infection model.

In conclusion the *tdc* gene may give advantages to *E. faecium* E1162, particularly in survival in low pH environments, in resistance to nisin, and possibly other antimicrobial peptides, and in virulence. Further investigation of the genetic determinants which were

identified in the work described in this chapter may lead to a better understanding the pathogenesis of *E. faecium*.

Chapter 4

The role of a serine threonine protein kinase in cell wall stress in *Enterococcus faecium*

4 The role of a serine threonine protein kinase in cell wall stress in *Enterococcus faecium*

4.1 Introduction

4.1.1 Bacterial Serine threonine protein kinases

Bacteria can sense and respond to environmental stimuli by using signal-transduction systems which may be comprised of one or two components (Ulrich et al., 2005). A two component signal transduction systems consist of a sensory, usually membrane-bound and a soluble response regulator which use phosphotransfer to transmit information across the cytoplasmic membrane. In a typical two-component systems, once an environmental stimulus is sensed by the input domain of the protein kinase, the kinase is autophosphorylated and subsequently transfers the phosphoryl group to the response regulator, which control the expression of target genes (Stock et al., 2000). In one component systems, the input and output domain are fused in a single protein (Lewis et al., 1996; Ulrich et al., 2005). Example of one component signal transduction systems is the serine threonine protein kinase. Serine threonine protein kinase (STPKs) has been intensively investigated and involved in adaptive bacterial responses (Olsen et al., 2006). STPKs transfer a phosphate group onto specific serine, threonine and/or tyrosine residues of a protein substrate. This activates the substrate to perform a specific activity in different bacteria (Table 4.1 and Table 4.2) (Huse and Kuriyan, 2002). Conversely, phosphatase removes a phosphate group from its substrate. The protein phosphatase P (PPP) family represents the largest source of protein serine/threonine phosphatases (Cohen, 1991; Barton et al., 1994).

Table 4.1 Selected functions of STPKs in bacteria

Function	Bacteria	Reference
Virulence[1]	*Staphylococcus aureus*	Debarbouille et al., 2009; Burnside et al., 2010; Sun et al., 2012
	Streptococcus pyogenes	Pancholi et al., 2010
	Streptococcus agalactiae	Rajagopal et al., 2003
	Streptococcus mutans	Banu et al., 2010
	Mycobacterium tuberculosis	Cowley et al., 2004
	Streptococcus pneumoniae	Beilharz et al., 2012
	E. faecalis	Kristich et al., 2007
Cell wall biosynthesis	*Mycobacterium tuberculosis*	Alderwick et al., 2006
	Staphylococcus aureus	Beltramini et al., 2009; Liebeke et al., 2010
	Streptococcus pneumoniae	Beilharz et al., 2012
	Streptococcus mutans	Hussain et al., 2006
	Streptococcus agalactiae	Rajagopal et al., 2003
Growth[2]	*Staphylococcus aureus*	Debarbouille et al., 2009
	Streptococcus pyogenes	Pancholi et al., 2010
	Streptococcus agalactiae	Rajagopal et al., 2003
	E. faecalis	Kristich et al., 2007
Biofilm	*Staphylococcus epidermidis*	Liu et al., 2011
	Streptococcus mutans	Hussain et al., 2006; Banu et al., 2010
	Bacillus subtilis	Madec et al., 2002
Acid resistance	*Streptococcus mutans*	Hussain et al., 2006
Genetic competence	*Streptococcus mutans*	Hussain et al., 2006
Antimicrobial resistance	*E. faecalis*	Kristich et al., 2007

[1]Virulence assay was performed in an animal model
[2]Growth defects was observed in complex media

In some bacteria, STPK substrates have been identified (Table 4.2).

Table 4.2 STPK substrates

Organism	Substrate	Function of the substrate	Reference
Mycobacterium tuberculosis	-putative transcriptional regulator, EmbR	Arabinan synthesis	(Molle et al., 2006; Jang et al., 2010)
	-beta-ketoacyl –acyl carrier protein (ACP) synthase III	Mycolic acids biosynthesis	(Veyron-Churlet et al., 2009; Thakur and Chakraborti, 2006)
	- chaperone, GroEL1	-essential for growth and necessary for folding of several essential proteins	(Canova et al., 2009)
	- UDP-N-acetylmuramoyl-L-alanine:D-glutamate-ligase, MurD	- peptidoglycan biosynthesis	(Thakur and Chakraborti, 2008)
	-Wag31,an ortholog of the cell division protein DivIVA	-cell shape/cell division	(Kang et al., 2005)
	- a conserved protein of unknown function, Rv1422	-unknown	
	- Two FHA domains on an ABC transporter, Rv1747	-growth	(Molle et al., 2006; Spivey, 2013)
	- multiple Forkhead-Associated (FHA) domains, Rv0020c	- growth	(Grundner et al., 2005)
	- a penicillin-binding protein required for cell division, PBPA	-cell division	(Dasgupta et al., 2006)

	- stress response regulon, SigH	Stress response and virulence	(Park et al., 2008)
	- glycogen accumulation regulator, GarA	-glutamate metabolism	(O'Hare et al., 2008)
	- penicillin-binding protein, DacB1 and transcriptional regulator, Rv0681	cell wall biosynthesis	(Zheng et al., 2007)
	-Pyruvate kinase A	-glycolysis	(Arora et al., 2010)
	- transcriptional regulator, VirS	-regulate the expression of the mycobacterial monooxygenase operon	(Kumar et al., 2009)
	- transporter, MmpL7	-virulence	(Perez et al., 2006)
	- two-component response regulator, DosR	-dormancy	(Chao et al., 2010; Kumar et al., 2012)
	- a protein of unknown function, Rv2175c	-unknown	(Canova et al., 2008)
	- Cell division under oxidative stress, FipA	-cell division	(Sureka et al., 2010)
	- Cell division, FtsZ		
Staphylococcus aureus	-Adenylosuccinate synthase, PurA	-Purine biosynthesis	(Donat et al., 2009)
	- Transcription regulator SarA	-virulence	(Didier et al., 2010)
	AFT-2	- Human transcription factor,	(Miller et al., 2010)

Streptococcus pneumoniae	hypothetical protein, Spr0334)	-unknown	(Nováková et al., 2010)
	cell division protein, DivIVA	-cell division	
	-phosphoglucosamine mutase,GlmM	-common precursor to cell envelope components	(Nováková et al., 2005)
	-repressor of iron transport, RitR;	-iron transport	(Ulijasz et al., 2009)
	-Cell division, FtsZ	-cell division	(Giefing et al., 2010)
	- the cell-division protein, DivIVA	-cell division	(Beilharz et al., 2012)
Bacillus subtilis	-Elongation factor, EF-Tu	- protein translation	(Absalon et al., 2009)
	- a small ribosome-associated GTPase,CpgA	-responsible for accumulation of peptidoglycan precursors in cells	
	stressosome protein, YezB	-general stress response	
	-Elongation factor, EF-G;	- protein translation	(Shah and Dworkin, 2010)
	-Acetolactate decarboxylase, AlsD	-metabolism	(Pietack et al., 2010)
	-Glutamine synthetase, GlnA		
	-Isocitrate dehydrogenase, Icd		
	-Transaldolase, YwjH		

STPKs comprise a C-terminal extracellular sensory domain containing multiple PASTA (penicillin binding protein and serine/threonine kinase associated) domains, a transmembrane region and an intracellular kinase domain (Yeats et al., 2002). PASTA domains are present in variable numbers in STPKs. The STPK of *B. subtilis* has three PASTA domains while the STPK (PknB) of *M. tuberculosis* has four PASTA domains in the extracellular portion (Shah et al., 2008; Yeats et al., 2002).

In this study, an ORF, EFF33865, encoding a predicted protein of 689 amino acids, annotated in the *E. faecium* E1162 genome sequence as a STPK, which we have named Stk1, was identified. Analysis of the predicted protein architecture of Stk1 shows an N-terminal serine/threonine kinase domain, a transmembrane domain and four PASTA domains at the C-terminus (Figure 4.1).

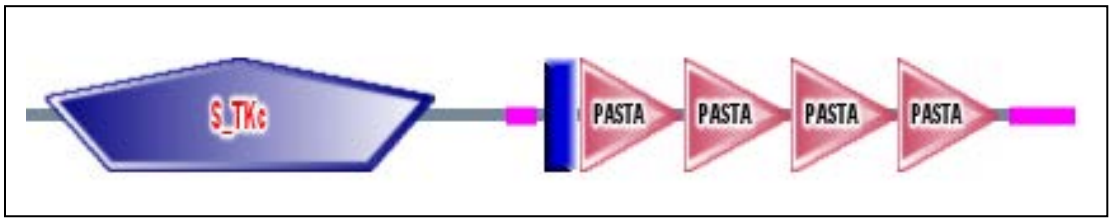


Figure 4.1 Predicted domain architecture of *E. faecium* Stk1; S_TKc, serine/threonine protein kinase domain (blue diamond); low complexity domain (pink square), transmembrane domain (blue square); PASTA domains (pink triangle). The domains were predicted identified using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de).

In *E. faecalis*, a STPK homologue with similar domain architecture is known as IreK (Kristich et al., 2011). The protein architecture of IreK has an N-terminal serine/threonine kinase domain, a transmembrane region and five PASTA domains at the C-terminus (Kristich et al., 2007). IreK has been implicated in intrinsic cephalosporin resistance (Kristich et al., 2007).

4.1.2 Peptidoglycan

Peptidoglycan surrounding the cytoplasmic membrane of bacteria is composed of glycan chains consisting of alternating units of an N-acetylglucosamine (GlcNac) and acetylmuramic acid (MurNac). The carboxyl group of N-acetylmuramic acid is substituted by a peptide subunit, L-alanyl-γ-D-glutamyl- L-lysyl or meso-diaminopimelyl (m-Dpm)-D-alanyl-D-alanine (Figure 4.2). The neighboring glycan chains are linked by a short peptide bridge between neighbouring peptide subunits or forming a link with a carboxyl group from D-ala in an adjacent tetrapeptide side (Schleifer and Kandler, 1972).

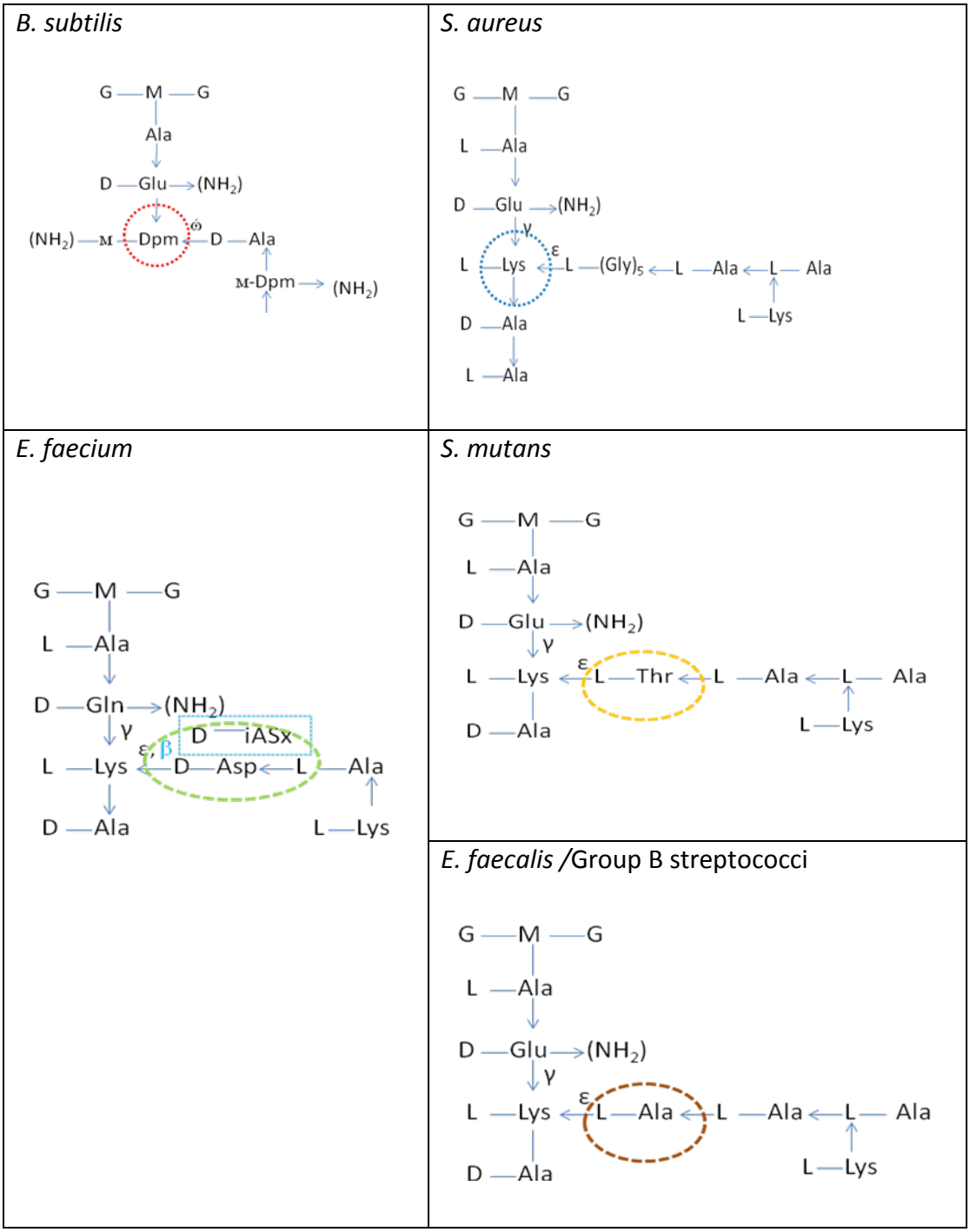


Figure 4.2 Variations of the peptide moiety of peptidoglycan in gram-positive bacteria; the red dotted line shows the m-Dpm residue in *B. subtilis*; the blue dotted line shows the L-lys residue in *S. aureus;* the orange dotted line shows the L-Thr residue in *S. mutans;* the chocolate dotted line shows the L-Ala residue in *E. faecalis/* Group B streptococci;the green dotted line shows the differences in the side chains of the peptidoglycan moiety in *E. faecium*.

Analysis of *E. faecium* peptidoglycan revealed the presence of a nucleotide precursor UDP-MurNAc-pentapeptide-Asp or pentadepsipeptide-Asp (Billot-Klein et al., 1997). By

using a D-aspartate ligase (Aslfm) and an aspartate racemase (Racfm), *E. faecium* incorporates D-isoasparagynyl or D-isoaspartyl (D-iAsx) residues into the side chain of the peptidoglycan precursor. Heterologous expression of both enzymes (D-aspartate ligase and racemase) in *E. faecalis* leads to the production of stem peptides substituted by D-Asp instead of L-Ala^{2} (Bellais et al., 2006).

Polymerization of bacterial peptidoglycan is executed by multienzyme complexes which are high-molecular-weight class A and B penicillin- binding proteins (PBPs). Class A PBPs consist of a single polypeptide chain that has two essential activities for peptidoglycan polymerization, glycosyltransferase and D,D-transpeptidase activity. Class B PBPs on the other hand are monofunctional D,D-transpeptidases that must cooperate with a glycosyltransferase to synthesize peptidoglycan (Scheffers and Pinho, 2005). Transpeptidation generates tensile strength by cross-linking the glycosidic backbone into a network of interlocking units (Yeats et al., 2002) and involves both D, D- transpeptidases and L,D-transpeptidase in *E. faecium*. D, D-transpeptidase catalysed the formation of 4 to 3 crosslinks (D-Ala^{4}→D-iAsx-L-Lys^{3}) while L, D-transpeptidase catalysed the formation of 3 to 3 crosslinks (L-Lys^{3}→D-iasx-L-Lys^{3}) (Mainardi et al., 2007; Mainardi et al., 2000).

It has been reported that the release of peptidoglycan fragments from the cell wall of growing bacteria into the extracellular environment are potent germinants of dormant *B. subtilis* spores (Shah et al., 2008). The PASTA domains of the STPK were shown to bind peptidoglycan fragments (Shah et al., 2008). In *M. tuberculosis*, the PASTA domain binds to specific peptidoglycan fragments and this domain is required for STPK localization (Mir et al., 2011). The PASTA domain binds to peptidoglycan fragments in the presence of specific amino acids at the second and third positions of the stem peptide (Mir et al., 2011). The PASTA domain also binds to exogenous peptidoglycan fragments and this caused

localisation of the STPK at the mid-cell and the cell poles (Mir et al., 2011). In *S. pneumoniae,* the PASTA domains bind to unlinked peptidoglycan (Beilharz et al., 2012).

In the present study the role of *E. faecium* Stk1 in peptidoglycan synthesis will be investigated. The specificity of Stk1 in sensing different peptidoglycan moiety will be tested. It was anticipated that the data obtained from this study would improve our understanding of the role of Stk1 in *E. faecium*.

4.2 Materials and methods

4.2.1 Bacterial strains, plasmids and oligonucleotides

Bacterial strains and plasmids used in this study are listed in Table 4.3. *E.coli* DH5α clones were grown in LB broth or agar supplemented with 100 µg/mL ampicilin. *Enterococcus* sp. were grown in Tryptic Soy Broth (TSB), Todd–Hewitt broth (THB) or Brain Heart Infusion (BHI) media. Strains of enterococci bearing plasmids were grown in broth or agar supplemented with 40 µg/mL erythromycin.

Table 4.3 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. faecium		
E1162	Clinical blood isolate; CC17; Amp^r Van^s Chl^s Gen^s Ery^s Esp	Willems et al., 2005
E. faecium 201c	E1162 ΔStk1 Amp^r Van^s Chl^r Gen^s Ery^s Esp	Nair (unpublished)
E. faecalis OG1RF	Ery^s Chl^s	Kristich et al., 2007
E. faecalis CK119	OGF1RF ΔIreK Ery^s Chl^s	Kristich et al., 2007
E. coli DH5α	Routine cloning host	
pTEX 5500ts	Chl^r	This study
pZ188	pSK236; Amp^r Chl^r	Ping (unpublished)
pSK236	Amp^r	Ping (unpublished)
Complementation plasmids		
pHFH9	pSK236; Amp^r Ery^r	This study
pHFH10	pHFH9 containing *stk1* with RBS	This study
pHFH11	pHFH9 containing *ireK* with RBS	This study

r: resistant, s: sensitive, Amp: Ampicillin, Van: Vancomycin, Chl: Chloramphenicol, Gen: Gentamicin, Ery: Erythromycin, Esp: enterococcal surface protein gene, RBS: ribosome binding site

4.2.2 Restriction enzyme digestion of genomic DNA

Genomic DNA was concentrated to 3 µg/µL using a SAVANT SPD 1010 SpeedVac concentrator (Temperature: 60˚C, heat time: 15 min, Radiant Cover: On). Genomic DNA was digested with HindIII (1 U/µg; NEB) in 1x NE buffer 2 at 37˚C overnight, purified using a PCR purification kit (NBS Biologicals) according to the manufacturer's instructions and concentrated using a SAVANT SPD1010 Speed Vac concentrator.

4.2.3 PCR reaction for generating probes for Southern hybridizations

Three probes were generated by PCR for the Southern blot hybridization, (EA 126≈1000 bp, EA1 27≈650 bp and EA 128≈620 bp) using the oligonucleotides. Probe EA 126 was specific for the *E. faecium stk1* gene, EA 127 was specific for the *cat* gene and EA128 was specific for the region of *stk1* targeted for deletion in the mutant. For PCR amplification of EA126 and EA 128, *E. faecium* genomic DNA was used as a template, and plasmid pTEX 5500ts (containing *cat* gene) was used as a template for EA 127.

The PCR reaction mixtures:

Components	Amount (µL)
Forward primer	0.5 µM
Reverse primer	0.5 µM
dNTP	0.2 mM
Standard Taq buffer	1 x
DNA template	~0.1 ng/µL
Taq DNA polymerase	0.05 U/µL

The amplification conditions were as follows: 1) Pre-denaturation: 95°C for 120 s, 2) Denaturation: 94°C for 30 s, 3) Annealing: The melting temperature-5°C for 30 s (Tm was calculated from an equation under section 2.6.3), 4) Elongation: 72°C for 1 kb/min and 5)

Final elongation: 72°C for 10 mins. Steps 2-4 were repeated for 30 cycles. The PCR was held at 4°C at the end of cycling.

4.2.4 Digoxigenin (DIG-) labeling of DNA probes

DIG-DNA labelling of PCR products was done according to the manufacturer's instructions using a DIG High Prime, DNA Labeling and Detection Starter Kit II (for chemiluminescent detection with CSPD) (Roche Applied Science). Prior to hybridization, the labelled probes were denatured by heating at 99˚C for 5 min and immediately cooled on ice.

4.2.5 Southern blot

HindIII digested genomic DNA was subject to gel electrophoresis overnight at 20 V on a 1.0 % agarose gel (see section 2.6.6). Twenty microlitres (~3 µg) of Dig-labelled DNA Molecular Weight Marker VII (Roche Applied Science), and 3 µg of each sample in 1 x gel loading buffer was loaded. All procedures were done according to the manufacturer's instructions using DIG High Prime, DNA Labeling and Detection Starter Kit II (for chemiluminescent detection with CSPD) (Roche Applied Science). The membrane was exposed to a Hyperfilm™ ECL X-ray film for an appropriate length of time.

4.2.6 Lysozyme sensitivity assays

Sixteen hour overnight cultures in TSB (Fluka Analytical, Sigma Aldrich) were diluted to an optical density of 0.010 at 600 nm (OD_{600} 0.010) in 20 mL of prewarmed medium. Cultures were incubated aerobically at 37°C with shaking at 200 rpm. Lysozyme sensitivity was assessed by adding lysozyme at a final concentration of 50, 100 or 200 µg/mL to exponential-phase-cells (OD_{600} 0.8), followed by incubation at 37˚C with shaking at 200 rpm for 48 hours.

4.2.7 Triton X-100-induced autolysis under non-growing conditions

Bacteria were grown to an optical density of 0.8 at 600 nm in the TSB. The cells were pelleted by centrifugation, washed in 20 mL of ice-cold sterile water, and resuspended in the same volume of 50 mM Tris-HCl, pH 7.2, containing 0.1 % Triton X-100 with or without 1 M NaCl. The bacterial suspension was then incubated at 30˚C with shaking and the OD_{600} were measured every 30 min. The percentage of lysed cells were calculated, i.e., percent lysis at time t= [(OD_{600} at time zero-OD_{600} at time t)/ OD at time zero] x 100.

4.2.8 Sample preparation for transmission electron microscopy (TEM)

Sixteen hour overnight cultures (100 µL) were dispensed on to 3 mm gold grids of 200 mm mesh (Agar Scientific R) for 5 min. The samples were then fixed with 1 % methylamine tungstate for 30 seconds and examined in a Philips CM12 TEM.

4.2.9 Samples preparation for scanning electron microscopy (SEM)

Sixteen hour overnight cultures were dispensed (120 µL) on to a glass slide for 1 hour, dried and fixed in 3 % gluteraldehyde (Agar Scientific, UK) in 0.1 M cacodylate buffer for 5 min. The fixed samples were then dehydrated by submerging the glass slide for 5 min in a series of graded ethyl alcohols (20 %, 50 %, 70 % and 90 %) at room temperature and

finally in absolute ethanol for 5 min (3 times). The samples were then immersed in hemathyldisilazane (TAAB Ltd, UK) in foil cups for 5 min and dried in a fume hood. The samples were mounted onto aluminium pin stubs with adhesive carbon tab (Agar Scientific, UK) and examined in a Cambridge 90B SEM (Leo, UK).

