# Biofilm formation and pathogenicity in Enterococci

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# Biofilm formation and pathogenicity in Enterococci.

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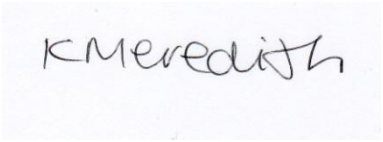

# Table of Contents

# List of Figures

# List of Tables

# Abbreviations

| | |
|---|---|
| Acm | Adhesion of collagen from *E. faecium* |
| AFLP | Amplified-fragment length polymorphism |
| AltA | Autolysin |
| Amp | Ampicillin |
| AS/Agg | Aggregation substance |
| ATP | Adenosine triphosphate |
| Bap | Biofilm associated protein |
| *bee* locus | Biofilm enhancer in enterococci |
| BHI | Brain-heart infusion broth |
| Bop | Biofilm on plastic |
| Caco-2-cells | Human colorectal adenocarcinoma cells |
| CAT/Chl | Chloramphenicol |
| CC17 | Clonal complex group 17 |
| c-di-GMP | Cyclic diguanylate monophosphate |
| CFU | Colony forming units |
| CGH | Comparative genomic hybridization |
| CLSM | Confocal Laser Scanning Microscope |
| CRISPR | Clustered, regularly interspaced short palindromic repeats |
| CV | Crystal violet |
| *Cyl* | Cytolysin |
| D | Aspartic acid |
| D-ala-D-ala | D-alanyl-D-alanine |
| D-Lac | D-lactate |
| DNA | Deoxyribonucleic acid |
| D-Ser | D-serine |
| *E. casseliflavus* | *Enterococcus casseliflavus* |

| | |
|---|---|
| *E. coli* | *Esherichia coli* |
| *E. faecalis* | *Enterococcus faecalis* |
| *E. faecium* | *Enterococcus faecium* |
| *E. flavescens* | *Enterococcus flavescens* |
| *E. gallinarum* | *Enterococcus gallinarum* |
| EARRS | The European antimicrobial resistance surveillance system |
| *ebp* | Endocarditis and biofilm associated pili |
| EcbA | *E. faecium* collagen binding protein A |
| ECM | Extracellular matrix |
| eDNA | extracellular DNA |
| Em | Erythromycin |
| Epa | Enterococcal polysaccharide antigen |
| EPS | Extracellular polymeric substances |
| ER | Endoplasmic reticulum |
| Esp | Enterococcal surface protein |
| FPLC | Fast performance liquid chromatography |
| GBAP | Gelatinase biosynthesis-activating pheromone |
| GBS | Group B streptococci |
| GelE | Gelatinase |
| ICE | Integrative conjugative element |
| IGF | Insulin-like growth factor |
| IgG | Immunoglobulin |
| Ipa | Invasion plasmid antigens |
| IPTG | Isopropyl-β-thiogalactopyranoside |
| IS | Insertion sequence |
| *L. monocytogenes* | *Listeria monocytogenes* |
| LB | Luria-Bertani |
| LPS | Lipolysaccharide |
| Lsp | Prolipoprotein signal peptidase |

| | |
|---|---|
| LTA | Lipoteichoic acid |
| MBC | Minimum bactericidal concentration |
| MIC | Minimum inhibitory concentration |
| MLST | Multilocus sequence typing |
| MRSA | Meticillin-resistant *Staphylococcus aureus* |
| MSCRAMM | Microbial Surface Components Recognising Adhesive Matrix Molecules |
| NICE | Nisin-controlled gene expression system |
| OD600 | Optical density at 600 nm |
| P | Prokaryotic |
| PBP | Penicillin-binding proteins |
| PBS | Phosphate buffered saline |
| PBS-BSA | PBS containing 0.05% Bovine Serum Albumin |
| PCR | Polymerase chain reaction |
| PFGE | Pulsed-field gel electrophoresis |
| PGC | Pilin gene cluster |
| P*nisA* | *nisA* promoter |
| P-t | Power-time |
| PVDF | Polyvinylidene difluoride membrane |
| R | Resistant |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal RNA |
| S | Sensitive |
| *S. aureus* | *Staphylococcus aureus* |
| *scrB* | Sucrose-6-phosphate hydrolase |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis |
| SgrA | serine-glutamate repeats containing protein A |
| SPase | Signal peptidase |

| | |
|---|---|
| SprE | Serine protease |
| ST | Sequence type |
| TAM | Thermal activity monitor |
| TBST | Tris-buffered saline containing Tween 20 |
| TGF-β | Transforming growth factor |
| THB | Todd-Hewit broth |
| tRNA | Transfer RNA |
| TSA | TSB agar |
| TSB | Tryptone soya broth |
| TSB-G | TSB containing 0.25% glucose |
| TSB-G-B | TSB containing 0.25% glucose and 5% blood |
| UTI | Urinary tract infection |
| UV | Ultra violet light |
| VRE | Vancomycin resistant enterococci |
| VRSA | Vancomycin resistant *Staphylococcus aureus* |

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I have really enjoyed my time in the University and living in Bath and I would not wish to change anything. The decision to come to Bath to do this PhD was initially a hard one as I was already settled in North Wales and would be leaving my family and most of all my fiancé, Andrew. I would like to thank them all for their support in this decision, especially encouraging me and making me laugh through all these years; research often does not go the way you expect it and so family and friends are important to keep you sane! This leads me onto giving a big thank you to all the friends I made in Bath; over the years we have had lots of fun, tea and wine and I'm sure that this isn't going to stop now I have finished!

I would like to dedicate this thesis to my daughter Isabelle Meredith Wynne (11 months old on submission of this thesis), she has brought lots of love and happiness to our family. I hope this shows her that you can do anything that you put your mind to.

# Declaration

The work presented in this thesis is original work conducted by myself under the supervision of Dr Albert Bolhuis. The research was funded by a BBRSC studentship. All sources of information have been acknowledged by means of references. None of this work has been used in any previous application for a degree at this or any other University or institute of learning.

# Abstract

Enterococci are opportunistic pathogens that are frequently a source of nosocomial infections and it is their resistance to antibiotics and their ability to form biofilms that represent important virulence traits. Normally, in healthy individuals it is a harmless commensal that is usually found in the intestine. This thesis firstly studies signal peptidases (SPases), which play an essential role in protein translocation. Interestingly, *E. faecium* was found to contain three type I SPases. Many proteins that are secreted are virulence factors, and the aim was to delete one or more of the SPases and study the effect of its removal on virulence. Unfortunately no mutants were obtained suggesting that the genes were essential. To establish if the genes were essential an inducible integration vector was constructed, but due to time constraints this could not be tested further.

Biofilm formation was studied in both *E. faecium* and *E. faecalis*. The presence of the Enterococcal Surface Protein (Esp) in *E. faecium* was shown to increase hydrophobicity, and therefore also increase biofilm formation. Similarly, *E. faecalis* isolates that were good biofilm formers were also more hydrophobic in nature. The expression of Esp in *E. faecium* was studied under different conditions; these studies indicated that the highest level of Esp expression was found in biofilms cells. This growth-dependent manner Esp expression was not observed in *E. faecalis* BS12297. Surprisingly, Esp in *E. faecium* was also shown to have a role in ampicillin resistance, which was identified using calorimetry. This method proved to be a sensitive and rapid method to analyse antibiotic resistance.

In the gut, bacteria encounter various adverse conditions, such as low pH and the presence of bile salts. Here we investigated the effects of bile salts on biofilm formation in *E. faecium* and *E. faecalis* and demonstrated that biofilm formation is induced at physiological concentrations of bile salts. In *E. faecium* the presence of bile salts caused an increase in initial attachment, microcolony formation and EPS production. Various factors were investigated, including hydrophobicity, cell growth, cell morphology, Esp expression and the production of extracellular polymeric substance (EPS). In *E. faecium*, only EPS production appeared to play a role, but the stimulation of biofilm formation due to bile salts is still to be fully explained.

# Chapter 1: Introduction

# Biofilm formation and pathogenicity in Enterococci.

## *1.1. General*

Enterococci are Gram-positive, facultative anaerobic bacteria; they are oval cocci that form chains of different lengths and belong to the lactic acid bacteria of the firmicutes phylum. They were first classified as streptococci, but with the introduction of Lancefield serological typing system in 1930 and, in addition, their ability to grow at a wide range of temperatures (10-45$^{o}$C), in 6.5% sodium salt (NaCl), in pH 9.6, in 40% bile and ability to survive 30 minutes at 60$^{o}$C, they were separated from other streptococci and given the genus name enterococci (Cetinkaya et al., 2000). The natural habitat of enterococci is in the intestine and oral cavity of humans and animals where they are usually harmless commensals, but they have also been found in water, soil, plants and birds (Gelsomino et al., 2002, Franz et al., 1999). They can, however, become opportunistic pathogens in humans when the host resistance is lowered i.e. by other diseases or drugs. The diseases they can cause include endocarditis, urinary tract infections, bacteraemia, and intra-abdominal and pelvic infections. There are two clinically relevant enterococcal species, *Enterococcus faecalis* and *Enterococcus faecium*. Importantly, enterococci are becoming an increasing problem in hospitals due to their resistance to many antibiotics (Mohamed and Huang, 2007).

## *1.2. Biofilm formation.*

Not only are enterococci resistant to many antibiotics, they are also able to produce biofilms. Biofilms are communities of organisms that are attached to a range of biotic and abiotic surfaces and are encased in exopolymeric substances (Mohamed and Huang, 2007). Such a community can contain single species or multiple species of micro organisms (O'Toole et al., 2000). Figure 1.1 shows a simplified model of biofilm formation.

### 1.2.1 Initial attachment

Bacteria, when they are not attached to a surface, are planktonic, free-floating bacteria (Hall-Stoodley et al., 2004). These planktonic cells may attach to a surface, but that is dependent on several key elements, such as the properties of the cell, the properties of the substratum and the environment. Initial attachment of bacterial cells requires electrostatic, Lewis acid-base interactions, Lifshitz-van der Waals and hydrophobic forces to overcome the repulsion of the usually net negative charge surfaces, and some of the above interactions are helped by the cell surface proteins (van Merode et al., 2006a). This attachment is initially reversible but eventually it becomes irreversible. There are also genetic changes occurring, due to possibly the sensing of a change in environment, which triggers a shift in expression of genes the products of which further stimulate attachment (Beloin and Ghigo, 2005, Monds and O'Toole, 2009). An example of this is the shift seen in *Escherichia coli* in which genes encoding flagella components are repressed as these are not needed anymore after attachment (Ren et al., 2004). Bacteria also have other surface structures that are important to initial attachment, which include fimbriae, lipoproteins, lipopolysaccharides, enzymes, and adhesins (such as Bap in *S. aureus* (Latasa et al., 2006)) (Lejeune, 2003). The properties of the substratum also has a role, as bacteria have been shown to attach better to rougher surfaces as well as those that are more hydrophobic (Donlan, 2002). The presence of a conditioning film on the surface can also effect initial attachment; the film can contain different organic and inorganic materials depending on the environment and can therefore effect attachment by altering the surface characteristics leading to better attachments (Palmer et al., 2007). The effect of conditioning films seen in industry has been shown to reduce and increase bacterial attachment to surfaces (Palmer et al., 2007). It has also been shown to increase bacterial attachment to tooth enamel and medical devices inserted into the body (Donlan, 2002, Habash and Reid, 1999). The environmental factors that can effects initial attachment include flow velocity, pH, temperature, cations and the presence of antimicrobials agents. All of these may affect attachment, which in some cases coincides with changes in gene expression as mentioned above (Beloin and Ghigo, 2005, Donlan, 2002).

### 1.2.2 Microcolony formation

Once attached, more bacteria add to the monolayer and bacteria already present divide forming a microcolony which contains approximately 100 cells in a cluster (Costerton, 1995, Hall-Stoodley and Stoodley, 2002, Monds and O'Toole, 2009). When microcolonies form on the surface there is also an increase in the production of extracellular polymeric substances (EPS; Hall-Stoodley and Stoodley, 2002), which is essential for the production of a biofilm as it holds cells closely together and further helps attachment to the surface. Components of EPS include polysaccharides, proteins (enzymes and structural proteins), extracellular DNA (eDNA), lipids, and biopolymers. The amount of EPS varies between biofilms, due to temperature, shear force, nutrients available and the organisms within the biofilm having the ability to form components of the EPS. These combined factors mean that even the composition of EPS produced by identical bacteria may vary considerably (Sutherland, 2001, Allison, 2003, Flemming and Wingender, 2010).

Biofilms tend to have a high cell density and this allows for quorum sensing (cell-cell communication) to occur. Quorum sensing is a cell-population density dependent gene regulation system that is controlled by the concentration of chemical signal molecules, autoinducers, which are released into the environment by the bacteria. Once these autoinducers reach a critical concentration, which only occurs at high cell densities, the bacteria collectively are able to switch on genes. Genes regulated through quorum sensing often includes those involved in symbiosis, virulence, biofilm formation, conjugation, motility, sporulation and antibiotic production (Miller and Bassler, 2001, Li and Tian, 2012).

### 1.2.3 Mature biofilm

In well-established mature biofilms, the microcolonies and EPS have developed into large 3-dimensional structure (macrocolony). Usually at this stage the amount of microorganisms are only 10% of the dry mass, with 90% of the dry mass represented

by the EPS matrix (Flemming and Wingender, 2010). At this stage the biofilms will have dense areas with many bacteria present and also some sparse areas which act as channels that transport of water, nutrients and oxygen (Donlan, 2002). The biofilm environment also allows for recycling of components, which includes the EPS matrix that can be degraded if required, and components from dead bacteria can be reabsorbed by other cells in the biofilm (Flemming and Wingender, 2010).

Biofilms also enable the transfer of DNA between bacteria, either through eDNA released by cells, conjugation or horizontal transfer (Montanaro et al., 2011, Donlan, 2002). Within the biofilm structure conditions can vary, for instance some areas with less oxygen or nutrients than in others. These differences in local conditions will not be advantageous for all the cells (Flemming and Wingender, 2010).

### 1.2.4 Dispersal

Particularly in older biofilms, dispersal of cells also starts to play a role. These dispersed cells can then, for instance, colonise or infect other sites of the body. Dispersal can be active being caused by cell signals or environmental changes, or physical dispersal in which flow forces cause shearing (Donlan, 2002, McDougald et al., 2012). Active dispersal is usually first noticed by the death of cells leaving voids in the biofilm; the dead cells then provide the nutrients for the other cells to detach and move to other areas. Inducers that cause active dispersal include nutrient levels (Carbon/oxygen limitation or iron availability), quorum sensing signals, c-di-GMP levels, D-amino acids, nitric oxide and EPS-degrading enzymes (McDougald et al., 2012). Physical dispersal has been studied mainly in biofilm reactors and it has been established that biofilms can be dispersed due to abrasion, which is when particles from detached biofilm already in the fluid collide into the biofilm causing parts to become detached. This type of removal is more likely to occur with the backwashing biofilters, these filters contain microorganisms which degrade and therefore clear pollutants from water systems and air systems (Morganroth and Wilderer, 2000). Biofilm removal can also occur by erosion or shearing, which happens over time

removing small sections of biofilm by the shear stress on the surface caused by the fluid flow (Derlon et al., 2008). Physical dispersal of biofilms in nutrient rich environments can also occur with sloughing; this removes the biggest amount of biofilm in the smallest amount of time and is due to nutrient and oxygen depletion (Donlan, 2002).

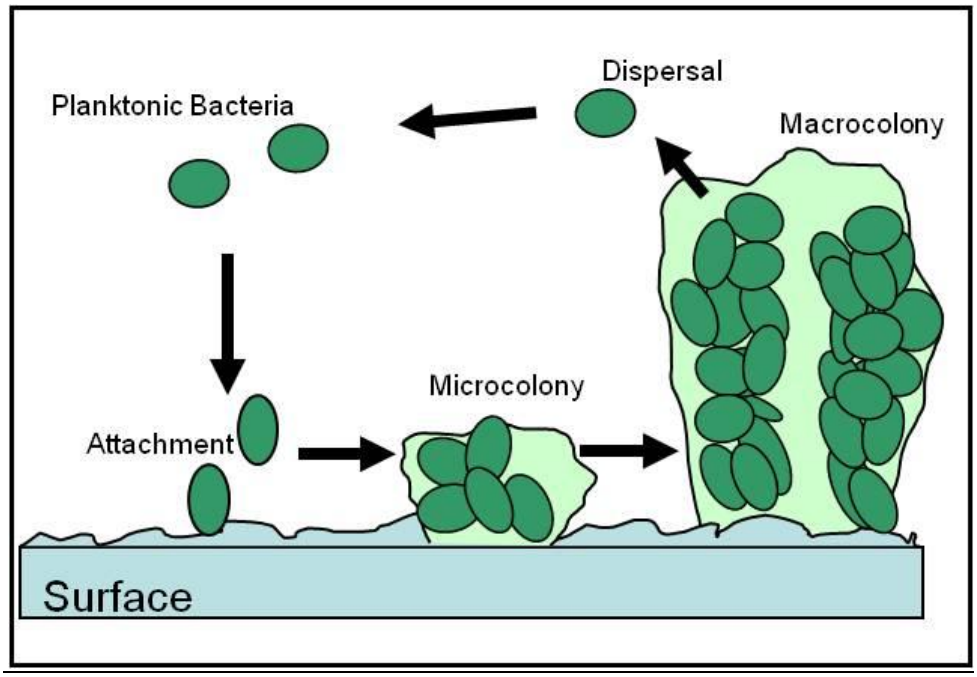



**Figure 1.1. Simplified schematic of the steps involved in biofilm formation. See text for details.**

## 1.2.5 Biofilms in nature

It has been found that bacteria are more often found in a biofilm as compared to the planktonic state (Davey and O'Toole G, 2000). Biofilms are found on most surfaces of the planet, such as on living tissues (e.g. mouth and gut), sea beds, hot springs, rock surfaces, soil, in sediments, industrial water systems and natural aquatic systems (Donlan, 2002, Davey and O'Toole G, 2000, Hall-Stoodley et al., 2004). Usually biofilms will have multiple species within it (Moons et al., 2009), although some clinically found biofilms can have a single species (Hall-Stoodley et al., 2004). The disadvantages to living in biofilms is similar to living in any environment with

many bacteria present, which include competition for nutrients, toxins produced by other bacteria and a lack of space (Moons et al., 2009). There are however many advantages to growing in biofilms. Firstly, biofilms provide protection from the harsh environments, such as UV exposure, host defence systems, antimicrobial agents and dehydration (Hall-Stoodley et al., 2004). Secondly, having a place to attach gives the cells a stable place to grow and by keeping cells close together it also enables cross-feeding of nutrients (Costerton, 1995, Moons et al., 2009, Hall-Stoodley et al., 2004).

### 1.2.6 Clinical relevance

It is now widely known that more than 60% of infections caused by micro organisms are in biofilms (Lewis, 2001). Human infections caused by biofilms can be divided in two groups: general infections i.e. periodontitis, otitis media, biliary tract infections and osteomyelitis, and infections involving a foreign body such as ventilation-associated pneumonia, cerebral spinal fluid-shunts, urinary catheter infections and orthopaedic prosthesis (Fux et al., 2005). One of the main clinical problems with biofilms in the clinical setting is that the bacteria in biofilms are 10-1000 times more resistant to antibiotics. They are also more resistant to the immune system and other stressful conditions (Hoiby et al., 2010). Resistance to antimicrobials is caused by several factors. Firstly, biofilm cells probably have features similar to cells grown to stationary phase in which the metabolic rate is strongly decreased. Many antibiotics block processes that are most active in rapidly growing cells, and these compounds are therefore not very active on cells that are in a near-dormant state (Fux et al., 2005, Lewis, 2001). Secondly, penetration through the biofilms (and EPS) is likely to play a role, although the evidence for this is conflicting. Some studies have shown that EPS does not inhibit penetration of antibiotics, but there are suggestions that the biofilm may bind or deactivate the antibiotics, creating an antibiotic gradient within the biofilm that could induce resistance genes (Hall-Stoodley and Stoodley, 2009). In contrast, other studies have shown that penetration of EPS of

antibiotics can occur, but also that penetration can depend on the antibiotic used (Zahller and Stewart, 2002, Singh et al., 2010b).

Cells in biofilms are also resistant to several host defence systems. Studies have shown that phagocytes are unable to attack bacteria in biofilms due to the protective layer of the EPS, which also prevents proper interaction of antibodies with cells as they can interact with the biofilm surface only. Furthermore, bacteria within the biofilms have been shown to produce toxins that kill polymorphic neutrophils, preventing biofilm clearance (Hall-Stoodley and Stoodley, 2009). It is not only toxins that help bacteria during colonisation or infection. Several other aforementioned factors may also help in this, including the production of adhesins and secretory proteins that enable attachment and invasion of host tissues. Finally, as mentioned before, some of the cells in biofilms grow very slowly (e.g. due to low levels of nutrients or oxygen) and behave like persister cells that are very tolerant to antibiotics. These type of cells lie dormant until a more favourable environment occurs, after which they then can reform the previously antibiotic-treated biofilm, causing a relapse in infection (Lewis, 2001).

### 1.2.7 Industrial relevance

Biofilm formation and other types of biofouling is a large problem in industry causing reduced levels of production, a decrease in product quality and instrument damage, all of which cost the industry a considerable amount of money. Areas affected include water treatment systems and food/beverage industries (Bixler and Bhushan, 2012). In the case of water-treatment systems, biofouling can cause damage leading to problems such as an increase in friction, an increase in power requirements, corrosion of stainless steel, reduced efficiency in heat exchangers, premature destruction of mineral materials, pipe-pressure drops and contamination of pharmaceutical products. This all leads to the quality of water decreasing as it allows re-growth of bacteria in the water (Coetser and Cloete, 2005, Bixler and Bhushan, 2012). Such problems can be prevented (partially) by using low adhesion materials,

by decreasing the amount of drag on the flow, and by using chemical methods (Bixler and Bhushan, 2012).

Food spoilage and disease transmission are also worries for the food industry. To prevent biofilm formation and microbial contamination, specific cleaning procedures are in place to ensure that attached bacteria are removed before they are able to produce biofilms. These procedures include the use of chemical agents and/or a high temperature (Chmielewski and Frank, 2003). Surfaces in the food industry are often made of stainless steel, which is resistant to damage caused by cleaning procedures and this helps to prevent bacterial attachment (Chmielewski and Frank, 2003, Kumar and Anand, 1998). Equipment design is also important, as a good design allows for good cleaning methods and prevents for instance dead ends for bacteria to attach and multiply (Kumar and Anand, 1998, Chmielewski and Frank, 2003). Routine testing of processing surfaces is important also to monitor microbial attachment, which is performed by swabbing, contact plates or ATP bioluminescence testing (Chmielewski and Frank, 2003).

It is also important to point out that biofilms can also be helpful in industry, with an example being their use in bioremediation. Biofilms can degrade toxic compounds and help reduce pollutants. They are also used in waste sewage management and water purification management, where they can be used for organic nutrient-trapping (Kumar and Anand, 1998).

## *1.3. Antimicrobial resistance in Enterococci*

Enterococci are becoming an increasing problem in hospitals due to their resistance to many antibiotics such as penicillins, glycopeptides (e.g. vancomycin) and aminoglycosides (Sood et al., 2008). The European Antimicrobial Resistance Surveillance system (EARSS) has been monitoring resistance in this organism across Europe and they have seen over the past 9 years that vancomycin resistance has been

increasing in many countries. Vancomycin resistant enterococci (VRE) pose a problem clinically, as 10-25% (Figure 1.2) of *E. faecium* clinical isolates were vancomycin resistant in the UK in 2010 (EARRS, 2010), and patients that acquire VRE are harder to treat. Another cause for concern is the transfer of this resistance to other bacteria such as meticillin-resistant *Staphylococcus aureus* (MRSA; Sood et al., 2008).

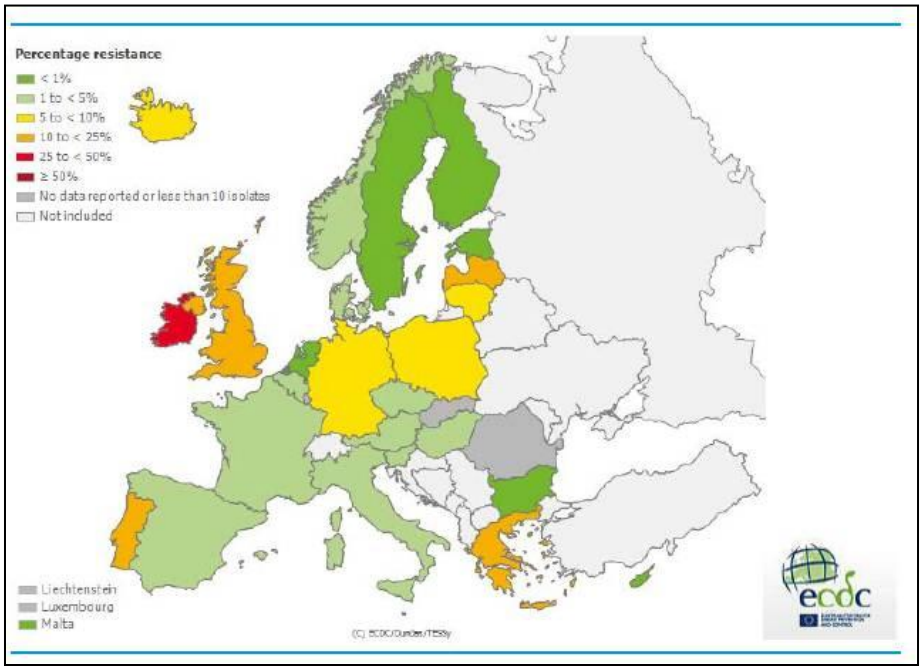



**Figure 1.2. Distribution of vancomycin-resistant *E. faecium* in EARS-Net countries in 2010. (EARRS, 2010)**

## 1.3.1 Intrinsic resistance

There are two forms of antimicrobial resistance: intrinsic resistance and acquired resistance. 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. All enterococci have up to nine different penicillin-binding proteins (PBP; Williamson et al., 1986) which have low affinity and therefore resistance to β-lactams such as penicillins, carbapenems and cephalosporins. The amount of

resistance varies between the β-lactams, with resistance the lowest for penicillins such as ampicillin (Top et al., 2008).

Enterococci also show resistance to aminoglycosides due to poor permeability of the cell wall to highly polar molecules (Arias and Murray, 2012). Aminoglycosides can be used in conjunction with β-lactams and glycopeptides (cell wall synthesis inhibitors), which can work together synergistically, allowing increased efficacy of the aminoglycosides to kill (Top et al., 2008). Unfortunately, *E. faecium* has a chromosomally encoded enzyme, aminoglycoside acetyltransferase AAC(6')Ii, that can stop the above synergism occurring, leading to resistance to tobramycin and kanamycin, but enterococci remain sensitive to gentamicin (Top et al., 2008, Chow, 2000).

### 1.3.2 Acquired resistance

Acquired resistance is accompanied by a change in the genetic material of the bacteria, either through mutation or by acquiring genetic material via plasmids, transposons or other mobile genetic elements. It is this type of resistance that has enabled enterococci to become highly resistance to ampicillin. *E. faecium* has mutations that cause the overproduction of PBP5 or decreased affinity for ampicillin, the decrease affinity is due to amino acid changes in the active site (Arias and Murray, 2012). Hospital-associated isolates of *E. faecium* are 90% likely to be resistant to ampicillin while, in contrast, it is rare to find *E. faecalis* isolates that are resistant to ampicillin. When ampicillin resistance is identified in hospital isolates of *E. faecalis* it has occurred due to the production of β-lactamases (Murray, 1992). Due to the increase in high level ampicillin resistance and vancomycin resistance (see below), *E. faecium* now causes between 38% and 75% of enterococcal infections, with the remainder caused by *E. faecalis*. This ratio has gradually changed from the early 1990s when *E. faecalis* (with only low level ampicillin and vancomycin resistance) caused 90% of infections and the remainder was caused by *E. faecium* (Willems et al., 2011).

