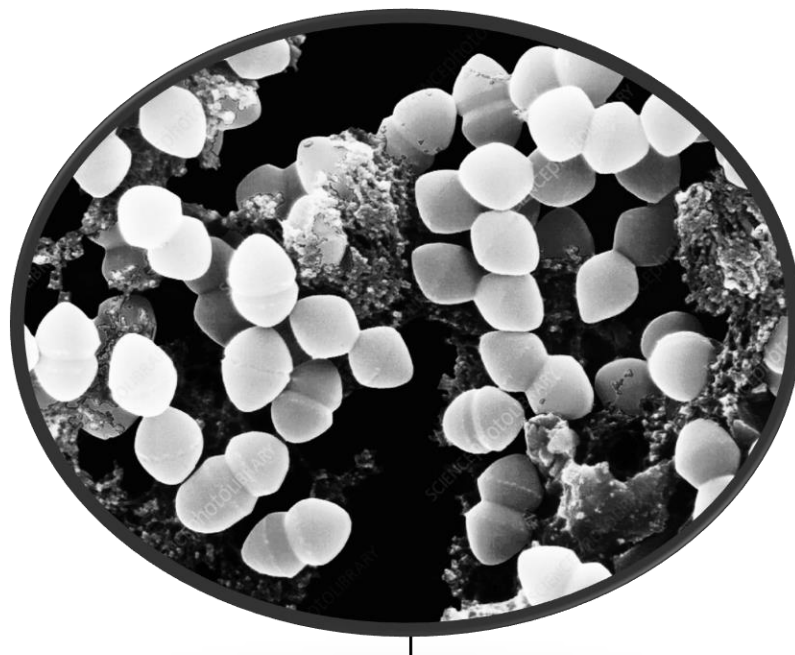


Identification of *Enterococcus faecium* genes involved in resistance to oxidative stress and virulence

Thesis submitted by
Hadeel Ghazi Ahmed Mahmoud

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**Faculty of Medical Sciences
Department of Microbial Diseases
Eastman Dental Institute
University College London
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“The journey. Not the destination matters.”

- T.S. Eliot

Declaration

I certify that the work described in this thesis entitled “Identification of *Enterococcus faecium* genes involved in resistance to oxidative stress and virulence” is my own unless stated otherwise.

Hadeel Mahmoud

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In the name of Allah, the Most Merciful and Beneficent

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Abstract

The *Enterococcus faecium* clonal complex (CC17) is an example of how a cumulative evolutionary process improves relative fitness of bacteria in the hospital setting. As a result, a better knowledge of pathogen resistance mechanisms is required to forecast and prevent its spread. The research described here was aimed to test if zebrafish larvae and planarian infection models could be used to study the survival rates, colonisation, and pathogenicity of *E. faecium* E1162 and its isogenic mutants: 201c, 59N, Mabc, 183N, and 47N, which had transposon insertion in a gene that encoded respectively one of the following a serine/threonine protein kinase, a putative muramidase, a putative permease component of an ABC transporter, a cystathionine beta-synthase domain protein, and a putative tyrosine decarboxylase. In zebrafish larvae, mutants 201c, 59N, Mabc, and 183N were less virulent than *E. faecium* E1162.

Killing was much later than *E. faecium* E1162 when planarians were co-incubated with mutants 59N, 47N and Δesp (deletion of the enterococcal surface protein gene). Also in this study the *E. faecium* E1162 transposon mutant library was screened for mutants with reduced oxidative stress sensitivity against superoxide generator menadione and/or the organic peroxide generator cumene hydroperoxide. Three mutants were identified: MS1 with an insertion in *EfmE1162_1492* encoding a putative tyrosine decarboxylase involved in acid adaptation, MS2 with an insertion in *EfmE1162_1516* encoding a putative conserved hypothetical protein, a tRNA-modifying enzyme, and MS3 with an insertion in *efmE1162_2615* encoding a

putative transposase a response stimulator. Mutant MS1 and MS2 were significantly more sensitive to 3.38 mM cumene hydroperoxide than *E. faecium* E1162. Compared to *E. faecium* E1162, the killing rate with MS2 decreased significantly in the first two days of infection in the *Galleria mellonella* ($p > 0.025$). In addition, mutant 59N was found to be more susceptible to menadione and cumene hydroperoxide than *E. faecium* E1162, and the wild-type virulence was restored in the *G. mellonella* infection model when mutant 59N was complemented *in trans* with the wild-type putative muramidase encoding gene.

In conclusion, the genes mentioned above may play an important role in the pathogenesis of *E. faecium* infections. In addition, zebrafish larvae and planarian models are useful for studying *E. faecium* pathogenicity.

Impact statement

Multidrug-resistant hospital-associated CC17 *Enterococcus faecium* can resist many antimicrobials owing to the acquisition of numerous antimicrobial resistance genes. *E. faecium* causes severe illness and death among immunocompromised hosts, this has driven the need to understand the pathogenicity of *E. faecium* and develop new therapeutics. Research on virulence factors and mechanisms of bacterial virulence could give new clues for other therapeutic options apart from antibiotics. The work described in this thesis aimed to identify genes contributing to the virulence of multidrug-resistant *E. faecium*. Different animal models have been used to imitate *E. faecium* infections, however, none of them are considered ideal for studying pathogenesis.

The research described in this PhD thesis examined the use of zebrafish larvae and planarian models to study innate immunity and to overcome the ethical and practical issues concerning the mammalian models. These two models are also low-cost and time-efficient models for the study of pathogenesis. Survival and colonisation in infected planarian and zebrafish larvae were studied using the parent strain *E. faecium* E1162 and isogenic mutants. In the majority of zebrafish and planarian infection models, infection is achieved by microinjecting bacteria. The research presented in this thesis used zebrafish larvae and planarian infection models to investigate the pathogenicity of *E. faecium* E1162 using static immersion. Static immersion takes less time and does not require any additional experience. Results showed that these models are quick and reproducible screening tools for *E. faecium* and its tested mutants.

A *Himar1 mariner* transposon mutant library of *E. faecium* E1162 was screened for mutants with decreased resistance to superoxide generators, menadione, and cumene hydroperoxide, three genes have been identified as mediating *in vitro* susceptibility to menadione or cumene hydroperoxide. The research described here was also demonstrated the role of the permease component of an ATP-binding cassette transporters (ABC transporter), serine/threonine protein kinase (Stk), a putative muramidase, and a cystathionine β -synthase domain containing protein in the pathogenicity of *E. faecium* E1162, these proteins could possibly be future therapeutic targets against multidrug-resistant hospital-associated CC17 *E. faecium*.

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List of Abbreviations

ABC transporters	ATP-binding cassette transporters
Acm	cell wall-anchored collagen adhesion
Amp ^r	Ampicillin-resistant
AsrR	Antibiotic and stress response regulator
BAPS	Bayesian analysis of genetic population structure
BHI	Brain heart infusion
CBS	Cystathionine beta synthase
CC17	Clonal Complex 17
CFU	Colony Forming Unit
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
diH ₂ O	deionised Water
Dpf	day post fertilisation
Dpi	day post infection
eDNA	Extracellular DNA
Esp	Enterococcal Surface Protein
GidA	Glucose-inhibited division protein A
hour/hours	hr/hrs
LB	Luria-Bertani
Minute	min
MIC	Minimum Inhibitory Concentrations
MHA	Mueller-Hinton agar
MLST	Multilocus Sequence Typing
MORN2	Membrane Occupation and Recognition Nexus-2
MS	Menadione-sensitive
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
O ₂ ⁻	Superoxide
OH ⁻	Hydroxide ion
•OH	hydroxyl radical
¹ O ₂	singlet oxygen

OD600 nm	Optical Density at 600 nanometer
ORF	Open Reading Frame
P	<i>p</i> -value
PAMPs	pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PBP	Penicillin-binding protein
Pmol	Picomole
PNK	Polynucleotide Kinase
Plac8	Placenta specific protein 8
PRRs	Pattern Recognition Receptors
PFGE	Pulsed-field gel electrophoresis
RepA ⁺	replication protein A
rRNA	ribosomal RNA
ROS	Reactive Oxygen Species
Rpm	Revolutions per minute
SagA	secreted antigen
SgrA	Serine-glutamate repeat-containing protein A
SOC	Super Optimal broth with Catabolite repression
Stk1	serine threonine protein kinase
STs	Sequence Types
TBE	Tris/Borate/EDTA
tdc	tyrosine decarboxylase
TLRs	Toll-like receptors
TSB	Tryptic soy broth
VAN	vancomycin
VRE _{fm}	Vancomycin-resistant <i>Enterococcus faecium</i>
v/v	volume/volume
w/v	weight/volume
WGS	Whole-Genome Sequencing
xg	Times gravity
A	Alpha
β	Beta

Ω	Omega
$::$	Complementation

Chapter I

General Introduction

1.0 General Introduction

In the developed world, life has improved rapidly from the time when penicillin was discovered (1928), thus, sicknesses like tuberculosis and pneumonia no longer commonly kill the young and healthy people (Leatherby, 2017). So, usually one can control infection by using antimicrobial drugs to kill or limit the growth of bacteria, viruses, parasites, or fungi and finally, fight off the infection (Tacconelli et al., 2017). However, microbes can develop resistance to the drugs that target them, a condition known as antimicrobial resistance (Fair and Tor, 2014). Failure to apply infection control practices, inappropriate prescribing, and the use of antimicrobials in agriculture is increasing antimicrobial resistance (Byarugaba, 2004; *Antibiotic Resistance*, 2020). Antimicrobial resistance is exacerbated when antibiotics may be purchased without a prescription for human or animal usage. Similarly, in countries without standard treatment guidelines, antibiotics are often over-prescribed by health workers and veterinarians and over-used by the public.

The world faces a post-antibiotic era with no immediate action, in which common infections and minor injuries can once again kill. Antimicrobial resistance is growing to high levels among all parts of the world. Resistance mechanisms are developing and spreading worldwide, limiting the ability to treat common infectious diseases. Pneumonia, tuberculosis, blood poisoning, gonorrhoea, and foodborne diseases are occasionally difficult to treat as antibiotics become less active (*Antibiotic Resistance*, 2020). Globally, antimicrobial resistance causes 700,000 deaths per year, this number could reach 10 million deaths annually by 2050 (Jim O'neill, 2016). Frequently, bacterial strains that had developed some form of antimicrobial resistance are

hospital-acquired infections or nosocomial-infections (Inweregbu et al., 2005; Sousa et al., 2021).

Numerous groups of drugs are regularly applied in the hospital to overcome infections by microorganisms, hence, microbial populations inhabiting such an environment are continuously exposed to antibiotics as well as disinfectants, consequently, this can lead to antibiotic resistance developing. It is worth noting that the prevalence of multidrug-resistant organisms in hospitals may be higher than in the general population, making it difficult for doctors to choose an antibiotic that will effectively treat the infection. Multiple drug resistance organisms can be hidden by using different antibiotics, infections caused by multiple drug resistance organisms are dangerous and can result in death (Fair and Tor, 2014). The WHO reports that antibiotic resistance has a negative impact on the health and the economy (*Global Action Plan on Antimicrobial Resistance*, 2021). The number of bacteria acquiring resistance to antibiotics is increasing (MacKinnon et al., 2021). Multidrug-resistant bacteria are now causing 75% of all infections among our hospitalized patients (Vaithiyam et al., 2020).

To slow antibiotic-resistance in hospital, some hospitals cycle the antibiotics they use to treat disease. Infections caused by antibiotic-resistant bacteria are harder to treat and no one can completely avoid getting an infection, including an antibiotic-resistant infection, but some people are at greater risk than others. Patients in a hospital are more susceptible since they are sick, for example, many of them might be recovering from surgery or immunocompromised or under prolonged antibiotic treatments (Inweregbu et al., 2005; Nathwani et al., 2014; Ventola, 2015).

Enterococcus faecium, is an opportunistic pathogen usually found as a commensal of human and animal intestines and can also survive in the environment. *E. faecium* can rapidly acquire resistance genes that, if expressed can enable the bacterium to escape the effects of many antibiotics. The hospital-adapted clone of *E. faecium* clonal complex 17 (CC17), is responsible for a significant amount of hospital-acquired infections and results in severe morbidity and mortality (Lee et al., 2019; De Oliveira et al., 2020; Erdem et al., 2020; Peng et al., 2022).

1.1 General features of *E. faecium*

E. faecium is low G+C Gram-positive cocci, which is a facultative anaerobe (Sghir et al., 2000; de Regt, 2010) and grows aerobically at a variety of temperatures from 5 to 50°C (Van den Berghe et al., 2006). However, as per Byappanahalli et al. (2012) it grows best at 35°C. *E. faecium* is non-mobile, non-sporulating, non-capsulated, non-pigmented, tolerant to pH between 4.8–9.6 and salt-resistant (up to 28% NaCl) (Lebreton et al., 2014; Li et al., 2018; Zommiti et al., 2018).

1.2 Habitat of *E. faecium*

E. faecium has been detected in the faecal material of most animals, from insects to mammals. In humans, *E. faecium* lives as a commensal in the gut, the oral cavity, and the vagina. It has been isolated from a variety of environments, including waste and surface water, plant environments, as well as from the intestinal microbiota of mammals, reptiles, birds, fish, and insects (Sghir et al., 2000; de Regt, 2010; Gilmore et al., 2014). It has also been recovered from foods such as milk and meat products such as fermented sausages, as well as in large quantities in foods such as cheese (Fisher and Philips, 2009).

1.3 The advent of *E. faecium* as a nosocomial-infection

The genus *Enterococcus* now has about 67 species the most common of which are *Enterococcus faecalis* and *E. faecium* in human faeces (Ramos et al., 2020; Aun et al., 2021). In 1899 enterococci were first identified in human fecal flora, they were considered part of the genus *Streptococcus* (Gram-positive bacteria which broadly spread through the normal flora of human and animals) until 1984 (Zhou et al., 2020). Members of this species can cause invasive infection if they present in a normally sterile site (Parks et al., 2015). In 1906, *Streptococcus faecalis* was first named when the bacteria were isolated from a patient with endocarditis, while *Streptococcus faecium* was first described in 1919. Later on, group D *Streptococcus* were divided into two groups based on differences in biochemical and nucleic acid (DNA-rRNA homology and 16S rRNA) (Schleifer and Kilpper-Bälz, 1984). Enterococcal infections in humans mainly caused by *E. faecalis* and *E. faecium*. Enterococci appeared as a primary cause of hospital-acquired infections in 1970s (Gilmore et al., 2013).

Enterococci are often one of the most typical species isolated from nosocomial blood stream infection in intensive care units, rating 2nd in the USA (Peel et al., 2012; Rosa et al., 2014) and 3rd in European intensive care units (Iosifidis et al., 2013). Globally in these patient groups, crude death rates range from 15% to 100% (Butler et al., 2010; Hayakawa et al., 2012; Kang et al., 2013; Rosa et al., 2014; Chang-Hua et al., 2016).

E. faecium has effectively adapted to conditions in a nosocomial situation and developed resistance against glycopeptides (Bonten et al., 2001; Top et al., 2008). *E. faecium* was first recognised as a nosocomial pathogen in the

United States in the 1980s, with an increase in infections caused by ampicillin-resistant *E. faecium*, which was quickly followed by the advent of vancomycin-resistant enterococci in the early 1990s (Murray, 2000). In the mid-1990s, a substantial surge in colonisation and infections with ampicillin-resistant enterococci preceded the advent of vancomycin-resistant enterococci in Europe (Top, Willems and Bonten, 2008). There are two subpopulation of *E. faecium*, the first subpopulation includes commensals of the gastrointestinal tract and generally not causing clinical infection, the second subpopulation includes nosocomial *E. faecium*. These subpopulations were recognized using amplified fragment length polymorphism (Zhou et al., 2020). Later, these two subpopulations confirmed and characterized according to sequence-based methods such as multi-locus sequence typing (MLST) and whole-genome sequencing (WGS) (Willems et al., 2000; Willems et al., 2005). These two subpopulations known as clade A and clade B, the hospital-associated lineages linked to a subclade of clade A and A-1 known as CC17 (Willems et al., 2012).

Often, *E. faecium* isolates causing hospital outbreaks are belonging to the hospital-associated subpopulation which are commonly resistant to ampicillin and contain pathogenicity islands (Willems et al., 2005). Because of acquiring and losing genes, hospital-associated isolates developed a number of traits such as increase in antibiotic resistance genes and virulence genes enhancing biofilm formation making them successful in the hospital atmosphere (Gao et al., 2018). These genes acquired through plasmid transfer and homologous recombination which mediated by insertion sequence elements (Leavis et al., 2007).

E. faecium developed resistance against β -lactams (penicillin), cephalosporines, lincosamides, streptogramins, aminoglycosides and glycopeptides {e.g., vancomycin (VAN)} or macrolides. This critical problem of resistance is linked to growing number of nosocomial-infections associated with VAN-resistant *E. faecium* (VRE_{fm}), owing to these reasons *E. faecium* belongs to member of the group of ESKAPE microbes (*E. faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), which are the main cause of nosocomial-infections and are particularly difficult to treat because of their resistance to many antibiotics (Nakonieczna et al., 2019). *E. faecium* can cause a range of nosocomial-infections of soft tissues, abscesses, urinary tract infections and endocarditis (Růžicková et al., 2020).

VRE_{fm} is putatively the lethal cause of health-care- associated infections worldwide. There was a 65.7% mortality rate after 30 days in 35 patients with VRE_{fm} identified between 2010-2012 in a major tertiary referral hospital in Southern Brazil, according to Rosa et al. (2014).

1.4 Molecular epidemiology of *E. faecium*

E. faecium can be split into genetic lineages known as sequence types (STs) using MLST, which characterizes the loci within seven *E. faecium* housekeeping genes (*atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, and *adk*) (Homan et al., 2002). The hospital-associated clade A-1, which has now been renamed CC17, was identified as the ancestral clone of ST17 (Top et al., 2008). Since then, the bulk of *E. faecium* isolates from hospitals have been identified as CC17 members (Figure 1-1). To better understand the genetic foundation of

the highly hospital adapted ST17, a sample ST17 genome of vancomycin-resistant strain Ef aus0004 was completely constructed (Lam et al., 2012). According to the analysis, the genome contained a chromosome, three plasmids, and several prophages. When the completely assembled genome was compared to a collection of ST17 draft genomes, it was found that this clone had a large accessory genome (38%) that demonstrated the lineage's potential to horizontally acquire new genetic material (Lam et al., 2012).

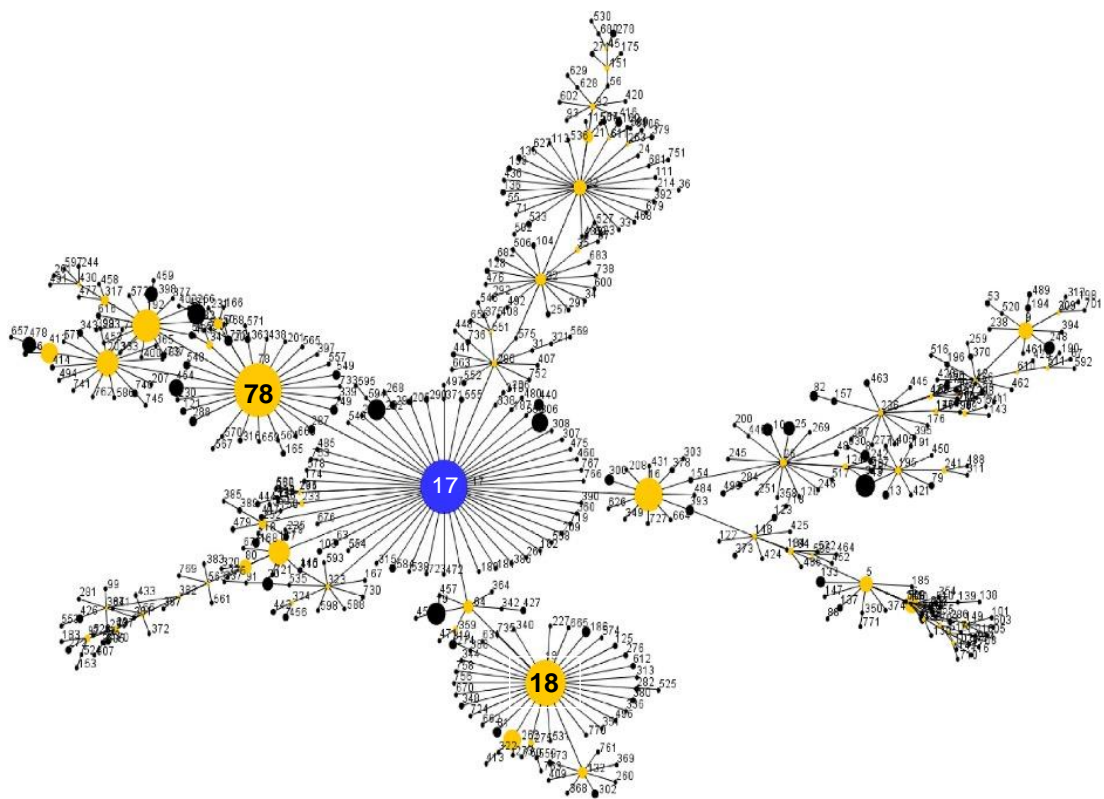


Figure 1-1 eBURST created a population snapshot of *E. faecium* sequence types linked with CC 17 around the world (derived from <http://e.faecium.mlst.net/>). December 15, 2017.

A black dot is used to represent each sequence type. The numbers correspond to a specific sequence type. The number of isolates inside a sequence type is represented by the size of each dot. A blue dot is used to signify a clonal complex's ancestral sequence type. The yellow dots signify a co-founder of a subgroup. Adapted from Lee et al. (2019).

MLST is an essential tool for typing isolates, but clinical isolates of *E. faecium* with the same sequence type have shown significant sequence diversity

(Johnson et al., 2010; Howden et al., 2013). Many *E. faecium* strains that could not be characterized by MLST due to the absence of the essential housekeeping gene *pstS* (Carter et al., 2016). According to WGS studies, genetic diversity within *E. faecium* may have already crossed a degree of divergence often associated with speciation (Lebreton et al., 2013). As a result, a more reliable typing technique that considers genetic changes across the entire genome would be more appropriate for typing *E. faecium* isolates. MLST is a method for determining STs strains into clonal complexes by analyzing DNA sequence variability in housekeeping genes (Homan et al., 2002). MLST data can be stored in the database for the collection and analysis of epidemiological data from all over the world. It is possible to track changes in the genome that occur gradually (Homan et al., 2002). At the moment, surveillance can still use MLST to detect the introduction of a novel *E. faecium* sequence type at a facility or location using MLST. To combine the allelic variation of the seven housekeeping genes with information obtained from comparative genomics research, the WGS and MLST approaches have been employed (Homan et al., 2002), although MLST has limitations when used to classify *E. faecium*, it is difficult to infer putative transmission in the absence of fine resolution, so analyses based on WGS data are increasingly being used alongside MLST (Reuter et al., 2013; Brodrick et al., 2016) to overcome this limitation and identify transmission events. As a result, other methods with strong discrimination capabilities are more appropriate for doing short-term epidemiological research. One of these techniques, known as Amplification of DNA surrounding rare restriction sites (ADSRRS-fingerprinting), relies on changes in the electrophoretic profiles produced after the selective

amplification of restriction fragments following the digestion of entire genomic DNA (Krawczyk et al., 2003).

Early detection of new *E. faecium* STs in a hospital could lead to infection control prevention, especially if the STs have previously been identified as highly pathogenic (Lee et al., 2019).

MLST of *E. faecium* isolates of human and animal origin showed the presence of host-specific genogroups associated with hospital isolates including CC17. Strains belonging to CC17 are typically ampicillin-resistant (Amp^r) and are enriched for putative virulence factors that may assist with hospital adaptation and spread. Studies have indicated that there is significant genetic variety among CC17 isolates causing them to exhibit various characteristics that allow them live in the hospital environment, colonise people, and cause infections such as bacteraemia, peritonitis, endocarditis, and urinary tract, wound, and device-related illnesses and to be resistant to powerful antibiotics (Sava et al., 2010). Initially, amplified fragment length polymorphism was used to determine the genetic similarity of strains from different hosts and areas (Willems et al., 2000). By using amplified fragment polymorphism, strains obtained from hospitalized patients formed specific clusters different from clusters of strains that were obtained from humans in the public and farm animals. But this method was not suitable for universal studies because of the differences in the data obtained from diverse laboratories due to methodology and reproducibility (Savelkoul et al., 1999).

The additional practice designated MLST (Homan et al., 2002; Ruiz-Garbajosa et al., 2006) was introduced, where gene sequences of fragments of several

housekeeping genes (seven housekeeping genes in the case of the *E. faecium* and *E. faecalis*) were used for strain typing, sequence data is straightforwardly compared through an on-line database. Currently there are 116, 832 allele sequences (Jolley et al., 2018). With the innovation of eBurst (electronic-Based Upon Related Sequence Types), studies of *E. faecium* MLST data established a definite clustering of strains originating from the hospital environment (Feil et al., 2004) with CC17 shown to have rapidly spread around the world (Willems et al., 2005; Top et al., 2008).

eBURST divides MLSTs into clonal complexes based on the identity of five of the seven MLST loci. However, this method is less reliable when determining genetic relatedness in highly recombining species like *E. faecium* (Turner et al., 2007; Zalipour et al., 2019). In summary, the model combines into BURST presumes that, because of selection or genetic drift, some genotypes will occasionally continue to occur in the population and then slowly expand through the formation of mutation(s) and/or recombinational replacements, resulting in minor variants of the founding genotype. Members of this arising clone will originally be indistinguishable by MLST in allelic profile, but over time, the clone will expand to produce a number of single locus variants in which one of the seven MLST loci has been changed. Additional evolution will result in dual locus variations of the parent sequence type, which differ at two of the seven loci. In this simplified model, bacterial populations are made up of clonal complexes that can be identified using the allelic profiles of strains in an MLST database (Feil et al., 2004). MLST is used to investigate the linkages of newly categorized strains to those in the database. By employing basic configuration, eBURST algorithm takes the STs and their allelic patterns as

input and divides the strains into groupings where each group member must share six of the seven loci with at least one other group member. At least three STs with a single-locus mutation made up a clonal complex. Sequence kinds that are not linked together in a clonal complex are called singletons (Li, Xin et al., 2018). However, when genetic variation within a population is directed by recombination in place of mutations (such as the case in *E. faecium* and *E. faecalis*), eBurst occasionally does not accurately allocate STs to clonal complexes (Turner et al., 2007).

The eBuster and Bayesian analysis of genetic population structure (BAPS) used to assign population structure disagree due to recombination of various microbial pathogens (Corander et al., 2012; Thomas et al., 2014). When MLST data of *E. faecium* is applied to BAPS, 519 STs (491 for *E. faecium* and 28 for *E. faecalis*) of 1720 *E. faecium* isolates separated into 13 non-comparable BAPS groups. BAPS 3-3 were significantly linked with isolates from hospitalized patients, whereas BAPS 2-1 and 2-4 were significantly associated with farm animals. In the hospital acquired CC17, ST78 (Figure 1-1) and its inheritor STs, were categorized in BAPS 2-1 together with farm animal isolates, whereas ST17 and ST18 (Figure 1-1) with their inheritor STs grouped in BAPS 3-3 indicating that nosocomial *E. faecium* isolates have not developed from a lone descender. Instead, nosocomial isolates sometimes have acquired adaptive components. Leaving aside the high levels of recombination in *E. faecium*, BAPS of MLST data showed that, hospital isolates relatively have a low-level of combination meaning that once strains have adapted to the various hospital niche become environmentally isolated and recombination with other populations lessens (Willems et al., 2012).

1.5 The clade structure of *E. faecium*

Genetic variability among strains can be explained by ratios of distance by detection of single nucleotide polymorphisms in core genome arrangement or by grouping allelic variants in hundreds to thousands of core genes using whole-genome sequencing-based epidemiology (Galloway-Peña et al., 2012). Pulsed-field gel electrophoresis (PFGE) was the gold standard for molecular typing of *E. faecium* until the emergence of whole-genome sequencing-based epidemiology (Turabelidze et al., 2000). The evolution of *E. faecium* was investigated in order to figure out why some strains are clinically relevant while others are considered commensals. Galloway-Peña et al. (2012) and Lebreton et al. (2013) discovered two distinct *E. faecium* clades, one for hospital isolates (A) and the other for community isolates (B). As per Montealegre et al. (2016) commensal clade B strains in the absence of antibiotics, outcompete clade A strains in the murine digestive tract. This could explain why clade B predominates in the community and why antibiotic-resistant hospital-associated *E. faecium* strains are frequently replaced by clade B strains after patients leave the hospital. This could be due to the adaptation of clade A to the abnormal environment of the antimicrobial-exposed gastrointestinal tract. The differential utilization of carbohydrates has been identified as one of the main drivers for the divergent evolution of clade A (Chilambi et al., 2020). Clade B frequently contain genes associated with complex carbohydrate utilisation, while clade A are more likely to contain genes which are more enriched in genes associated with amino-sugar metabolism as well as specific carbohydrate–phosphotransferase systems, indicating colonisation in antimicrobial-treated environments (Lebreton et al., 2013; Gao et al., 2018).

Galloway-Peña et al. (2012) calculated that the evolutionary divide happened 1–3 million years ago using a synonymous single nucleotide polymorphism molecular clock estimate with *Escherichia coli* parameters. Lebreton et al. (2013) developed a more complex evolutionary path with two divisions using Bayesian evolutionary analysis on sampled phylogenetic trees ignoring recombination's. Greater urbanization and animal domestication were considered to have caused the first split, which happened around 2776 ± 818 years ago and separated the species into human and animal dominating clades. The animal clade was separated further into an epidemic hospital clade (A-1) and a clade that causes sporadic infections in animals and humans in the community (A-2). The divide was assumed to have developed some 74 ± 30 years ago as a result of the development and use of antimicrobials in hospitals and livestock feed (Lebreton et al., 2013). Clade A-1 includes the vast majority of strains obtained in clinical settings and overlaps with the previously reported *E. faecium* subpopulation CC17 (Willems et al., 2005; Guzman Prieto et al., 2016). Strains obtained from household animals and cattle are overrepresented in the polyphyletic clade A-2 (Lebreton et al., 2013; Raven et al., 2016; Gouliouris et al., 2018). While bacteria from both clades A-1 and A-2 can be vancomycin-resistant, strains from clade A-1 are almost always ampicillin-resistant, whilst strains from other clades are generally ampicillin-susceptible (Willems et al., 2005). Additionally, there is evidence indicating that Clade A-2 is not a single clade, as previously believed. However, it can be divided into specialized clusters of strains based on the animal it inhabits (Arredondo-Alonso et al., 2019). In addition to mechanisms to help invade the body, evade the immune system, and adapt to the low level

of nutrients found in blood, strains from clade A1 have acquired various adaptations that assist them in their success as hospital-adapted pathogens (Zhang et al., 2017; Gao et al., 2018).

1.6 The differences between nosocomial and commensal *E. faecium*

In comparison to commensal *E. faecium*, nosocomial *E. faecium* strains have a larger genome and more than 100 genes, including carbohydrate metabolism genes, insertion sequence elements, and putative virulence elements, including a pathogenicity island, ICE*Efm1*, with the enterococcal surface protein (*esp*) gene as well as other genes (de Been et al., 2013; Zhang et al., 2017; Puchter et al., 2018). Commensal *E. faecium*, on the other hand, have small genomes (2.7 Mb) and auxotrophy for amino acids, vitamins, and other micronutrients (Palmer et al., 2012). Nosocomial *E. faecium* is more prone to develop ampicillin-resistance and ciprofloxacin-resistance. For example, vancomycin-resistance has been established in clades A-1 and A-2, but not in commensal bacteria from clade B. (Lebreton et al., 2013; Flor Duro, 2021). Another important feature of the genome of nosocomial *E. faecium*, is the absence of the clustered regularly interspaced short palindromic repeats (CRISPR) in the majority of strains. CRISPR-*cas* (CRISPR associated with *cas* genes) are used as acquired defenses against plasmids and phages (Palmer and Gilmore, 2010; van Schaik et al., 2010). Cas9 nuclease induces double-strand breaks in DNA that can be targeted by CRISPR, which is used to defend prokaryotic cells from bacteriophages and plasmid infections (Brouns et al., 2008; Marraffini and Sontheimer, 2008). The lack of a CRISPR-*cas* system could explain how mobile genetic elements encoding antibiotic resistance and virulent genes can be acquired by the genome of nosocomial

E. faecium strains (Palmer and Gilmore, 2010; Lam et al., 2012). Phosphotransferase systems, which sense and phosphorylate sugars before introducing them into the cell, were one of the genes found to be inserted in mobile genetic elements. These systems were enriched in clade A-1 strains, a nosocomial strains and some of them were found in mobile elements (Gilmore et al., 2013). Aside from sugar uptake, the phosphotransferase systems also regulate gene expression and mutagenic processes (Gilmore et al., 2013; Lebreton et al., 2013; Flor Duro, 2021).

1.7 *E. faecium* E1162

E. faecium strain E1162 is a blood isolate of the CC17, this clonal complex causes many hospital outbreaks (Heikens et al., 2007), this strain is Amp^r and contains an intact *esp* gene. The *esp* gene is involved in biofilm formation and adherence to abiotic materials (Heikens et al., 2007; Hashim, 2016). The *esp_{Efm}* gene is part of a cluster of six genes that are present on a pathogenicity island. Pathogenicity islands, a distinct class of genetic elements, are acquired by microorganisms through horizontal gene transfer, they are integrated into the genome of pathogenic organisms, while they are mostly absent from non-pathogenic strains of the same species or of closely related species. The *esp_{Efm}*PAI is an integrative conjugative element that has an integrase gene *intA*. The *intA* gene is involved in the excision and formation of circular intermediates of the *esp* PAI. The *esp* PAI is a self-transmissible element (Top et al., 2011). It has been confirmed that the *esp* gene of *E. faecium* is involved in urinary tract infections (Andrade et al., 2017) and enhances kidney and bladder infections in mice (Leendertse et al., 2009). The strain E1162 also contains a point mutation in the penicillin-binding protein

gene (*pbp5*) that confers resistance to ampicillin. Moreover, the strain possesses the cell wall-anchored collagen adhesin gene (*acm*) coding a collagen adhesion (van Schaik et al., 2010).

1.8 *E. faecium* can withstand harsh conditions and form biofilms

E. faecium can grow in 6.5% NaCl and live in higher salt concentrations, as well as tolerate chlorine and alcohol disinfectant treatments. Furthermore, three CC17 isolates were discovered to have lived *in vitro* for more than 5 years without sustenance (Wagenvoort et al., 2015). Further, they are found in the hands of healthcare workers, which accounts for their easy transmission (Reyes et al., 2016). *E. faecium* can tolerate prolonged dryness and if challenged with a nutrient-poor environment it can possibly enter a viable but non-culturable state and could exist in this form on non-living objects (Giard et al., 2001).

Information on factors that enable colonisation, virulence elements and cellular receptors are yet to be determined and it is recognised that this organism usually does not make powerful exotoxins unlike *Staphylococcus* genus or group A *Streptococcus*, but its outer cell wall contains lipoteichoic acids that can stimulate cytokine responses in mammalian species, while it is also not known to have enzymes to ease tissue invasion (Higuera and Huycke, 2014).

Virulence genes, which include alternative sigma factors of RNA polymerase and specific transcriptional regulators, can play a role in surviving environmental stresses such as high temperature, oxidative stress, carbon starvation, and low pH. In *E. faecium* 10 transcriptional regulators have been

identified in the genome and are thought to be involved in virulence and stress response in the closely related bacterium *E. faecalis* (Michaux et al., 2011). The resistance of *E. faecium* is increased by forming biofilms, when planktonic (floating) bacteria adhere to each other and/or to a surface *ex vivo* (e.g., on medical devices) as well as *in vivo* (inside the host) and are covered with exopolymeric substances. When microcolonies form on the surface more extracellular polymeric substances will be formed to keep cells closer together. Components of extracellular polymeric substances are polysaccharides, proteins, extracellular DNA (Sarquis et al., 2004), lipids, and biopolymers (Mohamed and Huang, 2007; Paganelli et al., 2012). Biofilms guard bacteria against phagocytosis, which makes it very difficult for the host to clear the bacteria and protect bacteria against antimicrobials (Hashem et al., 2017). Antibiotic resistance develops in biofilm communities as a result of a number of processes, including slow or partial antibiotic penetration into the biofilm and an altered chemical environment within the biofilm, slower reproduction, and the presence of an adaptive stress response (Sharma et al., 2019; Dincer et al., 2020). In biofilm antibiotic tolerance is mediated by bacterial persistence via dormant cells (Conlon et al., 2013) and biofilm morphological states (Tseng et al., 2013). Antibiotics that target actively metabolizing cells (such as cell wall synthesis) usually fail in the presence of latent and/or biofilm-associated bacterial populations. Biofilms usually have a high cell density, and this allows for cell-to-cell communication (quorum-sensing) where bacteria produce signal molecules, autoinducers which at a certain concentration allow bacteria to switch on genes. Genes regulated through quorum-sensing usually are those involved in virulence, biofilm formation, conjugation, antibiotic

production, motility, sporulation and symbiosis (Novick and Geisinger, 2008; Ng and Bassler, 2009; Williams and Camara, 2009).

1.9 Envelope structures in Gram-positive bacteria

Peptidoglycan made from Park's nucleotide, the amino sugar N-acetylmuramic acid (MurNAc) together with D- or L-amino acids (L-Ala-DiGlu-m-Dpm-D-Ala-D-Ala (AGDA2) creates UDP-MurNAc-AGDA2 in the bacterial cytoplasm. Park's nucleotide is joined to the lipid transporter undecaprenyl-pyrophosphate to make C55-PP-MurNAc-AGDA2 (lipid I), the latter is modified with UDP-GlcNAc to make C55-PP-MurNAc (AGDA2)-GlcNAc (lipid II). Lipid II is then polymerized by penicillin-binding proteins (transglycosylases and transpeptidases) to produce MurNAc (AGDA2)-GlcNAcMurNAc (AGDA2)-GlcNAc that are crosslinked with other strands (Strange and Kent, 1959; Higashi et al., 1967; Pratt and Govardhan, 1984; Parks et al., 2015). This peptidoglycan shields Gram-positive bacteria from cytolysis and acts as a wall against osmotic pressure, hydrolases, and some membrane toxic substances (Shockman and Barrett, 1983). Peptidoglycan also acts as a platform holding capsular polysaccharides, wall teichoic acids and bound proteins (proteins bound to envelop structure, covalently connected to peptidoglycan), gathered into pili or scattered within surface layer structures (Navarre and Schneewind, 1999; Hancock et al., 2014).

1.10 Clinical importance of *E. faecium*

E. faecium is one of the ESKAPE organisms which are the dominant cause of nosocomial-infections (Siné et al., 2017). The major causes of health-care-associated enterococcal infections in humans are *E. faecium* and *E. faecalis* (Higuita and Huycke, 2014). Due to its resistance to many antibiotics, *E.*

faecium has become a common hospital acquired infection (Willems and van Schaik, 2009; Arias and Murray, 2012; Lee et al., 2019). In the 1970s and 1980s, *E. faecium* developed ampicillin-resistance (Galloway-Peña et al., 2009), then become resistant to vancomycin (Arias and Murray, 2012; García-Solaches and Rice, 2019). In 2016, a high rate of VRE_{fm} infections occurred in Ireland, eastern and south-eastern Europe.

For infections caused by VRE_{fm}, it has been reported that the therapeutic options are more limited, with higher mortality rates and financial costs for the health system when compared with vancomycin-susceptible enterococci (Cheah et al., 2013; Tacconelli et al., 2017). Figure 1-2 shows percentage of VRE_{fm} invasive isolates by country/area, WHO European Region (World Health Organization, 2022).

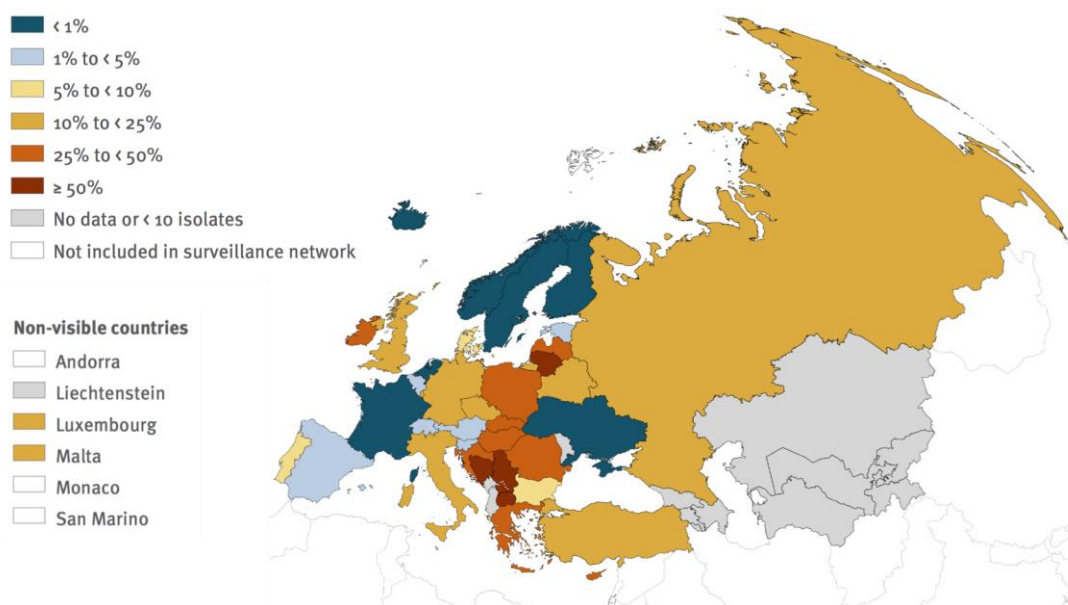


Figure 1-2 Percentage of vancomycin-resistant *E. faecium* invasive isolates by country/area, WHO European Region.

Note: Scotland and Wales are not included in the United Kingdom data for 2020. Adapted from World Health Organization (2022).

In Europe, hospital infection with ampicillin-resistant *E. faecium* developed in the 2000's (Top et al., 2007). According to European Antimicrobial Resistance Surveillance System data 2002-2008, the number of positive *E. faecium* blood cultures is 19.3% more than the increase of other pathogens as *E. coli*, *S. aureus*, *Streptococcus pneumoniae* and *E. faecalis* (De Kraker et al., 2013; Zhou et al., 2020). The development of *E. faecium* bloodstream infections was also seen in the University Medical Center Groningen's monitoring data. The Netherlands shows the ratio of positive blood cultures with *E. faecium* and *E. faecalis* in individual patients from 1998 to 2017 (Figure 1-3). While the rate of *E. faecalis* bloodstream infections remained relatively stable, the ratio of *E. faecium* to *E. faecalis* increased from 0.1 in 1998 to 1.6 in 2017. CC17 clones are linked to an upsurge in *E. faecium* bloodstream infections in hospitals across Europe, including Ireland, Spain, Poland, Denmark, and Switzerland (Gudiol et al., 2013; Pinholt et al., 2014; Ryan et al., 2015; Gawryszewska et al., 2016; Zhou et al., 2020).