4.2.10 Western blotting

Plate-grown bacteria were resuspended in PBS to an OD660 of 1.0 (1 x10^9 CFU/ml) (Gaspar and Ton-That, 2006). Cells were harvested by centrifugation (10,000 rpm; 5 min) and resuspended in 50 µL PBS. Proteins were quantitated using a Pierce bicinchoninic acid (BCA) protein assay kit. All procedures for western blotting were carried out according to Heikens et al. (2007).

4.2.11 Zymography

Exponential-phase cells were extracted with 100 µL of 4 % SDS (1 x 10^9 CFU/mL). Proteins were quantitated using a BCA protein assay kit, applied to the gels, and resolved electrophoretically. Autolysin activity was visualized on 8 % SDS-polyacrylamide gels containing lyophilized *Micrococcus lysodeikticus*. All procedures for zymography were carried out according to Tamber et al. (2010).

4.3 Results

4.3.1 Confirmation of the disruption of the *stk1* gene in *E. faecium*

In order to examine the function of the *stk1* gene in *E. faecium*, the gene was mutated by the in frame deletion of 2067 bp and insertion of a chloramphenicol acetyl transferase (*cat*) gene in frame with the coding sequence by allelic exchange following the protocol of Nallapareddy et al. (2006). The construct was verified by sequencing.

Southern blotting was performed on three clones of putative *stk1* mutants (201a, 201b and 201c), using three probes designated, EA 126, EA 127 and EA 128. Probe EA 126 was specific for nucleotides 246-266 bp of *stk1* and was expected to hybridize to two HindIII fragments in *E. faecium* E1162 and three fragments in the mutant (Figure 4.4).

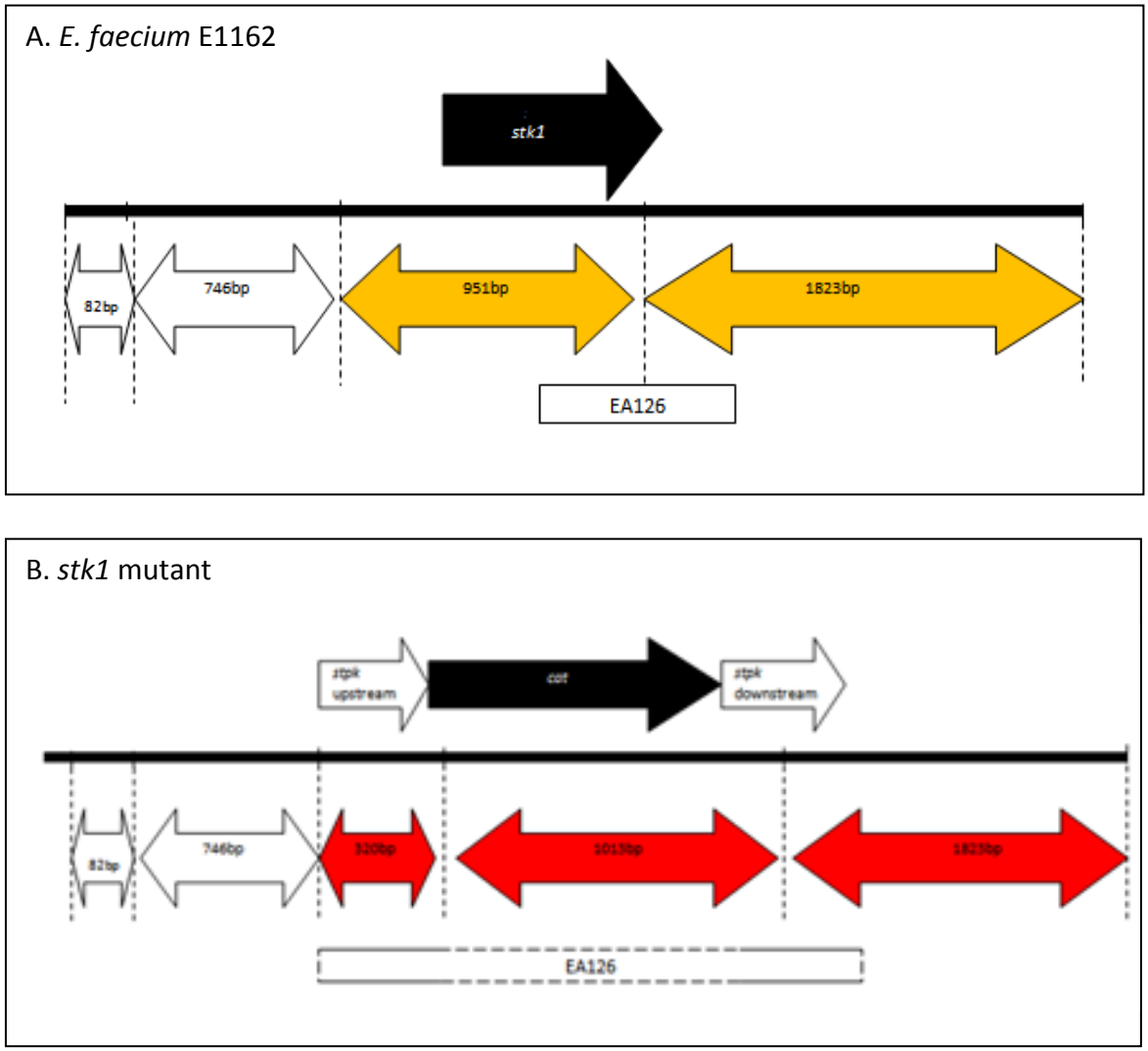


Figure 4.4 A. A diagram of HindIII fragments in *stk1* (yellow arrows) expected to hybridise with probe EA 126 in *E. faecium* E1162; B. A diagram of HindIII fragments (red arrows) expected to hybridise with probe EA 126 in a *stk1* mutant. The sizes of the fragments are given, dotted lines indicate HindIII sites.

Southern blot analysis of genomic DNA from the wild type and putative mutant strains using probe EA 126 is shown in Figure 4.5. As expected, the probe hybridised to two fragments of 1823 bp and 951 bp in the genomic DNA of the wild type (Lane 2). However,

there was an additional unexpected band at 700 bp which is assumed to have arisen due to star activity in the restriction digest (Tang et al., 2000). The genomic DNA from the three putative mutants gave three hybridising HindIII fragments as expected. The low signal intensity of the 1013 bp band was probably due to the low sequence complementarity with the probe.

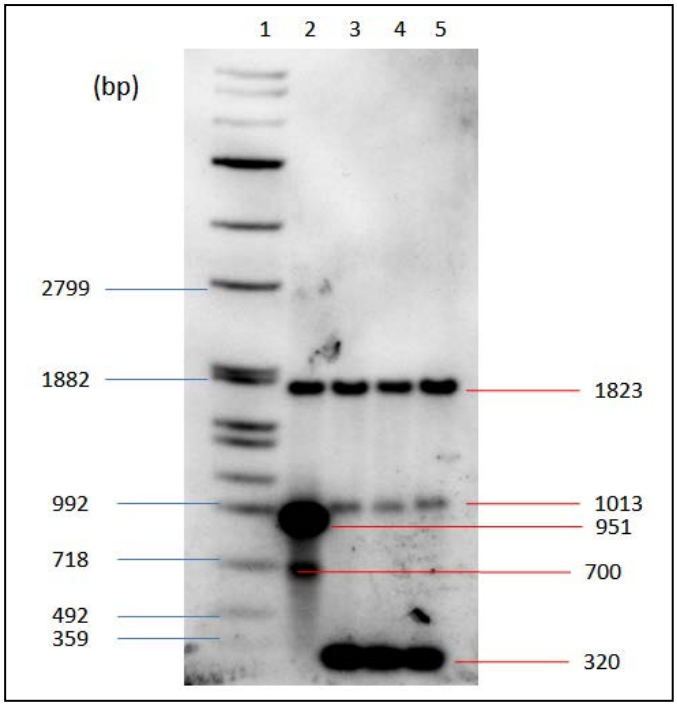


Figure 4.5 Southern hybridization of HindIII-digested genomic DNA with probe EA 126. Lane1: molecular weight marker; Lane 2: genomic DNA of *E. faecium* E1162; Lane 3: genomic DNA of the putative mutant strain, 201a; Lane 4: genomic DNA of the putative mutant strain, 201b and Lane 5: genomic DNA of the putative mutant strain, 201c.

Since the mutant was constructed by insertion of *cat*, probe EA 127, a 624 bp PCR amplified fragment of *cat* was also used in Southern hybridization. The probe was predicted to bind to a single fragment of 1013 bp in the mutants (Figure 4.6). As expected, a fragment of 1013 bp hybridised with EA 127 in all three putative mutants confirming that the *cat* gene

had been inserted on to the chromosome and the putative mutants were disrupted in the *stk1* gene (Figure 4.7).

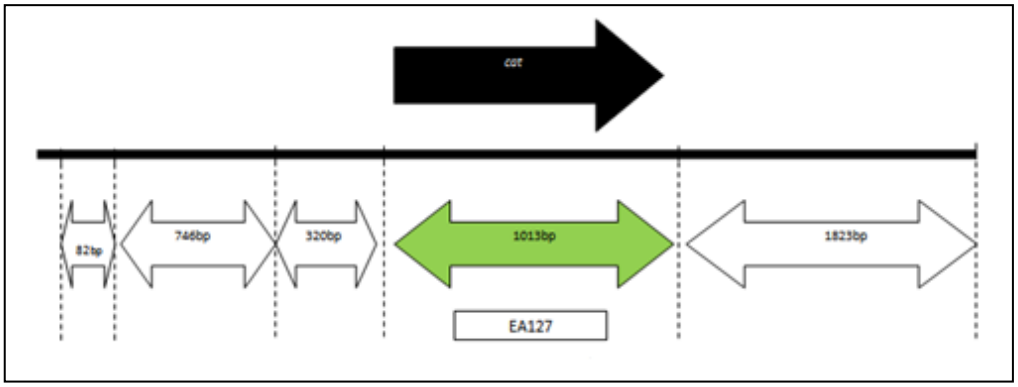


Figure 4.6 A diagram of HindIII fragments (green arrows) expected to hybridise with probe EA 127 in a *stk1* mutant.The sizes of the fragments are given, dotted lines indicate HindIII sites.

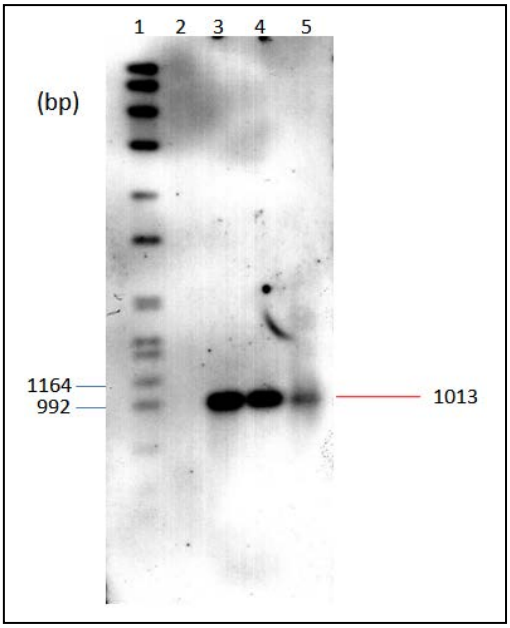


Figure 4.7 Southern hybridization of genomic DNA with probe EA 127 (*cat* gene). Lane1: molecular weight marker; Lane 2: genomic DNA of *E. faecium* E1162; Lane 3: genomic DNA of the putative *stk1* mutant, 201a; Lane 4: genomic DNA of the putative *stk1* mutant, 201b and Lane 5: genomic DNA of the putative *stk1* mutant, 201c.

The probe EA 128 was specific for the region of *stk1* gene which was deleted and was expected therefore to hybridise only to the wild-type strain. As expected (Figure 4.8), the probe hybridised to a 951 bp fragment in the wild-type strain and did not hybridise with the genomic DNA from the *stk1* mutants (Figure 4.9). Collectively, the Southern blot results show that a *stk1* mutant was successfully constructed.

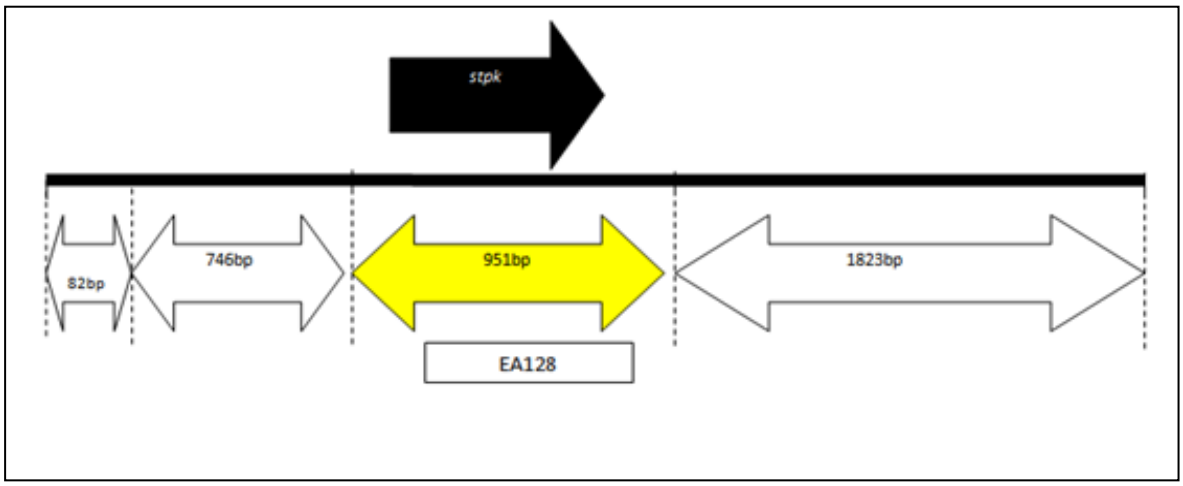


Figure 4.8 HindIII fragments expected to hybridise with probe EA 128 in the wild type strain, *E. faecium* E1162.

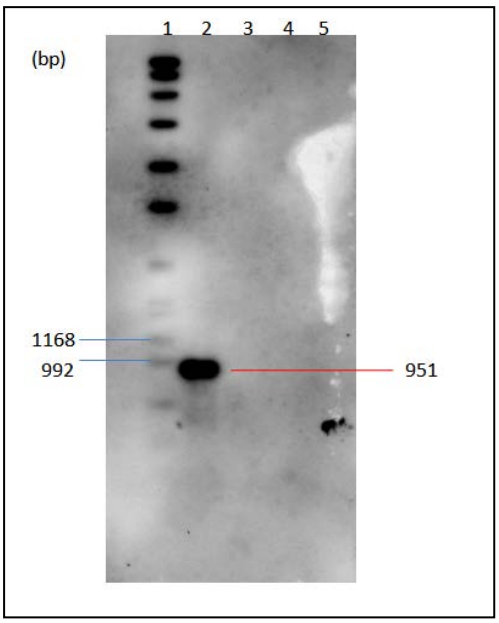


Figure 4.9 Southern hybridization of genomic DNA with probe EA 128 (*stk1* gene). Lane1: molecular weight marker; Lane 2: genomic DNA of *E. faecium* E1162; Lane 3: genomic DNA of the *stk1* mutant strain, 201a; Lane 4: genomic DNA of the *stk1* mutant strain, 201b and Lane 5: genomic DNA of the *stk1* mutant strain 201c

4.3.2 Growth of the *stk1* mutant

We analyzed the growth of the wild type and the isogenic *stk1* mutant, 201c, in TSB without dextrose as described by Kristich et al., 2007. The growth rate of *E. faecalis ireK* mutant was significantly reduced in TSB without dextrose (Kristich et al., 2007).

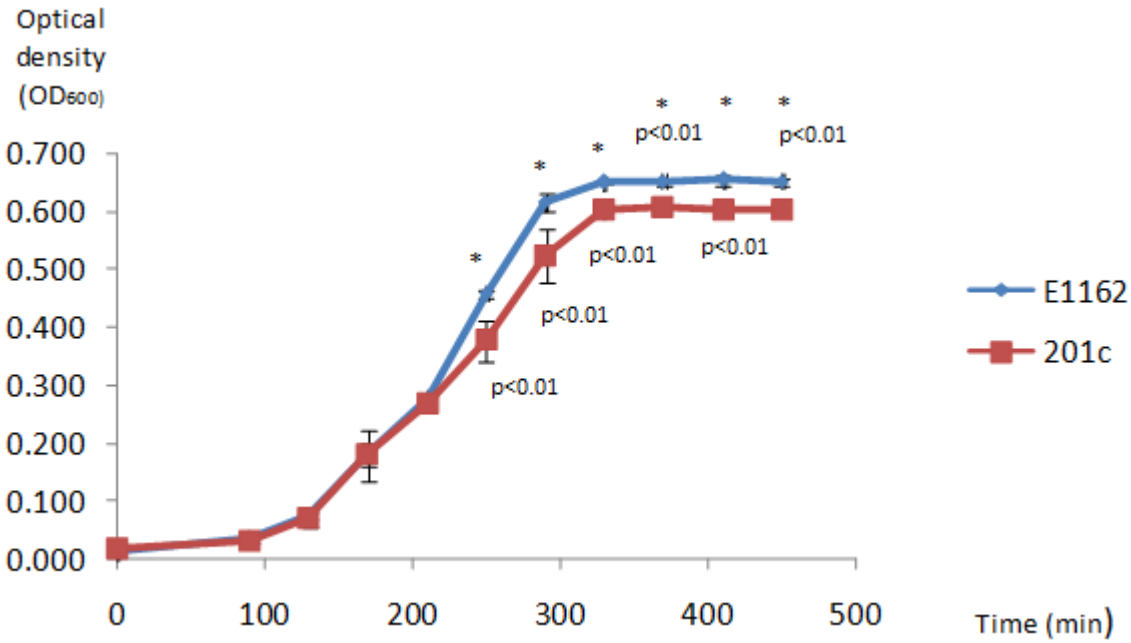


Figure 4.10 Analysis of growth kinetics of the *stk1* mutant compared to the wild-type strain in TSB without dextrose. Blue: *E. faecium* E1162 and Red: *stk1* mutant. Data presented is the mean of three independent experiments (n=9) with error bars showing the standard deviations. The significance of the difference between *E. faecium* E1162 ($p<0.01$) and *stk1* mutant (from 270 minutes onwards were analysed with the Mann-Whitney U test.

Figure 4.10 shows that the *stk1* mutant grew as rapidly as wild-type cells until it reached mid-exponential phase when culture density was lower than the parental strain (Figure 4.10).

4.3.3 Sensitivity to bile salts

To determine if the *stk1* gene plays a role in the capacity of the bacterium to survive in the human gut, we examined the sensitivity of the *stk1* mutant, 201c, to bile salts in

comparison with the wild type strain. The results showed that the *stk1* mutant was approximately two-fold more sensitive to both sodium deoxycholate and sodium cholate compared to the wild-type strain (Table 4.4).

Table 4.4 The minimum inhibitory concentrations of bile salts against *E. faecium* E1162 and the *stk1* mutant 201c. Data presented are the mean of three independent experiments.

Bile salt	***E.faecium* E1162**	***E. faecium* 201c**
Sodium deoxycholate	0.05-0.10 %	0.05 %
Sodium cholate	2.50-5.00 %	0.30-2.50 %

4.3.4 Antibiotic sensitivity

Antibiotic sensitivity testing was performed to see if there were differences between the wild type strain and the *stk1* mutant, 201c (Table 4.5). Antibiotics which target penicillin-binding proteins including ampicillin, imipenem and the broad-spectrum cephalosporins (ceftazidime, cefotaxime, ceftriaxone, cefoxitin and cefuroxime) were used. In addition, vancomycin that targets terminal D-Ala-D-Ala dipeptide, bacitracin that binds to C_{55}-isoprenyl pyrophosphate and nisin binds to lipid II were also used. Antibiotics which do not target the cell wall such as tetracycline and gentamicin that target the 30S ribosome were used. Erythromycin that targets 50S ribosome was also tested.

Table 4.5 Minimum inhibitory concentrations of antibiotics against *E. faecium*. Data presented are three independent experiments, each using three individual colonies as replicates.

Mode of Action	Antibiotic	Wild type	Stk1 Mutant
Cell wall synthesis	Ceftazadime	131 mg/mL	65.5 mg/mL
Cell wall synthesis	Cefotaxime	>1024 µg/mL	64-256 µg/mL
Cell wall synthesis	Ceftriaxone	>1024 µg/mL	16-32 µg/mL
Cell wall synthesis	Cefoxitin	256 µg/mL	256 µg/mL
Cell wall synthesis	Cefuroxime	>1024 µg/mL	>1024 µg/mL
Cell wall synthesis	Ampicillin	64 µg/mL	32-64 µg/mL
Cell wall synthesis	Vancomycin	1-2 µg/mL	0.5-1 µg/mL
Cell wall synthesis	Imipenem	64-256 µg/mL	32-128 µg/mL
30S subunit	Tetracycline	128 µg/mL	128 µg/mL
50S subunit	Erythromycin	2 µg/mL	2 µg/mL
30S subunit	Gentamicin	64 µg/mL	32-64 µg/mL
Cell wall	Bacitracin	128 µg/mL	128 µg/mL

The *stk1* mutant was, in general, more sensitive to the cell wall-active antibiotics compared to the wild-type strain, *E. faecium* E1162. Somewhat surprisingly, *E. faecium* E1162 was resistant to a very high concentration of ceftazadime, 131 mg/mL and interestingly, the *stk1* mutant displayed a two fold sensitization compared to the wild type strain. For both cefotaxime and ceftriaxone, whereas the wild type strain was resistant to more than 1024 µg/ml, an increase in susceptibility of approximately 8-fold and 32-fold was observed in the *stk1* mutant for cefotaxime and ceftriaxone, respectively. Further, a two

fold increase in susceptibility was observed in the *stk1* mutant for vancomycin, nisin and imipenem relative to the wild type strain.

4.3.5 Triton X-100-induced autolysis assays

Having shown that the *stk1* mutant displayed increased susceptibility to some cell wall-active antibiotics such as ceftriaxone, we sought to determine what other phenotypic defects the mutant might have. Autolysis is the result of peptidoglycan hydrolases action on the bacterial cell wall and resulting cellular lysis. Thus, Stk1 probably plays important role in protecting the peptidoglycans from autolysins. A Triton X-100-induced autolysis assay was done to compare the susceptibility of the *stk1* mutant and wild type strain (Figure 4.11). This was done in both the presence and absence of sodium chloride to identify the effect of sodium chloride on the autolysis (Gilpin et al., 1972). Sodium chloride produces changes in electrochemical properties in bacterial cell wall (Steen et al., 2005). The result showed that the *stk1* mutant was significantly more sensitive to triton X-100 in the presence of sodium chloride compared to the wild type strain showing 79 % lysis at 270 min compared to 67 % lysis for the wild type strain.