Enterococci can also acquire high level resistance of aminoglycosides, which prevents the synergistic effects they have with cell wall synthesis inhibitors such as ampicillin and vancomycin. This resistance is caused by aminoglycosides-modifying enzymes, which stop the antibiotic interfering with the recognition site of tRNA by rRNA. The most clinically important enzyme found in enterococci is Aac(6')-Ie-Aph(2'')-Ia, which cause the bacteria to be resistant to most of the aminoglycosides available (Denyer et al., 2004, Chow, 2000).

Other acquired resistances found in enterococci include resistance to macrolides by ribosomal methylation, resistance to chloramphenicol by CAT encoding enzymes, and resistance to quinolones by modification to DNA gyrase and Topoisomerase IV (Top et al., 2008).

### 1.3.3 Vancomycin resistance

As mentioned in the beginning of this section, vancomycin resistance is increasing causing clinical problems. Vancomycin resistance in enterococci was first identified in 1986 in Europe. Since being identified, its dissemination in European hospitals has been slow, whereas in the USA vancomycin resistance in hospitals is extremely high and dissemination was fast (Willems et al., 2011, Arias and Murray, 2012). Vancomycin resistance in Europe was initially more widespread in the community in meat products and animals; this was due to the use of avoparcin (which gives cross resistance to vancomycin) as a growth promoter in animal feed. However, the use of avoparcin was banned in 1996, which helped decrease this reservoir of VRE. In contrast to Europe, the USA has low vancomycin resistance in the community (Top et al., 2008, Arias and Murray, 2012).

Vancomycin acts by binding to the D-alanyl-D-alanine (D-ala-D-ala) portion of the peptidoglycan precursor, which stops the enzyme transglycosylase from attaching the peptidoglycan precursor to the cell wall (Denyer et al., 2004). There have been six types of resistance described in vancomycin, VanA, VanB, VanC, VanD, VanE and VanG (Top et al., 2008). Resistance occurs due to two different methods:

replacement of the terminal residue of the peptidoglycan precursor, or removal of target precursors with D,D-dipeptidases and carboxypeptidases (Werner et al., 2008). The peptidoglycan precursor, D-alanine is replaced with D-lactate (D-Lac), which is what is found in resistance types VanA, VanB and VanD, or with D-serine (D-Ser) in resistance types VanE (in *E. faecalis* only), VanG (in *E. faecalis* only) and VanC. The resistance types above are all acquired, except for VanC which occurs intrinsically and can be found in *E. gallinarum*, *E. casseliflavus* and *E. flavescens,* but not *E. faecium or E. faecalis*. VanA and VanB resistance are the most likely to be found in hospital isolates (Rice, 2006), and both are problematic clinically because of patient treatment. An additional problem is that their genes are found on mobile genetic elements, enabling the spread of resistance to other bacteria. Such horizontal gene transfer has already been shown to occur in group A and viridans streptococci, *Listeria monocytogenes* and most importantly *S. aureus* (Cetinkaya et al., 2000, Noble et al., 1992). The latter is critical as vancomycin is an important drug for the treatment of infections with MRSA, and therefore gaining this vancomycin resistance would be a significant clinical problem. There have already been 12 MRSA isolates identified in the USA that are also vancomycin resistant (VRSA), and all of these have gained vancomycin resistance from enterococci (Kobayashi et al., 2012).

### 1.3.4 Epidemiology of the highly resistant *E. faecium* and *E. faecalis* clones

As resistance to vancomycin is on the increase in *E. faecium*, it is clear that molecular techniques are required to study genetic changes and the epidemiology of the outbreaks. Initially the main molecular method of studying the genetics and epidemiology of *E. faecium* and *E. faecalis* was by using pulsed-field gel electrophoresis (PFGE), the gold standard method used by many laboratories (Arias and Murray, 2012, Top et al., 2008). It was found however that this method could be too discriminatory for epidemiological studies due to enterococci rapidly recombining, leading to a single strain having variations in the pattern of banding which caused misleading results (Morrison et al., 1999, Willems et al., 2011).

Various other techniques were tested to look at epidemiology and to see if there were differences between isolates from differing backgrounds, they included amplified-fragment length polymorphism (AFLP) analysis, which identified isolates from different hosts, such as human, pigs or calves (Top et al., 2008, Jureen et al., 2004) and comparative genomic hybridization (CGH). The latter showed that isolates causing hospital outbreaks and clinical isolates were placed in the same cluster, and therefore there appears to be a single hospital clade (Leavis et al., 2007). Both CGH and AFLP showed that the genetics of hospital and non-hospital isolates differed, and that it was likely that hospital isolates have evolved from a recent common ancestor (Leavis et al., 2007, Willems et al., 2011).

To give more evolutionary detail on the genotypes multilocus sequence typing (MLST) was performed on *E. faecium*. This method studies the differences in seven housekeeping genes giving them a numeric allelic profile which is assigned a sequence type (ST). This method identified that hospital isolates clustered in groups called clonal complexes (Top et al., 2008, Arias and Murray, 2012). It also showed that the hospital-outbreak isolates clustered in one clonal complex group 17 (CC17) and this cluster contained many polyclonal subpopulations (ST17, ST18, ST78 and ST192; Willems et al., 2011, Arias and Murray, 2012).

Using MLST and CGH while analysing *E. faecalis* isolates, it was noticed that hospital-associated isolates (including the most common clones, ST6, ST9, ST16, ST21, ST28, ST40 and ST87) are also found in the community, in animals and food products. There are therefore no distinct hospital clonal complexes, but some clones are more enriched for the hospital environment (ST6, ST9, ST28 and ST40). Virulence factors found in *E. faecalis* have also been shown to be present in non-hospital associated isolates (Willems et al., 2011).

The main reasons for the emergence of hospital-associated enterococci is due to their ability to gain new DNA readily and this means that their genomes are enriched with

insertion sequence (IS) elements, some of which are involved in antibiotic resistance and virulence. An example is *E. faecalis* V583, a strain in which a quarter of its genome contains mobile genetic elements such as plasmids, prophages, and pathogenicity islands (Paulsen et al., 2003, Shankar et al., 2002). Similarly, the *E. faecium* hospital-adapted clonal complex 17 isolates also contains many acquired genes and seem to be able to take up any amount of exogenous DNA (van Schaik et al., 2010, Leavis et al., 2007). It has also been observed that multidrug-resistant enterococci lack the clustered, regularly interspaced short palindromic repeats, CRISPR-cas loci, which are part of a genetic interference pathway that limits conjugation and transduction, and is therefore said to encode an adaptive immunity against incoming DNA. Due to the lack of these loci, it enables hospital-associated isolates to easily pick up antibiotic resistance and other virulence genes (Palmer and Gilmore, 2010, Marraffini and Sontheimer, 2010).

## *1.3. Biofilm formation and pathogenicity in Enterococci*

### 1.3.1 Enterococcal infections

As mentioned at the start of this chapter, enterococci are usual found in the GI tract were they are commensals and cause no harm to the host. Enterococci can become opportunistic pathogens and cause many nosocomial infections (Jett et al., 1994). Usually enterococcal colonisation of the GI tract increases due to use of antibiotics, which causes changes in the gut microbiota and then enables pathogenic enterococci such as VRE to colonise the GI tract (Ubeda et al., 2010, Donskey et al., 2000). One study showed that a decrease in Gram-negative bacteria caused by antibiotics lowers the stimulation of surface proteins on the epithelium cells of the small intestine. This decrease in stimulation in turn reduced production of REGIIIγ, which is a C-type lectin with activity against Gram-positive bacteria, and therefore allowing those bacteria to proliferate in the gut (Brandl et al., 2008, Kinnebrew et al., 2010). Enterococci have been shown to translocate from the lumen of intestine to the mesenteric lymph nodes, liver and spleen, and then this allows them to enter the bloodstream and cause infections such as endocarditis (Arias and Murray, 2012, Jett

et al., 1994). Enterococci can also infect other patients and spreads around the hospital by faecal contamination, which can cause UTIs and intravenous catheter infections (Arias and Murray, 2012).

### 1.3.2 Environmental factors effecting enterococcal biofilm formation

Enterococci live in the intestine, which is a hostile environment; they have to withstand varying conditions (nutrients, pH, oxygen levels), bile acids, digestive enzymes, and toxins from other bacteria (Wilson et al., 2002). Any number of these factors could affect biofilm formation.

One example that has been shown already is glucose, which has been shown to stimulate biofilm formation in many *E. faecalis* and *E. faecium* isolates (Tendolkar et al., 2004). In a separate study a sugar-binding transcriptional regulator, BopD, which is part of the *bop* operon (biofilm on plastic), was shown to be involved in biofilm formation in *E. faecalis*. It was suggested that the presence of oligosaccharides in food influences this operon and therefore biofilm formation (Hufnagel et al., 2004, Creti et al., 2006). Thus, sugars have an important effect on biofilm formation and so it is likely that, more generally, the composition of growth media have an effect. Indeed, a study by Kristch *et al* showed that some media promoted biofilm formation and maturation (M17, TSB and M9YE) and others (BHI and THYE) only promoted the initial stages of biofilm formation, after which dispersal of the cells occurred. They suggested that this was due to an unknown signal triggered by components in different media that caused biofilm maturation or dispersal (Kristich et al., 2004).

Biofilm formation in *E. faecalis* has also been shown to decrease with increasing osmolarity (Kristich et al., 2004). This has also been shown to occur in other bacteria, showing environmental control of biofilm formation (Loo et al., 2000). Another factor effecting biofilm formation is the presence of serum, which increases

*E. faecalis*'s ability to adhere to glass and silicone by increasing its hydrophobicity (Gallardo-Moreno et al., 2002). This was not found in all bacteria, and in for instance *Burkholderia cepacia* attachment varied in the presence of serum and depended on the type of conditioning film and the ionic strength (Hwang et al., 2012). Such variation was also seen in organisms found in the oral cavity, with for instance the presence of serum, decreasing *Fusobacterium nucleatum* attachment, but increasing *Phorphyromonas gingivalis* attachment. When inflammation occurs gavial margin there is an increased production of gingival crevicular fluid (GCF), which is type of serum based exudates, therefore in this study serum is used as a substitute for GCF (Biyikoglu et al., 2012). These factors suggest that serum affects the surface with conditioning films and changes in physiochemisty and these changes are not always advantageous for biofilm formation.

### 1.3.3 Role of Esp and other biofilm associated surface proteins

The Sec-dependent pathway is a system which contains many components that work together to transport proteins that contain a signal peptide out of the cell (Mori and Ito, 2001). Many of the virulence factors mentioned here are transported via this pathway. Once transported through the membrane, lipoproteins are retained at the membrane through a lipo-modified N-terminal cysteine residue (Kovacs-Simon et al., 2011, Hutchings et al., 2009). Several other cell wall proteins contain a C-terminal LPxTG motif and these proteins are attached to the cell wall by a sortase (Hendrickx et al., 2009b). This enzyme cleaves between the threonine and glycine residues in this motif and the protein is then covalently immobilized to peptidoglycan in the cell wall (Hendrickx et al., 2009b). Many of the virulence factors mentioned below, and are shown in figure 1.3, contain this motif and require attachment to the cell wall by sortase. There is also a summary table of virulence factors found in both *E. faecium* and *E. faecalis* at the end of this section (Table 1.1)

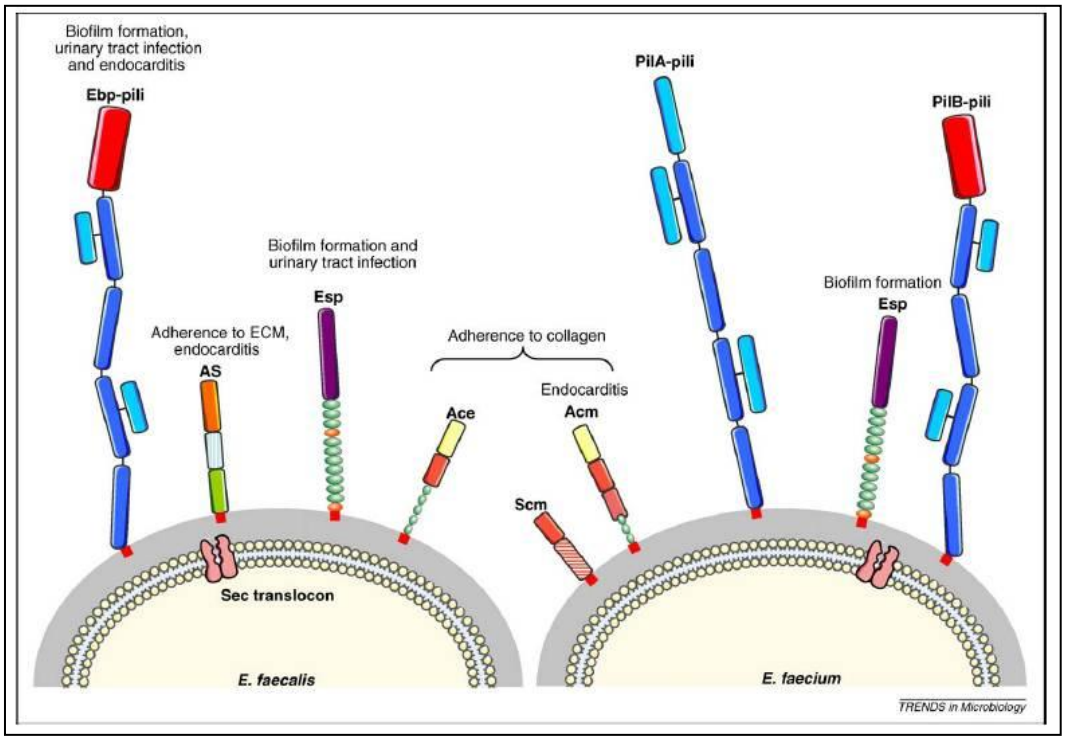



**Figure 1.3. Surface attached proteins involved in virulence for *E. faecalis* and *E. faecium*, more details in text (Hendrickx et al., 2009b).**

Enterococcal surface protein (Esp) is an approximately 202 kDa large cell wall protein found in both *E. faecium* and *E. faecalis* strains (Figure 1.3). The proteins in both strains are very similar and have a sequence identity of around 90% (Heikens et al., 2007). Esp has three regions (Figure 1.4): the N-terminal domain, which alone is sufficient to mediate biofilm formation (Tendolkar et al., 2005), a repeat domain, which contains repeat units, and the C-terminus which contains the cell wall anchor. Further details on structure of Esp are in chapter 6 (Shankar et al., 1999). Esp also shows similarities to other biofilm-associated proteins in other species, which include Bap from *S. aureus*, LapA from *Pseudomonas fluorescens* and BapA from *Salmonella enteritidis* (Lasa and Penades, 2006, Latasa et al., 2006).

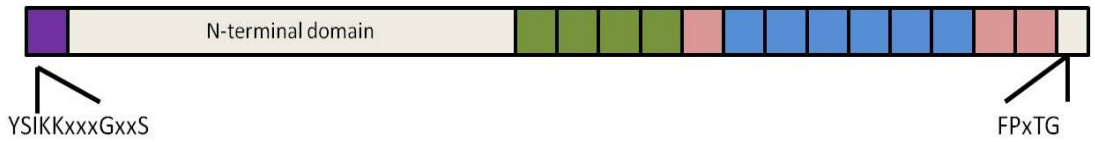



**Figure 1.4. *E. faecium* E1162 Esp structure. The signal peptide is represented by purple and also contains the YSIRK motif. A, B and C repeats are represented by blue, pink and green, respectively. FPxTG is the sortase-dependent cell wall-anchoring sequence. See text and chapter 6 for further details.**

In *E. faecalis* Esp is found on a pathogenicity island along with other virulence factors such as cytolysin (Shankar et al., 2002). The pathogenicity island has recently been identified as an integrative conjugative element (ICE) and is therefore self-transmissible, and enables transfer between its own species and other species (Laverde Gomez et al., 2011). *E. faecium* Esp is also found on pathogenicity island, ICE*Efm1*, which also been shown to be transmissible (Top et al., 2011).

*E. faecalis* biofilm formation has been shown to occur both dependent and independent of the presence of Esp, showing that there are also other biofilm-formation determinants involved (Kristich et al., 2004, Toledo-Arana et al., 2001, Tendolkar et al., 2004). Esp has also been shown to have a role in biofilm formation in *E. faecium* (Heikens et al., 2007), and isolates from hospitals are frequently found to have Esp. Importantly, although some strains can develop biofilms without the presence of Esp (usually non-clinical strains), strains with Esp have much thicker biofilms (Di Rosa et al., 2006). This study also observed that more *E. faecalis* isolates are able to produce biofilms compared to *E. faecium* isolates, and that *E. faecalis* is more likely to have Esp and gelatinase present compared to *E. faecium* isolates (Di Rosa et al., 2006).

The role of Esp in biofilm and virulence is still unclear. Research has shown that Esp is involved in initial attachment, colonisation and persistence (Heikens et al., 2007,

Shankar et al., 2001, Toledo-Arana et al., 2001, Van Wamel et al., 2007). The role of Esp in pathogenesis has been studied using human cell lines and in mammalian infection models. Studies in human bladder carcinoma T24 cells, Madin-Darby canine kidney (MDCK) epithelial cells and mice models have demonstrated that *E. faecium* Esp plays a role in urinary tract infections but not peritonitis by increasing attachment (Leendertse et al., 2009). Another study also showed Esp to be involved in renal parenchyma and persistence in the kidney (Sava et al., 2010). *E. faecalis* studies have been contradictory when it comes to a role for Esp in UTIs, with one study showing no role (Leendertse et al., 2009) and another study showing a role for Esp (Shankar et al., 2001). It has also been observed by one study that *E. faecalis* is involved in UTI's using mouse models (Kau et al., 2005). *E. faecium* isolates containing Esp were also shown to be recovered at higher levels than non-Esp producing isolates when studying endocarditis-induced vegetations from rat infection models (Heikens et al., 2011). Studies to see if Esp is essential to intestinal colonisation, showed in fact that there is no difference in adherence to human colorectal adenocarcinoma cells (Caco-2-cells) and that both *E. faecium* E1162 and an *esp* mutant strain were able to translocate to the mesenteric lymph nodes in mouse models (Heikens et al., 2009).

It has been observed that *E. faecium* and *E. faecalis esp* mutants are able to be complemented with plasmid-borne *esp*, thus restoring full biofilm formation (Tendolkar et al., 2005, Heikens et al., 2007). Interestingly, a study in which an *E. faecalis esp* was introduced in *esp* negative *E. faecium* or *L. lactis* strains did not show an increase in biofilm formation in these strains, even though expression levels of the protein on the cell surface were the same as observed for *E. faecalis*. Thus, it appears that *E. faecalis* Esp requires one or more additional factors, which are absent in *E. faecium* and *L. lactis*, to stimulate biofilm formation (Tendolkar et al., 2005).

Expression of Esp in *E. faecium* was shown to be dependent on growth conditions, as experiments showed increased expression at higher temperatures (37$^{o}$C compared to 21$^{o}$C), and under anaerobic conditions compared to aerobic. It was found that the

increase in Esp expression due to the temperature shift from 21$^{o}$C to 37$^{o}$C showed a correlation with increase initial attachment (Van Wamel et al., 2007). Similar to several other organisms, another growth condition that influences biofilm formation in *E. faecalis* was the presence of glucose in the media. This was, however, not due to Esp as its expression was not affected by the presence or absence of glucose (Tendolkar et al., 2004).

Aggregation substance (AS) was one of the first surface proteins to be discovered in *E. faecalis* and have not as yet been found in *E. faecium* (Figure 1.3; Hendrickx et al., 2009b, Hallgren et al., 2009). The genes for AS are found on pheromone-induced conjugative plasmids, and AS acts by allowing close contact between donor and recipient cells so virulence plasmids can be transferred (Hendrickx et al., 2009b). Several other roles for this protein have also been identified, including roles in adhesion and invasion of intestinal cells (Waters et al., 2004, Sartingen et al., 2000), binding to lipoteichoic acid (LTA; Waters et al., 2004), binding to the extracellular matrix (ECM; Rozdzinski et al., 2001), and stimulation of biofilm formation through cell aggregation (Chuang-Smith et al., 2010).

Pili are also found protruding outwards on the surface of the cell; they are made of major and minor pilin subunits. The genes for the production of pili are found on specific pilin gene clusters (PGCs) and include their own specific class C sortase that connects the subunits together. Once the structure is complete the housekeeping class A sortase attaches the pilus to the cell wall (Hendrickx et al., 2009b). *E. faecalis* has two PGCs, the biofilm enhancer in enterococci (*bee*) locus and the endocarditis and biofilm-associated pili (*ebp*) locus. The *bee* locus contains three *bee* genes (*bee-1* to -*3*) and two sortase genes, and this locus was identified to effect biofilm formation by inserting a transposon (Tn917) into the genes of the locus. The locus was also shown to be carried on a large extrachromosomal element, likely a conjugative plasmid, but it is not widely distributed among the isolates (Tendolkar et al., 2006).

The *ebp* locus has been studied in more detail (Figure 1.3). This locus has been identified in many clinical and environmental isolates and is thus widely spread in the *E. faecalis* population (Cobo Molinos et al., 2008). As the name suggests it has been linked with a role in endocarditis and biofilm formation (Nallapareddy et al., 2006b). It contains the genes *ebpA*, *ebpB*, *ebpC*, *srtC* and is regulated by *ebpR*. It is unknown what exactly the regulator responds to, but studies have shown increase expression in the presence of bicarbonate and/or carbon dioxide (Bourgogne et al., 2010, Bourgogne et al., 2007).

There have been found four PGCs in *E. faecium* that are similar to the *ebp* locus in *E. faecalis*. The two PGCs that have been studied the most are named PilA and PilB (Figure 1.3; Hendrickx et al., 2009b). Interestingly, expression of these genes is reduced at lower temperatures such as 21$^{o}$C, and pili are only observed on the surface when at 37$^{o}$C, and so the pili are controlled in a temperature dependent manner which allows them to take advantage if they are present in the body. Hospital-associated *E. faecium* isolates have been shown to be enriched in these pilus genes, again suggesting their role in pathogenesis. Research has also shown that cells can have both PilA and PilB present on the surface at any one time (Hendrickx et al., 2008) and PilB has also been associated with biofilm formation and virulence in UTI in mouse models (Sillanpaa et al., 2010).

Hendrix *et al* found five surface proteins that were found frequently in *E. faecium* Clonal complex 17 isolates. Some of these showed similarities with *E. faecalis* proteins involved in the aforementioned pili production, and some were of unknown functions (Hendrickx et al., 2007). One of the proteins was given the name SgrA (serine-glutamate repeat containing protein A). This protein consists of a non-repetitive region A, which may bind ligands, and it also contains a C-terminus with a repeating B-domain and a cell-wall anchor sequence. Similar B-domain repeats are also found in a *S. aureus* collagen binding protein, a protein belonging to the family of Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMM). However, SgrA is not an MSCRAMM as it does not contain

characteristic IgG-like folds. It was shown that this protein can bind to fibrinogen and human nidogens (glycoproteins associated to laminin) and also has a role in biofilm formation on abiotic surfaces (Hendrickx et al., 2009a).

As more enterococcal genomes become available, more genomic studies for virulence factors are being performed. In one such study on *E. faecalis* V583, a surface protein, EF3314, was identified. The gene encoding this protein was shown to have an above average GC content, suggesting that is was acquired through horizontal gene transfer. EF3314 has similarities to alpha-like proteins of group B streptococci as well as Esp. It too has been shown to effect biofilm formation and is involved in the early steps in attachment to epithelial cells. Also in a *Caenorhabditis elegans* infection model it was shown that the protein was a possible virulence factor, as there was attenuated killing with a deletion mutant (Creti et al., 2009).

### 1.3.4 Role of *fsr* two-component system and secreted proteins

*E. faecalis* has an *fsr* locus which consists of four genes, *fsrA*, *fsrB fsrC* and *fsrD*, and these genes are part of a quorum sensing two-component pathway. It involves an autoinducing peptide that increases in concentration outside of the cell. When the extracellular concentration of this peptide is high enough the expression of a number of other genes is up- or down-regulated (Hancock and Perego, 2004). The autoinducing peptide is a cyclic peptide called gelatinase biosynthesis-activating pheromone (GBAP), which is encoded by *fsrD*. Maturation of GBAP requires FsrB, which cyclises the peptide (Nakayama et al., 2006). Once GBAP accumulates, it is sensed by FsrC, a histidine kinase, and this leads to activation of the response regulator and transcription factor FsrA. The above process leads to the production of gelatinase (GelE) and serine protease (SprE; Qin et al., 2001, Hancock and Perego, 2004), the role of which in biofilm formation is outlined below. The *fsr* system has also been observed to affect other genes using both negative and positive regulation; genes included are involved in virulence and metabolism (Bourgogne et al., 2006).

GelE and SprE are encoded on the same operon; GelE is an extracellular zinc metalloprotease and SprE is a serine protease (Qin et al., 2001). Several studies have shown that mutants lacking *gelE* produce less biofilm (Mohamed et al., 2004, Hancock and Perego, 2004, Thomas et al., 2008). GelE has also been shown to effect virulence in mouse model peritonitis and endocarditis, endophthalmitis (Singh et al., 2005, Singh et al., 1998, Engelbert et al., 2004) and *Caenorhabditis elegans* models (Sifri et al., 2002). Gelatinase is able to degrade many proteins such as casein, gelatin, collagen fibrin, haemoglobin, plasmid conjugation factors and autolysins (Carniol and Gilmore, 2004). There have been many theories of how GelE is involved in biofilm formation, one being that that it is able to cleave at hydrophobic residues and therefore could cleave surface proteins on the cell, thereby allowing hydrophobicity to increase and stimulate attachment to surfaces (Carniol and Gilmore, 2004). However, the main theory now which has experimental evidence is that GelE-SprE locus is involved in fratricide, which is also known as allolysis ("sibling-killing-sibling" mechanism). This fratricidal mechanism allows the release of eDNA, which then contributes to the biofilm development. Thomas *et al* (2009) suggest that in biofilm cells, GBAP will switch on the *frs* system allowing the production of GelE and SprE. If a sibling cell (prey cell) has not as yet switched on their *frs* system, GelE from the producing cell (predator cell) will release an autolysin (AltA) from their membranes (it has a C-terminal cell wall anchor (Eckert et al., 2006)), leading to cell lysis and release of eDNA for biofilm formation. GelE from the predator cell is unable to act on its own AltA as SprE is also being produced; the latter acts as an immunity factor by stopping GelE attachment and therefore activation of AltA (Thomas et al., 2008, Thomas et al., 2009).

Initially it was suggested that SprE does not influence biofilm formation (Mohamed et al., 2004), but newer studies (mentioned above), showed that SprE does effect biofilm formation by modulating GelE's effect on fratricide (Thomas et al., 2009, Thomas et al., 2008). SprE has also been shown to effect virulence in a mouse peritonitis model (Qin et al., 2000), a rabbit endophthalmitis model (Engelbert et al., 2004) and in a *C. elegans* model (Sifri et al., 2002).

*E. faecalis* has several autolysins one of which, AltA (also mentioned above) if disrupted showed a decrease in biofilm formation, initial attachment and increased chaining (Kristich et al., 2008, Mohamed et al., 2004, Qin et al., 1998, Guiton et al., 2009). The role of autolysin in biofilm formation is to release eDNA into the environment (Thomas et al., 2009). eDNA has been shown to have a structural stabilising role in biofilms, and DNase I treatment has been shown to disrupt the biofilms of many bacteria (Montanaro et al., 2011). Experiments with DNaseI have also shown this to be the case in enterococcal biofilms; experiments showed that cells grown in the presence of DNaseI were able to attach to surfaces but were unable to form mature structured biofilms (Guiton et al., 2009). Barnes *et al* showed that eDNA also has a role in early biofilm formation, this study found that *E. faecalis* cells had thread like structures between them which were co localised with eDNA and suggested that the cells are able to secrete the eDNA (Barnes et al., 2012).