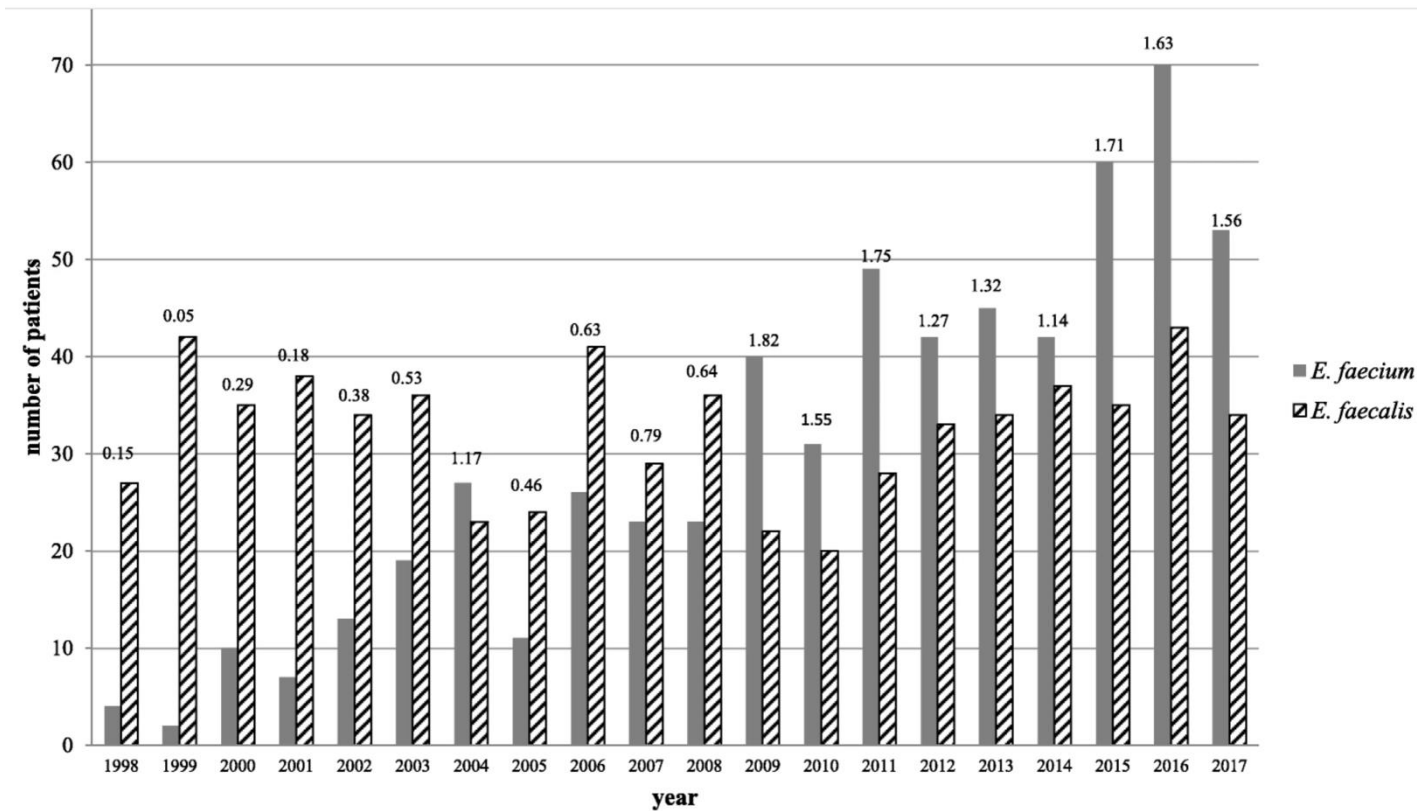


Figure 1-3 The ratio of *E. faecium* to *E. faecalis* has shifted during the years 1998–2017.

The University Medical Center Groningen recorded the number of patients who had blood cultures positive for *E. faecium* and *E. faecalis*, as well as the *E. faecium*/*E. faecalis* ratio. The ratio of *E. faecium* to *E. faecalis* has risen from 0.1 in 1998 to 1.6 in 2017. Adapted from Zhou et al. (2020).

Enterococci show intrinsic low to moderate levels of resistance to aminoglycosides such as streptomycin or gentamicin due to slow absorption of these highly polar molecules, the uptake of these highly polar molecules can be raised by the addition of cell wall-active components, in conjunction with penicillin plus vancomycin. Aminoglycosides can be used in combination with β -lactams and glycopeptides (such as vancomycin, teicoplanin, oritavancin, and dalbavancin) which can work together synergistically, permitting improved effectiveness of the aminoglycosides (Top et al., 2008). Aminoglycoside resistance in *E. faecium* is due to the chromosomal gene *efmM*, encoding the *E. faecium* methyltransferase plus *aac* (6')-Ib that encodes a 6'-N-aminoglycoside acetyltransferase (Galimand et al., 2011). Due to the acquisition of the aforementioned resistance genes, the rate of enterococcal infections has changed with *E. faecium* going from causing ~5% of enterococcal infections to causing ~35% of these infections (Galloway-Peña et al., 2009).

1.11 Factors associated with *E. faecium* virulence

To produce infections, enterococci need to colonise and attack host tissues, to be secure against host immune response and produce factors that facilitate persistence such as biofilm formation, secretion of extracellular enzymes and toxins (Giridhara Upadhyaya et al., 2009). Many factors that control the virulence of enterococci are now recognized, most of them enable colonisation of the gastrointestinal tract, competence to stick to different extracellular matrix elements such as lactoferrin, thrombospondin and vitronectin, and competence to attach to oral epithelial cells and human embryonic kidney cells (Fisher and Phillips, 2009). Many virulence factors control numerous virulence

features linked to adhesion, displacement, and evading host immunity (Johnson, 1994).

1.11.1 Adherence

1.11.1.1 Enterococcal surface protein

To establish an infection, it is a vital step for the bacteria to stick to the host tissues. Esp is a 200 kDa surface protein of *E. faecium* connected to the cell wall through a cell wall-anchored surface protein LPXTG-type motif (Leavis et al., 2004; Heikens et al., 2007). The *esp* gene is a portion of a pathogenicity island of ~60–100 Kbp in size which also contains additional genes required for mobilization plus others virulence elements (van Schaik et al., 2010; Top et al., 2011). Top et al. (2011) classified these pathogenicity island as ICE*Efm1* which are self-spreading among enterococci and other species. Heikens et al. (2007) experimentally demonstrated the role of the *esp* gene in the biofilm development in *E. faecium* and its role in mice with urinary tract infection (Leendertse et al., 2009) and mouse endocarditis models (Heikens et al., 2011).

Esp is also found on a pathogenicity island in *E. faecalis*, is expressed on the bacterium's surface, and is thought to be an adhesin that aids in the colonisation of urinary tract epithelial cells and biofilm formation. *Esp* of *E. faecium* is up to 90% homologous to *esp* of *E. faecalis* (Heikens et al., 2007). Complementation of an *esp*-negative strain of *E. faecium* with a plasmid carrying *esp* from *E. faecium* completely restored biofilm formation (Tendolkar et al., 2005, Heikens et al., 2007). Tendolkar et al. (2005) introduced *esp* from *E. faecalis* into *esp*-negative *E. faecium* E1162, and while *esp* was expressed on the *E. faecium* E1162 cell surface in comparable levels to *E. faecalis*, it did

not restore *E. faecium* E1162 biofilm formation. So, it may be that one or more unknown factors which may be present in *E. faecalis* could be required to impact biofilm formation in *E. faecium* (Tendolkar et al., 2005). In models of biofilm-associated infections, many *E. faecalis* OG1RF determinants of *in vitro* biofilm have been identified as virulence factors (Ch'ng et al., 2019; Willett et al., 2021). One such protein is the secreted metalloprotease GelE (gelatinase), which is controlled by the Fsr quorum sensing system, *gelE*-negative mutants exhibit biofilm formation abnormalities *in vitro* and have diminished effects in animal models. Peptidyl-prolyl isomerase or trigger factor disruption significantly changed the shape of the biofilm (Thurlow et al., 2010;- Willett et al., 2021).

1.11.1.2 Pili

Enterococci must make biofilm to cause urinary tract infection, endodontic infections and endocarditis (Singh et al., 2007). Adhesion, the first step in the biofilm process, is facilitated by pili expression on the bacterial cell surface. In Gram-positive organisms, pili are made through a complex process including the assembly of pilin subunits into a pilus polymer, which is then connected to the cell wall by housekeeping and specialized sortase enzymes (Dunny et al., 2014). Pilus biosynthetic genes are typically organized into operons called pilin gene clusters. Pilin protein subunits have MSCRAMM (microbial surface components recognizing adhesive matrix molecule) - like properties, as well as conserved pilin motifs (HLYPK) and E box motifs (ETxAPExY) that contribute in pilus assembly. Cell-cell interaction, host tissue colonisation, and biofilm formation all appear to be aided by Gram-positive pili (Telford et al., 2006; Dunny et al., 2014; Piepenbrink and Sundberg, 2016). Two distinct types

of pili can be found on the surface of a single *E. faecium* cell, and these pili are encoded by two different and physically isolated pilin gene clusters. Both pilA and pilB code for two unique pilin subunits (Mahlapuu et al., 2016) that polymerize form multimeric pilus structures. During biofilm formation, pilA and pilB play a role in adhesion (Leavis et al., 2006).

1.11.1.3 Enterococcal microbial surface components recognizing adhesive matrix molecules

Bacterial pathogens have surface-exposed proteins that bind tightly to ligands found on host tissues and biomaterials. These adhesins are important for infection in animal models, particularly during the early stages of attachment when the cells are under physical stress. Tensile stress activates adhesin–ligand interactions, which can be 10 times stronger than non-covalent biological connections. Gram-positive adhesins have peculiar stress-dependent molecular interactions that are critical for bacterial colonisation and spread (Dufrêne and Viljoen, 2020). MSCRAMMs are a protein family identified by the presence of two immunoglobulin G-like foldable subdomains that are next to each other. These increase ligand binding through methods involving significant conformational changes as seen in fibrinogen binding (Foster, 2019). In animal models, this adhesion is important for pathogenesis and is responsible for the majority of their adherence to collagen/laminin (Hancock et al., 2014; Somarajan et al., 2015). Several potential virulence or niche factors, notably MSCRAMMs and other chemicals related with adhesion and biofilm formation, bile tolerance, were found in hospital-adapted *E. faecium* (Hancock et al., 2014; Foster, 2019; Gouliouris, 2019). MSCRAMMs are involved in host-pathogen adherence. Host colonisation is thought to occur

via an interaction between defined proteins in the extracellular matrix and MSCRAMMs. Numerous *E. faecium* strains encode MSCRAMMs, particularly clinical isolates that can bind to fibrinogen, fibronectin, and collagen (Vengadesan and Narayana, 2011).

1.11.1.4 SgrA, EcbA and Scm

Serine-glutamate repeated-containing protein A (SgrA) and EcbA LPXTG-like cell wall-anchored proteins are associated with multidrug-resistance *E. faecium* isolates. EcbA is a unique *E. faecium* MSCRAMM that can attach to collagen type V and the gamma sequence of fibrinogen, whereas, SgrA can bind both the alpha and beta chains of fibrinogen and might have a role in adhesion in device-associated infections (Nagarajan et al., 2013). However, SgrA has no role in binding of *E. faecium* cells to biological surfaces such as human intestinal epithelial cells, human bladder cells, and kidney cells. EcbA is involved in *E. faecium* biofilm formation. The second collagen adhesin of *E. faecium* (Scm) is encoded by the *scm* gene and could bind to fibrinogen plus collagen type V (Sillanpää et al., 2008).

1.11.1.5 Adhesin of collagen of *E. faecium* (Acm)

Acm attaches to collagen type I and to a lower level with type IV (Nallapareddy et al., 2003; Nallapareddy et al., 2006). The occurrence of *acm* is prevalent in both clinical and non-clinical *E. faecium* isolates. The *acm* gene which presents in non-clinical isolates is usually non-functional- and mostly contains an insertion element, while in clinical isolates it is typically functional allowing adherence to collagen type I and IV. Acm participates in the pathogenesis of experimental endocarditis and interestingly, antibodies to Acm have been detected in sera of patients with *E. faecium* endocarditis, verifying that *acm* is

expressed *in vivo* and stimulates a human immune response (Nallapareddy et al., 2008).

1.11.1.6 A major secreted antigen (SagA)

Recombinant SagA (a major secreted *E. faecium* antigen) can bind different extracellular matrix proteins, for example fibrinogen, collagen type I and IV, fibronectin and laminin (Kalfopoulou and Huebner, 2020). The SagA protein contains three domains and is crucial for *E. faecium* growth (Kropec et al., 2011). In *Streptococcus mutans*, a protein with the similar role has been identified as participating in cell wall metabolism and virulence (Anantharaman and Aravind, 2003; Rigden et al., 2003). The analogous gene in *E. faecalis* is associated with stress resistance, biofilm and binding to collagen type I and fibronectin (Breton et al., 2002; Le Breton et al., 2003; Mohamed et al., 2006).

1.11.2 Autolysin

The autolysin AtIA_{Efm} is the main autolysin in *E. faecium* releasing in biofilms via cell lysis where extracellular DNA (eDNA) act as a matrix stabilizer (Paganelli et al., 2013). Disruption of the *atIA_{Efm}* gene results in cells with deficient attachment and causes a reduction in eDNA, biofilm formation, cell wall hydrolysis and chaining (Paganelli et al., 2013). Additionally, this disruption resulted in less binding to collagen types I and IV suggesting an association of AtIA_{Efm} with Acm and its role in biofilm and collagen binding in *E. faecium*.

1.11.2.1 Cytolysin

Cytolysin is a haemolysin that can also act as a bactericidal to other Gram-positive bacteria (Koch et al., 2004; Billström et al., 2008). Cytolysins are

commonly encoded by conserved conjugative plasmids such as pAD1, although they can be encoded chromosomally, they have a higher occurrence in clinical isolates compared to non-clinical isolates and are regulated by a quorum-sensing machinery two-component system (Clewell,1990; Fisher and Phillips, 2009).

1.11.3 Extracellular DNA (eDNA)

About 83 *E. faecium* strains of various phylogenetic sources (clade A-1 and B) were examined for their capability to form biofilm and the role of eDNA and released proteins. Despite no remarkable differences in biofilm formation among the tested strains from these two clades, the result of adding DNase I or proteinase K to biofilms revealed that eDNA is important for biofilm formation in numerous *E. faecium* strains, whereas proteolysis impacted primarily biofilms of clade A-1 strains. The SagA was the most abundant protein in biofilms from *E. faecium* clade A-1 and B strains. The *sagA* gene is found in all sequenced *E. faecium* strains, with a consistent difference in the repeat region among the clades, which correlated with the susceptibility of biofilms toward proteinase K. This indicates a relation between the SagA variable repeat profile and the localization and role of SagA in *E. faecium* biofilms (Paganelli et al., 2015).

1.11.4 Exoenzymes

1.11.4.1 Gelatinase

Gelatinase is a matrix metalloproteinase, encoded by the *gelE* gene and presumed to play a role in cleaning the bacterial cell wall of misfolded proteins. Gelatinase is capable of degrading various substrates such as haemoglobin, collagen, casein, gelatin and fibrin. Park et al. (2007) showed that in *E. faecalis* GelE break down cecropin and different other antimicrobial peptides secreted by *Galleria mellonella*. A study by Gaspar et al. (2009) showed that *E. faecalis* strains negative for *gelE* were less virulence when compared to the wild-type. *E. faecalis* produces gelatinase which contributes to the virulence of the organism in animals and humans, gelatinase damages the host tissue, facilitating the invasion and spread of bacteria by damaging the cellular wall, colonization and persistence by creating biofilms (Kiruthiga et al., 2020).

1.11.4.2 Hyaluronidase

Initially *hyl_{Efm}* was designated as hyaluronidase but later was suggested to be a putative glycosyl hydrolase. A *hyl_{Efm}* enables intestinal colonisation among different bacterial organisms (Freitas et al., 2010). The *hyl*-like gene is significantly linked with the hospital-associated clades, a majority of CC17 *E. faecium* clinical isolates from the United States (clinical strain A) and Colombia (clinical strain B) contain large plasmids carrying the *hyl_{Efm}* genes. Clinical strain A contained the largest plasmid (between 242.5 and 291 kb), while clinical strain B had the smallest plasmid (between 145 and 194 kb) (Arias et al., 2009), whereas the community-associated clade does not have very large plasmids containing a *hyl*-like gene (Rice et al., 2003; Arias et al., 2009). I-Ceul is an enzyme that exclusively digests bacterial chromosomes because it identifies certain sequences found in the 23S rRNA genes and may be used to distinguish the presence of extrachromosomal elements (such as plasmids)

using PFGE and hybridizations (Liu et al., 1993). I-Ceul digestion, PFGE, and hybridization with a *hyl*_{Efm} probe all confirmed the extrachromosomal location of *hyl*_{Efm}, I-Ceul recognizes a sequence in the genes encoding the 23S rRNA, which has six copies in *E. faecium*; thus, hybridization with a probe targeting the 23S rRNA results in six chromosomal bands in each strain. Hybridization with a *hyl*_{Efm} probe, on the other hand, produced bands that looked like smears and did not correspond to chromosomal bands, indicating the presence of different variants of the same plasmid (Arias et al., 2009). A phylogenetic lineage in a hospital setting could benefit from a *hyl*-like gene, since bacteria carrying plasmids with the *hyl* gene colonise the gastrointestinal tract of a murine peritonitis model and increase mortality rates (Rice et al., 2009; Panesso et al., 2011).

1.12 *E. faecium* antimicrobial resistance

Antimicrobial resistance in *E. faecium* can be classified as intrinsic or acquired resistance (Kristich et al., 2014). Intrinsic resistance is when the bacteria lack the target site for the antimicrobial or that the antimicrobial cannot penetrate the cell to reach the target site. The resistance of *E. faecium* to cephalosporin is dependent on the existence of class A PBPs (PonA, PbpF). PBP mutants display cephalosporin susceptibility that is reversible by exposure to penicillin and by selection on cephalosporin-containing medium (Desbonnet et al., 2016).

The hypermutable DNA of *E. faecium* allows for rapid adaptation to antimicrobials. Several recombinant regions accounting for up to 26% of the *E. faecium* genome had been identified (de Been et al., 2013). The lack of CRISPR-cas loci, which protect genomic DNA from extracellular DNA in other

bacteria, is thought to be the cause of the high recombination rates observed in *E. faecium* (Palmer and Gilmore, 2010). Furthermore, *E. faecium* is capable of rapidly acquiring and disseminating genes via mobile genetic elements such as plasmids and transposons, which are common among bacteria (Clewell, 1990; Pereira et al., 2018). Mobile genetic elements are typically accompanied by gene cassettes containing virulence factors and antimicrobial resistance genes.

Historically, *E. faecalis* was responsible for the majority of enterococcal infections (80–90%), but *E. faecium* is becoming more important because it is much more resistant to antimicrobials (García-Solaches and Rice, 2019). According to the European Antimicrobial Resistance Surveillance Network, the mean proportion of VRE_{fm} in invasive isolates in 2018 was 17.3% up from 10.4% in 2014 in European Union and European Economic Area countries (ECDC, 2019). Within the European Union and European Economic Area countries, the increasing proportion of VRE_{fm} has been reported at the country level (e.g., Germany, Italy, Slovakia, and Norway) (ECDC, 2019; Markwart et al., 2019).

In *E. faecium*, resistance to β -lactam antibiotics due to mutations in *pbp* genes have been reported (Zorzi et al., 1996; Rice et al., 2001; Zhang et al., 2012; Cattoir and Giard, 2014). Enterococci express a weakly binding PBP5 to β -lactam antibiotics (intrinsic low-level β -lactam resistance < 64 mg/l) (Murray, 1990). Ampicillin-resistance in *E. faecium* is mostly caused by increased PBP5 synthesis and/or mutations in the beta subunit of this protein (Gagetti et al., 2019). These PBPs are chromosomally encoded and transferable, implying

that the spreading of higher-level {minimum inhibitory concentration (MIC) \geq 128 mg/l} Amp^r (associated with hospital environment) could be because of both clonal spread of *pbp5* mutants plus horizontal gene transfer (Fontana et al., 1994; Gagetti et al., 2019). An ampicillin-resistant laboratory mutant strain of *E. faecium* (MIC > 2000 mg/l) has been found to have another β -lactam resistance mechanism that does not involve PBPs by removal of PBP5 caused a bypass of d,d-transpeptidation during the final stages of peptidoglycan synthesis, in addition mutations in genes encoding other species-specific proteins involved in cell wall synthesis may slightly increase MIC values (Mainardi et al., 2000; Sacco et al., 2014; Gagetti et al., 2019; Miller et al., 2020). Also, low level expression of β -lactamase has been confirmed in *E. faecium* with minor influence towards their ampicillin susceptibility (Sarti et al., 2012; Munita and Arias, 2016). Inherent lower-level ampicillin-resistance (< 64 mg/l) (community associated environment) is encoded by core genome chromosomal determinants and involves in the production of the low-affinity class B penicillin-binding protein 5, which binds weakly to β -lactam antibiotics (Kristich et al., 2014; Munita and Arias, 2016).

The rates of *VREfm* colonisation and infection have risen steadily since the 1980s (Werner et al., 2008). After 1990, *E. faecium*-resistance to vancomycin ranged from 58.4% in surgical site infection to 82.2% in catheter-associated urinary tract infection, while in *E. faecalis* resistance ranges from 3.5% to 9.8% in catheter-associated urinary tract infection and central line-associated bloodstream infections, respectively (Weiner et al., 2016). Vancomycin blocks cross-linking of peptidoglycan layers in the bacterial cell wall by binding to the D-alanine-D-alanine elements of the peptide chains that bond peptidoglycan.

Changes from D-alanyl-D-alanine to D-alanyl-D-lactate trigger high-level resistance toward vancomycin, while change from D-alanyl-D-alanine to D-alanyl-D-serine caused low-level resistance (Courvalin, 2006).

Currently, *vanA*, *vanB*, *vanD* and *vanM* are gene clusters that confer resistance to vancomycin in enterococci by the formation of D-alanyl-D-lactate. Whereas, a formation of D-alanyl-D-serine is generated by *vanC*, *vanE*, *vanG*, *vanL*, and *vanN* gene clusters (Courvalin, 2006; Boyd et al., 2008; Xu et al., 2010; Lebreton et al., 2011). Of these nine gene clusters, *vanA* and *vanB* are most frequently found among clinically important *E. faecium*. Both *vanA* and *vanB* are situated on transposons that contributes to the spreading of vancomycin-resistance among enterococci (Courvalin, 2006). The *vanA* operon is commonly associated with transposons (Tn), such as Tn1546, involves two elemental translocation genes (*orf1* and *orf2*) and one associated with teicoplanin resistance (*vanZ*) (Ahmed and Baptiste, 2018), although the genetic organization of *vanB* is similar to that of *vanA*, however, *vanB* is not structurally homologous to *vanZ* and instead encodes a protein called *vanW*, its role in drug resistance is not fully understood.

The *optrA* gene in *E. faecium* isolates of human and animal origin encodes resistance to linezolid and tedizolid (oxazolidinone) together with chloramphenicol and orfenicol (phenicols) (Cai et al., 2015; Wang et al., 2015; Cavaco et al., 2017). The ATP-binding cassette transporters (ABC-transporter) encoded by *optrA* has a role in decreasing the sensitivity to

oxazolidinone and phenicols. The *optrA* gene is present on a conjugative plasmid (Wang et al., 2015).

A study of 4,274 *E. faecium* isolates from USA hospitals found that 3.9 % of the strains were resistant to daptomycin (Edelsberg et al., 2014). Daptomycin is a last-line antibiotic used in the treatment of multidrug-resistant- *E. faecium* infections. However, daptomycin-resistant *E. faecium* has emerged *in vitro* by acquiring high-level daptomycin resistance due to genetic mutations as well as changes in the thickness and charge of the cell membrane (Zeng et al., 2022). Lev et al. (2022) demonstrated that bacteriophages combined with daptomycin and β -lactams can be an effective way to eliminate biofilm-mediated infections and prevent resistance to antibiotics and phages in cases of multidrug-resistant *E. faecium*.

Tigecycline is a synthetic derivative of a wide-spectrum tetracycline antibiotic minocycline, which prevents binding of the ribosome to its related aminoacyl-tRNAs. The appearance of resistance to tigecycline was described in *E. faecium* by Niebel et al. (2015). This resistance to tigecycline was due to upregulation of *tet(L)* (encoding an efflux pump) and *tet(M)* providing protection to ribosomes (Fiedler et al., 2016) and mutations in *rpsJ* the ribosomal protein (Beabout et al., 2015; Cattoir et al., 2015; Niebel et al., 2015). But this resistance is rare among *E. faecium* and *E. faecalis* (0.3% for both) (Hoban et al., 2015).

The expansion of *E. faecium* from commensal to hospital-adapted pathogen not only urges the need to develop new antibiotics but also to explore how hospital-acquired *E. faecium* strains can outgrow in the gut of hospitalised

patients and adapt to specific positions like the human urinary tract. A better understanding of virulence could provide new clues for other therapeutic options. The generation of antimicrobial drugs depends on our understanding of the interactions between host and microbe (Yuen and Ausubel, 2014).

As one of the new therapeutic strategies, anti-virulence therapy has emerged as a promising alternative since it works by depriving pathogens of their virulence factors instead of killing them. Therefore, the selective pressure exerted on pathogens should be lower than that exerted by conventional antibiotics, and the spread of resistant mutants should be less likely (Munguia and Nizet., 2017; Fleitas Martínez et al., 2019).

1.13 The oxidative stress response in *E. faecium*

Bacterial virulence is associated with the capability of pathogens to adapt to different stresses. To colonise the human host bacteria, need to survive under harsh environmental conditions of the human host (a limited range of temperature, oxygen and pH, specific nutrients, and low iron availability, stress from host innate immune attacks for example, reactive oxygen and nitrogen species, cationic host defence peptides as well as antibiotic-induced stresses) (de la Fuente-Núñez et al., 2013; Fang et al., 2016). To survive and grow, bacteria have developed molecular mechanisms for changing their lifestyles in response to changes in environmental conditions, for example, bacteria can alter their lifestyle to a virulent form, the virulent form rapidly colonises and disseminates in host tissues to cause infections (Desai and Kenney, 2019).

Partial reduction of O₂ produces reactive oxygen species (ROS); these oxidants include, superoxide (O₂⁻), H₂O₂ (hydrogen peroxide) and -OH hydroxyl radical (Figure 1-4). Bacteria have developed protection mechanisms

to guard themselves against ROS, it has 1st and 2nd lines of defence. The 1st line of defence consists of enzymatic proteins e.g., catalase, superoxide dismutase plus glutathione peroxidase and non-enzymatics such as vitamins and ascorbic acid. The 2nd line of defence is for example: induction of genes (transcriptional factors for ROS sensing enzymes such as superoxide dismutase, catalase, and peroxidase protect cells from the steady-state concentration of reactive oxygen (Kotova et al., 2014).

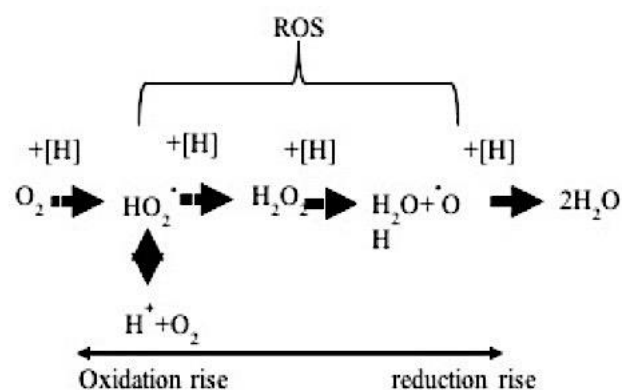


Figure 1-4 Different types of Reactive Oxygen Species, each have the potential to cause oxidation. Adapted from Das and Roychoudhury (2014).

Hydroxyl radical, -OH , reacts with nucleic acids, carbonylates proteins, and causes peroxidation of lipids. -OH can be generated from H_2O_2 and cause a large amount of the damage via the Fenton reaction (Figure 1-5), which needs iron (or another divalent metal ion, such as copper) and NADH (reducing equivalents) to regenerate the metal (Humphries et al., 1998).

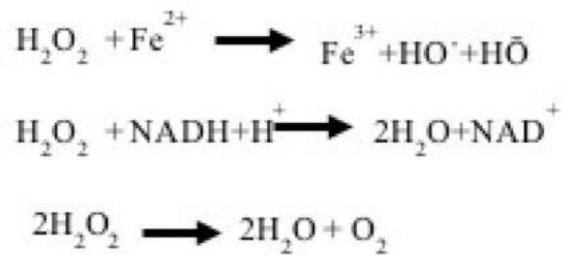


Figure 1-5 Fenton's reactions.

Iron and hydrogen peroxide, their reaction together will produce some hydroxyl radicals as shown in the above reaction. Adapted from Pinto (2012).

During oxidative stress, lipids are a major target. To establish a successful infection, a pathogen must be able to rapidly adapt to a changing environment, which is achieved by changes in gene expression levels of regulators and effectors of the stress response. These stress response genes are vital for bacterial virulence (Riboulet et al., 2007).

Free radicals can attack polyunsaturated membrane fatty acids and cause lipid peroxidation, peroxidation decreases membrane fluidity. Thus, altering the integration of membrane constituents and can cause significant membrane-bound protein disruption. These effects induce the formation of more radicals, and polyunsaturated fatty acids are degraded to a range of products, such as aldehydes, which are very reactive and can damage molecules such as proteins. Relative to free radicals, aldehydes are relatively long-lived and for that reason can diffuse and attack targets situated far-off from the original free-radical event, acting as additional toxic messengers (Humphries et al., 1998).

E. faecium has an oxidative stress sensor and response regulator belonging to the multiple antibiotic resistance regulators family, named antibiotic and stress response regulator (AsrR), this regulator has a cysteine residue and

utilizes cysteine oxidation to sense H₂O₂. Oxidative stress alters the binding affinity of AsrR to its promoter and eventually results in detachment of AsrR (Lebreton et al., 2012).

1.14 Oxidative stress generator used in this study

1.14.1 Menadione

Menadione, also known as 2-methyl-1,4-naphthoquinone, is a vitamin K analog that can undergo redox-cycling via one-electron reduction (O₂) to O₂^{•-} by enzymes such as microsomal NADPH–cytochrome P450 reductase and mitochondrial NADH–ubiquinone oxidoreductase (complex I) (Halilovic et al., 2016; McBee et al., 2017). It is a bright yellow crystalline powder with a molecular weight of 172.18. It is water insoluble, but soluble in ethanol, benzene, chloroform, carbon tetrachloride, and vegetable oils, among other things. It is unstable in the air and decomposes when it comes into contact with iron. It can be volatilized in the same way as vapor may. It is harmful to the skin and has a significant irritating effect (Nigam et al., 2012). Menadione is a redox-cycling chemical that raises superoxide levels inside cells. Menadione has been shown to produce large quantities of 8-oxoG residues, suggesting that it may have a role in DNA damage. Because single-stranded breaks and lethal double-stranded breaks can be caused by oxidized nucleotides (Oka et al., 2008). ROS have the ability to destroy biological components like mitochondrial DNA and genomic DNA. Oxidized DNA can cause deadly double stranded breaks if not repaired adequately (Senejani et al., 2019).

1.14.2 Cumene hydroperoxide

Cumene hydroperoxide is a noxious-smelling colorless to light yellow liquid. The flash point is 175°F. It boils at 153°C and 100°C at a decreased pressure of 8 mm Hg (millimetres of mercury). It has a little higher density than water and is marginally soluble in water. As a result, it sinks when submerged in water. Soluble in alcohols, acetones, esters, hydrocarbons, and chlorinated hydrocarbons (*Cumene Hydroperoxide*, 2022). It is toxic when inhaled or absorbed through the skin. It is an oxidizing compound that induces oxidative stress in bacterial cells. Cumene hydroperoxide boosts the production of free radicals, which damage the cell membrane causing the cell to burst and die.

1.15 Zebrafish larvae as an infection model for *E. faecium* E1162

With the completion of numerous microbial genome studies over the previous decade, databases currently contain a huge number of "hypothetical proteins." These annotations are based on open reading frames (ORFs) that have been found, sometimes with projected functions based on bioinformatics analysis, but often without a function that has been experimentally proven. Many of these unique proteins are thought to be virulence factors and identifying their functions will help us better understand disease mechanisms and, in turn, pave the way for the development of new therapeutics. Understanding enterococcal colonisation and pathogenesis pathways is essential for finding novel approaches to controlling and interfering with these infections. *E. faecium* can colonise and infect the host in a different locations (Figure1-6). For all enterococcal infections, there is no single complete model system. Despite the fact that mammalian models are well-suited to addressing knowledge gaps during infections in a variety of infectious situations, large-scale investigations involving large numbers of animals are technically problematic. As a result, a

high-throughput mix of non-mammalian models for preliminary and large-scale screening, followed by confirmation testing in an animal model, may be advantageous (Goh et al., 2017). It is also obvious that enterococcal pathogenesis is complex, with virulence factors playing redundant roles in different infection sites (Goh et al., 2017). In addition, several *in vitro* phenotypes used as surrogate models for infection do not correlate with virulence *in vivo*, emphasizing the significance of investigating the role of specific virulence components in infection using a range of host-based model systems.

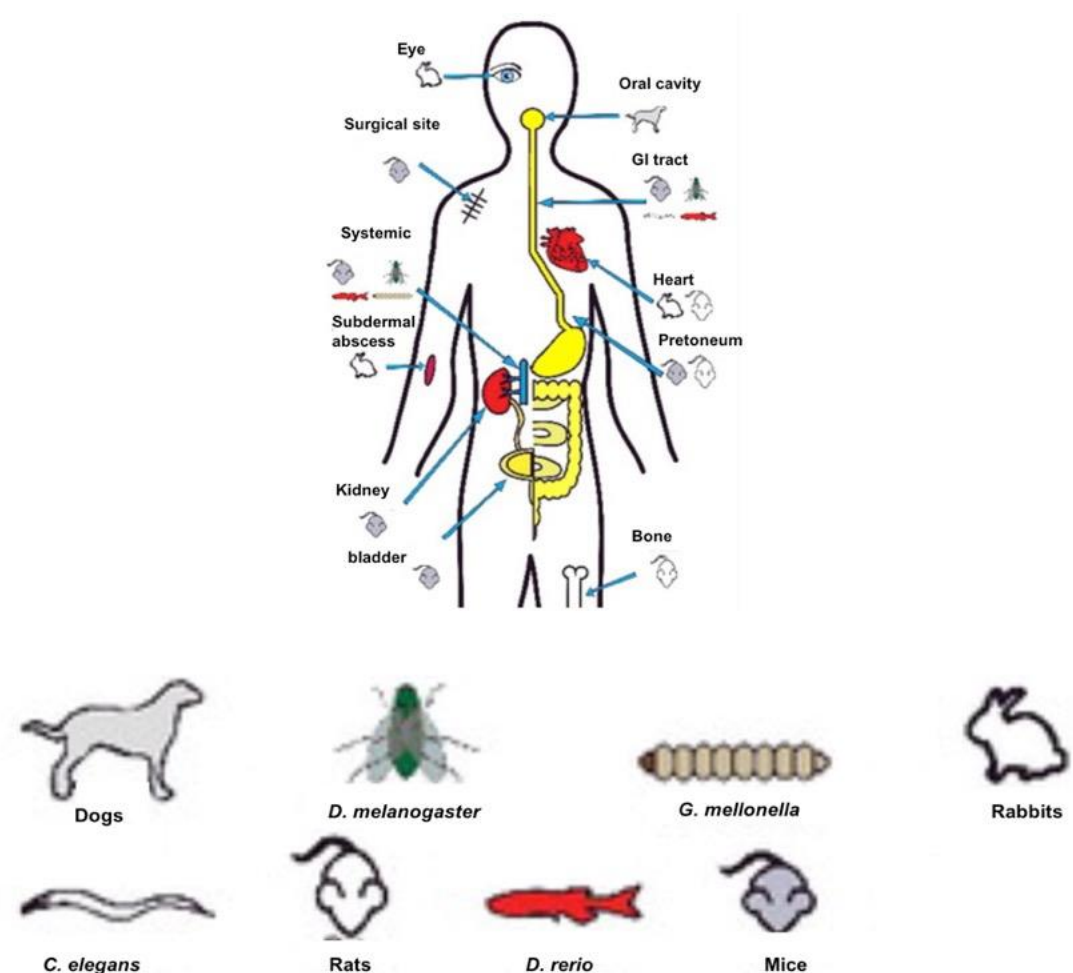


Figure 1-6 A cartoon depicts the different host model systems that have been used to investigate niche-specific Enterococcal infections.

Depicted from Goh et al. (2017).

Zebrafish is a teleost belonging to the Cyprinidae family. The innate immune system of the zebrafish embryo or larvae includes macrophages and neutrophils which are present at 1 day post fertilization (dpf) plus functional complement factors and antimicrobial enzymes which are present in the embryo before or just after hatching (Herbomel et al., 1999; Le Guyader et al., 2008). Zebrafish larvae have been used extensively as an animal infection model because zebrafish have a small size, short generation time, high fertility, and ease of genetic manipulation. Furthermore, the development and the physiology of zebrafish's digestive system are close to those of mammals. At 1 dpf, zebrafish embryos display normal bacteria-phagocyte interactions and as zebrafish larvae are optically transparent, most cell types, including macrophages and neutrophils can be tracked after infection with fluorescent bacteria (Prajsnar et al., 2013).

The zebrafish digestive system is homologous to that of mammals, including a liver, pancreas, gall bladder, and a linearly fragmented intestinal track with absorptive and secretory utilities. The intestinal epithelium shows proximal-distal functional specification and comprises several epithelial cell types with similar functions to those found in mammals, including absorptive enterocytes, goblet cells, and enteroendocrine cells. Previous microbial studies on zebrafish gastrointestinal tract showed a pathological effect inside it due to the infection, for example with *Vibrio cholerae* (Manneh-Roussel et al., 2018). To my knowledge, zebrafish larvae have not been used as a model to study pathogenicity of *E. faecium* by using an immersion infection method.

Phagocytes are a class of cells capable of ingestion and destruction of microorganisms and viruses. First to gather around the invaders and initiate the phagocytic process are neutrophils (known as polymorphonuclear leukocytes). Later, local, and blood-borne macrophages (also called mononuclear phagocytes) migrate to the tissue site and initiate phagocytosis. Bone marrow stem cells give rise to promonocytes which develop into monocytes that are released into the blood stream. The monocyte is actively phagocytic and bactericidal (Herbomel et al., 1999; Renshaw et al., 2006).

Macrophages are more active in phagocytosis than monocytes and develop many more granules containing hydrolytic enzymes. Although macrophages can develop by cell division under inflammatory stimuli, most macrophages are matured blood monocytes. Compared to neutrophils, macrophages are long-lived cells. Neutrophils play a key role during acute stages of infection, while during chronic stages of infection macrophages play a key role. Their innate immune response release chemicals which destroy microorganisms. These products consist of proteins such as lysozyme, peroxidases, and elastase as well as ROS such as O_2^- , H_2O_2 , hypohalous acid, and hydroxide ions (OH^-). It has been proved that the superoxide-generating NADPH oxidase, a multisubunit enzyme originally identified in immune cells, is involved in innate immunity and bacterial killing (Quinn et al., 2006).

1.16 *G. mellonella* as an infection model for *E. faecium*

Invertebrates are a valuable model system for researching conserved antimicrobial immune responses because they are extremely basic, lack an adaptive immune system, have immune-competent cells that mimic vertebrate phagocytes in some ways, have the ability to perform high-throughput

screenings (Flannagan et al., 2012; Gold and Bruckner, 2015; Abnave et al., 2017; Hartenstein and Martinez, 2019). Invertebrates' cellular immune response to pathogens is linked to the presence of macrophage-like cells, phagocytes, or cells with immunological characteristics comparable to macrophages from vertebrates (e.g., amebocytes, hemocytes, and coelomocytes) (Abnave et al., 2017; Hartenstein and Martinez, 2019).

G. mellonella belongs to the Lepidoptera order and the Pyralidae family of insects (snout moths) (Maguire et al., 2017). The caterpillar larvae, often known as wax worms, are employed as an animal model rather than the adult moth. *G. mellonella* larvae are less expensive to generate and maintain than typical mammalian model hosts because they do not require specialized laboratory equipment. Furthermore, *G. mellonella* does not require ethical approval for usage, and their short life history makes them excellent for high-throughput investigations (Ramarao et al., 2012). *G. mellonella* larvae, unlike other invertebrate models can survive at 37°C, allowing researchers to study temperature-dependent microbial virulence factors (Smoot et al., 2001). *G. mellonella* has become a popular infection model for studying bacterial and fungal infections as well as evaluating the efficiency of new antimicrobial medicines. The *G. mellonella* immune system contains plasmatocytes and granular cells, which function in a similar manner to phagocytes in the innate immune system of mammals (Browne et al., 2013). In *G. mellonella* once pathogens are phagocytosed, they can be killed by numerous mechanisms including antimicrobials and ROS. *G. mellonella*'s cuticle serves as a barrier to confine immune cells known as hemocytes, which are immune cells that are

similar to mammalian neutrophils in terms of their ability to phagocytose and kill infections via superoxide generation (Renwick et al., 2007).

Phagocytosis is a key biological action in which hemocytes release enzymes by degranulation to eliminate the pathogen (Kavanagh and Reeves, 2004; Pereira et al., 2018). Plasmocytes mediate phagocytosis in *G. mellonella* (Figure 1-7 C). Granulocytes have a role in the process in an indirect way (Pereira et al., 2018). The phagocytosis of hemocytes is mediated by complement-like proteins that opsonize pathogens and activate the enzymatic system (Browne et al., 2013). Pathogens are also recognized by hemocytes via calreticulin or apolipoprotein receptors. Calreticulin is a protein found on the surface of hemocytes that plays a role in cell adhesion, phagocytosis, antigen presentation, and the inflammatory process in animals. The protein apolipoprotein III is related to the mammalian apolipoprotein E, and both are connected with innate immunity binding to Gram-positive bacteria and lipoteichoic acid, which promotes phagocytosis (Whitten et al., 2004; Browne et al., 2013). Prophenoloxidase is produced by granular cells and oenocytes, and when phagocytic cells come into contact with a pathogen, this membrane-bound enzyme system is triggered (Figure 1-7).

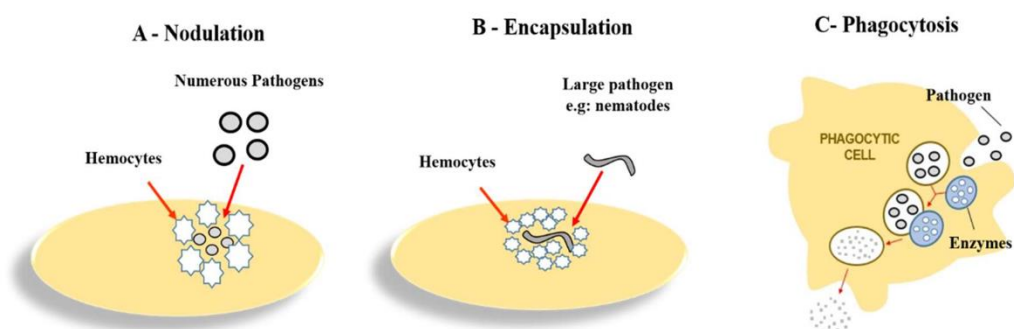


Figure 1-7 Hemocytes in *G. mellonella* use a variety of defense mechanisms:

(A) Nodulation, hemocytes create a layer of cells surrounding a collection of microorganisms. **(B)** Large pathogens such as protozoa, nematodes, and parasitic insect eggs or larvae are encapsulated by plasmocytes and granulocytes. **(C)** Phagocytosis, pathogens are destroyed by enzymes produced by plasmocytes and granulocytes. Adapted from Pereira et al. (2018).