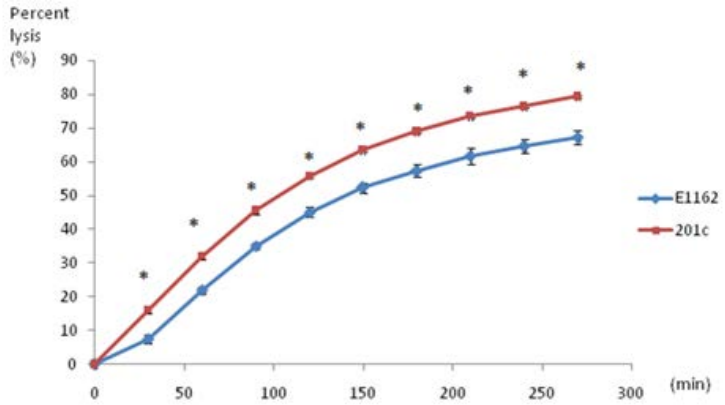


Figure 4.11 Autolysis assay in 0.1 % triton X-100 and 1 M NaCl. Data presented are the mean of three independent experiments with error bars showing the standard deviations. The significance of the difference between Blue: *E. faecium* E1162 and Red: the *stk1* mutant was determined using the Mann-Whitney U test.*= $p < 0.01$

Susceptibility to triton X-100 was also determined in the absence of sodium chloride (Figure 4.12). This data showed that the *stk1* mutant was significantly more resistant to autolysis compared to *E. faecium* E1162. After 300 min, 20 % of the *stk1* mutant population was lysed compared to 27 % of the wild type population.

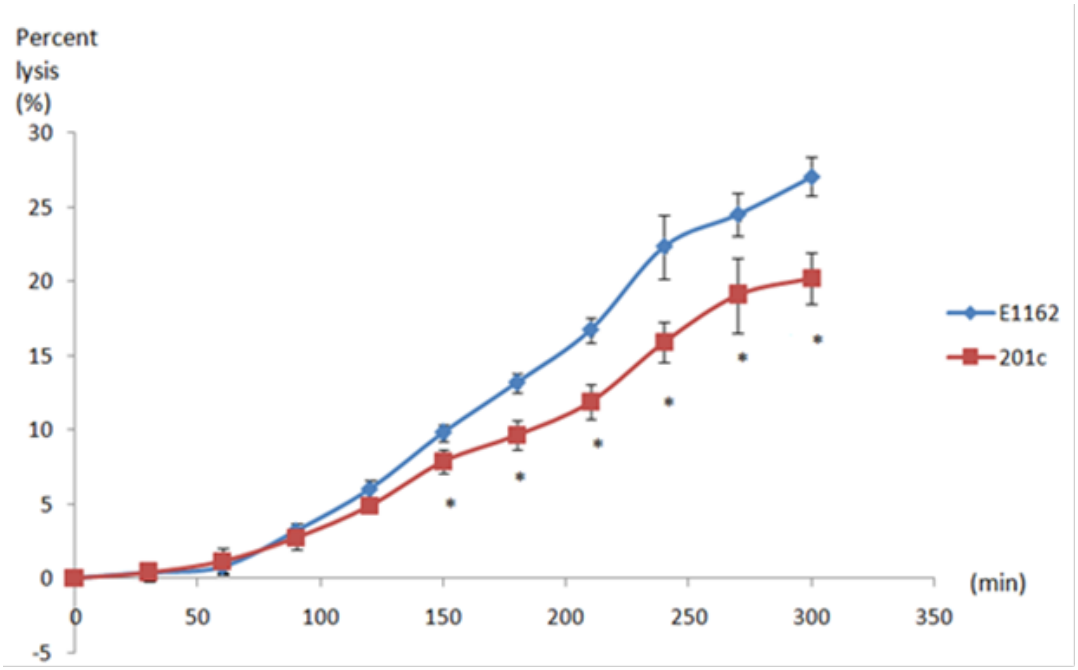


Figure 4.12 Autolysis assay in 0.1 % triton X-100 without 1 M NaCl. Data presented are the mean of three independent experiments with error bars showing the standard deviations. The significance of the difference between Blue: E1162 and Red: the *stk1* mutant was determined using the Mann-Whitney U test. *= $p < 0.01$

4.3.6 Lysozyme sensitivity

Lysozyme is found in tears, mucus, milk and saliva. Lysozyme attacks bacterial peptidoglycan by hydrolysis of the N-acetylmuramic acid (NAM) and N-acetyl-glucosamine (NAG) residues (Bera et al., 2005). Thus, to survive in hostile environments comprising high concentrations of lysozyme, bacteria have to developed mechanisms to resist lysozyme. To determine the lysozyme sensitivity of the *stk1* mutant and *E. faecium* E1162, lysozyme was added to exponential phase cultures (see 3.2.7) to a final concentration of 100 µg/mL, and

its effect on cell density was monitored. The percentage of lysis was calculated using the formula: present OD_{600} /highest OD_{600} x 100.

It was observed that the deletion of *stk1* confers resistance to lysozyme (Figure 4.13). The lysis of *stk1* mutant cells was reduced in the presence of lysozyme after 24 hours incubation (3.6 %), while, *E. faecium* E1162 was 7.1 %. After 48 hours incubation, the lysis of *stk1* mutant cells was 68.7 % and the wild type was 85.2 %.

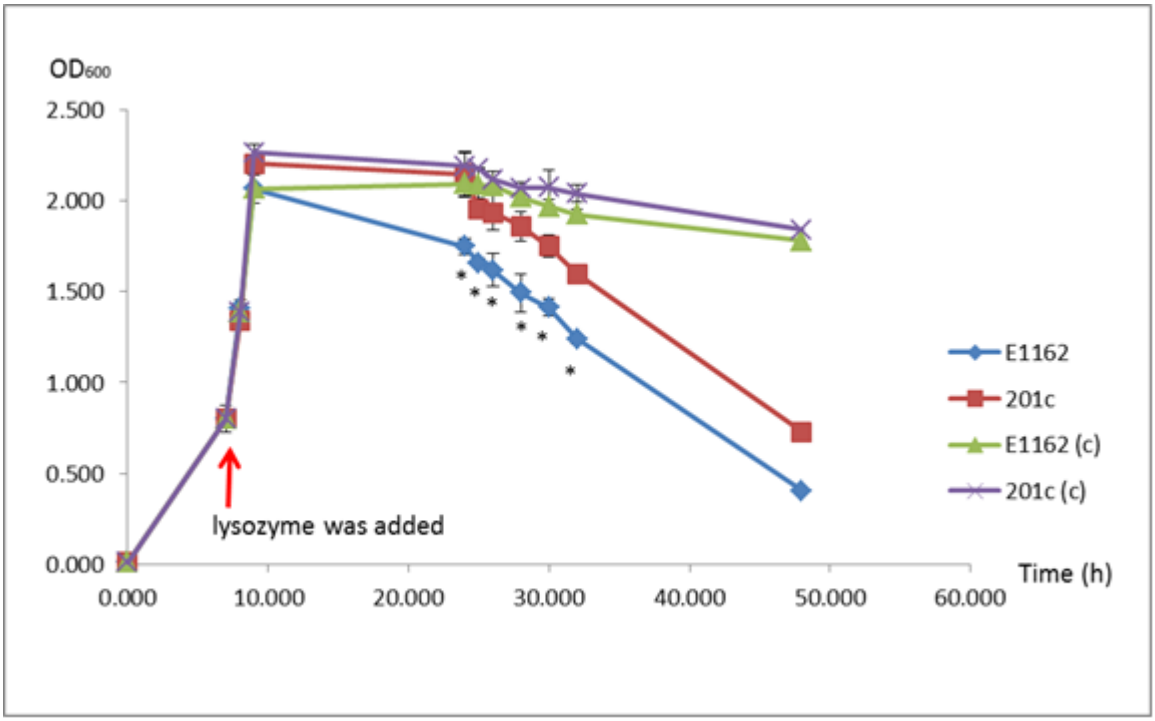


Figure 4.13 Comparison of cell lysis in the presence of 100 µg/mL lysozyme of the wild type E1162 and *stk1* mutant 201c. The symbol (c), represents the growth of the strain in tryptic soy broth. Blue: E1162 in TSB+100 µg/mL lysozyme; Red: *stk1* mutant in TSB+100 µg/mL lysozyme; Green: E1162 in TSB and Purple: *stk1* mutant in TSB. Data presented are the mean of three independent experiments with error bars showing the standard deviations. The significance of the difference between E1162 and the *stk1* mutant was determined using the Mann-Whitney U test. *= $p < 0.01$

Next, it was investigated whether a lower concentration of lysozyme (50 µg/mL) could increase the cell lysis (Metcalf and Deibel, 1969; Wilcox Jr and Daniel, 1954) of these strains (Figure 4.14). Antimicrobials have a contrasting effect at low and high concentrations (Linares et al., 2006). Lower concentrations of antimicrobial induce a niche-specific response

in bacteria (Linares et al, 2006). In this cell lysis assay, a final concentration of 50 µg/mL lysozyme was added at the mid exponential growth phase, and its effect on cell density was monitored.

The lysis of the *stk1* mutant cells was 20.8 %, while, the lysis *E. faecium* E1162 was 54.1 % in the presence of 50 µg/mL lysozyme (after 24 hours incubation). After 48 hours incubation, the lysis of *stk1* mutant cells was 65.1 % and *E. faecium* E1162 was 80.4 %.

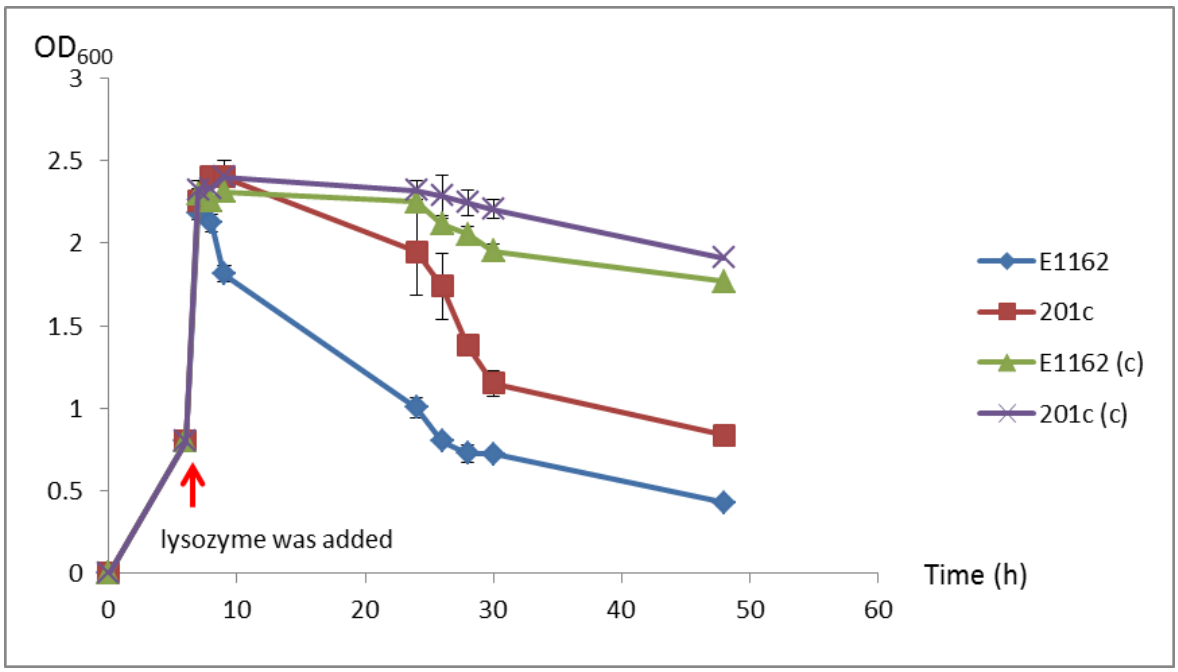


Figure 4.14 Comparison of cell lysis in the presence of 50 µg/mL lysozyme of *E. faecium* E1162 and the *stk1* mutant 201c. The symbol (c), represents the growth of strains in TSB. Blue: E1162 in TSB+50 µg/mL lysozyme; Red: *stk1* mutant in TSB+50 µg/mL lysozyme; Green: E1162 in TSB and Purple: *stk1* mutant in TSB. Data presented are the mean of three experiments with error bars showing the standard deviations. The significance of the difference between E1162 and the *stk1* mutant was determined using the Mann-Whitney U test.

4.3.7 Morphological phenotype of *E. faecium* E1162 and the *stk1* mutant

Since the *stk1* mutant showed a reduction of culture density during the stationary phase, we hypothesised that the interruption of *stk1* might have a morphological effect on the bacterial cells. The wild type and *stk1* mutant were grown in TSB without dextrose to

stationary phase (330 mins) and examined by scanning electron microscopy (SEM). No obvious morphological changes (Figure 4.15), including the size and appearance of the cell surface were observed. Bacterial cultures were also examined by transmission electron microscopy (TEM). At a higher magnification, no differences in cell size, surface and shape were observed (Figure 4.16).

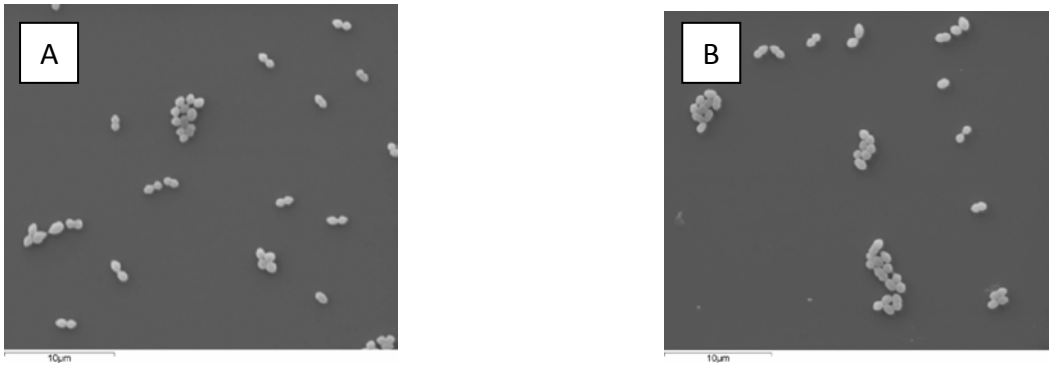


Figure 4.15 SEM images of *E. faecium.* A: wild type and B: *stk1* mutant at stationary phase. Image is from one experiment.

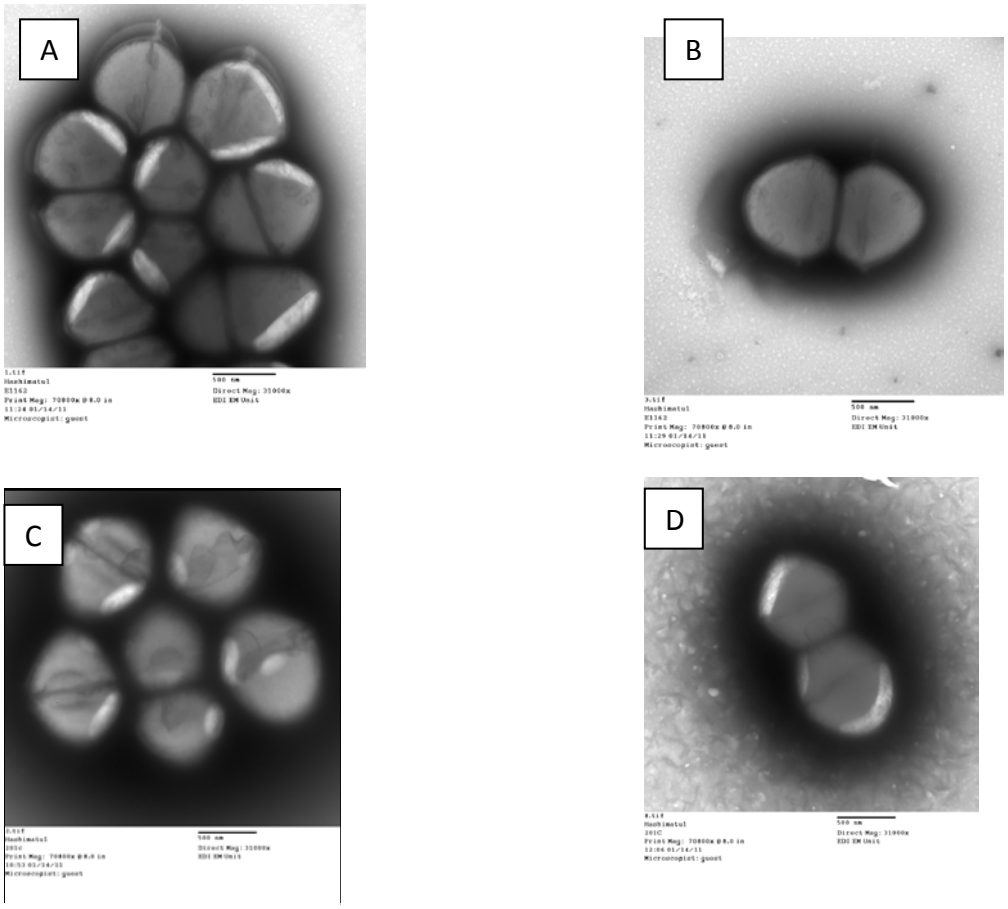


Figure 4.16 TEM images of *E. faecium.* A and B: wild type and C and D: *stk1* mutant at stationary phase. Image is from one experiment.

4.3.8 *Galleria mellonella in vivo* infection model

To study the role of *stk1* in the potential virulence of *E. faecium*, infection of *G. mellonella* wax worms with the wild type and the *stk1* mutant was evaluated (Figure 4.17). It was observed that killing was significantly ($p<0.01$) delayed for wax worms injected with *stk1* mutant compared to *E. faecium* E1162. After 96 hours incubation, 60 % of the larvae injected with *stk1* mutant were killed, versus 100 % of those infected with the wild type. This result indicates that *stk1* contributes to the virulence of *E. faecium* in the *G. mellonella* infection model.

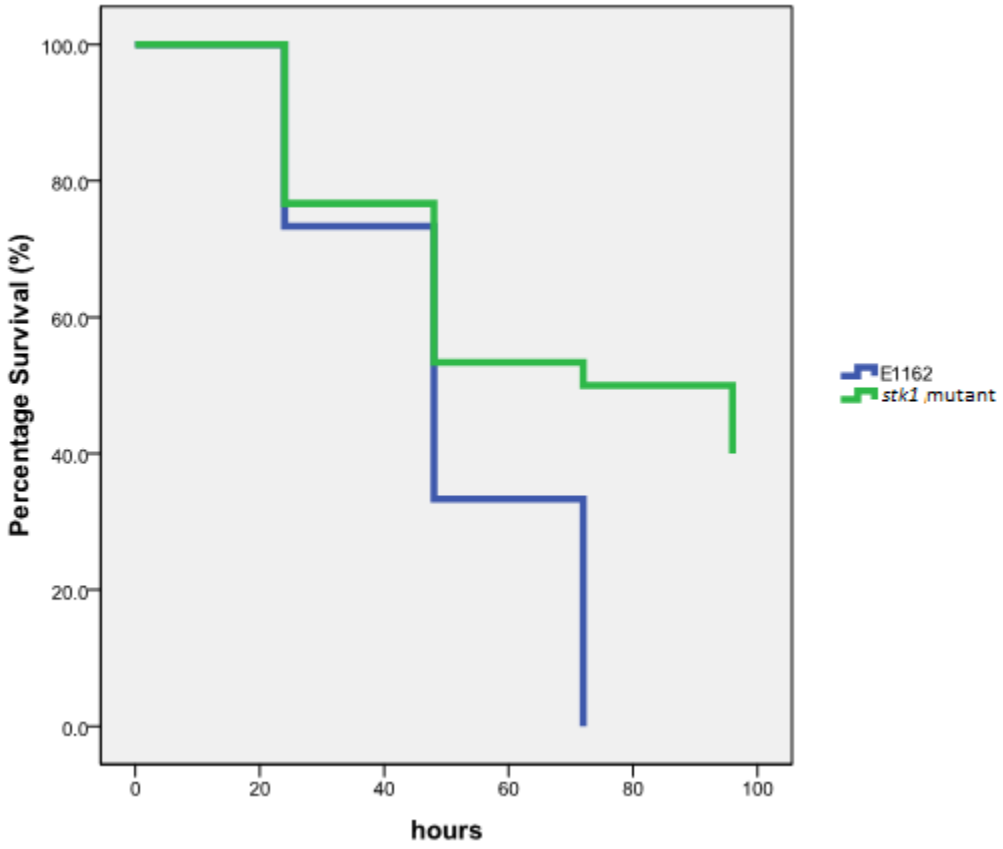


Figure 4.17 Kaplan-Meier survival curves of *Galleria mellonella* wax worms inoculated with *E. faecium*. Blue: *E. faecium* E1162 and Green: *stk1* mutant. Data presented are the percentage survival of 30 wax worms which were tested in groups of 10 on three different days.

4.3.9 Genetic complementation of *stk1* mutant and *ireK* mutant with the *E. faecium stk1* gene under the control of a kanamycin resistance gene promoter

A complemented strain was constructed in which *stk1* was expressed under the control of a constitutive kanamycin resistance gene promoter in plasmid, pHFH9. The kanamycin resistance gene resides on plasmid pJH1. The plasmid pJH1 was obtained from Dr Haitham Hussain (unpublished). The *stk1* gene was amplified with primers (refer to Table 4.3) EfmSTPKF and EfmSTPKR using genomic DNA from *E. faecium* E1162 as template. The DNA fragment obtained (2250 bp) was cloned into pHFH9. The construct was verified by sequencing. Subsequently, the purified plasmid containing the construct was introduced into the *stk1* and *ireK* mutant by electroporation.

Analysis of the *stpk* gene in the closely related species, *E. faecalis* using the online Simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/) (Letunic et al., 2009) revealed interesting similarities and differences in the predicted *stk1* of *E. faecium* and *ireK* of *E. faecalis*. Both the *stk1* of *E. faecium* and and *ireK* of *E. faecalis* have catalytic domains, a region of low complexity and a transmembrane domain. However, the numbers of PASTA domains are different in the two proteins. Four PASTA domains exist in *E. faecium* Stk1 while five PASTA domains are found in *E. faecalis* IreK protein (Figure 4.18).

Given the differences in the PASTA domains between the *E. faecalis* and *E. faecium* STPKs, and the differences in peptidoglycan structure (Figure 4.18), it was decided to see if *E. faecalis* IreK could complement the *E. faecium stk1* mutant. Description of the plasmid used in the present study is shown in Table 4.3. The *ireK* gene was amplified with primers EfcSTPKF and EfcSTPKR using genomic DNA from OG1RF as template. The DNA fragment

obtained (2302 bp) was cloned into pHFH9 under the control of the kanamycin promoter. The construct was verified by sequencing. Subsequently, the purified plasmid containing the *stk1* or *ireK* was introduced into the *stk1 and ireK* mutant by electroporation.

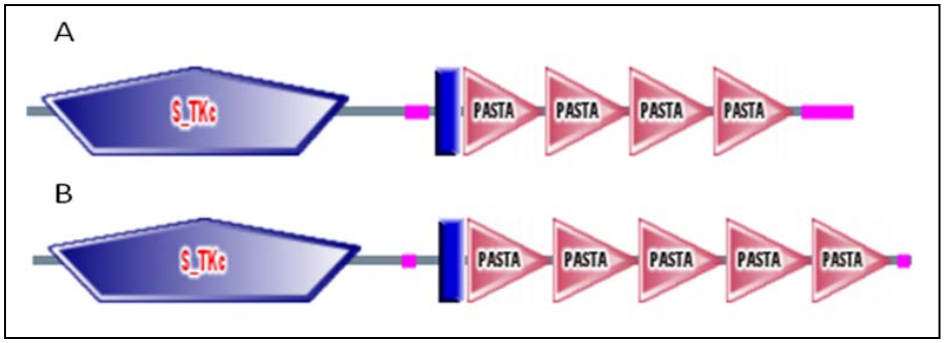


Figure 4.18 Architecture of the STPKs in (A) *E. faecium*; (B) *E. faecalis*; S_TKc, serine/threonine protein kinase domain (blue diamond); low complexity region (square pink), transmembrane domain (blue square); PASTA, domains (pink triangle). The domain was identified using SMART (http://smart.embl-heidelberg.de).