A major secreted antigen, SagA, was identified in *E. faecium* isolates from endocarditis patients. It was shown to be able to bind to many ECM components including fibrinogen, collagen I and IV and fibronectin, but it was also suggested that it has a role in cell metabolism (Teng et al., 2003). Homologues of *sagA* were identified in *E. faecalis*, *salA* and *salB*; these were shown when deleted to have reduced amounts of biofilm formation. SalB was shown to bind collagen I and fibronectin but SalA did not bind any of the ECM proteins tested (Mohamed et al., 2006). Interestingly, even though biofilm formation was decreased in a *salB* mutant under standard conditions, when the mutant was grown in presence of horse serum and fibronectin its biofilm formation was actually more than the wild type strain (Mohamed et al., 2006). SalB has also been shown to be involved in cell shape (Breton et al., 2002), and it was therefore suggested that perhaps the lack of SalB allows easier binding of other proteins, in the presence of ECM proteins, due to the change in cell shape, therefore increasing biofilm formation under these conditions (Mohamed et al., 2006).

### 1.3.5 Role of other virulence factors

MSCRAMMs are types of surface associated adhesions that are involved in the attachment to components within the ECM of the host. These proteins can attach to fibrinogen, fibronectin and collagen (Vengadesan and Narayana, 2011). One well studied MSCRAMM is Cna in *S. aureus*, which contains an A domain that consists of single and multiple sequences of an immunoglobulin (IgG)-like fold, an N-terminal signal peptide, a B-repeat domain and a C-terminal contain the LPxTG cell wall anchor sequence (Hendrickx et al., 2009b, Vengadesan and Narayana, 2011). Seven MSCRAMMs have been identified in *E. faecalis* (Sillanpaa et al., 2004) with similar structure to Cna. The main one studied is denoted Ace (Figure 1.3), a protein that has the ability to bind collagen type I and VI as well as laminin (Rich et al., 1999, Nallapareddy et al., 2000a). Ace has been shown to be expressed during human infection (Nallapareddy et al., 2000b) and has a role in urinary tract infections (Nallapareddy et al., 2011b) and endocarditis (Singh et al., 2010a). Recently there has been evidence to suggest that the *E. faecalis* MSCRAMMs expression on the surface is regulated by the Frs system and GelE (Pinkston et al., 2011).

Several *E. faecium* strains, and in particular clinical isolates, also encode MSCRAMMS, all of which share homology with Ace (Nallapareddy et al., 2008b). There have been three studied so far: Acm (Figure 1.3; adhesion of collagen form *E. faecium*), which interacts with collagen type I and has been shown to be involved in the pathogenesis of endocarditis (Nallapareddy et al., 2008a, Nallapareddy et al., 2003); Scm (Figure 1.3; second collagen adhesion of *E. faecium*) which interacts with collagen type V and fibrinogen (Sillanpaa et al., 2008); and EcbA (*E. faecium* collagen binding protein A) that binds to collagen types I to V, fibrinogen and laminin, and is detected in exponential and late exponential phase of growth (Hendrickx et al., 2009a).

A gene cluster called *epa* (Enterococcal polysaccharide antigen) has also been linked to *E. faecalis* biofilm formation. This cluster of genes has been shown to be involved in polysaccharide biosynthesis, and it contains many genes including glycosyl transferases, dTDP-4-dehydrorhamnose 3,5-epimerase, ABC transporter permease protein and hypothetical membrane proteins (Teng et al., 2009). A disruption mutant strain at the glycosyl transferase EpaB showed a 73% decrease in biofilm formation (Mohamed et al., 2004). Similarly, deletions in other genes in the locus have also been shown to decrease in biofilm formation (Teng et al., 2009). There has also been evidence that the locus is involved in UTIs and peritonitis pathogenesis (Teng et al., 2009, Singh et al., 2009). However, the mechanisms in virulence is unclear as these protein have not been identified on the surface, but there is a suggestion that they may be buried in the cell wall (Hancock and Gilmore, 2002).

As mentioned earlier many of the virulence factors mentioned contain the cell wall anchor motif and therefore require attachment to the cell wall by sortase. Therefore it is unsurprising that sortase A (*srtA*) has been implicated in biofilm formation. Deletion of *srtA* has indeed been shown to cause a decrease of 30% and 60% respectively in biofilm formation in two separate studies (Guiton et al., 2009, Kristich et al., 2008), but further studies into which SrtA-dependent proteins are involved in biofilm formation is required (Guiton et al., 2009). Similarly, deletions in *srtC* (encoding sortase C) also result in a decrease in biofilm formation. This sortase is part of the *ebp* locus, which is involved in attachment and cross-linking pillus subunits, as mentioned above in more detail (Nallapareddy et al., 2006b).

***Table 1.1 Virulence factor summaries for E. faecium and E. faecalis***

| Protein | Role in virulence |
|---|---|
| *Surface associated proteins:* | |
| Esp (*E. faecium* and *E. faecalis*) | • Biofilm formation<br>• Experimental UTI/endocarditis |
| AS (*E. faecalis*) | • Role in plasmid conjugation<br>• Biofilm formation<br>• Adhesion & invasion of intestinal cells<br>• ECM attachment |
| Pilus production: *bee* locus (*E. faecalis*) | • Biofilm formation |
| Pilus production: *ebp* locus (*E. faecalis*) | • Biofilm formation<br>• Endocarditis |
| Pilus production: PilA and PilB (*E. faecium*) | • Biofilm formation<br>• Experimental UTI |
| EF3314 *(E. faecalis)* | • Biofilm formation<br>• Attachment to epithelial cells<br>• Virulence in *C. elegans* |
| MSCRAMMS: Ace *(E. faecalis)* | • ECM attachment<br>• Experimental UTI and endocarditis |
| MSCRAMMS: Acm, Scm & EcbA *(E. faecium)* | • ECM attachment<br>• Experimental UTI |
| Secreted proteins: | |
| GelE and SprE *(E. faecalis)* | • Biofilm formation<br>• Experimental models: peritonitis, endocarditis, endophthalmitis<br>• Virulence in *C. elegans* |
| Autolysin *(E. faecalis and E. faecium)* | • Biofilm formation |
| SagA (*E. faecium*)<br><br>SalA and SalB *(E. faecalis)* | • ECM attachment<br>• Biofilm formation |
| *Other factors:* | |
| *epa* locus | • Biofilm formation<br>• Experimental UTI and peritonitis |
| SrtA | • Biofilm formation |
| *bop* locus | • Biofilm formation |

## *1.4. The aims of the thesis*

Enterococci are increasingly becoming a problem clinically with their increase in antibiotic resistance and ability to form biofilms. Initially the thesis will consider a possible antimicrobial target, the signal peptidase; this enzyme is a component of the Sec-dependent pathway. This pathway is responsible for exporting many enterococcal virulence factors, and so by interfering with this pathway there is a possibility that it would make enterococci less virulent. There has already been an inhibitor for signal peptidases identified, Penem (Harris et al., 2009), and so if signal peptidases are found to be a good target here it would allow further investigation into this inhibitor. The above will be performed by producing a signal peptidase mutant which can then be studied for virulence differences.

The next section of the thesis will study antimicrobial resistance in *E. faecium* E1162 using the method of calorimetry to establish the merits of the method. This method has been shown to identify between meticillin sensitive and resistant *S. aureus* isolates in hours compared to traditional antimicrobial methods that can take days (von Ah et al., 2008). Here we address whether this is also the case for enterococci.

Further experiments will include studies on the effect of bile salts on biofilm formation in both *E. faecium* and *E. faecalis* isolates. A particular focus is on the role of Esp in these organisms. Its function is still not fully understood, and the role of Esp in relation to the effect of bile on biofilm formation will be analysed further.

Research has shown that *E. faecalis* shows pathogenicity in a *C. elegans* infection model (Garsin et al., 2001), whereas the *E. faecium* strains that have been tested are not (Moy et al., 2006, Moy et al., 2004, Garsin et al., 2001). However, only a limited number of *E. faecium* strains have been tested, and for that reason several clinical isolates will be analysed to establish if a lack of pathogenicity to *C. elegans* applies to all *E. faecium* strains.

# Chapter 2:
# Materials and Methods.

## *2.1. Bacterial strains.*

All bacterial strains used in this thesis are listed in Table 2.1.

***Table 2.1. Bacterial strains.***

| Strain | Information | Reference |
|---|---|---|
| *E. coli:* | | |
| *E. coli* JM109 | F´ *traD36 proA*$^{+}$*B*$^{+}$ *lacI*$^{q}$ *Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14*$^{-}$ *gyrA96 recA1 relA1 endA1 thi hsdR17* | Yanisch-Perron *et al*. 1985 |
| *E. coli* C41 (DE3) | F$^{-}$ *ompT hsdSB* ($r_B^-$ $m_B^-$) *gal dcm* (DE3) | Miroux and Walker 1996 |
| *E. coli* NovaBlue | *Used for routine cloning, K-12 endA1 hsdR17* ($r^-$ $m^+$) *supE44 thi-1 recA1 gyrA96 relA1 lac F'[proA+B+ lacIqZΔM15::Tn10] (TetR)* | Novagen |
| *E. coli* HB101 | F$^{-}$, *hsd*S20( $r_B^-$ $m_B$ ), *xyl*5, l-, *rec*A13, *gal*K2, *ara*14, *sup*E44, *lac*Y1, *rps*L20(strp$^{R}$), *leu*B6, *mtl*-1, *thi*-1 | Lacks and Greenberg 1977 |
| *E. coli* OP50 | Uracil requiring mutant | Brenner 1974 |
| *L. lactis:* | | |
| *L. lactis* NZ9700 | Progeny of the conjugation between nisin producer strain NIZO B8 with MG1614 (Rif$^{R}$, Strp$^{R}$ derivative of MG1363) | Kuipers *et al.* 1998 |

***Table 2.1. Bacterial strains continued...***

| Strain | Information | Reference |
|---|---|---|
| *E. faecium:* | | |
| E1162 | Clinical blood isolate. $Amp^R$, $Esp^+$ | Heikens *et al*. 2007 |
| E1162Δ*esp* | E1162 strain with *esp* gene deleted | Heikens *et al*. 2007 |
| TX1330 | Healthy volunteer faecal isolate. $Amp^S$, $Esp^-$ | Nallapareddy *et al*. 2003 |
| E1162Δ*ebrB* | E1162 strain with *ebrB* gene deleted. | Not Published |
| *E. faecium* clinical isolate 1 | Intensive care unit*. Asctric fluid‡. $Van^R$, $Pen^R$, $Ery^R$, $Chl^R$, $Tet^R$, $Amp^R$ | Health protection agency. Southampton general hospital |
| *E. faecium* clinical isolate 2 | Surgical ward*. Intra-abdominal drain swab‡. $Van^R$, $Pen^R$, $Ery^R$, $Chl^R$, $Amp^R$ | As above |
| *E. faecium* clinical isolate 3 | Leukaemic ward*. Blood culture‡. $Van^R$, $Pen^R$, $Ery^R$, $Chl^R$, $Tet^R$, $Amp^R$ | As above |
| *E. faecium* clinical isolate 4 | Neonatal unit*.Gastric aspirate‡. $Van^R$, $Pen^R$, $Ery^R$, $Chl^R$, $Tet^R$, $Amp^R$ | As above |
| *E. faecium* clinical isolate 5 | Intensive care unit*. Central venous catheter swab‡. $Van^R$, $Pen^R$, $Ery^R$, $Chl^R$, $Tet^R$, $Amp^R$ | As above |
| *E. faecalis:* | | |
| ATCC19433 | Reference strain- $Esp^+$ | ATCC |
| BS12297 | Isolate from clogged biliary stents. $Esp^+$, $GelE^-$ | van Merode *et al*. 2006 |
| BS11297 | Isolate from clogged biliary stents. $Esp^+$, $GelE^+$ | van Merode *et al*. 2006 |
| BS385 | Isolate from clogged biliary stents. $Esp^-$, $GelE^-$ | van Merode *et al*. 2006 |

*Source of sample. ‡Sample type.

## *2.2. Plasmids*

All plasmids used and constructed are listed in Table 2.2.

***Table 2.2. Plasmids used during this thesis.***

| Plasmid | Details | Reference |
|---|---|---|
| *Plasmids involved in making the signal peptidase mutant:* | | |
| pTEX5500ts | Shuttle plasmid, temperature sensitive in Gram-positive hosts; $Chl^R$, $Gen^R$ | Nallapareddy *et al*. 2003. |
| pSP1 | pTEX5500ts with TX1330/E1162 S0713 upstream fragment | This thesis |
| pSP2 | pS0713U with TX1330/E1162 S0713 downstream fragment | This thesis |
| pSP3 | pTEX5500ts with TX1330 S1233 upstream fragment | This thesis |
| pSP4 | pTXS1233U with TX1330 S1233 downstream fragment | This thesis |
| pSP5 | pTEX5500ts with E1162 S0133 upstream fragment | This thesis |
| pSP6 | pE1162S0133U with E1162 S0133 downstream fragment | This thesis |
| pSP7 | pTEX5500ts with E1162 S1233 upstream fragment | This thesis |
| pSP8 | pE1162S1233U with E1162 S01233 downstream fragment | This thesis |
| *Plasmids involved in making the integration:* | | |
| pNZ8148 | Broad host range vector with *nisA*-promoter and multiple cloning site and a *Nco*I site used for translational fusions, standard vector; $Chl^R$ | Kuipers *et al*. 1998 |
| pMUTIN4 | pBR322-based vector containing *lacZ*, *lacI*, and *Pspac* inducible promoter; $Amp^R$, $Em^R$. | Vagner *et al* 1998. |

| | | |
|---|---|---|
| pINT1 | pMUTIN4 with no *lacZ*, *lacI* and an inserted *Nco*I site. | This thesis |
| pINT2 | Combination of pNZ8148 and pMutin4. ColE1 replication sequence, *nisA*-promoter, $Amp^R$, $Em^R$ and $Chl^R$ | This thesis |
| pINT3 | pNZpMUT with *nisRK* | This thesis |
| pINT4 | pNZpMUTnisRK with SPase713 fragment | This thesis |
| *Esp Expression:* | | |
| pN-tEsp | pCRT7/CT-TOPO TA expression vector encoding the Esp N-terminal domain | Van Wamel *et al.* 2007. |

## *2.3. Chemicals and bacterial culture media.*

All chemicals were purchased from Fisher Scientific or Sigma unless stated otherwise. All culture media were purchased from Oxoid.

## *2.4. Cell culture methods.*

### 2.4.1 *E. coli* growth conditions

*E. coli* was grown at 37°C in Luria-Bertani (LB) broth, unless by otherwise stated. Antibiotics were added at the following concentrations: chloramphenicol 10 µg/ml (Fisher Scientific), gentamicin 25 µg/ml (Sigma), or ampicillin 100 µg/ml (Sigma).

### 2.4.2 Enterococcal growth conditions

*E. faecalis* and *E. faecium* strains were cultured in Tryptone soya broth (TSB), TSB containing 0.25% glucose (TSB-G), Brain heart infusion (BHI) broth or LB broth at

37°C unless stated differently. Antibiotics were added at the following concentrations: chloramphenicol 10 µg/ml, gentamicin sulfate 200 µg/ml (Fisher Scientific) or gentamicin 125 µg/ml.

### 2.4.3 Viable counts

To establish the number of viable bacteria inoculated or present, the optical density at 600 nm (OD600) was determined of exponentially growing cultures using an Eppendorf Bio-Photometer. The number of colony forming units (CFU) were determined by serial dilution and plating on TSB agar (TSA) plates.

### 2.4.4 Growth rate of *E. faecium* in the presence of bile salts

*E. faecium* strains were cultured overnight at 37°C in TSB. Next, cultures were diluted in fresh TSB-G containing 0%, 0.5%, 1.5% or 5% bile salts, and the OD600 was determined at regular intervals for 8 hours.

## *2.5. Nucleic Acid techniques*

### 2.5.1 Small scale plasmid purification.

All plasmids were isolated using the Nucleospin Plasmid Kit (Macherey-Nagel) following manufacturer's instructions.

### 2.5.2 Chromosomal extraction of DNA

Chromosomal DNA extraction from *E. faecium* strain E1162 was performed as described previously (Leenhouts et al., 1990).

### 2.5.3 Agarose gel electrophoresis and gel purification

Agarose gel electrophoresis was performed using standard methods (Sambrook and Russel, 2001). 0.8% agarose gels containing 0.5 μg/ml ethidium bromide were run at 120V and were visualised under UV light. Purification of DNA from agarose gels was performed using the Nucleospin Extract II Kit (Macherey-Nagel) following the manufacturer's instructions.

### 2.5.4 DNA quantification

DNA was quantified at 260 nm using an Eppendorf Bio-Photometer, or by comparison with standard amounts of DNA on agarose gels.

### 2.5.5 DNA sequencing

Sequencing was performed by Eurofins MWG Operon, UK. Samples were prepared by following Eurofins instructions.

## *2.6. Cloning techniques.*

### 2.6.1 Polymerase chain reaction (PCR)

PCR was performed with a Mastercycler gradient machine (Eppendorf). The KAPA2G robust or KAPA Hifi PCR kit was used (KAPA biosystems), following the manufacturer's instructions. All primers were synthesised by Invitrogen or Sigma and are noted in the relevant chapters. PCR products were purified using the NucleoSpin Extract II kit (Macherey-Nagel).

### 2.6.2 Restriction enzyme digestion

Restriction enzyme digests were performed as recommended by the manufacturer, New England BioLabs. DNA for ligations or other cloning steps was always digested overnight unless stated otherwise. After digestion, DNA was purified using PCR clean-up gel extraction kit, NucleoSpin Extract II (Macherey-Nagel).

### 2.6.3 Ligation of DNA.

Two methods were used for ligation of DNA. These were either using T4 DNA ligase (New England Biolabs) as described (Sambrook and Russel, 2001), or using the In-fusion advantage PCR cloning kit (Clontech), following the manufacturer's instructions. Primers for the latter were designed as recommended by Clontech; these were designed as normal with an additional 15 nucleotides that share sequence homology with the linearized vector. Primers used are stated in the relevant chapter.

## *2.7. Transformation of competent cells with plasmid DNA*

### 2.7.1 Transformation of *E. coli*

*E. coli* strain JM109 and C41 (DE3) were made competent for DNA uptake by treatment with $CaCl_2$ (Sambrook and Russel, 2001), following this cells were used for transformations. Commercial *E. coli* NovaBlue GigaSingles competent cells were used for some transformations, following the manufacturer's guidelines. After transformation bacteria were plated out on LB plates with the relevant antibiotic.

### 2.7.2 Transformation of electro competent Enterococci cells.

Electrocompetent enterococcal cells were prepared and transformed as previously stated (Heikens et al., 2007). In brief, 5 µl of plasmid was added to 100 µl of electro

competent enterococcal cells in a 1mm cuvette (Invitrogen). Cells were electroporated at 1.25kV, 25μF and 200Ω, using a BioRad Gene Pulser, giving a time constant of 4-6 msec. Cells were allowed to recover at 28$^{o}$C for 4 hours for the SPase mutant protocol (Chapter 3, Section 3.3) or at 37$^{o}$C for 2 hours for the integration plasmid protocol (Chapter 3, Section 3.4), before being plated onto Todd-Hewit broth (THB) containing 20% sucrose and the relevant antibiotics.

## *2.8. Protein gel techniques*

### 2.8.1 SDS-Polyacrylamide gel electrophoresis (PAGE) and Western blotting

Western blotting was performed using a semi-dry western blotting system. Proteins were firstly resolved by SDS-PAGE (Laemmli, 1970). Samples were prepared by adding a 1:1 volume Laemmli loading dye and boiling for 3 minutes. For full length Esp a 7.5% gel was used, while for the analysis of the N-terminal domain of Esp a 12.5% gel was used. For all gels the EZrun Pre-stained Rec protein ladder (Fisher) was used to establish size of bands. The gel and polyvinylidene difluoride membrane (PVDF; Immobilon-P; Millipore) were sandwiched in between several layers of Whatman 3MM chromatography paper (Schleicher & Schuell). The buffers used for transfer were Towbin transfer buffer (25mM Tris, 192 mM glycine, 20% Methanol) or a buffer with SDS for the transfer of large proteins such as Esp (48mM Tris, 39mM glycine, 0.1% ml SDS and 10% methanol). The membrane and gel were blotted for 2-3 hours at 20V and 150mA.

Following blotting membranes were blocked with Tris-buffered saline containing Tween 20 (TBST; 150 mM NaCl, 20mM KCl, 25mM Tris pH 7.4 and 0.05% Tween-20/litre) and 5% skimmed milk (Marvel) for 2 hours. Next, the membrane was incubated at 4$^{o}$C overnight with the primary antibody or pre-immune sera at the appropriate concentration (as stated in the chapter). The membrane was washed and the secondary antibody, horseradish peroxidase- conjugated to goat anti-rabbit (Promega), added in all cases at concentration of 1 in 5000. Blots were washed with TSBT prior to performing signal detection with the Supersignal West Pico-chemiluminescent Substrate Kit (Pierce), following the manufacturer's guidelines.

### 2.8.3 Coomassie staining

Proteins were separated by SDS-PAGE, and the gel was fixed (40% methanol and 10% acetic acid) for 25-20 minutes on a rocker at room temperature. Next, the gel was stained for 1 hour (in 0.025% Coomassie brilliant blue G250 in 10% acetic acid), and then destained (20% methanol and 10% acetic acid) until the bands were clearly visible. For long-term storage gels were dried in cellophane wrap (BioDesign GelWrap).

## *2.9. Antibiotic sensitivity.*

### 2.9.1 Isothermal Calorimetry.

Cultures for calorimetry were grown overnight at 37$^{o}$C in TSB. 15% glycerol was added and aliquots of 1 ml were dispensed and frozen at -80$^{o}$C. Viable counts were performed to establish the numbers of cells after freezing. These were then used for continuous culture calorimetry, using a Thermometric 2277 Thermal activity Monitor (TAM-Therometric AB, Jarfalla, Sweden) that was calibrated between 0-1000µW (the experiment range) at 37$^{o}$C.

The strain of choice was thawed and diluted to give approximately 1 x $10^4$ CFU/ml of TSB. This was then incubated in an external water bath at 37$^{o}$C. Tubing from the calorimeter ampoule was placed in the culture and a peristaltic pump (flow-rate: 1ml/minute) was used to pump the growing culture through the calorimetry cell and the power-heat out-put was measured over time by the thermopile arrays.

Cells were grown in the absence or presence of ampicillin. Ampicillin (64µg/ml, twice the MIC of *E. faecium* E1162) was added when the output of the culture had reached 10 µW (corresponding to approximately 1.2 x $10^5$ cfu/ml).

Before and after use of the calorimeter the tubing and ampoule was washed by firstly by turning the peristaltic pump to 90% speed and washing through a one molar

solution of sodium hydroxide for 5 minutes. Sterile distilled water was then washed through at the same speed for 20 minutes.

### 2.9.2 Minimum inhibitory concentration (MIC) tests and Minimum bactericidal concentration (MBC) tests.

MIC tests using a microdilution protocol were performed as described (Andrews, 2001). Tests were performed in TSB-G, 200 µl cultures were grown for 18 hours at 37$^{o}$C in 96-well plates, with ampicillin ranging in concentration from 1-256 µg/ml, and an inoculum of $10^5$ cells/ml. MIC determinations for each strain were performed in triplicate test. The MIC is defined as the lowest concentration without visible growth.

Following the above MBC for the strains were determined. From the MIC 96-well plate 10µl was taken from each of the wells with concentrations of ampicillin that were higher than the MIC. The 10µl sample was added to 90µl TSB and this was then plated onto a TSA plate. The plates were incubated for 24 hours at 37$^{o}$C. The MBC was taken as the lowest concentration where no colonies grew.

### 2.9.3 M.I.C.Evaluator strip test and Antimicrobial susceptibility discs test.

The M.I.C.Evaluator strip (Oxoid) or discs (10 µg; Oxoid) with ampicillin were used. Tests were performed in triplicate on Mueller Hinton agar (Lab M) as recommended by the manufacturer. The plates were incubated at 37$^{o}$C for 24 hours.

## *2.10. Crystal violet biofilm assay*

### 2.10.1 Biofilm growth

Strains were grown overnight in TSB-G. The following day they were diluted in TSB-G to $10^8$ cfu/ml suspension, and 100 µl of the suspension was then added to

wells of a 96-well microtitre plate (Costar). 100 µl of the relevant concentration of the compound (diluted in TSB-G) being assayed was added to the well, or in the case of the controls, TSB-G. Compounds included Bile salts (0-5%), Sodium taurocholate (0-2%), Sodium glycocholate (0-2%; Calbiochem), Sodium taurocholate plus Sodium glycocholate (0-2%), Sodium dodecyl sulphate (SDS; 0.00975-0.03%), Triton X100 (0.01-1%). The microtitre plates were incubated for 24 at 37$^{o}$C on a 3D plate rotator (Grant-Bio; 30 rpm). If 48 hour biofilms were being studied, after 24 hours planktonic cells were removed and replaced with fresh media containing the compound being assayed.

### 2.10.2 Crystal violet assay

After incubation the cell suspension was removed, the plate was washed twice with 0.9% NaCl, and then inverted to dry for 1 hour at room temperature. Following this 150 µl of Crystal violet solution (CV; PROLAB Diagnostics) was added and was allowed to stain the biofilm for 15 minutes. Next, CV was removed and the wells were washed 3 times with 0.9% NaCl. The CV was solubilised with 200 µl of ethanol-acetone (80:20 v/v). The CV absorbance of the wells was read at 595 nm on a Versa max Tunable microplate reader. Each condition was tested at least three times in three independent tests.

## *2.11. Initial polystyrene adherence assay.*

The initial adherence was performed as described previously (Baldassarri et al., 2001). Briefly, bacterial strains were grown overnight at 37$^{o}$C on Tryptone soya agar (TSA). The following day they were diluted in TSB-G to an OD600 of 0.5 (5 x $10^{8}$ CFU/ml). 50µl of the suspension was added in triplicate to the wells of a 96 well plate. 50 µl of TSB-G containing the relevant amount of bile salts (double the required amount) was added to the wells. The plate was then placed on the 3D rotator (Grant-Bio; 30 rpm) and incubated for 2, 4 and 6 hours at 37$^{o}$C. Following incubation, wells were washed twice with 200µl of 0.9% NaCl and then allowed to dry for 15 minutes at 60$^{o}$C. 50 µl Crystal violet solution was added to the wells and was allowed to stain for 15 minutes. The stain was washed three times with 0.9%

NaCl, plates were allowed to dry for 15 minutes at room temperature, and then absorbance was read at 595nm on the plate reader (Versa max Tunable microplate reader). Each condition was tested three times in three independent tests.

## *2.12. Cell surface hydrophobicity determination*

Bacterial cell surface hydrophobicity was performed using MATHS, as previously described (Rosenberg et al., 1981). Briefly, bacterial cultures were grown in TSB-G overnight at 37$^{o}$C. Next, the culture was diluted 50-fold in TSB-G or TSB-G containing the compound being assayed. Each culture was incubated for 4 hours at 37$^{o}$C, and the log-phase cells were harvested by centrifugation and washed 3 times with PUM Buffer (0.15M potassium phosphate, 0.3M Urea, 6.7mM $MgSO_4$, pH 7.1). Cell pellets were resuspended in PUM buffer to an optical density at 400 nm (OD400) of 1. To 1 ml of the cell suspension, 200µl of n-hexadecane was added this was incubated for 10 min at 30$^{o}$C. Following this, the mixture was mixed thoroughly by vortexing for 2 min. Next, the mixture was allowed to stand for 15 min at room temperature to ensure complete separation of the organic and aqueous phases. The absorbance of the aqueous layer was measured at 400nm. The percentage of cell surface hydrophobicity, which is a measure of the percentage of bacterial cells partitioning into the organic phase, was calculated by using the following formula: [1-(final OD400/initialOD400) x100]. At least three independent tests were performed.

## *2.13. Confocal Laser Scanning Microscope (CLSM).*

### 2.13.1 Biofilm preparation

Cells were cultured overnight in TSB-G. The following day they were diluted in TSB-G to $10^8$ cfu/ml suspension and 2 ml of the suspension was added to wells of a 6-well microtitre plate (Costar). 2 ml of TBS-G containing different concentrations of bile salts was added to each well. Vinyl coverslips (Electron Microscopy Sciences, EMS, UK) were added to each well so that the biofilm could form on these

and used for visualisation. Every 24 hours medium was replaced with fresh medium (containing the relevant concentration of bile salts). Biofilms were grown for 72 hours.