During a microbial infection, oenocytoids release the prophenoloxidase cascade, which is then activated by serine proteases (this cascade is triggered by bacteria or fungi). Phenoloxidase, also called tyrosinase, when activated, converts tyrosine to dihydroxyphenylalanine and oxidizes phenolic substances to quinones and melanin, darkening infected larvae (Lu et al., 2014). Invading pathogens in the hemolymph of *G. mellonella* are detected as foreign substances by proteins, just as they are in mammals, and this detection is followed by the activation of the prophenoloxidase cascade. These recognition proteins attach to compounds like Lipopolysaccharides, peptidoglycan, and 1,3-glucan, which are components of Gram-negative, Gram-positive, and fungal cell walls, respectively (Park et al., 2005). Components of the phenoloxidase system are kept dormant in oenocytoids in unchallenged larvae (Li et al., 2002) and are released after identification of invasive bacteria that commence the process of melanization. This happens as part of the cellular immune response and during wound healing. This process initiates pathogen encapsulation at the site of injury, followed by coagulation and opsonization, and is analogous to the formation of abscesses in mammalian infections (Tang, 2009). Jorjão et al. (2018) investigated the melanisation of *G. mellonella* in the presence of *S. aureus* and *E. coli* infections. The researchers found that after 24 hrs of inoculation with *S. aureus* or *E. coli*, the melanisation of larvae increased, demonstrating that these bacteria can activate the phenol oxidase enzyme.

1.17 Aims of this study

E. faecium is intrinsically resistant to many antibiotics and it has been described as an emerging pathogen that causes a significant number of nosocomial-infections, which present a big threat for patients all over the world. Owing to its clinical significance, the WHO designates VRE*Efm* as a high priority pathogen on the global priority list of antibiotic-resistant bacteria. It is important to understand the biology of *E. faecium* infections to stop the increasing rates of nosocomial-infections. By using an *E. faecium* transposon mutant library, Hashim (2016) identified genes that showed resistance to antimicrobial peptides, nisin and lysozyme, in *E. faecium* E1162 and studied their role in virulence by using a *G. mellonella* larvae model. So, this PhD work aims; firstly to look at other possible infection models, zebrafish larvae and a planarian infection model and evaluate the use of these infection models to study the pathogenesis of *E. faecium* E1162; secondly, use of these infection models to compare virulence, survival, and colonisation between *E. faecium* E1162 and its mutant strains which were shown to be attenuated in *G. mellonella* larvae infection model; thirdly, screen an *E. faecium* E1162-*mariner*-transposon mutant library to identify genes involved in oxidative stress resistance by using menadione as superoxide generator and cumene as a peroxide generator to examine virulence of the identified sensitive mutants by using a *G. mellonella* model; fourthly, construct an *E. faecium* marker-less deletion mutant with deletion of *efmE1162_1543* and to compare the virulence of *E. faecium* E1162, mutant 59N and the *efmE1162_1543* deletion mutant by using a *G. mellonella* infection model and lastly, to see whether *trans-*

complementation of the 59N mutant with *efmE1162_1543* could restore the wild-type virulence in the *G. mellonella* infection model.

Chapter II

Materials and Methods

2.0 Materials and Methods

2.1 Bacteriological methods

2.1.1 Bacterial culture media, chemicals and antibiotics

All culture media were purchased from Oxoid. All chemicals and antibiotics were purchased from Sigma unless stated otherwise. All broth media were prepared according to the manufacturer's instructions unless specified. Media was prepared in deionized water and sterilized by autoclaving for 15 min at 121°C at 15 psi (pounds per square inch), unless otherwise stated. Bacterial culture media and chemicals that were used in this study are listed in Table 2-1.

Table 2-1 Bacterial culture media and chemicals

Media	Components
Agar Bacteriological (Agar No 1)	As supplied by Oxoid (LP0011)
Brain Heart Infusion	As supplied by Oxoid (CM1135)
Tryptic Soy broth	As supplied by Oxoid (CM0129)
Luria Bertani	As supplied by Sigma (L3022)
M9 Minimal salts medium	As supplied by Fluka (63011)
Minimal Medium Yeast Extract Glucose (MM9YEG)	11.28 g/l, M9; 2.5 g/l, yeast extract; 15 g/l, Agar; 20 g/l, Glucose
Todd–Hewitt broth	As supplied by Oxoid (CM0189)
4-Chloro - DL- phenylalanine	As supplied by Sigma (C6506)
Super Optimal broth with Catabolite repression (SOC) medium	As supplied by Invitrogen
Glycerol for molecular biology	As supplied by Sigma (G5516-IL)
Yeast extract granulated	As supplied by MERCK
Menadione (vitamin k3)	As supplied by Cayman Chemical Company NSC4170

Cumene hydroperoxide solution	As supplied by Sigma-Aldrich (247502)
Deeside still natural mineral water	(Pannanich Wells/ Scotland) purchased from Amazon
Ultra-low gelling temperature agarose	As supplied by Sigma-Aldrich A5030
Red food coloring	Waitrose Essential

2.2 Growth conditions

Unless stated otherwise, *E. faecium* E1162 and the isogenic mutants were grown in Tryptic Soy Broth (TSB) broth/agar or Brain Heart Infusion (BHI) broth/agar (Oxoid) for 16 hrs at 37°C and shaking condition (200 rpm). Luria-Bertani (LB) broth/agar (Sigma) media were used to grow the *E. coli* strains at 37°C. When needed, antibiotics were supplemented at the concentrations as specified in Table 2-2, and an optical density at 600 nm (OD_{600 nm}) was used to determine the growth.

2.3 Antibiotics

Table 2-2 shows the antibiotics used in bacterial culture media for selection. Concentrations required for the selection of bacteria were prepared by dissolving each antibiotic in the specified solution then filter-sterilization using 0.22 µm filters (Starlab, UK, Ltd) unless stated otherwise.

Table 2-2 Antibiotics used for culturing *E. faecium* and *E. coli* strains

Antibiotic	Solution	Working concentration µg/ml
Kanamycin	Filtered deionised Water (diH ₂ O)	50 µg/ml for <i>E. coli</i>
Ampicillin	Filtered diH ₂ O	100 µg/ml for <i>E. coli</i>
Gentamicin	Filtered diH ₂ O	300 µg/ml for <i>E. faecium</i> and 25 µg/ml for <i>E. coli</i> , (Guzman Prieto et al., 2015)
Chloramphenicol	95% Ethanol	4 µg/ml for E1162 (De Kraker et al., 2013)

2.4 Bacterial strains/plasmids and storage

Bacterial stocks were preserved in bacteriological media containing 20% (v/v) sterilized glycerol and stored at -80°C. Glycerol is diluted in deionized water.

All bacterial strains used in this study are listed in Table 2-3.

2.5 Determination of growth curves

Except where indicated, the *E. faecium* E1162 and its isogenic mutants were grown for 16 hrs in BHI. At the beginning cells were inoculated at initial OD₆₀₀ nm of 0.05 into 10 ml BHI, followed by incubation at 37°C with continuous agitation (220 rpm) and absorbance of OD₆₀₀ nm was recorded every 60 min for up to 8 hrs, the experiment was conducted in triplicate for three independent biological repeats.

Table 2-3 Bacterial strains and plasmids

Strain or plasmid	Information	Reference
<i>E. faecium</i> E1162	Clinical blood isolate; CC17; Amp ^r Van ^s Chl ^s Gen ^s Ery ^s Esp ⁺	Heikens et al., 2007
E1162 Δ esp	E1162 Amp ^r Van ^s Chl ^r Gen ^s Ery ^s Esp ⁻	Heikens et al., 2007
<i>E. faecium</i> 201c	E1162 Δ stk1 Amp ^r Van ^s Chl ^r Gen ^s Ery ^s Esp ⁺	Hashim, 2016
<i>E. faecium</i> Mabc	Deletion of the <i>E. faecium</i> E1162 putative permease component <i>efmE1162_0264</i> of ABC transporters	Hashim, 2016
<i>E. coli</i> DH5 α	F- ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (rk ⁻ , mk ⁺) <i>phoA supE44</i> <i>thi-1 gyrA96 relA1</i> λ -	Invitrogen
TOP10 <i>E. coli</i> cells	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 araD139</i> Δ (<i>araleu</i>)7697 <i>galU galK rpsL</i> (StrR) <i>endA1 nupG</i>	Invitrogen
EC1000	<i>E. coli</i> cloning host; MC1000 containing <i>pWV01 repA</i> on chromosome	Leenhouts et al., 1996
pHFH4	pSK236; Kan promoter; Amp ^r Clm ^r	Hashim, 2016
pSK236	Shuttle vector containing pUC19 cloned into the <i>HindIII</i> site of pC194	Gaskill and Khan, 1988
pCR 2.1- TOPO	Amp ^r	Invitrogen
pHOU1	Gen ^r	Panesso et al., 2011
<i>E. faecium</i> E1162 transposon mutant strains		
Strain	Transposon insertion site	Reference

83N	Conjugative transposon protein encoding gene <i>efmE1162_1535</i>	Hashim, 2016
183N	Cystathionine beta synthase (CBS) domain protein encoding gene <i>efmE1162_0514</i>	Hashim, 2016
59N	A putative muramidase encoding gene <i>efmE1162_1543</i>	Hashim, 2016
64N	Conserved hypothetical protein encoding gene <i>efmE1162_0147</i>	Hashim, 2016
95N	Transposase encoding gene <i>efmE1162_1389</i>	Hashim, 2016
139N	Tyrosine decarboxylase encoding gene <i>efmE1162_1492</i>	Hashim, 2016
47N	Tyrosine decarboxylase encoding gene <i>efmE1162_1492</i>	Hashim, 2016
MS1	Tyrosine decarboxylase encoding gene <i>efmE1162_1492</i>	This study
MS2	Conserved hypothetical protein encoding gene <i>efmE1162_1516</i>	This study
MS3	Transposase encoding gene <i>efmE1162_2615</i>	This study
59N:: <i>mur</i>	<i>Trans</i> -complementation of mutant 59N with an intact <i>efmE1162_1543</i>	This study
59N containing pHFH4	<i>efmE1162_1543</i> ligated with pHFH4 and then transformed into <i>E. coli</i> DH5 α , and plasmids were purified from transformants designated pHFH4- <i>mur</i>	This study

Van^s: vancomycin-sensitive, **Cm^r**: chloramphenicol-resistance, **Gen^s**: gentamicin-sensitive, **Ery^s**: erythromycin-sensitive, Δ **stk1**: deletion of a serine threonine protein kinase coding gene, **CC17**: clonal complex 17, **Esp⁺**: contain the enterococcal surface protein gene, Δ **esp**: deletion of the enterococcal surface protein gene, **N**: nisin-sensitive, **MS**: menadione-sensitive, **repA⁺**: replication protein A. Bacterial strains and plasmids were selected using appropriate growth conditions and antibiotics according to manufacturer's and bacterial stock note book instructions.

2.6 Experimental animals

Zebrafish and planaria were used as infection models to study colonisation, survival, and virulence of E1162 and some of its mutants. *G. mellonella* as used to compare the virulence between E1162 and its oxidative stress sensitive mutants. Hashim (2016) showed that 59N was significantly weakened in killing *G. mellonella* in comparisons to E1162 so *G. mellonella* was used also to see whether *trans*-complementation of the 59N mutant with *efmE1162_1543* could restore the wild-type virulence in this infection model or not. Devices and reagents used for the experimental animal models are shown in Table 2-4.

2.6.1 Ethical Statement

Pre-feeding zebrafish larvae (up to 5 dpf) are exempt from mammalian model and adult zebrafish ethical home office laws (Nüsslein-Volhard and Dahm, 2002; Westerfield, 2007). There are no ethical limitations for invertebrate animals for example; insects (*G.mellonella*) and planarian worms (*Dugesia japonica*) {*Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, 2014*}.

Table 2-4 Devices and reagents used for the experimental animal models

Reagent/Device	Manufacture
Used for planaria (<i>D. japonica</i>)	
Deeside still natural mineral water	Pannanich Wells / Scotland) purchased from Amazon
Red food coloring	(Essential Waitrose)
Ultra-low gelling temperature agarose	(Sigma-Aldrich) A5030
Calf liver	Coombe Farm Organic UK

Surgical blades sterile	Carbon Steel (Swann-Morton, 10 REF 0201)
Cooled incubator SciQuip which provide controllable temperature uniformity throughout (range between 4°C to 65°C)	SciQuip
Axygen™ Tissue Grinder	Thermo Fisher scientific

Used for *G. mellonella*

Transfer pipette 3.5 ml	Sarstedt
Phosphate buffered saline (PBS)	Oxoid (BR0014G)
1 ml syringe	VanishPoint®
Automated syringe pump injector	New Era Pump System, Inc
Incubator (12°C)	Thermo Scientific
Ethanol 70%	Sigma-Aldrich
Used for zebrafish larvae (<i>Danio rerio</i>)	
Zebrafish water	0.6 g/l aquarium salt in deionized water
Falcon Polystyrene Microplates (6–well tissue culture plate)	Thermo Fisher Scientific
Bench top shaker	Thermo Fisher Scientific
Tissue culture incubator (28°C)	Thermo Fisher Scientific

2.7 Recombinant DNA and genetic techniques

2.7.1 Isolation of genomic and plasmid DNA

Genomic deoxyribonucleic acid (DNA) from *E. faecium* E1162 and its isogenic mutants was isolated using the Puregene Yeast/Bact. Kit (Qiagen) with slight modification. 5 ml of the 16 hrs old culture was centrifuged at 17900 xg (Eppendorf 5417R) for 1 min and 550 µl of suspension buffer, 10 µl of 100 U mutanolysin (M9901 from Sigma), and 40 µl of 25 mg/ml lysozyme (Sigma) were added and incubated for 30 min at 37°C followed by centrifugation at

17900 xg (Eppendorf 5417R) for 1 min. The supernatant was discarded, the rest of the procedure was as per the kit protocol. Then the quality and quantity of the genomic DNA was assessed using Nanodrop spectrophotometer (Thermo Scientific). Plasmid DNA purification from *E. coli* performed using QIA amp DNA Mini Kit (Qiagen) as per the manufacturer's instruction.

2.7.2 Primers used in this study

Primers were designed using Primer3-plus software. All primers used in this study (Table 2-5) were synthesized by Sigma Aldrich at a concentration of 100 μmol .

Table 2-5 List of primers used in this study

Primers	Sequence 5'→3'
Linker I- <i>Taq</i> I	TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACAT
LinkerII- <i>Taq</i> I	CGATGTCCCCGTACATCGTTAGAACTACTCGTACCATCCACAT
Linker I- <i>As</i> I	TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACAAT
Linker II- <i>As</i> I	TAATTGTCCCCGTACATCGTTAGAACTACTCGTACCATCCACAT
Y- linker primer	CTGCTCGAATTCAAGCTTCT
Transposon primer	GGAATCATTGAAGGTTGGTA
Del- <i>mur</i> -F1- <i>Eco</i> R1	AAGAATTCAGCTTTGGATGATAGTAC
Del- <i>mur</i> -R1	GTCAGATTGATTACCTTTTTTACATTTGATTTACCTCTCTT
Del- <i>mur</i> -F2	AAGAGAGGTAAATCAAATGTAAAAAAGGTAATCAATCTGA
Del- <i>mur</i> -R2- <i>Not</i> I	AAGCGGCCGCCTATTTCTCTTTAGAACG
<i>mur</i> (F)	CTTGCAATCAGCAGTTATATTAATGTCC
<i>mur</i> (R)	TTAGTCTCCGAATAATAAGGATCAAC
Comp- <i>mur</i> _F1467- <i>Bam</i> H1	CGGATCCAAAAGAGAGGTAAATCAAATG
Comp- <i>mur</i> _R1467- <i>Sph</i> I	CGCATGCTTAGTCTCCGAATAATAAGG

2.7.3 Molecular biology kits

Molecular biology kits which were used in this work are listed in Table 2-6.

Table 2-6 The molecular biology kits routinely used

Molecular biology kit	Manufacture
Genra Puregene Yeast/Bact. Kit	Qiagen/ (Cat No./ID: 158567)
Purelink Quick Gel Extraction kit	Qiagen (Cat.No. 28706)
TOPO TA Cloning Kit, with pCR2.1-TOPO, One Shot TOP10 Chemically Competent <i>E. coli</i> , and PureLink Quick Plasmid Miniprep Kit	Invitrogen K450002
PCR Purification Kit (50)	Qiagen (Cat No./ID: 28104)

2.7.4 Agarose gel electrophoresis

Genomic/plasmid DNA and PCR amplicons were examined by loading 2.0-3.0 µl of product accompanied by a standard DNA marker where necessary on a 1.0% w/v agarose gel containing GelRed or GelGreen Nucleic Acid Gel Stains (Table 2-7), and electrophoresed at 70 volts for 45 min. An Alpha Imager (Alpha Innotech) was used to visualize the DNA bands. When required- DNA band (s) were excised from the gel with a clean disposable razor blade and purified using an agarose gel purification kit as per the manufacturer's instructions.

For gel purification, GelRed stain and GelGreen stain were used since both are compatible with subsequent applications like cloning, ligation, and sequencing (Biotium, 2021).

Table 2-7 Solutions and buffers for agarose gel electrophoresis

Designation	Components	Comments
5X TBE buffer	Tris base: 54 g boric acid: 27.5 g (EDTA) 0.5 M pH 8: 20 ml diH ₂ O: up to 1 litre	dilute 1:5 before use to obtain TBE 1X
Agarose	As supplied by Bioline (BIO - 41025)	
Agarose gel	agarose: 1% in TBE	1 g agarose in 100 ml TBE 1X GelRed/GelGreen at 1: 10,000
Loading dye	Bioline loading dye 6X (200 mM EDTA, pH 8, 50% (v/v) glycerol, 0.1 % (w/v) bromophenol blue	added 20% of the sample volume
Molecular weight marker	Hyper ladder 1 (1kb) Bioline/Hyper ladder11 50 (bp) (Bioline)	
GelGreen nucleic acid gel stain; 10,000X in water, 0.5 ml Promega cat 41005		Dilute the GelGreen™ 10,000X stock reagent into the molten agarose gel solution at 1: 10,000
GelRed nucleic acid gel stain; 10,000X in water, 0.5 ml		Dilute the GelRed™ 10,000 X stock reagent into the molten agarose gel solution at 1: 10,000
1X TAE buffer		40 mM Tris-acetate, 1.0 mM EDTA, pH 8.0

2.7.5 Polymerase chain reaction

Usually, the polymerase chain reaction was done using a Biometra Uno Thermoblock Thermal Cycler. DNA Primers were purchased from Sigma. Unless mentioned otherwise, the programs were used as follows (Table 2-8).

Table 2-8 PCR programs used in this study

Step	Temperature	Time in min
Initiation	94°C	4
Denaturation	94°C	0.5
Annealing	X	0.5
Elongation	72°C or 68°C	Y
Termination	72°C or 68°C	5 or 7

Both the annealing temperature (X) and the time (Y) required for polymerization were selected according to the fragment to be amplified. The annealing temperature in the PCR was in most cases about 5°C lower than the lowest T_m of the primers, elongation usually 1 min for each 1 kb. The PCR was cycled 30 times between (94°C, 0.5 min; 72°C or 68°C, Y min). The PCR reaction comprised 25 µl of Q5 High-Fidelity 2X Master Mix (NEB, UK) (contains Q5® High-Fidelity DNA polymerase for high-fidelity amplification), 5.0 ng of genomic DNA as a template with 20 pmol/µl of both the forward and reverse primer (Table 2-5), the total volume of PCR mixture was adjusted to 50 µl using sterile DNase-free water. PCR products were examined using agarose gel electrophoresis.

2.7.5.1 Colony PCR

The same PCR protocol as in section 2.7.5 was used to do colony PCR with the change, that a single bacterial colony was used as the DNA template in place of purified genomic DNA. Individual colonies picked up using a sterile toothpick, and each immersed into separate PCR tubes. Once the solution becomes cloudy, the toothpick was removed, and the PCR process continued. Each

tooth-picked colony was gently pushed up and down in the PCR mix tube before being touched by a sterile agar plate containing the proper media and incubated at 37°C for 16 hrs. On the plates, at least 75% of the selected colonies were grown.

2.7.6 DNA digestion with restriction enzymes

DNA digestion was conducted in either 10 µl reaction volumes or in at a preparative scale (70 µl). All restriction enzymes were obtained from New England Bio-labs (NEB). Restriction reactions were as per the manufacturer's instructions.

2.7.7 DNA ligation reactions

Unless mentioned otherwise, ligations were set in a 12 µl ligation volumes containing vector: insert molar ratio of 1: 3, 1.2 µl T4 DNA Ligase Buffer (NEB) and 1.0 µl T4 DNA Ligase (NEB), mixed and incubated at 16°C for 16 hrs, 3-5 µl of ligation mixture were used to transform calcium chloride competent *E. coli* EC1000, *E. coli* DH5α, and One Shot™ TOP10 cells (Invitrogen) and the transformants were selected on LB agar plates supplemented with 25 µg/ml gentamicin, 100 µg/ml ampicillin and 50 µg/ml ampicillin, respectively.

2.8 Statistical analysis

Origin Pro (OriginPro 2018 v9.5.1) for windows was used for data analysis. The significance of the difference between the percentage survivals of *G. mellonella*/ zebrafish larvae/planarians infected with different strains (wild-type *E. faecium* E1162 and isogenic mutants) was analysed using the log-rank test. Data with a $p \leq 0.05$ significance level were considered statistically significant. The significance of the difference between a group of strains was determined using one-way ANOVA combined with Tukey's *post-hoc* test for multiple comparisons

for data with a normal distribution. When the samples are small a non-parametric test, the Mann Whitney U test, was used to compare outcomes between two independent groups. A linear regression (Excel, 2016) was used to relate the units between the optical density measurements and it's approximate bacterial cell density of the wild-type E1162, in statistics, the r-squared (R²) value is used to show how well the data fit the regression model.

2.9 DNA sequencing and bioinformatics analysis

DNA sequences were made using Sanger sequencing method at GeneWiz (formerly known as Beckman Coulter Genomics), UK.

The basic local alignment search tool-nucleotide (Nucleotide BLAST_BLASTn) was used to compare two nucleotide or protein sequences and identify homologous regions, (BLASTx) was used to translate the nucleotide sequence into a predicted amino acid, and BLAST-protein (BLASTp) was used to query a protein database (Altschul et al., 1990).

Primers are designed using the Primer3+ software. While SMART (a simple modular architecture retrieval tool) (<http://smart.embl-heidelberg.de>) was used in the identification and analysis of protein domains within proteins (Letunic et al., 2012).

Chapter III

Development of zebrafish larvae and planarian infection models to study survival, colonisation and virulence of *E. faecium* E1162

3.0 Development of zebrafish larvae and planarian infection models to study survival, colonisation and virulence of *E. faecium* E1162

3.1 Introduction

E. faecium is an opportunistic human nosocomial pathogen that has arisen as a prominent cause of multiple drug resistance infections. Treating infections caused by it can be difficult and there are limited therapeutic options against multiple-drug resistant enterococci. Host of an infected gastrointestinal tract serves as a reservoir from which the organism is disseminated (Hidron et al., 2008).

In this chapter, zebrafish larvae and planaria were used to study colonisation-, survival, and virulence of the wild-type *E. faecium* E1162 and its isogenic mutants which were shown to be attenuated for virulence in *G. mellonella* larvae infection model (Hashim, 2016). Zebrafish larvae have been used extensively as an animal infection model to study bacterial infections including Gram-positive and Gram-negative bacterial pathogens. *E. faecalis*, *Streptococcus* species, *Listeria monocytogenes*, *Mycobacterium marinum*, *Salmonella* as well as *Mycobacterium marinum* successfully have been studied using this model (Harvie et al., 2013; Zaccaria et al., 2016).

3.1.1 Zebrafish Innate immune response

During the innate immune response, host cells identify conserved molecular structures on pathogens called pathogen-associated molecular patterns (PAMPs) (Figure 3-1) via germline-encoded pattern recognition receptors (PRRs). PAMPs which are conserved among all classes of pathogens, are

crucial for the pathogen survival and enable host cells to differentiate 'self' from 'non-self' (Medzhitov et al., 2000).

The zebrafish larvae's innate immune system primarily consists of neutrophils and macrophages. Neutrophils are the first to respond to an injury; macrophages are then recruited to inflamed tissues to phagocytose pathogens and tissue debris. Neutrophils rapidly accumulate at wounds (Renshaw et al., 2006) and this chemotactic activity is critical in responding to tissue injury and infections. Phagocytically active macrophages are the first leukocytes to appear in the zebrafish embryo (Herbomel et al., 1999; Lieschke et al., 2002) and exhibit phagocytosis of cellular debris and bacteria (Herbomel et al., 1999; Redd et al., 2006).

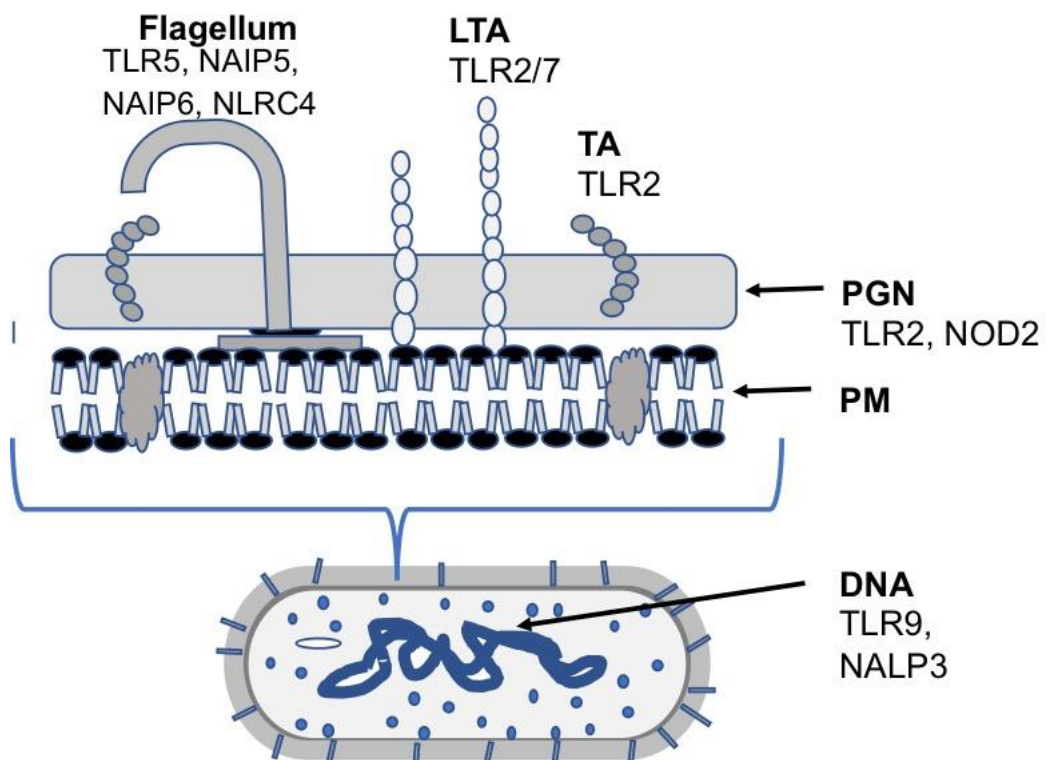


Figure 3-1 Pathogen-associated molecular patterns expressed by Gram-positive bacteria and the host receptors.

For example: **DNA** [TLR9, NALP3]. **PM** (periplasmic membrane). **PGN** (peptidoglycans) [TLR2, NOD2]. **TA** (Teichoic acid) [TLR2], **LTA** (Lipoteichoic acid) [TLR2/7] and **Flagellum** [TLR5, NAIP5, NAIP6, NLRC4]. This Figure was adapted from Kumar et al. (2013).

Zou et al. (2007) identified antimicrobials as part of the innate immunity of zebrafish. Also, peptidoglycan recognition proteins with peptidoglycan-lytic amidase activity and a broad spectrum of bactericidal activity have also been recognized in zebrafish (Chang et al., 2007; Li et al., 2007).

Using a zebrafish infection model would have numerous advantages including high genetic and organ system homology to humans compared to other non-mammalian systems, the transparency that enables powerful imaging modalities, small size, short generation time, high fertility, and ease of genetic manipulation. The zebrafish intestine has some differences when compared to mammalian organs, however, many studies have reported the high conservation of cellular composition, and function of the digestive organs (Zhao and Pack, 2017). Additionally, much of the molecular pathways that initiate and promote the development of the digestive organs and regulation of repair following an injury of the mature system have been conserved (Carten and Farber, 2009; Brugman, 2016; Zhao and Pack, 2017). Therefore, zebrafish have become a useful model vertebrate in which to study digestive development, physiology, and disease. At 1 dpf, zebrafish embryos display normal phagocyte bacteria interactions. Neutrophils and natural killer cells are the main components of its innate immune system, which form the first line of defense against a wide range of pathological challenges. Natural killer cells have a significant capability to adapt to their surroundings, for example the presence or absence of MHC class I molecules. At a molecular level, zebrafish and human immune systems shows approximately 75% similarity (Hsu et al., 2007), with

Toll-like receptors (TLRs) (Stokes et al., 2015), nucleotide oligomerization domain receptors NOD1 and NOD2 (Goldsmith and Jobin, 2012), and the complement system. As zebrafish larvae are optically transparent, most cell types, including macrophages (Ellett et al., 2011) and neutrophils (Renshaw et al., 2006), can be microscopically imaged in real-time upon injection of fluorescent bacteria into the host.

The zebrafish digestive system is homologous to that of mammals, including a liver, pancreas, gall bladder, and a linearly fragmented intestinal track with absorptive and secretory utilities (Ng et al., 2005). The intestinal epithelium shows proximal-distal functional specification and comprises several epithelial cell types with similar functions to those found in mammals, including absorptive enterocytes, goblet cells, and enteroendocrine cells (Ng et al., 2005). In the last decade, numerous gastrointestinal pathologies have been exhibited in the zebrafish.

3.1.1.1 Pattern Recognition Receptors (PRRs)

PRRs consist of TLRs, the retinoic acid-inducible gene I (RIG-I) like receptors, nucleotide-binding oligomerization domain receptors (NOD-like receptors), and C-type lectin receptors which recognize microorganisms that have entered the phagolysosome degradation pathway. Attachment of the microbe to the phagocyte results in signaling cascades that stimulate phagocytosis. Alterations in membrane remodeling and the actin cytoskeleton take place causing the development of pseudopods that engulf the microorganisms (Flannagan and Grinstein, 2010), followed by association and dissociation of lipids from the phagosome's membrane and Rho (**R**as **h**omologous) GTPases, Rac (**R**as-related **C**3 botulinum toxin substrate 1) is a family of related proteins which is

expressed in all animal cell lineages and organs), Ras (Rat sarcoma is a superfamily of small GTPases-mediating the responses to various extracellular stimuli) and Cdc42 (Cell division control protein 42), which are vital regulators of the actin cytoskeleton, become activated and move to the forming phagosome leading to depression of the membrane (phagocytic cup) (Mao and Finnemann, 2015). These trigger ingestion of the microbe, so that it is eventually taken inside the cytoplasm of the phagocyte, engulfed in a membrane vesicle called a phagosome, the phagosome containing the microorganism migrates into the cytoplasm and soon fuses with lysosomes forming a phagolysosome. The PRRs role in sensing is crucial, and in mammals, it is well known that all TLRs except TLR3, signal through MyD88 (myeloid differentiation primary response protein 88) (Meijer and Spaink, 2011), which is well-known for its vital role as an adaptor molecule in interleukin 1 receptor and TLR signaling (van der Vaart et al., 2013). Except for TLR4 which recognises lipopolysaccharide, an almost complete set of TLRs has been described in the zebrafish (Roach et al., 2005; Sepulcre et al., 2009). As per Phelan et al. (2005), zebrafish TLRs are orthologs of mammalian TLRs that sense invading pathogens and they have a similar adaptor for triggering nuclear factor kappa-light-chain-enhancer of activated B cell (NF- κ B) signaling. By using transgenic reporter lines, Hall et al. (2009) confirmed that in zebrafish MyD88 modulates innate immune responses to microbes.

Development of the adaptive immune system is not complete until almost three-weeks post-fertilization (Lam et al., 2004), given that fact there are at least two weeks in which the innate immune response can be studied in the absence of T- and B-cell responses. The key components of the TLR signaling pathway, including MYD88, TIRAP (Toll-Interleukin 1 Receptor (TIR) Domain Containing

Adaptor Protein), TIR-domain-containing adapter-inducing interferon- β , TNF Receptor Associated Factor 6, Interferon Regulatory Factor 3 and IRF7 (Interferon Regulatory Factor 3) have been described in zebrafish (Purcell et al., 2006), and were found to be vastly expressed in one-day-old zebrafish embryos at which stage functional macrophages are first present (Jault et al., 2004). On the other hand, zebrafish TLR4 paralogs, TLR4a and TLR4b do not recognize lipopolysaccharide may be due to differences in their extracellular structure (Sepulcre et al., 2009).

3.1.1.2 Toll-like receptors (TLRs) and Nod-like receptors (NLRs)

TLRs are type-I transmembrane proteins spanning the membrane once, with their N-terminus on the extracellular side of the membrane after cleavage of its signal sequence and have an ectodomain, which contains leucine-rich repeats that mediate the recognition of PAMPs, a transmembrane region, and cytosolic **Toll-IL-1** receptor domains that activate downstream signaling pathways. They are expressed either on the cell surface or associated with intracellular vesicles (Kawai and Akira, 2011). Pathogens that escape the surveillance of cell surface and endosomal PRRs may end up in the cytosol, where nucleotide-binding oligomerization domain-like receptors (NOD-like receptors, in brief NLRs) detect their presence by intracellular PAMPs. These resemblances between the innate immune system of humans and zebrafish suggest that zebrafish are a good model for vertebrate innate immunity.

3.1.2 Planarians as a model for studying host-pathogen interactions

Planarians of the Turbellaria class (phylum Platyhelminthes) are non-parasitic flatworms found in freshwaters. They are hermaphrodites, can replicate sexually or asexually via splitting and regeneration and known for their unlimited capacity to regenerate. This capability to regenerate arises from a population of stem cells distributed throughout their body. They are small, resistant to infections by bacteria that are pathogenic to humans including Gram-positive bacteria (*S. aureus*, *L. monocytogenes*, *Mycobacterium tuberculosis*, *M. avium* and *M. marinum*) and Gram-negative bacteria (*Salmonella enterica* serovar Typhimurium and *Legionella pneumophila*). They are easy to work with, cheap and genetically identical (Abnave et al., 2014; Hamada et al., 2016), and they do not raise the ethical concerns of working with vertebrate animals.

The planarian digestive system consists of three divisions, two dorsal and one central (Bueno et al., 1997), their intestine is connected to extensile pharynges that functions as a mouth (Elliott and Sánchez Alvarado, 2013). In the head region, there is light sensitive structures as well as chemoreceptors sited in the sensory lobes or auricles. Planarian intestines are involved in digestion, nutrient circulation as well as in immune response. Secretory goblet cells and absorptive phagocytes are the two main types of planarian intestine epithelium cells which are involved in bacterial clearance (Abnave et al., 2014). Planarian stem cells efficiently regenerate tissues or even organs from the three germ layers (ectoderm, mesoderm and endoderm) that can travel to the site of the injury and restore the injured tissue or just substitute old tissue when there is no injury (Elliott and Sánchez Alvarado, 2013).

Histological and functional studies in *Schmidtea mediterranea* have identified active phagocytic cells surrounding areas of wounding. Microbial adhesion to mucus reduces colonisation by pathogens and acts as a defense mechanism

towards infection. Planarians are protected by a thin layer of mucus which may act as a defensive wall. During wounding, planarians produce mucus that may have a dual role in preventing pathogen entry and aid in healing (Gao et al., 2017). Bocchinfuso et al. (2012) found that planarian derived mucus proteins exhibited similarity with mucosal secretions (thick protective fluids secreted by the mucus membrane) from humans.

Introducing bacterial lipopolysaccharide into wound sites of planarians resulted in induction of potential antimicrobials (Altincicek and Vilcinskas, 2008). Antimicrobials are small, positively charged, evolutionarily conserved, often secreted by phagocytic cells and are known to have immunomodulatory functions (Mahlapuu et al., 2016). Abnave et al. (2014) observed that when planarians were fed with bacteria, they were able to destroy the bacteria. They found that planarians have a *MORN2* gene (Membrane occupation and recognition nexus-2) which is conserved in humans and involved in light chain 3-assisted phagocytosis. In human macrophages, *MORN2* enables the phagocytosis-mediated killing of *M. tuberculosis*, *L. pneumophila* and *S. aureus*. *MORN2* recruits microtubule-associated protein 1A/1B light chain 3 (an autophagy protein involved in phagocytosis) to *M. tuberculosis*-containing phagosomes which later mature to degradative phagolysosomes. Abnave et al. (2014) identified 18 genes required for bacterial clearance in planarians, most of these genes were transcriptionally upregulated in the planarian's intestine during infection and some of these genes were crucial for resistance against infection by Gram-positive and Gram-negative pathogens, including a dual-specificity phosphatase and progesterin and adiponQ receptor family member 3. In vertebrates, Placenta specific protein 8 (Plac8) has been well studied. In *D. japonica*, a gene encoding a homolog of Plac8 was found to be upregulated

during regeneration for the pharynx, the epidermis, and the intestine of adult planarians. In a different study, Pang et al. (2017) found that *DjPlac8* expression was upregulated upon lipopolysaccharide challenge and *Plac8* showed antibacterial activity against *E. coli* and *P. aeruginosa*.

3.1.3 Aims of this chapter

The aims of the work described in this chapter were to evaluate the use of zebrafish larvae and planarian models to study the pathogenesis of *E. faecium* E1162; use of these models to compare virulence, survival and colonisation between the *E. faecium* E1162 parental strain and isogenic mutants which have been shown by Hashim (2016) to be attenuated in the *G. mellonella* larvae infection model.

3.2 Materials and methods

3.2.1 Bacterial strains

The bacterial strains used in this chapter are listed in Table 2-3 of Chapter II. Unless otherwise stated bacterial stocks were preserved in 20% (v/v) glycerol in BHI at -80°C. Zebrafish larvae, zebrafish water, Deeside still natural mineral water, ultra-low gelling temperature agarose, reagents and devices were as in Table 2-4 of Chapter II.

3.2.2 Growth conditions

E. faecium strains were grown either in TSA (broth/agar) or BHI (broth/agar) media at 37°C with shaking (200 rpm) unless stated otherwise.

3.2.3 Growth curves of *E. faecium* E1162 and the nisin-sensitive mutants in BHI

Growth curves of the wild-type *E. faecium* E1162 and the isogenic nisin-sensitive mutants were compared in BHI medium. Overnight cultures of *E.*

faecium E1162 and mutant strains were diluted to OD600 nm of 0.05 in 10 ml BHI. Cultures were incubated at 37°C in a shaking incubator. OD600 nm was measured every hour until stationary-phase.

3.2.4 Preparation of the wild-type *E. faecium* E1162 dosage for infection in the animal models

Different OD600 nm (0.5, 1.0, and 3) were prepared in BHI by using an overnight culture and incubated at 37°C in order to plot a standard growth curve for the wild-type *E. faecium* E1162 that displayed different OD600 nm_s and colony forming unit per milliliter (CFU/ml). At regular intervals, samples were taken, and the amount of live bacteria were enumerated.

3.2.5 Virulence of *E. faecium* E1162 strains in a zebrafish larvae infection model

The eggs of wild-type zebrafish were obtained from the UCL zebrafish fish facility (University College London). Eggs were obtained on the same day of fertilization. Embryos were incubated in zebrafish water (0.6 g/l aquarium salt in deionized water) in 6-well tissue culture plate (polystyrene, non-pyrogenic REF 353046) at 28°C, for 3 days until hatching. Following hatching, embryos were dispensed into each well of 6-well plates containing 5 ml of fish water (10 embryos per well) (Dlugos and Rabin, 2007), all procedures were performed in a laminar microbiological cabinet. *E. faecium* strains were grown in BHI medium at 37°C for 16 hrs with agitation at 200 rpm. Bacterial cells were collected by centrifugation at 960 xg for 10 min, washed twice with 10 ml zebrafish water, and suspended in 1 ml of the same fish water to give 5.32×10^9 CFU/ml. The zebrafish's water decreased the CFU/ml of the *E. faecium* E1162 from 10^6 to 10^5 in the course of 5 days (this study, data not shown). Dilutions of the bacterial suspensions were made in zebrafish water to infect the zebrafish larvae with

different doses (1×10^7 , 5×10^7 , 1×10^8 , 2.5×10^8 , 5×10^8 , 7.5×10^8 CFU/ml), zebrafish control groups were treated with PBS (without infection). The numbers of surviving larvae were recorded each 24 hrs following infection (the absence of a heartbeat was used to quantify the number of dead embryos). The experiment was repeated 3 separate times, and the percentage survival of larvae inoculated with different strains was analyzed using Kaplan-Meier curves. The log-rank test was used to analyze different Kaplan-Meier curves.

3.2.6 Planarian infection model

D. japonica strain G1 was maintained in darkness at 22°C in Deeside water and they were fed organic ox liver from Coombe Farm Organic UK (once every 2 weeks). To increase the rate of replication, planarians were cut at the head and tail and kept for two weeks. Infections were restricted to planarians that had starved for 7 days (Peiris et al., 2016). As starvation adaptations follow conserved signaling, gene regulation, and metabolic mechanisms (Baugh and Hu., 2020).

3.2.7 Planarian infection with *E. faecium* strains using the feeding method

Planarians (30 planarians per test group) were washed at least 3 times using filter-sterilized Deeside water, then placed in petri-dish in 15 ml of the same water and bacteria mixed with planarian food (100 µl) was added. Planarian food was made by adding ox liver paste (300 µl), 150 µl low gelling temperature agarose, 7 µl of red colouring, a bacterial pellet, mixed and left in eppendorf tubes on ice till solidified. Bacterial pellets were prepared to the needed OD600 nm which was 3×10^8 CFU/ml and 6×10^9 CFU/ml. The control group were fed food without bacteria. Planarians were left with the food for 2 hrs at 22°C before washing several times with filter-sterilized Deeside water, then transferred to a

new petri-dishes at 22°C. Experiments continued up to 96 hrs. At least 3 independent replicates of each experiment were made (Newmark et al., 2003).

3.2.7.1 Determination of the colony forming units within planarians following feeding

Immediately after feeding and washing as stated above (3.2.6), bacteria were recovered and counted at 2 hrs post-infection (day0) by collecting 5 planarians and putting them in a tube contained 25 µl PBS. Then planarians were homogenized using a tissue grinder, serial 10-fold dilution was plated onto BHI plates. The plates were incubated overnight at 37°C and CFUs/5 planarians were determined. The remaining infected planarians were kept at 22°C and CFUs/5 planarians at day 1, 2, and 4 were quantified.