E. faecium E1162 containing the vector pHFH9 (without an insert) was resistant to more than 1024 µg/mL ceftriaxone (Table 4.6). As expected, the *stk1* mutant, 201c, containing the vector pHFH9 was sensitive to 32 µg/mL. The *stk1* mutant, 201c, containing plasmid pHFH10 expressing the *E. faecium stk1* gene under the control of the constitutive kanamycin resistance gene promoter had an MIC of 1024 µg/mL confirming that the increased sensitivity of the mutant to cephalosporins was due to disruption of the *stk1* gene. *E. faecium* 201c containing plasmid pHFH11 expressing the *E. faecalis ireK* gene had a restored resistance to ceftriaxone.

Table 4.6 Complementation of *E. faecium* 201c with the *E. faecium stk1* gene and *E. faecalis ireK* gene

Antibiotic	E1162+pHFH9	201c+ pHFH9	201c+ pHFH10	201c+ pHFH11
ceftriaxone	>1024 µg/mL	32 µg/mL	1024 µg/mL	1024 µg/mL

pHFH9=empty vector, pHFH10: *stk1* from *E. faecium;* pHFH11= *ireK* from *E. faecalis*

4.3.10 Genetic complementation of *ireK* mutant, *E. faecalis* CK119 with the *E. faecalis ireK* gene and *E. faecium stk1* gene

The wild type *E. faecalis* OG1RF containing the the vector pHFH9 (without an insert) was resistant to more than 512 µg/mL ceftriaxone. As expected, the *ireK* mutant *E. faecalis* CK119 containing the vector pHFH9 was sensitive to 32 µg/mL. Mutant *E. faecalis* CK119 containing plasmid pHFH10 expressing the *E. faecalis ireK* gene and *E. faecium stk1* gene, had a restored resistance to ceftriaxone (Table 4.7)

Table 4.7 Complementation of *E. faecialis* CK119 with the *E. faecium stk1* gene and *E. faecalis ireK* gene

Antibiotic	OG1RF+ pHFH9	CK119+ pHFH9	CK119+ pHFH10	CK119+ pHFH11
ceftriaxone	512 µg/mL	32 µg/mL	1024 µg/mL	512 µg/mL

pHFH10= *stk1* from *E. faecium;* pHFH11= *ireK* from *E. faecalis*

The plasmid pHFH11, which coded for the IreK of *E. faecalis* restored the resistance of the *E. faecium stk1* mutant, increasing the MIC of this strain to 1024 µg/mL ceftriaxone (Table 4.7).

4.3.11 Examination of the role of *stk1* in controlling the Esp, PilA and PilB proteins

We also evaluate if disruption of the *stk1* gene in *E. faecium* had an effect on protein expression of virulence determinants in *E. faecium* E1162. Three virulence proteins were tested in this study, which are Esp, PilA and PilB. The ability of Stk1 in controlling the expression of murein hydrolases was also tested. The SDS-polyacrylamide gel electrophoresis of *E. faecium* strains tested is shown Figure 4.19 shows the equal loading of proteins in all samples.

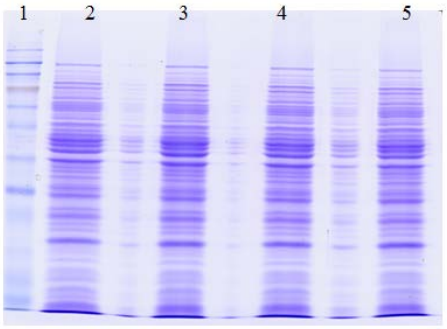


Figure 4.19 SDS-polyacrylamide gel electrophoresis of *E. faecium* strains tested. This shows the equal loading of proteins in all samples. Lane 1: ColorPlus™ Prestained Protein Ladder, Broad Range (10-230 kDa); Lane 2: E1162+pHFH9; Lane 3: stk1 mutant +pHFH10; Lane 4: stk1 mutant +pHFH9 and Lane 5: Δesp

4.3.11.1 Expression of enterococcal surface protein (Esp) in *E. faecium*

The expression of Esp in the wild type and the *stk1* mutant was evaluated (Figure 4.20). The western blot analysis revealed no obvious differences in the expression of Esp protein (212, 630 daltons) in all strains tested.

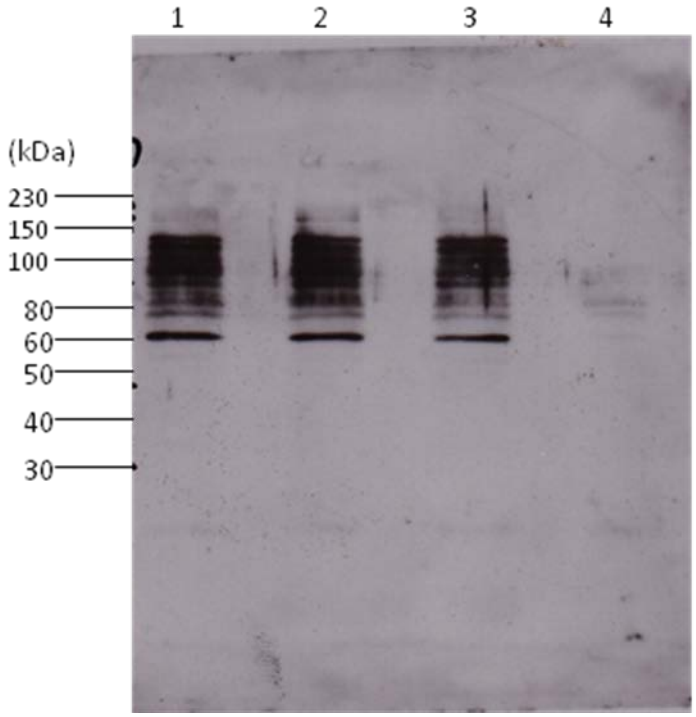


Figure 4.20 Western blots of surface protein extracts of *E. faecium* using rabbit anti-esp immune serum. Lane 1: E1162+pHFH9; Lane 2: 201c+pHFH10; Lane 3: 201c+pHFH9 and Lane 4: Δ*esp*

4.3.11.2 Expression of Pil-A and Pil-B type pili

Two distinct pilus like structure (PilA and PilB), were identified on the surface of a hospital-adapted *E. faecium* bloodstream isolate (Hendrickx et al., 2008). The expression of PilA and PilB in wild type and *stk1* mutant was evaluated (Figure 4.21 and Figure 4.22).

The western blot analysis indicated that the PilA (64, 800 daltons) and PilB (61, 000 daltons) expression were similar in all strains tested. Non-specific protein binding were present in the wild type, in the complementation strain and in the PilA mutant. This non-specific protein binding was absent in the *stk1* mutant *E. faecium* 201c.

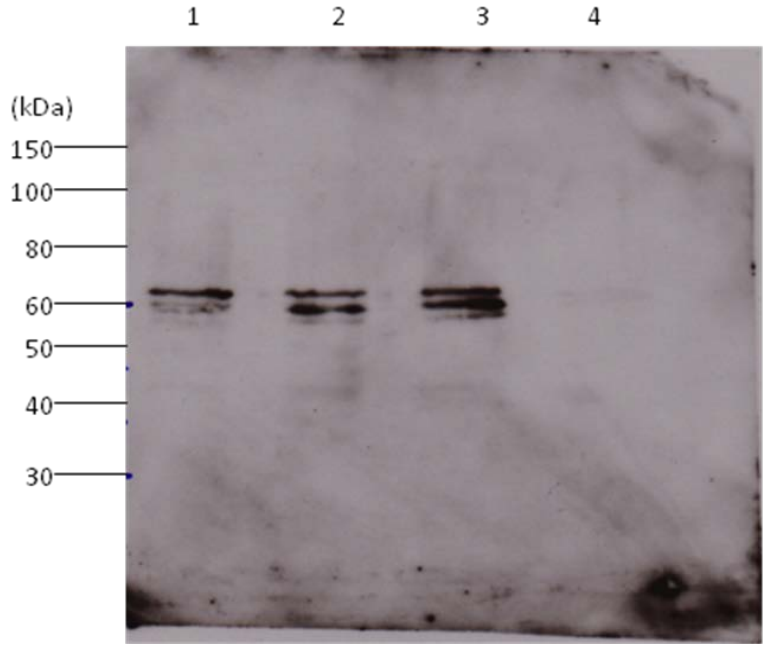


Figure 4.21 Western blots of surface protein extracts of *E. faecium* using rabbit anti-pilA immune serum. Lane 1: E1162+pHFH9; Lane 2: 201c+pHFH9; Lane 3: 201c+pHFH10 and Lane 4: Δ*pili*A::*ermC*

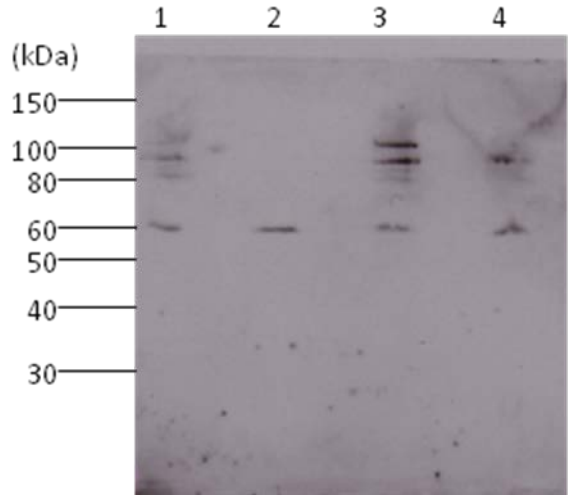


Figure 4.22 Western blots of surface protein extracts of *E. faecium* using rabbit anti-pilB immune serum. Lane 1: E1162+pHFH9; Lane 2: 201c+pHFH9; Lane 3: 201c+pHFH10 and Lane 4: Δ*pili*A*::ermC*

4.3.11.3 Expression of murein hydrolases

The expression of murein hydrolases or autolysins in *E. faecium* was also tested. Zymography analysis was done in the presence of 1 mg/mL of *M. lysodeikticus* cells. Two catalytically active protein bands of were observed (80, 000 daltons and 50, 000 daltons)

(Figure 4.23). The zymographic analysis indicated that the expression of murein hydrolases was similar in all strains tested.

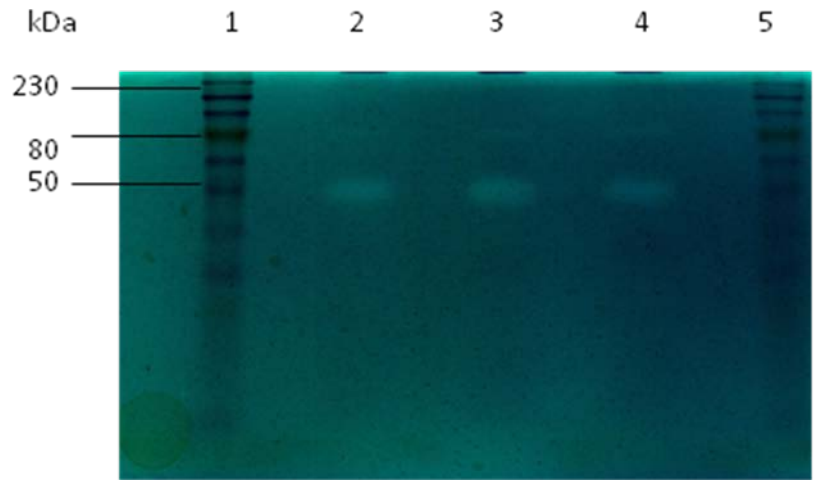


Figure 4.23 Zymographic analysis of extracellular murein hydrolases. Lane 1: ColorPlus™ Prestained Protein Ladder, Broad Range (10-230 kDa); Lane 2: E1162+pHFH9; Lane 3: 201c+pHFH9; Lane 4: 201c+pHFH10 and Lane 5: ColorPlus™ Prestained Protein Ladder, Broad Range (10-230 kDa)

4.4 Discussion

While the *E. faecium stk1* mutant was being constructed, a study on a STPK (annotated as IreK) of *E. faecalis* was published (Kristich et al., 2007). According to Kristich, the disruption of *ireK* in this bacterium led to a growth defect under glucose limiting conditions (Kristich et al., 2007). Thus, in the present study, analysis of the bacterial growth was done in the same medium (TSB in the absence of dextrose). Similar growth defects have been reported in other bacteria where the *stk1* gene has been knocked out, as for example in *S. pyogenes* (Jin and Pancholi, 2006). In the present study, a growth defect was detected in *stk1* mutant demonstrating that the STPKs of these two bacteria might have different regulatory roles. We also found that the *stk1* mutant was resistant to a high level of ceftazadime but displayed a two fold sensitization relative to the wild type. This was in

contrast to *E. faecalis* where the *ireK* mutant had at least a 32-fold increase in sensitivity to ceftazadime. It was observed that collapsed cells and cells with lesions in the cell wall were present when *E. faecalis ireK* mutant was cultivated in tryptic soy broth without dextrose (Kristich et al., 2007). However, none of these morphological defects were observed in the *stk1* mutant when cultured in the same medium but some caution is required with these results since they were done with stationary phase cells. The exponential phase cultures should be examined as these may have morphological defects. We decided to examine a number of other phenotypes that had been shown to be affected by the disruption of the *ireK* gene in *E. faecalis* (Kristich et al., 2007). We investigated if there were other differences in the function of the Stk1 and IreK in these two closely related species. The presence of bile salts is a stress experienced by this bacterium in its environmental niche. In fact, sodium deoxycholate and sodium cholate are the two principal antimicrobial components in the bile salts of the gastrointestinal tract (Begley et al., 2005). Our result showed that the *stk1* mutant displayed an increased sensitivity to both sodium deoxycholate and sodium cholate compared to the wild type *strain.* This indicates that *stk1* may play a role in promoting the survival of *E. faecium* in the intestinal tract.

It is well known that beta-lactam antibiotics are poorly active against enterococci (Murray, 1990) and so it was not a surprise to find that the wild type was resistant to all of the cephalosporins examined. In general, the *stk1* mutant was more sensitive to the antibiotics tested compared to *E. faecium* E1162. It was reported that an *E. faecalis* mutant lacking the *ireK* gene shows an enhanced sensitivity to antibiotics that target PBPs (Kristich et al., 2007). The intrinsic resistance to cephalosporins in this bacterium was regulated by both *ireK* and *ireP*. The *ireP*, which encodes a protein phosphatase is located upstream of *ireK*. The deletion of *ireP* showed hyperresistance to cephalosporins and this

hyperresistance phenotype in this mutant was mediated by highly phosphorylated *ireK*. Thus, the *ireK* activity was negatively controlled by IreP (Kristich et al., 2007).

The result of complementation *in trans* showed that the *stk1* mutant, *E. faecium* 201c containing plasmid pHFH10 expressing *E. faecium stk1* gene and plasmid pHFH11 expressing *E. faecalis ireK*, had restored resistance to ceftriaxone. *E. faecalis ireK* mutant containing plasmid pHFH10 and pHFH11 also showed restored resistance to ceftriaxone. This data indicate that the *stk1* was able to complement *ireK* mutant *E. faecalis* and *ireK* was able to complement *stk1* mutant. This suggests that serine threonine protein kinase in both strains are able to produce the same signal or molecule, which plays a role in cephalosporins resistance.

A two-fold sensitization to vancomycin, imipenem and nisin of the *stk1* mutant was observed relative to *E. faecium* E1162. The bypass of the DD-transpeptidases by the LD-transpeptidase (Ldtfm) contributes to high level of resistance to ampicillin in *E. faecium* (Mainardi et al., 2000). The Ldtfm catalysed the formation of L-lysyl$_3$-D-alanine$_4$ crosslinks instead of the classical D-lysyl$_3$-D-alanine$_4$ crosslinks (Mainardi et al., 2000). In that study, they found that LD-transpeptidase is not as sensitive to β-lactam antibiotics as DD-transpeptidase. Later, they found that Ldtfm was inihibited by imipenem (Mainardi et al., 2007). Recent study demonstrates that *stk1* involved in the activation of the L,D-transpeptidation pathway (Sacco et al., 2014). Although our results revealed that *E. faecium* E1162 and *stk1* mutant have similar sensitivity to ampicillin, two fold sensitivity of *stk1* mutant to imipenem was observed in this study. Our result suggests an altered cell wall in *stk1* mutant (but not detected using TEM and SEM). The *stk1* mutant was more susceptible to nisin. It has been shown in other gram-positive bacteria such as *Lactococcus lactis* that higher D-Asp amidation levels in peptidoglycan decreases the charge of the cell wall and

causes higher sensitivity to the cationic antimicrobials nisin (Veiga et al., 2009). However, further analysis is still required to analyze the peptidoglycan structure of both strains tested by reverse phase high performance liquid chromatography and mass spectrometry.

Our result showed that the *stk1* mutant has higher susceptibility to ceftriaxone, it might suggest that there may be differences in the cell walls of these two strains. We therefore examined other possible phenotypic cell wall deficiencies that might be found in the *stk1* mutant. We tested this by performing Triton X-100-induced autolysis assays. *E. faecium* E1162 was more resistant to autolysis compared to the *stk1* mutant in the presence of NaCl. The NaCl was added to evaluate the physiological conditions on autolysis. However, in the absence of NaCl, the *stk1* mutant was less susceptible to autolysis. Higher rates of autolysis in both strains were observed in the presence of NaCl. In other gram-positive bacteria such as *S. aureus*, the presence NaCl has different effects on murein hydrolase activity (Tobin et al., 1994). In that study, higher rate of autolysis were observed in the presence of 1.5 M NaCl since the activities of cell wall-bound murein hydrolases were inhibited (Tobin et al., 1994). In contrast to this finding, other researchers had demonstrated that the addition of NaCl actually enhanced the activity of N-acetylmuramy-L-alanine amidase (Foster, 1992). Our zymographic analysis indicated that the expression of autolytic enzymes was similar in *stk1* mutant compared to the wildtype. We do recognize the limitation of our study that this method might have failed to detect the expression of murein hydrolases quantatively. Therefore, to elucidate further the capability of *stk1* in controlling autolysins of *E. faecium*, the expression levels of genes encoding autolysins should be evaluated in cells grown in the presence of subinhibitory concentrations of ceftriaxone.

Lysozyme constitutes a first line of defence against infection where it is found in other human body fluids such as tears, saliva and milk (Masschalck and Michiels, 2003). Lysozyme (an *N*-acetylglucosamyl-*N*-acetylmuramidase) acts on the invading bacteria by hydrolyzing the β—1, 4 glycosidic bonds between *N*-acetylmuramic acid (MurNac) and N-acetylglucosamine (GluNac). Thus, peptidoglycan degrades and this causes subsequent cell lysis (Jolles and Jolles, 1984). Besides having muramidase activity, lysozyme also acts as a cationic antimicrobial peptide (CAMP) (Meehl et al., 2007). We also found that the deletion of *stk1* confers resistant to lysozyme compared to *E. faecium* E1162. This result might indicate that the interruption of the *stk1* gene might affect the cell wall formation of this bacterium.

The ability of *stk1* in controlling the expression of autolysins was evaluated. The expression of autolysin was similar in all strains. In another study, major autolysin in this strain was identified and functionally characterized (Paganelli et al., 2013). Inactivation of major autolysin (E1162_2692) resulted in a reduction of extracellular DNA, reduced early adhesion and less biofilm formation. The mutant was resistant to lysis and possessed a chaining phenotype. It was also observed that this autolysin was involved in a collagen-binding adhesin, Acm surface localization. Less binding to collagen types I and IV was observed. In zymography analysis, the major autolysin (75, 000 daltons) was absent in major autolysin mutant (Paganelli et al., 2013). The second autolysin (50, 000 daltons) and third autolysin (37, 000 daltons) were also identified. In that study, zymography analysis was done in the presence of heat-killed *E. faecium* E1162 cells (Paganelli et al., 2013). Our study revealed only two different sizes of autolysins when zymography analysis was done in the presence of 0.2 % *M. lysodeikticus* cells (80, 000 daltons and 50, 000 daltons). The

third autolysin was not observed in our study. Thus, indicates that the third autolysin might be specific to *E. faecium*.

Based on the western blot analysis, no differences in the Esp, pilA and pilB type pili expression were observed in the wild type and the *stk1* mutant. The mutant of this gene also displayed significant impaired in killing *G. mellonella* larvae compared to the wild type *E. faecium* E1162. Thus, indicates that this gene plays an important role in virulence mechanisms and persistence of this bacterium during infections. However, the virulence profile of the complementation strain was closer to the *stk1* mutant. This might be due to the instability of the plasmid, which could result in a significant loss in expression of *stk1 in trans*.

We conclude that *stk1* plays important role in cell-wall stresses, antibitiotics resistance and virulence in *E. faecium* E1162.

Chapter 5

The Role of an Uncharacterized ABC Transporter in Resistance to Antimicrobial Peptides and Virulence of *Enterococcus faecium*

5 The Role of an Uncharacterized ABC Transporter in Resistance to Antimicrobial Peptides and Virulence of *Enterococcus faecium*

5.1 ABC transporters

ATP-binding-cassette (ABC) transporter consists of four domains; two transmembrane domains (TMD) and two nucleotide-binding domains (NBD). NBD are hydrophilic and responsible for the ATP binding and hydrolysis to power the transport. NBD also contain the highly conserved Walker A and B consensus motifs. TMD consists of membrane-spanning helices which are hydrophobic and form a translocation pathway through the lipid bilayer. The membrane-spanning helices sometimes is referred to as a permease or transporter. (Higgins, 1992; Tomii and Kanehisa, 1998). Certain ATP-binding-cassette (ABC) transporters have been recognized to play a role in the resistance to antimicrobial peptides (AMP) among other bacteria (Dintner et al., 2011) such as In *Bacillus subtilis* (Staron et al., 2011), *Streptococcus mutans* (Ouyang et al., 2010) and *Staphylococcus aureus* (Yang et al., 2012; Meehl et al., 2007). ABC transporters and two component systems are co-involved in antimicrobial peptide resistance (Falord et al., 2012). Two component systems involve two different proteins, which consist of histidine kinase and response regulator. Usually the histidine kinase acts as a sensor protein and activates its cognate response regulator. In ABC transporter AMP resistance modules, the histidine kinase is unable to detect the presence of antimicrobial peptides and it requires the ABC transporter for signalling (Staron et al., 2011; Yang et al., 2012; Meehl et al., 2007). Many of the intramembrane-sensing histidine kinases are located next to neighbourhood genes encoding ABC-transporters (Mascher, 2006). Phylogenetic analysis of the permeases and sensor kinases indicates a tight evolutionary correlation between the components. Phylogenetic tree analysis of the

transport permeases identified eight distinct groups (I-VII). Only permeases from Group VI are not associated with a two component systems (Dintner et al., 2011).

Susanne Gebhard (Gebhard, 2012) classified ABC transporters involved in resistance to antimicrobial peptides in the Firmicutes into five distinct groups based on their domain structure (Table 5.1).