If early attachment was being studied biofilms were only allowed to grow for 6 hours prior to staining.

### 2.13.2 Coverslip preparation and staining

After incubation the coverslips were removed and washed twice with 0.9% NaCl on a horizontal plate shaker (Heidolph). 1 ml of BacLight (Invitrogen) or acridine orange (Sigma) was added to each coverslip, and these were then placed in darkness for 10 minutes. Following staining the coverslips were rinsed twice with 0.9% NaCl. If EPS was also being studied then calcofluor white stain (600 µl) was added for 10 minutes (in darkness), followed by two wash steps. Coverslips were then mounted onto glass slides with nail varnish.

### 2.13.3 Confocal Laser Scanning Microscope

Images were collected using a LSM510META Zeiss Confocal laser scanning microscope, lasers included the argon laser (488nm) and the helium/neon laser (543 nm). A Plan-Apochromat 63x/1.4 oil objective was used. Images were prepared using Zeiss LSM software (release 4.0). Quantitative analysis of maximum thickness and bio volume of the CLSM biofilm images was measured using Comstat 2 software (Heydorn et al., 2000). At least three independent tests were performed.

## *2.14 Cell chain length determination.*

*E. faecium* strains were grown overnight in TSB broth and diluted 100 fold in fresh medium in the presence or absence of 0.5% bile salts. Cells were then cultured until mid-exponential growth phase (OD600 ~0.5-0.6) and examined by light microscopy. The number of cells in each chain observed was counted in at least 4 fields of view.

## *2.15 Protein expression and purification of the N-terminal Esp.*

### 2.15.1 Induction of *E. coli* C41 (DE3) cells.

*E. coli* C41 (DE3) cells were used to express the N-terminal domain of Esp. A culture was grown in LB broth until an OD600 of 0.6, at which point the cells were induced with 1 mM isopropyl-β-thiogalactopyranoside (IPTG). Cells were then grown for 16 hours before harvesting

### 2.15.2 *E.coli* cell lysis

Cells were collected by centrifugation and resuspended in a 1/20th volume of buffer A (50mM sodium phosphate pH 7.5, 300mM NaCl) containing 0.2 mg/ml lysozyme (Sigma). Cells were incubated for 10 minutes on ice and then lysed by sonication (Branson; model 250/450). Cell debris was removed by centrifugation (8000 g, 10 minutes). A protease inhibitor cocktail (complete- EDTA free, Roche Molecular Biochemicals) was added to prevent protein degradation.

### 2.15.3 His-tagged protein purification

A HisPur Cobalt Resin (Pierce) was used for purification, following the manufacturer's instructions.

### 2.15.4 Fast performance liquid chromatography (FPLC)

FPLC Akta Prime system (GE Healthcare) was used to further purification using a 5 mL Q-sepharose column (Amersham Biosciences) and a NaCl gradient from 50-1000 mM.

### 2.15.5 Protein dialysis

Fractions containing N-terminal Esp protein (as judged by analysis with SDS-Page) were pooled and dialysed using Snakeskin dialysis membrane (10kDa MWCO, Pierce) overnight in 50mM Tris, pH 8.

### 2.15.6 Protein concentration

Purified protein was concentrated using Viva spin filters 15R (Sartorius; 10,000 MWCO), following the manufacturer's instructions.

### 2.15.7 Determining protein concentration

Protein concentration was determined using the BCA assay (Pierce), following the manufactures guidelines.

### 2.15.8 Raising polyclonal antibodies

Polyclonal antibodies were raised by immunizing two rabbits with slices of SDS-PAGE gel containing 200 µg of the purified Esp protein per injection (Eurogentec, Belgium). A 3 month programme was used, with rabbits being injected at day 0, 14, 28 and 56 days, with a final bleed on day 87. This was all performed according to regulations on animal experiments. The antiserum was purified using protein A Sepharose (GE Healthcare).

### 2.15.9 Esp Expression using flow cytometry

#### *2.15.9.1 Bacterial growth*

Bacteria were grown in various conditions: on TSA plates, in broth till stationary phase (aerobically (with shaking) and anaerobically (no shaking)) or grown in broth until log phase. In all cases cells were resuspended in phosphate buffered saline

(PBS) containing 0.05% Bovine Serum Albumin (PBS-BSA; Sigma) to an OD600 of 1.

Biofilm formation and biofilm planktonic cells were prepared differently; bacteria were grown for 24 hours in 6 well microtitre plates (Costar). Planktonic bacteria were removed, washed and resuspended in PBS-BSA to an OD600 of 1 for testing. Cells in the biofilm were washed twice with 0.9% NaCl, and following this PBS-BSA was added to wells and cells were removed by scraping the surface and resuspended to a final OD at 600 nm of 1.

*2.15.9.2 Flow cytometry*

The expression of Esp on enterococcal cells were determined by flow cytometry with FACSCanto$^{TM}$ (B-D Biosciences, San jose. Calif.), equipped with an argon ion laser (488nm). Samples were prepared as stated above and then 300µl of each sample were pelleted by centrifugation (6,500*g*, 1 min) and resuspended in 50µl PBS-BSA containing 20-fold diluted anti-Esp serum. E1162Δ*esp* was used as the main negative control for the *E. faecium* experiments, but the pre-immune serum was used as an additional control. For *E. faecalis* Esp experiments the pre-immune serum was used as the main negative control. Samples were incubated at 4$^{o}$C for 16 hours, washed once in PBS-BSA, resuspended in PBS-BSA containing a 1/50 dilution of goat anti-rabbit antibody labelled with Alexa Fluor 488nm (Invitrogen) and incubated on ice for 1 hour. Finally, samples were washed twice and resuspended in 500µl of PBS-BSA for flow cytometry measurements. 10,000 cells were counted in each experiment. BD FACSDiva software version 5.0.3 was used to determine the number of *esp* expressing cells, using EfmE1162Δ*esp* (for *E. faecium)* or the pre-immune sera (for *E. faecalis*) as non-expressing negative control cells. Tests were performed at least three times.

### 2.16.9 Esp Expression using SDS-PAGE

#### *2.16.9.1 Bacterial growth*

Bacteria were grown under aerobic and anaerobic conditions (using an anaerobic jar with carbon dioxide generators and indictors (Becton, Dickinson (BD)) on TSA-G or TSB-G containing 5% sheep blood agar plate (TSA-G-B, TSB-G-B). Samples were incubated at 37$^{o}$C overnight. Next, bacteria were washed with PBS and made to an OD600 of 1. Mutanolysin (50U) and lysozyme (5mg/ml) was added to the bacteria and incubated at 37$^{o}$C for 1 hour. Samples were prepared for SDS-PAGE as stated in section 2.8.

## *2.16. C. elegans*

### 2.16.1 *C. elegans* maintenance

NGM plates (Brenner, 1974) were prepared and overnight culture of the *E. coli* HB101 strain was added to the centre of the plate and allowed to grow overnight at 37$^{o}$C. The *C. elegans* strain AU37 (*glp-4(bn2)*, *sek-1 (km4)*) used was obtained from the Caenorhabditis Genetics Centre (CGC) (Moy et al., 2006); this strain has mutations making the worms unable to produce progeny at 25$^{o}$C and, in addition, is more sensitive to pathogens. This strain was maintained on NGM plates with an *E. coli* lawn at 15$^{o}$C, as described previously (Brenner, 1974). NGM plates usually contained nystatin (Fisher; 125 U/ml) to inhibit fungal contaminations.

### 2.16.2 Synchronising *C. elegans*

Worms were transferred to fresh NGM plates containing *E. coli* HB101. Plates were then incubated for 7-8 days (depending on seeding) at 15$^{o}$C until the presence of many adults were observed and most of the *E. coli* was consumed. Worms were removed from the plates and washed 3 times with M9 (3 g/L $KH_2PO_4$, 6 g/L $Na_2HPO_4$, 5 g/L NaCl, 0.25 g/L $MgSO_4.7H_2O$). Eggs were released from the adult

worms using an alkaline bleach solution (2 ml 10% NaOCl, 5 ml 1M NaOH and 2 ml water) as described (Burns et al., 2006) then washed 4 times with M9. 200-500 eggs were seeded on agar plates with lawns of *E. coli* HB101 and incubated at 25$^{o}$C for 72 hours to allow the eggs to hatch and mature to L4/young adult stage. Worms were collected from the plates and washed 3-4 times in cold M9 to remove *E. coli*.

### 2.16.3 *C. elegans* Infection

BHI agar plates containing 80 µg/ml kanamycin (Sigma) and a lawn of the relevant pathogen to be tested were prepared prior to requirement. For anaerobic killing assays bacteria were grown on plates in an anerobic GasPak jar (Becton Dickinson) prior to requirement. NGM agar plates containing spectinomycin (50 µg/ml; Sigma) were made for the *E. coli* HB101 controls. Antibiotics and nystatin were added to stop contamination but also in the case of the pathogen plates stop *E. coli* HB101 growing. A ring of palmitic acid (10 mg/ml in ethanol) was also added around the edge of the plates; the palmitic acid is not soluble and forms crystals that prevent worms escaping from the plates. 30-50 synchronised L4/young adult worms were placed on a least 3 plates. These were incubated at 25$^{o}$C and worm survival was scored daily. Worms that lost their characteristic sigmoidal shape and were insensitive to mild agitation by tapping the plate or gentle touch with a platinum wire were considered dead.

## *2.17. Statistical analysis*

Differences between conditions were analysed using Students T-test. Significance was defined as a P-value of $<0.05$, evaluations were performed using SPSS 14.0.

# Chapter 3: Results Section I

# Construction of Signal peptidase mutants

## *3.1. Background and objectives*

Bacteria transport proteins across the cytoplasmic membrane using a number of distinct processes. Two of these are general secretion pathways, being able to translocate a wide variety of proteins. These are the Sec-dependent pathway, in which proteins are transported unfolded across the membrane, and the Tat pathway, in which proteins fold in the cytoplasm prior to translocation (Palmer and Berks, 2012). In addition Gram-positive bacteria also have a number of more specialist pathways that are involved in the translocation of only a very limited number of substrates. Two examples include the 'Accessory Sec system' which is found in staphylococci and streptococci. This pathway can contain an additional SecA2 and SecY2 which are able to transport proteins, usually virulence factors, across the cell membrane independently of the Sec-dependent pathway. The second example is the Type VII/WXG100 system, which is the most recent secretion pathway discovered. It has most widely been studied in *Mycobacterium tuberculosis* and other high-GC diderm (two membranes) Gram-positive bacteria (Freudl, 2013). This chapter deals with the Sec pathway only as enterococci do not have the Tat pathway.

Enterococci transport the majority of their proteins across the cell membrane using the Sec-dependent pathway (Figure 3.1). In this, secretory proteins are synthesised as pre-proteins with a signal peptide at the amino terminus. This signal peptide is an indicator to the Sec machinery that this protein has to be transported across the cytoplasmic membrane. The pre-protein is targeted by chaperones to a membrane-bound translocase which contains several components that recognise the pre-protein and transports it through the cytoplasmic membrane. At the *trans* side of the membrane the signal peptide is cleaved off by a signal peptidase, and the protein folds into its active configuration. Many of the components for protein transport are essential to cell survival (van Wely et al., 2001).

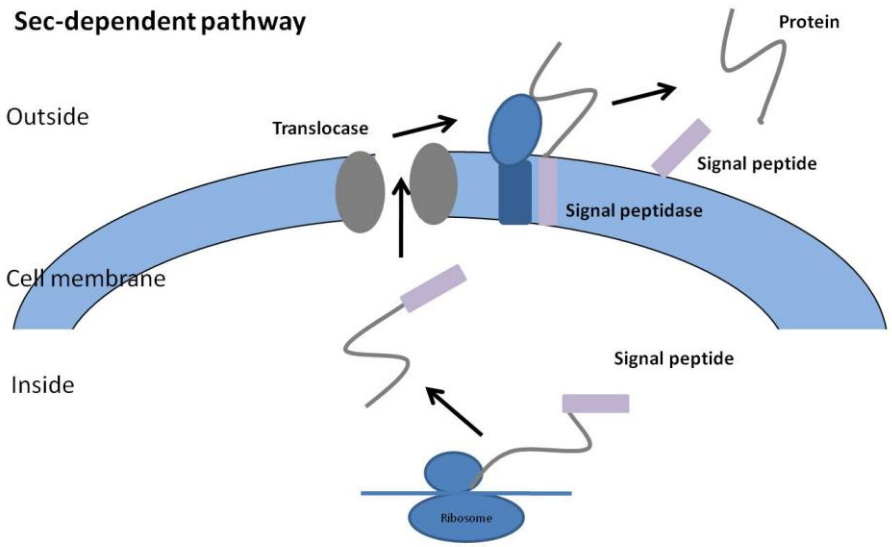



**Figure 3.1. Sec-dependent pathway. See text for details.**

There are four types of bacterial signal peptides: archetypal Sec-type, lipoprotein, Tat and prepillin (Paetzel et al., 2002), and only the first two will be discussed here. Signal peptides are divided into three regions: the N-terminal, H - region and C-region (Figure 3.2). The N-terminal consists of positively charged amino acid residues; these help orientate the signal peptide in the membrane. The H-region consists of hydrophobic amino acid residues, usually 10-15, which form a α-helix in the membrane. The C- region contains the signal peptidase recognition site, 'Ala-X-Ala', at -1 and -3 relative to the cleavage site in the pre-protein (van Roosmalen et al., 2004). The signal peptides for lipoproteins vary from the above; they too contain the three regions, but their H-domain is shorter and their signal peptidase recognition site is a conserved Lipobox: L(AS)(GA)C (Babu et al., 2006).

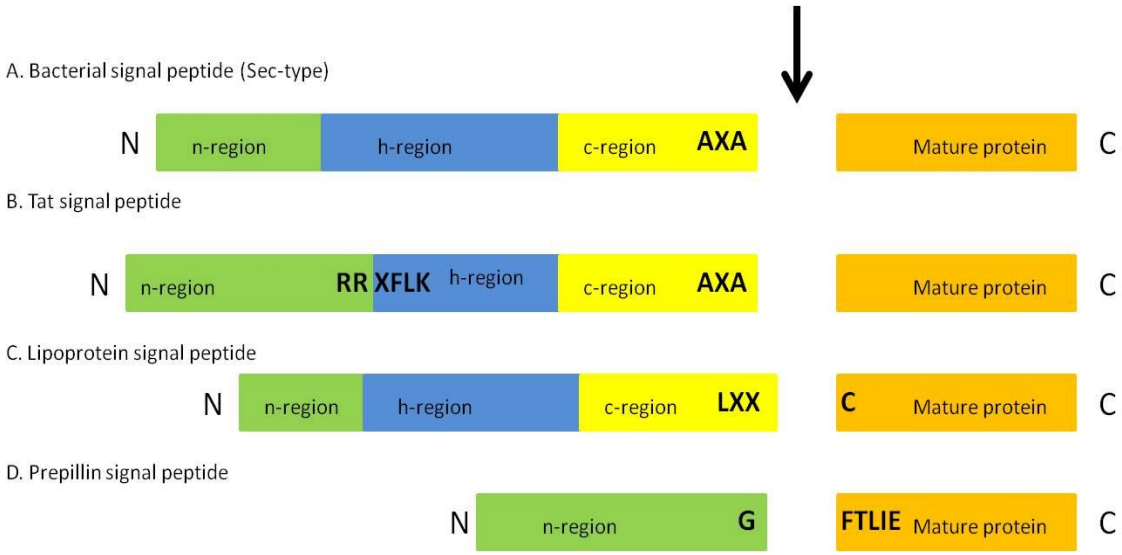



**Figure 3.2. Types of bacterial signal peptides. A. Bacterial signal peptide (Sec-type). B. Tat signal peptide. C. Lipoproteins signal peptide. D. Prepillin signal peptide. Black arrow indicates cleavage site. Bold letters that are not X represent conserved amino acid residue. Bold letter X represents non-conserved amino acid residues (Paetzel et al., 2002).**

There are two types of signal peptidases (SPase), being type I and type II, both of which have their active site at the *trans* side of the membrane (Paetzel et al., 2002). Type II SPases, also called prolipoprotein signal peptidases (Lsp), cleave glyceride-modified prolipoproteins that contain the Lipobox. Those lipoproteins are retained at the membrane after processing (Pragai et al., 1997). This chapter will focus on type I signal peptidase, of which there are two subtypes: the prokaryotic (P)-type and the endoplasmic reticulum (ER)-type. P-type SPases are found in bacteria and organelles of eukaryotes (mitochondria and chloroplasts), while ER-type SPases are found in all the three domains of life (van Roosmalen et al., 2004).

Using bioinformatics to study the genomes of enterococci (Qin et al., 2009, van Schaik et al., 2010, Qin et al., 2012), some of which are not fully annotated, it was

observed that *E. faecium* has three putative type I signal peptidases Interestingly, many bacteria (including *Escherichia coli*) have only one type I SPase (Dalbey and Wickner, 1985), but amongst the Gram positive bacteria there are a number of examples of organisms with several SPases, such as *Bacillus subtilis*, *Clostridium perfringens, S. aureus* and *Staphylococcus epidermidis* (van Roosmalen et al., 2004). Studies in *B. subtilis* have shown that the type I SPases may have different specificities, albeit that there is some overlap in activity as the deletion of one signal peptidase is not lethal to the bacteria (Tjalsma et al., 1997). Research has not clearly established why there are more type I SPases in these bacteria; it has been suggested that they may be used for increased cell-processing capacity, as a back-up signal peptidase activity if one is lost, or as a way to adapt to changing environments by regulating which signal peptidase is expressed and, therefore, which pre-proteins are being processed (van Roosmalen et al., 2004)

Several proteins involved in biofilm formation and virulence in enterococci are predicted to be Sec-dependent substrates. One such protein, the Enterococcal surface protein (Esp) has been shown to enhance biofilm formation in some clinical isolates (Heikens et al., 2007, Tendolkar et al., 2004). Another example is Gelatinase E (GelE), an extracellular zinc metalloprotease that is able to hydrolyse gelatine, collagen and casein; research has shown that it is also involved in biofilm formation (Thomas et al., 2008). Other examples are SalA and SalB, which are believed to be major secreted antigens. SalA is likely to be involved in binding to the extracellular matrix and whereas SalB plays a role in the resistance to stressful conditions (Nallapareddy et al., 2006a, Mohamed et al., 2006).

Although there are many components of the Sec-dependent pathway, the intention of the work here was to produce knockouts of the signal peptidases found in *E. faecium* to analyse possible substrate specificities and, in addition, whether any of the signal peptidases has a specific role in virulence. This is important as there are signal peptidase inhibitors available (Harris et al., 2009) and further research on these could be encouraged with more information if the importance of signal peptidases in pathogens such as enterococci is clearly demonstrated.

## *3.2. Bioinformatics*

### 3.2.1 Identification of the signal peptidase genes of *E. faecium*

Type I SPases can be split into two groups: the P-type, and ER-type. All type I SPases contains 5 conserved regions (boxes A-E; Tjalsma et al., 1997). Region A (not illustrated in the alignments shown in figure 3.4) represents the anchor region that inserts into the membrane. Region B contains a serine and region D contains a lysine residue, both of which form an essential catalytic dyad. The first serine residue in region E helps to position these two residues. Residues in region C and the arginine residue in region D have been suggest to be involved in structural roles involved in substrate attachment (Paetzel et al., 2002).

ER-type SPases have the same conserved serine but have a histidine residue instead of the lysine residue for catalytic activity. ER-type and P type SPases also differ in the sequences between the conserved domains. In ER type SPases, regions B and C are only separated by one residue, whereas P-type SPases can be separated by 19-42 residues. In addition, regions D and E are separated by 2-11 residues in ER-type SPases, whereas P-type SPases are separated by 23-118 residues. There is also an additional domain C' in ER-type SPases (van Roosmalen et al., 2004).

The typical P-type SPase 1 SipS of the Gram-positive bacterium *B. subtilis* was used in a Blast search to find SPase sequences in the *E. faecium* DO genome. Three SPases were identified, denoted: S0133DO (ZP_00605020.1), S0713DO (ZP_00604101.1) and S1233DO (ZP_00603941.1). Further Blast searches found similar genes in the *E. faecium* E1162 genome: S0133E, S0713E and S1233E (ZP_06677521.1, ZP_06677866.1 and ZP_06676098.1) and TX1330 genome: S0133TX, S0713TX and S1233TX (ZP_03982526.1, ZP_07860942.1 and ZP_03980473.1).

To study the relationships between the enterococcal SPases, multiple sequence alignments were made using the protein sequences encoded by the genes mentioned above as well as *B. subilis* SPases SipS (Figure 3.3). Alignment percentages for identical and similar residues are stated in Table 3.1. All alignments except S1233 show moderate similarity between the proteins and SipS of *B.subtilis*. Alignment analysis of the amino acids shows that SPases S0713 and S0133 from all *E. faecium* strains (Figure 3.3A) contain the essential residues for activity. All the alignment percentages were low for S1233 to SipS for all *E. faecium* strains (alignments not shown), with identities between 11-16% (Table 3.1). It was observed that the S1233 sequence for *E. faecium* E1162 starts short of the other S1233 sequences. This could be due to incorrect annotation, and could indicate that there is a start codon further upstream that can be used instead. However, no other potential start codon with a ribosomal binding site could be found upstream.

A phylogenetic tree was constructed (Tamura et al., 2011) with the sequences used for the alignments but also an additional *B. subtilis* SPase SipW, which is an ER-type SPase (Figure 3.4). It can be seen that each of the three enterococcal strains contains three different types of SPases that cluster together. Two of those clusters (containing the SPases S0713 and S0133) are similar to *B. subtilis* SipS, while those in the third cluster (SPases S1233) are closely related to SipW. Therefore multiple sequence alignments were performed comparing the SPases S1233 with SipW. The percentage identity of the *E. faecium* S1233 SPases to SipW were higher than when compared to SipS (Table 3.1), although the percentage was still relatively low (16-24% identity). Alignments for SPases S1233DO and S1233TX, but not S1233E (due to residues missing at the beginning) with *B. subtilis* SipW are shown in Figure 3.2B. The transmembrane helices spanning domains were also analysed using the software TMHMM 2.0 (Krogh et al., 2001). These results showed that there are two membrane spanning domains, with one N-terminal anchor and one C-terminal anchor. In contrast, type 1 SPases usually only have one N-terminal anchor (van Roosmalen et al., 2004), again showing that the SPases S1233 are more alike to SipW than SipS *B. subilis* (Figure 3.4B). All the *E. faecium* SPases S1233 show some consensus in Regions C, C' and E between each other. It is also noted that there is one residue between regions B and C and 20 residues between regions C and

D, which is similar to what has been found for SipW. The consensus sequence in region B for ER-type SPases is usually vlsgSMePxf (Capital letters being highly conserved) (Tjalsma et al., 2000b). Surprisingly, none of the *E. faecium* SPases S1233 contains the serine residue in domain B required for activity. In S1233E and S1233DO (but not S1233TX) there is a serine residue, but it is not in the usual place. Instead, it is located between the conserved residues methionine and proline, not prior to the methionine. The other essential active residue, a histidine which is usually found in Region D is also not present in the *E. faecium* SPases S1233; together with the lack of serine it suggests that the SP1233 SPases are not active.

***Table 3.1. Alignment percentage identity and similarity comparing the E. faecium SPases to two B. subtilis SPases.***

| | | ***B.subtilis SPase genes*** | | | |
|---|---|---|---|---|---|
| | | ***SipS*** | | ***SipW*** | |
| ***SPase*** | ***E. faecium strain*** | ***Identical (%)*** | ***Similar (%)*** | ***Identical (%)*** | ***Similar (%)*** |
| ***S0133*** | ***DO*** | ***30.9*** | ***9*** | ***-*** | ***-*** |
| | ***TX1330*** | ***39.6*** | ***4.0*** | ***-*** | ***-*** |
| | ***E1162*** | ***39.6*** | ***19.6*** | ***-*** | ***-*** |
| ***S0713*** | ***DO*** | ***36.4*** | ***20.6*** | ***-*** | ***-*** |
| | ***TX1330*** | ***36.4*** | ***20.1*** | ***-*** | ***-*** |
| | ***E1162*** | ***39.7*** | ***26.1*** | ***-*** | ***-*** |
| ***S1233*** | ***DO*** | ***16.3*** | ***23.9*** | ***23.7*** | ***22.1*** |
| | ***TX1330*** | ***14.7*** | ***23.4*** | ***23.2*** | ***21.0*** |
| | ***E1162*** | ***11.4*** | ***15.8*** | ***16.3*** | ***17.4*** |

A.


BOX B
S0713TX ----------MLRGFLFIPVPVEGNSMENVLKQ 23
S0713E -MTKKQRYINRFWLVFKYLLVSVFLAFMLRGFLFIPVPVEGNSMENVLKQ 49
S0713DO ----------MLRGFLFIPVPVEGNSMENVLKQ 23
SipS BAC MKSENVSKKKSILEWAKAIVIAVVLALLIRNFIFAPYVVDGDSMYPTLHN 50
BOX C
BOX D
S0713TX GDMVVMEK----FSEIRRFDIVVFQLADGTIYIKRVIGLPGENVSYQNDQ 69
S0713E GDMVVMEK----FSEIRRFDIVVFQLADGTIYIKRVIGLPGENVSYQNDQ 95
S0713DO GDMVVMEK----FSEIRRFDIVVFQLADGTIYIKRVIGLPGENVSYQNDQ 69
SipS BAC RERVFVNMTVKYIGEFDRGDIVVLN-GDDVHYVKRIIGLPGDTVEMKNDQ 99
BOX E
S0713TX LKINGKVVKEPYLTKNLKSDHANASYTTDFTLQELTGQSKLPEDNYFVLG 119
S0713E LKINGKVVKEPYLTKNLKSDHANASYTTDFTLQELTGQSKLPEDNYFVLG 145
S0713DO LKINGKVVKEPYLTKNLKSDHANASYTTDFTLQELTGQSKLPEDNYFVLG 119
SipS BAC LYINGKKVDEPYLAANKKRAKQDG---FDH-LTDDFGPVKVPDNKYFVMG 145
BOX E
S0713TX DNRRVSKDSRS-FGTINKTDILGKARFVYYPLDEIKWIQ 157
S0713E DNRRVSKDSRS-FGTINKTDILGKARFVYYPLDEIKWIQ 183
S0713DO DNRRVSKDSRS-FGTINKTDILGKARFVYYPLDEIKWIQ 157
SipS BAC DNRRNSMDSRNGLGLFTKKQIAGTSKFVFYPFNEMRKTN 184


**Figure 3.3. Signal peptidase alignments. SPases S0713 compared with *B. subtilis* SipS (A) and SPases S1233 compared with SipW (B). Box B-E show conserved areas of the signal peptidases in *B. subtilis*. The symbols at the bottom of the line indicate regions of perfect (*), good (:), and moderate (.) conservation. Residues highlighted in green for A are essential for stability/activity in SipS of *B. Subtilis* (van Dijl et al., 1995). Residues highlighted in green for B are conserved in ER-type SPases (Tjalsma et al., 2000b). Colour lines indicate transmembrane helices spanning domains. More details in the text.**

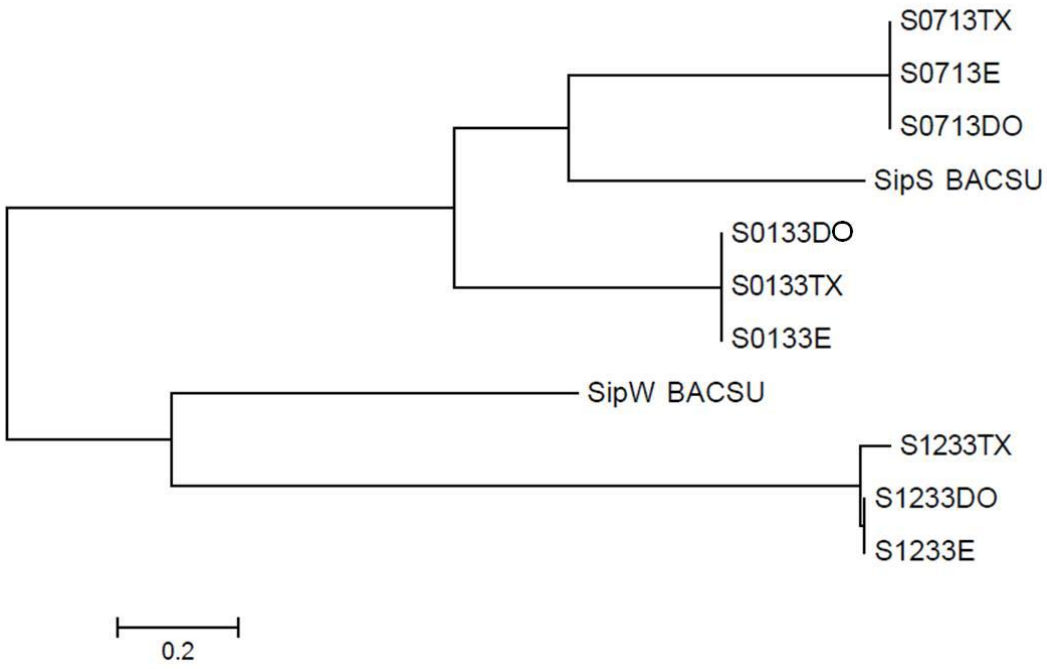



**Figure 3.4. Phylogenetic tree for the *E. faecium* SPases S0713, S0133 and S1233. They are also compared to *B. subtilis* SPases SipS (SipS BACSU) and SipW (SipW BACSU). E- *E. faecium* E1162, DO- *E. faecium* DO and TX-*E. faecium* TX1330. The scale bar represents the number of substitutions per position. The tree was constructed with Mega5.10 software using maximum likelihood method (Tamura et al., 2011).**

## 3.2.2 Identification of proteins secreted via the Sec-dependent pathway.

As mentioned before, Sec-dependent proteins contain a signal peptide that is essential for translocation. Using SignalP (V.4.0) software (Petersen et al., 2011) and LipoP (V.1.0) software (Juncker et al., 2003) we firstly identified proteins that contained possible signal peptides. To ensure that only secreted proteins were to be studied the proteins were analysed for transmembrane helices spanning domains using software TMHMM Server, v 2.0 (Krogh et al., 2001); any protein with 3 or more transmembrane domains was disregarded, even though there was still a

possibility they contain a cleavable signal peptide. Table 3.2 shows the possible secreted proteins and lipoproteins identified.