3.2.8 Planarian infection with *E. faecium* strains by co-incubation

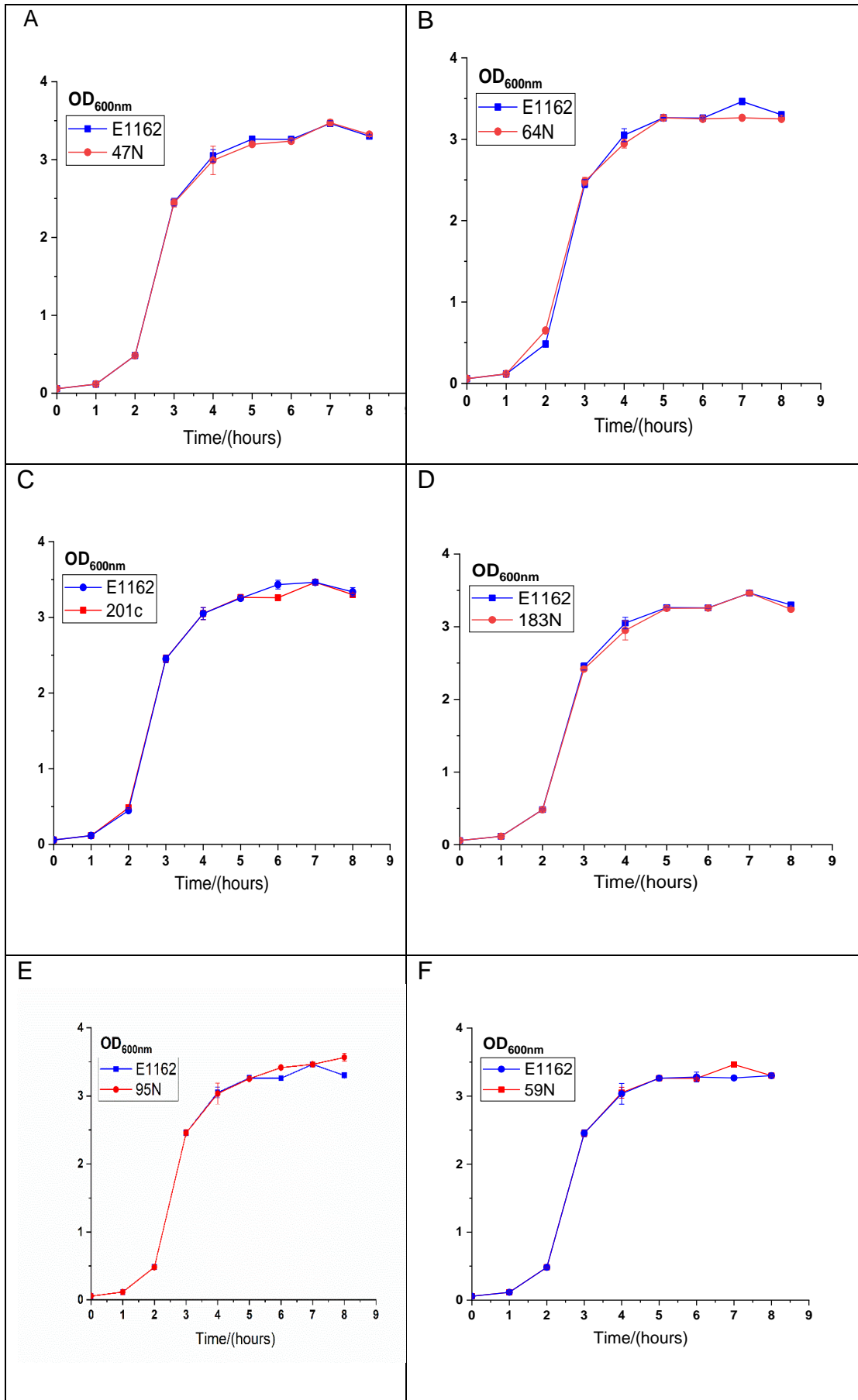
Planarians were dispensed into petri-dish plates containing 15 ml of Deeside mineral water (10 planarians per plate), *E. faecium* strains were grown in BHI medium at 37°C for 16 hrs with agitation at 200 rpm. Bacterial cells were collected by centrifugation at 960 xg for 10 min, washed twice with 10 ml Deeside mineral water, and suspended in 1 ml of the same water. Dilutions of the bacterial suspensions were made in Deeside mineral water to infect the planaria with different doses (1×10^9 , 3×10^9 , 6×10^9 , and 1×10^{10} CFU/15ml) (Arnold et al., 2016), the planarians control groups were treated with PBS (without co-incubation). The numbers of surviving planaria were recorded each 24 hrs following infection. The experiment was repeated 3 separate times, and the percentage survival of planaria inoculated with different strains were

analyzed using Kaplan-Meier curves. The log-rank test was used to analyze different Kaplan-Meier curves.

3.3 Results

3.3.1 Comparison of growth curves of the wild-type *E. faecium* E1162 and its mutants

The growth curves of the nisin-sensitive mutants: 64N with a transposon inserted in *efmE1162_0147* annotated in the *E. faecium* E1162 genome as encoding a putative conserved hypothetical protein of unknown function; 95N with an insertion in *efmE1162_1389* annotated as encoding a putative transposase; 183N with an insertion in *efmE1162_0514* annotated as encoding a putative CBS domain protein; 59N with an insertion in *efmE1162_1543* annotated as encoding a putative muramidase; 47N with an insertion in *efmE1162_1492* annotated as encoding a tyrosine decarboxylase, the *E. faecium* E1162 (wild-type), an isogenic *stk1* mutant with a chloramphenicol-resistance cassette inserted in the *stk1*, E1162 Δ *esp* and mutant Mabc which had a deletion in a gene encoding a putative permease component of an ABC transporter (EFF34617) were compared in BHI. There were no major differences between the wild-type and the mutants during growth in BHI medium (Figure 3-2). Each assay was run in triplicate with an average of three independent experiments.



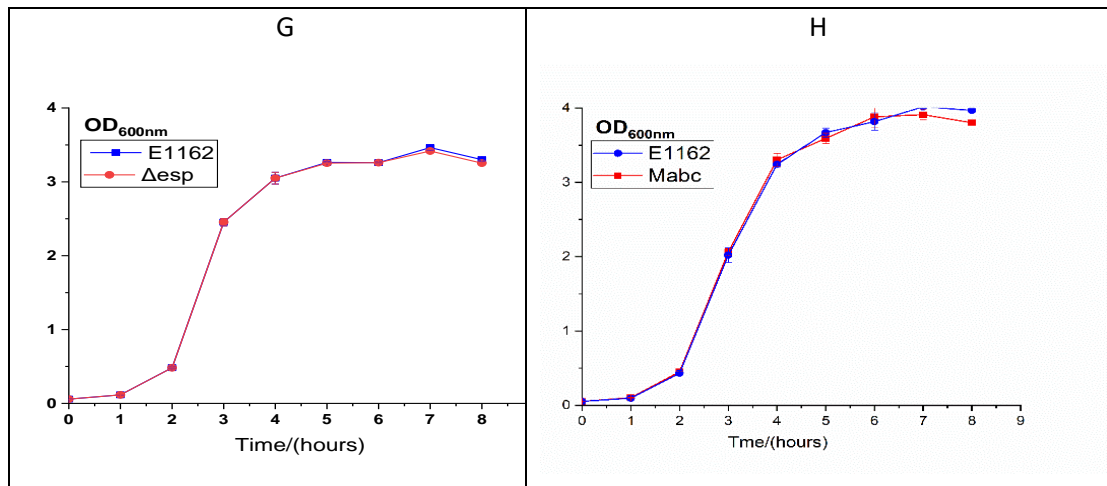


Figure 3-2 Growth curves of wild-type *E. faecium* E1162 and isogenic mutants obtained during growth in BHI medium.

A. mutant 47N which had an insertion in a gene coding for a putative tyrosine decarboxylase, **B.** mutant 64N which had an insertion in a gene encoding for a putative conserved hypothetical protein of unknown function, **C.** *stk1* mutant 201c (deletion mutant of a serine threonine protein kinase encoding gene), **D.** mutant 183N which had an insertion in a gene coding for CBS domain protein (a putative cystathionine β-synthase), **E.** mutant 95N with an insertion in *efmE1162_1389* annotated as encoding a putative transposase, **F.** mutant 59N which had an insertion in a gene coding for a putative muramidase, **G.** E1162 Δesp (enterococcal surface protein gene), **H.** mutant Mabc which had a deletion in a gene encoding a putative permease component of an ABC transporter (EFF34617). Data represent mean of three biological replicates performed in triplicates with error bars showing the standard deviations.

3.3.2 Development of a zebrafish infection model for *E. faecium* E1162

Until 5 dpf, pre-feeding of zebrafish larvae is exempt from UK Home Office laws that applies to adult zebrafish and mammalian models. In this study, zebrafish larvae were infected at 3 dpf when the embryos were developing into larvae (zebrafish embryos were used to hatch 72 hours post fertilization (3 dpf), which means 1 dpi. If the experiments go beyond 3 dpi (5 dpf), a UK Home Office license is required, so the larval death monitoring in this study was halted at 3 dpi. A logarithmic growth curve for E1162 was shown (Figure 3-3), along with various OD600 nm_s and their corresponding CFUs/ml. There were not any infected zebrafish larvae killed by the tested different doses (1 x 10⁷, 5 x 10⁷, 1 x 10⁸, 2.5 x 10⁸ CFU/ml) at 3 dpi, only 2.5 x 10⁸ CFU/ml data were reported in

this study (Figure 3-4), in contrast, 5×10^8 CFU/ml killed 15/30 (50%) of the infected larvae at 3 dpi, whereas wild-type E1162 at dose 7.5×10^8 CFU/ml killed 19/30 (63.33%) (Figure 3-4). *E. faecium* E1162 doses 5×10^8 and 7.5×10^8 CFU/ml were more effective in killing the infected larvae compared to the lower dose 2.5×10^8 CFU/ml. Zebrafish larvae exposed to varying concentrations of *E. faecium* E1162 CFU/ml were found to be killed regardless of dose.

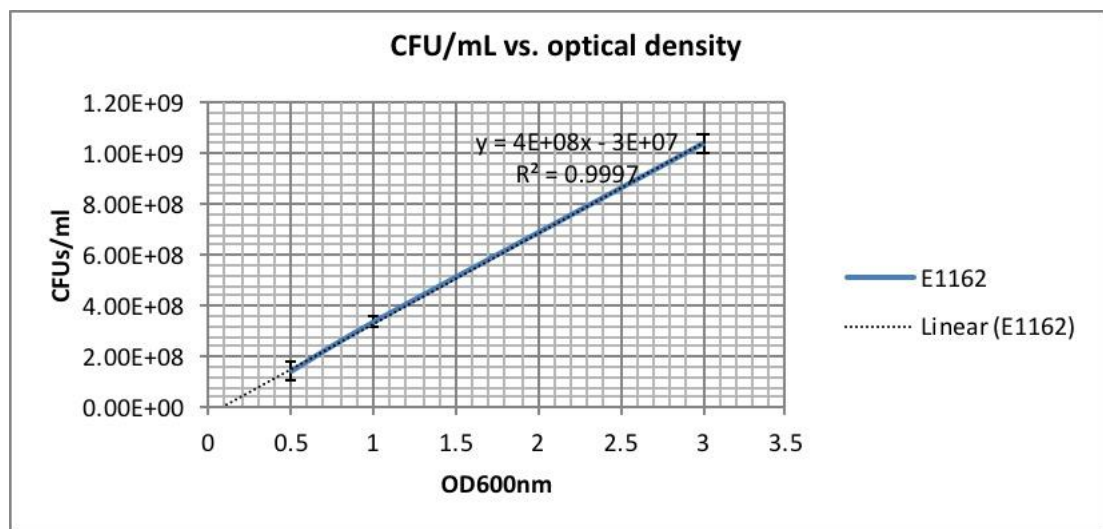


Figure 3-3 A graph showing the wild-type E1162's concentration (CFU/ml) in relation to the optical density measurements

The data have been fitted with a line using Excel's trendline tool; the equation for the line and coefficient of determination R2 values are displayed on the graph.

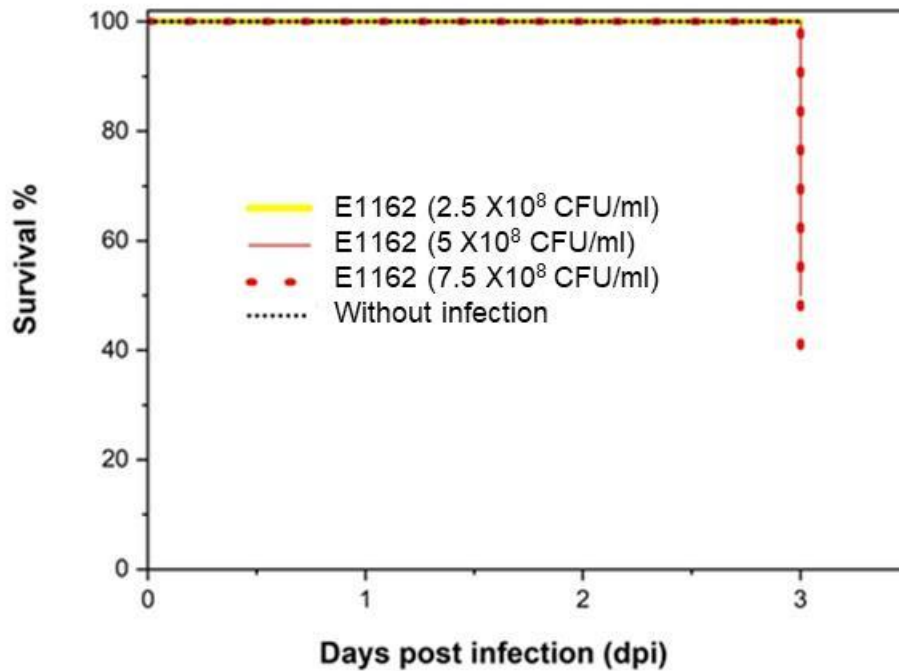


Figure 3-4 Survival of zebrafish larvae following infection with the wild-type *E. faecium* E1162 at doses 2.5×10^8 , 5×10^8 , and 7.5×10^8 CFU/ml.

Experiments were done in triplicate. For each test group 30 larvae were tested in groups of 10 which was carried out at three biological replicates performed in triplicate.

3.3.3 Comparison of the virulence of the wild-type *E. faecium* E1162 and its isogenic mutants in zebrafish larvae model

Mutant 47N showed the same killing effect as E1162 in the infected zebrafish larvae (this study, data not shown). Figures 3-5A and 3-5B show the survival of zebrafish infected with the mutant Mabc which had a deletion in a gene encoding a putative permease component of an ABC transporter (EFF34617). Infection of zebrafish larvae with 5×10^8 CFU/ml wild-type *E. faecium* E1162 killed 14/30 (46.66%) of the larvae at 3 dpi (Figure 3-5A), while infection of larvae with the same dose of mutant Mabc did not kill any of the infected larvae (Figure 3-5A). After 2 dpi, at 7.5×10^8 CFU/ml *E. faecium* E1162 did not kill any infected zebrafish larvae, whereas the Mabc mutant was more pathogenic than E1162 and killed 1/30 (3.3%) on the same day (2 dpi), though Mabc did not increase the killing at 3 dpi, whereas E1162 killed 17/30 (56.6%) larvae (Figure 3-5B).

These results showed that killing of zebrafish larvae by Mabc mutant was significantly decreased ($p < 0.0001$) at 3 dpi relative to the wild-type E1162.

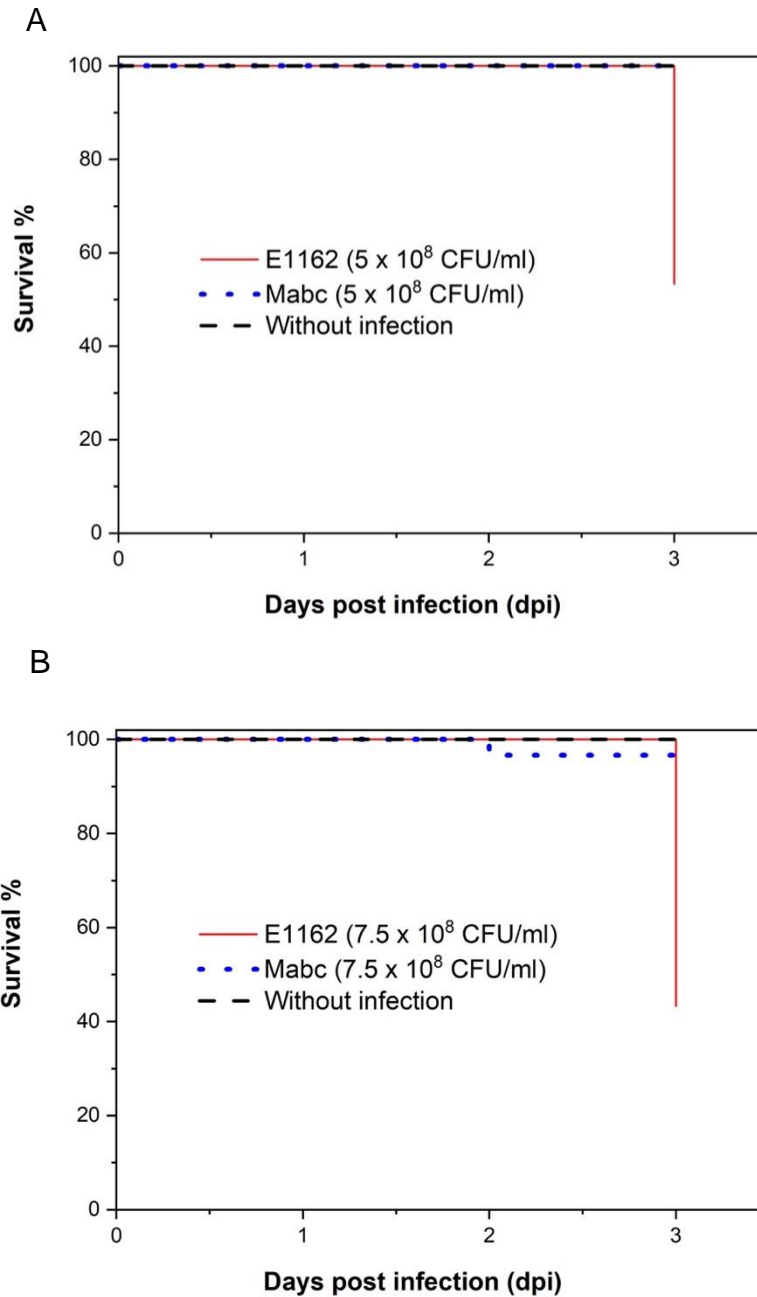
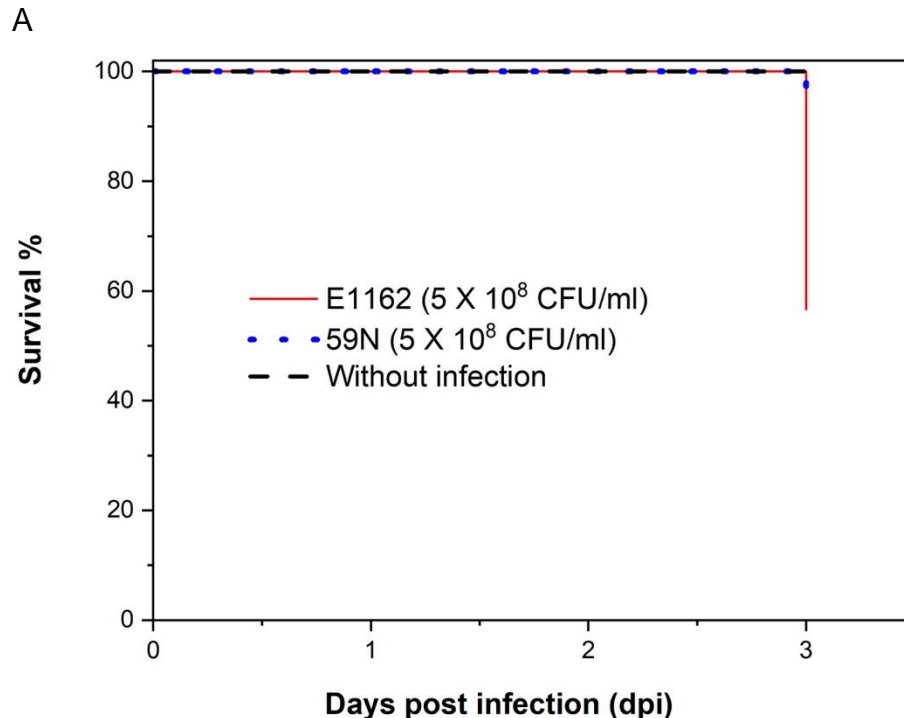


Figure 3-5 Survival of zebrafish larvae following infection with the wild-type *E. faecium* E1162 and the Mabc mutant.

Bacteria adjusted to **(A)** 5×10^8 CFU/ml and **(B)** 7.5×10^8 CFU/ml. Killing of zebrafish larvae by Mabc mutant was significantly decreased ($p < 0.0001$) at 3 dpi relative to the wild-type. Significance testing was performed by the log-rank test. For each test group 30 larvae were tested in groups of 10 on three biological replicates.

Figures 3-6A and 3-6B show the survival of zebrafish larvae infected with the mutant 59N, which had a transposon insertion in *efmE1162_1543* annotated in the *E. faecium* E1162 genome as encoding a putative muramidase (EFF33496). Mutant 59N at either 5×10^8 CFU/ml or 7.5×10^8 CFU/ml was significantly impaired in its ability to kill zebrafish larvae ($p < 0.0001$) compared to the wild-type *E. faecium* E1162. The two different doses (5×10^8 CFU/ml or 7.5×10^8 CFU/ml) showed that *E. faecium* E1162 killed 13/30 (43.3%) and 17/30 (56.6%) of the infected larvae respectively compared to 59N that killed 1/30 (3.33%) at 5×10^8 CFU/ml but interestingly did not kill any infected larvae at 7.5×10^8 CFU/ml (Figure 3-6B). These results demonstrate that the gene coding for the putative muramidase contributes to *E. faecium* virulence in zebrafish larvae infection model.



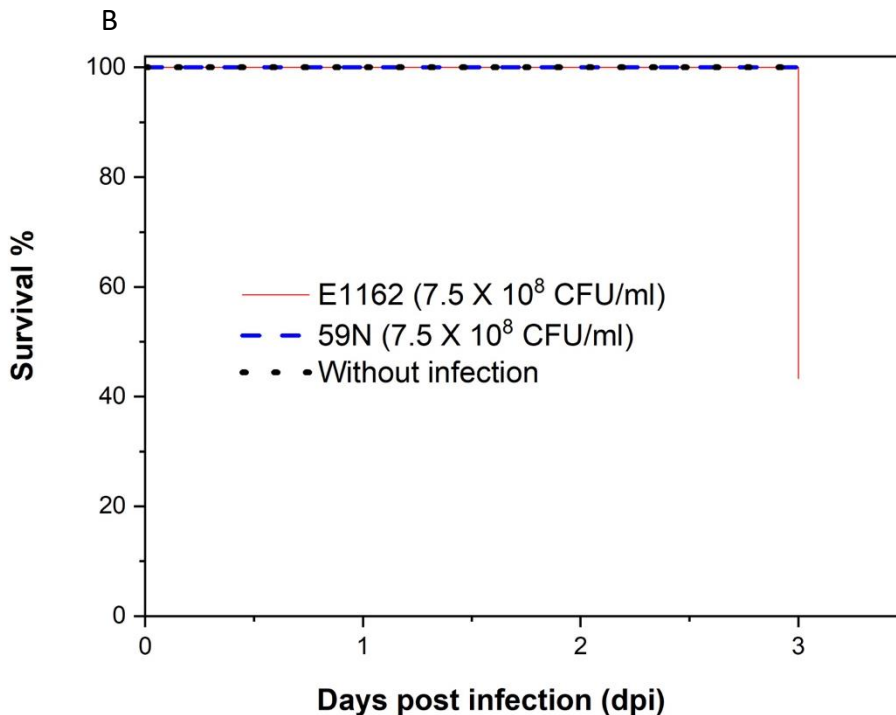


Figure 3-6 Survival of zebrafish larvae following infection with the wild-type *E. faecium* E1162 and mutant 59N.

Bacteria adjusted to **(A)** 5×10^8 CFU/ml and **(B)** 7.5×10^8 CFU/ml. Zebrafish larvae killing by 59N was significantly decreased ($p < 0.0001$) relative to the wild-type. Significance testing was done by the log-rank test. Data are the percentages of 30 larvae which were tested in groups of 10 on three biological replicates.

The killing of zebrafish larvae infected with either 5×10^8 CFU/ml or 7.5×10^8 CFU/ml of the mutant 183N, which had a transposon inserted in *efmE1162_0514* encoding a putative CBS domain protein, was significantly attenuated when compared with the same dose of the wild-type *E. faecium* E1162 (Figure 3-7). At 5×10^8 CFU/ml bacteria, the wild-type *E. faecium* E1162 killed 12/30 (40%) of the infected larvae at 3 dpi, while Infection of larvae with the same dose of mutant 183N did not kill any of the infected larvae ($p < 0.0001$) (Figure 3-7A). At 7.5×10^8 CFU/ml bacteria, the wild-type killed 17/30 (56.6%) of the infected larvae at 3 dpi, while Infection of larvae with the same dose of mutant 183N killed 6/30 (20%) at 3 dpi ($p < 0.003$) (Figure 3-7B). These results

showed that the gene encoding a putative CBS domain protein contributes to *E. faecium* virulence in the zebrafish larvae infection model.

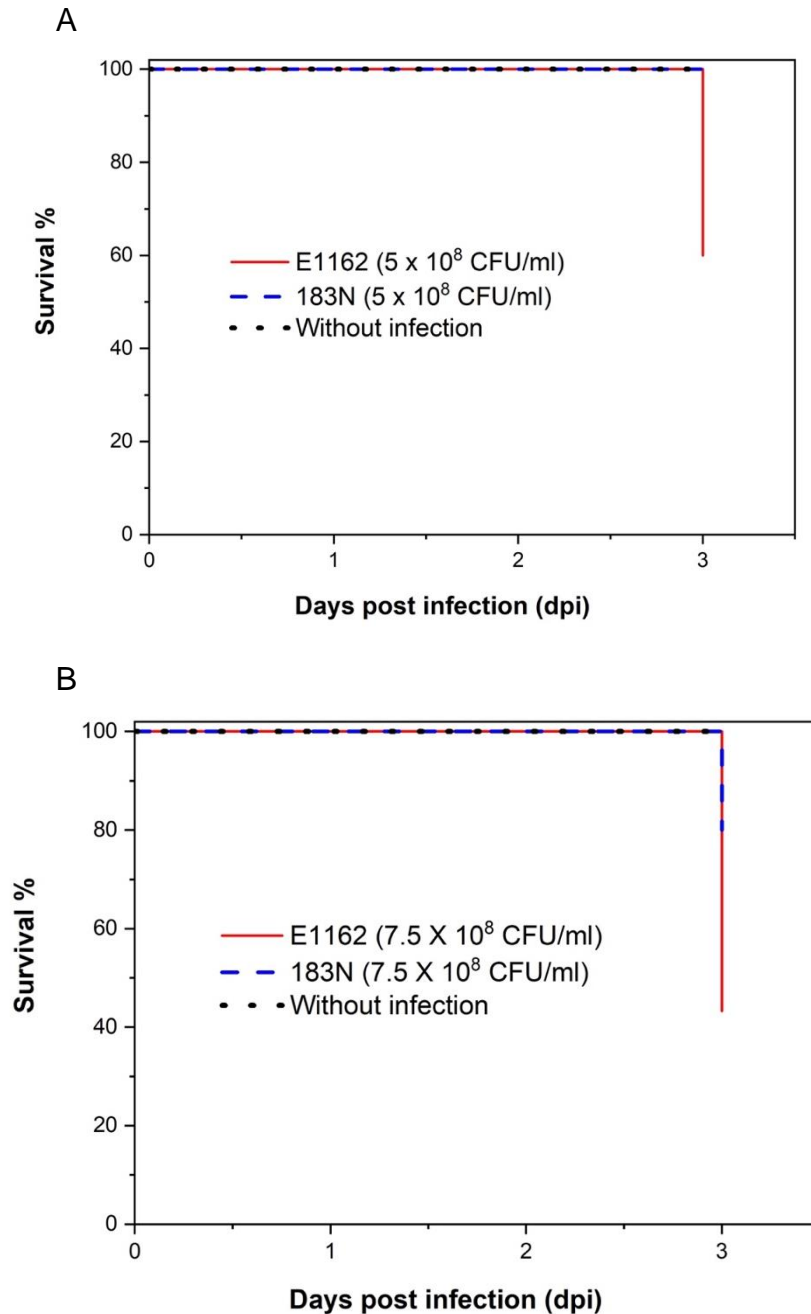
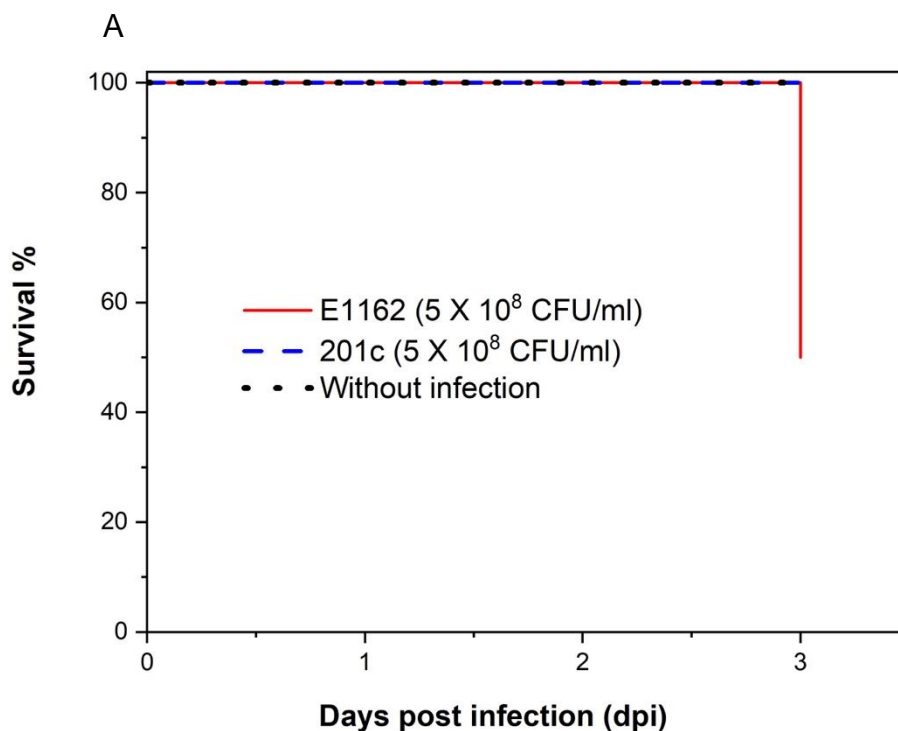


Figure 3-7 Survival of zebrafish larvae following infection with the wild-type *E. faecium* E1162 and mutant 183N.

Bacteria adjusted to **(A)** 5×10^8 CFU/ml and **(B)** 7.5×10^8 CFU/ml. This mutant did not kill zebrafish larvae at doses of 5×10^8 CFU/ml whereas it showed a reduction in killing capacity at 3 dpi at 7.5×10^8 CFU/ml ($p < 0.003$) when compared to the wild-type *E. faecium* E1162. Data are the percentages of 30 larvae which were tested in groups of 10 on three biological replicates.

The killing of zebrafish larvae was significantly attenuated in the *stk1* mutant, 201c, ($p < 0.0001$) compared to wild-type *E. faecium* E1162 as determined by the log-rank test (Figure 3-8A and 3-8B). The wild-type *E. faecium* E1162 at 5×10^8 CFU/ml killed 50% of the infected larvae at 3 dpi, while larvae infected with the same dose of *stk1* mutant, 201c, showed no larvae killing at 3 dpi (Figure 3-8A). By infecting the larvae with 7.5×10^8 CFU bacteria, 60% infected with the wild-type *E. faecium* E1162 were killed at 3 dpi (Figure 3-8B), while infection of the larvae with the same dose of the *stk1* mutant, 201c, did not kill the larvae (Figure 3-8B). These results showed that the gene encoding a serine-threonine kinase contributes to *E. faecium* virulence in the zebrafish larvae infection model.



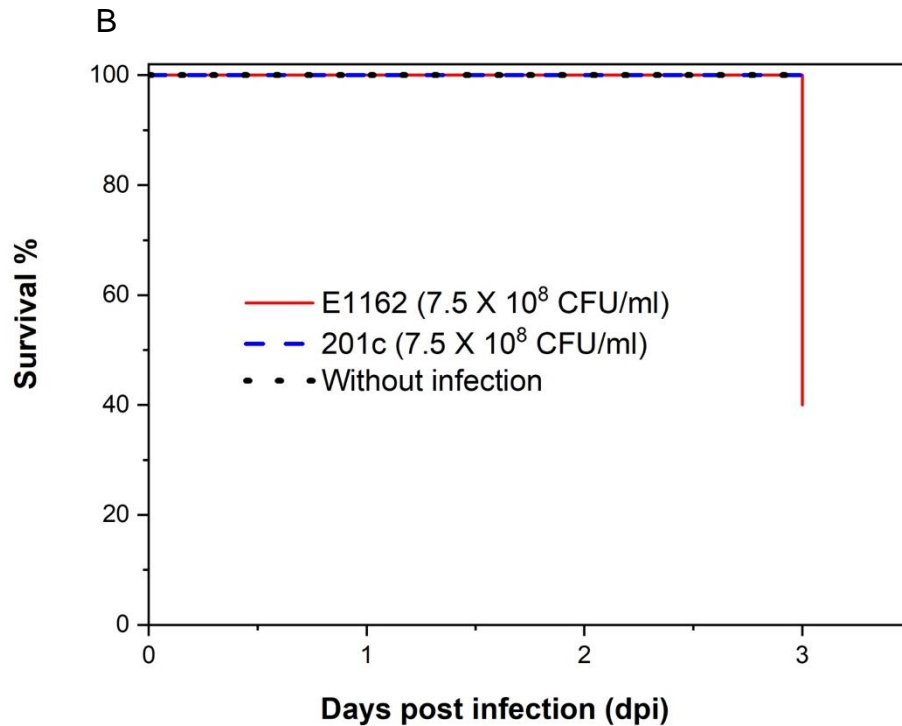


Figure 3-8 Survival of zebrafish larvae following infection with the wild-type *E. faecium* E1162 and the *stk1* mutant, 201c.

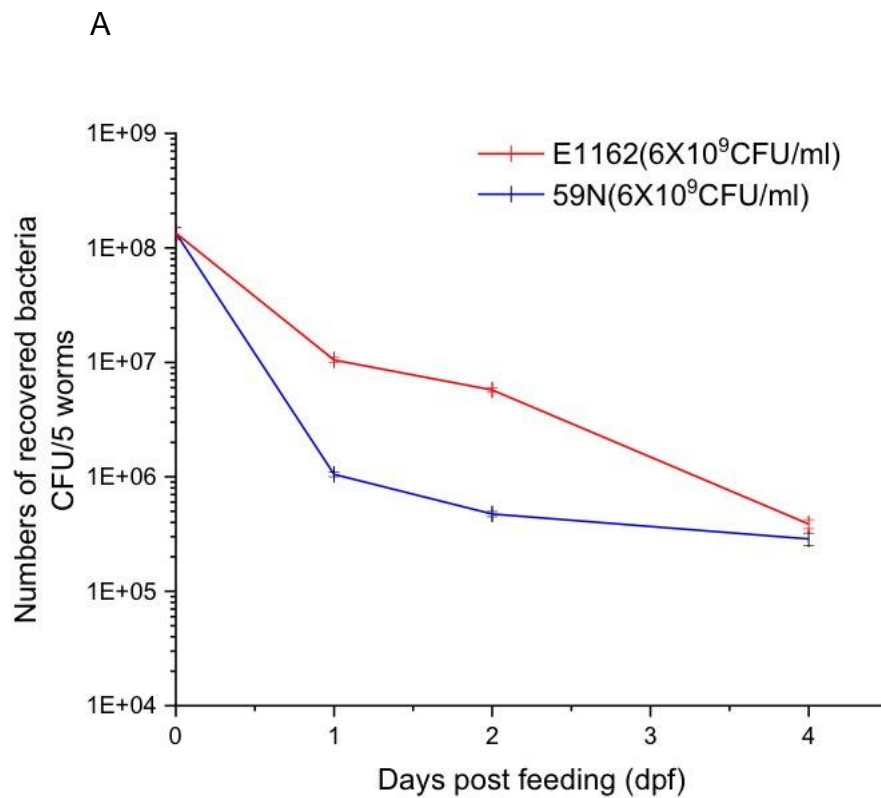
Bacteria adjusted to **(A)** 5×10^8 CFU/ml and **(B)** 7.5×10^8 CFU/ml. This mutant was significantly attenuated in killing zebrafish larvae ($p < 0.0001$) compared to the wild-type *E. faecium* E1162. Data are the percentages of 30 larvae which were tested in groups of 10 on three biological replicates.

3.3.4 Virulence and survival of *E. faecium* E1162 and isogenic mutants in a planarian infection model

3.3.4.1 Virulence and survival of *E. faecium* E1162 and isogenic mutant 59N in a planarian infection model

The capacity of the mutant 59N to colonise and survive *in vivo* was studied using a planarian infection model. Planarians were infected with either *E. faecium* E1162 or mutant 59N at doses of 3×10^8 CFU/ml and 6×10^9 CFU/ml by feeding planarians bacteria mixed with fresh organic ox liver paste, the planarian control group was run with no *E. faecium* added. On the day of feeding the bacterial load in the fed planarians was similar for the wild-type *E. faecium* E1162 and isogenic mutant 59N, being on average 1×10^7 CFUs/5 worms and 1×10^8 CFUs/5 worms when planarians were fed with 3×10^8 CFU/ml and 6×10^9

CFU/ml, respectively (Figure 3-9A and 3-9B). In Figure 3-9A, the number of CFU/ml recovered from planarians fed with 6×10^9 CFU/ml of 59N at 1 and 2 dpf was significantly less ($p < 0.05$) using the Mann Whitney statistical test compared to those recovered from planarians fed with the same number of *E. faecium* E1162 at 1 and 2 dpf, though the overall killing rate at 4 dpf was the same. However, in Figure 3-9B, there were no significant differences between CFUs recovered from planarians fed with 3×10^8 CFU/ml of *E. faecium* E1162 or isogenic mutant 59N.



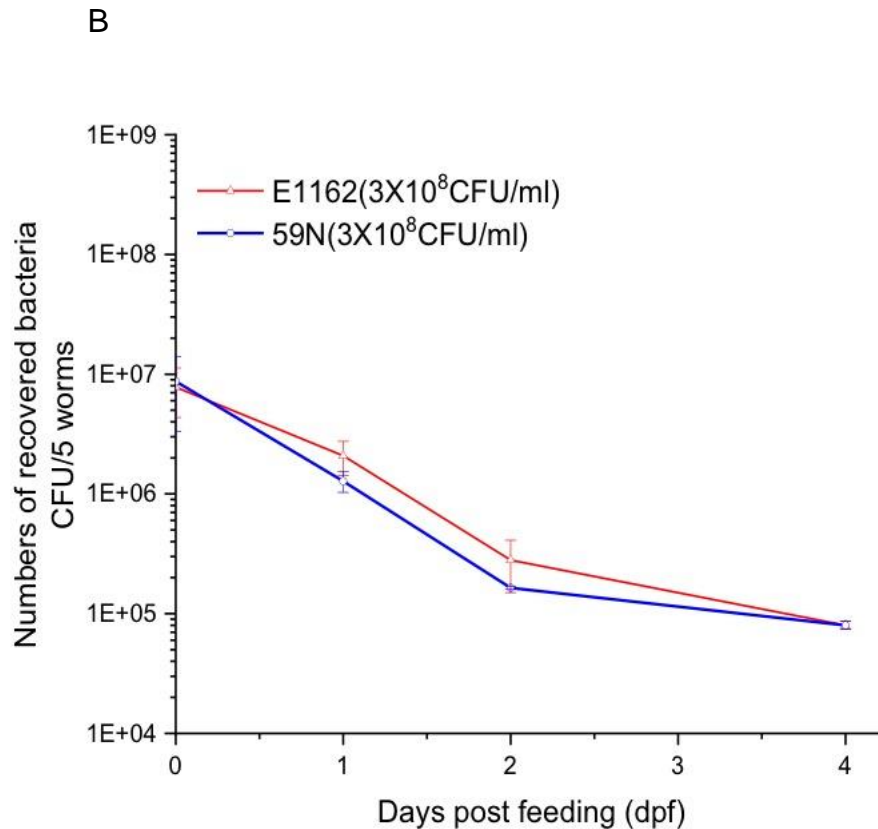


Figure 3-9 Comparison of clearance of the wild-type *E. faecium* E1162 and mutant 59N from planarians.

Planarians were fed with *E. faecium* E1162 or mutant 59N which contained a transposon insertion in a gene coding for a putative muramidase (EFF33496). **(A)** Bacteria adjusted to 6×10^9 CFU/ml and **(B)** 3×10^8 CFU/ml, the CFUs/5 worms in each group were measured over 4 days. The values represent the mean of three biological replicates performed in duplicate. Significance testing was performed using the Mann Whitney statistical test.

The planarian model was also evaluated to see if it could predict the virulence of *E. faecium* E1162 and the mutants 59N, which harboured a transposon within *efmE1162_1543* annotated as coding for a putative muramidase, 47N which had a transposon inserted in *efmE1162_1492* annotated as coding for tyrosine decarboxylase and E1162 Δ *esp*, *Esp* it is the first documented determinant in *E. faecium* CC17 that plays a significant role in biofilm formation, which is a critical factor in infection pathogenesis (Heikens et al., 2007; Cui et al., 2020).

which had a disruption in the enterococcal surface protein gene. To determine the optimal CFU of *E. faecium* E1162 to use in the experiments, planarians were co-incubated with different numbers of CFU/15 ml (1×10^9 , 3×10^9 , 6×10^9 , and 1×10^{10}) and their survival was monitored and recorded over time (Figure 3-10). Planarians co-incubated with 1×10^9 and 3×10^9 CFU/15 ml wild-type *E. faecium* E1162 displayed slower killing when compared with planarians co-incubated with the higher doses of 6×10^9 and 1×10^{10} CFU/15 ml. The percent survival plot of the planarians co-incubated with 1×10^{10} CFU/15 ml of E1162 showed that 28/30 (93.33%) and 30/30 (100%) of the planarians were killed at day 2 and day 3 post co-incubation respectively. By co-incubating planarians with 6×10^9 CFU/15 ml of E1162, 8/30 (26.6%) and 30/30 (100%) of the tested planarians were killed at 2 and 3 days post co-incubation respectively. At day 7 post co-incubation, all planarians co-incubated with 3×10^9 CFU/15 ml of E1162 were killed, while it took 11 days for E1162 at dose 1×10^9 CFU/15 ml to kill all the co-incubated planarians (Figure 3-10).