Table 5.1 Types of ABC transporters that have been implicated in resistance to AMPs in Firmicutes (Gebhard, 2012)

Type of AMP transporter	**Characteristics of the transporters**	**Size of permease/s(number of amino acids)**	**Number of predicted transmembrane helices in the permease**
Sun-T	-Large transporters -ATPase domain fused to the C-terminus of the permease domain -N-terminal C39 peptidase domain	700	5 or 6
Nis-T	-Similar to Sun-T type transporters -Absence of peptidase domain	550-600	5 or 6
LanFEG	-Two separate permeases -One ATPase	200–250	6
BceAB	- One ATPase - One permease	650	10 (with a large extracellular domain located between helices VII and VIII)
BcrAB	-1 ATPase -1 permease	230	6

Among the ABC transporters listed in Table 5.1, the BceAB-like two component systems are widespread in firmicutes and constitute part of antimicrobial peptide-resistance modules (Dintner et al., 2011). The best-characterized example of an antimicrobial peptide-

resistance module is BceRSAB in *Bacillus subtilis* (Tetsch and Jung, 2009; Dintner et al., 2011). In this module, the two-component system, BceRS consist of histidine kinase, BceS, and its cognate response regulator, BceR. The proteins BceAB form an ATP-binding cassette transporter which consists of a NBD, BceA and a TMD, BceB. The BceRSAB proteins in this module are important for sensing and transducing of the signal. This module is responsible for conferring resistance to several peptide antibiotics such as bacitracin, plectasin, mersacidin and actagardine (Staron et al., 2011). It has been observed that the BceAB-type permeases share a distinctive domain architecture with 10 transmembrane helices and a large extracellular domain between transmembrane helices 7 and 8 that constitutes about 200 amino acids. The large extracellular loop was identified as the actual binding site for the AMPs in the *S. aureus* BceB (Hiron et al., 2011). This distinctive domain architecture is highly conserved among all BceB-like proteins (Dintner et al., 2011). The BceAB-like ABC transporters are classified in the peptide-7 exporter (Pep7E) family in the Transport Classification Database (TCDB). Currently there is no published information on the transport mechanism of Pep7E-type systems (Saier, Jr. et al., 2009).

In this study, two putative permeases that may be involved in antimicrobial peptide resistance have been identified in the genome sequence of *Enterococcus faecium* E1162 (EfmE1162_0264 and EfmE1162_1458). Both of these permeases are not associated with neighboring two-component systems. EfmE1162_0264 has a large extracellular domain between helices 8 and 9 while EfmE1162_1458 has a large extracellular domain and between helices 7 and 8. Since the large extracellular loop was identified as the actual binding site for the AMPs in *S. aureus* (Hiron et al., 2011) it is possible large extracellular domain of EfmE1162_0264 and EfmE1162_1458 have similar function. Protein sequences of EfmE1162_0264 and EfmE1162_1458, share only 19 % overall protein sequence identity.

Further comparison of these putative permease components is shown in Table 5.2. These two putative BceAB-type permeases are classified in group VI since they are not associated with two-component system (orphan ABC) (Dintner et al., 2011). According to Sebastian Dintner, group I-VII permeases (except for group VI, BceAB-type permeases) have a tight evolutionary correlation with neighboring two-component systems (TCS) and form self-sufficient detoxification modules against antimicrobial peptides (Dintner et al., 2011).

Since there are no neighboring two-component system genes associated near the genes for these permeases, it was hypothesized that the predicted large extracytoplasmic domain of permease component might play a role as a sensory module of an orphan ABC transporter and may be involved in antimicrobial peptides resistance. A markerless EfmE1162_0264 mutant was constructed and its phenotype compared to the parental strain. We showed that EfmE1162_0264 contributes to the AMP resistance of *E. faecium* E1162.

5.2 Results

5.2.1 Bioinformatic analysis of the two putative ABC transporters that may be involved in AMP resistance

In the present study, two putative BceB-like permeases (EfmE1162_0264 and EfmE1162_1459) were identified in *E. faecium*. These permeases are predicted as BceB-like permease since they have a large extracellular domain located between the transmembrane helices (Table 5.1). The first ABC transporter of *E. faecium* consists of a putative permease, EfmE1162_0264 and a putative nucleotide binding domain protein (EfmE1162_0265) (Figure 5.1). Using the online prediction tools available in SMART (http://smart.embl-heidelberg.de), the membrane-spanning domain for the putative permease,

EfmE1162_0264, is predicted to have 11 transmembrane domains (Table 5.1). The EfmE1162_0264 is located downstream of EfmE1162_0265, which encodes a putative ATPase component. A large extracellular domain between helices 8 and 9 that consists of 238 amino acids was identified in this putative permease EfmE1162_0264. The *dlt* (D-Alanylation of lipoteicoic acids) operon is located downstream of the orf EfmE1162_0264. The *dlt* operon in this bacterium consists of five genes (*dltX*, *dltA*, *dltB*, *dltC* and *dltD*). The genetic organization of the *dlt* operon is similar to *E. faecalis* V583, where the *dltX* is located upstream of *dltA* (Le Jeune et al., 2010). In *Bacillus subtilis*, the *dlt* operon consists of five genes (*dltA*-*dltE*) (Perego et al., 1995). The first gene in the *dlt* operon (*dltA*) is predicted to code for a D-alanine-D-alanyl carrier protein ligase (also known as D-alanine-activating enzyme),while *dltC* is predicted to code for the alanyl carrier protein (Dcp) and DltB which is identified in other organisms is responsible for transportation of activated D-alanine. The *dltD* gene is required for the incorporation of D-alanine into lipoteichoic acid (LTA) and wall teichoic acid (WTA) (Perego et al., 1995).

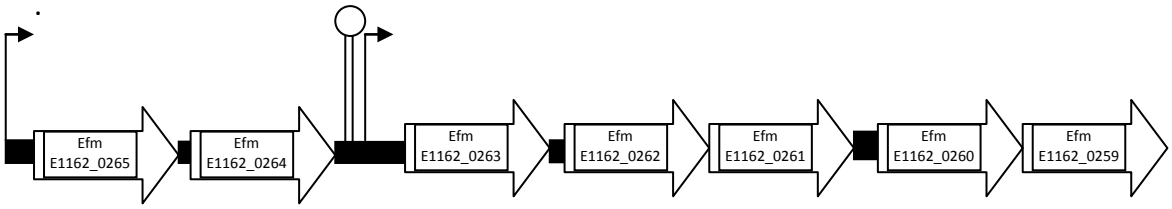
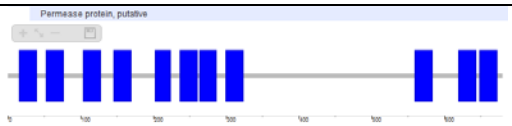
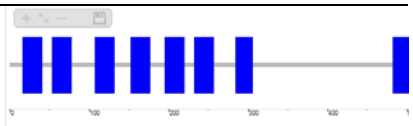


Figure 5.1 Genetic organization of the region downstream of the orf coding for a putative permease, EfmE1162_0264 of *E. faecium* E1162. EfmE1162_0263=putative D-Ala-teichoic acid biosynthesis protein; EfmE1162_0262= D-alanine-activating enzyme; EfmE1162_0261= DltB protein; EfmE1162_0260 = D-alanyl carrier protein; EfmE1162_0259= DltD protein and E1162_0265 = putative ATPase component.

Table 5.2 Comparison of the domain structure of the permease components. The domains were identified using SMART (http://smart.embl-heidelberg.de)

Characteristics	**EfmE1162_0264**	**EfmE1162_1458**
Predicted domain		
Number of transmembrane helices	11	10
Location of the large extracellular loop	Between transmembrane helices 8 to 9	Between transmembrane helices 7 to 8
Number of amino acids residues of the extra/intracellular loop	238 residues	179 residues

Blue box: transmembrane helices

A second putative ABC transporter which may be involved in AMP resistance in *E. faecium* was also identified. The second ABC transporter consists of a putative nucleotide binding domain protein (EfmE1162_1459) and a putative permease (EfmE1162_1458). Using the online prediction tools available in SMART (http://smart.embl-heidelberg.de), the putative EfmE1162_1458 is predicted to have 10 transmembrane domains (Figure 5.2). The putative permease, EfmE1162_1458 which is located downstream of the gene encoding a putative nucleotide binding domain protein, is predicted to have a large extracellular domain between helices 7 and 8 that consists of 179 amino acids (Table 5.2).

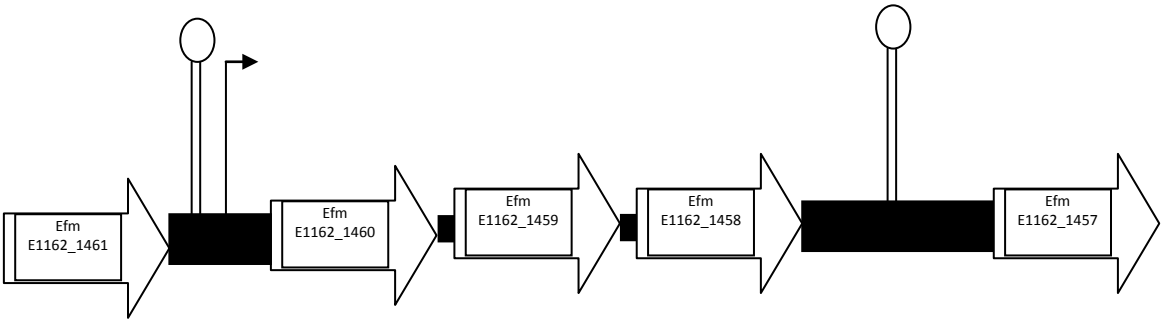


Figure 5.2 Genetic organization of the region next to the orf coding for a putative permease EfmE1162_1458 of *E. faecium* E1162. Smooth line indicates a promoter/terminator predicted by software or manually. EfmE1162_1457= putative aldose 1-epimerase; EfmE1162_1459= putative nucleotide binding domain protein; EfmE1162_1460=conserved hypothetical protein and EfmE1162_1461=putative Band 7 protein.

5.2.2 Construction of an *E. faecium* markerless mutant with a deletion of the putative permease component EfmE1162_1458

Allelic exchange is the strategy that was used to replace EfmE1162_1458, which codes for a putative permease. A 1076 bp DNA fragment upstream of EfmE1162_1458 consisting of the coding sequence of EfmE1162_1457 (Figure 5.2) and 35 bp of upstream sequence was amplified by PCR with primers MabcPUF and MabcPUR (refer to the Table 2.4; Figure 5.3). A 773 bp fragment downstream of EfmE1162_1458 consisting of the coding sequence of EfmE1162_1459 and 32 bp of upstream sequence was PCR amplified with primers MabcPDF and MabcPDR (Table 2.4; Figure 5.3). The primers used for amplification had an overlap of 13 bp between two adjacent fragments to enable them to anneal together in a subsequent overlapping PCR reaction using primers MAbcPUF and MAbcPDR. This strategy allows the 3' overlap of each fragment to serve as a primer for the 3'extension of the complementary strand (Figure 5.4). The construct was verified by sequencing. The amplified fragment was cloned into pHOU1 (Figure 5.5).

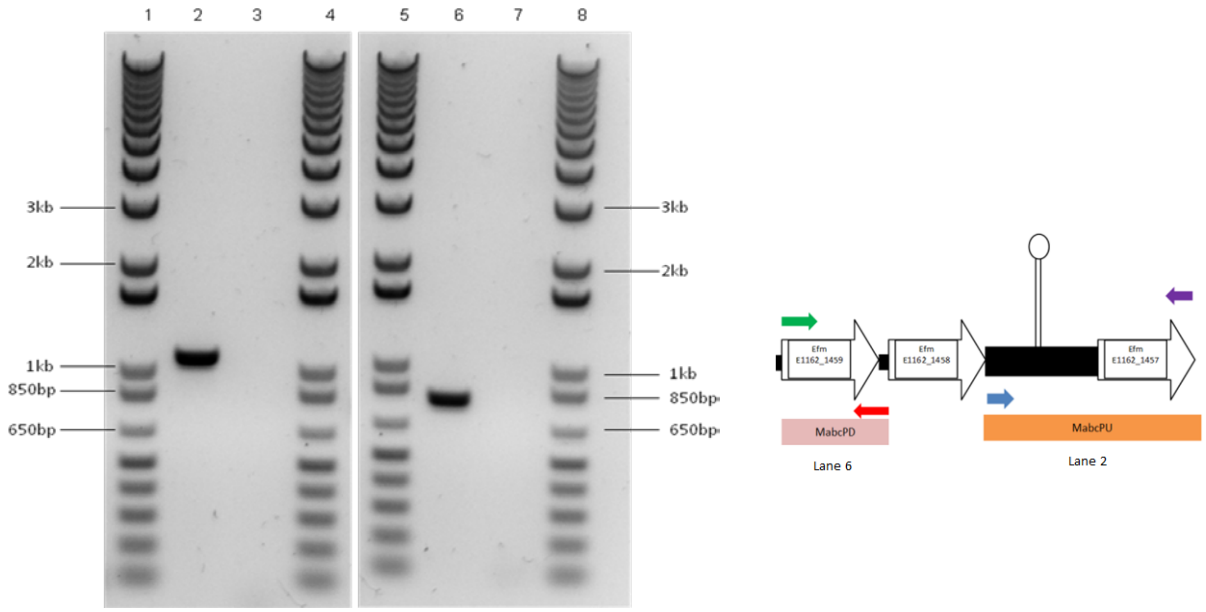


Figure 5.3 Image of the electrophoresis gel of amplified upstream and downstream regions of EfmE1162_1458. Lane1: 1 kb plus DNA ladder (Invitrogen), Lane 2: upstream fragment of EfmE1162_1458 (Orange: MabcPU); Lane 3: negative control (dH2O) , Lane 4: 1kb plus DNA ladder (Invitrogen); Lane 5: 1 kb plus DNA ladder (Invitrogen); Lane 6: downstream fragment of EfmE1162_1458 (Pink: MabcPD); Lane7: negative control (dH2O) ; Lane 8: 1 kb DNA ladder (Invitrogen); Green: MabcPDR; Red: MabcPDF; Blue: MabcPUR and Purple: MabcPUF.

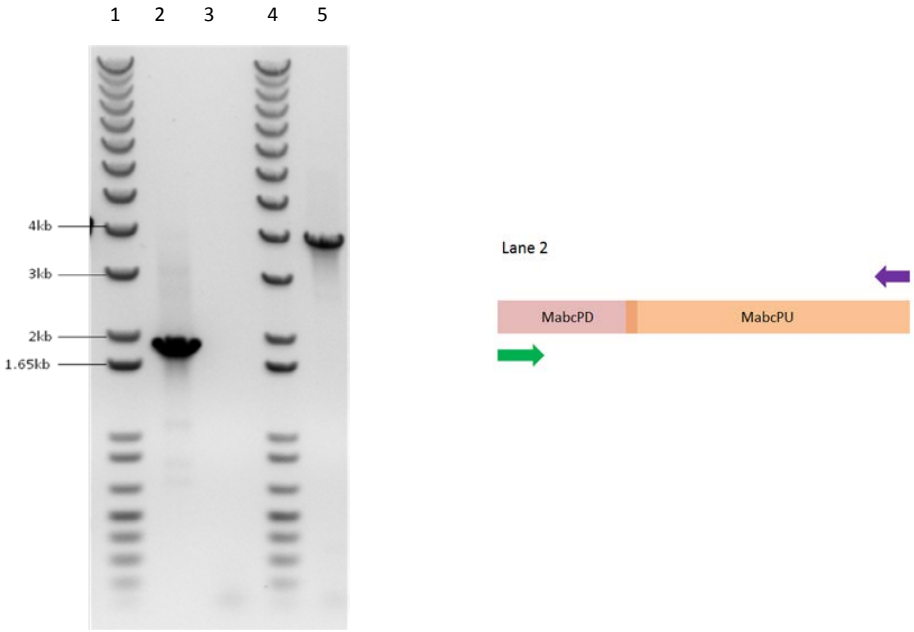


Figure 5.4 Image of the electrophoresis gel of the amplicon obtained after the amplicons of the upstream and downstream region of EfmE1162_1458 were amplified by the overlapping PCR technique. Lane1: 1 kb plus DNA ladder (Invitrogen), Lane 2: combination of amplified upstream and downstream fragment of EfmE1162_1458 using MAbcPUF and MabcPDR from the *E. faecium* E1162; Lane 3: negative control (dH2O) , Lane 4: 1 kb plus DNA ladder (Invitrogen); Lane 5: amplified gene from the *E. faecium* E1162 using primers MAbcUPF and MabcDPR (containing EfmE1162_1458 gene); Green: MabcPDR and Purple: MabcPUF

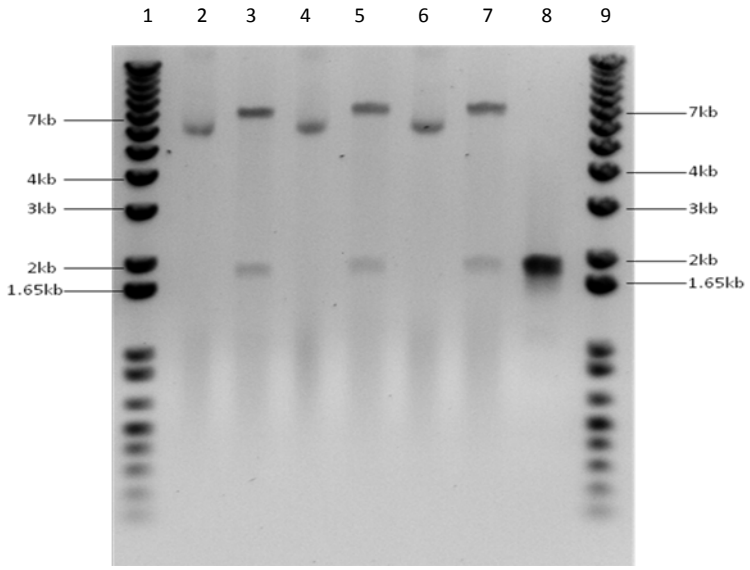


Figure 5.5 Image of the electrophoresis gel of restriction digests of the plasmid preparations from clones. Lane1: 1 kb plus DNA ladder (Invitrogen); Lane 2, 4 and 6: uncut plasmid pHOU1 containing EfmE1162_1458; Lane 3, 5 and 7: combination of upstream and downstream fragment of EfmE1162_1458 which was cloned in pHOU1, digested by NotI and EcoRI restriction enzymes; Lane 8: positive control (combination of amplified upstream and downstream fragment of EfmE1162_1458 using MAbcPUF and MabcPDR from *E. faecium* E1162 and Lane 9: 1 kb plus DNA ladder (Invitrogen)

The pHOU1 counter selection system has previously been used in *E. faecium* E1162 to make strains with deletions in hyl_{Efm} (Panesso et al., 2011) and also to make markerless deletion mutants (as described in my studies in Chapter 2.10). However, after introduction of the construct into *E. faecium* no deletion mutants of EfmE1162_1458 were obtained.

Therefore another attempt was made to construct a mutant lacking EfmE1162_1458, using an erythromycin gene, *erm*(C), as a marker of gene disruption. The strategy was to introduce the *erm*(C) gene in between regions upstream and downstream of EfmE1162_1458 thereby deleting the gene. To amplify the genetic region comprising the upstream region (EfmE1162_1457), AbcPUermCF2 and AbcPUermCR3 primers were used. All primer sequence can be found in Table 2.4. The downstream region (EfmE1162_1459)

was amplified with AbcPDermCF3 and AbcPDermCR2 primers. To introduce the *erm*(C) gene between EfmE1162_1457 and EfmE1162_1459, the *erm*(C) gene was excised from plasmid pZ118 using AflII and PstI. The insertion of *erm*(C) gene expectedly would lead to erythromycin resistance in mutants. The pHOU1 counter selection system was used to select the excisants (as described in section 2.8). However, no corresponding colonies of EfmE1162_1458 mutants were obtained.

5.2.3 Construction of an *E. faecium* markerless mutant with a deletion of the putative permease component EfmE1162_0264

The pHOU1 counter selection system was used (as described in section 2.8) to construct EfmE1162_0264 mutant. Allelic exchange was used to replace EfmE1162_0264 which codes for a putative permease. A 773 bp DNA fragment upstream of EfmE1162_0264 consisting of the coding sequence of EfmE1162_0263 (Figure 5.6) and 626 bp of upstream sequence was amplified by PCR with primers MabcUF and MabcUR (refer to the Table 2.4; Figure 5.6). A 840 bp fragment downstream of EfmE1162_0264 consisting of the coding sequence of EfmE1162_0265 and 45 bp of upstream sequence was PCR amplified with MabcDF and MabcDR primers (Table 2.4; Figure 5.7). The primers used for amplification of the fragments had an overlap of 13 bp to enable the two fragments to anneal in a subsequent overlapping PCR reaction using primers MAbcUF and MAbcDR. This allows the 3' overlap of each fragment to serve as a primer for the 3'extension of the complementary strand (Figure 5.4). The construct was verified by sequencing. The amplified fragment was cloned into pHOU1 using NotI and EcoRI restriction enzymes (Figure 5.8). The pHOU1 counter selection system was used (as described in section 2.8). The excisants of EfmE1162_0264 mutants were obtained. PCR amplification was carried out using genomic

DNA of excisants as a template where the expected regions were predicted to span 1613 bp and 3923 bp for EfmE1162_0264 mutant and wild type, respectively. The corresponding EfmE1162_0264 mutant where the amplified fragment size was expected to be 2310 bp smaller. Agarose gel of the PCR products (Figure 5.9) showed that amplification of the fragments reflected the expected sizes in the EfmE1162_0264 mutant. The EfmE1162_0264 mutant showed a band of the same size as the control (Lane 22 in Figure 5.9), suggesting that the EfmE1162_0264 region was deleted successfully.

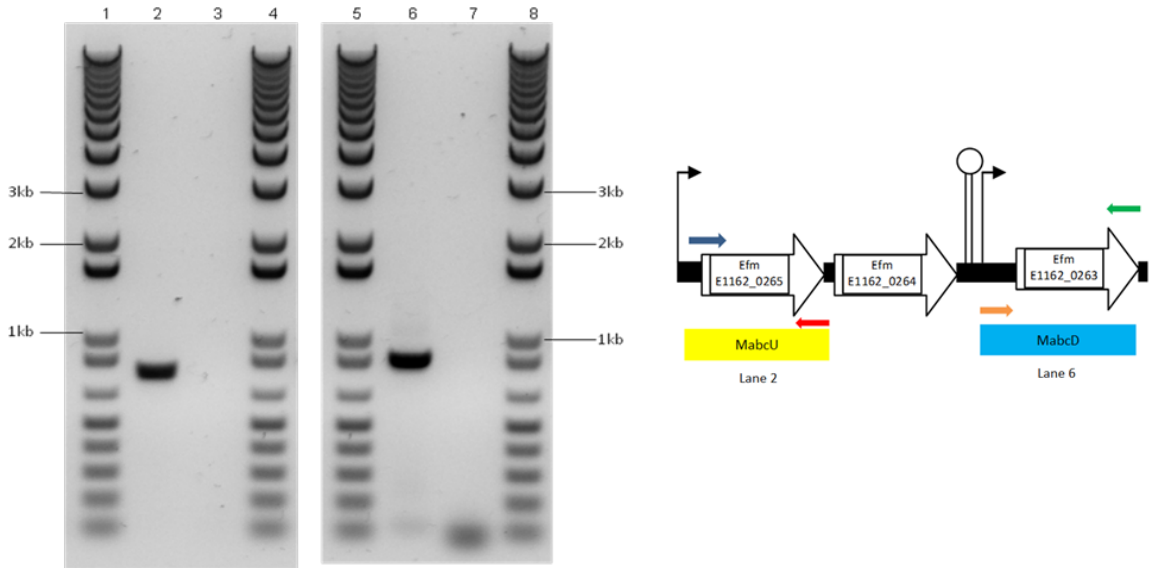


Figure 5.6 Image of the electrophoresis gel of the amplified upstream and downstream regions of the EfmE1162_0264. Lane1: 1 kb plus DNA ladder (Invitrogen); Lane 2: upstream fragment of the EfmE1162_0264 (Yellow:MabcU); Lane 3: negative control (dH2O); Lane 4: 1 kb plus DNA ladder (Invitrogen); Lane 5: 1 kb plus DNA ladder (Invitrogen); Lane 6: downstream fragment of EfmE1162_0264 (Blue: MabcD); Lane 7: negative control (dH2O); Lane 8: 1 kb DNA ladder (Invitrogen); Dark blue: MabcUR; Red: MabcUF; Orange: MabcDR and Green: MabcDF.