The proteins encoded by the *E. faecium* E1162 genome were analysed with the above software to establish if they are secreted. There are in total 2964 proteins encoded by the *E. faecium* E1162 genome; of these proteins 2.3% (69) have a Sec-type signal peptide and are therefore putatively secreted (Table 3.2). This is slightly lower compared to another Gram-positive bacteria; for instance, 4% of *B. subtillis* proteins contains Sec-type signal peptides (Tjalsma et al., 2000a). In *E. faecium*, there is an array of proteins secreted: hydrolases, various enzymes, extracellular binding proteins and many cell wall surface proteins. There are also many virulence factors, which is important for the aim of this chapter, these include: Esp, autolysins, pili B subunit protein, two *bee3* genes and a collagen adhesion proteins. There are also 16 hypothetical secreted proteins with no known function (23% of the total number of secretory proteins).

Lipoproteins are a group of membrane proteins that have many roles in virulence, signal transduction, adherence and conjugation (Kovacs-Simon et al., 2011). Whilst analysing the genome for proteins with signal peptides, 55 lipoproteins (1.8%) were identified with LipoP, but the percentage found compares well with other research which suggests lipoproteins usually represent 2-3% of the genome (Reffuveille et al., 2011, Babu et al., 2006). The lipoproteins identified include: three PrsA foldase proteins, a lactose transport system, zinc binding, ABC transporters, thiamine biosynthesis and a pheromone. In addition, there are 12 lipoproteins with unknown functions (20% of the total lipoproteins).

***Table 3.2. Predicted Sec-type signal peptides and lipoprotein signal peptides of E. faecium E1162.****

| NCBI reference sequence | Sec-type signal peptides ↓ | Predicted function |
|---|---|---|
| ZP_06675853.1 | MQELKTRSARHHRPKRKMVINKKAIAPLIGTSLLVGPMMVTAVPQHVHADEQ | Autolysin |
| ZP_06675922.1 | MKKFVSGILVGSLATAAAV | Conserved hypothetical protein |
| ZP_06675936.1 | MKVKKAVIPAAGLGTRFLPATKAMAKEM | UTP-glucose-1-phosphate uridylyltransferase |
| ZP_06675999.1 | MKKMMFGVAALIVIGITGMTGLLNAAFH | Hypothetical protein EfmE1162_0154 |
| ZP_06676032.1 | MRKKRPFLLFWLFGFFLLLILSAAFSGKMTADLAQK | Hypothetical protein EfmE1162_0187 |
| ZP_06676055.1 | MNQCVKKLIASILFVFPLLMVTSVFADVE | Hypothetical protein EfmE1162_0210 |
| ZP_06676058.1 | MKKAVIICALGIGFVGGNAMSSE | Hypothetical protein EfmE1162_0213 |
| ZP_06676089.1 | MKNKKTIRLLAATWLIAPSVLSTNTVFAVDNQ | Putative glycosidase GlyA |
| ZP_06676104.1 | MKKKLFAIFGPVVLAV | DltD protein |
| ZP_06676125.1 | MMKKFFLFFLLLVSVGWPVATHADTATV | Conserved hypothetical protein |
| ZP_06676221.1 | MRYSTILISTFAALSLSLTINQTVHTEEI | Surface protein, putative |
| ZP_06676288.1 | MKKRLLWCLLSLSLSLTSQPVFAEET | Hypothetical protein EfmE1162_0443 |
| ZP_06676292.1 | MSIKKTRLVLLLASLILCLASFLPAVAVQAEDTF | D-alanyl-D-alanine carboxypeptidase |
| ZP_06676329.1 | MNKRRWAAVLIAAGLFVVSLVSASLT | Putative signal peptide peptidase sppA |
| ZP_06676381.1 | MKLWKKLAFTGVSALLLGTLAACGGSGSKDQAEASNS | Extracellular solute-binding protein |
| ZP_06676418.1 | MNKRKWLFVCVAALFSTVAYSQLSFADSS | Hypothetical protein EfmE1162_0573 |

| NCBI reference sequence | Sec-type signal peptides ↓ | Predicted function |
|---|---|---|
| ZP_06676420.1 | MENKIHCTTGRVITLVLFLFLLLFARGTAVQALEK | Hypothetical protein EfmE1162_0575 |
| ZP_06676421.1 | MKLFNPKTVLAIGTFAASLSLAGSVDAAST | Conserved hypothetical protein, putative |
| ZP_06676433.1 | MENKTLKIVLTAAISVSLIGNIALFWQLKEEQAANT | Hypothetical protein EfmE1162_0588 |
| ZP_06676517.1 | MKKKTFKKLLFLG | Putative secreted protein |
| ZP_06676528.1 | MTSLKTLLFGTTLAAGAAFFMGTTAHADEA | Peptidoglycan-binding LysM |
| ZP_06676542.1 | MYNLILGIVIVLSIVMVIAIMMQPSKQNSAASA | Preprotein translocase, SecG subunit |
| ZP_06676554.1 | MKRNYLVISIIALSVLLFFEITSVASLITE | Polysaccharide deacetylase family protein |
| ZP_06676595.1 | MLLQNKFSFFRSFFFTSLFVLSIFIFSPSAEAVGA | Hypothetical protein EfmE1162_0750 |
| ZP_06676596.1 | MKTSILLGGIIASLLFFSWQEPVQADTK | Hypothetical protein EfmE1162_0751 |
| ZP_06676597.1 | MNQSKRLFVSFFSILSLFFIFPSISQAKDS | Cell wall surface anchor family protein, putative |
| ZP_06676598.1 | MKKNMIGTLLLSTLLLGGLATPAFAEGQ | Extracellular protein |
| ZP_06676846.1 | MKLWMKILLGVALALIVALVFAGNY | Hydrolase of the alpha/beta superfamily |
| ZP_06676864.1 | MAKKTIMLVCSAGMSTSLLVTKMQKAAEA | Cellobiose-specific phosphotransferase enzyme iib component |
| ZP_06676885.1 | MKKKITITAMSLLTALFLLPINTFAYTI | N-acetylmuramoyl-L-alanine amidase |
| ZP_06676947.1 | MAKKTIMLVCSAGMSTSLLVTKMQKAAEE | Cellobiose-specific phosphotransferase enzyme iib component |
| ZP_06676996.1 | MKKTATVLFVILGLSIIAAMIGISTSANER | Cell wall anchored protein SgrA |
| ZP_06677002.1 | MRTKQVLKGALVILFAVSTSVPQWTVYGESI | Hydrolase of the alpha/beta superfamily |
| ZP_06677099.1 | MLGVLFLILPLLTNSFGAKKVFAEET | Putative pilus subunit protein PilB |
| ZP_06677101.1 | MTTTGKKLKVIFMLIILSLSNFVPLSAIADTT | von Willebrand factor type A domain protein |

| NCBI reference sequence | Sec-type signal peptides ↓ | Predicted function |
|---|---|---|
| ZP_06677130.1 | MKTSTKVTIGLSVAAAASVATA VVV | Conserved hypothetical protein |
| ZP_06677152.1 | MEKKTIMLVCSAGMSTSLLVTKMQKA AEA | Cellobiose-specific phosphotransferase enzyme iib component |
| ZP_06677175.1 | MRPKQQLFITLLVILGALFNNISVSASA FNF | Cell surface protein |
| ZP_06677177.1 | MMKKEIVLTAAMMLFSMVASTTFVSA TEV | Cell surface protein |
| ZP_06677181.1 | MKHCVILGILGTCLAGIGTGIDVDA ATY | Class IIa sec-dependent bacteriocin |
| ZP_06677271.1 | MKKTKNVRVDIFAVLFFILLVFFIGSPTVSA RES | Cell wall surface anchor family protein |
| ZP_06677273.1 | MKNIKTKIWTFLGTAIMLLPFVLGLGTAEVSA AVS | Bee3 |
| ZP_06677349.1 | MGKVMKKRMKPLIWPIAVSSIFLLGQTAFA ETS | Lpxtg-motif cell wall anchor domain protein |
| ZP_06677373.1 | MKKTMLKVFVGLFLLTGIGGIFSKTVDA GSY | Hypothetical protein EfmE1162_1528 |
| ZP_06677388.1 | MKMGIWLRKAVLFAAVLGALGAVSKTVEASA SEF | Arabinogalactan endo-1,4-beta-galactosidase |
| ZP_06677519.1 | MRKVAPILKPIAVLTISTIVATTTGIVNS SSY | Conserved hypothetical protein |
| ZP_06677537.1 | MKKCRNIILSMLFIITNITSLIPVHVYA DAG | Collagen adhesin |
| ZP_06677582.1 | MKVKKAVIPAAGLGTRFLPATKAMA KEM | UTP-glucose-1-phosphate uridylyltransferase |
| ZP_06677610.1 | MLDIVPSNTHRSGSLINFAAIFACASVILASPVVVDA SST | Hypothetical protein EfmE1162_1850 |
| ZP_06677646.1 | MTRSSHRKKSKHVALLSVLGVLLVLISFYTYR STY | Beta-lactam-insensitive peptidoglycan transpeptidase |
| ZP_06677763.1 | MKKISTLLFIGLVSLGLFGFSIPGFAH GYI | Extracellular protein |

| NCBI reference sequence | Sec-type signal peptides ↓ | Predicted function |
|---|---|---|
| ZP_06677965.1 | MNKKMIVTGMSVLLLVTAGIGIFGNGKLVK AEE | Bee3 |
| ZP_06677966.1 | MKKLLCLMLQLLVLLPLAAVLLPHAMSA EES | Lpxtg-motif cell wall anchor domain protein |
| ZP_06677994.1 | MNSFKKIVLGTTFAAGATAMFVGTTNAHA DEV | LysM domain protein |
| ZP_06678100.1 | MNKLNILFVCGAGLGSSFA AQM | PTS sysytem, mannitol-specific enzyme II, B component |
| ZP_06678134.1 | MKKFNPNKNIIITLVIVIIVVTILSVTAAQ RAN | Cell shape-determining protein MreC |
| ZP_06676032.1 | MRKKRPFLLFWLFGFFLLLILSAAFSGKMTADL AQK | Hypothetical protein EfmE1162_0187 |
| ZP_06676052.1 | MKKTIALSTVLAGSLLLCTQSVFA ENK | Cell surface protein |
| ZP_06678263.1 | MKKPRFKNWRLLATLSLLCQTIGGSLGPTIAFA DEI | Cell wall surface anchor family protein EcbA |
| ZP_06678366.1 | MMRTMLISLSVGVYMHVPVQA TTQ | Conserved hypothetical protein |
| ZP_06678378.1 | MKKALIICAAGMSSSMMASK TTD | Cellobiose-specific PTS system IIB component |
| ZP_06678390.1 | MENIARKERRRLEQTKRFRKAKRGAAIVGTAMVGCSVAAPLVQPVPVQA DET | Autolysin |
| ZP_06678401.1 | MFERKGRGKMKKRASLLVLVALLFNIFSYGVAVSA ETY | Cell surface protein |
| ZP_06678454.1 | MVSKNNKRVFLEKTKKRVLKYSIKKLSVGVASVLIGVGLVLGTTELVKA QDE | ESP |
| ZP_06678456.1 | MKKSLKLASAVILMSQLLIATPFHVLA SEN | Putative muramidase |
| ZP_06678482.1 | MYKKQKKKLCVLTILSLAVQAIVGAIA PTI | Cell wall surface anchor family protein EcbA |
| ZP_06678511.1 | MKAKNLLFSVILLGNLAVANTTVLA EES | Muramidase-2 |
| ZP_06676054.1 | MNRIWNKSHRNNCSLLMILLLGFILWQTPVHA SEF | Cell surface protein |
| ZP_06677100.1 | MKKLGWLSMCLFLLLFKPAFTQVA TET | Cell wall surface anchor family protein |

| NCBI reference sequence | Lipoprotein signal peptides ↓ | Protein function |
|---|---|---|
| ZP_06676023.1 | MKKIIRFVVITFVAIIWLAG CSQG | Lipoprotein, YaeC family |
| ZP_06676035.1 | MKKLIVLFILSAGLLTLTG CDLF | Lipoprotein, putative |
| ZP_06676079.1 | MKRLIGILAMLVLAGMLTA CGAS | Zinc-binding lipoprotein AdcA |
| ZP_06676179.1 | MKKWLLLLIGMGILFTG CTSE | Lipoprotein |
| ZP_06676194.1 | MKKIIYSLLAMSVSILILTS CSTT | Lipoprotein, putative |
| ZP_06676209.1 | MKKTVIYTSLFAALFLAA CQSN | Lipoprotein |
| ZP_06676404.1 | MKKATLVKLGLSFAGILALAA CGSS | Lipoprotein |
| ZP_06676423.1 | MKKKQLWFVSMFAIFFIILSG CSSN | Lipoprotein, putative |
| ZP_06676455.1 | MRAIFSSISVVGLFFLLAG CGSA | Lipoprotein, putative |
| ZP_06676682.1 | MKKLGFILLLVSLFLVG CTSP | Lipoprotein, putative |
| ZP_06677023.1 | MIKKLFLLSILTLAMAG CSDS | Lipoprotein, putative |
| ZP_06677159.1 | MKKRILVTIGLAFLLAG CTNG | Lipoprotein, putative |
| ZP_06677913.1 | MKKFAIILTCALLIGTISG CTTS | Lipoprotein, putative |
| ZP_06677997.1 | MKTKKSLFLILAVSFLVLAG CGKQ | Manganese ABC transporter substrate-binding lipoprotein |
| ZP_06678109.1 | MKKYLLTATVMIGALLFAA CGNT | Zinc-binding lipoprotein AdcA |
| ZP_06678268.1 | MRKKMLFIPFALLLSA CSSV | Lipoprotein, putative |
| ZP_06678395.1 | MRKKLLFSLITLGISFMVLTG CSNS | Thiamine biosynthesis membrane-associated lipoprotein |

| NCBI reference sequence | Lipoprotein signal peptides ↓ | Protein function |
|---|---|---|
| ZP_06678396.1 | MKKKRFVTGFAVLAFSALVLGACGAD | Pheromone cAD1 lipoprotein |
| ZP_06678431.1 | MMRKWKAVLGSLGILIALFIFGACSTN | Manganese ABC transporter substrate-binding lipoprotein |
| ZP_06678445.1 | MKKISVGLIGIAALGLLGACSST | Zinc-binding lipoprotein AdcA |
| ZP_06675911.1 | MKLKTKVFTLSSILLLAGVLSACSPS | Iron compound ABC transporter, substrate-binding protein |
| ZP_06676052.1 | MKKTIALSTVLAGSLLLCTQS | Cell surface protein |
| ZP_06676057.1 | MKKRFLALAIVLGTGLLSGCTNA | Foldase protein PrsA |
| ZP_06676206.1 | MKKKRLFALITSAFFILSACGTA | ABC-type amino acid transport system, periplasmic component |
| ZP_06676214.1 | MKKRSVKQLGLGLISLGILGGLAACGNG | Maltose/maltodextrin ABC transporter, binding protein |
| ZP_06676363.1 | MTEPIKNLCKRATAAALVLISLFALSGCQTT | Alpha/beta hydrolase superfamily protein |
| ZP_06676381.1 | MKLWKKLAFTGVSALLLGTLAACGGS | Extracellular solute-binding protein |
| ZP_06676416.1 | MAINRYVKKAGMLVGMLGIIASCFQG | Putative pilus subunit protein PilA |
| ZP_06676484.1 | MNIRKLIAGVVCLAAASGFLTGCGSA | Lactose transport system |
| ZP_06676647.1 | MKKRYKLVIALLNITLLFSLSACGKT | Bacterial extracellular solute-binding protein, |
| ZP_06676773.1 | MKRILKMTMVLFSLLILASCSFP | Glycine betaine/carnitine/choline-binding protein |
| ZP_06676784.1 | MKKTSLLFAASSLSLGLLGLSGCGNN | Trap dicarboxylate transporter, dctp subunit |
| ZP_06676994.1 | MGNFLKKQLLIMCLSACAILLFSGCSKS | Foldase protein PrsA |
| ZP_06677218.1 | MKKKSIILAATSALAVLTLAACSGD | Foldase protein PrsA |
| ZP_06677456.1 | MKKTIFGFAALMSAVVLAACGGG | Peptide ABC transporter, peptide-binding protein |

| NCBI reference sequence | Lipoprotein signal peptides ↓ | Protein function |
|---|---|---|
| ZP_06677467.1 | MKKLVVGILILASFG<u>LAA C</u>GNS | Iron compound ABC transporter, substrate-binding protein |
| ZP_06677478.1 | MKGKKWLIGSLLVAATG<u>ILGA C</u>GGG | Sugar ABC transporter, sugar-binding protein, putative |
| ZP_06677542.1 | MKKTSFLFALIAGLLL<u>FAG C</u>SNK | Amino acid ABC transporter, amino acid-binding protein |
| ZP_06677550.1 | MKKKFTFGLVAVCGLV<u>LAG C</u>YGG | Peptide ABC transporter, peptide-binding protein |
| ZP_06677901.1 | MKKLWLLLPLLLL<u>LSA C</u>GTA | ABC transporter, substrate-binding protein |
| ZP_06677927.1 | MKKKIFGFAAALLLTVG<u>LAA C</u>GNN | D-methionine-binding lipoprotein MetQ |
| ZP_06677973.1 | MKKLLFTGLLASSALFL<u>LGG C</u>GSS | ABC transporter substrate-binding protein, putative |
| ZP_06678159.1 | MKNKFRKIVGTIAILTASL<u>LSA C</u>GNS | Multiple sugar transport system |
| ZP_06678161.1 | MKRVLFFLALVVIAYQ<u>MMG C</u>TPN | ABC transporter periplasmic-binding protein YtfQ |
| ZP_06678184.1 | MKKRMRIILALSVMLFF<u>LTA C</u>GQE | Bacterial extracellular solute-binding protein, putative |
| ZP_06678309.1 | MKKVLFTLGMMGIL<u>LAG C</u>GSG | Phosphate-binding protein |
| ZP_06678369.1 | MKKLVAGLLTAVALLA<u>MSA C</u>GAD | Fumarate reductase flavoprotein subunit |
| ZP_06678407.1 | MKKNIIIGLGAALLL<u>ALG G C</u>AQK | ABC transporter permease protein |
| ZP_06678412.1 | MKKIIVTTCLLATGFL<u>LTA C</u>GES | ABC transporter, substrate-binding protein |
| ZP_06676236.1 | MKKNKVILSISLAAALLF<u>AAG C</u>SAN | Peptidyl-prolyl cis-trans isomerase |
| ZP_06676873.1 | MKKLKLFLPIIGLAILL<u>SG C</u>SKW | Phosphate ABC transporter, phosphate-binding |
| ZP_06677595.1 | MRRLKKIRLIFLLGIAFL<u>ALAG C</u>KGN | Glutamine-binding protein |
| ZP_06676062.1 | MEKPFVKSLTAVAVGAGA<u>IAI C</u>LFG | Hypothetical protein EfmE1162_0217 |
| ZP_06677397.1 | MKKAKLFGLGAVALAAGL<u>FLGA C</u>GNN | Basic membrane protein family |
| ZP_06676588.1 | MKKIIGAFVFAIII<u>III C</u>FQI | N-acetylmuramoyl-L-alanine amidase |

* SPase cleave recognition site is illustrate by underling. Positivity charged residues are indicated in bold letter and the H-region is indicated in grey shading.

## *3.3. Construction of type I SPase mutants.*

### 3.3.1 Summary of the method

Using methodology previously developed (Nallapareddy et al., 2006a); the objective was to make SPase mutants as shown in Figure 3.5. Fragments upstream and downstream of the signal peptidase gene of choice (in blue) were introduced into the pTEX5500ts vector (A). The vector was then used to transform *E. faecium* E1162 or TX1330 (B). Colonies obtained with the vector were screened for single-crossover occurrences. Integration can occur through either the upstream or downstream fragment (C). A second step in which single crossover integrants will undergo a double crossover via homologous recombination leads then to the deletion of the gentamicin and signal peptidase gene (D-E). The correct mutants are thus gentamicin sensitive and chloramphenicol resistant.

### 3.3.2 Making the vector constructs (A)

Using the In-fusion advanced PCR cloning kit, upstream and downstream fragments were inserted into the plasmid. Primers used to amplify the fragments are listed in Table 3.3. Upstream fragments were inserted in the *Nhe*I and *Hind*III sites of pTEX5500ts, while downstream fragments were inserted into *Pvu*I and *Pst*I sites following the manufacturer's guidelines for the infusion method.

Constructs were made for all three *E. faecium* E1162 SPases (E0133, E0713 and E1233), producing the three SPase mutant constructs (pSP2, pSP8, pSP6). Constructs for *E. faecium* TX1330 were only produced for only two of the SPases, TX0713 and TX1233 (pSP2 and pSP4, respectively). All constructs were verified with PCR, restriction digests, and sequencing (see methods and materials section). The vector construct for *E. faecium* TX0133 was not made as this gene appeared to contain some of the restriction sites used for cloning, even though these were absent from the sequence in the database.

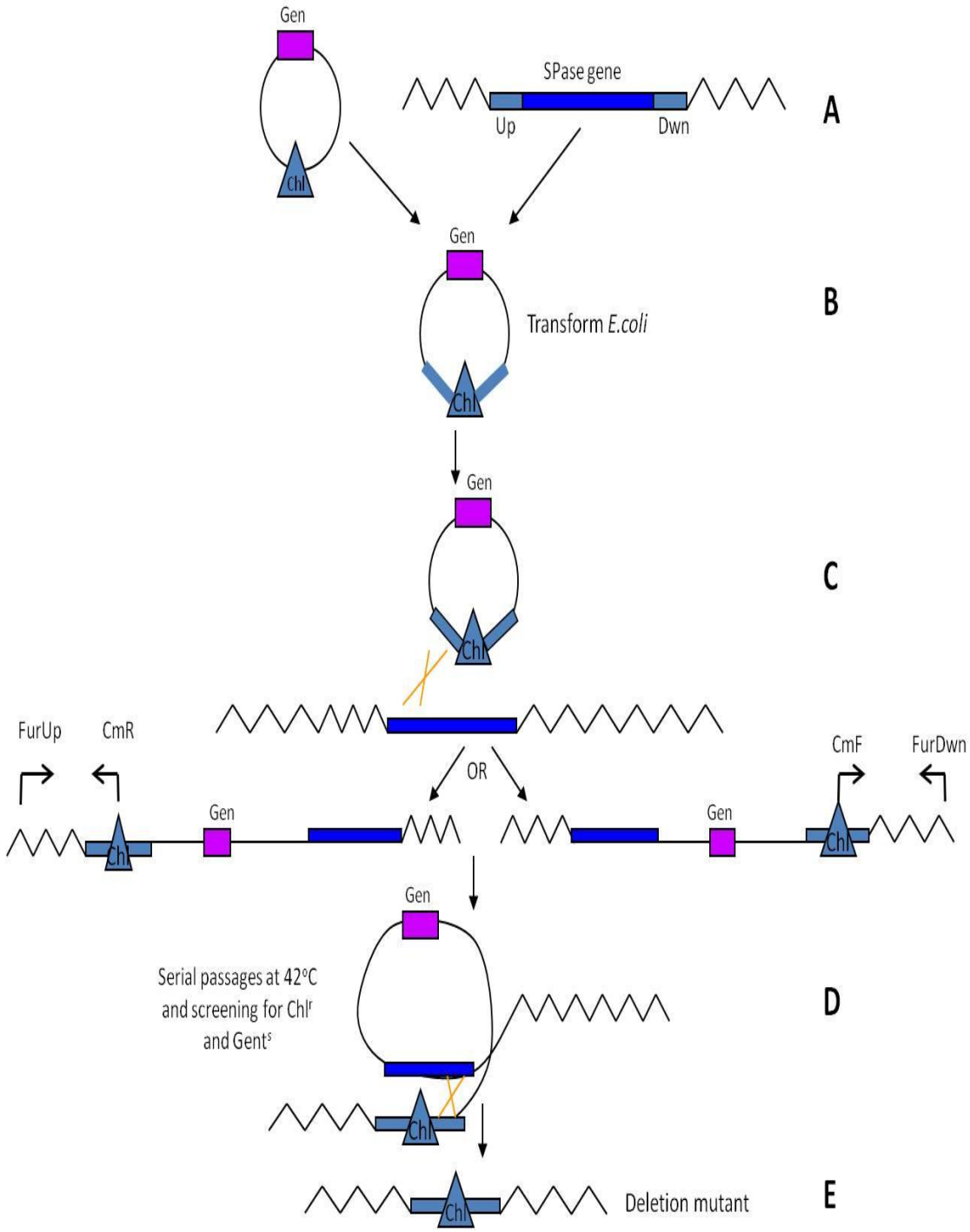



**Figure 3.5. Procedure to produce the signal peptidase mutant. See text for details**

***Table 3.3. Primers used for producing vector constructs.***

| Name | Type | Sequence (5' to 3')* | Reference |
|---|---|---|---|
| ***E. faecium* TX1330- to make the pTEX5500ts constructs** | | | |
| TX713UpFW | Forward | TTGTACAATTGCTAGCGCTTGCGGAAAGATTCAAAG | This thesis |
| TX713UpRev | Reverse | AACGAAAATCAAGCTTCTGCTTTTGGAACACCGTTT | This thesis |
| TX713DwnFw | Forward | GCGCGCCATGGGCCCTGCAGCAATAAAGCGTGCAGTCTGTT | This thesis |
| TX713DwnRev | Reverse | CGGGTACCGAATTCGATCGGCCCGAAATGAATGCAAGAT | This thesis |
| TX1333UpFw | Forward | TTGTACAATTGCTAGCTGGATGGATTTCTGGTCGAT | This thesis |
| TX1333UpRev | Reverse | AACGAAAATCAAGCTTCCTCCTACTTGTATTTTCCTCGTT | This thesis |
| TX1333DwnFw | Forward | GCGCGCCATGGGCCCTGCAGAGCGAAGCGTTTTTGTTCAT | This thesis |
| TX1333DwnRev | Reverse | CGGGTACCGAATTCGATCGACAGCCACCAAAAAGTCCTG | This thesis |
| TX1233UpFw | Forward | TTGTACAATTGCTAGCCGTCCCAATAGCAATGAAAA | This thesis |
| TX1233UpRev | Reverse | AACGAAAATCAAGCTTTGCGCATAAAATGGAGCATA | This thesis |
| TX1233DwnFw | Forward | GCGCGCCATGGGCCCTGCAGAGGTGGCGTTTTGATGGTAG | This thesis |
| TX1233DwnRev | Reverse | CGGGTACCGAATTCGATCGCAAGAATCAGCGGAACATCA | This thesis |
| ***E. faecium* E1162- to make the pTEX5500ts constructs** | | | |
| E713UpFw | Forward | TTGTACAATTGCTAGCGCTTGCGGAAAGATTCAAAG | This thesis |
| E713UpRev | Reverse | AACGAAAATCAAGCTTCTGCTTTTGGAACACCGTTT | This thesis |
| E713DwnFw | Forward | GCGCGCCATGGGCCCTGCAGCAATAAAGCGTGCAGTCTGTT | This thesis |
| E713DwnRev | Reverse | CGGGTACCGAATTCGATCGGCCCGAAATGAATGCAAGAT | This thesis |
| E1333UpFw | Forward | TTGTACAATTGCTAGCTGGATGGATTTCTGGTCGAT | This thesis |
| E1333UpRev | Reverse | AACGAAAATCAAGCTTCCTCCTACTTGTATTTTCCTCGTT | This thesis |
| E1333DwnFw | Forward | GCGCGCCATGGGCCCTGCAGAGCGAAGCGTTTTTGTTCAT | This thesis |
| E1333DwnRev | Reverse | CGGGTACCGAATTCGATCGACAGCCACCAAAAAGTCCTG | This thesis |
| E1233UpFw | Forward | TTGTACAATTGCTAGCGGATGATCCATCACAACACG | This thesis |
| E1233UpRev | Reverse | AACGAAAATCAAGCTTGGTGAGCTAGGCAAACTGGA | This thesis |
| E1233DwnFw | Forward | GCGCGCCATGGGCCCTGCAGAGGTGGCGTTTTGATGGTAG | This thesis |
| E1233DwnRev | Reverse | CGGGTACCGAATTCGATCGCAAGAATCAGCGGAACATCA | This thesis |

* Primers synthesised by Invitrogen

### 3.3.3 Production of the single crossover integrants (B-C).

Vector constructs were introduced into the relevant strain of *E. faecium* (TX1330 or E1162) by electroporation. The presence of plasmids in *E. faecium* was confirmed by re-isolating the plasmid followed by the relevant restriction enzyme digests (used above), or by PCR using a primer located in the chloramphenicol resistant gene on the vector, CmR or CmF (Table 3.4) and a primer used to amplify one of the SPase fragments (Table 3.3).