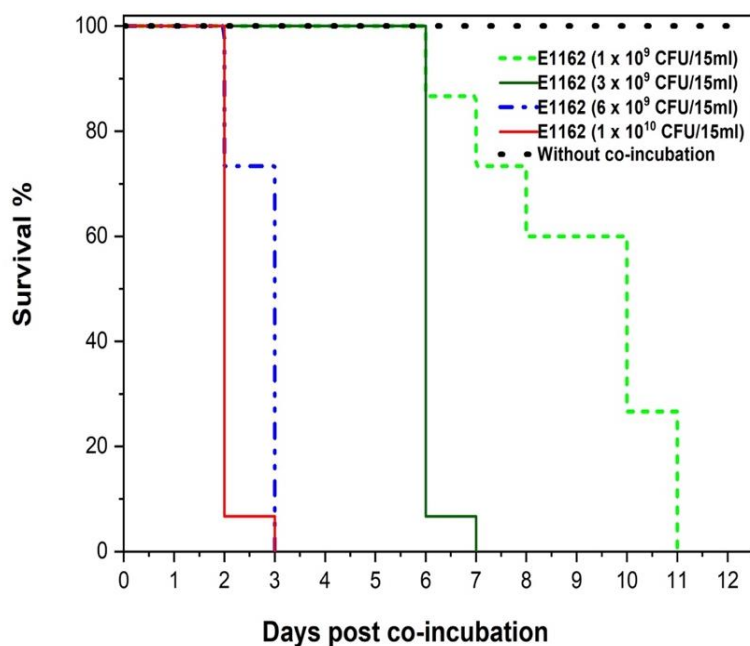


Figure 3-10 Planarians survival after co-incubation with different CFU/15 ml wild-type *E. faecium* E1162.

Planarians were co-incubated with *E. faecium* E1162 at doses 1×10^9 , 3×10^9 , 6×10^9 , and 1×10^{10} CFU/15 ml. For each test 30 planarians were examined in groups of 10 on three biological replicates performed in triplicate.

Planarians were co-incubated with different numbers of CFU/15 ml (1×10^9 , 3×10^9 , 6×10^9 , and 1×10^{10} CFU/15ml) to compare the survival and colonization of *E. faecium* E1162 parental strain and isogenic mutants 59N, 47N, and Δesp . Only in this study, the concentrations that showed delayed killing between the wild type and the mutants were shown. At 1×10^{10} CFU mutant 59N showed ($p < 0.001$) delayed killing of planarians compared to *E. faecium* E1162 (Figure 3-11). At 2 and 3 days post co-incubation, *E. faecium* E1162 killed 28/30 (93.33%) and 30/30 (100%) of the planarians, respectively, while planarians infected with the same dose of mutant 59N killed 6/30 (20%) at 2 days post co-incubation and 30/30 (100%) at 3 days post co-incubation (Figure 3-11).

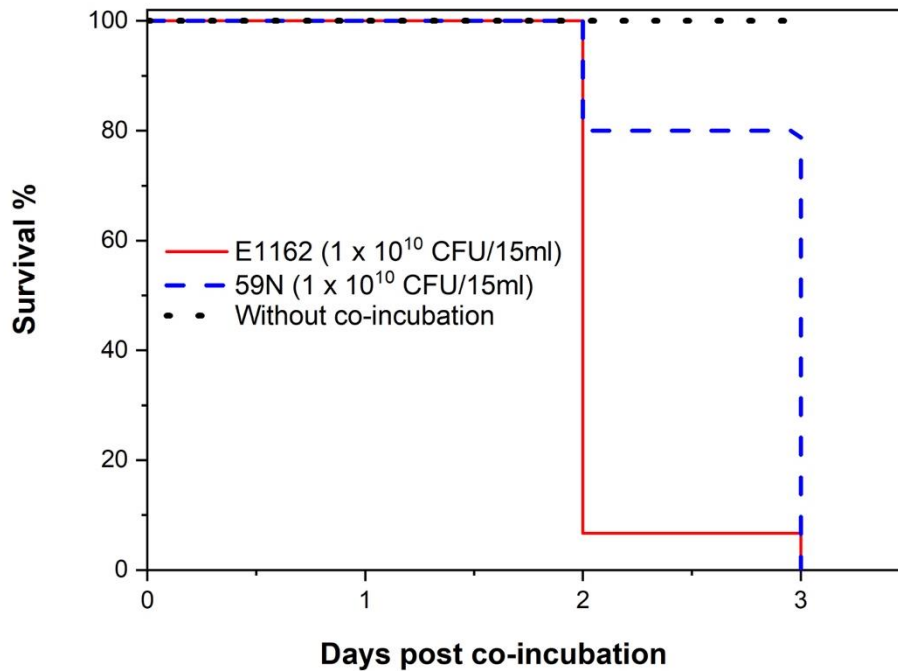


Figure 3-11 Planarians survival after co-incubation with 1×10^{10} CFU/15 ml of wild-type *E. faecium* E1162 and mutant 59N.

At 2 days post co-incubation, the mutant 59N delayed in killing planarians significantly ($p < 0.001$) than *E. faecium* E1162, while both the wild type E1162 and the mutant 59N killed 100% at day 3. For each test 30 planarians were examined in groups of 10 on three biological replicates performed in triplicate. Significance testing was performed by the log-rank test.

Planarians co-incubated with 6×10^9 CFU/15 ml of either mutant 59N or Δesp had significantly ($p < 0.001$) delayed killing action compared to the wild-type (E1162). The wild-type (E1162) killed 8/30 (26.6%) and 30/30 (100%) of the infected planarians at 2 and 3 days post co-incubation, respectively. Mutant 59N killed only 2/30 (6.6%) and 30/30 (100%) of the infected planarians at 3 and 4 days post-co-incubation, respectively. While Δesp killed 15/30 (50%) and 30/30 (100%) of the infected planarians at 3 and 4 days post-co-incubation, respectively (Figure 3-12).

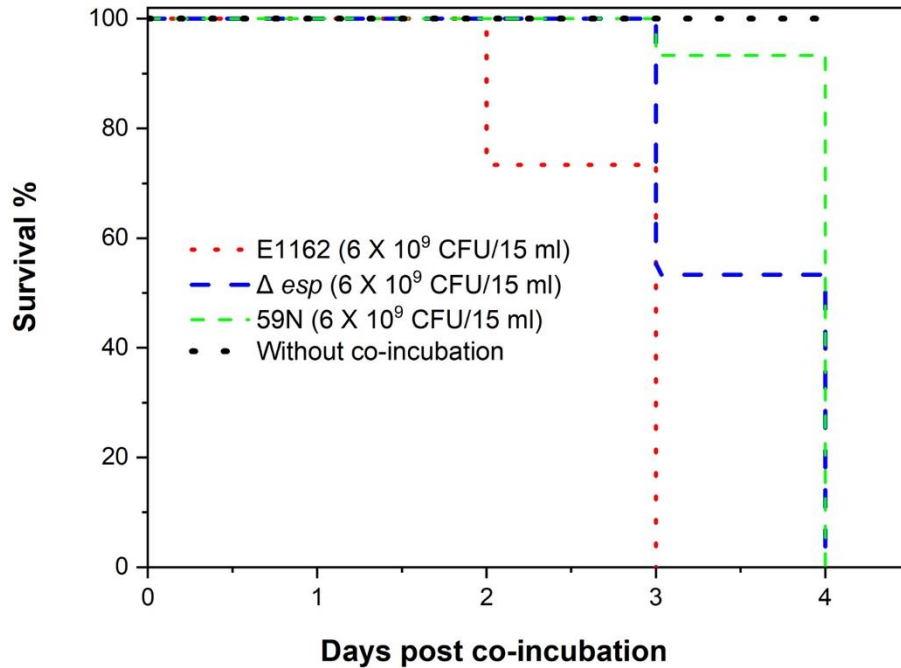


Figure 3-12 Planarians survival after co-incubation with 6 x 10⁹ CFU/15 ml wild-type E1162, mutant 59N, and E1162Δesp.

Co-incubating planarians with 6 x 10⁹ CFU/15 ml of either mutant 59N or E1162Δesp resulted in significantly less killing of planarians compared to wild-type *E. faecium* E1162. For each test 30 planarians were examined in groups of 10 on three biological replicates performed in triplicate. Significance testing was performed by the log-rank test.

Mutant 47N which had a transposon inserted in the *tdc* gene (tyrosine decarboxylase) was significantly attenuated ($p < 0.05$) for its ability to kill planarians compared to the wild-type *E. faecium* E1162, as 30/30 (100%) of planarians co-incubated with *E. faecium* E1162 at dose 1 x 10⁹ CFU/15 ml were killed after 13 days post-co-incubating, while it took 15 days for mutant 47N to kill all planarians, this reduced virulence indicates that the tyrosine decarboxylase is important for *E. faecium* E1162 virulence using this model (Figure 3-13).

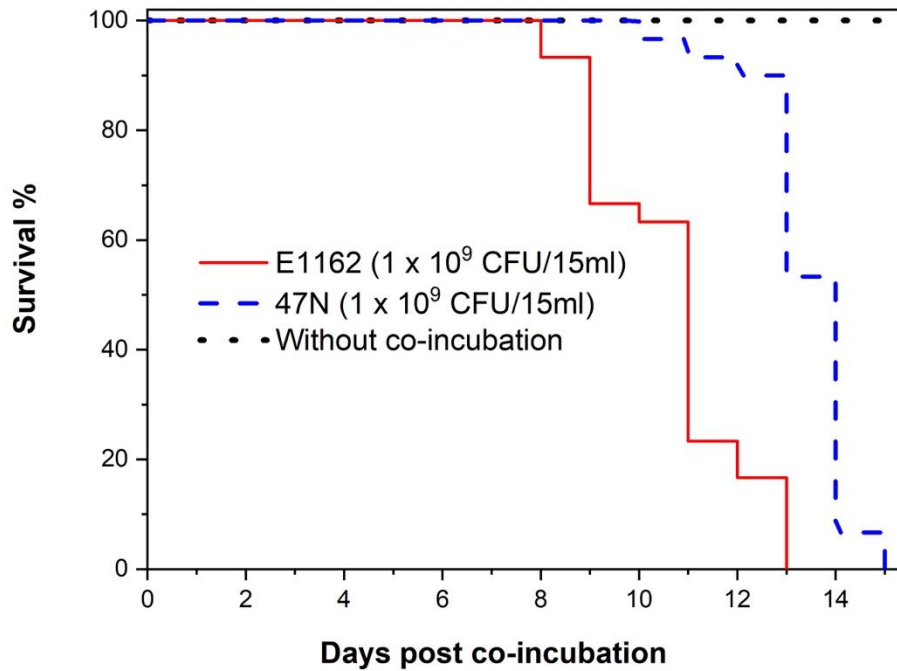


Figure 3-13 Planarians survival curves after co-incubation with 1 x 10⁹ CFU/15 ml of wild-type *E. faecium* E1162 and mutant 47N.

Mutant 47N was significantly ($p < 0.05$) weakened in its ability to kill planarians relative to parent strain *E. faecium* E1162. For each test 30 planarians were examined in groups of 10 on three biological replicates performed in triplicate. Significance testing performed by the log-rank test.

3.4 Discussion

The purpose of the research described in this chapter was to assess the use of zebrafish larvae and planarians infections models to investigate the pathogenicity of *E. faecium* E1162 via static immersion rather than time-consuming microinjection method. The static immersion method mimics the natural conditions under which bacteria cause infectious diseases in animals, assesses the precise contribution of the bacterial virulence factor to *E. faecium* pathogenesis, does not require specialized knowledge, and is not time-consuming, in contrast to Zhang et al. (2017), who published their findings on the pathogenicity of multiple *E. faecium* strains in zebrafish larvae via microinjection into the circulation of dechorionated zebrafish embryos. The transparent zebrafish larvae have proven themselves as an excellent model to

study many infectious diseases caused by human pathogens (Sullivan and Kim, 2008; Tobin et al., 2010; Volkman et al., 2010; Meijer and Spaik, 2011; van der Vaart et al., 2012). The larvae have phagocytic cells against invading microbes as early as one day following fertilization and can mount an innate immune response comparable to the mammalian innate immune system (Lam et al., 2004; van der Vaart et al., 2012).

In the present work, the growth curves of *E. faecium* E1162 wild-type parental strain and the isogenic mutant strains in BHI medium were compared to confirm that the mutations in the *E. faecium* E1162 isogenic mutant strains did not affect gene(s) essential for metabolism. *In vitro* growth curves in BHI medium confirmed the mutant strains lack of growth defects. This suggests that the mutant strains virulence defects in zebrafish larvae and *G. mellonella* infection models are not due to a general metabolic defect *in vivo*.

Zebrafish larvae were not killed with the *stk1* mutant 201c. This finding is in line with previous research, which found that mutant 201c was significantly slower to kill *G. mellonella* larvae, killed 18/30 (60%), of the infected larvae at 4 dpi compared to (30/30) 100% killing of larvae infected with the same dose of the wild-type at 3 dpi (Hashim, 2016). A UK Home Office license is required if zebrafish experiments go beyond 3 dpi (5 dpf), so the larval death monitoring in this study was halted at 3 dpi (5 dpf). Hashim (2016) on the other hand, continued work on monitoring *G. mellonella* death until 4 dpi because there are no ethical constraints for invertebrates such as insects (*G. mellonella*), so it did not require UK Home Office Licence. In the same study, Hashim found that mutant 201c is more sensitive to sodium deoxycholate, sodium cholate, bile salts, and antibiotics that target the PBPs. *E. faecalis* serine-threonine

eukaryote-like kinase (*ireK*) controls *E. faecalis* inherent antimicrobial resistance and intestinal persistence. In the absence of antimicrobial stress, an *E. faecalis* mutant lacking *prkC* grows at a wild-type rate but is more sensitive to cell-envelope-active compounds, such as antibiotics that target cell-wall biogenesis and bile detergents. The mutant was also impaired at persistence in the intestine of mice, which was consistent with its bile sensitivity. Ceftriaxone-resistance was restored in *E. faecium* *stk1* mutant 201c with plasmid pHFH10 expressing the *E. faecium* *stk1* gene and pHFH11 expressing the *E. faecalis* *ireK* gene. *E. faecalis* *ireK* mutants with pHFH10 and pHFH11 plasmids also showed restored ceftriaxone-resistance (Hashim, 2016). This research shows that *E. faecium* *stk1* gene can complement *ireK* mutant *E. faecalis* and that *ireK* can complement *stk1* mutant *E. faecalis*. This suggests that both strains of serine threonine protein kinase can produce the same signal or molecule, which is involved in cephalosporin-resistance. The *S. aureus* *stk1* mutant had a significantly lower survival rate in the kidneys of intravenously infected mice, this finding was confirmed in two different *S. aureus* strains, 8325-4 and SH1000, indicating that *stk1* is required for full virulence (Débarbouillé et al., 2009). These findings show that *stk1* is required for complete pathogenesis in *S. aureus*. In this study, there was no difference between the growth curves of mutant 201c and the wild-type parent strain *E. faecium* E1162 in BHI medium, however, growth curves using TSB without dextrose showed reduced growth of mutant 201c compared to parental wild-type strain (Hashim, 2016), suggesting that *stk1* gene might have a role in cell growth and/or division. This is consistent with what has been found in previous research where Labbe and Kristich (2017) found that IreK of *E. faecalis* play a role in cell growth and/or division.

The mutant 183N did not kill zebrafish larvae at doses of 5×10^8 CFU/ml, but it did show killing at 7.5×10^8 CFU/ml at the same time with E1162 (3 dpi) with a lower killing level ($p < 0.003$). On *G. mellonella* larvae, Hashim (2016) compared the killing effects of 5×10^8 CFU/ml wild-type *E. faecium* E1162 and mutant 183N. When compared to the wild-type, killing was significantly delayed ($p < 0.05$) with mutant 183N. These findings indicate that the gene encoding a putative CBS domain protein contributes to *E. faecium* virulence in both the zebrafish and *G. mellonella* models. This implies that *E. faecium* may use this gene to survive in zebrafish and *G. mellonella* larvae infection models. *Bifidobacterium breve* UCC2003 utilizes cystathione β -synthase (CBS) to defend against bile and to maintain bile tolerance (Ruiz et al., 2012). Thus, it is likely *E. faecium* utilizes this gene or other neighbouring genes that are essential to surviving in the host's gastrointestinal tract. Further, the ability of *Yersinia pestis* to form biofilm is regulated by the CBS domain within YrbH protein (a multifunctional protein required for biofilm formation). The CBS domain within the OpuA in *Lactobacillus lactis* is involved in sensing ionic strength, a protein with ability to sense environmental stress and/or the energy status of the cell and could possibly regulate DNA replication (Bryant et al., 2010). As such, *E. faecium* might use the similar mechanisms and utilise this gene or other neighbouring genes that might help it survive in zebrafish and *G. mellonella*'s gastrointestinal tract.

In this study, an *E. faecium* E1162 with a deletion of a putative permease component of an ABC transporter (Mabc) was found to be more pathogenic than the wild type because it killed 1/30 (3.3%) of infected zebrafish at 2 dpi, whereas E1162 shows no killing. However, Mabc showed a significant decrease in killing

zebrafish larvae after 3 dpi compared to the parent strain *E. faecium* E1162 (this study). The outcome was that the mutant Mabc strains was more able to multiply at the beginning of an infection allowing the mutant strains that can grow under stress conditions to rule until the environment changes. However, the wild-type strains E1162 performed better than Mabc after 3 dpi and became more pathogenic. It has previously been shown that the absence of this gene also impaired resistance to the antimicrobials nisin, bacitracin, colistin, and changed the net charge of the bacterial cell surface, however, when evaluated in *G. mellonella* infection model, the parental strain *E. faecium* E1162 and the killing rate of mutant Mabc did not differ significantly (Hashim, 2016). The difference in body mass between zebrafish larvae (24-72 hpf) and *G. mellonella* is big and likely account for the differences in mutant Mabc lethal dose between the two different developmental stages for the same/different model. Also, the same factor may not necessary play the same role in different animal models or in different niches within the same animal, therefore, there is no single comprehensive model system for all enterococcal infections (Goh et al., 2017).

Mutant 47N which had a transposon inserted in a gene putatively encoding a tyrosine decarboxylase was significantly attenuated in killing planarians compared to the *E. faecium* E1162. It has previously been shown that mutant 47N was significantly attenuated in its capacity to kill *G. mellonella* larvae compared to the *E. faecium* E1162 ($p < 0.01$) (Hashim, 2016). Tyrosine is decarboxylated to tyramine by tyrosine decarboxylase (TdcA), Tyramine has a positive charge and is secreted by the tyrosine-tyramine antiporter at the same time as further tyrosine is taken in. The amino acid/amine antiport system, accompanied by decarboxylation functions to generate proton motive force.

Amino acid decarboxylase systems have been described as the major cellular mechanisms allowing extreme acid adaptation. Perez et al. (2015) constructed a non-tyramine-producing *E. faecalis* mutant to study the role of the tyramine biosynthesis pathway. Wild-type strain exhibited higher survival in a system that mimics gastrointestinal stress, signifying that the tyramine biosynthetic pathway has a role in acid tolerance.

Interestingly, mutant 59N, a nisin-sensitive mutant with a transposon inserted in a gene putatively encoding a muramidase, failed to kill zebrafish larvae at 7.5×10^8 CFU/ml but killed 1/30 (3.33%) at 5×10^8 CFU/ml. The reason behind why the lower dose of 59N (5×10^8 CFU/ml) was more infectious than its higher dose (7.5×10^8 CFU/ml) might be attributed to the various biochemical mechanisms that pathogens employ to infect hosts which could account for the variation in infective dose, numerous molecules that are secreted by pathogens make it easier to suppress and/or evade host immune responses, which supports the development of bacterial. Infections can be started from a small number of pathogenic cells if these molecules act locally, close to the pathogenic cell, in which case only small numbers of molecules may be needed for successful growth while large numbers of molecules might be needed to avoid the host immune system, however, if the pathogenic molecules diffuse and therefore act remotely so in these situations, a higher density of pathogenic cells may be required to start an infection (Schmid-Hempel and Frank, 2007; Schmid-Hempel, 2011; Leggett et al., 2012). Hashim (2016) found that a dose of 5×10^8 CFU/ml of 59N delayed killing in *G. mellonella* larvae ($p < 0.01$). It also showed delayed killing in planarian infection models (this study, Chapter III) and the CFU/ml recovered from planarians fed with 6×10^9 CFU/ml mutant

59N differed significantly ($p = 0.05$) in the first 3 dpf from CFU/ml recovered from planarians fed with the same dose of *E. faecium* E1162. The result was that mutant 59N strains are less able to multiply at the start of an infection, allowing clonal bacteria populations (E1162) to rule until the environment changes, allowing mutant strains that can grow under stress to grow, however, wild-type strains perform better during the first step of an infection than mutant strains, but not during phase two (Faucher et al., 2021). Therefore, if the wild-type strains initiate the infection, they should grow faster during the early phases of infection than 59N mutants. These results indicated that the mutation in 59N impaired the bacteria's ability to multiply during an early stage of infection. This suggests that the putative muramidase might have a role in colonisation in these models. As per Paiva et al. (2011) β -N-acetylmuramidases cleave the β -(1 \rightarrow 4) glycoside bond between N-acetylmuramic acid and N-acetylglucosamine, given this is a putative muramidase it may participate in remodeling the cell wall, and that the cell-wall remodeling could be a stress resistance factor relevant to successful colonisation. It is notable that some bacterial cell-wall proteins are essential in host-pathogen interactions, virulence, adhesion, and colonisation and a normal cell-wall acts as a docking station for such surface proteins. Thus, cell-wall remodeling is important in host-pathogen interactions, virulence, and antibiotics resistance (Khan et al., 2019).

The goal of the work described in this chapter was to assess the suitability of zebrafish and planarian infection model as a screening models for comparing the virulence and colonisation of the *E. faecium* E1162 and its isogenic mutant strains. The results revealed that *E. faecium* E1162 and its isogenic mutant strains did not kill zebrafish larvae in a dose-dependent manner because mutant

59N appeared to kill zebrafish larvae at 5×10^8 (Figure 3-6A) more than the higher dose at 7.5×10^8 (Figure 3-6B). The *stk1* (201c) mutant did not appear to kill zebrafish larvae at the doses 5×10^8 and 7.5×10^8 (Figure 3-8), while Mabc really struggles to kill zebrafish at dose 7.5×10^8 (Figure 3-5B). Based on the evidence, these genes appear to be critical for E1162 pathogenicity in zebrafish larvae model. The killing of zebrafish larvae by mutant Mabc, 59N, 183N, and *stk1* was significantly reduced at 7.5×10^8 CFU/ml when compared to the wild-type strain. CFUs/5 worms recovered after 1 and 2 days of infection by feeding with 6×10^9 CFU/ml mutant 59N were significantly lower than those recovered after infection with the wild-type *E. faecium* E1162. When planarians were co-incubated with mutant strains 59N, 47N, and Δesp , delayed killing action was shown compared to the wild-type E1162. This study shows that an alternative host, zebrafish larvae and planarians, can be applied to understand *E. faecium* infections. The *E. faecium* *tdc* and *mur* genes seem to play role in the survival of *E. faecium* E1162 within the planarian and colonisation in planarians.

Chapter IV

The identification of *E. faecium* genes which are involved in oxidative stress resistance

4.0 The identification of *E. faecium* genes which are involved in oxidative stress resistance

4.1 Introduction

The development of *E. faecium* from commensal to hospital-acquired pathogen has driven the need to develop new antibiotics and find how hospital-acquired *E. faecium* strains can persist in the gut of hospitalized patients and adapt to specific sites like the human urinary tract. To cause an effective infection, a pathogen must be able to adapt to a changing environment, this is achieved by changes in gene expression levels of regulators and effectors of the stress response (Yuen and Ausubel, 2014). The most notable character of *E. faecium* isolates is their ability to colonise both healthy people and patients, to tolerate host defenses and to expand across the hospital environment, generating outbreaks (Lebreton et al., 2012). *E. faecium* CC17 can infect or colonise human hosts indefinitely due to progressively acquired genetic elements that confer selective advantages. Plasmids and transposons are two types of genetic elements that typically carry acquired genes like antimicrobial resistance and virulence. In addition to antimicrobial resistance genes, colonisation and virulence genes are important for the adaptability and spread of *E. faecium* CC17 to hospital environments and/or patient niches. These colonisation and/or virulence genes primarily include the *fms* gene, which encode microbial surface components that recognize adhesive matrix molecules, the *esp_{Efm}* gene, which encode surface proteins responsible for biofilm formation, and the *hyl_{Efm}* gene, which encode putative glycoside hydrolases that aid in intestinal colonisation and peritoneal invasion (Gao et al., 2018).

Understanding the interaction between the host and the bacteria may aid in the development of new antimicrobial medications (Yuen and Ausubel, 2014). Mutant library screens have a lot of potential for identifying genes that cause certain phenotypes in bacteria and uncovering genetic determinants that allow bacteria to thrive in a variety of environments. Random transposon insertions (Platnich and Muruve, 2019) can cause gene or regulatory region functions to be disrupted (Picardeau, 2010). To create mutant libraries in bacteria, several different transposon-based gene delivery systems have been used (Lampe, 2010; Hoffmann et al., 2012; DeJesus et al., 2017).

The *Himar1 mariner* transposon has been used in different bacterial species, including low-GC Gram-positive bacteria (Le Breton et al., 2006; Zhang et al., 2012; Hashim, 2016). This transposon was initially isolated from *Haematobia irritans* and belongs to the *Tc1/mariner* superfamily of transposable elements (the most-widespread transposons in nature) (Plasterk et al., 1999). In low G+C content bacteria such as *E. faecium* TA dinucleotides is the only recognition signal required for insertion of the *Himar1 mariner* transposon. By inserting these sequences, genes and functional elements are knocked out or altered. A *Himar1 mariner* transposon requires only its own transposase for efficient transposition (Lampe et al., 1999) and this makes it a good option for transposition to make random mutant libraries in low G+C content (~38%) bacteria like *E. faecium* E1162. Randomly inserting *mariner* transposons into a mutant library allowed unbiased screening of all non-essential genes under a variety of biologically relevant conditions.

Some other transposons, for example, Tn917 have been found to be less random and are incorporated at numerous hot spot regions making them non-

ideal for the creation of random libraries (Garsin et al., 2004). Tn917 mutagenesis exhibits regional preference in *E. faecalis* and *Bacillus subtilis* (Shi et al., 2009). Both the transposition and the orientation of the *Himar1 mariner* transposon are more random when compared with Tn917 (Cao et al., 2007). Cao et al. (2007) also reported that a *Himar1 mariner*-based transposon has a higher transposition efficacy and generated about 10-fold more mutants when compared to Tn917-based transposon delivery systems.

A plasmid containing a *Himar 1 mariner*-based transposon designated as pJAWTRASH2 (Figure 4-1) (Wright, unpublished) from a previously described delivery system, pMC38 (Cao et al., 2007). This was used as a transposon delivery vector for *in vivo* transposition in *E. faecium* E1162 (Nair, unpublished), and the subsequent *mariner*-based transposon mutant library screened for impaired resistance to lysozyme and using nisin as a model of antimicrobial (Hashim, 2016).

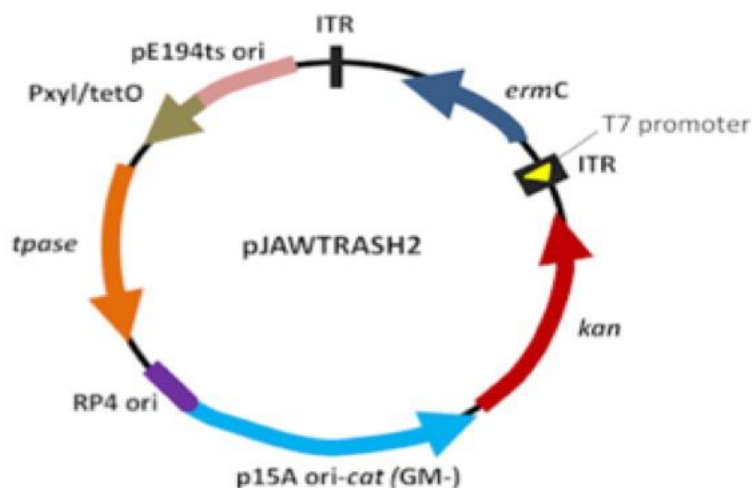


Figure 4-1 *Mariner*-based mutagenesis delivery vector pJAWTRASH2

p15A = *E. coli* low-copy number replication origin: **RP4 ori** = origin for conjugative transfer: **pE194ts ori** = a temperature sensitive replication origin for Gram-positive: **(cat)** = chloramphenicol-resistance gene for Gram-negative: **ermC** = constitutive erythromycin-resistance gene for Gram-positive: **(Kan)** = Kanamycin-resistance gene

for Gram-positive (as a screening marker for loss of the plasmid): (**tpase**) = the *Himar1 mariner* transposase gene. Adapted from Hashim (2016).

Bacteria can defend themselves against acute or chronic oxidative stress, caused by ROS formed as bactericidal weapons by specific cells in the immune system of mammals, by regulating gene expression through signal transduction pathways that detect changes in oxidant levels, these responses combat ROS, repair oxidative damage to cell components (Blanchard et al., 2007). Generation of intracellular oxygen radical to a level over the bacterium's defense ability by redox-cycling agents like menadione can produce oxidative stress. Macromolecules such as DNA, RNA, proteins, and lipids are the main biological targets for these ROS. Lipid peroxidation initiated when free radicals attack polyunsaturated fatty acids in membranes leads to a reduction in membrane fluidity and changes membrane properties and can disrupt membrane-bound proteins, resulting in the formation of more radicals (Ayala et al., 2014). Multiple antibiotic resistant hospital-acquired *E. faecium* strains have an AsrR belonging to the multiple antibiotic resistance regulator family. The AsrR regulator utilize peroxide-sensitive cysteine residues to sense H₂O₂ which ends in its detachment from promoter DNA (Lebreton et al., 2012). Organic-oxidizing agents such as cumene hydroperoxide have a peroxy-functional group and in the presence of the transition metal, iron, generates cumoxyl radicals which remove hydrogen from lipids causing lipid peroxidation (Figure 4-2) (Ayala et al., 2014). Moreover, cumene hydroperoxide reacts with amino acids and proteins triggering oxidation of side-chains, backbone aggregation, fragmentation and dimerization (Gračanin et al., 2009). It was hypothesized in this study that by screening an *E. faecium Himar1 mariner*-based transposon mutant library for mutants with increased sensitivity to menadione and/or

cumene hydroperoxide, it would be possible to identify genes involved in oxidative stress resistance in *E. faecium*.

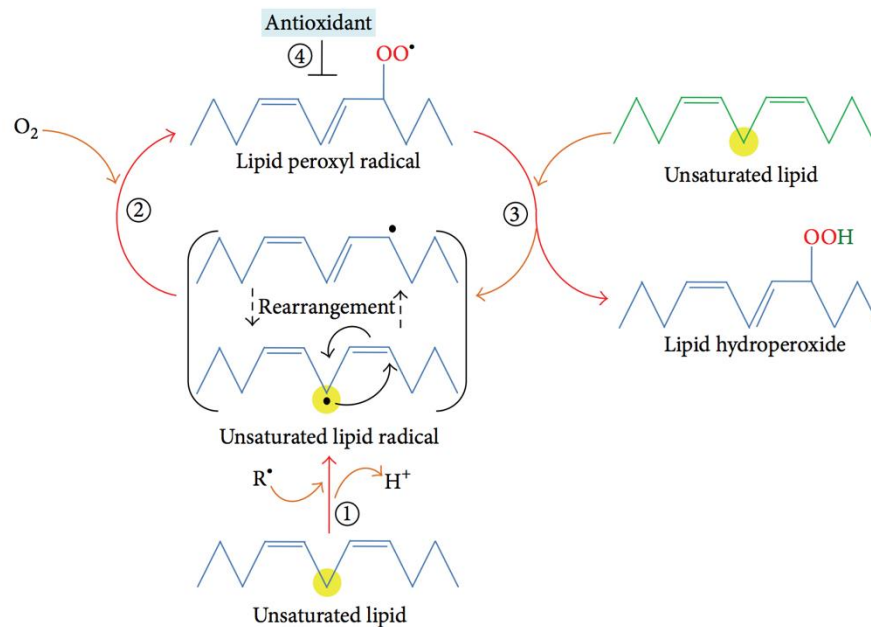


Figure 4-2 Process of lipid peroxidation.

(1) Prooxidants extract the allylic hydrogen during initiation, generating a carbon-centered lipid radical; the carbon radical is stabilized by a chemical rearrangement, forming a conjugated diene. **(2)** During the propagation phase, a lipid radical combines quickly with oxygen to generate a lipid peroxy radical. **(3)** which then takes a hydrogen from another lipid molecule, resulting in the formation of a new lipid radical and lipid hydroperoxide. **(4)** Antioxidants contribute a hydrogen atom to the lipid peroxy radical species in the termination reaction, resulting in the creation of non-radical products. Adapted from Ayala et al. (2014).

4.2 Aims of this chapter

The work described in this chapter aimed to screen an *E. faecium* E1162-*mariner* transposon mutant library to identify genes involved in oxidative stress resistance by using menadione as superoxide generator, cumene hydroperoxide as a peroxide generator and to examine virulence of the identified sensitive mutants using a *G. mellonella* model. The second aim was to examine sensitivity of mutant *efmE1162_1543* (59N) a nisin-sensitive mutant (Hashim, 2016) against menadione and cumene hydroperoxide. The *efmE1162*

1543 gene is located immediately adjacent to the putative NADH oxidase gene *efmE1162* 1544 (*nox*) and is part of a 150 kb putative pathogenicity island. NADH oxidase is involved in oxidative stress tolerance in other bacteria such as *Streptococcus suis* (Zheng et al., 2017). As a result, it was proposed in this study that the *mur* gene is involved in *E. faecium* oxidative stress tolerance.

4.3 Materials and methods

4.3.1 Menadione as a superoxide generator

Menadione is a superoxide generator, which induces the formation of superoxide inside the cells, is an air-stable, light and heat sensitive and soluble in 95% ethanol. A 10 mg/ml menadione stock solution was freshly prepared by adding 0.2 g of the menadione powder to 20 ml absolute ethanol (95%), the contents were mixed well until dissolved by using a vortexer (Labnet VX100). The MIC of menadione against *E. faecium* E1162 was determined using the agar-dilution method in TSA rather than BHI (Lebreton et al., 2013) because the Clinical and Laboratory Standards Institute (2009) stated that TSB is recommended for use as the inoculum broth for disc diffusion and agar-dilution antimicrobial susceptibility testing due to its growth promotion, whereas the Clinical and Laboratory Standards Institute (CLSI) (Ferraro, 2000) recommended Mueller-Hinton agar (MHA) media for antimicrobial susceptibility because it contains starch, which is known to absorb toxins released by the tested bacteria, and it is a loose agar, which allows for more effective antibiotic diffusion than other types of media, so a more precise zone of inhibition results from improved diffusion. It was thought that using TSA instead of MHA to determine the MIC of menadione against E1162 and its mutants was a study limitation.

4.3.2 Cumene hydroperoxide as a peroxide generator

Cumene hydroperoxide is a peroxide generator, which is soluble in water and was stored at 4°C (Palyada et al., 2009). The MIC of cumene hydroperoxide against *E. faecium* E1162 was determined using the method described by Lebreton et al. (2012) on TSA instead of BHI using the agar-dilution method (Patel et al., 2015). Because of its growth-promoting properties, TSB used as an inoculum broth for disc diffusion and agar-dilution antimicrobial susceptibility testing, as defined by the Clinical and Laboratory Standards Institute (*Clinical and Laboratory Standards Institute*, 2009; Patel et al., 2015).

4.3.3 Determination of minimum inhibitory concentrations of menadione and cumene hydroperoxide.

To identify genes involved in resistance to oxidative stress in *E. faecium* E1162 the *Himar1 mariner* transposon mutant library was screened for mutants with reduced resistance to menadione (superoxide generator, Cayman chemical NSC4170). The sensitivity of the selected mutants was also assessed against an organic peroxide, cumene hydroperoxide (Sigma-Aldrich, 247502). The MICs for menadione and cumene hydroperoxide were determined by using broth and agar-dilution methods (Andrade et al., 2017). For broth dilution, serial 2-fold broth dilutions starting at 256 µg/ml down to 2.0 µg/ml menadione and cumene hydroperoxide were made in TSB using 96-well microtiter plates. *E. faecium* E1162 from stationary-phase cultures (16 hrs old cultures) were diluted to an OD_{600 nm} of 0.5 in TSB growth medium and inoculated into each well to give 10⁵ CFU/ml (50 µl). Microtiter plates were then incubated at 37°C for 48 hrs. The lowest concentration of the tested compound that prevented growth was recorded as the MIC. The procedure for agar-dilution assays was similar to that for broth dilution (starting at 256 µg/ml to 2.0 µg/ml menadione and cumene

hydroperoxide) except that the test compounds were added into TSA medium (molten) using serial 2-fold dilutions, then colonies from replica's master-plates were copied onto the agar plate surface, incubated at 37°C in 5% CO₂ for 48 hrs. The MICs were recorded as the lowest concentration of compound that completely inhibited the growth of *E. faecium* E1162. This concentration was then used for screening the *E. faecium* E1162 transposon mutant library.

4.3.4 Screening *E. faecium* E1162 *Himar1* mariner transposon mutant library for menadione-sensitive mutants

The *E. faecium* E1162 transposon mutant library was screened for growth impairment via replica plating (Bio 101 system). To select menadione-sensitive mutants, the library was plated out onto TSA plates (master-plates) and colonies allowed to grow overnight (16 hrs). Master-plates were then replica plated (Dale and Park, 2010) in triplicate onto TSA plates with or without 64 µg/ml menadione (one 2-fold dilution lower than the MIC of the wild-type *E. faecium* E1162), followed by incubation at 37°C in 5% CO₂ for 72 hrs. Those colonies which grew on TSA plates but not on the TSA containing menadione plates were putative menadione-sensitive mutants.

4.3.5 Spot-plate assay to test the sensitivity of the transposon mutants to menadione and cumene hydroperoxide

Cells of *E. faecium* E1162 menadione-sensitive transposon mutant strains MS1, MS2 and MS3 (described later) and the wild-type *E. faecium* E1162, were grown in TSB at 37°C for 16 hrs. Diluted cells suspensions of the wild-type *E. faecium* E1162 at an OD_{600 nm} of 0.5 = ~ 1.5 x 10⁸ CFU/ml (Chapter III, Figure 3-3), subsequently, cells of the wild-type and the mutant strains at an OD_{600 nm} of 0.5 were six 2-fold diluted in sterile PBS. Unless otherwise stated, 5.0 µl of each dilution was then spotted onto TSA plates supplemented with menadione or

cumene hydroperoxide. These plates were incubated for 16 hrs at 37°C in 5% CO₂ (Song et al., 2015; Wan et al., 2015). Besides transposon mutants, other two mutants (59N and 47N) were also studied in an attempt to determine the sensitivity of these mutants against menadione using a spot-plate assay.

4.3.6 Susceptibility of the transposon mutants to cumene hydroperoxide using a disk diffusion assay

Bacterial suspensions of *E. faecium* E1162 (control), the menadione-sensitive transposon mutant strains; MS1, MS2, and MS3, and the nisin-sensitive mutant 59N, which contains a transposon inserted in a gene encoding a putative muramidase, were prepared equivalent to a 0.5 McFarland and were used to inoculate TSA agar plates via spreading method by using sterile cotton swabs. Ten microliters of cumene hydroperoxide (3.38 M) were spotted onto sterile filter paper discs (6 mm diameter) and placed on the plates and then incubated at 37°C in 5% CO₂ for 16 hrs. The inhibitory-zone diameters for the wild-type and mutants were measured and compared (Ruangpan and Tendencia, 2004).

4.3.7 Growth curves of *E. faecium* E1162 and menadione-sensitive mutants MS1, MS2 and MS3 in TSB

To confirm that the mutations that occurred in the mutants MS1, MS2, and MS3 did not affect gene(s) required for bacterial metabolism and growth, a 16 hrs culture of *E. faecium* E1162 and menadione-sensitive mutants were used to inoculate 10 ml TSB (cultures were adjusted to an OD_{600 nm} of 0.05) for each strain then incubated at 37°C with continuous agitation (220 rpm) and the absorbance at OD_{600 nm} was recorded every 60 min for up to 8 hrs, each experiment was performed in triplicate.

4.3.8 Menadione and cumene hydroperoxide sensitivity of the nisin- sensitive mutant 59N

In *E. faecium*, the muramidase and NADH oxidase encoded by the muramidase and *nox* genes, respectively, which are located on the same putative pathogenicity island, ICE*Efm1* (Leavis et al., 2004; Top et al., 2013). The NADH oxidase of *E. faecalis* is engaged in glycolytic metabolism. However, because similar enzymes in *Streptococcus pyogenes*, *S. mutans*, and *S. pneumoniae* are virulence factors involved in adaptive responses to O₂, allowing these bacteria to grow in O₂-rich environments (Leavis et al., 2004; Zheng et al., 2017). Given the foregoing, it was suggested in this study that *E. faecium* NADH oxidase is engaged in the adaptive response to O₂, and since the muramidase gene is situated right downstream of the *nox* gene in the ICE*Efm1*, it may be involved in the response to O₂ in *E. faecium*. In order to determine if the mutation changed *E. faecium* E1162's susceptibility to ROS produced by the host phagocyte cell, sensitivity of mutant 59N to the peroxide stress or cumene hydroperoxide and the superoxide stress or menadione was assessed. The concentration of cumene hydroperoxide to be used in the spot-plate assay was first determined. *E. faecium* E1162 was grown on TSA containing different cumene hydroperoxide concentrations then incubated at 37°C for 24 hrs.

4.3.9 Identification of the disrupted genes in mutants that had altered sensitivity to menadione

To identify disrupted genes in *E. faecium* E1162 *Himar1 mariner* transposon mutants, a Y-linker PCR method was used to amplify transposon-flanking regions using chromosomal DNA from the mutants as described by Kwon and Ricke (2000) (Figure 4-3). The genomic DNA isolated from transposon mutants was digested with *Asel* 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 primers (Transposon-

specific primer and Y-linker primer) can selectively amplify DNA fragments containing transposon-specific sequences (Chapter II, Table 2-5). The Y-linker was made in the following manner: first, linker-II was phosphorylated at the 5' end with T4 polynucleotide kinase (PNK). The reaction mixture consisted of 20 μ l, including 9 μ l of linker-II (350 ng/ μ l), 6 μ l of buffer (3 mM Tris-HCl, 0.2 mM EDTA, pH 7.5), 2 μ l of 10 X PNK buffer, 2 μ l of 10 mM ATP, and 10 units (1.0 μ l) of PNK (New England Biolabs) incubated for 1 hr at 37°C followed by heating for 10 min to inactivate the PNK. Then 9 μ l of linker-I were added to give a final volume of 29 μ l. The mixture was heated at 95°C for 2 min and slowly cooled to room temperature.