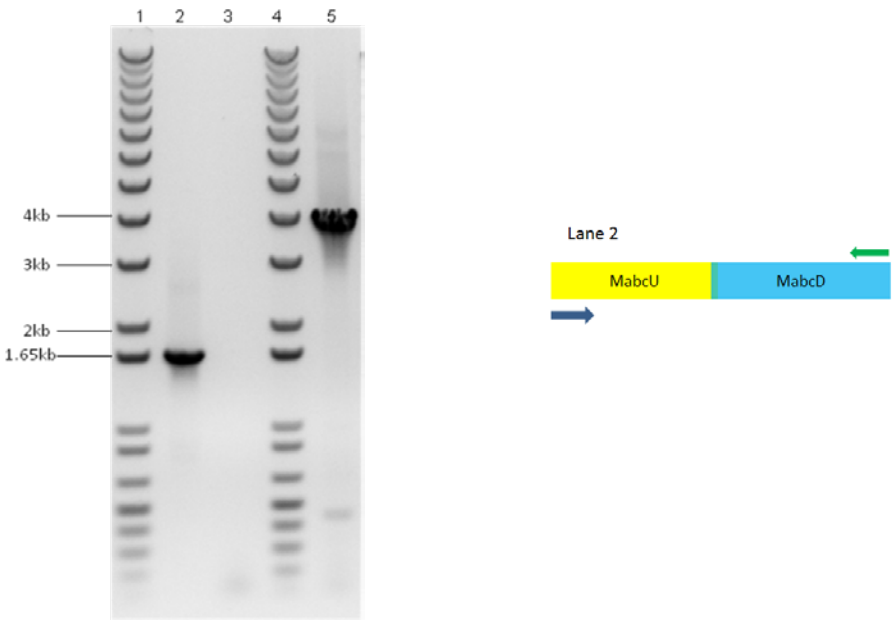


Figure 5.7 Image of the electrophoresis gel of the amplicon obtained after the amplicons of the upstream and downstream regions of EfmE1162_0264 were amplified by the overlapping PCR technique. Lane1: 1 kb plus DNA ladder (Invitrogen); Lane 2: combination of upstream and downstream fragment of EfmE1162_0264 using MAbcUF and MabcDR; Lane 3: negative control (dH2O); Lane 4: 1 kb plus DNA ladder (Invitrogen); Lane 5: amplified gene from the *E. faecium* E1162 using MAbcUF and MabcDR (containing EfmE1162_0264); Dark blue: MabcUR and Green: MabcDF.

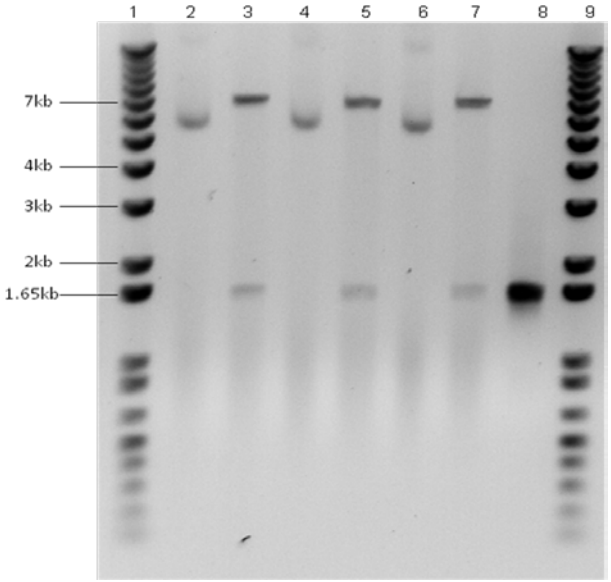


Figure 5.8 Image of the electrophoresis gel of restriction digests of the plasmid preparations from clones. Lane1: 1 kb plus DNA ladder (Invitrogen); Lane 2, 4 and 6: uncut plasmid of pHOU1 containing markerless EfmE1162_0264 mutants ; Lane 3, 5 and 7: plasmid of pHOU1 containing markerless EfmE1162_0264 mutants digested with NotI and EcoRI restriction enzymes

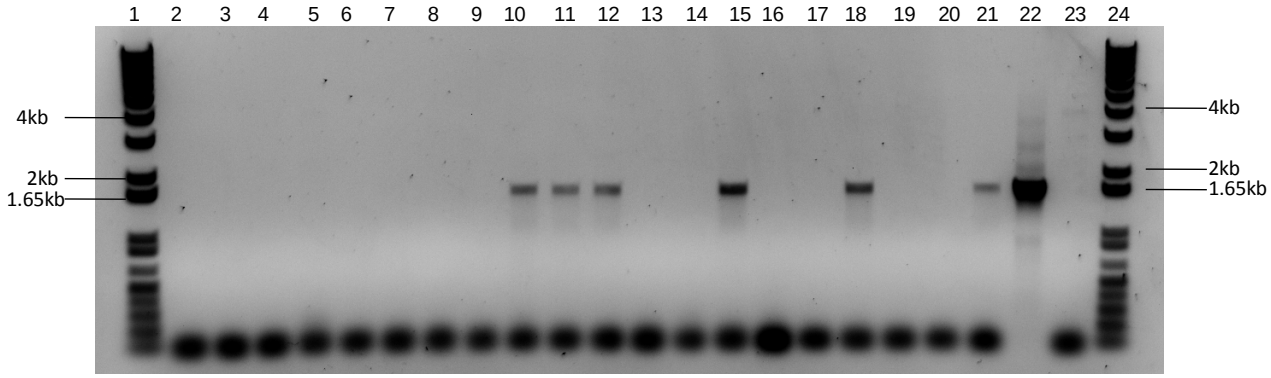


Figure 5.9 Amplification of the fragments reflected the expected sizes in the EfmE1162_0264 mutants. Lane1: 1 kb plus DNA ladder (Invitrogen); Lane 2 negative control (dH2O); Lane 10, 11,12 ,15 and 18: amplification on genomic DNA of the excisants EfmE1162_0264; Lane 21: plasmid of pHOU1 containing amplicon obtained after the amplicons of the upstream and downstream regions of EfmE1162_0264 were amplified by the overlapping PCR technique; Lane 22: amplicon resulting from combination of upstream and downstream fragments of EfmE1162_0264 by overlap PCR; Lane 23: amplified gene from the *E. faecium* E1162 using primers MAbcUF and MabcDR (containing EfmE1162_0264); Lane 2-9, 13, 14, 16, 17, 19, 20: amplification on genomic DNA of the wildtype and Lane 24: 1 kb plus DNA ladder (Invitrogen).

5.2.4 Growth of *E. faecium* E1162 and the mutant with a deletion of EfmE1162_0264 in tryptic soy broth and brain heart infusion broth

Having confirmed that a gene coding for the putative permease EfmE1162_0264 had been successfully deleted in *E. faecium*, as described in section 5.2.3, the impact of deletion on the growth of the bacterium was evaluated by comparing the growth of the *E. faecium* E1162 and the isogenic EfmE1162_0264 mutant in TSB. Analysis of bacterial growth was done by measuring the optical density at 600 nm of bacterial cultures at time intervals.

It was found that in comparison to *E. faecium* E1162 the isogenic EfmE1162_0264 mutant (red line) had an extended lag phase (Figure 5.10). It was found that the growth of the isogenic EfmE1162_0264 mutant (red line) was nearly identical to that of *E. faecium* E1162 (blue line) when grown in BHI (Figure 5.11). This data demonstrates that the deletion of EfmE1162_0264 does not produce a general growth defect.

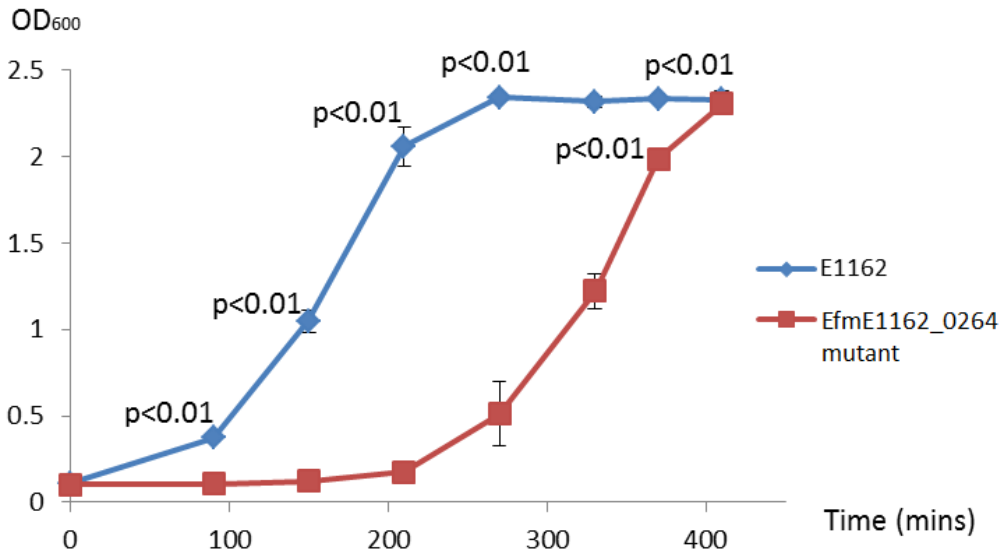


Figure 5.10 Growth of *E. faecium* E1162 and the mutant with a deletion of EfmE1162_0264 in the tryptic soy broth. Dark blue: E1162 and Dark red: Δ EfmE1162_0264 mutant. Data presented are the mean of three independent experiments with error bars showing the standard deviations. Significance of the difference between E1162 and the EfmE1162_0264 mutant in TSB were analyzed with the Mann-Whitney U test.

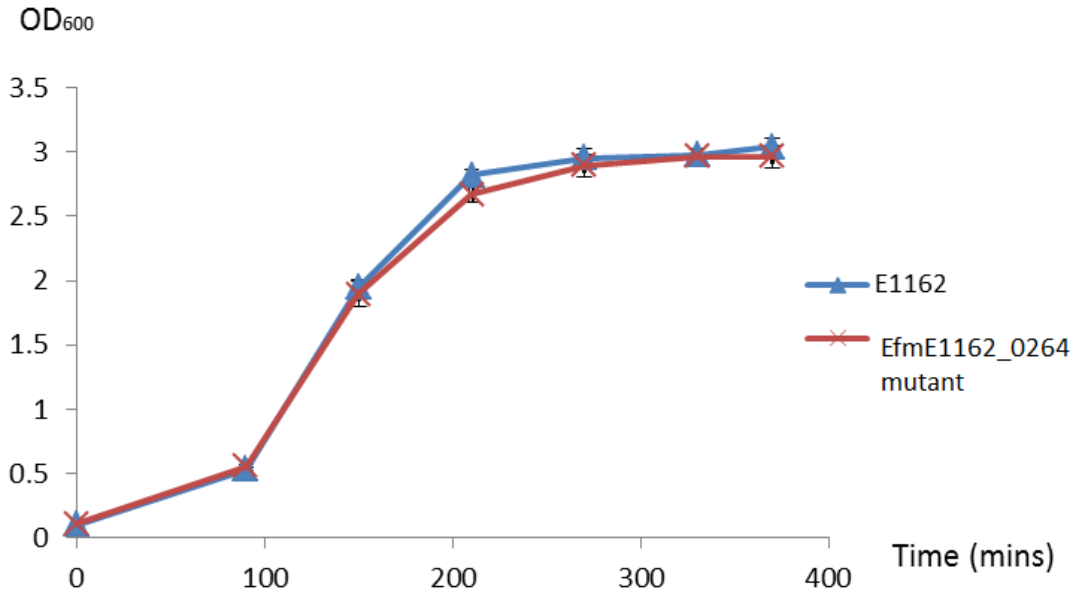


Figure 5.11 Analysis of growth kinetics in brain heart infusion. Blue: E1162 and Red: mutant with a deletion of EfmE1162_0264. Data presented are the mean of three independent experiments with error bars showing the standard deviations.

We sought to investigate why the EfmE1162_0264 mutant exhibited a severe growth defect in TSB but not BHI. We hypothesised that pancreatic digest of casein (Tryptose), which is one of the components in TSB may contain one or more antimicrobial peptides. Therefore the same quantity of Tryptose (17 g/L) as present in TSB was added into the brain heart infusion broth.

It was found that the isogenic EfmE1162_0264 mutant (red line) exhibited a growth defect compared to *E. faecium* E1162 (blue line) when BHI supplemented with tryptose was used for culture (Figure 5.12).

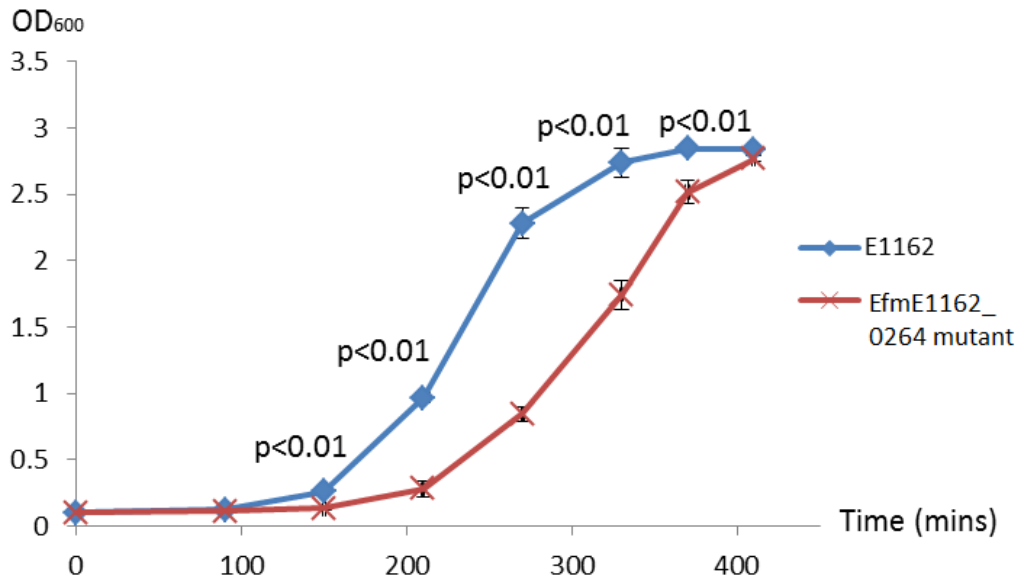


Figure 5.12 Analysis of growth kinetics in BHI supplemented with tryptose. Blue: E1162 and Red: EfmE1162_0264 mutant. Data presented are the mean of three independent experiments with error bars showing the standard deviations. Significance of the difference between E1162 and EfmE1162_0264 mutant in BHI supplemented with tryptose were analyzed with the Mann-Whitney U test.

5.2.5 Antimicrobial peptide sensitivity of *E. faecium* E1162 and the EfmE1162_0264 deletion mutant

We investigated whether there were any differences in susceptibility to antimicrobial peptides between *E. faecium* E1162 and the isogenic EfmE1162_0264 mutant.

It was found that the EfmE1162_0264 mutant was more sensitive to the antimicrobial peptides tested compared to the *E. faecium* E1162 (Table 5.3). *E. faecium* E1162 was resistant to more than 1024 µg/mL of colistin (polymyxin E) while the isogenic EfmE1162_0264 mutant was sensitive to 512 µg/mL of this antimicrobial peptides. There was a 16 fold and a 4 fold reduction in the resistance of the isogenic EfmE1162_0264 mutant compared to *E. faecium* strain E1162 for bacitracin and nisin respectively.

Table 5.3 Comparison of the antimicrobial peptide sensitivity of *E.faecium* E1162 and the EfmE1162_0264 mutant.

strain	***E.faecium* E1162**	**EfmE1162_0264 mutant**
MIC of nisin (µg/mL)	128	32
MIC of bacitracin (µg/mL)	256	16
MIC of colistin (µg/mL)	>1024	512

5.2.6 Evaluation of the bacterial cell surface charge

Cytochrome c is a cationic peptide and therefore can bind to negative charges on the bacterial cell surface and therefore can indicate changes in bacterial cell surface charge. The cytochrome c binding assay was performed by measuring the quantity of cytochrome c that did not bind to the cells and subtracting that value from the amount added to give the amount bound (Matsuo et al., 2011).

The data presented in Figure 5.13 shows that the percentage of cytochrome c bound to the isogenic EfmE1162_0264 mutant (47.5 %) was higher than that which bound to the *E. faecium* E1162 (23.2 %). This result indicates that the cell surface of the isogenic

EfmE1162_0264 mutant was more negatively charged than *E. faecium* E1162. This assay was previously done with *S. aureus* LS-1 in the Nair lab and therefore, this strain was used as a positive control in this study.

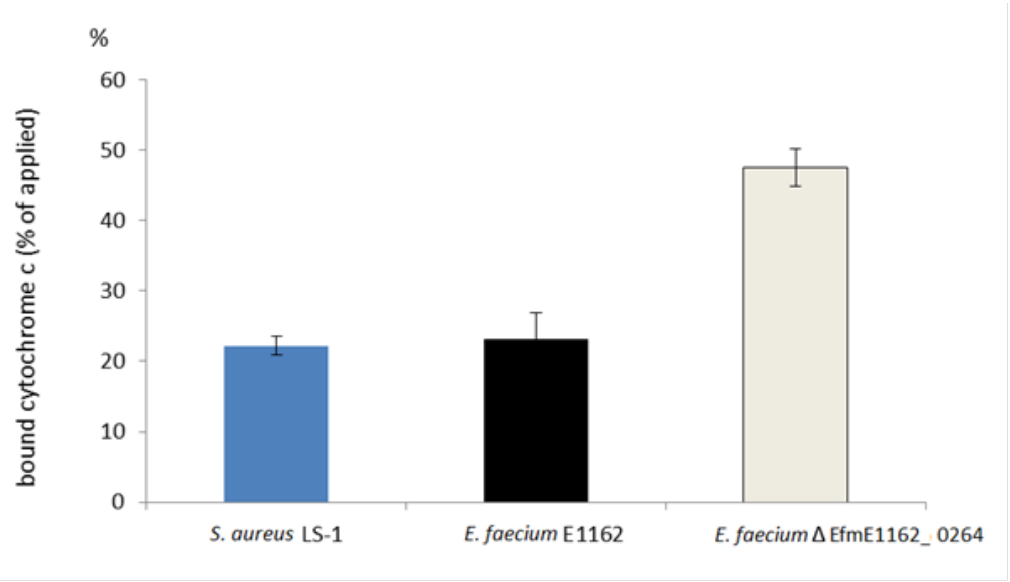


Figure 5.13 Percentage of cytochrome c bound to the cells. Blue: *S. aureus* LS-1; Black: *E. faecium* E1162 and Grey: *E. faecium* EfmE1162_0264 mutant. Data presented are the mean of three independent experiments with error bars showing the standard deviations. Significance of the difference between E1162 and the EfmE1162_0264 mutant were analyzed with the Mann-Whitney U test. , **p<0.01

Next, we evaluated the cell surface changes of *E. faecium* in the exponential and stationary phase to see if growth phase affected surface charge. Additionally, we used colistin (polymyxin E) which is a cationic antimicrobial peptide to determine if EfmE1162_0264 was capable of sensing cationic peptides in general. The reason to choose polymyxin E was it is nontoxic to *E. faecium*. Polymyxin E is cationic and binds to anionic lipopolysaccharide, thus leading to disruption of membrane integrity. Since *E. faecium* is a gram-positive, it does not have lipopolysaccharide like gram-negative bacteria. Therefore, polymyxin E does not cause toxicity to *E. faecium* cells. We analysed the growth kinetics (data is not shown) of *E. faecium* E1162 and the isogenic mutant with a deletion of EfmE1162_0264 in BHI broth in the absence or presence of varying concentrations of

polymixin E (0.5 µg/mL, 1 µg/mL, 2 µg/mL, 5 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL polymyxin E). It was found that in comparison to the wildtype, the isogenic EfmE1162_0264 mutant had an extended lag phase in the presence of 10 µg/mL to 100 µg/mL polymyxin E (data not shown). Hence, 5 µg/mL was chosen for further experiments since this concentration had no significant effect on the growth of these two strains (Figure 5.14).

We investigated the sensing ability of *E. faecium* E1162 and the isogenic EfmE1162_0264 mutant in the middle of exponential and stationary phase. *E. faecium* E1162 and the isogenic EfmE1162_0264 mutant were collected at mid-exponential and stationary phase. The overall surface charge at these growth phases were determined by using cytochrome c. As mentioned above, the assay was done using 5 µg/mL polymyxin E, since at this concentration had no significant effect on the growth of these two strains.

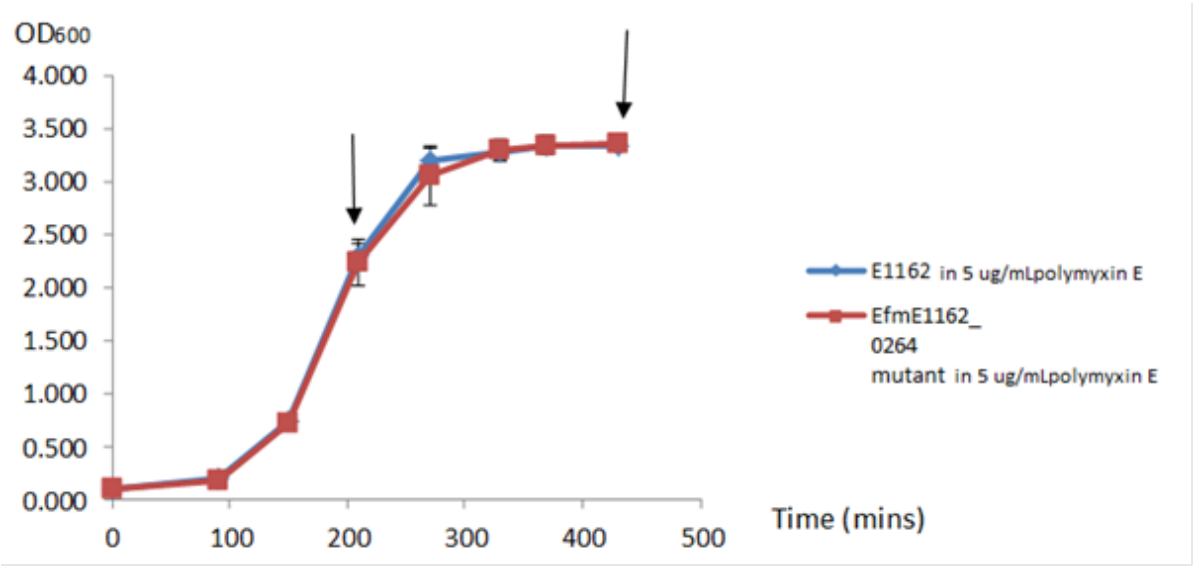


Figure 5.14 Growth of *E. faecium* E1162 and the isogenic EfmE1162_0264 mutant in the presence of 5 µg/mL polymyxin E. The arrow indicates the time point when the strains were harvested to determine the overall surface charge at these growth phases by using cytochrome c. Dark blue: E1162 and Dark red: the isogenic EfmE1162_0264 mutant. Data presented are the mean of three independent experiments with error bars showing the standard deviations.