After incubation at 42$^{o}$C to force integration of the plasmids, confirmation of single crossover integration was performed by PCR with primers listed in Table 3.4. PCR reactions performed included primer CmR or CmF paired with primers that are located on chromosome upstream (FurUp) or downstream (FurDwn) the cloned fragments (Figure 3.3). Results for the positive PCR reactions are shown in Figure 3.6; if a single cross over integration has occurred only one of the above PCRs will work (the negative control PCR is not shown), and this is the case for these.

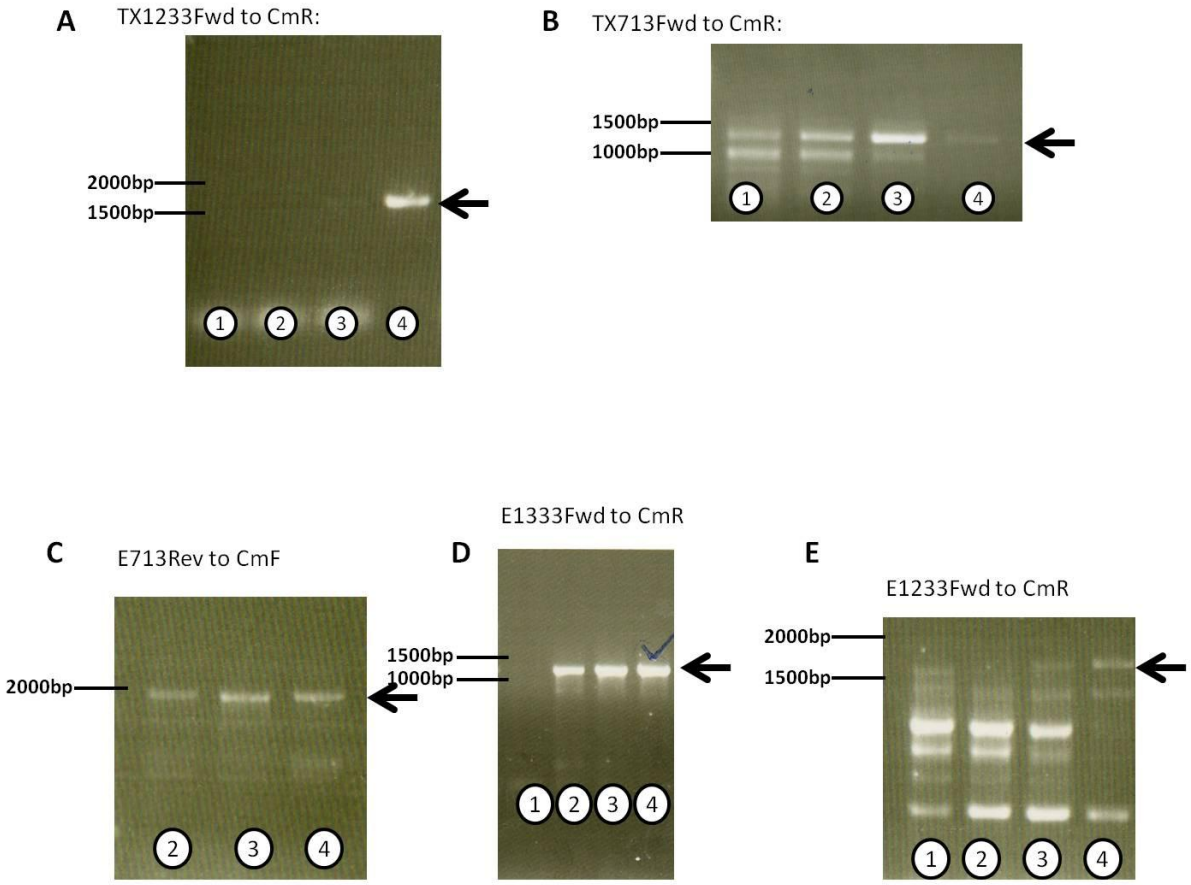



**Figure 3.6. PCR reactions to test for positive single cross over integrants to created mutations in the following genes: A. TX1233. B. TX1713. C. E713. D. E1333 and E. E1233. Primers used are stated at the top of the image and expected band size is indicated by the black arrow. Numbers in circles represent the different annealing temperature gradient for the PCR reactions (*1*. 45°C. *2*. 50.4°C. *3*. 58.5°C and *4*. 65.6°C).**

***Table 3.4. Primers for electroporation confirmation and crossover confirmation***

| Name | Description | Sequence (5' to 3') | Reference |
|---|---|---|---|
| pTEX5500ts | | | |
| CmF | Forward primer found in the Chl gene | GAATGACTTCAAAGAGTTTTATG | (Nallapareddy et al., 2006a) |
| CmR | Reverse primer found in the Chl gene | AAAGCATTTTCAGGTATAGGT | (Nallapareddy et al., 2006a) |
| Used for identifying the integrants: | | | |
| *E. faecium* TX1330 | | | |
| TX713Fwd | Primer upstream of the upstream fragment | TACTGAAAGCAAGACAAAAG | This thesis |
| TX713Rev | Primer downstream of the downstream fragment | AAGTGAAAGTGAACCAGTCCT | This thesis |
| TX1233Fwd | Primer upstream of the upstream fragment | GCTGTCGCAGTTGCTCATAA | This thesis |
| TX1233Rev | Primer downstream of the downstream fragment | TAGCGTCACAGGAGGGATTC | This thesis |
| *E. faecium* E1162 | | | |
| E713Fwd | Primer upstream of the upstream fragment | GTTGGATCAATTCCGAATGC | This thesis |
| E713Rev | Primer downstream of the downstream fragment | ATTCCCCGGCTTTACTTGTT | This thesis |
| E1333Fwd | Primer upstream of the upstream fragment | GCGTTCAACATCCAGACAGA | This thesis |
| E1333Rev | Primer downstream of the downstream fragment | TTGATTCAGGAAGCCCAAAC | This thesis |
| E1233Fwd | Primer upstream of the upstream fragment | CAAGAATCAGCGGAACATCA | This thesis |
| E1233Rev | Primer downstream of the downstream fragment | AATGCCAAAGCAAAGCAACT | This thesis |
| Primers for identifying possible mutant in the TX1330 SPase0713 | | | |
| TXseqfwd | Designed outside the area that has been manipulated | ACGACTGGACATACGGAAGC | This thesis |
| TXseqRev | | TTTTTGTAAATCGCCAAGGTG | This thesis |

### 3.3.4 Production of a mutant (D-E).

After the single crossover integrants were produced, eight rounds of passage at 42$^{o}$C, under chloramphenicol selective pressure was performed to allow the double crossover step to occur. If a double crossover event did occur, then colonies would be gentamicin sensitive and chloramphenicol resistant. For all the single crossover integrants 1000 colonies or more were screened. Although all single crossover integrants were passaged and screened for the double crossover occurrence only one colony was obtained (for *E. faecium* TX1330 SPase713) that was gentamicin sensitive and chloramphenicol resistant. PCR using primers TXseqfwd and TXseqRev (Table 3.4) were used to check whether the mutant was correct. The expected band size for the TX1330 control is 1980bp and for the SPase713 mutant 2484bp; the obtained image showed both bands to be at approximately 2000bp (Figure 3.7) and a SPase713 mutant was thus unfortunately not obtained. Further PCR reactions were performed using the above primers in combination with primers CmR and CmF, and again these showed that no mutant had been obtained.

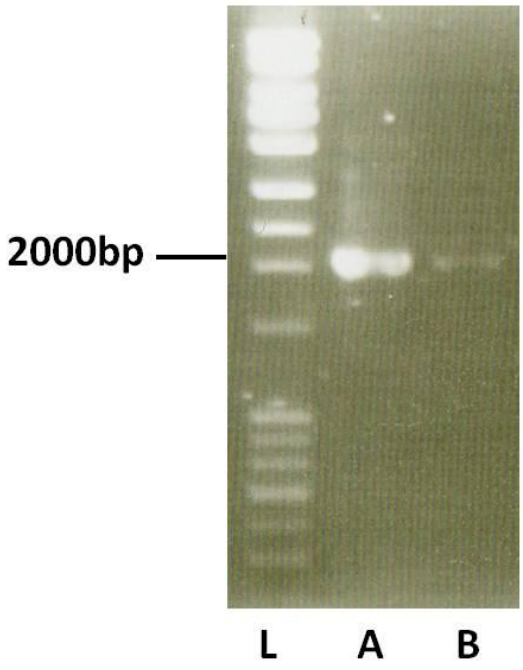



**Figure 3.7. PCR results for the possible TX713 mutant. L. DNA ladder. A. TX1330 control and B. Possible TX713 mutant.**

## *3.4. Nisin-inducible integration plasmid.*

### 3.4.1 Summary

The results above suggested that the SPases of *E. faecium* might be essential for viability, and a different strategy to analyse the role of the signal peptidases was thus required. A potential route is to control the expression of genes of interest with an inducible promoter. However, such a system does not exist for use in enterococci and a new vector had to be constructed for that purpose.

The vector would need (a) the ability to replicate in *E. coli* but not in *E. faecium*; (b) suitable antibiotic resistance markers; and (c) an inducible promoter. It was decided to base the construct on pMutin4, a commonly-used integration vector for *B. subtilis*, and combine this with a nisin-inducible system.

pMutin vectors are used in *B. subtilis* as an integration plasmid controlled by a Pspac promoter, which is tightly controlled by levels of IPTG in the media. The vector is introduced into the genome thereby placing the target gene under control of the Pspac promoter (Vagner et al., 1998). A similar system is required for enterococci, but a drawback is that the Pspac promoter does not work in these organisms (Bryan et al., 2000).

An alternative to the Pspac promoter is the nisin-controlled gene expression system (NICE). It has primarily been developed for *Lactococcus lactis*, but can also be used in other Gram-positive bacteria including enterococci (Zhou et al., 2006). Nisin is a lantibiotic which acts by causing cytoplasmic membrane leakage, and it controls its own expression via a quorum sensing system. This involves a two-component signal transduction system which is used here as the mechanism to control gene expression. To generate a nisin-inducible system the vector needs to contain the *nisA* promoter (P*nisA*) for control of the expression of the gene interest, and the *nisRK* regulatory

genes. Variable (sub-inhibitory) amounts of nisin to the culture medium will then determine the level of expression of the gene of interest, as the histidine kinase (NisK) senses the nisin, resulting in autophosphorylation. The phosphate group from NisK is then transferred to the response regulator NisR, which in turn results in translational activation of the P*nisA* promoter (Mierau and Kleerebezem, 2005, Zhou et al., 2006).

### 3.4.2. Construction of the nisin-inducible integration vector.

Firstly, using PCR (KAPA HiFi PCR kit), the section of pMutin4 vector containing the ori and antibiotic resistance genes (but not the region containing the *lacZ* and *lacI* genes) was amplified, with the primers being used (pMUT4NCOfwd and pMUTNCO1rev) also introducing an *Nco*I site into the vector. The PCR product was then digested with *Nco*I and ligated, resulting in pINT1.

Plasmid pNZ8048 was digested with *Nco*I and *Sal*I and the fragment containing the chloramphenicol gene and PnisA was ligated into the *Nco*I and *Sal*I sites of pINT1. The resulting plasmid was denoted pINT2. This plasmid was checked by sequencing using primers VecCombiP1 and VecCombiP4 (Table 3.5).

The *nisRK* region was amplified using the *L. lactis* NZ9700 genome as a template (Table 3.5; primers: NisRKHindIIIfwd and NisRKBamHIrev). The amplified product was then cloned into the *BamH*I and *Hind*III sites of pINT2 using the infusion method, resulting in pINT3.

Next, using the Infusion system, the region encoding the N-terminal domain of SPase713 was amplified from the *E. faecium* E1162 genome (Table 3.5; primers: Sig713fwd and Sig713rev) and then was cloned into the vector pINT3, resulting in pINT4. The SPase713 gene is small, being only 551bp and therefore the fragment required for the vector is even smaller. Studies with pMUTIN have shown that 150

bp can be enough for homologous recombination and therefore the insertion of the vector into the genome (Vagner et al., 1998). To aid optimal insertion, primers were designed, one located 100bp upstream of the start codon of the gene and a second primer between the active sites of the *spase713* gene, resulting in a 280 bp product. The construct made was verified by restriction digests (with *Nar*I and *Nco*I) and sequencing (Table 3.5; primers: SPinsfwd and SPinsrev). Primers used here are listed in Table 3.5. The final plasmid is shown in Figure 3.8.

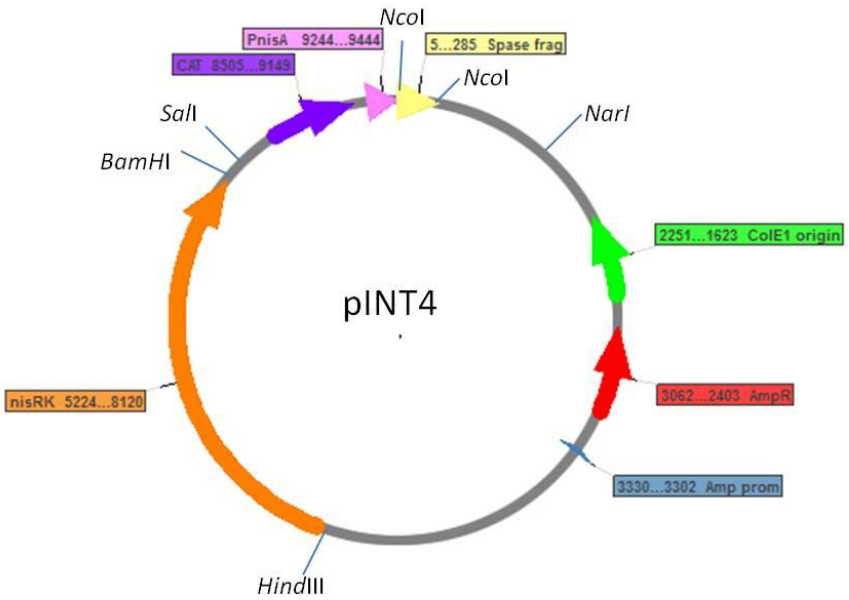



**Figure 3.8. Final integration vector containing the SPase 713 fragment (pINT4). ColE1 origin- *E. coli* origin of replication. AmpR- ampicillin resistance gene. Amp prom- ampicillin resistance promoter. nisRK- *nisRK* genes. CAT- chloramphenicol resistance gene. PnisA- *nisA* promoter. Spase frag- SPase713 fragment. Restriction sites used are also shown. The map was made with Serial cloner 2.1 software.**

***Table 3.5. Primers used to make the integration plasmid.***

| Name | Sequence (5' to 3') | Reference |
|---|---|---|
| pMUT4NCO1fwd | AAAACCATGGTGTGAGTTAGGCATCGCATC | This thesis |
| pMUT4NCO1rev | AAAACCATGGGTGCTGCAAGGCGATTAAGT | This thesis |
| NisRKHindIIIfwd | AAAAAAGCTTTGACTTCTCAGCAGGAGCAA | This thesis |
| NisRKBamHIrev | AAAAGGATCCAACTGCCTTCAATGGCAAAC | This thesis |
| Sig713fwd | GAGGCACTCACCATGGTGTCCAATAGCCTAATTGAAAGAA | This thesis |
| Sig713rev | CTTGCAGCACCCATGGCCATCACAACCATATCACCTTG | This thesis |
| *Sequencing primers:* | | |
| VecCombiP1 | AATGGTTCGGGGAAATTGTT | This thesis |
| VecCombiP2 | AACAATTTCCCCGAACCATT | This thesis |
| VecCombiP3 | GGATCCCCAGCTTGTTGATA | This thesis |
| VecCombiP4 | AACCGTATTACCGCCTTTGA | This thesis |
| SPinsfwd | TGCCCCGTTAGTTGAAGAAG | This thesis |
| SPinsrev | TTCTACCATCGACACCACCA | This thesis |

## 3.4.3 Introduction of pINT4 to *E. faecium*

Vector pINT4 was used to transform both *E. faecium* E1162 and TX1330; the latter was included as it is easier to transform. As the vector does not contain a Gram-positive origin of replication, the vector will integrate into the genome by a single crossover (Campbell-type integration), in this case at the SPase713 gene. Insertion of this vector results in the *spase713* promoter being followed by an incomplete copy of the *spase*713 gene (leading to an inactive protein), and a complete copy of the *spase713* gene that is under control of the PnisA promoter (Figure 3.9).

Before transforming the strains with pINT4, the tolerance of *E. faecium* E1162 and TX1330 to nisin was determined. In both cases they were able to grow in the

presence of at least 40 µg/ml nisin (maximum concentration tested), which should be sufficient to achieve good expression levels. One study suggested that 20 µg/ml was the optimal concentration for induction in *E. faecalis* (Eichenbaum et al., 1998). Therefore, following transformation with pINT4, *E. faecium* cells were grown on agar plates with a range of nisin concentrations (0, 1, 2, and 20 µg/ml), and 10 µg/ml chloramphenicol for selection of the plasmid. The expectation was that, if the SPase713 gene is indeed essential, cells would only be able to grow in the presence of nisin.

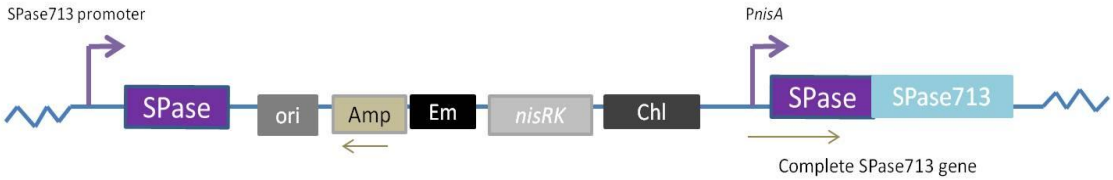



**Figure 3.9. Schematic of the genome once the integration has occurred. SPase713- Genomic SPase713. SPase- SPase713 fragment. Chl- chloramphenicol resistance gene. Em-erythromycin resistance gene. Amp- ampicillin resistance gene. Ori- *E. coli* origin of replication. PnisA- nisA promoter. *nisRK*- *nisRK* genes. See text for further detail.**

Plates were incubated for 48 hours at 37°C and very few colonies (of varying sizes) of *E. faecium* TX1330 were observed both on plates with and without nisin. Transformed *E. faecium* E1162 cells did not result in any colonies. All colonies were tested by PCR to establish if the vector had integrated (data not shown). Primers used included CmF (found in the chloramphenicol gene) with either TXseqRev (Table 3.3) or TX713DwnRev (Table 3.2), found on in or outside of the SPase713 gene. Both negative controls (using *E. faecium* TX1330 and the above primers) and positive controls (using primers used to amplify the SPase713 fragment) were included, and PCR reactions were performed at different temperatures to establish the best annealing temperature. Unfortunately, a correct integrant was not identified. Due to time constraints, it was also not possible to repeat this experiment.

## *3.5. Discussion*

Using bioinformatics, three possible SPases were found in the genome of *E. faecium* and alignments were performed against *B. subtilis* SPases. Interestingly it was observed that S0713 is likely to be the main SPase for *E. faecium*, as all the strains were highly conserved for this gene. S0133 was also likely to be active for all *E. faecium* strains. Based upon multiple sequence alignments, membrane topology, and a phylogenetic tree it also seems likely that SPase1233 is possibly a SipW-type (ER-type) SPase, although due to the lack of active residues they are likely to inactive. Alternatively, these SPases could represent a novel class of enzymes with a different catalytic mechanism. SipW is relatively uncommon in other bacteria except *Bacillus* spp. where it has been shown to have differing substrate specificity to the other SPases found. It has been linked to involvement in processing spore-associated proteins (van Roosmalen et al., 2004, Tjalsma et al., 2000b), but it should be noted that enterococci do not produce spores. SipW in *B. subtilis* has also been shown to be required for biofilm formation (Hamon et al., 2004) and it is part of an operon (*tapA-SipW-tasA*). Both TasA and TapA are processed by SipW, after which they are transported to the extracellular matrix of the biofilm. TasA is major protein component in the EPS and it is attached and assembled at the cell wall with the help of TapA (Terra et al., 2012). SipW not only functions in the above mechanism but it also appears to have a regulatory role in biofilm formation as well (Terra et al., 2012). Further research is required to establish if this is also the case in enterococci and therefore it could be a possible antimicrobial target.

This chapter has illustrated that many proteins (2.3%) and lipoproteins (1.8%) are secreted via the Sec-dependent pathway, some of which have already been shown to be involved in virulence, but there are also many hypothetical proteins with unknown functions. Further studies, both bioinformatics and wet-lab experiments are needed to analyse the functions of these proteins. Analysis of *E. faecalis* V385 showed that its genome contained 2.7% liproproteins; this is much more than seen here for *E. faecium* (Reffuveille et al., 2011). It is conceivable that it is these extra lipoproteins that make *E. faecalis* more prevalent in hospitals. It was interesting to

observe that some virulence related genes had paralogues in the genome, which included *bee-3*, a gene that is usually found as a part of a locus in *E. faecalis* responsible for the production of a pilus (see introduction). However, this locus has only been identified in 1% of *E. faecalis* clinical isolates. Although the *bee-3* gene is present in the *E. faecium* E1162 genome, the remainder of the locus is absent. The function of *bee-3* has not as yet been studied in *E. faecium* (Nallapareddy et al., 2011a). As mentioned in the introduction *E. faecium* have a large capacity to gain new genetic material. Perhaps in the case of the *bee* locus most of the locus was lost again, due to the fact that *E. faecium* already has four pilus clusters.

Following a previous study method, which used pTEX5500ts to produce a deletion of particular gene (Nallapareddy et al., 2006a); we attempted to produce the SPase mutants for all three SPases in *E. faecium* E1162 and TX1330 (except for S0133TX). Other researchers have used this method successfully deleting their required gene (Nallapareddy et al., 2006a, Heikens et al., 2007, Hendrickx et al., 2010). In this study no mutants were found and there could be two reasons for this. Firstly, it could suggest that the genes were all essential for the bacteria and, secondly, some mutants are difficult to obtain for various reasons. For example, Nallapareddy *et al* (Nallapareddy et al., 2006a) found only 4 colonies in 5000 containing the correct mutation in the gene of interest, and perhaps with analysing more colonies a mutant could still be identified.

The inducible integration plasmid was designed as the next logical step, to establish whether the genes were essential. Although the integration vector was successfully made and a SPase713 fragment inserted into it, time restrictions meant that successfully integrating the vector into *E. faecium* strains were not met and therefore studying the control of SPase713 expression was not achieved, this also meant that the vector's ability to work was not tested.

Further research work on this section would include continuing to look for mutants with insertional inactivation mutagenesis and to test the integration vector.

# Chapter 4: Results section II:

# The Enterococcal Surface Protein Esp affects antibiotic sensitivity in *Enterococcus faecium*

## *4.1. Background and objectives*

Enterococci are emerging pathogens, with many clinical isolates developing resistance to antibiotics such as penicillins, glycopeptides (e.g. vancomycin) and aminoglycosides. This can make the treatment of enterococcal infections with antimicrobials very difficult (Sood et al., 2008). Resistance to antimicrobials can be split into intrinsic and acquired resistance. Intrinsic resistance occurs when bacteria are inherently resistant to a particular compound, for example because of structural features that prevent uptake of the compound, or lack of a target of the compound. In contrast, acquired resistance is created by mutations or gain of exogenous DNA, with the latter most frequently through genetic mobile elements such as transposons or plasmids from other bacteria. In the case of the β-lactam ampicillin, both intrinsic and acquired resistance has been found in enterococci. The former may due to low affinity of enterococcal penicillin-binding proteins (PBP) to β-lactams, whereas acquired (high level) ampicillin resistance is usually due to overproduction of PBP5 or mutations in this protein, or β-lactamases that are encoded by transferable plasmids. However, β-lactamases producing enterococci are not often isolated and are usually in *E. faecalis* hospital isolates (Top et al., 2008, Sood et al., 2008).

In the pharmaceutical industry calorimetry is frequently used to study purity, decomposition and also to study the types of interactions that occur between drugs i.e. synergic or antagonistic interactions, which can then lead on to being able to develop combination therapies for clinical use (Giron, 1986, Vine and Bishop, 2005). In brief, a calorimeter (Figure 4.1) works by detecting heat flow from the sample being tested (within the ampoule) via thermopile arrays on the outside of the ampoule. The arrays are kept at a constant temperature as they are in a thermostatic water bath that serves as a heat sink. The energy required to keep the temperature constant is converted into a voltage signal (Power, μW; von Rege and Sand, 1998).

Cultures of bacteria produce heat as they metabolise nutrients, and this heat can be monitored by calorimetry and provide real-time data on the growth. Thus, calorimetry could be used as a tool in the pharmaceutical industry to identify bacterial contamination of products, identifying the ideal growth conditions for the production of genetically engineered therapeutics, and as a high-throughput

screening method to identify effective antimicrobials (Vine and Bishop, 2005). Calorimetry has also been used in a clinical setting with a number of studies evaluating bacterial growth, such as the analysis of meticillin resistance in *Staphylococcus aureus* (Baldoni et al., 2009, von Ah et al., 2008). Calorimetry can also be used to identify bacteria, as each bacterium has a characteristic output signal (Beezer, 1980).