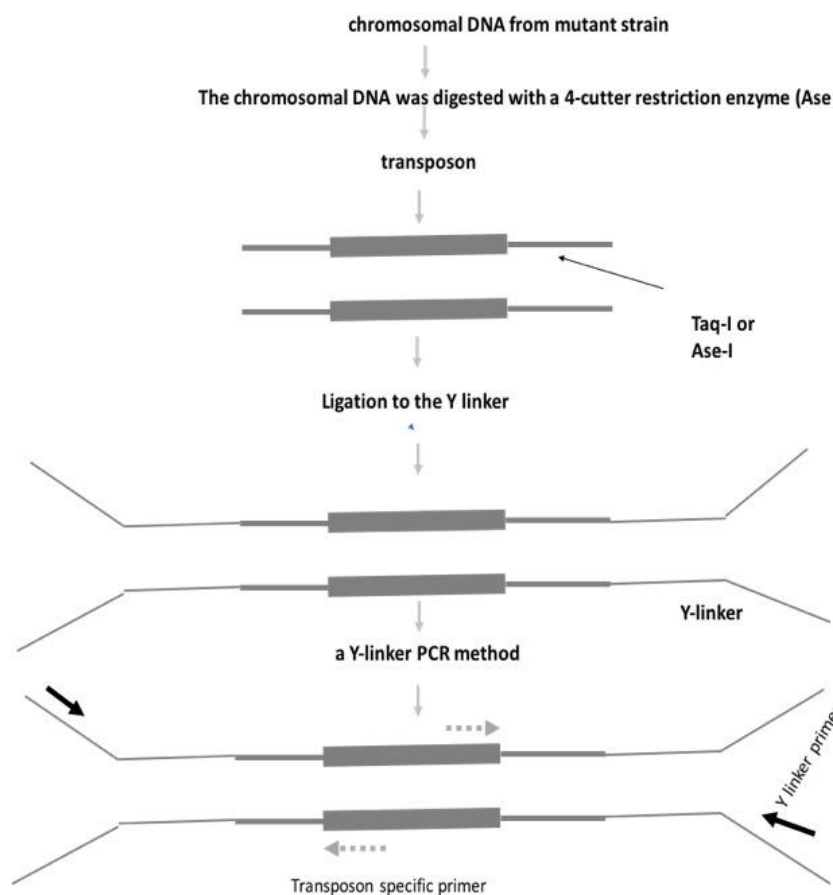


Figure 4-3 The diagram of the Y-linker PCR method used to amplify transposon – flanking regions in transposon mutant chromosomal DNA as described by Kwon and Ricke (2000). Adapted from Hashim (2016).

4.3.9.1 Ligation of the *Asel* or *TaqI* digested genomic DNA of transposon mutants to Y-linker

A reaction mixture (20 µl) including 5 µl of Y-linker (200 ng/µl), 2 µl of 10X ligase buffer, 2 µl of sterile deionised H₂O, 10 µl (50 ng/µl) of digested DNA, and 1 unit of ligase were incubated overnight at 16°C, then diluted 10-fold using diH₂O, and the ligase inactivated by heating for 10 min at 65°C. These ligation products were then used as a template in the Y-linker PCR reaction.

4.3.9.2 Amplification of transposon – flanking sequences

A 5.0 µl aliquot of the ligation was used as a template for a PCR reaction containing 1 µl of 20 µM transposon primer, 1 µl of 20 µM Y-linker primer, 10 µl NEBNext Hot start Q5 HiFi PCR master mix and 6- µl sterile diH₂O. The PCR mix was initially heated to 95°C for 4 min and then cycled 30 times between (94°C, 1 min; 57°C, 1 min; 72°C, 1 min); final extension of 5 min at 72°C and cooled at 4°C until required. Following amplification, agarose gel electrophoresis was performed to check the PCR products. To identify the disrupted genes in the menadione-sensitive mutants, the DNA bands were excised from the agarose gel, purified and their nucleotide sequences were determined by an external company. The sequencing results were then compared against sequences in GenBank using the Nucleotide-nucleotide Basic Local Alignment Search Tool, which performed sequence similarity searches using a nucleotide query and against the data from the whole- genome shotgun-contigs of *E. faecium* E1162 strain (accession number ABQJ000000000).

4.3.10 The susceptibility of *G. mellonella* larvae to infection with different *E. faecium* strains

4.3.10.1 Preparation of *E. faecium* strains for larval infections

To prepare *E. faecium* strains for *G. mellonella* larval infection. First *E. faecium* strains were grown overnight (16 hrs) on BHI agar at 37°C prior to inoculation in BHI broth. Following 16 hrs growth in BHI broth (37°C and 220 rpm), cultures were collected by centrifugation at 960 xg for 10 min, washed 3 times by repeated gentle pipetting in 10 ml PBS (freshly prepared) for 2 min, following each washing step in PBS cells were centrifuged at 960 xg for 10 min. Cells that were obtained diluted in PBS buffer to the needed OD600 nm (Hashim, 2016).

4.3.10.2 Virulence of *E. faecium* strains in the *G. mellonella* larvae infection model

G. mellonella larvae were obtained from Cornish Crispa, Co. (UK) and stored in darkness at 12°C. Larvae, with body weights of between 200-290 mg were used. The larvae are large enough to be easily injected, to obtain hemolymph and hemocytes, and to isolate other organs for further study when they are 2 cm long and 250 mg in weight before pupation (Ramarao et al., 2012). For each bacterial strain, an allotted random set of 10 *G. mellonella* larvae were placed in a petri-dish on ice for 5-10 min, till no movement could be spotted, then injected with 10 µl of bacterial suspension using an automated syringe pump injector (New Era Pump System, Inc) by inserting the needle into the last left pro-leg (into the haemocoel). Infected caterpillars were then incubated in the darkness at 37°C. Two control groups (10 larvae per-group), were included in each experiment, one group was injected with sterile PBS and the other group left without injection. Experiments continued for up to 96 hrs on 3 different occasions. Infected larvae were checked daily, and the number of live larvae was noted (Hashim, 2016).

4.4 Results

4.4.1 Menadione sensitivity of *E. faecium* E1162

In reaction to microbial invaders, host phagocytes respond and generate ROS as one of the main early defenses against bacterial infections. These ROS are highly toxic to microorganisms and used to prevent the colonisation of host tissues by pathogens. To identify genes involved in resistance to oxidative stress in *E. faecium*, an E1162 *Himar1 mariner* transposon mutant library was screened for mutants with reduced resistance to menadione (superoxide generator). It was determined the concentration of menadione to be used in screening the transposon mutant library. It was found that the MIC of *E. faecium* E1162 against menadione was 128 µg/ml menadione; the transposon library was screened on TSA supplemented with 64 µg/ml menadione to identify mutants with increased sensitivity. Approximately 12,970 colonies from the transposon library were screened for increased menadione sensitivity on TSA supplemented with 64 µg/ml menadione. Three mutants were found in this screen designated as menadione-sensitive MS1, MS2 and MS3.

4.4.2 Determination of menadione sensitivity using a spot-plate assay

Superoxide sensitivity of an *E. faecium* E1162, mutants MS1, MS2, MS3 and 59N towards a different levels of the intracellular superoxide generator menadione was quantitatively assayed using the spot-plate assay described by Wan et al. (2015). Menadione concentrations of 64, 80, 100 and 128 µg/ml were used. Menadione concentrations above 128 µg/ml and below 64 µg/ml were either too toxic (no growth) or had no effect on the strains MS1, MS2, MS3 (growth comparable to that seen in the absence of menadione), but this was not the case for 59N, which displayed no growth at 64 µg/ml menadione (this study,

data not shown), which was thought to be its MIC. Accordingly, 32 µg/ml menadione was used to study 59N's sensitivity against menadione, which demonstrated very weak growth on it in comparison to the wild type E1162 therefore, the sensitivities of each strain of MS1, MS2 and MS3 were tested with menadione concentrations of 64, 80, and 100 µg/ml. The wild-type and the mutant strains grew similarly in the spot-plate assay on TSA containing 64 µg/ml menadione (Figure 4-4). In the spot-plate assay, the *E. faecium* E1162 and menadione-sensitive mutant strains (MS1 and MS2) grew similarly on TSA containing 64 µg/ml menadione, a 2-fold lower than the *E. faecium* E1162 MIC.

MS3 mutant showed low growth at 1×10^6 to 2.5×10^5 CFU/ml or no growth at 1×10^5 CFU/ml on TSA containing 64 µg/ml menadione (Table 4-1, Figure 4-4). When compared to the wild-type *E. faecium* E1162 and isogenic mutants MS1 and MS3, mutant MS2 showed a little improvement in growth on TSA containing 80 µg/ml and 100 µg/ml menadione at 1×10^5 and 2.5×10^6 cells/5 µl, respectively (Table 4-1 and Figure 4-4). There was no significant difference between the growth of *E. faecium* E1162 and its isogenic mutants MS1, MS2 and MS3 when grown in TSB (Figure 4-5).

The results of this study showed no difference in the growth patterns of E1162, MS1 and 47N grown on TSA containing 64 µg/ml menadione by using spot-plate assay (this study, data not shown).

The presence of 64, 80, and 100 µg/ml menadione on TSA suppressed the growth of 59N in the spot-plate experiment (this study, data not shown). Thus, 32 µg/ml menadione was used with 59N (Figure 4-7B).

Table 4-1 Growth effect of menadione on *E. faecium* E1162 and its isogenic mutants (MS1, MS2 and MS3) using spot-plate assay.

		Amount of the growth on TSA				
		Bacterial cells per 5 μ l	E1162	MS1	MS2	MS3
Control		5×10^6	+++	+++	+++	+++
		2.5×10^6	+++	+++	+++	+++
		1×10^6	+++	+++	+++	+++
		5×10^5	+++	+++	+++	+++
		2.5×10^5	+++	+++	+++	+++
		1×10^5	+++	+++	+++	+++
Menadione 64 μg/ml		5×10^6	+++	+++	+++	+++
		2.5×10^6	+++	+++	+++	+++
		1×10^6	+++	+++	+++	++
		5×10^5	++	++	++	+
		2.5×10^5	++	++	++	+
		1×10^5	+	+	+	-
Menadione 80 μg/ml		5×10^6	++	++	++	++
		2.5×10^6	++	++	++	++
		1×10^6	+	+	++	+
		5×10^5	-	-	-	-
		2.5×10^5	-	-	-	-
		1×10^5	-	-	-	-
Menadione 100 μg/ml		5×10^6	++	++	++	++
		2.5×10^6	-	+	++	-
		1×10^6	-	-	-	-
		5×10^5	-	-	-	-
		2.5×10^5	-	-	-	-
		1×10^5	-	-	-	-

Symbols: (-): no growth; (+): low growth; (++) : moderate growth; and (+++) : high growth

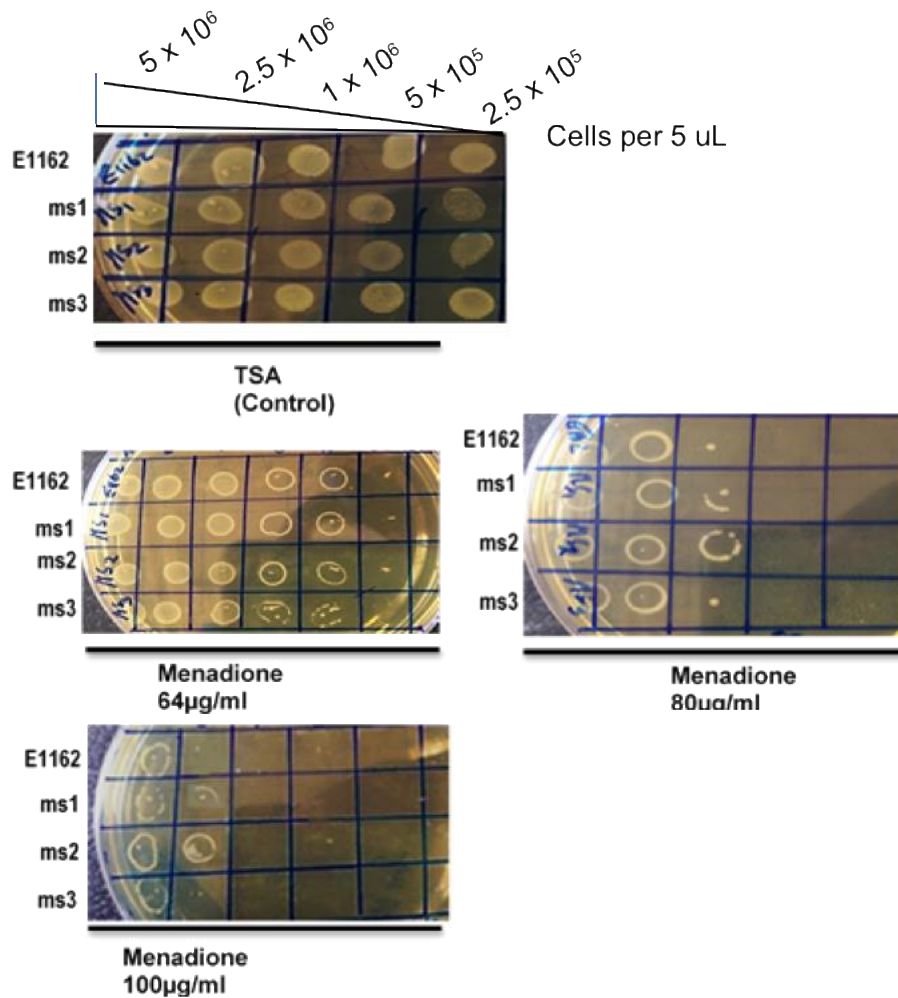


Figure 4-4 Sensitivity of *E. faecium* E1162 and mutants MS1, MS2 and MS3 to different concentrations of menadione.

All cells were grown overnight in TSB. Plates were spotted with inocula ranging from 5×10^6 to 2.5×10^5 cells of each strain in a volume of 5 µl. Plates were supplemented with 64, 80 and 100 µg/ml menadione or without (control). The density of bacterial growth was similar for all strains in the absence of menadione. Data are representative of 3 biological replicates and pictures show a representative plate for each condition.

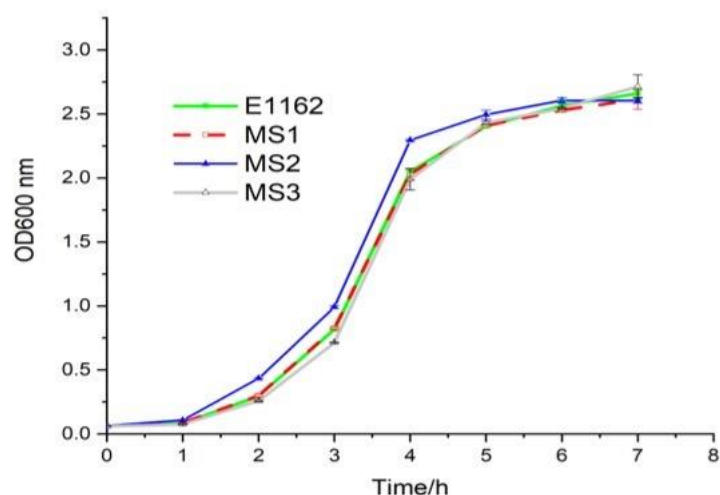


Figure 4-5 The growth of *E. faecium* E1162 and mutants MS1, MS2, and MS3 in TSB.

Green line: E1162; **Red dots:** MS1; **Blue line:** MS2; and **Grey line:** MS3. Data presented are the mean of three biological replicates performed in triplicate and error bars show standard deviations.

4.4.3 Cumene hydroperoxide susceptibility of *E. faecium* E1162 and mutant MS1, MS2 and MS3

Cumene hydroperoxide disc diffusion assays were performed to test if the mutated genes in mutants MS1, MS2 and MS3 similarly mediated resistance to peroxide generated by 3.38 mM cumene hydroperoxide (a peroxide stress generator). The wild-type *E. faecium* E1162 and the mutant strains were found to be sensitive to 3.38 mM cumene hydroperoxide. The inhibitory-zone diameters for *E. faecium* E1162 and its mutant strains (MS1, MS2, and MS3) were measured and compared. Tukey's *post hoc* analysis combined with analysis of variance was used to provide precise information on differences between the *E. faecium* E1162 mutant strains (MS1, MS2, and MS3) and the wild-type parental strain *E. faecium* E1162 (Kim, 2014). The analysis showed significant differences ($p = 0.05$) between *E. faecium* E1162 and MS1, *E. faecium* E1162 and MS2, MS1 and MS3, and MS2 and MS3, but there was no significant difference between the inhibitory-zone of *E. faecium* E1162 and MS3 as illustrated in Figure 4-6.

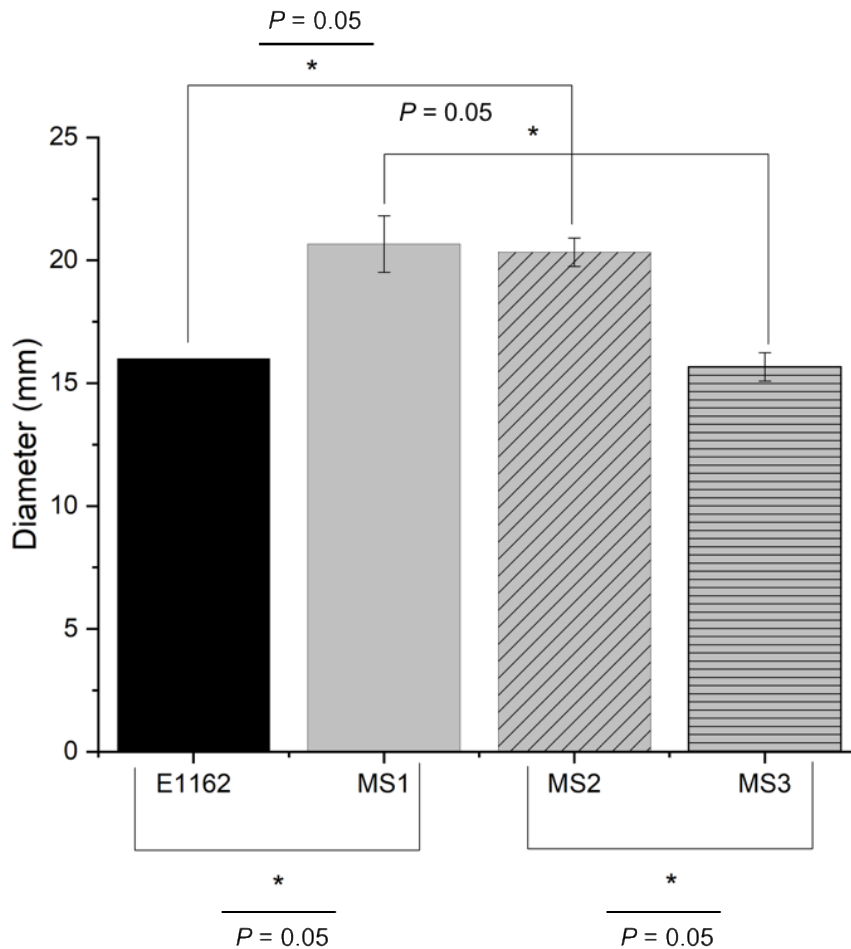


Figure 4-6 Effects of 3.38 mM cumene hydroperoxide on *E. faecium* E1162 and mutants MS1, MS2, and MS3.

Data presented are the mean of three biological replicates. Statistical analysis was performed by one-way analysis of variance combined with Tukey's *post hoc* test for multiple comparisons.

4.4.4 Menadione and cumene hydroperoxide sensitivity of the putative muramidase mutant 59N

The concentration of cumene hydroperoxide to be used in the spot-plate assay was first determined to see if the putative muramidase mutant 59N of an *E. faecium* E1162 which had been identified by Hashim (2016) would also be more responsive to menadione and/or cumene hydroperoxide stress. *E. faecium* E1162 was grown on TSA containing different cumene hydroperoxide concentrations. After 24 hrs incubation, it was found that *E. faecium* E1162 was

sensitive to 32 mM cumene hydroperoxide (this study, data not shown). Therefore, 16 and 24 mM cumene hydroperoxide was used in the spot-plate assay. In the presence of menadione 64, 80, and 100 µg/ml, mutant 59N growth is entirely suppressed (this study, data not shown). Thus, in the spot-plate experiment 32 µg/ml menadione was used for this mutant (59N).

In BHI medium, all the nisin-sensitive mutants tested had similar growth curves to E1162 (Chapter III, Figure 3-2). The growth of strain 59N on TSA was same as the E1162 (Table 4-2, Figure 4-7A). On TSA containing 32 µg/ml menadione at cells densities of 5×10^6 to 1×10^5 cells per 5 µl, the growth of strain 59N was less when compared to *E. faecium* E1162, at a cell density of 2.5×10^5 CFU/ml, strain 59N was at least 3-4-fold more sensitive to menadione when compared to *E. faecium* E1162. On TSA containing 16 mM cumene hydroperoxide at cell densities of 1×10^6 , 5×10^5 , and 2.5×10^5 cells per 5 µl, the growth of strain 59N was 3-4 times less than that of strain E1162 (Table 4-2, Figure 4-7C). On TSA containing 24 mM cumene hydroperoxide, the growth of 59N at a cell density between 1×10^6 to 1×10^5 cells/5 µl was at least 3-4-fold lower than E1162 (Table 4-2, Figure 4-7C).

Table 4-2 Growth effect of menadione and cumene hydroperoxide against *E. faecium* E1162 and mutant 59N using spot-plate assay.

	Bacterial cells per 5 μ l	Amount of the growth on TSA	
		E1162	Mutant 59N
Control	5 x 10 ⁶	+++	+++
	2.5 x 10 ⁶	+++	+++
	1 x 10 ⁶	+++	+++
	5 x 10 ⁵	+++	+++
	2.5 x 10 ⁵	+++	+++
	1 x 10 ⁵	+++	+++
Menadione 32 μg/ml	5 x 10 ⁶	+++	+++
	2.5 x 10 ⁶	+++	++
	1 x 10 ⁶	+++	++
	5 x 10 ⁵	+++	++
	2.5 x 10 ⁵	+++	+
	1 x 10 ⁵	+++	+
Cumene hydroperoxide 16 mM	5 x 10 ⁶	+++	++
	2.5 x 10 ⁶	+++	++
	1 x 10 ⁶	+++	+
	5 x 10 ⁵	+++	+
	2.5 x 10 ⁵	+++	+
	1 x 10 ⁵	+++	+
Cumene hydroperoxide 24 mM	5 x 10 ⁶	+++	+++
	2.5 x 10 ⁶	+++	++
	1 x 10 ⁶	+++	+
	5 x 10 ⁵	+++	+
	2.5 x 10 ⁵	+++	-
	1 x 10 ⁵	+++	-

Symbols: (-): no growth; (+): low growth; (++) : moderate growth; and (+++): high growth

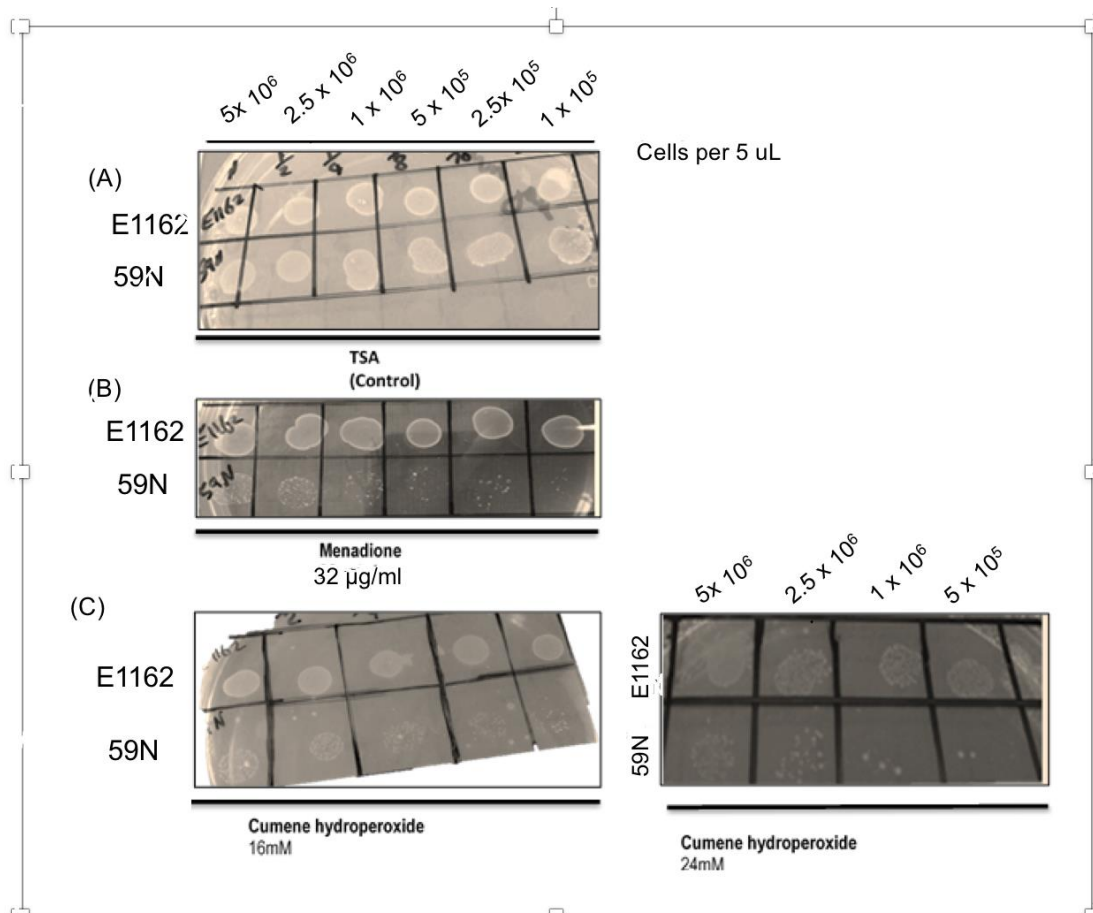


Figure 4-7 Sensitivity of *E. faecium* E1162 and nisin-sensitive mutant 59N to different concentrations of menadione and cumene hydroperoxide.

Plates were supplemented with menadione or cumene hydroperoxide or not supplemented (control): **(A)** E1162 and 59N on TSA. **(B)** E1162 and 59N on TSA + 32 µg/ml menadione. **(C)** E1162 and 59N on TSA + 16 mM and 24 mM cumene hydroperoxide. Plates were spotted with inocula ranging from 5×10^6 to 1.0×10^5 cells in a volume of 5 µl. Data are representative of 3 biological replicates performed in triplicates and pictures show a representative plate for each condition.

4.4.5 Y-linker PCR strategy for detecting transposon insertions

Y-linker PCR technique was used to amplify transposon-flanking regions using genomic DNA from the menadione-sensitive mutants. The amplified fragments were separated by agarose gel electrophoresis. Figure (4-8) shows a gel electrophoresis images of the Y-linker PCR products from mutant MS1, MS2 and MS3 where the Y-linker PCR gave a single PCR product. Sample in lanes 2 and 3 are Y-linker PCR products for MS1 and MS2, respectively, done by

using *TaqI*, while the sample in lane 5 is Y-linker PCR product for MS3 done by using *Asel*.

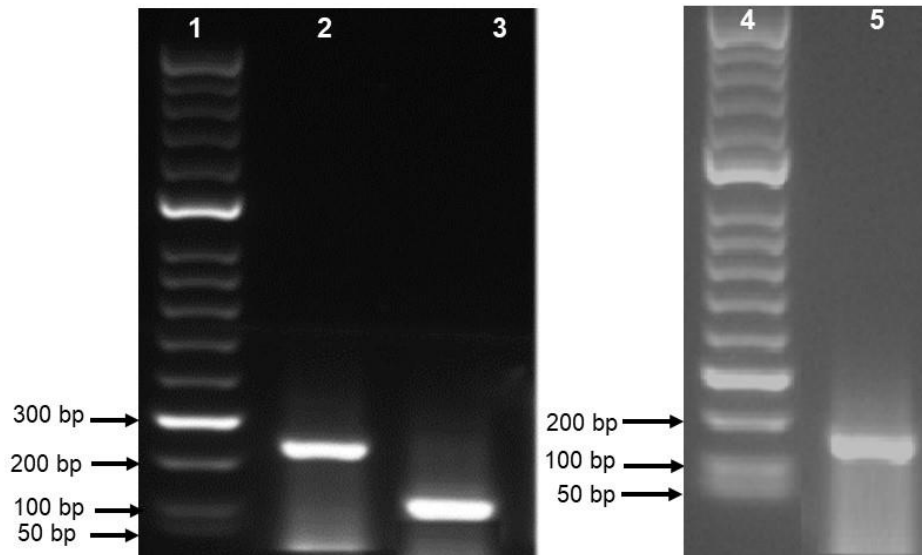


Figure 4-8 Images of electrophoresis gel in which the Y-linker PCR products from mutant MS1, MS2 and MS3 were separated.

Lane 1 and 4 molecules DNA ladder (Hyper-Ladder II Bioline), **lane2**, MS1, **lane 3**, MS2, and **lane5**, MS3.

4.4.6 Identification of mutants with increased sensitivity to menadione

Mutant MS1 had the transposon inserted at 1421 bp in reverse orientation within an ORF EFF34651 (*EfmE1162_1492*) encoding a protein of 625 aa annotated in the *E. faecium* E1162 genome sequence as tyrosine decarboxylase, which might contribute to acid resistance ((*UniProtKB - P0DTQ4 (TYRDC_ENTF3)*, 2018). Figure 4-9 shows the genetic architecture of the area upstream and downstream of EFF34651 (*EfmE1162_1492*), EFF34649 (*EfmE1162_1490*): annotated as encoding Na⁺/H⁺ antiporter. Na⁺/H⁺ antiporters are membrane proteins that play an important role in pH and Na⁺ homeostasis in organisms from bacteria to humans and higher plants and implicated in the resistance to oxidative stress (Padan et al., 2001; Katiyar-Agarwal et al., 2006; Khan et al., 2021); EFF34650 (*EfmE1162_1491*): annotated as encoding antiporter; and

EFF34652 (*EfmE1162_1493*): annotated as encoding tyrosyl-tRNA synthetase. The aminoacyl-tRNA synthetase family identify information such as concurrent tRNA molecules and amino acid structures, which are required for translating coded information into protein structures in nucleic acids (Mirande, 2017).

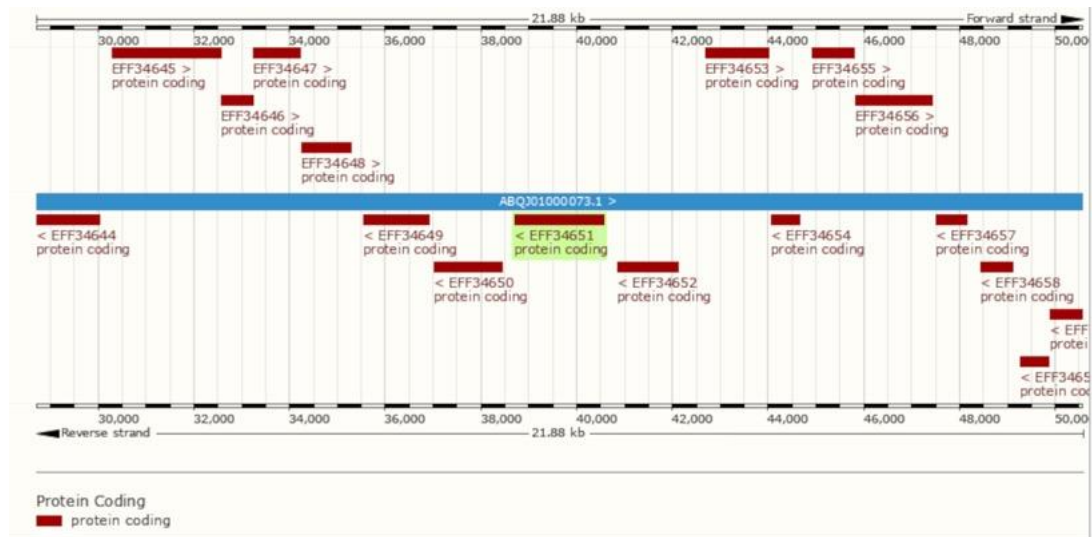


Figure 4-9 Genetic organization of the region upstream and downstream of EFF34651 (*EfmE1162_1492*).

EFF34651 (*EfmE1162_1492*) annotated as encoding TDC; EFF34649 (*EfmE1162_1490*): annotated as encoding Na⁺/H⁺ antiporter; EFF34650 (*EfmE1162_1491*): annotated as encoding antiporter; EFF34652 (*EfmE1162_1493*): annotated as encoding tyrosyl-tRNA synthetase.

In mutant MS2, a transposon was inserted in contig00077 3860 to 3873 (+) within an ORF EFF34605 (*Efm E1162_1516*) in reverse orientation. *Efm E1162_1516* encodes a protein of 417 amino acids annotated in *E. faecium* E1162 genome sequence as a conserved hypothetical protein (Figure 4-10). Analysis of the predicted protein architecture of EFF34605 shows an N-terminal Pyr_redox_2 domain (FAD flavoproteins belonging to the family of pyridine nucleotide-disulfide oxidoreductases), and GidA (Glucose-inhibited division protein A) a tRNA-modification enzyme domain at the C-terminus (Figure 4-11).



Figure 4-10 Genetic organization of the region upstream and downstream of EFF34605 (*EfmE1162_1516*).

EfmE1162_1516: annotated as encoding a conserved hypothetical protein, *EfmE1162_1517*: annotated as encoding Prolyl-tRNA editing protein, YbaK/ebcC; *EfmE1162_1518*: annotated as encoding 3-dehydroquinate dehydratase type I; *EfmE1162_1519*: annotated as encoding methyltransferase; *EfmE1162_1515*: annotated as encoding NAD(P)-specific glutamate dehydrogenase; *EfmE1162_1514*: annotated as encoding glucose-6-phosphate isomerase; *EfmE1162_1513*: annotated as encoding a conserved hypothetical protein.



Figure 4-11 Predicted domain architecture of the *E. faecium* EFF34605.

Pyr_redox_2: FAD flavoproteins belonging to the family of pyridine nucleotide-disulfide oxidoreductases, **GidA**: a tRNA-modification enzyme. The domains were predicted using the Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de>).

Mutant MS3 had the transposon inserted within an ORF EFF33659 (*efmE1162_2615*) encoding a protein of 303 aa annotated in *E. faecium* E1162 genome sequence as transposase (**HATPase_c (PF02518)**) family with two domains, the first domain is integrase, a catalytic core which starts at 170 and end at 265 and the second domain is ribonuclease H-like domain which starts at 160 and end at 303. Analysis of the predicted protein architecture of EFF33659 shows an N-terminal Histidine kinase-like ATPases (Figure 4-12).

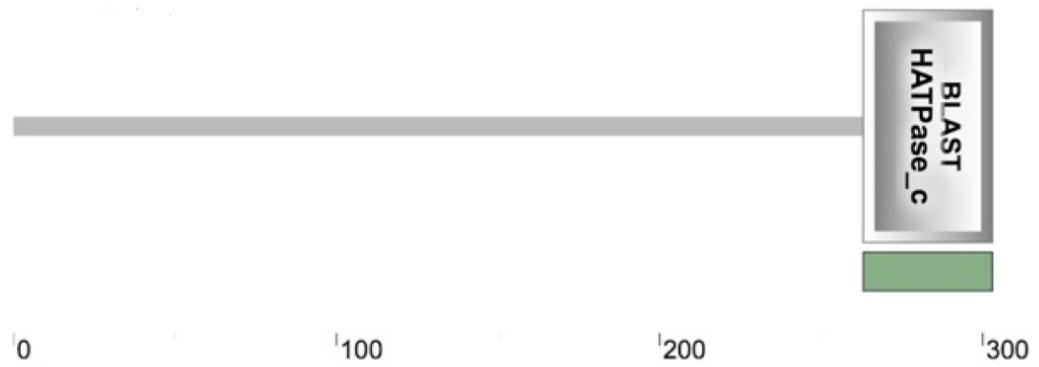


Figure 4-12 Predicted domain architecture of mutant MS3 (EFF33659). Histidine kinase-like domain.

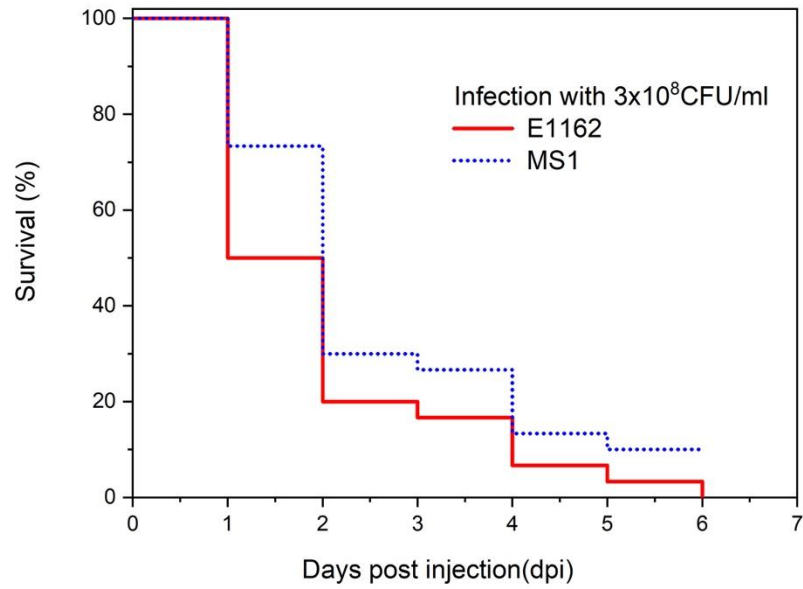
The domain was predicted using the Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de>).

4.4.7 Survival of *G. mellonella* following infection with *E. faecium* E1162 and the mutants MS1, MS2, and MS3

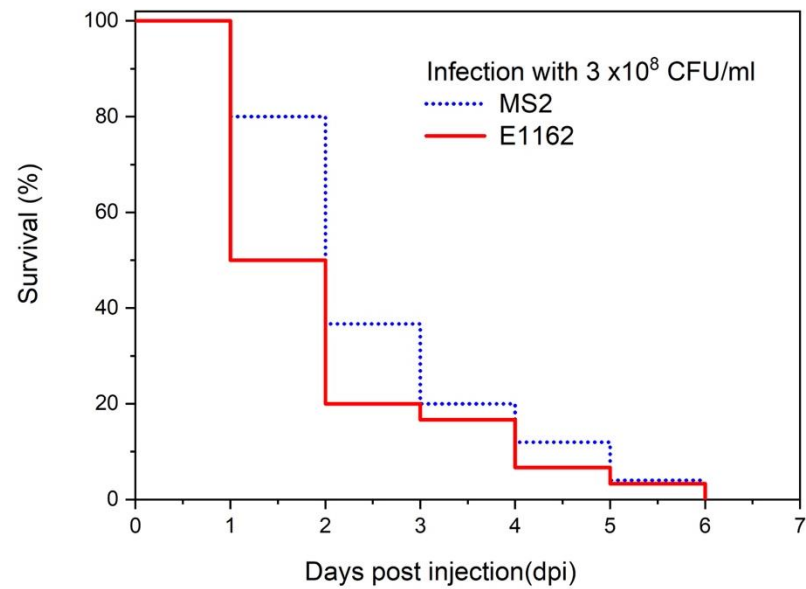
One of the aims of the work was to screen an *E. faecium* Himar 1 mariner-based transposon mutant library for mutants with an increase susceptibility to menadione and/or cumene hydroperoxide, as these mutants might have disruptions in genes involved in resistance to oxidative stress in the host. If they did then such mutants might have impaired virulence in the *G. mellonella* larvae model. *G. mellonella* larvae were infected with the wild-type E1162 and the isogenic mutants MS1, MS2, and MS3 at 3×10^8 CFU/ml and their survival was monitored over time. E1162 killed 15/30 (50%), 24/30 (80%) at 1 dpi and 2 dpi respectively, while mutant MS2 killed 6/30 (20%) and 19/30 (63.3%) at 1 dpi and 2 dpi respectively which was significantly different (Figure 4-13B). The log-rank test was used to determine the significance of differences in survival rates between MS1, MS2 and MS3 compared to the wild-type *E. faecium* E1162 and the *p*-values were $p < 0.076$, 0.025, and 0.07, respectively. A value of $p < 0.05$ was considered statistically significant. Across all the three mutants of MS1,

MS2, MS3, there were no significant differences compared to wild-type E1162 in the overall killing of *G. mellonella* at 6 dpi.

(A)



(B)



(C)

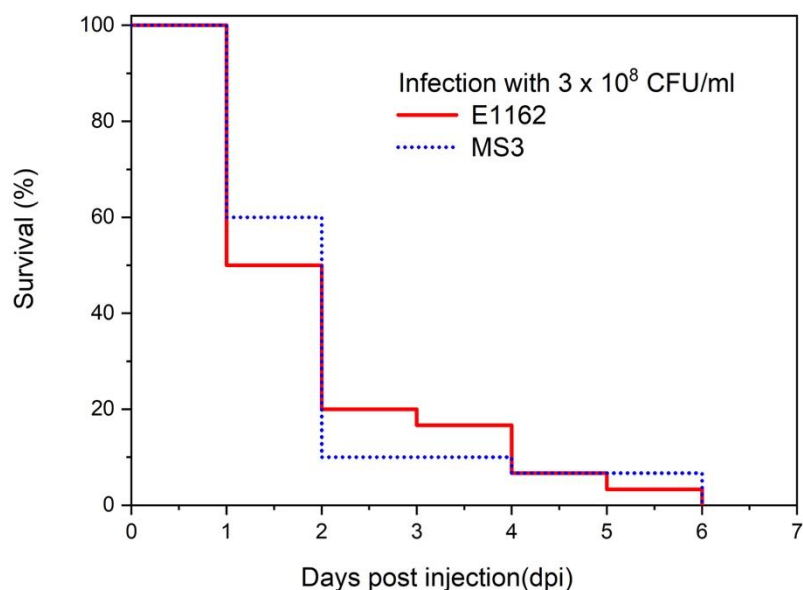


Figure 4-13 Kaplan-Meier survival plots of *G. mellonella* larvae after infection with *E. faecium* E1162 wild-type and its isogenic mutants MS1, MS2 and MS3.

A: E1162 (solid-black line), MS1 (Black-dot). **B:** E1162 (solid -black line), MS2 (Black-dot). **C:** E1162 (solid -black line), MS3 (Black-dot). Data presented are the percentage survival of 30 worms which were tested in groups of 10 on three biological replicates performed in triplicate.

4.5 Discussion

To investigate genes that may play roles in oxidative stress resistance in *E. faecium*, *E. faecium* E1162 *Himar1 mariner* transposon mutant library was screened on TSA supplemented with 64 $\mu\text{g/ml}$ menadione (2-fold lower than the MIC of 128 $\mu\text{g/ml}$) for mutants with increased sensitivity to menadione (a superoxide producer). Of 12,970 transposon mutants, 3 mutants were identified as menadione-sensitive and designated MS1, MS2 and MS3. Menadione concentrations of 64, 80, and 100 $\mu\text{g/ml}$ were used to test the sensitivity of each strain. No phenotypic differences were observed during growth in TSB medium between *E. faecium* E1162 and the mutant strains.