It was observed that the amount of cytochrome c binding to the isogenic EfmE1162_0264 mutant was higher ($p<0.01$) during exponential phase and stationary phase compared to the *E. faecium* E1162 (Figure 5.15). This indicates that the cell surface charge of the isogenic EfmE1162_0264 mutant was more negative than that of *E. faecium* E1162.

In *E. faecium* E1162, the cell surface charge was more negative during stationary phase compared to exponential phase ($p<0.01$). In the EfmE1162_0264 mutant the cell surface charge was more negative during exponential phase compared to stationary phase ($p<0.01$).

In the presence of 5 µg/mL polymyxin E, the cell surface charge of *E. faecium* E1162 was more positive during exponential and stationary phases compared to in the absence of polymyxin E ($p<0.01$). This suggests that *E. faecium* can sense polymxyin E and alters its cell surface charge. In the presence of polymyxin E the cell surface charge of EfmE1162_0264 mutant was more positive during exponential phase compared to in the absence of polymxyin E ($p<0.01$). This suggests that EfmE1162_0264 is not involved in sensing polymyxin E during growth in exponential phase. However the cell surface charge of EfmE1162_0264 mutant was more negative during stationary phase in the presence of 5 µg/mL polymyxin E compared to without polymyxin E. It is therefore possible that EfmE1162_0264 is involved in sensing polymyxin E during stationary phase. Given the proximity of the *dlt* operon to the EfmE1162_0264 gene it is possible to wonder if EfmE1162_0264 sensing of polymyxin E somehow effects expression of the operon to alter surface charge.

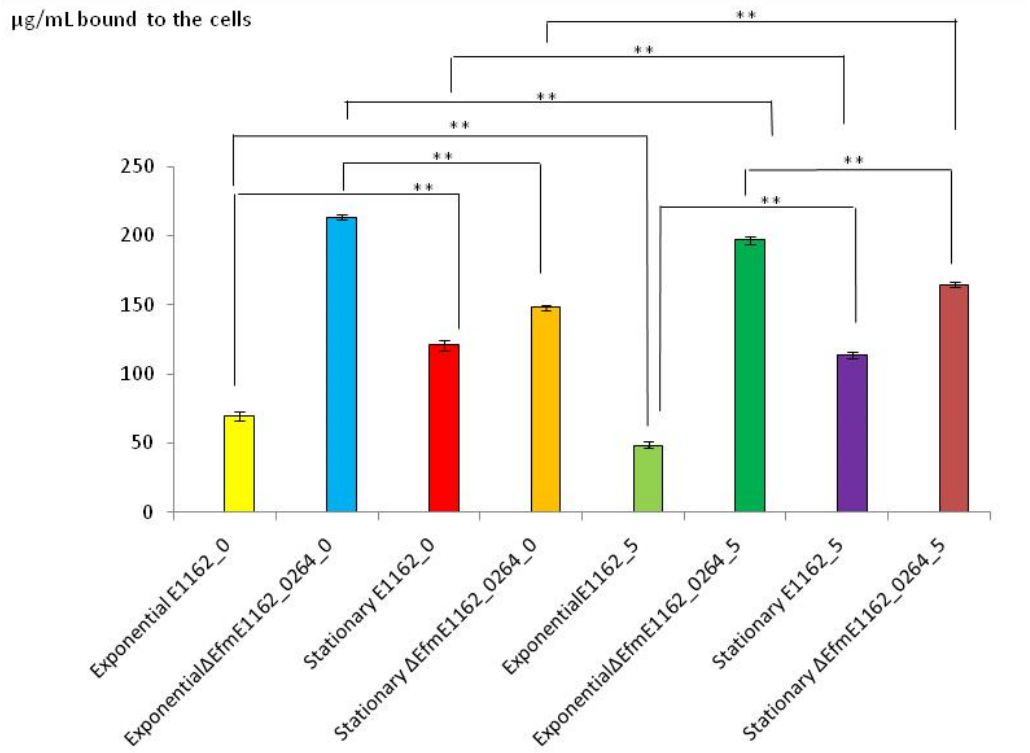


Figure 5.15 Amount of cytochrome c bound to cells of *E. faecium* E1162 and the isogenic ΔEfmE1162_0264 mutant at exponential and stationary phase in the presence and absence of 5 ug/mL polymyxin E. Yellow: Exponential phase of E1162 in BHI; Blue: Exponential phase of EfmE1162_0264 mutant in BHI; Red: Stationary phase of E1162 in BHI; Orange: Stationary phase of EfmE1162_0264 mutant in BHI; Green: Exponential phase of E1162 in BHI in the presence of 5 µg/mL; Dark green: Exponential phase of EfmE1162_0264 mutant in BHI in the presence of 5 µg/mL; Purple: Stationary phase of E1162 in l BHI in the presence of 5 µg/mL and Brown: Stationary phase of EfmE1162_0264 mutant in BHI in the presence of 5 µg/mL. Data presented are the mean of three independent experiments with error bars showing the standard deviations. Significance of the difference between E1162 and EfmE1162_0264 mutant were analyzed with the Mann-Whitney U test. , **p<0.01

5.2.7 Role of EfmE1162_0264 in virulence in the *Galleria mellonella* infection model

In order to evaluate the role of EfmE1162_0264 in virulence, the EfmE1162_0264 mutant was compared with the wild-type strain in the *Galleria mellonella* infection model (Figure 5.16). After 48 hours post-injection, 40 % of the larvae survived when injected with *E. faecium* E1162 while only 20 % of the wax worms injected with the EfmE1162_0264 mutant survived after the same time. However the difference in killing was not statistically

significant (p>0.05) different, suggesting that EfmE1162_0264 is not involved in *E. faecium* virulence in this model.

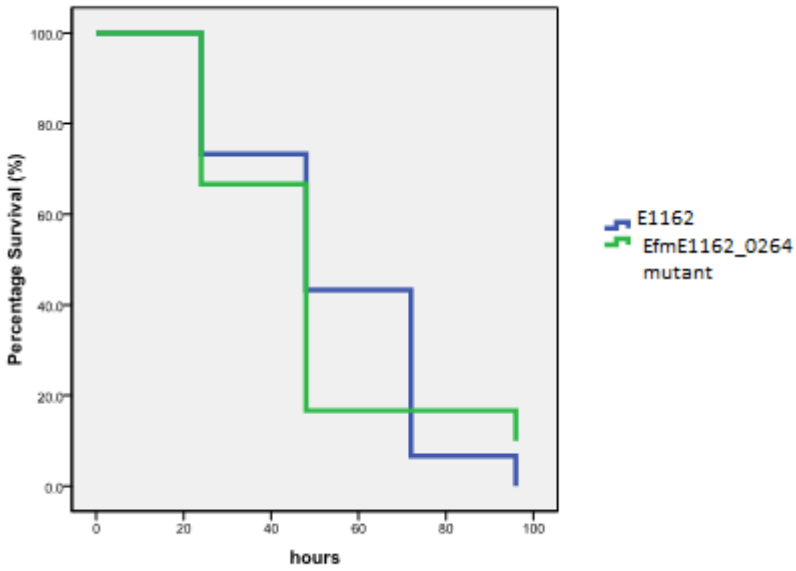


Figure 5.16 Kaplan-Meier survival curves of *Galleria mellonella* wax worms inoculated with *E. faecium*. Blue: *E. faecium* E1162 and Green: EfmE1162_0264 mutant. Data presented are the percentages of 30 wax worms which were assayed in groups of 10 on three different days.

5.2.8 Complementation of the EfmE1162_0264 mutant with the *E. faecium* EfmE1162_0264 gene

To confirm that the different amounts of cytochrome c which bound to the EfmE1162_0264 mutant and wild-type cells was due to the permease component, complementation with the wildtype EfmE1162_0264 *in trans* was done (Figure 5.17). The EfmE1162_0264 gene was amplified with primers AbcF and AbcR using genomic DNA from E1162 as template. This was to include the ribosomal binding site and terminator of EfmE1162_0264. The DNA fragment obtained (2325 bp) was cloned into pHFH4.

Recombinant plasmid was purified from *E.coli* DH5α grown on Luria-Bertani agar containing ampicillin (100 µg/mL). The construct was verified by sequencing. Subsequently, the purified plasmid was introduced into the EfmE1162_0264 mutant by electroporation

and colonies were recovered on brain heart infusion (BHI) agar plates containing chloramphenicol (20 µg/mL).

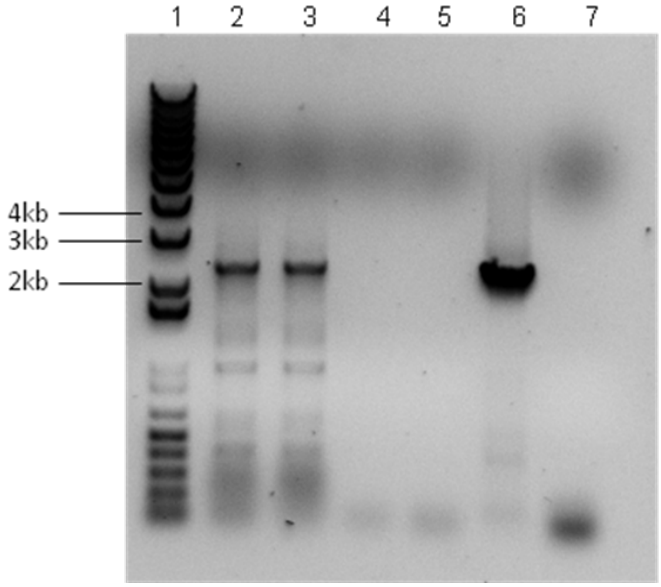


Figure 5.17 Image of the electrophoresis gel of amplified DNA coding for EfmE1162_0264 that was cloned into pHFH4 (pHFH13). Lane1: 1 kb plus DNA ladder (Invitrogen); Lane 2 and Lane 3: PCR of colonies of *E. faecium* containing pHFH13 and Lane 6: plasmid pCR2.1 containing EfmE1162_0264 isolated from *E. coli* DH5α

5.2.8.1 Studies with the complemented strain to confirm the difference in surface charge between *E. faecium* E1162 and the EfmE1162_0264 mutant was due to loss of EfmE1162_0264

We have shown that the percentage of cytochrome c bound to the EfmE1162_0264 mutant was higher than wild-type cells. In the work described in this section it was observed that the concentration of cytochrome c bound to the EfmE1162_0264 mutant containing plasmid pHFH4 without any insert (153.10 µg/mL) was higher ($p<0.01$) compared to the *E. faecium* E1162 (123.74 µg/mL) (Figure 5.18). The plasmid pHFH12, which coded for the EfmE1162_0264 partially restored the level of cytochrome c binding to the EfmE1162_0264 mutant cells (128.79 µg /mL). However the bacterial cell surface charge of the complementation strain was slightly more negative ($p<0.01$) compared to the *E. faecium*

E1162. This complementation study confirmed that the increased negative charge of the EfmE1162_0264 mutant cells was due to disruption of the EfmE1162_0264 gene.

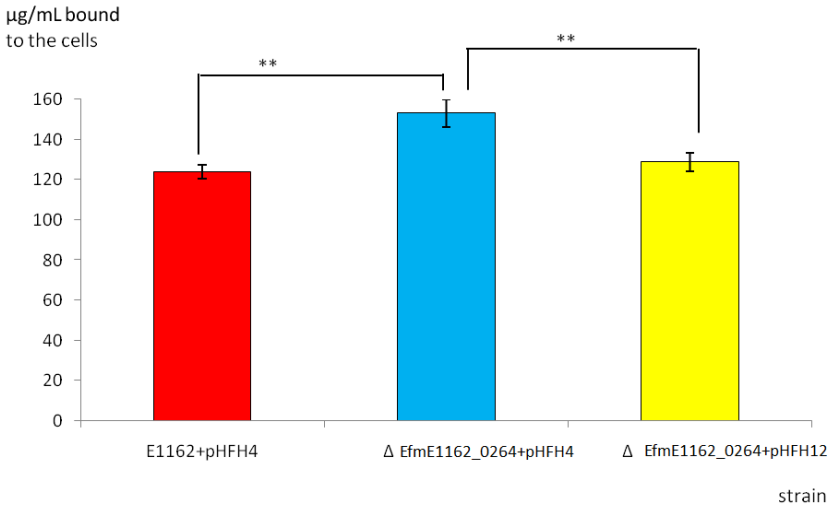


Figure 5.18 Amount of cytochrome c bound to cells of *E. faecium*. Red: *E. faecium* E1162+pHFH4; Blue: EfmE1162_0264 mutant+pHFH4 and Yellow: EfmE1162_0264 mutant+pHFH12. Data presented are the mean of three independent experiments with error bars showing the standard deviations. Significance of the difference between E1162, EfmE1162_0264 mutant and complementation strain were analyzed with the Mann-Whitney U test. , **p<0.01

5.2.8.2 Studies with the complemented strain examining the surface charge at exponential and stationary phase in the presence and absence of polymixin E

In section 5.2.6 it was reported that the patterns of binding of cytochrome c to the wild-type and the EfmE1162_0264 mutant were different in mid-exponential and stationary phase. The growth rate of the wild-type E1162+pHFH4, the EfmE1162_0264 mutant containing pHFH4 and the complementation strain EfmE1162_0264 containing pHFH12 was similar in the BHI (Figure 5.19) and in BHI containing 5 µg/mL polymyxin E (Figure 5.20).

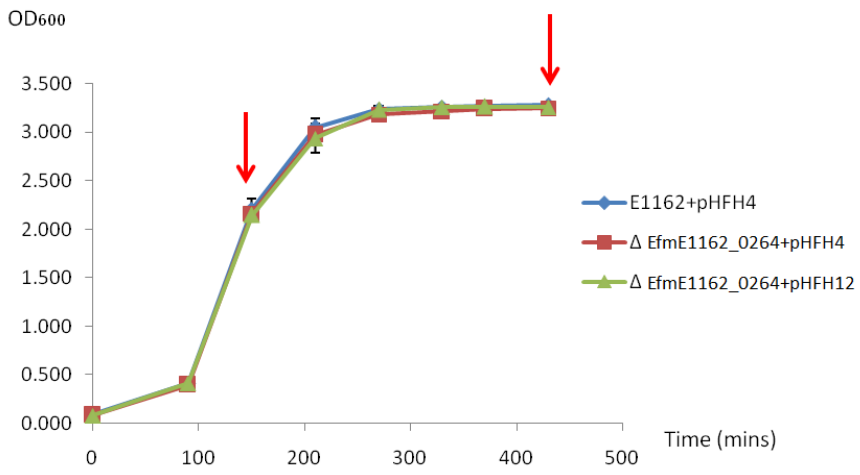


Figure 5.19 Growth of wild-type *E. faecium* E1162, the EfmE1162_0264 mutant and the complementation strain in BHI. The arrow indicates the time point when the strains were harvested to determine the overall surface charge at these growth phases by using cytochrome c. Dark blue: E1162+pHFH4, Dark red: ΔEfmE1162_0264 +pHFH4 and Green: ΔEfmE1162_0264+pHFH12 (EfmE1162_0264). Data presented are the mean of three independent experiments with error bars showing the standard deviations.

It was shown earlier that the cell surface charge of the *E. faecium* E1162 cells was more positive during exponential phase compared to the EfmE1162_0264 mutant. In the complementation experiment (Figure 5.21), the concentration of cytochrome c bound to the EfmE1162_0264 mutant containing the plasmid pHFH4 without an insert, the EfmE1162_0264+pHFH4 (166.12 µg/mL) was higher compared to *E. faecium* E1162 containing the plasmid pHFH4 without an insert (110.16 µg /mL) in exponential phase. Therefore, the cell surface charge of the wild-type E1162+pHFH4 was more positive than the mutant ΔEfmE1162_0264+pHFH4 during the exponential phase. The EfmE1162_0264 mutant containing plasmid pHFH12 expressing the *E. faecium* EfmE1162_0264 gene, partially restored the cell surface positive charge (135.78 µg /mL) to that of wildtype.

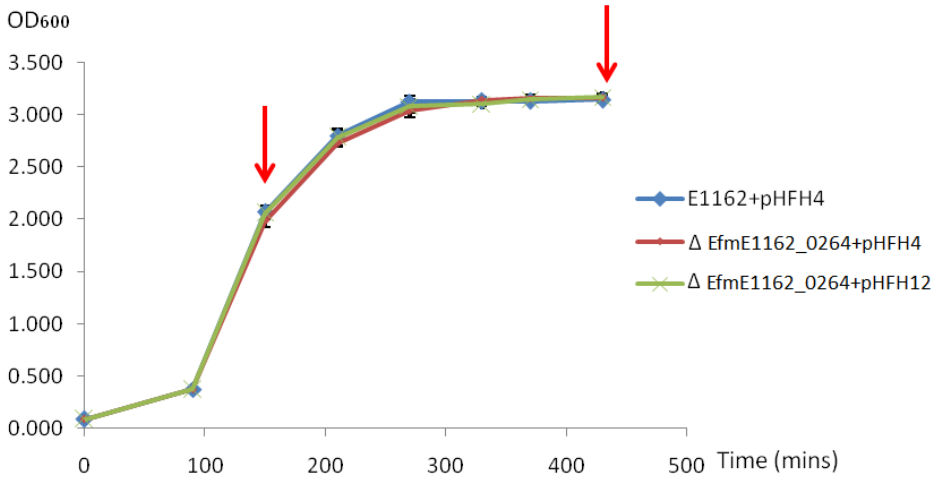


Figure 5.20 Growth of *E. faecium* E1162, the EfmE1162_0264 mutant and the complementation strain in the presence of 5 µg/mL polymyxin E. The arrow indicates the time point when the strains were harvested to determine the overall surface charge at these growth phases by using cytochrome c. Dark blue: E1162+pHFH4, Dark red: Δ EfmE1162_0264+pHFH4 and Green: Δ EfmE1162_0264 +pHFH12 (EfmE1162_0264 Data presented are the mean of three independent experiments with error bars showing the standard deviations.

The cell surface charge of the *E. faecium* E1162+pHFH4 cells was more negative charge during the exponential phase in the presence of 5 µg/mL polymyxin E (Figure 5.22). The concentration of cytochrome bound to the wild-type E1162+pHFH4 cells (83.41 µg/mL) was lower than without the addition of polymyxin E (110.16 µg /mL) ($p<0.01$). The concentration of cytochrome c bound to mutant Δ EfmE1162_0264 +pHFH4 during the same phase was lower (142.48 µg/mL) than without the addition of polymyxin E (166.12 µg/mL) ($p<0.01$). The EfmE1162_0264 mutant containing plasmid pHFH12 expressing the *E. faecium* EfmE1162_0264 gene, had restored positive cell surface charge (83.48 µg /mL). These complementation studies confirmed that the increased negative charge of the EfmE1162_0264 mutant cells was due to disruption of the EfmE1162_0264 gene.

During stationary phase, the *E. faecium* E1162+pHFH4 (74.61 µg /mL) had increased positive charge in the presence of 5 µg/mL polymyxin E compared to that of without the

addition of polymyxin E (120.14 µg /mL) (p<0.01). During the same phase, the Δ EfmE1162_0264+pHFH4 had increased positive charge in the presence of 5 µg/mL polymyxin E compared to that of without the addition of polymyxin E (140.89 µg /mL) (p<0.01). The Δ EfmE1162_0264 mutant containing plasmid pHFH12 expressing the *E. faecium* EfmE1162_0264 gene, had restored positive cell surface charge (75.19 µg/mL). These complementation studies confirmed that the increased negative charge of the EfmE1162_0264 mutant cells was due to disruption of the EfmE1162_0264 gene.

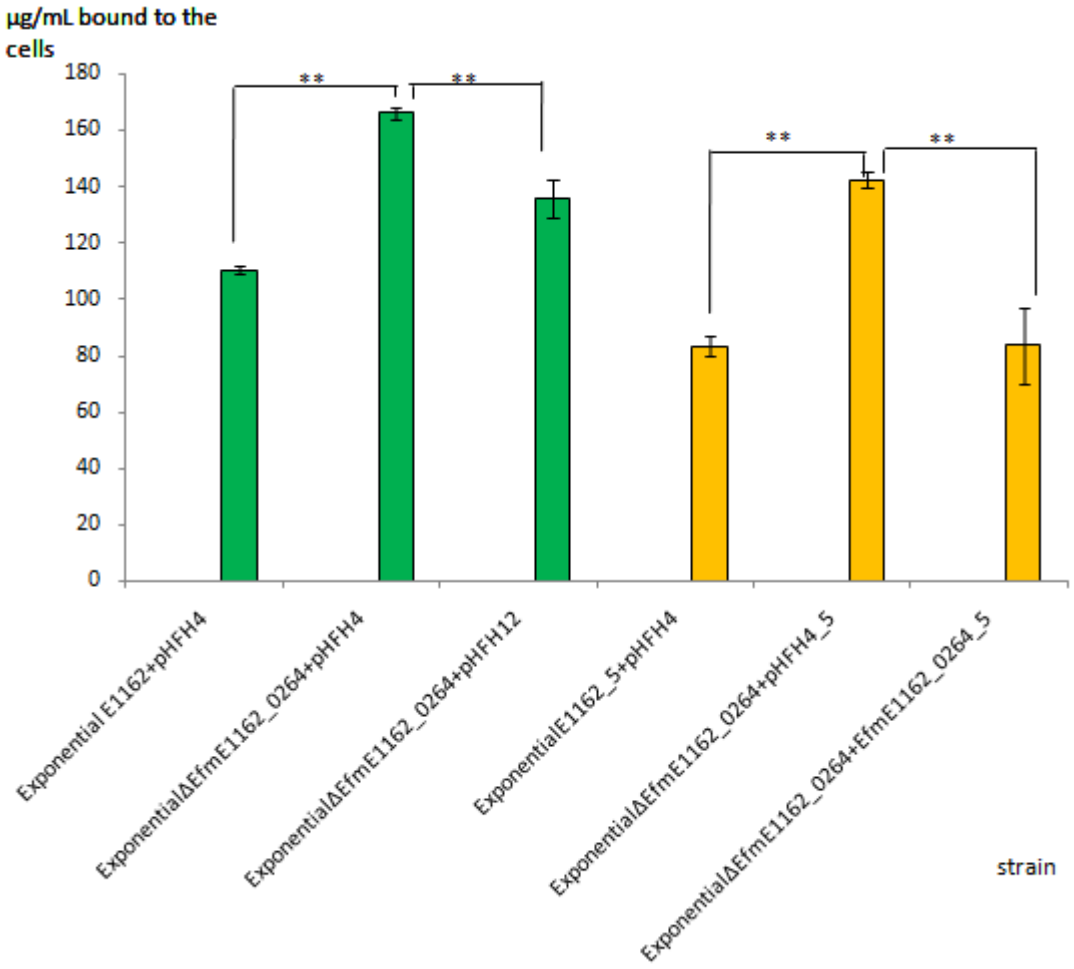


Figure 5.21 Amount of cytochrome c bound to cells of *E. faecium* E1162+pHFH4, Δ EfmE1162_0264 +pHFH4 and complementation strain (Δ EfmE1162_0264 +pHFH12) at exponential phase in BHI and in the presence of 5 µg/mL polymyxin E. Green: Exponential phase in BHI and Yellow: Exponential phase in BHI in the presence of 5 µg/mL polymyxin E: Stationary phase. Data presented are the mean of three independent experiments with error bars showing the standard deviations. Significance of the difference between E1162 and Δ EfmE1162_0264 were analyzed with the Mann-Whitney U test. **p<0.01