There are two types of calorimetric techniques: batch and continuous flow calorimetry (Vine and Bishop, 2005). Batch calorimetry is when the media and bacteria to be tested are placed in an enclosed ampoule and measurements of temperature change are then recorded. Flow calorimetry is when the bacteria being tested are cultured outside the calorimeter, in a water bath kept a constant temperature, which is pumped at a continuous flow rate through the calorimeter's ampoule. This has many advantages over batch calorimetry as the sample is continuously stirred reducing sedimentation, also aerobic bacteria can be tested (batch calorimetry is a closed system and the environment becomes anaerobic) and, more importantly, flow calorimetry allows the addition of nutrients or antimicrobial compounds to the media during testing (Vine and Bishop, 2005). These advantages make flow calorimetry the ideal method for these trials.

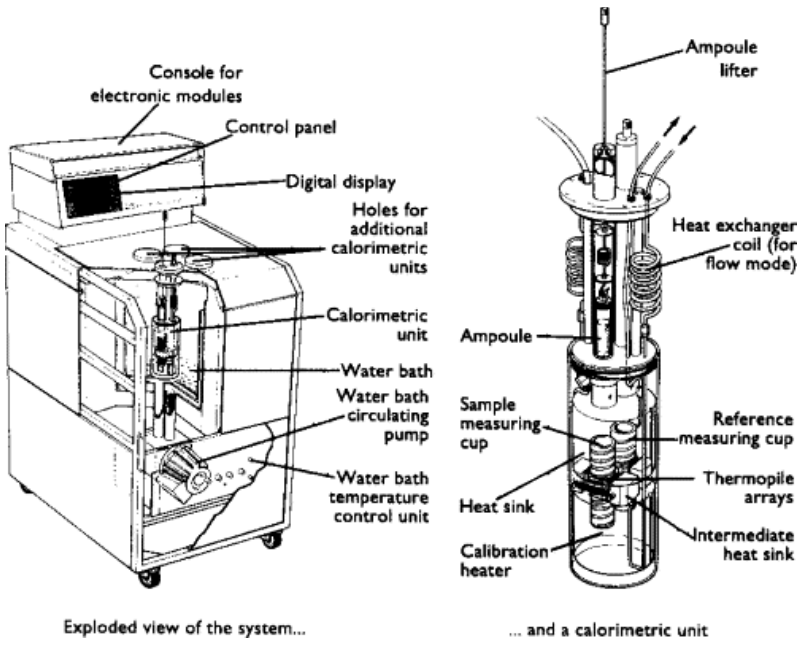



**Figure 4.1. Schematic of the 2277 Thermal activity monitor (TAM; Thermometric AB; O'Neill et al., 2003).**

In this chapter the antibiotic resistance of *E. faecium* strain E1162 was analysed. This is a single clonal lineage of the Complex 17 (CC17), a clinical blood isolate that causes many hospital outbreaks (Heikens et al., 2007). The strain is resistant to ampicillin and contains the gene encoding the enterococcal surface protein Esp. The resistance of this strain was compared to that of *E. faecium* E1162Δ*esp*, an *esp* deletion mutant of E1162 (Heikens et al., 2007), and *E. faecium* TX1330, an ampicillin sensitive and *esp* negative strain that was isolated from a healthy individual (Nallapareddy et al., 2003). The initial objective of this study was to test the use of isothermal calorimetry as a tool to measure antibiotic resistance, and to compare this with traditional methods such as the disc susceptibility and minimal inhibitory concentration (MIC) measurements (Andrews, 2001).

## *4.2. Traditional antimicrobial susceptibility tests.*

Disc susceptibility tests with ampicillin showed small clearing zones with *E. faecium* E1162 and E1162Δ*esp*, demonstrating its resistance to ampicillin, while a larger zone of inhibition was observed around the disc on *E. faecium* TX1330 cells (Table 4.1).

As determined by microbroth dilution, both *E. faecium* E1162 and E1162Δ*esp* have an MIC of 32 µg/ml, while MIC Evaluator strips showed a lower MIC at 16 µg/ml. Corroborating the disk tests, the control strain *E. faecium* TX1330 showed a higher sensitivity to ampicillin with an MIC of 4 µg/ml as determined by microbroth dilution or 0.75 µg/ml as determined by the MIC Evaluator strip (Figure 4.2). Using the standard antimicrobial susceptibility tests, it is therefore clear that the different methods (broth dilution, disc susceptibility, and MIC Evaluator strips; Table 4.1) do not show a difference between *E. faecium* E1162 and E1162Δ*esp* strains.

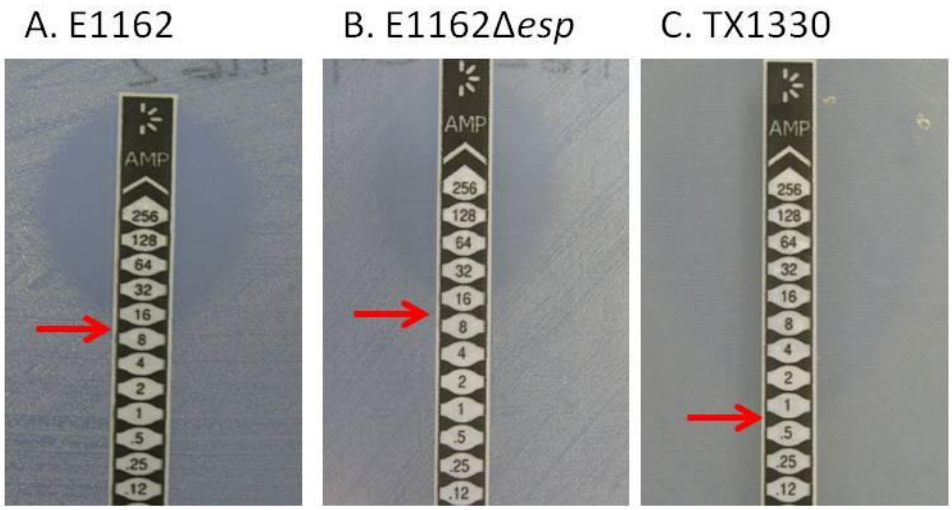



**Figure 4.2. Representative images of the MIC Evalulator strip results. A. E1162. B. E1162Δ*esp*. C. TX1330.**

## *4.3. Calorimetry*

### 4.3.1. Optimisation of the method

Initial tests showed that calorimetry is a very sensitive method that led to variation in curves obtained. Effects could be observed, for instance, from using different batches of media preparations or the length of time ampicillin was stored (data not shown). For consistency, one large batch of culture medium was prepared that was frozen and defrosted one day before use, while ampicillin was prepared fresh every week.

### 4.3.2 Control curves

As bacteria grow and metabolise nutrients in the broth, more heat is produced, which was recorded by the calorimeter as power-time (P-t) curves. P-t curves in the absence of antibiotics for *E. faecium* E1162, *E. faecium* E1162Δ*esp* and *E. faecium* TX1330 are shown in Figure 4.3A. As the bacteria metabolise nutrients in the media and multiply, the amount of heat (measured in μWatt) increases. The peaks and troughs observed are probably due to the sequential use of nutrient sources in the complex culture medium (O'Neill et al., 2003), which emphasises the importance of keeping the media contents consistent. With all three strains, three distinct peaks were observed at similar times and with similar heat outputs, implying that they metabolise the medium in a similar way. *E. faecium* strain TX1330 has a slightly different growth curve, probably due to it being a different isolate of *E. faecium* than the other two strains. The fact that *E. faecium* E1162 and E1162Δ*esp* display nearly identical P-t curves suggests that Esp does not have an effect on metabolism.

### 4.3.3 Effect of ampicillin on P-t curves

To test the effect of ampicillin on the three strains, a final concentration of 64 μg/ml was added when the cultures reached 10 μW (Figure 4.3B), which was equivalent to approximately 1.2 x $10^5$ cfu/ml. The concentration chosen was double the MIC of the

*E. faecium* E1162 and E1162Δ*esp* strains, but still half the value MBC, thus ensuring a strong response without killing all the cells immediately. As expected, *E. faecium* E1162 responded differently to the addition of ampicillin compared to the control strain *E. faecium* TX1330. Whereas the maximum output of the E1162 strain reached 36 μW, the heat output from the TX1330 strain, which has an MBC of only 8 μg/ml, almost immediately stopped after addition of ampicillin, reaching a maximum of only 12 μW.

Unexpectedly, the addition of ampicillin to *E. faecium* E1162Δ*es*p resulted in a consistently lower heat output than E1162, with an average peak value of 24 μW (Figure 4.3B; Table 4.1). Consequently the output of the strain lacking Esp was only 64% of that of its parental strain, this was an unexpected result as the traditional antimicrobial susceptibility tests showed no difference in MIC between *E. faecium* E1162 and its *esp* mutant.

Similar results were obtained when a lower ampicillin concentration of 32 μg/ml was added during testing (data not shown). Under those conditions, the maximum output of the *E. faecium* E1162 strain was considerably higher (maximal peak value around 90 μW). However, these data were less reproducible, probably because the concentration of ampicillin was close to the MIC. However, a significant difference between *E. faecium* E1162 and E1162Δ*esp* was still observed, with the output of the latter being, on average, 50-60% lower (data not shown).

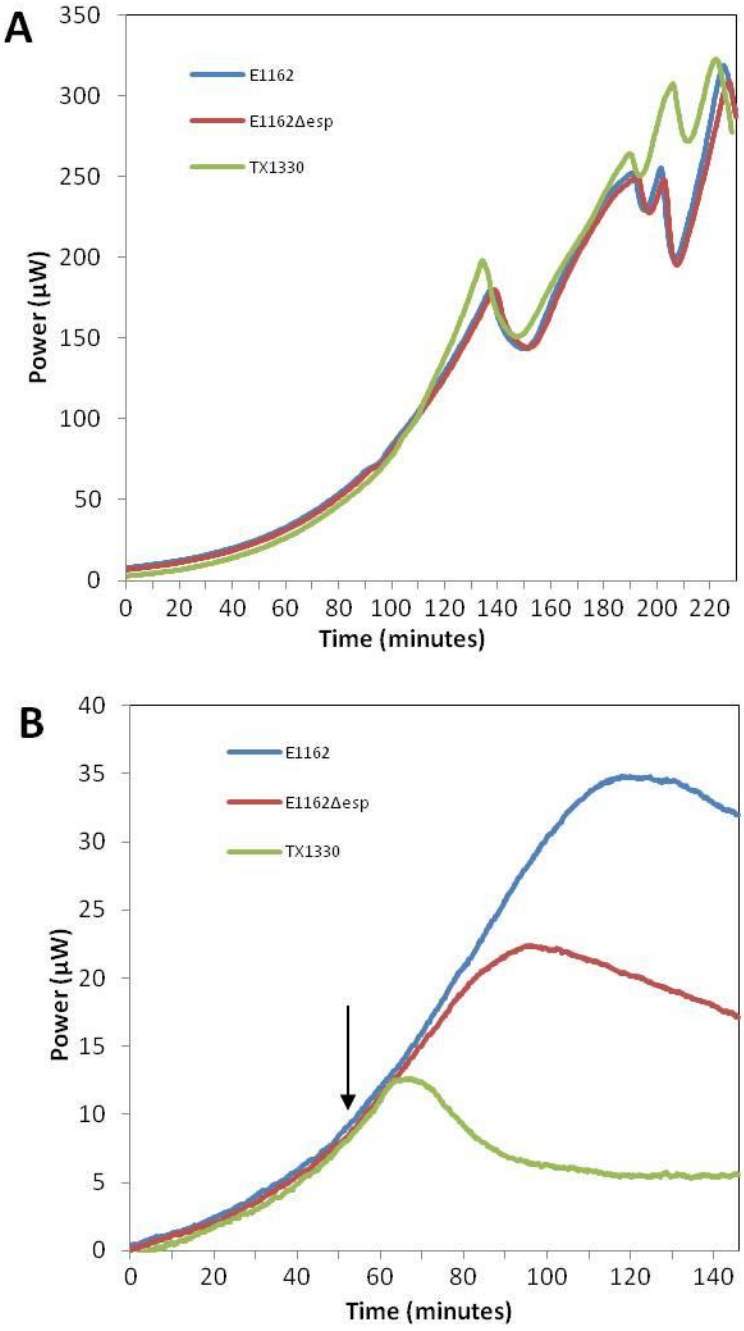



**Figure 4.3. Response of *E. faecium* cultures to addition of ampicillin. Representative Power-time curves for E1162 (blue line), E1162Δ*esp* (red line) and TX1330 (green line). A. Control conditions in the absence of ampicillin. B. 64*µ*g/ml ampicillin was added when cultures reached an output of 10*µ*W (shown by the black arrow).**

***Table 4.1. Response of E. faecium strains to ampicillin using different test methods.***

| | *MIC* | | | *MBC* | *Isothermal Calorimetry* |
|---|---|---|---|---|---|
| *Stain* | *Broth dilution (µg/ml)* | *Disk susceptibility (mm)* | *M.I.C.Evalulator (µg/ml)* | *Broth dilution (µg/ml)* | *Mean peak height (µW)*[1,2] |
| *E1162* | *32* | *1.5* | *16* | *128* | *36 ± 4.4* |
| *E1162Δesp* | *32* | *1.5* | *16* | *128* | *23 ± 1.5* |
| *TX1130* | *4* | *10* | *0.75* | *8* | *12* |

[1] From an average of three measurements, with the exception of *E. faecium* TX1330 (n=1).

[2] Standard deviation stated

## *4.4. MIC, broth dilution method using stepwise concentrations for ampicillin.*

Although there was no difference in ampicillin resistance using the standard broth dilution method (which uses doubling concentrations), it was decided to determine the MIC using a narrower range of concentrations, which in this case was between 16 and 32 µg/ml. This method is somewhat more laborious and would not be realistic to be performed in a clinical setting, but was performed to see if a difference between *E. faecium* E1162 and E1162Δ*esp* could be observed. This method was indeed sensitive enough to observe a difference: *E. faecium* E1162 gave an MIC value of 22 µg/ml, while the MIC for *E. faecium* E1162Δ*esp* was 18 µg/ml (Table 4.2).

Adjacent to the *esp* gene is a gene encoding EbrB, which has recently been shown to be a regulator that controls expression of *esp* (see chapter 5 for more details; personal communication, Dr Janetta Top). It was therefore of interest to test whether an *EbrB* mutant was also more sensitive to ampicillin as shown above for the *esp* mutant. To this end we compared sensitivity of *E. faecium* E1162, with *E. faecium* E1162*ebrB* (kindly provided by J Top) containing either pAT18 (empty vector) or pAT18*ebrB* (expressing *ebrB*). In the *E. faecium* E1162 *ebrB* mutant, *ebrB* is disrupted by a chloramphenicol gene leading to a lack of expression of Esp (J. Top, unpublished data). Strikingly, it was also shown that EbrB does not only affect Esp but also a number of other genes including the NADH oxidase Nox, a putative muramidase, a hypothetical protein, and a drug resistance transporter (EmrB/QacA). Ampicillin sensitivity was tested using the stepwise method and, similar to the *E. faecium* E1162 *esp* mutant, inactivation of *ebrB* lead to an increased sensitivity (MIC 18 µg/ml; Table 4.2). Presence of pAT18*ebrB*, but not the empty vector, partially restored ampicillin resistance (Table 4.2).

## *4.5. Minimum inhibitory concentrations (MICs) for SDS*

Esp is a protein anchored to the cell wall, and the difference in ampicillin sensitivity could be due to a difference in cell wall integrity in the strains with and without Esp. If so, E1162Δ*esp* cells would be expected to be more sensitive to cell lysis. To establish this hypothesis, sensitivity to the detergent SDS was determined using a standard broth dilution method. For both strains the MIC for SDS was however the same (0.0156%), suggesting that there was no difference in cell wall integrity. Other detergents were also tested, i.e. Triton X100 and bile salts. However, MIC values were not determined for these as *E. faecium* appeared to be very resistant to these (MIC for bile salts >50%, MIC for Triton X100 >1%).

As a difference was seen for ampicillin using the stepwise broth dilution method it was decided to test this for SDS as well using a range between 0.0156% and 0.0078%. No difference was seen between E1162 and E1162Δ*esp*, with their MIC value being 0.0137%.

## *4.6. MIC, broth dilution and broth dilution using stepwise concentrations for Vancomycin.*

Ampicillin is an antibiotic that inhibits transpeptidase, which is involved in synthesis of the cell wall, and it was decided to test another antibiotic that also acts on the cell wall. Vancomycin was chosen, which is an antibiotic that binds to the terminal D-Ala-D-Ala residues in the cell wall peptide chains (Gholizadeh and Courvalin, 2000). A normal MIC using broth dilution was performed using vancomycin on the *E. faecium* strains, which gave an MIC value of 2 µg/ml (standard method) or 1.2 µg/ml (stepwise method) for both strains (Table 4.2).

***Table 4.2. MIC values of E. faecium strains to ampicillin, SDS and vancomycin.***

| | *MIC: Broth dilution* | | | | |
|---|---|---|---|---|---|
| *Strain* | *E1162* | *E1162Δesp* | *E1162ΔebrB* | *E1162ΔebrB-pATebrB* | *E1162ΔebrB-pAT* |
| *Ampicillin (µg/ml)* | *22* | *18* | *18* | *20* | *18* |
| *SDS (%)* | *0.0137* | *0.0137* | - | - | - |
| *Vancomycin (µg/ml)* | *1.2* | *1.2* | - | - | - |

## *4.7. Discussion*

Initially when testing calorimetry as a method to monitor bacterial growth, it was surprising to discover how sensitive this was to small differences in culture conditions. Calorimetry has been used previously to differentiate between growing cultures of meticillin-susceptible and meticillin-resistant *S. aureus*; it was able to do this in 4-5 hours while standard methods take 24 hours, showing it to be a rapid method (von Ah et al., 2008, Baldoni et al., 2009). Calorimetry tests using *E. faecium* E1162 (ampicillin resistant) and TX1330 (ampicillin sensitive) have illustrated that differentiation and resistance to ampicillin can be shown in hours by calorimeter, which is a significant improvement over traditional methods used here, some that can take days. Calorimetry also showed the unexpected difference between the resistance of *E. faecium* E1162 and E1162Δ*esp*, which was not observed with traditional antimicrobial resistance tests. This illustrates that calorimetry is a sensitive and fast method. When the MIC value was studied more closely using the broth dilution method in a stepwise manner, a difference could be observed, but this method is more laborious than the standard method. Calorimetry in the setup used here is also rather laborious and limited by the number of samples that can be tested at once, but this could be improved by automation. Chip calorimeters are now widely used, with samples set up in 96 well plates that only require small quantities and enable the testing of many samples at once (Braissant et al., 2010). There are some automated antimicrobial susceptibility testing systems on the market such as Vitek2, MicroScan Walk-away and Phoenix. These systems work on a broth based methods i.e. the isolate is diluted to a specific optical density; it is then used to inoculate cards or 96 well plates containing various antibiotics at different concentrations. The result times once tests are setup are 9, 20 and 12 hours respectively, illustrating again the speed of calorimetry (Sellenriek et al., 2005). There are also genotypic methods, using PCR and DNA hybridization as a bases, which can be used to identify resistance genes in isolates (Fluit et al., 2001), but these methods do involve more preparation (DNA isolation) and can be time consuming. One other big advantage that calorimetry has over these other methods is that it is being adapted for use into studying biofilms. Biofilms are grown within the ampoule and then treatments

against the biofilms can be tested to establish efficient removal, this can be used for industrial problems or clinical problems by testing antibiotics (Buchholz et al., 2010b).

It was interesting to note that the difference in sensitivity between E1162 and E1162Δ*esp* was only observed with ampicillin, but not with another antibiotic that acts on the cell wall (vancomycin), or a detergent (SDS). β-lactams (such as ampicillin) act by inhibiting the carboxy/transpeptidase or penicillin-binding proteins, which are involved in the late stages of peptidoglycan biosynthesis during peptide cross-linking. Glycopeptides (such as vancomycin) act on a different stage of the cell wall synthesis; they inhibit the peptidoglycan precursors from inserting into the cell wall by the transglycosylase enzyme through binding to the D-alanyl-D-alanine residue on the precursor (Denyer et al., 2004). It is conceivable that the lack of differences in sensitivity with vancomycin could be related to the differences in mode of action of these antibiotics, but further testing is required to investigate this. The role of Esp in this is unclear at present. As it is a large cell-wall protein it was thought that the lack of Esp could cause instability in the cell wall and, if that is the case, it would be expected that cells lacking Esp would be more sensitive to SDS (which disrupts the cell membrane), however, as previously noted, this was not observed. Esp has been connected to several different processes in enterococci, including initial adherence, colonisation (Shankar et al., 2001) and biofilm formation (Tendolkar et al., 2004, Heikens et al., 2007). Our findings suggest that Esp could also have a role in ampicillin sensitivity, but in view of the many potential roles that Esp may have, it is conceivable that the effects we observe are indirect. More generally, we showed that isothermal calorimetry is a method far more sensitive than traditional methods for analysing susceptibility to antibiotics. The method is also very rapid and could therefore be of real benefit if used clinically.

# Chapter 5: Results Section III:

# The effect of bile salts on biofilm formation in *E. faecium*.

## *5.1. Background and objectives*

Enterococci are commonly found in the intestine, which is a hostile environment with varying conditions (pH, oxygen levels), bile acids, digestive enzymes, or toxins from other bacteria (Wilson et al., 2002).

Bile is produced in the body to help metabolise lipids found in food in the gastrointestinal tract (GI tract), by emulsifying and solubilising them. There are several components in bile which include: sodium, potassium, chloride, cholesterol, phospholipids and bile acids. Bile acids are one of the main constituents providing 50% of the organic compounds of bile (Begley et al., 2005). They are a water-soluble group of steroids that can be strongly cytotoxic, as they are able to permeabilise membranes (Jenkins, 2008). Therefore bile is considered to also help the body by being a bactericidal agent (Merritt and Donaldson, 2009). Bile acids are usually present at physiological concentrations of between 0.2-2% in the small intestine (Hofmann, 1998).

The enterohepatic circulation is the means by which cholesterol in the liver is converted to bile acids for the use in the body and is summarised in Figure 5.1 (Begley et al., 2005). Cholic acid and chenodeoxycholic acid (primary bile acids) made from cholesterol in the liver are usually conjugated with taurine (sodium taurocholate) or glycine (sodium glycocholate), which makes these compounds more soluble in aqueous solutions. This conjugation allows the bile acids to increase in concentration in the lumen of the small intestine, as it reduces their membrane permeability and helps the formation of micelles, which allows the emulsification and absorption of lipids. In the lower intestine (caecum and colon) these conjugated bile acids can be cleaved by bacterial enzymes into free bile acids (secondary bile acids) such as deoxycholic acids (Jenkins, 2008) these are then readily reabsorbed and transported to the liver for recycling, where they are reconjugated and stored in the gallbladder until required for the next meal. This cycle can occur 10 times a day and is 95% efficient at recycling the bile acids (Begley et al., 2005, Jenkins, 2008).

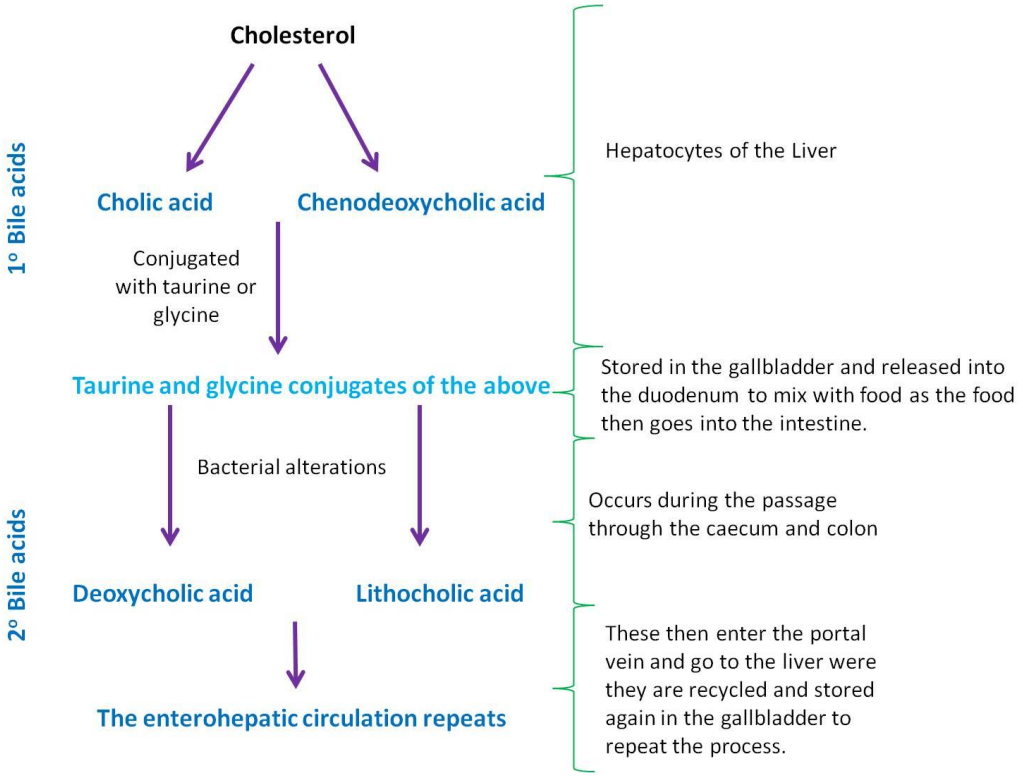



**Figure 5.1. Simplified version of the enterohepatic circulation. (Jenkins, 2008, Begley et al., 2005)**

Some bacteria have become well adapted to survive in the presence of bile salts. Several factors involved in bile resistance have been identified. These include, in the case of Gram negative bacteria, the lipopolysaccharide (LPS) which has been shown to aid resistance by merely providing an extra barrier, and Tol proteins, which are outer membrane pore proteins that are important for membrane integrity and stop bile access (Begley et al., 2005, Gunn, 2000). Furthermore, both Gram negative and Gram positive bacteria contain efflux pumps, which are the best characterized method of removing bile from the cytoplasm (Thanassi et al., 1997, Pumbwe et al., 2007). Finally, several bacteria contain bile salt hydrolases, which are generally intracellular enzymes used by the intestinal microflora (including enterococci) to deconjugate the bile salts; they are not normally found in pathogenic species, with the exception of *L. monocytogenes* (Dussurget et al., 2002). Their significance is not fully understood, but there are three main theories: (i) deconjugation provides additional nutrients; (ii) deconjugation adds cholesterol or bile into their cell membranes which can then help them evade the host defence; or (iii) deconjugation has a role in bile tolerance. The evidence is conflicting, but there are clear examples

were deletion of the genes encoding these hydrolases to give rise to bile sensitive mutants (Begley et al., 2005).

As well as having mechanisms to cope with the presence of bile, some pathogens also use bile as a trigger to activate or suppress virulence factors as a sensor for being in the right place for invasion or colonisation of the host. The most widely studied are enteric pathogens. For instance, *Salmonella typhimurium* down-regulates its type III secretion system until the bile concentration has decreased, which usually occurs when it has passed through the mucus layer and is at the epithelial cell surface (Prouty and Gunn, 2000). In *Shigella* spp. researchers have observed an increase in secretion of invasion plasmid antigens (Ipa), proteins that are involved in increased attachment to HeLa cells in the presence of bile (Pope et al., 1995).

Here we analysed the effect of bile salts on biofilm formation in *E. faecium* strains, with a particular focus on *E. faecium* E1162, which is a clinical isolate and CC17 clonal strain.