Mutant MS1 was determined to have a transposon inserted at 1421 bp in reverse orientation within a gene coding for a putative ORF EFF34651

(*efmE1162_1492*), encoding a protein of 625 amino acids, annotated in the *E. faecium* E1162 genome sequence as a tyrosine decarboxylase. When compared to *E. faecium* E1162, mutant MS1 is significantly more sensitive to cumene hydroperoxide ($p = 0.05$), but there was no difference in killing *G. mellonella*. When comparing mutant MS1 and 47N an *E. faecium* E1162 nisin-sensitive mutant (Table 2-3), the transposon was found within *efmE1162_1492*. Since, MS1 and 47N have the same gene *tdc* (*efmE1162_1492*), so the spot-plate assay for 47N on 64 µg/ml menadione was performed and the result showed similar growth to E1162 and MS1 (this study, data not shown). No other menadione concentrations were administered for 47N, the spot-plate assay was conducted using 5×10^6 to 2.5×10^5 CFU/5 µl instead of 1×10^3 CFU/ml as recommended by Wang et al. (2017) on TSA instead of MHA, this should be done in the future by following the correct procedure to detect the differences between E1162, MS1 and 47N against menadione sensitivity. The ability of mutant 47N to kill *G. mellonella* differed significantly more slowly ($p > 0.01$) from that of *E. faecium* E1162 and the complementing mutant 47N with *efmE1162_1492* from *E. faecium* E1162 restored the wild-type phenotype (Hashim, 2016). However, there was no significant difference in the mortality of *G. mellonella* larvae between wild-type E1162 and mutant MS1 infections (Chapter IV, section 4.4.7), inferring that the location of the transposon insertion in mutant MS1 (1421 bp) compared to the position insertion in mutant 47N (630 bp) within an ORF EFF34651 which encoding a protein of 625 amino acids annotated in the *E. faecium* E1162 genome sequence as a tyrosine decarboxylase (Hashim, 2016), so the different transposon location within the same *tdc* gene in both mutants MS1 and 47N could be the reason behind the differences in the phenotypic characters of MS1 and 47N. Therefore, the *G.*

mellonella killing assay using *E. faecium* E1162, mutant 47N, and mutant MS1 under the same conditions, as well as complementation of mutant MS1 with *efmE1162_1492* from *E. faecium* E1162, are necessary to investigate the theory that different transposon insertion sites within the base pairs of the same gene result in distinct behavioral traits.

Mutant MS2 was determined to have a transposon inserted in an ORF EFF34605 (*EfmE1162_1516*) encoding a predicted protein of 417 amino acids, annotated in the *E. faecium* E1162 genome sequence as a conserved hypothetical protein. In replica plate print screening, MS2 mutant was found to be sensitive to 64 µg/ml menadione on TSA. However, using the spot-plate assay method on TSA supplemented with the same concentration of menadione (64 µg/ml), there was no difference in the growth of this mutant and *E. faecium* E1162. As a result, MS2 sensitivity to 80 and 100 µg/ml menadione on TSA was compared to that of wild-type *E. faecium* E1162. MS2 was significantly (Mann-Whitney U Test Exact Prob>UII = 0.02) more resistance to 80 and 100 µg/ml menadione compared to *E. faecium* E1162 and the other mutant strains. The explanation for the MS2 mutant that was initially screened and found to be sensitive to 64 µg/ml menadione and then became not only capable of growing on that concentration but also capable of growing on concentrations of menadione (80 and 100 µg/ml) on TSA higher than the wild type E1162 could be due to the size of the initial colony on the master plate thus showing the sensitivity of MS2 to menadione in the replica plate print screening, in addition to the fact that in the spot-plate assay, cells/5 µl of 5×10^6 to 2.5×10^5 bacteria were used instead of 1×10^3 CFU/ml as recommended by Wang et al. (2017). Also, the spot-plate assay was done on TSA agar, however, MHA is

recommended for antimicrobial susceptibility by the Clinical & Laboratory Standards Institute (CLSI) (Ferraro, 2000) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (*EUCAST Disk Diffusion Test Methodology*, 2022) because it is a 'loose' agar, which helps to mediate the rate of antimicrobial diffusion more effectively than other types of media. As a result, the sensitivity of mutant MS2 as well as mutant MS1 to 64, 80, and 100 µg/ml menadione needs to be examined again using 1×10^3 CFU/ml on MHA in future research .

Mutation in mutant MS2 conferred sensitivity to cumene hydroperoxide compared to the wild-type *E. faecium* E1162. Analysis of the predicted protein architecture of EFF34605 shows an N-terminal Pyr_redox_2 domain and GidA domain at the C-terminus. The Pyr_redox_2 domain is found in FAD-flavoproteins belonging to the pyridine nucleotide-disulfide oxidoreductases family. Mutant MS2 was also significantly decreased ($p < 0.025$) in its capacity to kill *G. mellonella* larvae in the first two days compared to the wild-type. Thus, it is possible for E1162 to initiate an infection and can be favored until the environment changes and mutants that can survive in stressful environments become dominant (Faucher et al., 2021). Furthermore, mutant MS2 killed *G. mellonella* at the same rate as wild-type E1162 strains and their ability to kill is not determined by how fast they multiply in *G. mellonella* larvae model. Several tRNA-modification enzymes have been identified with GidA protein domains and are involved in the biosynthesis of 5-methylaminomethyl-2-thiouridine, deletion of *GidA* impairs virulence of several bacterial pathogens (Shippy and Fadl, 2014). Any deficiencies in a tRNA-modification enzyme under oxidative stress might cause accumulation of shorter and defective tRNAs, thus, triggering a

harmful effect on cells (Campos Guillen et al., 2017), this suggest that *E. faecium* might use *efmE1162_1516* to survive during oxidative stress.

Mutant MS3 showed an increased sensitivity to menadione compared to wild-type E1162. This mutant was determined to have an insertion within an ORF EFF33659 (*efmE1162_2615*). Analysis of the predicted protein architecture showed a histidine kinase-like domain at the C-terminus of EFF33659. All forms of life require the translation of environmental stimuli into cellular behavior. This process typically occurs in bacteria, where a sensor histidine kinase autophosphorylates in response to a stimulus before transferring the phosphoryl group to a response regulator that controls downstream effectors (Dikiy et al., 2019). As a result, the *E. faecium* E1162 could be using this gene to detect oxidative stress in the environment.

In the nisin-sensitive mutant 59N, the transposon was inserted in an ORF *efmE1162_1543* encoding a putative muramidase (EFF33496), this strain showed an increased susceptibility to both menadione and cumene hydroperoxide compared to *E. faecium* E1162 and it was found that 59N was suppressed at 64 µg/ml menadione when other mutants (MS1, MS2, MS3) were not, which could be because the *mur* and *nox* genes in *E. faecium* are located on the same putative pathogenicity island (Leavis et al., 2004; Top et al., 2013). It was hypothesized that because the *mur* gene is located directly downstream of the *nox* gene in the pathogenicity island (Leavis et al., 2004; Top et al., 2013) which is involved in the bacterial tolerance against the oxidative stress so *mur* might have the same role as *nox*. Also, the putative muramidase may play a role in bacterial cell wall remodeling, which may be an important stress resistance

factor. As a result, the mutant 59N, which is thought to lack peptidoglycan editing enzymes due to the transposon insertion in *mur* gene so this may find it difficult to respond to the oxidative stress, which may explain why 59N was sensitive to 64 µg/ml mendiane while other mutants were not. Hashim (2016) reported that strain 59N was significantly impaired ($p < 0.01$) in virulence towards *G. mellonella* compared to the wild-type. This strain was significantly attenuated ($p < 0.0001$) in its ability to kill zebrafish larvae compared to *E. faecium* E1162 (this study Chapter III, Figure 3-6). Peptidoglycan-degrading enzymes such as muramidase are tightly regulated to maintain the structural integrity of the bacterial cell wall during growth. By altering their peptidoglycan structures, many bacterial pathogens have acquired methods to fight various host defense measures (Yadav et al., 2018). In Gram-positive bacteria, peptidoglycan recycling is critical for survival in the stationary-phase (Davis and Weiser, 2011; Borisova et al., 2016). Changes in the peptidoglycan structure affect a variety of functions, including lysozyme-resistance, host immunological response, and antibiotic resistance. Bacteria with unaltered peptidoglycan are lysed when they come into contact with lysozyme, which releases peptidoglycan fragments that can be detected by pattern recognition receptors in the host (Davis and Weiser, 2011; Yadav et al., 2018). These changes are significant not only as a means of adapting to specific stresses, but also because the cell wall is chemically edited. These changes will almost certainly have an impact on the activity of other peptidoglycan-associated enzymes, as well as their physical interactions with these new muropeptides. In this case, the lack of peptidoglycan editing enzymes would not only make it difficult for bacteria to adjust to stress, but it would also initiate a cascade of peptidoglycan alteration that would impair cell wall integrity (Yadav et al., 2018). Strain 59N has a mutation in an ORF

efmE1162_1543 encoding a putative muramidase, so compared to *E. faecium* E1162 the mutant 59N may have an altered ability to modify its peptidoglycan backbone.

In conclusion, the current study described in this chapter identified some determinants which might mediate ROS resistance in *E. faecium* E1162 by screening an *E. faecium* E1162-*mariner* transposon library for mutants with impaired resistance to a superoxide generator menadione. Three mutants were identified and their sensitivity toward cumene hydroperoxide, a peroxide generator was also examined. Relative to the parental strain *E. faecium* E1162, mutants MS1 and MS2 were significantly more sensitive to cumene hydroperoxide. MS1 and MS3 had no difference in virulence toward *G. mellonella* when compared to wild-type *E. faecium* E1162, whereas MS2 had a significantly reduced ability to kill *G. mellonella* larvae ($p < 0.025$) in the first two days post injection (Figure 4-13B). The research described in this chapter also tested the sensitivity of mutant 59N (a putative muramidase mutant) to menadione and cumene hydroperoxide (oxidative stress generator). Mutant 59N was found to be more sensitive to menadione and cumene hydroperoxide than wild-type *E. faecium* E1162.

Chapter V

Construction of an *E. faecium* marker-less deletion mutant of *efmE1162_1543* and a *trans*- complemented strain

5.0 Construction of an *E. faecium* marker-less deletion mutant of *efmE1162_1543* and a *trans*-complemented strain

5.1 Introduction

Pathogenicity island is a genomic island harbouring genes that participate in the pathogenic effects of the harbouring bacteria and are generally deficient in non-pathogenic strains of the same species (Hacker and Kaper, 2000). Most PIAs are attained from outside the species via horizontal gene transfer. Hospital-acquired *E. faecium* lineages including ST17, belonging to the CC17 and ST78 have multiple drug resistance, for example, ampicillin and quinolones and have many virulence genes (Bourafa et al., 2016). *E. faecium* has a putative pathogenicity island associated with hospital-acquired outbreaks, this pathogenicity island is transferable and has the characteristics of an integrative conjugative element and hence is called ICE*Efm1*, previously called the *esp* PAI (Hendrickx et al., 2007; Sava, Heikens, Kropec et al., 2010; Top et al., 2011; Willems et al., 2012; Top et al., 2013; Gawryszewska et al., 2016). The ORF *efmE1162_1543*, which codes for a putative muramidase (EFF33496), is part of an operon of five genes located on the ICE*Efm1* in *E. faecium* E1162 (Figure 5-1A), which also includes *efmE1162_1544*, *efmE1162_1542*, *efmE1162_1541*, and *efmE1162_2353*, respectively, had been annotated as a putative NADH oxidase, a hypothetical protein, a putative drug resistance transporter and *esp* (Top et al., 2013).

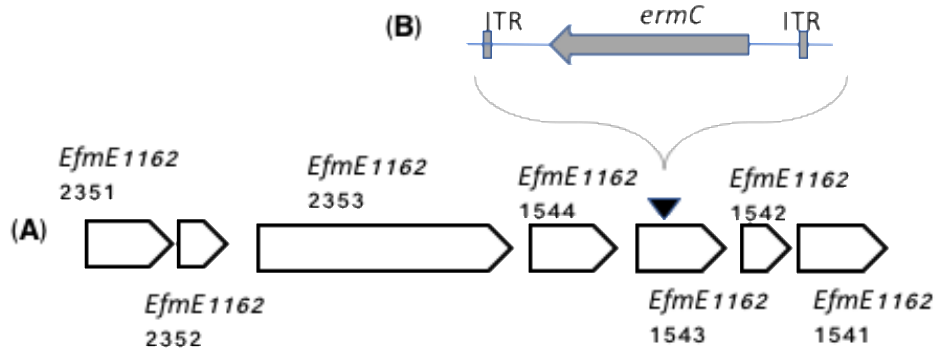


Figure 5-1 (A): Diagram shows the genetic organization of the genes upstream and downstream of *efmE1162_1543*, coding for a putative muramidase.

efmE1162_1541 a putative drug resistance transporter gene; *efmE1162_1542*, a conserved hypothetical protein-coding gene; *efmE1162_1544*, a putative NADH oxidase gene (*nox*); *efmE1162_2353*, enterococcal surface protein; *efmE1162_2352*, a conserved hypothetical protein-coding gene; *efmE1162_2351*, *ebrB*. **(B):** A ~1.4 kb *Himar1 mariner* transposon composed of a Gram-positive erythromycin-resistance gene, *ermC* bordered by a 5'ITR and a 3'ITR of the *Himar1 mariner* inserted in the *efmE1162_1543*.

The mobilization of the *mariner* transposon is a non-replicative, cut-and-paste process that includes the formation of a single-end-complex, two transposase monomers recognise the *mariner* inverted terminal repeats, bind to them via the helix-turn-helix motif (Figure 5-2A) then the 5' ends of the inverted terminal repeats are cleaved by transposase to release the non-transferred strands and the two transposase monomers assemble together generating paired-end complex and transposase dimer (Figure 5-2B). The paired-end complex recognises any TA dinucleotide and binds forming the target capture complex (Figure 5-2C) at which insertion can take place (Muñoz-López and García-Pérez, 2010).

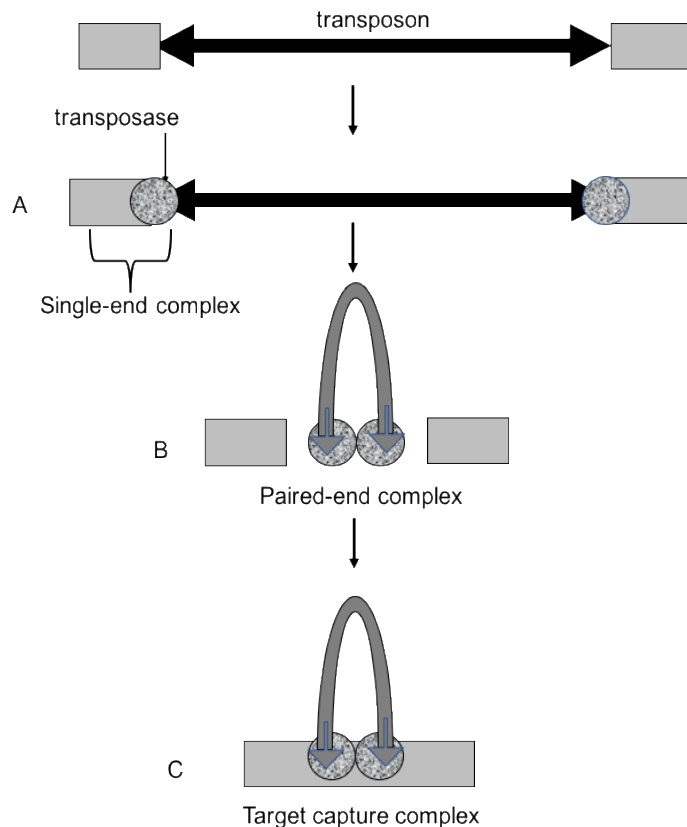


Figure 5-2 Transposition mechanism of the *mariner* transposon.

(A): The transposition starts when two transposase molecules bind to the toll-interleukin 1 receptor, making the single-end-complex. **(B):** Followed by joining the transposon ends via both transposase molecules create a transposase dimer and generating the pair-ends complex, then excision occur. **(C):** Finally, the transposase dimer goes and bind to a random TA dinucleotide forming a target capture complex. The Figure was drawn and adapted from Muñoz-López and García-Pérez, 2010.

Hashim (2016) screened the *E. faecium* transposon mutant library for mutants with altered susceptibility to nisin. Screening of the library identified several mutants that had reduced susceptibility to nisin. The nisin-sensitive mutant 59N which had a transposon inserted in *efmE1162_1543* annotated as muramidase was selected for further studies. The *mariner* transposon inserted in an orientation (Figure 5-1B) within the *efmE1162_1543* in which the erythromycin promoter and the erythromycin gene were in the opposite orientation to the direction of transcription of *efmE1162_1543* and the operon which may have had a polar effect. Thus, the impaired phenotypes that were detected in the

mutant 59N may have been due to disruption of the muramidase gene, but they could also have resulted from polar effects of the transposon on *efmE1162_1544*.

Peptidoglycan is a ubiquitous component of the bacterial cell wall, with a few exceptions, and thus a primary target of various host-produced antimicrobials (Yadav et al., 2018; Naclerio and Sintim, 2020). The epithelium secretes a number of antimicrobial enzymes to protect the host mucosal surfaces from infection. Lysozyme, a host antimicrobial enzyme with muramidase and cationic antimicrobial peptide activity, is one of the most common proteins on the mucosal surface (Davis and Weiser, 2011; Naclerio and Sintim, 2020). The epithelium secretes lysozyme, which is also a key component of the granules of professional phagocytes, where it may aid in the killing of bacterial pathogens within phagolysosomes. Lysozyme's muramidase activity causes hydrolysis of the peptidoglycan backbone's -1,4 glycosidic link between the C-1 carbon of N-acetylmuramic acid (MurNAc) and the C-4 carbon of N-acetylglucosamine (GlcNAc) residues (Davis and Weiser, 2011; Yadav et al., 2018). Bacteria have peptidoglycan-degrading enzymes that cleave at the same locations as antimicrobial enzymes in the host, but they help with regular cell wall growth and turnover.

Figure 5-3 depicts three types of glycosidases that cleave the glycan backbone. Lytic transglycosylases (A) glucosaminidases (B) muramidases (C) muramidases and lytic transglycosylases cleave between the MurNAc and GlcNAc residues and are referred to as N-acetyl-D-muramidases, muramidases produce terminal reducing MurNAc residues, whereas lytic transglycosylases produce a 1,6-anhydro ring (not depicted in Figure 5-3). GlcNAc residues are

hydrolyzed by N-acetylglucosaminidases (D and F) from adjacent sugar residues. The amidases (E) are divided into N-acetylmuramyl-L-alanine amidases and endopeptidases, which cleave the peptide moiety. The amide bond between MurNAc and L-alanine is hydrolyzed by N-acetylmuramyl-L-alanine amidases, which separates the glycan strands from the peptides. Endopeptidases are enzymes that cleave the bonds in stem peptides and peptide bridges. Carboxypeptidases, in particular, cleave the peptide chain's C-terminal amino acid (D-alanine).

PGN lytic enzymes

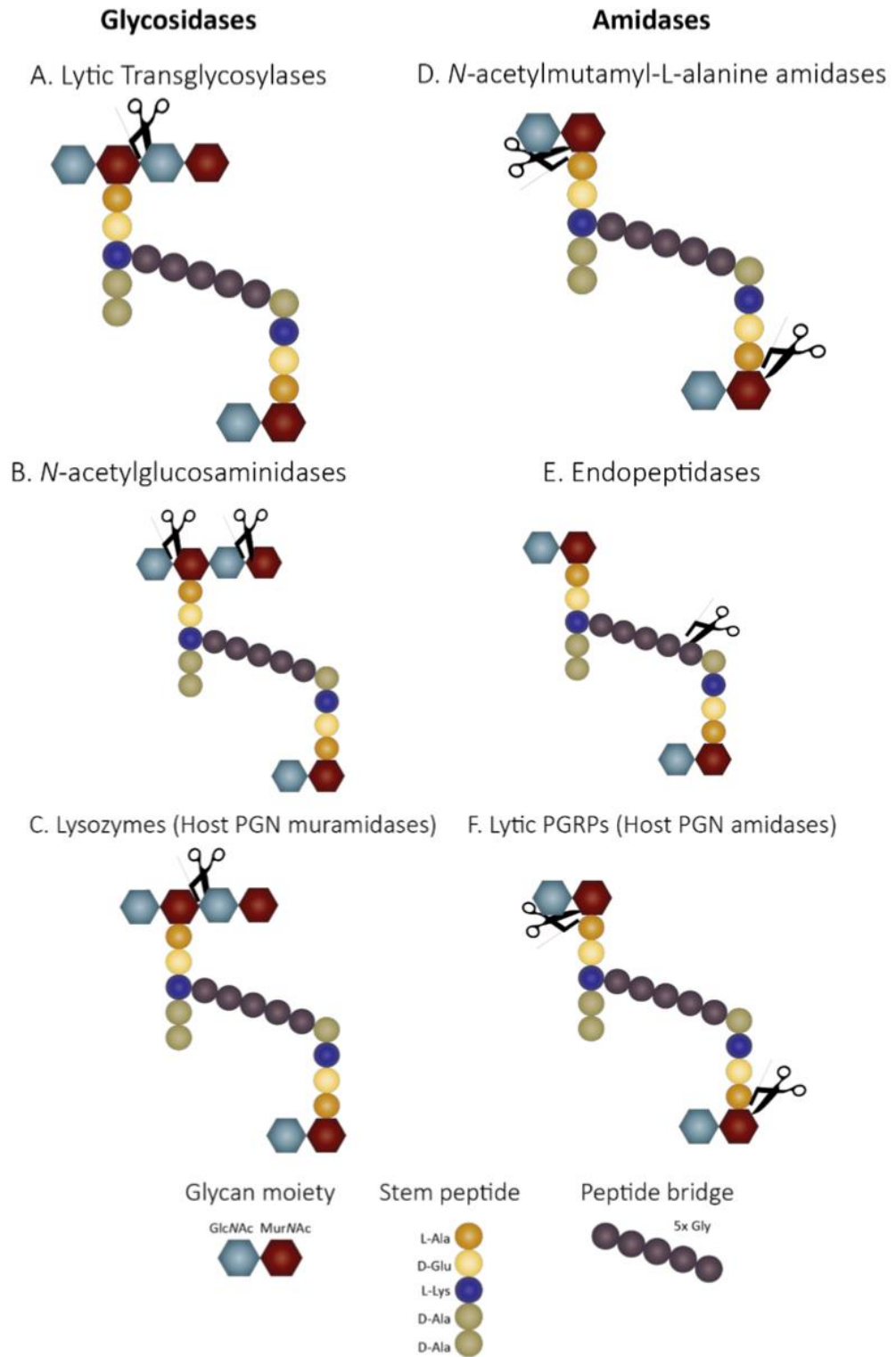


Figure 5-3 A peptidoglycan model depicting the hydrolytic actions of peptidoglycan by glycosidases and amidases. Adapted from (Vas, 2016).

5.2 Aims of this chapter

Generating mutants to determine how genes contribute to any relevant phenotype is necessary for understanding microbial behaviour so the aims of the work described in this chapter were to construct an *E. faecium* markerless deletion mutant with deletion of *efmE1162_1543* and to compare the virulence of the *E. faecium* E1162, the mutant 59N and the constructed deletion mutant of *efmE1162_1543* using a *G. mellonella* infection model; to see whether *trans*-complementation of the 59N mutant with *efmE1162_1543* could restore the wild-type virulence in the *G. mellonella* infection model or a transposon polar effect may explain the attenuated phenotypic of 59N in *G. mellonella*.

5.3 Materials and methods

5.3.1 Preparation of *E. coli* calcium chloride competent cells

A single colony of *E. coli* EC1000 cells was grown for 16 hrs at 37°C with shaking at 220 rpm in SOC medium (Invitrogen). The next day, the 16 hrs old bacterial culture was inoculated into fresh SOC (1:100) and grown at 37°C with shaking at 220 rpm until log phase. The cultures were incubated until they reached an OD_{600 nm} of 0.4. 40 ml of this culture was then centrifuged at 6000 xg for 5 min at 4°C and the cell pellet was resuspended in 20 ml ice-cold 0.1 M CaCl₂ (1/2 dilution of the culture). The cell suspension was incubated on ice at 4°C for 1 hr, followed by centrifugation at 6000 xg for 5 min at 4°C. The cell pellet was again resuspended in 4.0 ml ice-cold 0.1 M CaCl₂ (1/10 dilution of the culture), then sterile ice-cold glycerol was added to a final concentration of 20%. Finally, aliquots each of 100 µl were made and stored at -80°C till used (Chang et al., 2017).

5.3.2 Transformation of chemically competent *E. coli* cells

One Shot® TOP10 and EC1000 chemically competent *E. coli* strains were transformed using the quick chemical transformation protocol as described by the manufacturer's instruction (Invitrogen). Usually, 3-5 µl of the ligation reaction mixture was used to transform one vial of competent cells.

5.3.3 Blue-white screening system in *E. coli*

To distinguish between recombinant plasmids and re-ligands the blue-white selection was used prior to a transformation reaction. This procedure was limited to unique *E. coli* strains (encoding the C-terminal part of the β-galactosidase) and specifically vectors (harbouring the multiple cloning sites within the *lacZ* gene). For this selection, One Shot™ TOP10 cells that do not require isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce expression from the lac promoter were transformed. The LB agar plates contained 50 µg/ml ampicillin and 40 mg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside on which the transformations were plated, then incubated with 40 mg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside overnight at 37°C. White colonies, which indicate the existence of recombinant plasmids were chosen for more investigation (Padmanabhan et al., 2011).

5.3.4 Electroporation of *E. faecium* E1162 and isogenic mutant 59N

As described by Zhang et al. (2012) *E. faecium* E1162 was grown in 10 ml BHI (16 hrs, 220 rpm and 37°C), cell culture was then diluted 1000-fold in 25 ml fresh BHI in the presence of 1% glycine and 200 mM sucrose and again incubated for 16 hrs at 37°C. The next day, cells were washed by repeated gentle pipetting in 15 ml BHI (ice-cold) for 2 min and then centrifuged at 2900 xg for 15 min at 4°C. The wash procedure was repeated 3 times and then cells resuspended in 25 ml

of BHI, followed by incubation at 37°C with shaking at 220 rpm for 1 hr. Subsequently, cells were resuspended in 1.25 ml wash buffer (ice-cold). About 0.1–1 µg of purified plasmid was mixed with an aliquot of 100 µl of the cell suspension and placed into an ice-cooled 2-mm gap electroporation cuvette and left on ice for 30 min prior to electroporation. Electroporation was done using 2.5 kV, 25 µF capacitance and 400 Ω resistance. 900 µl of BHI were added to the electroporation mixture, incubated for 1 hr (25°C, 200 rpm) then transferred to a 28°C shaking incubator, 200 rpm for 1 hr. Subsequently, 100 µl aliquots were plated out on TSA supplemented with 300 µg/ml gentamicin then incubated at 28°C and were monitored for 72 hrs, random colonies were chosen for more investigation. For recovery of electrocompetent mutant 59N cells after transformation 900 µl of BHI was added to the electroporation mixture, incubated for 1 hr (37°C, 200 rpm) then 100 µl aliquots were plated out on TSA containing 10 µg/ml chloramphenicol, incubated at 37°C and were monitored for 72 hrs, resultant colonies were selected for additional confirmation.

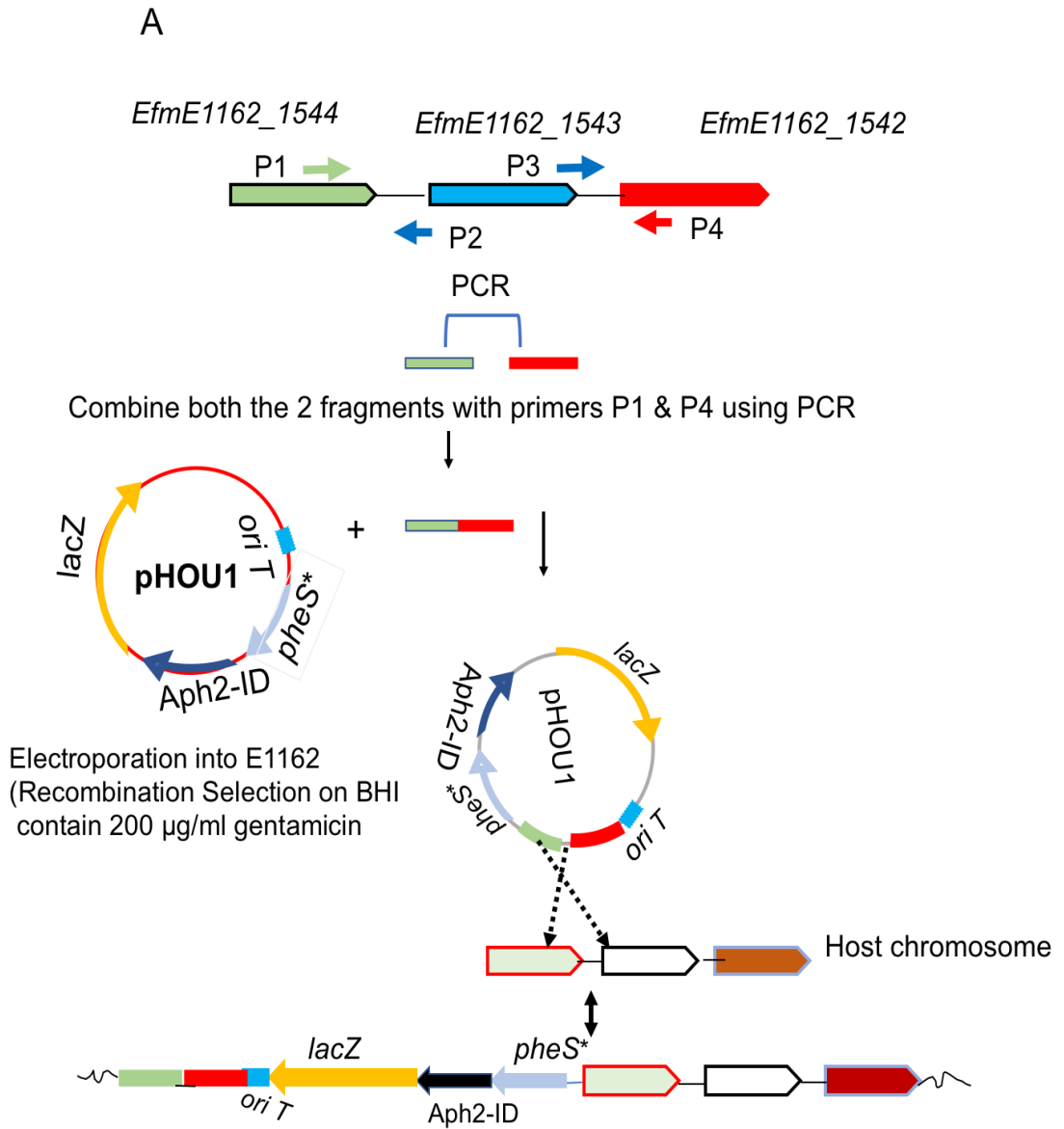
5.3.5 Construction of an *E. faecium* markerless deletion mutant of the gene for the putative muramidase (*efmE1162_1543*)

Bacterial strains, plasmids and primers used in this chapter are presented in Chapter II (Table 2-3 and Table 2-5). Allelic exchange is an important genetic technique that can be used to exchange a wild-type chromosomal allele of a target gene with a gene or DNA sequence that is different or has been altered *in vitro* (Johnson et al., 2003; Pritchett et al., 2004; Panesso et al., 2011; Faulds-Pain and Wren, 2013). Allelic exchange has the advantage that mutant alleles are positioned in their natural chromosomal context, which makes genetic analysis of their effects easier (Kristich et al., 2005). Additionally, multiple

mutations can be serially introduced into the same strain with markerless exchange without the need for distinct antibiotic resistance markers that might not be available or might otherwise interfere with subsequent genetic analysis. The homologous recombination procedure involves first introducing the knockout plasmid into the target strain, which must be incubated at high temperatures to insure plasmid integration. In order to excise the plasmid and parts of the gene of interest, growth at very low temperatures is required, without antibiotic selection (Schuster et al., 2019). The pHOU1 counter-selection system was used to attempt to generate an *E. faecium* markerless deletion mutant of the putative muramidase gene, *efmE1162_1543*. The suicide plasmid pHOU1 contain the counter-selectable marker *pheS**, a mutation in *pheS** (G294A) relaxed the substrate specificity of phenylalanine-tRNA synthetase allowing toxic phenylalanine analogs like p-chlorophenylalanine to be incorporated into proteins instead of phenylalanine, by inhibiting the replication of cells with pHOU1 plasmids in their chromosome by using toxic compounds would allow the selection for the loss of the plasmid backbone (counter/negative-selection) (Schuster et al., 2019). As pHOU1 is a suicide plasmid because it lacks an origin of replication that identifies it for replication by the host cell. When cells divide, plasmids without an origin of replication will not replicate and will become diluted away after subsequent generation in BHI broth over 7-9 days until the single cross-over integrants turn to double cross-over integrants. To construct an *E. faecium* E1162 deletion mutant of *efmE1162_1543*, a 1000 bp upstream and downstream of the *efmE1162_1543* locus, including the start and stop codon of *efmE1162_1543* were PCR amplified using genomic DNA from *E. faecium* E1162 and the following primers: (Del-*mur*-F1-*Eco*R1, Del-*mur*-R1 and *mur*-F2, Del-*mur*-R2-*Not*I). Then, the

downstream and upstream regions were joined using PCR and primers *Del-mur-F1-EcoR1* and *Del-mur-R2-Not1* through the overlapping sequence included in the primers. Subsequently, the resultant PCR construct was cloned into pCR2.1 to give pCR2.1- Δ *mur*-construct (Figure 5-4A). The correct insert was confirmed by sequencing, then released from pCR2.1 with *EcoR1-Not1* and ligated with pHOU1 digested with the same enzymes, the resultant plasmid was designated pHOU1- Δ *mur*-construct. The pHOU1- Δ *mur*-construct ligation was transformed into *E. coli* EC1000, a replication protein A (RepA⁺) strain which allows replication of pHOU1 in this strain. The transformants were selected on LB agar supplemented with gentamicin (25 μ g/ml). The plasmid was purified and introduced into *E. faecium* E1162 via electroporation then plated onto BHI agar supplemented with 300 μ g/ml gentamicin as described in section 5.3.4, then selected for single-crossover integrants since the plasmid cannot replicate in *E. faecium* and carried the *aph2''-ID* gene which confers resistance to gentamicin. Colonies were individually transferred into 50 μ l of 0.85% NaCl and plated on MM9YEG media in the presence of 7 mM *p*-Cl-phenylalanine (Chapter II, Table 2-1). Plasmid pHOU1 carries a counter-selective marker, a mutated *pheS** allele, this mutation yields a mutated phenylalanine tRNA synthetase (Gly294Asp) with a distorted substrate specificity allowing incorporation of *p*-chloro-phenylalanine (a toxic phenylalanine analogue), accordingly, *pheS** positive bacteria would be killed if grown on *p*-chloro-phenylalanine containing media. Therefore, following 48 hrs incubation at 37°C on media supplemented with 7 mM *p*-Cl-phenylalanine colonies that grew should be excisants lacking plasmid. These colonies were confirmed by replica plating on BHI supplemented with and without 300 μ g/ml gentamicin (Guzman Prieto et al., 2015). Finally,

sensitive excisants were chosen and checked for the deletion in the gene by PCR.



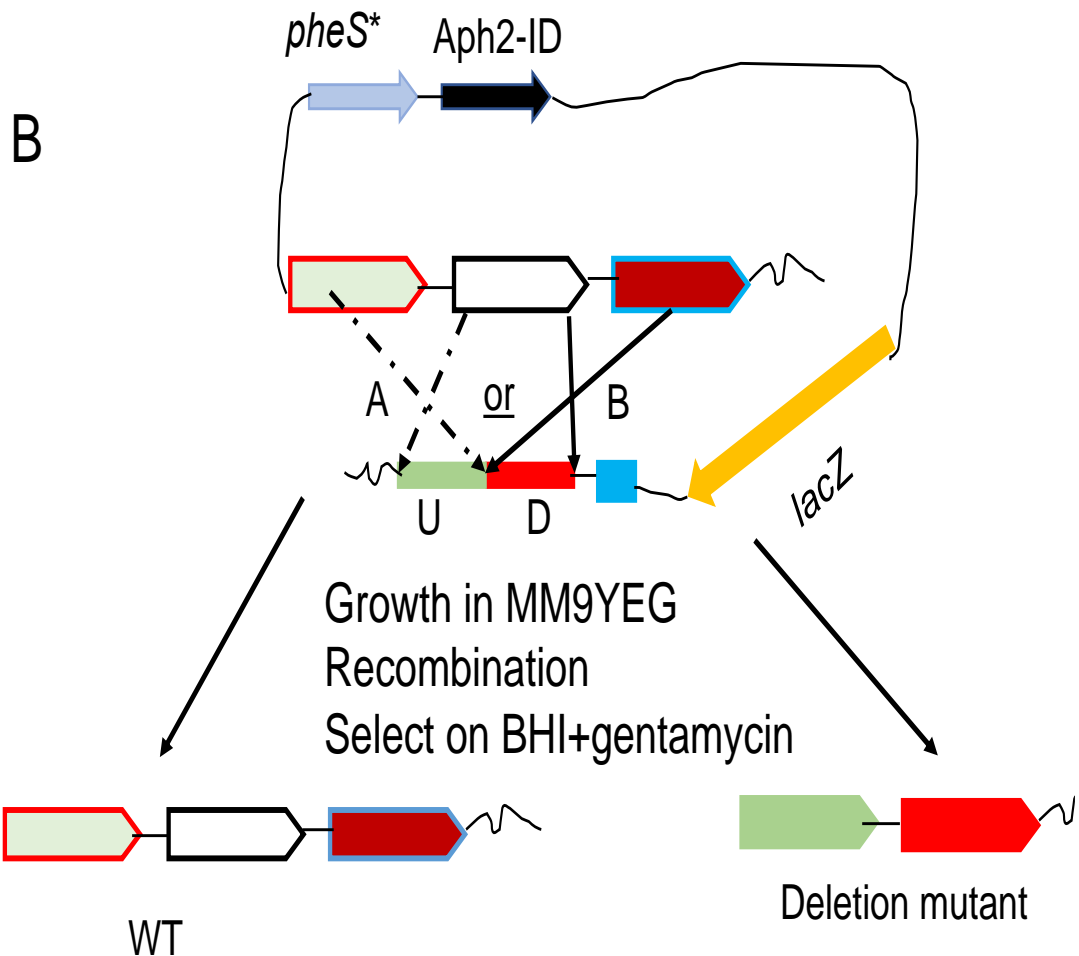


Figure 5-4 Diagram shows the allelic exchange strategy applied to generate a deletion of the *efmE1162_1543* in *E. faecium* E1162.

(A) Shows the joined fragment of the PCR products ligated in plasmid pCR2.1 followed by electroporation of *E. faecium* E1162 with pHOU1- Δmur construct, the entire plasmid is integrated into the *E. faecium* E1162 chromosome by a single-crossover between the homologous genes, producing a chromosomal duplication. The excised plasmid cannot replicate, thus was lost by segregation. The chromosomal duplication is segregated by homologous recombination. (B) After leaving one copy of the gene, loss of the plasmid can result genetic complementation of mutant 59N with an intact *efmE1162_1543* gene coding for the putative muramidase in the restoration of original wild-type allele or the desired allelic exchange mutation depending on where the recombination event occurs. Diagram adapted from Lehman et al. (2016), pCR2.1 adapted from <https://www.xenbase.org/reagents/vectorAction.do?method=displayVectorSummary&vectorId=1221309>.

Bacterial strains, plasmids and primers that were utilized in this work are described in Chapter II (Table 2-3 and Table 2-5). For in *trans*-complementation of mutant 59N with its native *efmE1162_1543* (*mur*), the *efmE1162_1543* gene was PCR amplified from *E. faecium* E1162 chromosomal DNA by using Q5 High-Fidelity DNA polymerase (NEB) and primers (Comp-*mur*_F1467-*Bam*H, Comp-*mur*-R1467-*Sph*1) (Chapter II, Table 2-5) and cloned into the expression vector pHFH4 under the control of a kanamycin promoter. First, the PCR product was cloned into the pCR2.1 vector as outlined by the manufacturer's instructions, using the TOPO TA Cloning Kit, with pCR2.1-TOPO (Chapter II, Table 2-6) to give pCR2.1-*mur*. Transformation of pCR2.1-*mur* into *E. coli* was as detailed in this chapter (Figure 5-4). The plasmids isolated from resultant colonies were screened for the presence of *efmE1162_1543* by restriction digestion. The insert was verified by sequencing and following digestion with *Bam*H1-*Sph*1 was gel purified, ligated to pHFH4 digested with the same enzymes, downstream of the kanamycin promoter, and then transformed into *E. coli* DH5 α . The plasmid pHFH4-*mur* was isolated from *E. coli* DH5 α grown on LB broth supplemented with ampicillin 100 μ g/ml and introduced into *E. faecium* strain 59N (Hashim, 2016) generating 59N::*mur*.

5.3.6 Virulence of *E. faecium* E1162, mutant 59N and 59N::*mur* in the *G. mellonella* infection model

G. mellonella larvae were injected with bacterial suspensions as outlined in Chapter IV, section 4.3.10.2 with mutant 59N which had the transposon inserted in *efmE1162_1543* encoding putative muramidase, strain 59N::*mur* and 59N containing pHFH4, information about these strains are listed (Chapter II, Table 2-3). Larvae survival was observed over time. Experiments continued up to 5 days using 10 worms on three different occasions. Infected larvae were checked

daily, and the number of live larvae was noted (Ramarao et al., 2012; Hashim, 2016).

5.4 Results

5.4.1 Construction of markerless deletion mutant of *efmE1162_1543*

Figure 5-5 shows amplified PCR products of the downstream (1000 bp) and upstream (1000 bp) regions of the *efmE1162_1543* and their PCR combination (~2.0 kb), while Figures 5-6 and 5-7 show the release of the Δ *efmE1162_1543* PCR construct from pCR2.1- Δ *efmE1162_1543* and pHOU1- Δ *efmE1162_1543* respectively, following digestion with *EcoR1-Not1*. The pHOU1- Δ *efmE1162_1543* construct plasmid was then used to electroporate *E. faecium* E1162 as described in section 5.3.5. Colonies which grew in the presence of 300 µg/ml gentamicin were considered as single-crossover integrants. Single-crossover integrants which grew in the presence of 7 mM *p*-Cl-Phe (para-chlorophenylalanine) and did not grow on media contained 300 µg/ml gentamicin were selected for confirmation of deletion *efmE1162_1543* by PCR. However, when PCR was carried out no deletion mutants of *efmE1162_1543* were obtained and this could be due to a low frequency of the events leading to the desired deletion. For additional confirmation, a mixture of small and large colonies were observed when the mutant cells were plated onto BHI agar plates containing 7 mM *p*-chloro- phenylalanine, large colonies are presumably expected to lose the plasmid, while small colonies have probably retained the plasmid, which confers gentamicin resistance to these bacteria. To demonstrate that large colonies were indeed plasmid-free, 15-20 large and 15-20 small colonies per experiment were patched onto gentamicin-containing BHI plates. All small colonies isolated from BHI *p*-chlorophenylalanine plates were

gentamicin-resistant, indicating that the pHOU1 pheS* plasmid was still present. Large colonies, on the other hand, were mostly gentamicin-sensitive, indicating that the knockout plasmid had been lost.