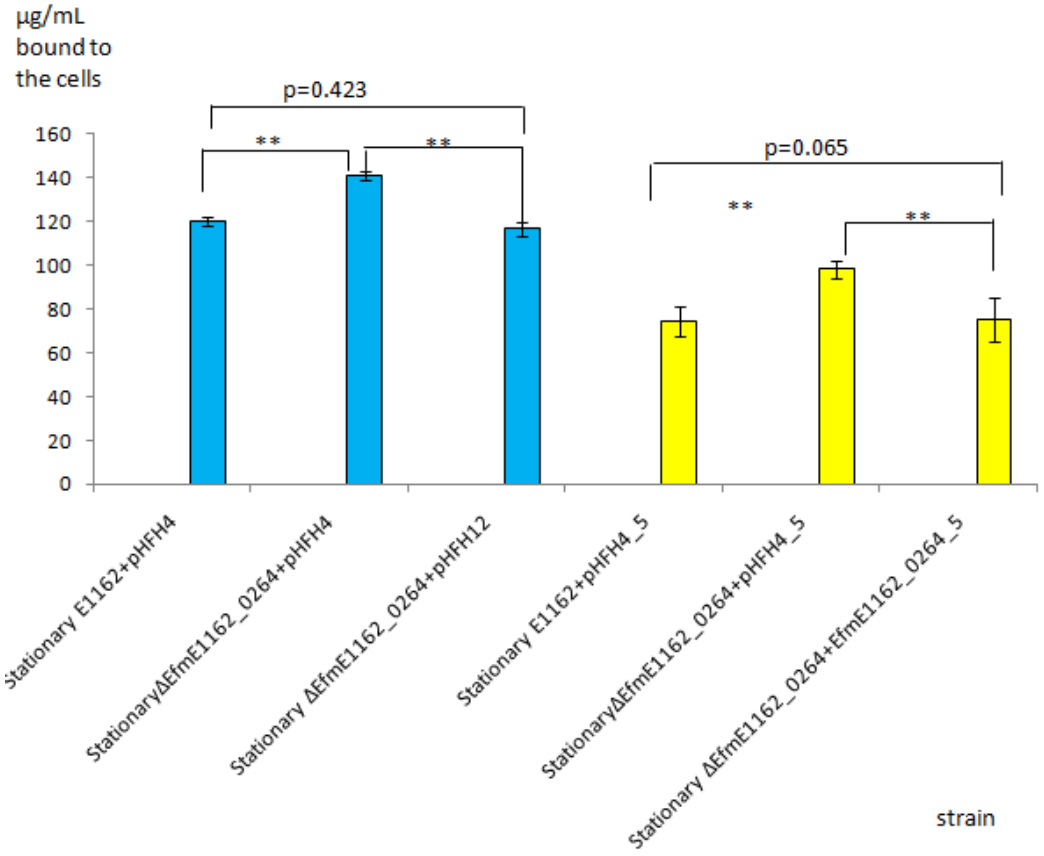


Figure 5.22 Amount of cytochrome c bound to cells of *E. faecium* E1162+pHFH4, mutant Δ EfmE1162_0264+pHFH4 and complementation strain (Δ EfmE1162_0264+pHFH12 (EfmE1162_0264) at stationary phase in BHI and in the presence of 5 µg/mL polymyxin E. Blue: Stationary phase in BHI and Yellow: Stationary phase in BHI in the presence of 5 µg/mL polymyxin E. Data presented are the mean of three independent experiments with error bars showing the standard deviations. Significance of the difference between E1162 and Δ EfmE1162_0264 were analyzed with the Mann-Whitney U test. **p<0.01

5.2.9 Discussion

The involvement of ABC transporters in resistance to AMPs has been widely reported in other firmicutes. For example using a transposon mutagenesis technique disruption of a gene that encodes a permease component of an ABC in transporter, *anrB*, in *Listeria monocytogenes* demonstrated that it plays a role in lantibiotic nisin resistance in that bacterium. Disruption of this gene also resulted in higher sensitivity to bacitracin,

gallidermin and various cell wall acting antibiotics (cefuroxime, cefotaxime, ampicillin, imipenem, and ceftazidime) (Collins et al., 2010).

In several other bacteria such as *Staphylococcus aureus*, two operons coding for the ABC transporters, BraDE and VraDE, have been demonstrated to be co-involved in bacitracin and nisin resistance. The BraDE senses bacitracin and activates signalling through BraSR, which is located upstream of BraDE. Subsequently, the VraDE plays a role by being a detoxification module via a large extracellular loop in VraE (Hiron et al., 2011). Another gram positive organism that possesses a similar mechanism is *Bacillus subtilis*, where disruption of the ABC transporter BceAB caused hypersensitivity to bacitracin (Ohki et al., 2003). It was found later that the ABC transporter, BceAB is important for bacitracin detoxification and sensing in this bacterium (Rietkotter et al., 2008). Later, another similar module for peptide antibiotic sensing and detoxification (PsdRS-AB), which responds and confers resistance against nisin was identified in the same bacterium (Staron et al., 2011). In other bacteria like *Clostridium difficile*, disruption of ABC transporter, *cprABC* resulted in higher susceptibility to nisin and gallidermin but not polymyxin B (McBride and Sonenshein., 2011).

In this study, a putative permease component of ABC transporter, EfmE1162_0264, which might have been important in AMP resistance and is not associated with neighboring two-component systems was identified. This permease has a large extracellular domain between helices 8 and 9. The results reported in this study showed that deletion of the permease component of the ABC transporter in *E. faecium* led to higher sensitivity to nisin, bacitracin and polymixin E than that observed in *E. faecium* E1162. These results

demonstrate that the putative EfmE1162_0264 permease component of an ABC transporter in *E. faecium* E1162 plays a role in the resistance to antimicrobial peptides.

We have also demonstrated that the absence of this permease led to growth defects in TSB medium with the EfmE1162_0264 mutant having an extended lag phase. We hypothesised that this growth defect might due to the presence of the enzymatic digest of casein in TSB. It is known that digestion of casein releases bioactive peptides. To test this, the strains were grown in BHI medium with and without the addition of casein hydrolysate. The *E. faecium* EfmE1162_0264 mutant had similar growth to *E. faecium* E1162 in BHI medium but was defective in growth in the presence of casein hydrolysate. Several casein-derived peptides have been found to have antimicrobial activity against various gram-negative and gram-positive pathogens (Szwajkowska et al., 2011) so this was the first indication that EfmE1162_0264 may be involved in resistance to antimicrobial peptides.

One of the mechanisms of antimicrobial resistance developed by bacteria is to modulate cell surface charge. The initial binding of cationic antimicrobial peptides to the anionic bacterial cell surface is mediated by electrostatic attraction (Peschel and Sahl, 2006). In general, there are two different polyanionic teichoic acids present on the gram-positive bacterial cell surface, lipothechoic acid (LTA) and a wall teichoic acid (WTA). LTA is noncovalently inserted into the cellular membrane and WTA is covalently linked to the N-acetylmuramic acid residues of peptidoglycan of the bacterial cell wall. D-alanyl residues are covalently linked to these chains and alter the net charge of the polyanionic teichoic acids (Neuhaus and Baddiley, 2003). In *Bacillus subtilis*, the absence of D-alanine from both LTA and WTA was observed when *dltA-dltD* were inactivated (Perego et al., 1995).

The results reported in this study demonstrate that the putative permease component of an ABC transporter somehow regulates the bacterial cell surface charge. The

percentage of cytochrome c bound to the *E. faecium* EfmE1162_0264 mutant was higher compared to the *E. faecium* wild-type E1162. This result indicates that the deletion of EfmE1162_0264 led to an increase in the net negative charge of the bacterial surface. It was also observed that a *dlt* operon is located downstream of the gene for EfmE1162_0264. Given our findings one could speculate that the EfmE1162_0264 is involved in AMPs resistance by in some way controlling expression of the *dlt* operon.

It has been shown in other bacteria such as *Staphylococcus aureus* that inactivation of the *dlt* operon results in higher sensitivity to cationic antimicrobial peptides. This mutant also had an increased negative surface charge due to absence of D-alanine, thus mutant cells bound more positively charged proteins like gallidermin, nisin, protegrins and human defensin HPN1-3 (Peschel et al., 1999) than the parent strain. In *Clostridium difficile*, a mutation in the first gene, *dltD* resulted in inactivation of the *dlt* operon. The mutant with a defective *dlt* operon showed higher susceptibility to cationic antimicrobial peptides including polymyxin B, nisin, gallidermin and vancomycin. This indicated that this operon plays an important role in protecting this bacterium from host defences or indigenous microbiota (McBride and Sonenshein, 2011). In a study performed on *E. faecalis*, deletion of *dlt*A resulted in a lack of D-alanine esters on the teichoic acids and caused a higher net negative charge on the bacterial cell surface (Fabretti et al., 2006). In that study, they observed that the Δ*dltA* formed less biofilm on polystyrene surfaces and had reduced opsonophagocytic killing compared to the wild-type. The *dltA* mutant also showed higher susceptibility to cationic antimicrobial compounds (colistin, polymyxin B and nisin) and had more enhanced susceptibility if *dltB* was also deleted (Fabretti et al., 2006).

Involvement of ABC transporters in modulating bacterial surface charge is not exclusive to *E. faecium*. The involvement of ABC transporters that are co-involved in the control of the cell envelope charge by controlling *dlt* operon has been previously described in other firmicutes. For example, in *S.aureus*, one of its permease component of an ABC transporter VraG alters the sensitivity of this bacterium to cationic AMP by modulating the charge of the bacterial cell surface (Meehl et al., 2007). In *Lactobacillus casei* BL23, the ABC transporter, ABC12 controls the expression of *dlt* operon (Revilla-Guarinos et al., 2013).

The present study used a subinhibitory concentration of polymyxin E to determine EfmE1162_0264 was involved in sensing AMPs. Although the result showed that this ABC transporter is not required for sensing polymyxin E in exponential phase it is possible that it may play a role in stationary phase. It may also be that this ABC transporter might be responsive as a sensor for specific AMPs such as nisin and bacitracin. It is also possible that there is cross-talk between ABC transporters or other two-component systems like in *S. aureus*. The two-component system, BraRS controlled the expression of an orphan ABC transporter, VraDE, thus conferring resistance against nisin and bacitracin (Hiron et al., 2011). While my research was going on, another researcher found that *E. faecalis* exhibits different regulatory mechanisms that mediates bacitracin resistance where an ABC transporter acts as a sensor, BcrR and activates the two-component system, VanSB-VanRB. Subsequently, the two-component system activates a second ABC transporter, BcrAB and this mediates resistance against bacitracin (Gebhard et al., 2014).

The result also demonstrate that the virulence of the *E. faecium* E1162 and the EfmE1162_0264 mutant was not significantly different in a *G. mellonella* wax worms model, thus suggesting that ABC transporter might not be involved in *E. faecium* virulence.

In summary, we have identified a permease component of an ABC transporter that plays a role in AMPs resistance. We have also observed that the permease component of ABC transporter also plays an important role in modulating the net charge of the bacterial cell surface of this bacterium.

Chapter 6

General Discussion and Future Work

6 General Discussion and Future Work

6.1 General discussion

The work described in this thesis aimed to identify genes involved in resistance to host stresses and virulence as these could potentially be future therapeutic targets. The studies presented in this thesis mainly focused on (i) identifying genes that are involved in lysozyme and nisin resistance; (ii) the role of tyrosine decarboxylase in acid tolerance; (iii) the role of a serine threonine protein kinase in cell wall stress; (iv) the role of an ABC transporter in antimicrobial peptide resistance.

Genes involved in antimicrobial peptide resistance were identified using a transposon mutagenesis and sensitivity screening protocol. The mariner-based transposable element was used in *Enterococcus faecalis* (Kristich et al., 2008). In my studies a mariner-based transposable element was used since it has been reported to only require a TA site for insertion (Lampe et al., 1996) . When the presented study was conducted, the mariner-based transposable element had not been used but, in 2012, Zhang and colleagues used similar technique to identify ampicillin resistance determinants (Zhang et al., 2012).

Since *E. faecium* is a low GC organism many such sites exist in the bacterial genome. This TA-specific mariner-based transposable element should theoretically allow for full genome coverage with insertions in all open reading frames. A mariner-based transposable element was also used in the same strain (*E. faecium* E1162) to identify genetic determinants in other phenotypes (Zhang et al., 2012; Zhang et al., 2013).

Using the transposon mutagenesis technique, the insertion of the transposon within a gene, results in the gene being disrupted and potentially giving rise to an inactive gene product. Transposon mutants with an insertion within an essential gene will not be viable

and viable mutants that were obtained using this approach will have transposons inserted into genes that are not essential for the organism to grow in laboratory. However, sometimes it is still possible to obtain a viable mutant even for essential genes since the insertion of a transposon in genes does not always completely eliminate the activity of the gene product (Blades and Broman, 2002).

In chapter 3 an *E. faecium* transposon mutant library was screened for altered resistance to lysozyme and to nisin as a model antimicrobial peptide. This approach led to the identification of several genes that contribute to lysozyme and nisin resistance.

The results revealed that a gene coding for a conserved hypothetical protein EFF34598 was likely to be involved in lysozyme resistance although polar effects on adjacent genes was not ruled out as accounting for this phenotype. It is possible that *E. faecium* E1162 utilise the toxin-antitoxin systems as *S. aureus* which might involved in maintenance and in global regulation of virulence (Bukowski et al., 2013).

Several genes involved in nisin resistance were also identified. Genes coding for a tyrosine decarboxylase (EFF34651), putative muramidase (EFF33496), cystathionine β-synthase (CBS) domain containing protein (EFF35601) and conjugative transposon protein (EFF33504) were identified as the nisin resistance determinants. The data showed that mutants strains were also significantly impaired in killing *G. mellonella* wax worms compared to the *E. faecium* E1162 wild type strain. The present result demonstrates that these genes potentially involved in virulence of this pathogen.

The mutant which had a transposon integrated into genes predicted to code for a conserved hypothetical protein (EFF35914) and putative transposase were involved in nisin resistance and significantly less virulent in the *G. mellonella* wax worms model compared to the wild type *E. faecium* E1162. This indicates that disruption of this genes or polar effects

on neighbouring genes, due to the transposon insertion, might be important in virulence of this bacterium.

The work described in that Chapter 3 served to further characterize the role of tyrosine decarboxylase in acid tolerance. Our results using a transposon mutant with a disruption in the *tdc* gene more robustly demonstrate that tyrosine decarboxylase is involved in acid resistance. Our result shows that the tyrosine decarboxylase pathway is also involved in *E. faecium* virulence in the *Galleria mellonella* wax worms infection model. Thus has improved our insights that tyrosine decarboxylase mediates the adaptability of this pathogen in adaptation for survival within the host.

In Chapter 4, a targeted genetic approach was used to examine the role of the *E. faecium* serine threonine protein kinase (Stk1) in antimicrobials resistance and virulence. In *E. faecalis* the *stpk* gene (*ireK*) is involved in antimicrobial resistance and intestinal persistence (Kristich et al., 2007). Similar to the study by Kristich et al. (2007), our result showed that disruption of the *stk1* gene in *E. faecium* led to higher sensitivity to antibiotics that target cell wall biogenesis and resistance to bile salts compared to the wild type. Our results showed that the *stk1* gene is also required for intrinsic cephalosporin resistance in *E. faecium.*

Providing the *stk1* gene from *E. faecium* or the *ireK* gene from *E.faecalis* in trans restored the resistance of the Stk1 mutant to ceftriaxone. Ceftriaxone belongs to a class of β-lactam antibiotics. The β-lactam antibiotics have a β-lactam ring in their structure which has structural similarity to the PBP transpeptidation substrate, the D-alanyl-D-alanine carboxy terminus of peptidoglycan peptides. The β-Lactams inhibit cell wall biosynthesis by inhibiting the transpeptidase (final-crosslinking) activity by acylating the penicillin-binding domain of PBPs and forming a stable acyl-enzyme (Goffin and Ghuysen, 1998; Massova and

Mobashery, 1998). Apart from Stk1, resistance to β-lactam antibiotics in *E. faecium* is conferred by the low affinity of the membrane-bound PBP5fm for β-lactam antibiotics (Zorzi et al., 1996). The β-lactams are less effective at disrupting cell wall synthesis of the bacterium because PBP5fm is able to replace the role of other high-molecular-mass (HMM) PBPs in peptidoglycan synthesis when the PBPs are inhibited by the antibiotic (Fontana et al., 1994; Rice et al., 2001; Sifaoui et al., 2001). The architecture of the Stk1 protein of *E. faecium* and IreK from *E.faecalis* are different with respect to the PASTA domains. *E. faecium* has four PASTA domains and *E. faecalis* has five PASTA domains in its STPK protein. Kristich and colleagues (2007) suggested that the IreK is a transmembrane receptor that monitors cell wall integrity and mediates adaptive responses in *E. faecalis* (Kristich et al., 2007). Other authors suggested that the PASTA domains may sense the presence of β-lactam antibiotics and these molecules might serve as extracellular signals of STPKs (Yeats et al., 2002). Although the number of PASTA domains between *E. faecalis* and *E. faecium* are different, it is tempting to speculate that the *stk1* gene of *E. faecium* and *ireK* gene of *E. faecalis* may directly or indirectly modulate the expression of PBP5fm since providing *stk1* gene *in trans* restored the resistance of the *stk1* mutant and *ireK* mutant to ceftriaxone. The result in the present study also showed that *stk1* gene contributed to *E. faecium* virulence in the *G. mellonella* infection model.

In chapter 5 putative permease (EfmE1162_0264) that may be involved in antimicrobial peptide resistance were been identified in the genome sequence of *Enterococcus faecium* E1162. A large extracellular domain between helices 8 and 9 in EfmE1162_0264 was identified using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de). No genes for two-component sensing systems were identified neighbouring the permease gene thus suggesting that the large extracellular

domain in the permease component may act as a sensor. A markerless EfmE1162_0264 mutant had reduced resistance to the antimicrobial peptides tested and altered the net charge of the bacterial cell surface.

The absence of this gene also led to growth defects in TSB medium. One of the components in TSB medium is casein-derived peptides that might contain bioactive peptides that have antimicrobial activity against *E. faecium*. Several casein-derived peptides have been found to have antimicrobial activity against various gram-negative and gram-positive pathogens (Szwajkowska et al., 2011) thus this could have been the first indication that EfmE1162_0264 may be involved in resistance to antimicrobial peptides.

To further characterize the role of EfmE1162_0264 in antimicrobial peptide resistance, the cell surface changes of *E. faecium* in the exponential and stationary phase in the presence and absense of polymyxin E was evaluated. Polymyxin E was used in the study to observe the ability of EfmE1162_0264 in sensing antimicrobial peptides during growth. Polymyxin E is cationic and binds to anionic lipopolysaccharide. Since *E. faecium* is a gram-positive bacteria, it does not have a lipopolisacharide and the polymyxin E (5 µg/mL) does not cause harm to this bacteria. Cytochrome c shows a high affinity for strongly negatively charged cells (Peschel et al., 1999). Therefore, to evaluate the cell surface charge, the cytochrome c binding affinity of the *E. faecium* E1162 and EfmE1162_0264 mutant were evaluated. The result showed that this ABC transporter is not required for sensing polymyxin E at the concentration used. However, it is possible that this ABC transporter might be responsive and potentially could be a sensor for other AMPs such as nisin and bacitracin. It is also possible that EfmE1162_0264 might regulate the *dlt* operon and mediates resistance to cationic antimicrobial peptides.

6.2 Future work

Several genetic determinants that were putatively involved in nisin resistance were identified. Although the present result demonstrate that several genes potentially involved in nisin resistance and virulence of this pathogen. However, this hypothesis is still to be confirmed. It was shown that these genes (tyrosine decarboxylase (EFF34651), putative muramidase (EFF33496), cystathionine β-synthase (CBS) domain containing protein (EFF35601) and conjugative transposon protein (EFF33504)) involved in virulence but the mechanism of activation and its impact on *E. faecium* pathogenesis is still to be clarified.

The glutamate decarboxylase in *Listeria monocytogenes* mediates nisin resistance by contributing intracellular ATP pools (Begley et al., 2010). Hence, it could be hypothesized that tyrosine decarboxylase of *E. faecium* might contribute to the intracellular ATP pools and make this bacterium more resistant to this lantibiotics. To confirm this, additional experimental work is needed to further analyse the intracellular ATP levels and determine the mechanism of activation of this tyrosine decarboxylase gene in acid tolerance.

The *stk1* mutant was, in general, more sensitive to the cell wall-active antibiotics compared to the wild-type strain, *E. faecium* E1162. For both cefotaxime and ceftriaxone, whereas the wild type strain was resistant to more than 1024 µg/mL, an increase in susceptibility of approximately 8-fold and 32-fold was observed in the *stk1* mutant for cefotaxime and ceftriaxone, respectively. Our results also suggest that there is an altered cell wall in the *stk1* mutant. Further studies are still required to study the Stk1 interactions with other genes or proteins that are involve in the presence of antibiotics to clarify the mechanism of action of this serine threonine protein kinase to maintain the cell wall

integrity of this bacterium. Further work could be done to analyse the proteins and genes by analysing its phosphoproteomics and transcriptomics.

Although the zymographic analysis indicated that the expression of autolytic enzyme was similar in the *stk1* mutant compared to *E. faecium* E1162. This method might fail to detect the expression of all the autolysins produced by *E. faecium* or may not be very quantitative. Therefore deeper investigation of the biological role of the *stk1* to influence autolysin expression levels in *E. faecium* E1162 could permit the identification of the signalling pathway influencing the *stk1* to clarify its role in the in cell wall metabolism of *E. faecium*.

Since our result showed that the *E. faecium* markerless mutant with a deletion of the putative EfmE1162_0264 permease component of the ABC transporter had a severe growth defect in TSB medium, future work could investigate which peptides that exist in the casein digest have antimicrobial activity towards this bacterium. Our result suggests that the ABC transporter is not a sensor and it might be a positive regulator of the *dlt* operon. However, further analysis is still required to confirm these possiblities.

Since *E. faecium* E1162 is resistant to a broad range of antibiotics, targeting the putative virulence genes that have been identified in this study is one of the alternative methods to treat infections due to this bacterium. Some virulence inhibitors such as antibacterial molecules and antibodies could be used to treat or prevent infections by this bacterium in future.

To conclude, this work has extended the current knowledge of genes involved in acid tolerance, antimicrobial resistance and virulence of *E. faecium*. It is hoped that the research undertaken could expand our knowledge about enterococcal pathogenesis and thus

contribute to future development of effective treatments for life-threatening infections caused by this pathogen.

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Appendix A

strains	24 hours			48 hours			72 hours			96 hours		
	E 1	**E 2**	**E 3**	**E 1**	**E 2**	**E 3**	**E 1**	**E 2**	**E 3**	**E 1**	**E 2**	**E 3**
E1162	3	3	2	5	7	8	8	10	10	10	10	10
47N	1	2	1	3	3	4	5	5	5	6	6	6
59N	1	0	0	1	0	3	1	0	3	3	1	6
64N	0	1	0	2	0	1	3	0	1	4	1	3
83N	2	1	1	4	3	8	4	5	9	5	5	10
95N	1	3	2	6	5	7	8	7	8	9	8	10
183N	2	3	2	5	3	8	7	7	9	7	7	9
Δ EfmE1162_0264	2	3	4	9	6	10	9	6	10	10	7	10
ΔEfmSTPK	2	2	3	2	3	4	3	7	5	4	8	6
ΔAGSA1	2	2	3	7	4	5	9	9	8	10	10	9

E: Independent experiments