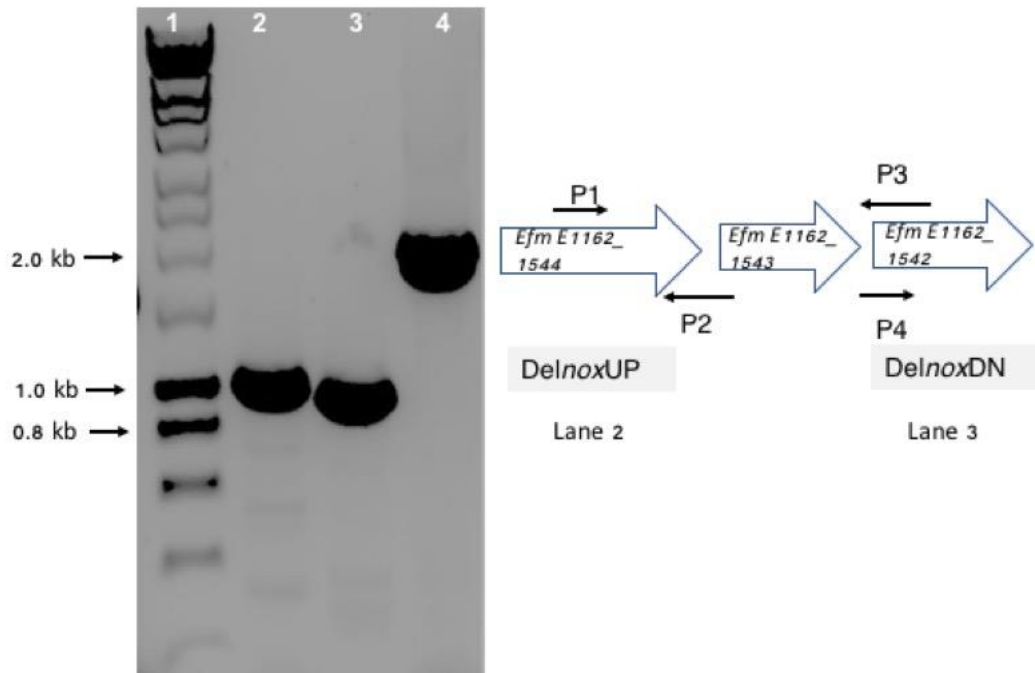


Figure 5-5 Gel electrophoresis picture of amplified downstream and upstream regions of *efmE1162_1543*.

Lane 1: HyperLadder 1 kb Plus (Bioline), **lanes 2 and 3:** downstream and upstream fragments of *efmE1162_1543* respectively, **lane 4:** combination of amplified downstream and upstream of *efmE1162_1543*.

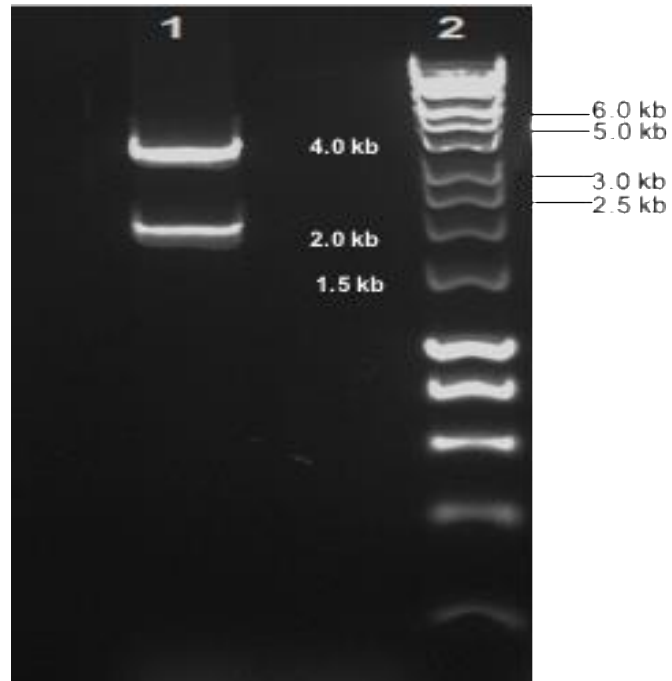


Figure 5-6 Gel electrophoresis picture confirming cloning of $\Delta efmE1162_{1543}$ construct into plasmid pCR2.1.

Lane 1, *EcoRI-NotI* restricted pCR2.1- $\Delta efmE1162_{1543}$ (expected digestion pattern: two fragments at sizes ~2.0 kb and ~3.9 kb); **lane 2**, HyperLadder 1 kb Plus (Bioline).

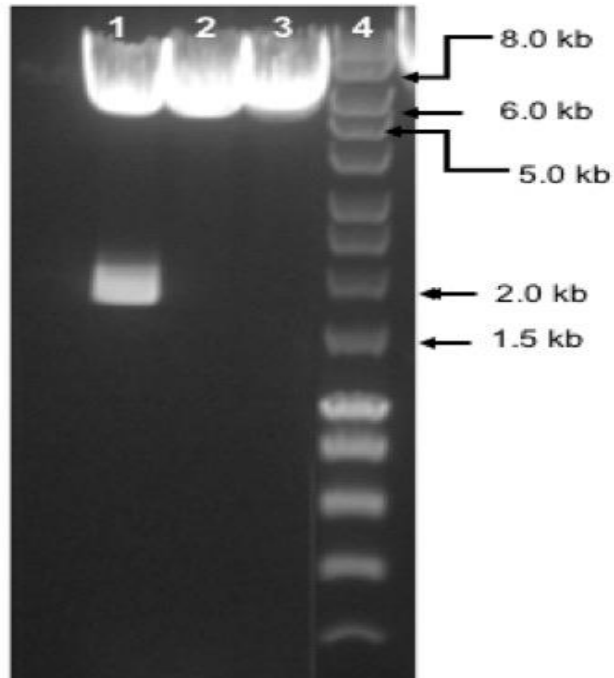


Figure 5-7 Gel electrophoresis picture confirming cloning of $\Delta efmE1162_{1543}$ construct into the deletion vector pHOU1.

Lanes 1,2,3: plasmids potentially containing the $\Delta emE1162_{1543}$ construct were digested with *EcoRI-NotI* (expected digestion pattern: two DNA fragments one ~ 6.9 kb and one ~2.0 kb), **lane 4**: HyperLadder 1 kb Plus.

5.4.2 Trans-complementation of mutant 59N with an intact *efmE1162_1543*

The *efmE1162_1543* gene was PCR amplified from *E. faecium* E1162 chromosomal DNA by using primers comp-*mur*_F1467-*Bam*HI and comp-*mur*_R1467-*Sph*I, the PCR product was confirmed by agarose gel electrophoresis as shown in Figure 5-8.

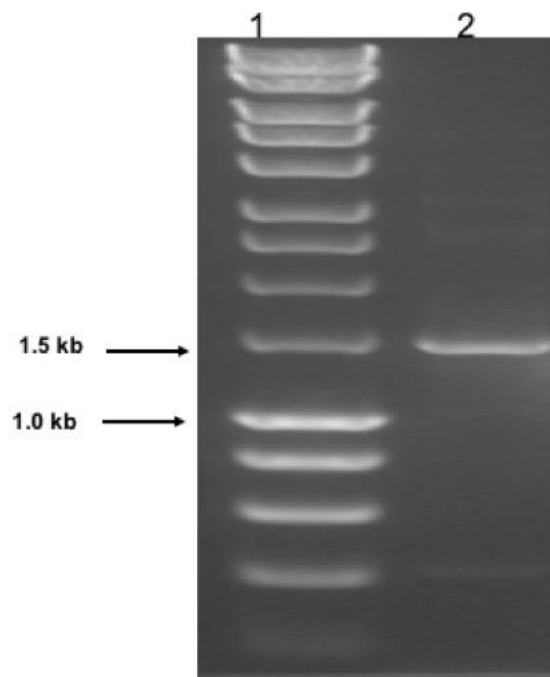


Figure 5-8 PCR amplified product of *E. faecium* E1162 *efmE1162_1543* encoding a putative muramidase (EFF33496) fractionated on 1% (w/v) agarose gel electrophoresis.

Lane 1: HyperLadder 1 kb Plus (Bioline), **lane 2:** amplified product of *efmE1162_1543* (expected size 1467bp).

The PCR product was ligated with the pCR2.1 vector and the ligation mix were transformed into *E. coli*. Plasmids were purified from transformants and verified by digestion with *Bam*HI-*Sph*I. Figure 5-9 shows the plasmid pCR2.1-*mur* generated fragments one of about 1.5 kb corresponding to the size of the *efmE1162_1543* and one of about 3.9 kb corresponding to the size of pCR2.1 vector after digestion with *Bam*HI-*Sph*I.

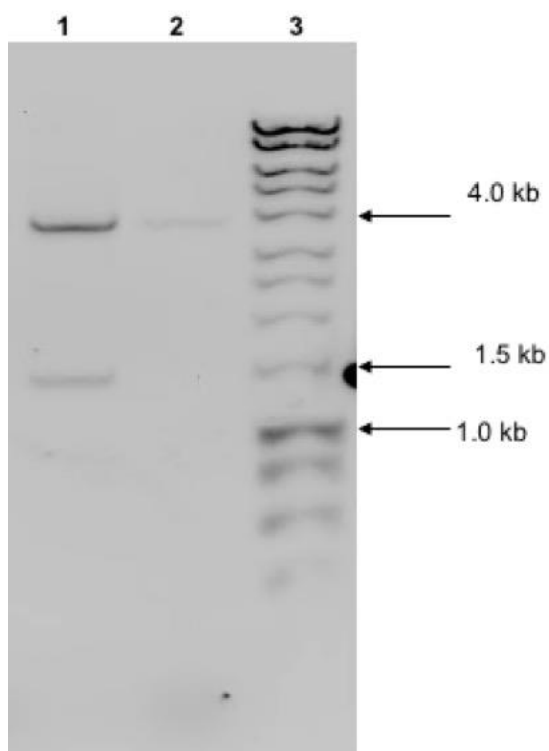


Figure 5-9 Restriction digestion of pCR2.1-*efmE1162_1543*.

Lane 1: pCR2.1-*efmE1162_1543* after digestion with *Bam*HI-*Sph*I (expected digestion pattern: two DNA fragments one of ~ 3.9 kb and one of ~ 1.467 kb), **lane 2:** an empty pCR2.1 restricted with the same enzymes *Bam*HI-*Sph*I (expected digestion pattern: one fragment of ~ 3.9 kb), **lane 3:** HyperLadder 1 kb Plus (Bioline).

The 1.5 kb band containing *efmE1162_1543* was extracted from the gel, purified, and ligated with similarly digested-gel-purified pHFH4 and then transformed into *E. coli* DH5 α , plasmids were purified from transformants and designated pHFH4-*mur*. Digesting of the pHFH4-*mur* with *Bam*HI-*Sph*I generated a fragment of about 1.5 kb, corresponding to the size of the *efmE1162_1543* and one of about 5.0 kb corresponding to the size of the pHFH4 vector (Figure 5-10). The construct was verified by sequencing. subsequently, the purified plasmid construct pHFH4 containing *mur* was introduced into the mutant 59N by electroporation as described in 5.3.5 and colonies were recovered on BHI agar plates containing chloramphenicol (10 μ g/ml), the obtained transformants (5 colonies) each was grown in BHI broth containing chloramphenicol (10 μ g/ml) for plasmids isolation, then the PCR was

carried using the primers *comp-mur_F1467-BamHI*, *comp-mur-R1467-SphI* (Chapter II, Table 2-5) and the isolated plasmids. The PCR generated fragments of about 1.5 kb corresponding to the size of the *efmE1162_1543* (Figure 5-10).

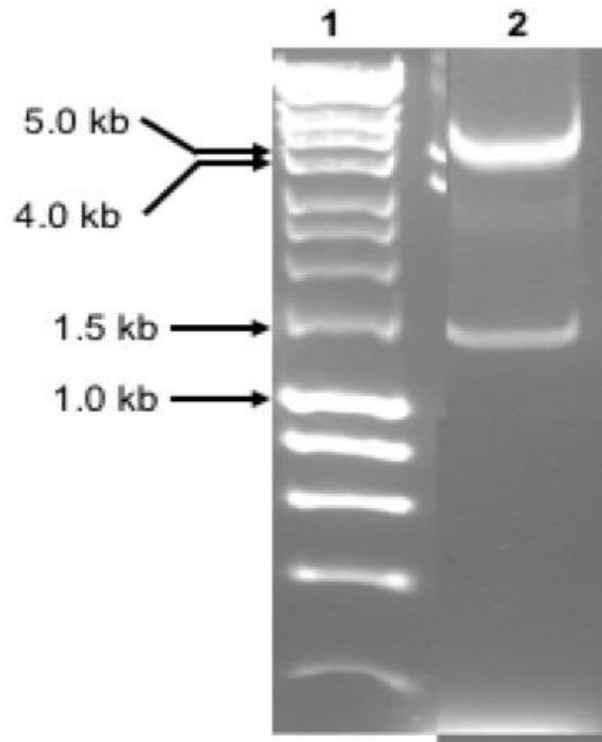


Figure 5-10 Gel electrophoresis of restriction enzyme digested plasmid pHFH4-*efmE1162_1543* for confirmation of insert.

Lane 1: Hyper-Ladder 1 kb Plus. **Lane 2:** pHFH4-*efmE1162_1543* digested with *BamHI-SphI* (*efmE1162_1543*~1467 bp) (expected digestion pattern: two DNA fragments one ~ 5.0 kb and one ~1467 bp).

5.4.3 Comparison of survival of *G. mellonella* larvae infected with *E. faecium* E1162, mutant 59N and 59N::*mur*

No deaths were seen in the control PBS-injected larvae over the 5 day time course, the mutant 59N containing empty pHFH4 did not kill any of the infected worms after 1 dpi while strain 59N::pHFH4-*mur* killed all the infected larvae after 1 dpi (Figure 5-11).



Figure 5-11 Picture of the wax worm larvae challenged with 10 μ l of 5×10^8 CFU/ml.

A: 59N::pHFH4-*mur* displaying complete melanisation (indicating the larvae's mortality) after one day post injection. **B:** Larvae injected with mutant 59N containing empty pHFH4.

Infection of *G. mellonella* larvae with 3×10^8 CFU/ml E1162 (empty pHFH4) killed 3/30 (10%), 20/30 (66.6%), 26/30 (86.6%), 29/30 (96.6%), and 30/30 (100%) after 1 dpi, 2 dpi, 3 dpi, 5 dpi and 6 dpi, respectively. Infection of larvae with the same dose (3×10^8 CFU/ml) of 59N (empty pHFH4) killed 3/30 (10%), 14/30 (46.6%), 19/30 (63.3%), 21/30 (70%) at 1 dpi, 2 dpi, 3 dpi, and 4 dpi, respectively thus there was no increase in mortality until 6 dpi. In contrast, infection with 3×10^8 CFU/ml 59N::*mur* resulted in 100% mortality of the infected larvae after 1 dpi signifying an increased virulence of the complemented strain 59N::*mur* in this model (Figure 5-12). The log-rank test was used to determine the significance of differences in survival rates, and the *p*-values for larvae infected with 59N (empty pHFH4) at 3×10^8 CFU/ml) was $p > 0.009$. The reduced virulence of the 59N (Empty pHFH4) and the increased virulence of the 59N::*mur* indicate that the *E. faecium* *efmE1162_1543* is required for *E. faecium* virulence in *G. mellonella* larvae model.

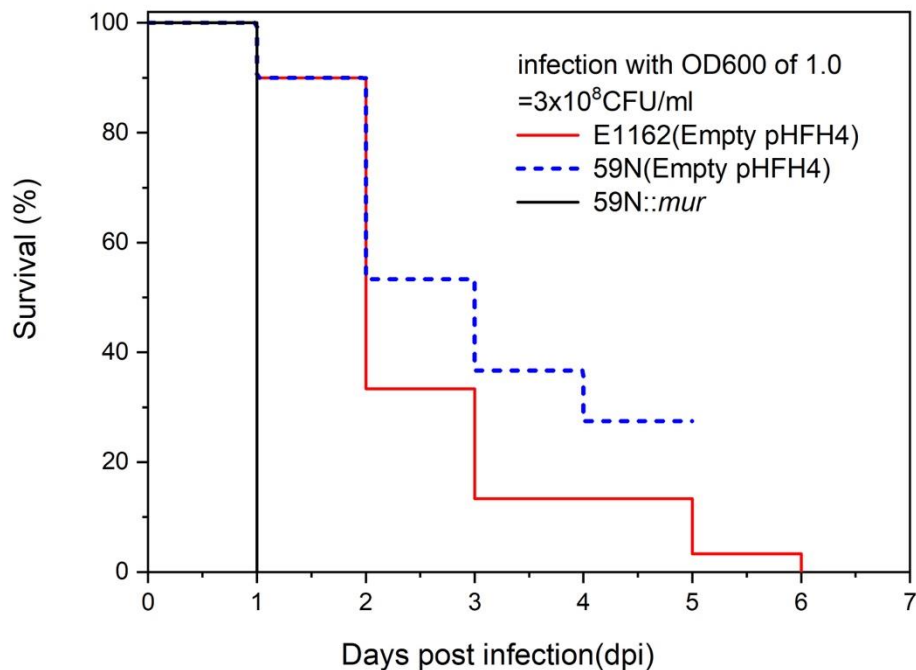


Figure 5-12 Kaplan-Meier survival curve of wax worms over 5 days post-infection with *E. faecium* E1162-empty pHFH4, 59N-empty pHFH4 and 59N::mur.

Infections were done with 3×10^8 CFU/ml. Ten worms were used for every test group and each test repeated at least 3 times on different occasions. **Red solid** = E1162 (Empty pHFH4), **blue short dashes** = 59N (Empty pHFH4), **black solid** = 59N::mur.

5.5 Discussion

E. faecium is a common Gram-positive commensal bacterium in the human gastrointestinal tract, is a cause of nosocomial-infections and a raising health concern owing to its growing resistance to multiple antibiotics. As per Zhang, de Maat et al. (2017) the mechanisms by which *E. faecium* can persist and grow in the host have not yet been identified.

The *E. faecium* strain 59N contains a transposon inserted in a predicted ORF EFF33496 annotated in the *E. faecium* E1162 genome sequence as *efmE1162_1543* encoding a putative muramidase. This strain was sensitive to the antimicrobial nisin and was impaired in its ability to kill *G. mellonella* larvae compared to *E. faecium* E1162 (Hashim, 2016). The *efmE1162_1543* gene is a

part of 150 kb putative pathogenicity island. To further see whether the impaired phenotypes seen in this strain were due to inactivation of *efmE1162_1543* or because of an indirect effect of the transposon insertion on neighbouring genes on this pathogenicity island, we aimed to construct a strain harbouring a markerless deletion of *efmE1162_1543*. The pHOU1 counter-selection system, however, after the introduction of the deletion construct into *E. faecium*, no deletion mutants of *efmE1162_1543* were obtained and this might depend on where the recombination event occurs, plasmid loss can result in the restoration of the original wild-type allele or the desired allelic exchange mutation so if the second event happened at the same region of homology as the first event, therefore, the cell reverts to the wild-type (Faulds-Pain and Wren, 2013) or could be due to a low frequency of the events leading to the desired deletion.

β -N-acetyl-muramidases hydrolysing the β -N-glycosidic bonds that linking N-acetylglucosamine and N-acetylmuramic acid in peptidoglycan, this hydrolysis is an important step in bacterial cell wall remodelling which is an essential step for bacterial cell growth and division. The impaired phenotypes seen in mutant 59N which had the transposon inserted within *efmE1162_1543* annotated in *E. faecium* E1162 as encoding putative muramidase may be due to impaired cell wall remodelling in this strain because of the transposon disruption.

Peptidoglycan is the primary structural component of the bacterial cell wall and hosts hydrolytic enzymes target peptidoglycan causing bacterial cell lysis. To escape killing by hydrolytic enzymes, bacteria could modify their peptidoglycan backbones, these modifications maintain bacterial cell wall rigidity which allows bacteria to persist inside the host. Peptidoglycan modification can also minimize

the release of peptidoglycan fragments which have an important role in initiating the host immune response. Host lysozyme is a major component inside the granules of professional phagocytes, where it may help in eliminating bacterial pathogens within phagolysosomes (Markart et al., 2004). Bacteria lacking certain muramidases are lysed upon interaction with host lysozyme, causing releases of peptidoglycan fragments that can be detected by host PRRs. Peptidoglycan that is fully modified is resistant to lysozyme and does not generate fragments that can be sensed by the host prior degradation by lysozyme, while unmodified peptidoglycan is easily broken down by lysozyme and releases proinflammatory fragments (Davis and Weiser, 2011; Irazoki et al., 2019). The disruption of *efmE1162_1543* by *Himar1 mariner* transposon insertion was found to be involved in *E. faecium* E1162 survival, colonisation, and virulence in *G. mellonella* larvae, zebrafish larvae and planarian infection models suggesting that *efmE1162_1543* may also contribute to virulence in mammals.

To summarize, the creation of an *E. faecium* E1162 markerless mutant with a deletion of the putative muramidase component *efmE1162_1543* failed, whereas *trans*-complementation of mutant 59N with the putative muramidase gene from wild-type *E. faecium* E1162 using plasmid pPHF4 under a constitutive kanamycin promoter succeeded. The complemented strain was more virulent than the parent strain E1162 in the *G. mellonella* infection model and this could be due to the use of a constitutive promoter.

Chapter VI

General discussion, Limitations and Future work

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6.0 General discussion, Limitations and Future work

6.1 General discussion

Owing to its intrinsic and acquired resistance to different antimicrobial agents *E. faecium* has emerged as a problematic cause of human clinical infections (urinary tract infection, nosocomial bloodstream infections, endocarditis, abdominal and pelvic blisters, and chronic periodontitis) (Silva et al., 2011; Akhter et al., 2014; Bhardwaj et al., 2017). Nosocomial *E. faecium* isolates can colonise both carriers and patients, tolerate host defences and to survive in the hospital environment (Fiore et al., 2019). To establish an infection, a pathogen must be able to adapt to a changing environment by changing gene expression levels of regulators and effectors of the stress response (Yuen and Ausubel, 2014). The ability of *E. faecium* to persist within the host, to adapt and responses to environmental stress are not fully understood, thus, identification of the genes necessary for *E. faecium* host-persistence, adaptation and responses to environmental stress is required.

Random transposon mutagenesis is a genetic tool that permits random mutation of genes across the genome (Kalindamar et al., 2019) and can lead to identification of genes which are important for growth, survival, and virulence. Several different transposon-based gene delivery systems have been used to make mutant libraries in bacteria (Lampe, 2010; Hoffmann et al., 2012; DeJesus et al., 2017). The *Himar 1 mariner* transposon has been used in different bacterial species, including low-GC Gram-positive bacteria (Le Breton et al., 2006; Cummins et al., 2013; Hashim, 2016; Zhang et al., 2017). The TA

dinucleotides are the only recognition signal for insertion of the *Himar1 mariner* transposon (Lampe et al., 1999).

In this study, zebrafish larvae, planarians, and *G. mellonella* were used as infection models to study pathogenicity, virulence, and the colonisation ability of *E. faecium*. The transparent zebrafish larvae infection model represents an excellent vertebrate model to study the pathogenesis caused by human pathogens (Sullivan and Kim, 2008; Tobin et al., 2010; Volkman et al., 2010; Meijer and Spaink 2011; van der Vaart et al., 2012; Fehr et al., 2015). The innate immune system develops with 24 hrs following fertilization with primitive macrophages then neutrophils at 32-48 hours post-fertilization. The adaptive immune system completes only 4-6 weeks post-fertilization, and this enables a chance to examine autonomously the larvae's innate immunity in the first day post fertilization (Lam et al., 2004; van der Vaart et al., 2012).

Planarians are small free-living invertebrates that have capacities for whole body regeneration and are extremely resistant to infections by bacteria that are very pathogenic to humans, either Gram-positive or Gram-negative, are easy to handle, have low maintenance costs and they are cheap (Abnave et al., 2014; Hamada et al., 2016). They can be genetically identical animals because of asexual reproduction, allowing direct comparison of the potential effects of bacterial strains on the worms without possible complications from the variability of genetic factors, and they do not raise any ethical concerns (Mummery et al., 2014).

The larvae of *G. mellonella*, can grow over a wide temperature range of 18 - 37°C. This is useful in studies performed using human pathogens that most

often require incubation at 37°C. Another advantage of this model is the size of the larvae of these insects when compared to, for example, *Drosophila melanogaster*. Greater wax moth larvae reach 12 - 25 mm in length, facilitating insect manipulation, easy application of the pathogen under investigation, or application of the tested active substance at the correct dose. It is also advantageous for researchers because they can get results faster, even within 24 - 48 hrs. The results of the experiment are easy to observe and usually appear as physical changes (color change, melanization, larval shape, slow movement) compared to insects from control. Studies of mammalian innate immunity have shown similarities to the invertebrate immune system (Salzet, 2001).

The use of static immersion to infect zebrafish larvae at a dose of 5×10^8 CFU/ml resulted in a significant delay ($p < 0.0001$) in the killing of zebrafish larvae after 3 dpi by the *stk1* mutant of *E. faecium* E1162 (deletion mutant of a serine-threonine protein kinase encoding gene), this is in line with previous findings by Hashim (2016) using the same *stk1* mutant strain 201c which infected *G. mellonella* larvae at a dose of 5×10^8 CFU/ml, the results showed that the strain 201c demonstrated significantly attenuated ability to kill *G. mellonella* which killed 18/30 (60%) of the infected larvae at 4 dpi compared to 0/30 (0%) the killing of zebrafish larvae infected with the same dose of the *stk1* mutant strain 201c at 3 dpi ($p < 0.0001$), different infection methods and different temperatures of incubation may be responsible for the difference in killing of zebrafish larvae and *G. mellonella* larvae.

In *Streptococcus suis*, the STK is essential in stress response and pathogenicity. Kristich et al. (2007) have demonstrated that the serine/threonine-protein kinase PrkC of *E. faecalis* was important for intestinal persistence of *E. faecalis* and for antimicrobial resistance. In prokaryotes, signal transduction done by histidine protein kinases leads to regulation of gene expression (Sasková et al., 2007). Stress responses, biofilm formation and virulence have been shown to be regulated by prokaryotic STPK (Hussain et al., 2006). Our results suggest that *E. faecium* E1162 STPK might be utilized to facilitate phosphorylation of some proteins associated with virulence, stress responses and/or virulence.

In the work described in Chapter III, it was found that *E. faecium* isogenic mutant 183N, which had an insertion in a gene annotated as encoding a CBS domain protein, did not kill zebrafish larvae at doses of 5×10^8 CFU/ml, but it did kill at 7.5×10^8 CFU/ml on the same day of E1162 (3 dpi) with a lower killing level ($p < 0.003$). Hashim (2016) showed that the gene for the CBS domain protein mediated the virulence of strain 183N in *G. mellonella* larvae ($p < 0.05$) at doses of 5×10^8 CFU/ml which might be because the body mass difference between zebrafish larvae (24-72 hpf) and *G. mellonella* is significant, which likely accounts for the differences in mutant 183N lethal dose between the two different models. Furthermore, the same factor may not play the same role in different animal models or different niches within the same animal, so there is no single comprehensive model system for all enterococcal infections (Goh et al., 2017).

In 2016, Hashim showed that the same mutant (59N) was significantly impaired in its ability to kill *G. mellonella* larvae. This mutant was found to be 3- to 4-fold less sensitive to menadione at a cell density of 5×10^2 CFU/ml than wild-type (this study), strain 59N was also significantly more sensitive to 16 mM cumene hydroperoxide at cell density ranged between 5×10^6 to 1×10^5 cells in a volume of 5 μ l (Chapter IV, section 4.4.4). The *mur* gene in mutant 59N impaired planarian colonisation dose-and-time dependently, and 59N killed 1/30 (3.33%) of the infected zebrafish larvae at lower dilutions 5×10^8 CFU/ml but did not kill at higher dilutions (7.5×10^8 CFU/ml). This could be because 59N might be one of the pathogens that use locally acting infection pathways which require a lower infective dose than pathogens that use distantly acting molecules and require higher infective dose. The ORF EFF33496 is part of a 150 kb putative pathogenicity island. When mutant 59N complemented in *trans* with intact ORF EFF33496, the resultant complemented strain 59N::*mur* showed more virulence in a *G. mellonella* larvae infection model compared to the wild-type strain *E. faecium* E1162, this indicates that the *efmE1162_1543* contribute to the virulence of *E. faecium* E1162 and it seems that putative muramidase is essential for modification of peptidoglycan in *E. faecium* E1162 which prevent peptidoglycan degradation and release of proinflammatory fragments which can be recognized by host receptors and initiate an immune response that will aid in clearing infection.

In the work described in Chapter III, the results showed that deletion of *EfmE1162_0264* encoding a putative permease component of an ABC transporter was significantly impaired in its ability to kill zebrafish larvae compared to the wild-type *E. faecium* E1162 at 3 dpi. Some of the ABC

transporters had been shown to have a role in resistance to antimicrobials (Hashim, 2016). This author reported that deletion of the permease component of the ABC transporter in *E. faecium* E1162 resulted in increased sensitivity toward nisin, bacitracin and polymixin E compared to the parental strain *E. faecium* E1162. It has been reported that zebrafish can express antimicrobials during early development, and they are inducible by microbial challenge (Caccia et al., 2017), and it is possible that the impaired virulence has seen in mutant Mabc in the zebrafish larvae infection model was due to higher sensitivity of mutant Mabc to zebrafish antimicrobials compared to the wild-type E1162.

In the work described in Chapter IV, a mutant library of the E1162-*mariner*-transposon was screened for mutants sensitive to two different oxidative stressors, menadione and cumene hydroperoxide. Three mutants (MS1, MS2, and MS3) were showed resistance to menadione with different levels when using spot-plate assay method. MS1, which had a *mariner* transposon inserted within *efmE1162 1492* annotated as encoding *tdc*, and MS2, which had the transposon inserted in *efmE1162 1516* annotated as encoding a conserved hypothetical protein, were significantly more sensitive to 3.38 mM cumene hydroperoxide when compared to wild-type cells.

The *E. faecium* E1162 Mutants; MS1 (this study) and 47N identified by Hashim (2016) both had the transposon inserted within *EfmE1162_1492* encoding a putative tyrosine decarboxylase (*tdc*). The research described here showed that the same number of planarians were killed upon infection with 1×10^9 CFU/15ml of E1162 or mutant 47N; however, mutant 47N took slightly longer time to kill all infected planarians (Chapter III, Figure 3-13). In 2016, Hashim tested the

survival of *G. mellonella* larvae against E1162 and mutant 47N using 5×10^8 CFU/ml, the number of larvae killing was significantly less with mutant 47N ($p < 0.01$). The differences in survival when using planarians or *G. mellonella* larvae could be attributed to dose differences, infection methods, and susceptibility to infection, animal physiological status. During this study, only planarians starved for 7 days were selected for infection. Qian et al. (2021) showed that fasting may enhance innate immunity when it is intermittently intensive, increased autophagy and decreased apoptosis after intensive fasting can promote leucocyte survival, a significant increase in neutrophil degranulation occurred and cytokines that regulate immune cells increased primarily released by neutrophils. Although mutant MS1 killed nearly the same number of *G. mellonella* larvae as the wild-type *E. faecium* E1162 ($p > 0.076$) after infection with 3×10^8 CFU/ml via injection, this mutant showed a delay in killing *G. mellonella* larvae compared to the E1162. The differences in killing of infected *G. mellonella* with MS1 and 47N could be attributed to dose differences: MS1 at 1×10^8 CFU/ml and mutant 47N at 5×10^8 CFU/ml. On a spot-plate assay at $5 \times 10^6 - 2.5 \times 10^5$ cells/5 μ l, mutant 47N showed no sensitivity to menadione 64 μ g/ml (data not shown), as did mutant MS1 (Chapter IV, Table 4-1, Figure 4-4). However, the mutant 47N was not tested for sensitivity to other menadione concentrations or cumene hydroperoxide. Complementation of the *tdc* gene in mutant 47N in *cis* with the intact *EfmE1162_1492* restored the wild-type phenotypes (Hashim, 2016) signifying that the impaired phenotypes seen in mutant 47N were due to *tdc* disruption. The sensitivity to MS1 to cumene hydroperoxide 3.38 mM can not be explained by a mutation in the *tdc* gene unless knock out the gene and study its effect on cumene hydroperoxide 3.38

mM then *trans*-complement it to see if this would restore back the phenotype or not.

Mutant MS2 which had the transposon within *EfmE1162_1516* (EFF34605), annotated in *E. faecium* E1162 genome sequence as a conserved hypothetical protein has nearly the same overall killing capacity as the wild-type E1162, however, there is a significant decrease ($p > 0.025$) in the killing rate between the mutant and the wild-type in the first two days. Analysis of the predicted protein architecture of EFF34605 showed an N-terminal Pyr_redox_2 domain and RNA modification enzymes named GidA domain at the C-terminus. In bacteria, RNA modification enzymes are involved in many biological pathways associated with different cell processes. GidA protein is involved in the biosynthesis of 5-methylaminomethyl-2-thiouridine, deletion of *GidA* has impaired virulence of several bacterial pathogens (Shippy and Fadl, 2014). Any deficiencies in a tRNA-modification enzyme under oxidative stress might cause accumulation of shorter and defective tRNAs leading to a harmful effect on cells (Campos Guillen et al., 2017) these suggest that *E. faecium* might use *EfmE1162_1516* -to survive during oxidative stress both *in vivo* and *in vitro*.

Mutant MS3 had the transposon inserted in an ORF EFF33659 (*efmE1162_2615*) putatively encoding a transposase. When compared to E1162, the sensitivity of this mutant to menadione and cumene hydroperoxide was not significantly different compared to the wild-type E1162 (Chapter IV, Table 4-1, Figure 4-4, and Figure 4-6). Furthermore, at 3×10^8 CFU/ml, mutant MS3 killed *G. mellonella* in a manner similar to that of wild-type E1162 (Chapter

IV, Figure 4-13C). Analysis of the predicted protein architecture for EFF33659 shows a histidine kinase-like domain at the C-terminus.

In replica plate print, mutant MS2 did not grow on TSA containing 64 µg/ml menadione (the MIC for wild-type *E. faecium* E1162). However, in the spot-plate assay, this mutant was able to grow on TSA plate supplemented with 64, 80 and 100 µg/ml menadione (Chapter IV, Figure 4-4). This may be because the CFU/ml utilized in the spot-plate assay (5×10^6 to 2.5×10^5 cells in a volume of 5 µl), which led to greater growth after 16 hrs of incubation, was higher than what Wang et al. (2017) suggested, which is 1×10^3 CFU/ml. Furthermore, the results of the spot-plate assay may have been influenced by the use of TSA agar rather than MHA, as recommended by the Clinical & Laboratory Standards Institute (CLSI) (Ferraro, 2000) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (*EUCAST Disk Diffusion Test Methodology*, 2022). When the disc diffusion assay was used to determine the sensitivity of mutant MS2 to 3.38 mM cumene hydroperoxide. Tukey's *post hoc* test revealed that MS2 was significantly more sensitive to 3.38 mM cumene hydroperoxide ($p = 0.05$) (Chapter IV, section 4.4.3).

In constructing an *E. faecium* markerless deletion mutant of the gene encoding a putative muramidase (*efmE1162_1543*), the addition of *p*-chlorophenylalanine to the growth medium (MM9YEG) reduced the growth of the *pheS**-carrying strain of E1162 and it did not lead to the complete growth arrest of it, which was sufficient to effectively discriminate plasmid-free strains in the last step of the homologous recombination procedure, the MM9YEG plates supplemented with *p*-chlorophenylalanine showed a few large colonies and many small colonies

which might mean that the strains should be passaged more frequently to increase the chances of obtaining the desired mutant strain. An alternative explanation for the failure to construct a markerless deletion mutant of the gene encoding a putative muramidase (*efmE1162_1543*) was might be due to the second event of homologous recombination occurred in the same region of homology as the first event, thus allowing cells to revert to the wild type. This may be due to the low frequency of events leading to the desired deletion. Also the 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 so *mur* gene might be an essential gene that is why it couldnot be deleted by markerless gene deletion.

In conclusion, *E. faecium* E1162 and its isogenic mutant did not cause zebrafish death in a dose-dependent manner. The genes encoding, a serine-threonine protein kinase (201c), a putative permease component of an ABC transporter (Mabc), a putative muramidase (59N), a cystathionine β -synthase domain-containing protein (183N), contribute to *E. faecium* virulence in a zebrafish larvae model. CFUs/5 planarians after 1 and 2 days of feeding with 6×10^9 CFU/ml mutant 59N were significantly lower than CFUs/5 planarians after infection with wild-type *E. faecium* E1162. Delayed killing actions in planarians were shown when co-incubated with mutant strains 59N, 47N (*tdc* mutant), and Δesp than when incubated with the wild-type *E. faecium* E1162. When

compared to *E. faecium* E1162 mutant 59N was at least 3-4-fold more sensitive to menadione and cumene hydroperoxide. In this study, successfully mutant 59N was complemented *in trans* with the intact a putative muramidase gene from *E. faecium* E1162. In the *G. mellonella* infection model, the complemented strain was more virulent than the parent strain E1162, which could be owing to the use of a constitutive promoter in the plasmid pHFH4 which used in *trans*-complementation. The *E. faecium* *tdc*, *mur* and *esp* genes seem to be involved in the survival and colonisation of *E. faecium* E1162 in planarians. When compared to *E. faecium* E1162 the sensitivity of mutant MS1 (had the transposon inserted in a gene encoding a putative tyrosine decarboxylase) and MS2 (had the transposon inserted in a gene encoding a conserved hypothetical protein) to cumene hydroperoxide was significantly higher.

6.2 Limitations

TSA was used in the MICs for menadione and cumene hydroperoxide as well as the spot-plate assay in Chapter IV, sections 4.3.2. 4.3.3 and 4.3.5. BHI was used in the spot-plate assay for *E. faecium* by Lebreton et al. (2013) and Andrade et al. (2017). MHA, on the other hand, is recommended for antimicrobial susceptibility testing by the European Antimicrobial Sensitivity Testing Commission and the Clinical Laboratory Standards Association (Ahman et al., 2020).

Based on the results of the spot-plate assay to test the sensitivity of the transposon mutants MS1, MS2, MS3 to menadione and cumene hydroperoxide (Chaper IV, section 4.3.5) the used number of bacterial cells in a volume of 5 μ l ranged from 5×10^6 to 2.5×10^5 , which resulted in more growth after 16 hrs of

incubation at 37°C, whereas Wang et al. (2017) recommends starting at 1×10^3 CFU/ml. Using 5×10^6 to 2.5×10^5 cells/5 μ l in a spot-plate assay is thus a limitation of this study.

BHI and TSB were used to culture *E. faecium* E1162 but it ought to be one sort of media to be used for consistency because totally different medium part may result on the downstream processes (study limitation).

Mutant 59N was delayed in killing zebrafish larvae, *G. mellonella* larvae, and planarian infection models compared to wild-type *E. faecium* E1162. The *trans*-complementation of 59N mutant with *efmE1162-1543* should be investigated using zebrafish larvae or planarians for their ability to restore wild-type virulence in these infection models, limiting the study to the exclusive use of *G. mellonella* for the complementation study is considered during this study.

6.3 Future work

Due to the variety of infections caused by *E. faecium* many infection models have been used, to mimic *E. faecium* infections, however, none of them are easy or very fast, so for monitoring *E. faecium* pathogenesis it would therefore be interesting to study pathogenesis in zebrafish larvae or planarians using the *E. faecium* E1162 and its isogenic mutants with the aid of genetically encoded fluorescent reporters to analyse lethality and bacterial dissemination in infected zebrafish larvae or planarians.

6.3.1 Further identification of *E. faecium* E1162 genes involved in resistance to oxidative stress

To comprehensively identify genes responsible for resistance to oxidative stress, mutants with reduced resistance to menadione can be identified using Transposon sequencing (Tn-Seq Illumina), which allows the sequencing of many DNA fragments simultaneously (Hernandez et al., 2008; Holt et al., 2008). Briefly, the *E. faecium* E1162 transposon mutant libraries could be incubated with or without oxidative stress generator (menadione, cumene hydroperoxide or H₂O₂) then genomic DNA of the mutants recovered from the samples with or without oxidative stress generator treatment can be prepared and processed for Illumina sequencing. In brief, the genomic DNA is randomly sheared to about 300 bp, then will be ligated with designed short oligonucleotide linkers. This pool of DNA is then PCR amplified by using a transposon specific forward primer and Illumina reverse primer that is specific to the short oligonucleotide linker (Langridge et al., 2009). The amplified DNA is then cleaned and subjected to Illumina sequencing using the transposon specific primer and the Illumina primer. The resultant sequence data from each sample can be analysed to identify the mutants that are absent from the sample with antioxidant treatment compared to that without antioxidant treatment. The genes disrupted in mutants with reduced resistance to oxidative stress represent potential drug targets. For example, inhibitors of these genes could help the host response to be more effective by making the bacteria more sensitive to oxidants.

6.3.2 Construction of stable fluorescent reporter plasmids for use in *E. faecium* E1162

To study the differences in phagocytosis uptake and survival within phagosomes between the *E. faecium* wild-type and mutant strains fluorescent reporter strains could be constructed.

In this study, it was showed that the serine-threonine protein kinase-encoding gene (201c), the permease component of the ABC transporters encoding gene, a putative muramidase-encoding gene (59N), a putative permease component of an ABC transporter-encoding gene (Mabc) and a gene encoding a cystathionine β -synthase domain protein (183N) significantly contributed to *E. faecium* virulence in zebrafish larvae, so further experiments are needed to compare *in situ* adaptation of the wild-type *E. faecium* E1162 and each of these mutants in zebrafish larvae or planarians using genetically encoded fluorescent reporters to investigate the route and organs involved in the infection due to their transparency and easy at imaging.

6.3.3 Sensitivity of the mutant 59N to lysozyme

The impaired phenotypes observed in mutant 59N due to transposon insertion into *mur* (*efmE1162_1543*) gene were thought to be due to a lack of peptidoglycan modification. Because modified peptidoglycans are resistant to lysozyme hydrolysis while unmodified peptidoglycans are easily hydrolyzed, the lysozyme sensitivity of the 59N mutant and *E. faecium* E1162 will be investigated. Lysozyme will be added to exponential phase cultures at a final concentration of 100 $\mu\text{g/ml}$, and the effect on cell density will be studied. The lysis percentage will be calculated using the method described by Hashim (2016) as follows: $\text{present OD600 nm} / \text{highest OD600 nm} \times 100$.

The studies included in this thesis showed the potential role of genes in nisin-sensitive mutants of an *E. faecium* E1162 in the pathogenesis of E1162 using zebrafish larvae as infection model and studied the survival and colonisation in a planarian infection model. When strain 59N was complemented, a nisin-sensitive mutant which had the transposon inserted within putative muramidase EFF33496, in *trans* with the intact *efmE1162_1543* from *E. faecium* E1162 the wild-type phenotype was restored in the *G. mellonella* larvae infection model. Also, an *E. faecium* E1162 *mariner* transposon mutant library was screened for mutants with increased sensitivity to menadione, a superoxide generator, three mutants were identified which were designated as MS1 (had the transposon inserted in an *efmE1162_1492* encoding a putative tyrosine decarboxylase), MS2 (had the transposon inserted in an *efmE1162_1516* encoding conserved hypothetical protein) and MS3 (had the transposon inserted within an *efmE1162_2615* encoding a putative transposase. The transposon insertion within *efmE1162_1516* in mutant MS2 resulted in a significant decreased in its capacity to kill *G. mellonella* larvae in the first two days of infection ($p > 0.025$) compared to *E. faecium* E1162, mutant MS1 and mutant MS3.

Overall, this work has contributed to the current understanding of genes involved in resistance to oxidative stress and virulence in *E. faecium*. Hopefully, the research undertaken will contribute to the understanding of enterococcal pathogenesis and medicine.

Chapter VII

References

7.0 References

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