## *5.2. Optimising the crystal violet biofilm assay for Enterococci*

The assay for biofilm formation of *E. faecium* isolates was initially tested to optimise the procedure and obtain reliable and consistent results. Several factors were tested, including the composition of the media, growth of biofilms in the wells of 96-well plates or on pegs, the length of incubation, method of shaking (speed and horizontal vs. 3D rotator), and the number of washing steps. The optimal method involved 24 hour biofilm growth in 96 well plates, shaken on a 3D rotator (data not shown), using TSB medium containing 0.25% glucose. Details of the method are given in the Materials and Methods section.

## *5.3. Control biofilm assays for the E. faecium isolates*

Initial tests were performed using the crystal violet biofilm assay to confirm that, as previously noted (Heikens et al., 2007), *E. faecium* E1162Δ*esp* produces approximately 50% less biofilm than the parental strain (Figure 5.1A). The difference was shown to be significant using a Student T-test ($p<0.0001$). As shown before (Nallapareddy et al., 2003), *E. faecium* TX1330 was a relatively poor biofilm former; the amount of biofilm formed was similar or slightly less than formed by *E. faecium* E1162Δ*esp*.

## *5.4. Hydrophobicity testing for the E. faecium isolates*

An important factor in biofilm formation on a surface such as polystyrene is hydrophobicity of the bacteria, with hydrophobic cells adhering better (Donlan, 2002). It was tested whether the lack of Esp and the concomitant reduced ability to form biofilms was due to reduced hydrophobicity. This appeared to be the case as shown in Fig 5.1 B, with *E. faecium* E1162 being significantly more hydrophobic than E1162Δ*esp* ($p<0.0001$) and TX1330 ($p<0.0001$). These results corroborate similar findings for *E. faecalis* (Tendolkar et al., 2004), in which mutants lacking Esp are also less hydrophobic.

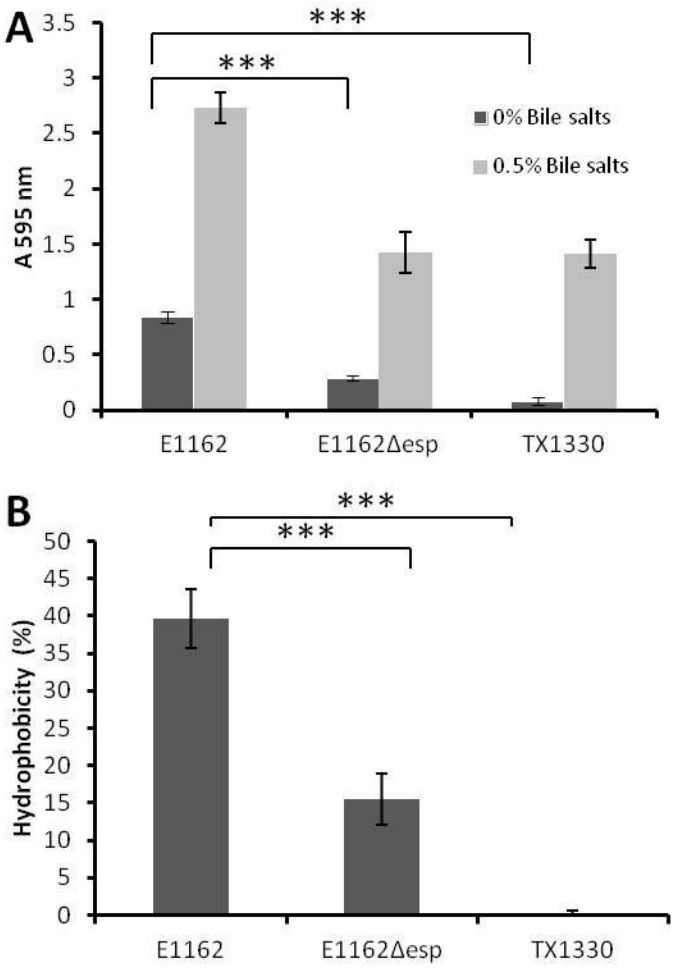



**Figure 5.2. Biofilm assay and hydrophobicity results for the *E. faecium* isolates. (A) Biofilm formation and (B) Hydrophobicity. The error bars represent the mean ± the standard error. *** P<0.0001**

## *5.5. The effect of bile salts on E. faecium.*

Prior to performing tests with bile salts an MIC test and growth curves were performed for each isolate. All strains were shown to be resistant to bile salts when grown in concentrations up to 50% (data not shown). Growth curves did show that in the presence of bile salts there was an increase in doubling time from approximately 36 minutes to 60 minutes (Table 5.1). The optical densities taken after overnight culture were the same for all *E. faecium* strains and therefore unaffected by the presence of up to 5%.

***Table 5.1. Average growth rates in the presence and absence of bile salts***

| % of bile salts | E1162 | E1162Δ*esp* | TX1330 |
|---|---|---|---|
| *Average growth rate (min):* | | | |
| 0 | 36 ± 0.5 | 39 ± 3 | 48 ± 8 |
| 0.5 | 53 ± 5 | 54 ± 10 | 56± 4 |
| 1.5 | 60 ± 7 | 60 ± 0.6 | 56 ± 3.5 |
| 5 | 48 ± 9 | 54 ± 6 | 56 ± 4 |

It was observed that chains of cells were, on average, longer in the presence of bile salts (Table 5.2). In the absence of bile, *E. faecium* cells usually appear as pairs, but when bile is present this increases to 4-20 cells per chain (Figure 5.3).

***Table 5.2. Average chain length in the absence and presence of 0.5% bile salts.***

| | Average chain length (±sd) | |
|---|---|---|
| *Strain:* | *- Bile salts* | *+ Bile salts* |
| E1162 | 2.44±0.99 | 4.61±2.51 |
| E1162Δ*esp* | 2.40±0.86 | 8.34±4.88 |
| TX1330 | 2.32±0.76 | 5.47±3.49 |

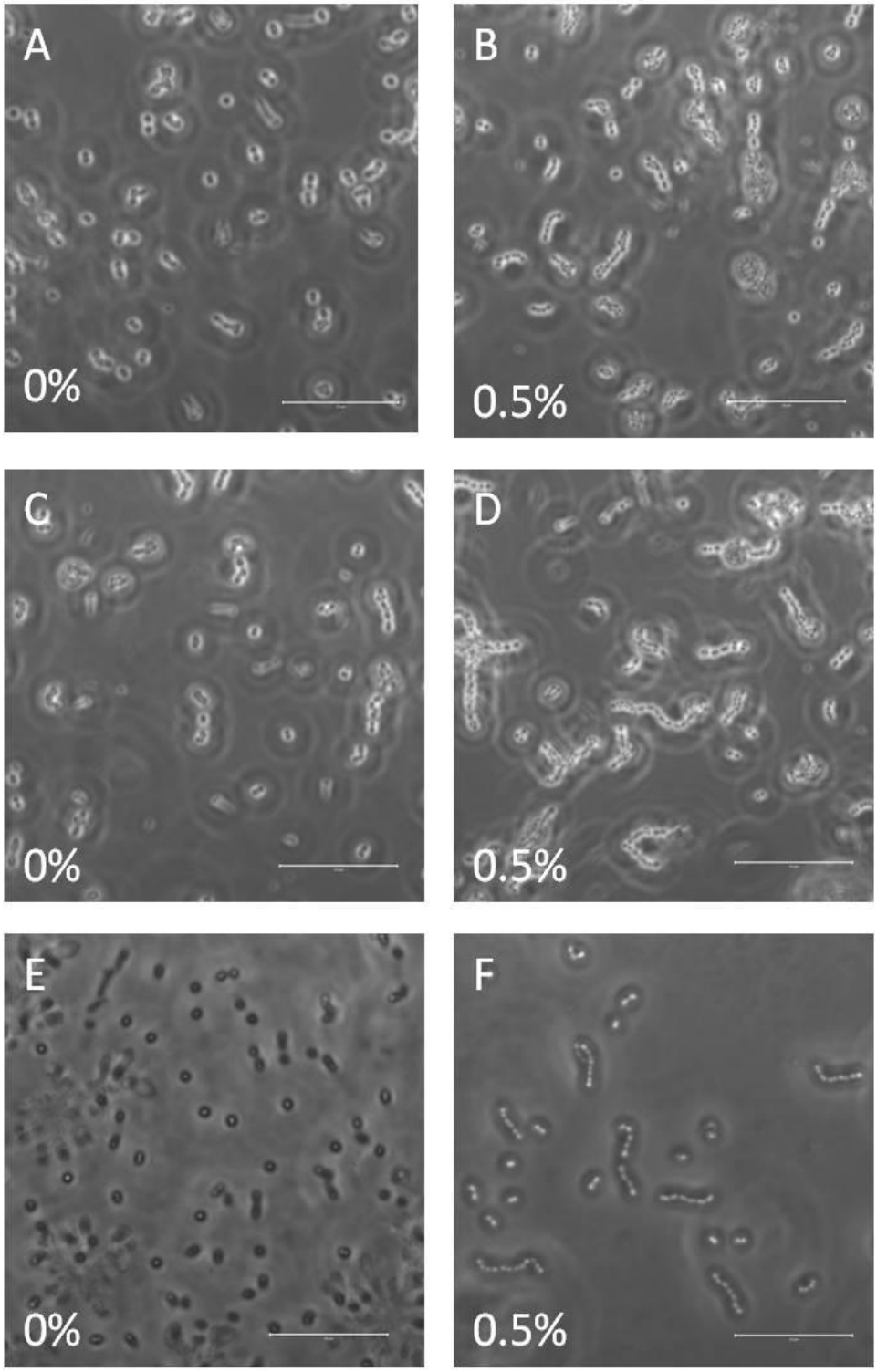



**Figure 5.3. Light microscopy images of *E. faecium* isolates: E1162 (A and B), E1162Δ*esp* (C and D) and TX1330 (E and F) in the presence and absence of 0.5% bile salts.**

## *5.6. The effect of bile salts on E. faecium biofilm formation.*

The effect of the bile salts on 24 hour biofilm formation in *E. faecium* strains E1162, E1162Δ*esp* and TX1330 is shown in Figure 5.4A. Interestingly, at increasing levels of bile salts all three strains formed significantly more biofilm, with the best biofilms observed at bile concentrations between 0.5 and 1.5% for E1126 and TX1330. Strikingly, this concentration is similar to the approximate physiological concentration in the small intestine (Hofmann, 1998). For E1162Δ*esp* the range of bile salt concentrations at which it forms better biofilms was more narrow, with maximum values observed at 0.5%. Higher concentrations of bile (above 2-3%) were detrimental to biofilm formation, with all strains forming biofilms very poorly at 5%. For biofilms grown for 48 hours, similar results were observed (Figure 5.4B). However, for E1162Δ*esp* and TX1330 the maximum biofilm formation is significantly higher than that seen for 24 hours, about 1.3 and 2 fold more at 0.5% bile salts, respectively.

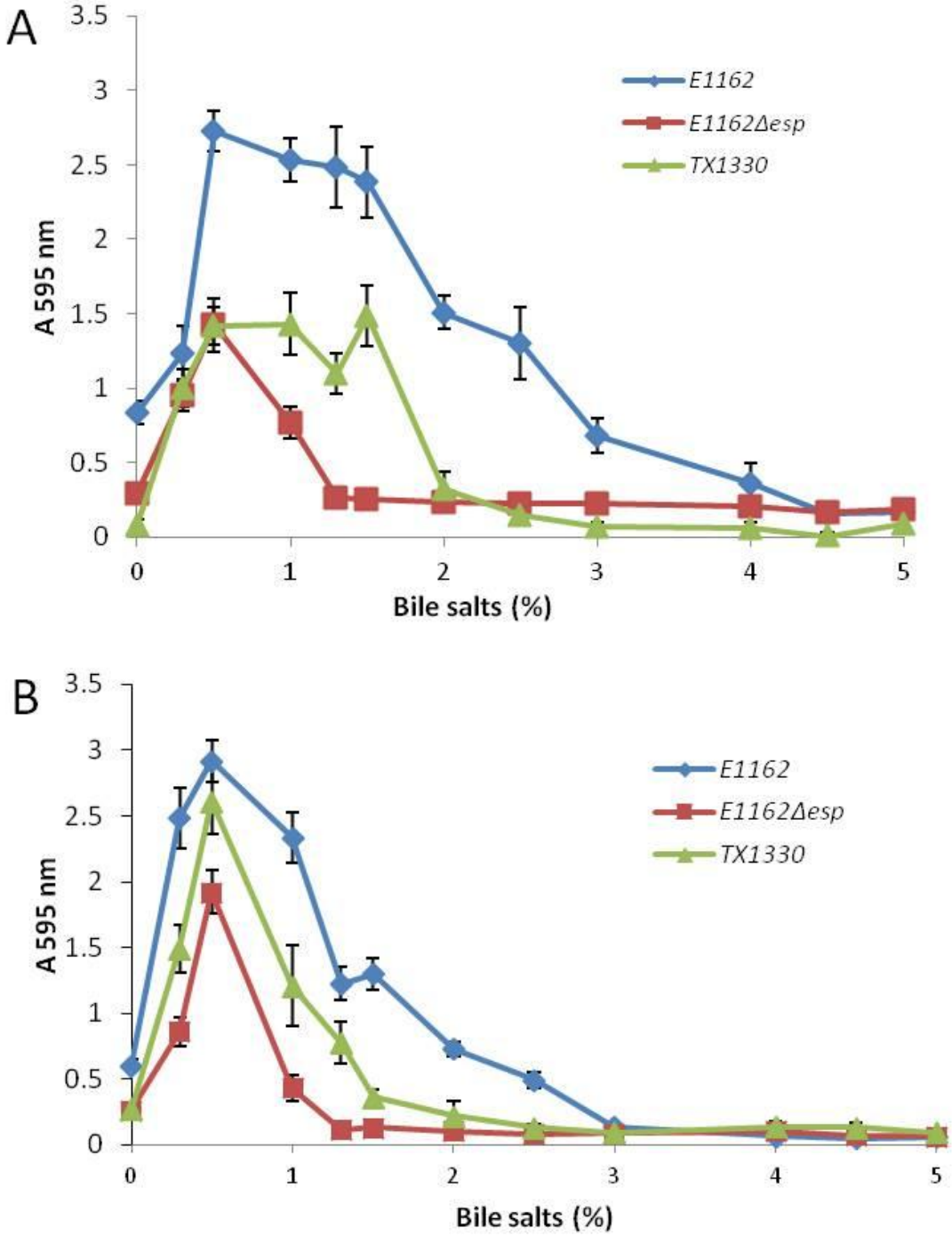



**Figure 5.4. Biofilm formation in the presence of bile salts for 24 hours (A) or 48 hours (B). With the latter fresh media was provided after 24 hours. The error bars represent the mean ± the standard error.**

## *5.7. Biofilm formation in the presence of other detergents*

Bile salts function as detergents, and it was therefore important to test whether other detergents had similar effects. To that purpose, biofilm assays were also performed in the presence of non-inhibitory concentrations of SDS (0.00975% to 0.03%) and Triton X100 (0.01% to 1%). However, neither of these stimulated biofilm formation (see appendix A.1), showing that the effects of bile salt are specific and not due to their detergent-like nature.

## *5.8. Biofilm formation in the presence of sodium glycocholate, sodium taurocholate and a 1:1 mix of the two.*

Bile salts contain mostly sodium taurocholate and sodium glycocholate. To investigate whether either of these components is important in the stimulation of biofilm formation, assays were performed in the presence sodium taurocholate, sodium glycocholate and a 1:1 mixture of the two. Sodium glycocholate did not stimulate biofilm formation in the three strains (Figure 5.5A). In contrast, sodium taurocholate clearly stimulated biofilm formation in *E. faecium* E1162, but not in E1162Δ*esp* or TX1330 (Figure 5.5B). Maximum biofilm formation by E1162 was found at 0.5% sodium taurocholate. The increase was less than with bile salts but still approximately 2-fold more than in the absence of sodium taurocholate. Interestingly, when a 1:1 mixture of sodium glycocholate and sodium taurocholate was used, *E. faecium* E1162 formed biofilms to a level similar to that observed when using crude bile salts (Figure 5.5C). This indicated that a mixture of the two salts is necessary for maximum biofilm formation. Surprisingly, the taurocholate/glycocholate mixture did not stimulate biofilm formation in either E1162Δ*esp* or TX1330, suggesting that the crude bile salts used before still contain other components that stimulate biofilm formation in these strains.

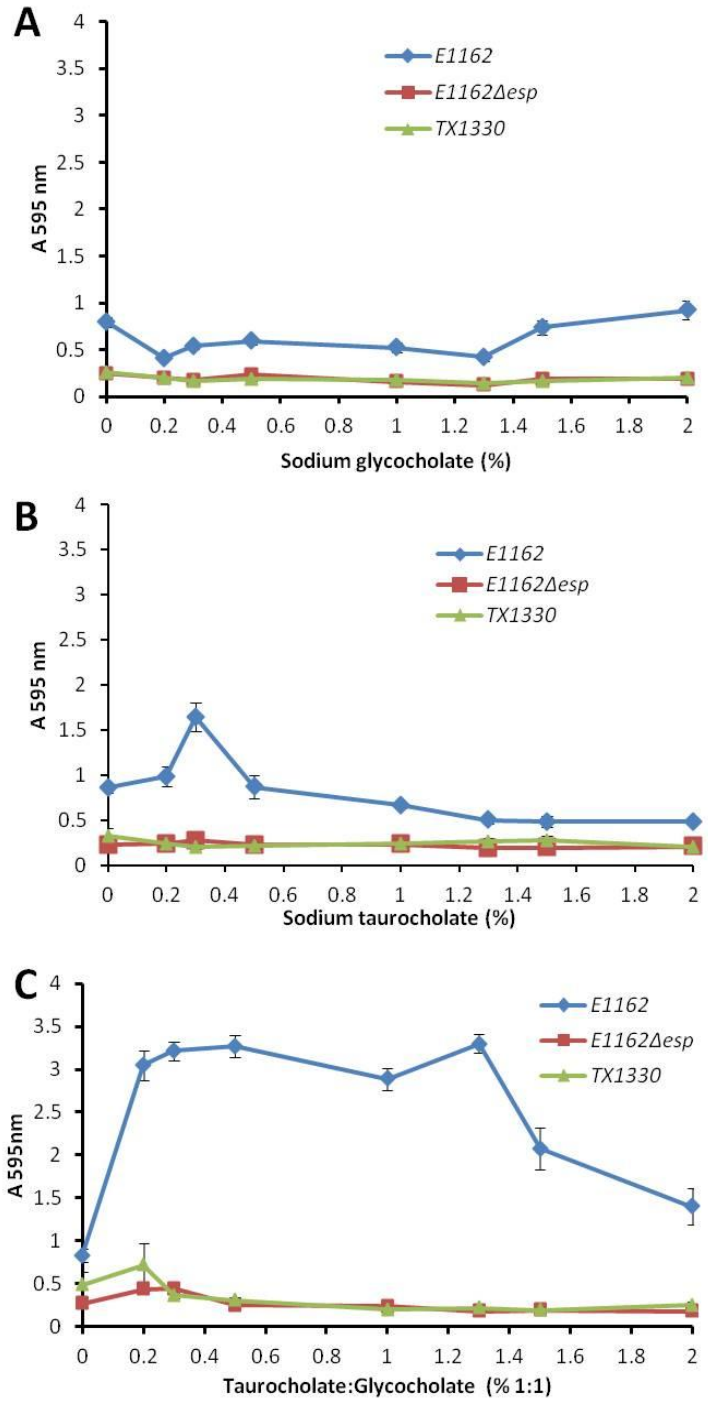



**Figure 5.5. Biofilm assays in the presence of (A) sodium glycocholate, (B) sodium taurocholate, and (C) Sodium taurocholate: Sodium glycocholate (1:1). The error bars represent the mean ± the standard error.**

## *5.9. Effect of bile salts on hydrophobicity.*

As shown before, hydrophobicity is an important factor in biofilm formation. It was therefore tested whether the effects of bile salts on biofilm formation was due to increased hydrophobicity. Surprisingly, in the presence of bile salts there was actually a slight decrease in hydrophobicity for both *E. faecium* E1162 and E1162Δ*esp*, but this decrease was statistically not significant ($p>0.086$ and $p>0.511$ respectively; Figure 5.6). Thus, the improved biofilm formation in the presence of 0.5-1.5% bile salts is clearly not caused by an increase in hydrophobicity. Similarly, there was also no effect on hydrophobicity of cells when grown in the presence of sodium taurocholate or glycocholate (data not shown).

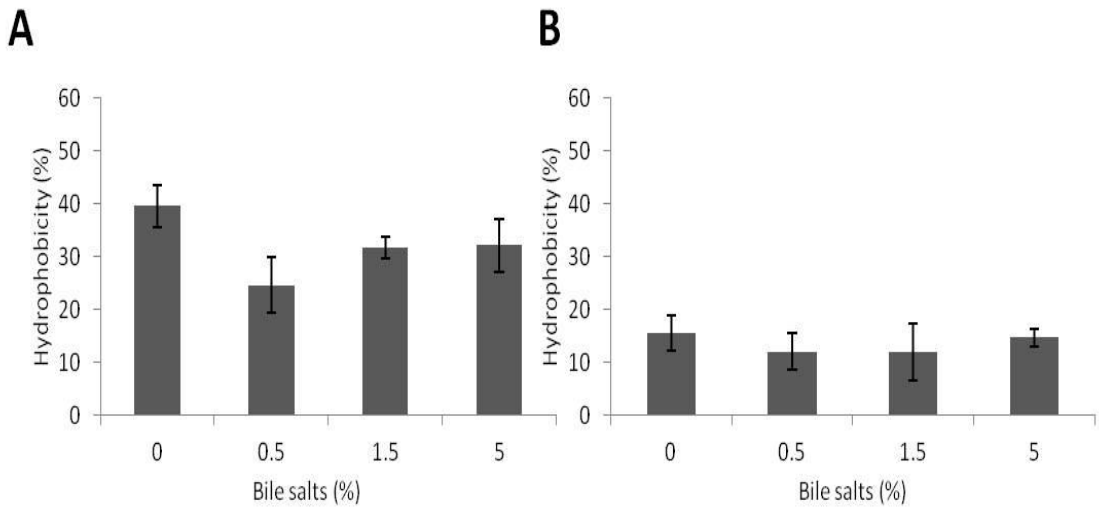



**Figure 5.6. Hydrophobicity in the presence of bile salts. (A) *E. faecium* E1162 and (B) *E. faecium* E1162Δ*esp*. The error bars represent the mean ± the standard error.**

## *5.10. Effect of bile salts on initial attachment.*

The first step in biofilm formation is direct attachment of cells to a surface, and we tested whether it was this stage that was stimulated by bile salts. To analyse this, attachment of cells was measured after 2, 4 and 6 hours in the presence or absence of

bile salts. Initial attachment after 2 and 4 hours showed no significant increase in attachment due to the presence of bile salts in *E. faecium* E1162 (Figure 5.7A). After 4 hours, a 2-fold increase in attachment could be observed comparing 0% with 1.5% bile salts, while after 6 hours (a stage at which microcolonies start to form) that difference was 3-fold. These differences were not observed in 0.5% bile salts. Similar trends were observed with E1162Δ*esp* (Figure 5.7B) and TX1330 (Figure 5.6C), although with these strains the effect was the strongest in 0.5% bile salts. With *E. faecium* TX1330 a significant increase in attachment was only observed after 6 hours. From the results it is also clear that the lack of Esp affects initial attachment, which has been established previously (Heikens et al., 2007).

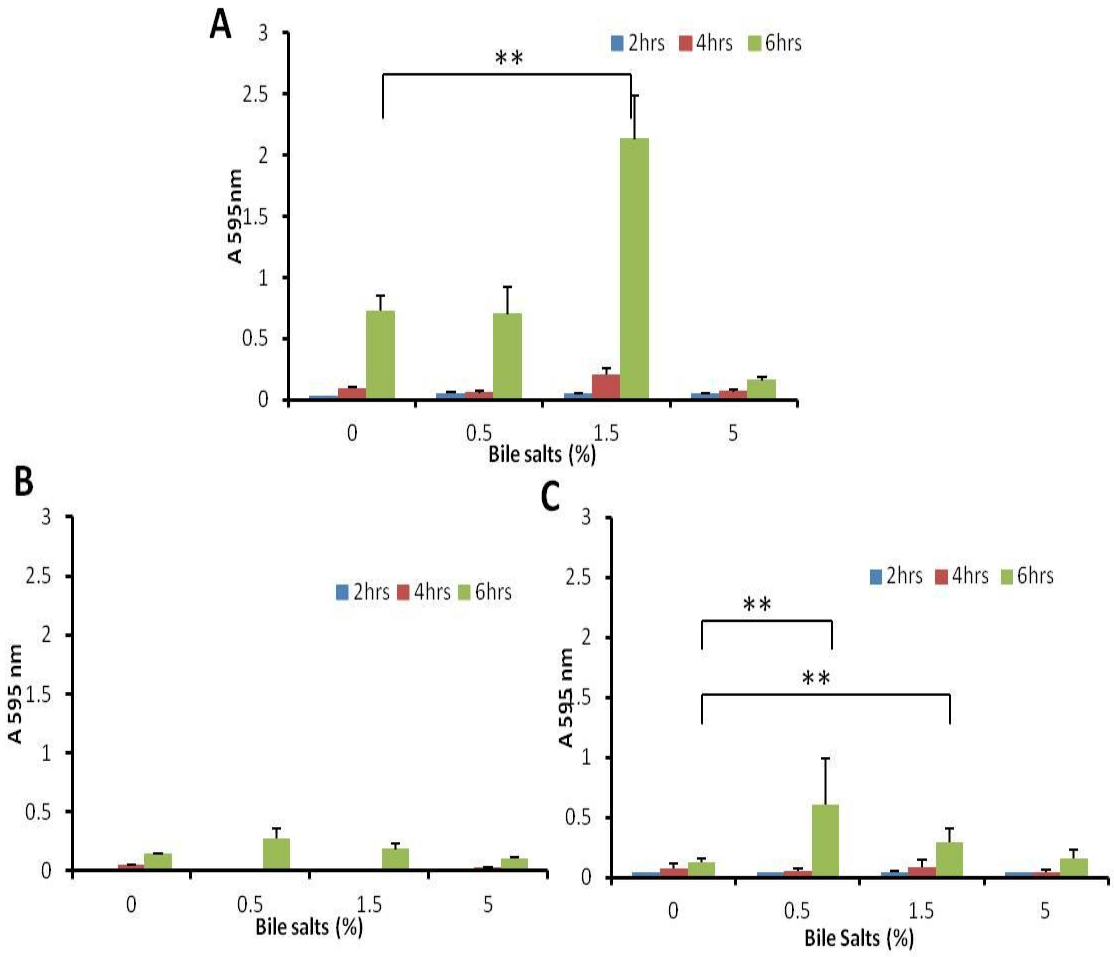



**Figure 5.7. Initial attachment in the presence of bile salts for the *E. faecium* isolates. (A) *E. faecium* E1162; (B) E1162Δ*esp*; and (C) TX1330. The error bars represent the mean ± the standard error. ** P<0.005.**

## *5.11. Effects of the pH and bile salts on biofilm formation*

In the GI tract it is not only the concentration of bile salts that changes, but also the pH. For instance, the pH in the small intestine is about 6, while that gradually increases to pH 8.5 in the distal part of the large intestine (Khan et al., 1999). We therefore analysed the effects of the pH on biofilm formation and combined this with the studies on the effects of bile salts. As shown in Figure 5.8, at pH 6 both *E. faecium* E1162 and E1162Δ*esp* produced slightly more biofilm (but not significantly) compared to pH 7. In contrast, there was a significant ($p<0.01$) decrease in biofilm formation when grown at pH 8 or 8.5. With increasing concentrations of bile salts, *E. faecium* E1162 forms similar amounts of biofilm at pH 6 or 7. However, with an alkaline pH biofilm formation is largely inhibited, even in the presence of bile salts, showing only a slight increase in biofilm formation around 0.5% to 1%. Interestingly, in the presence of 0.5% bile salts *E. faecium* E1162Δ*esp* forms significantly ($p<0.05$) better biofilms at pH 6 compared to pH 7. Again, an alkaline pH appears to inhibit biofilm formation. Hydrophobicity was also tested for both strains at the different pH values, but no difference was observed (data not shown).

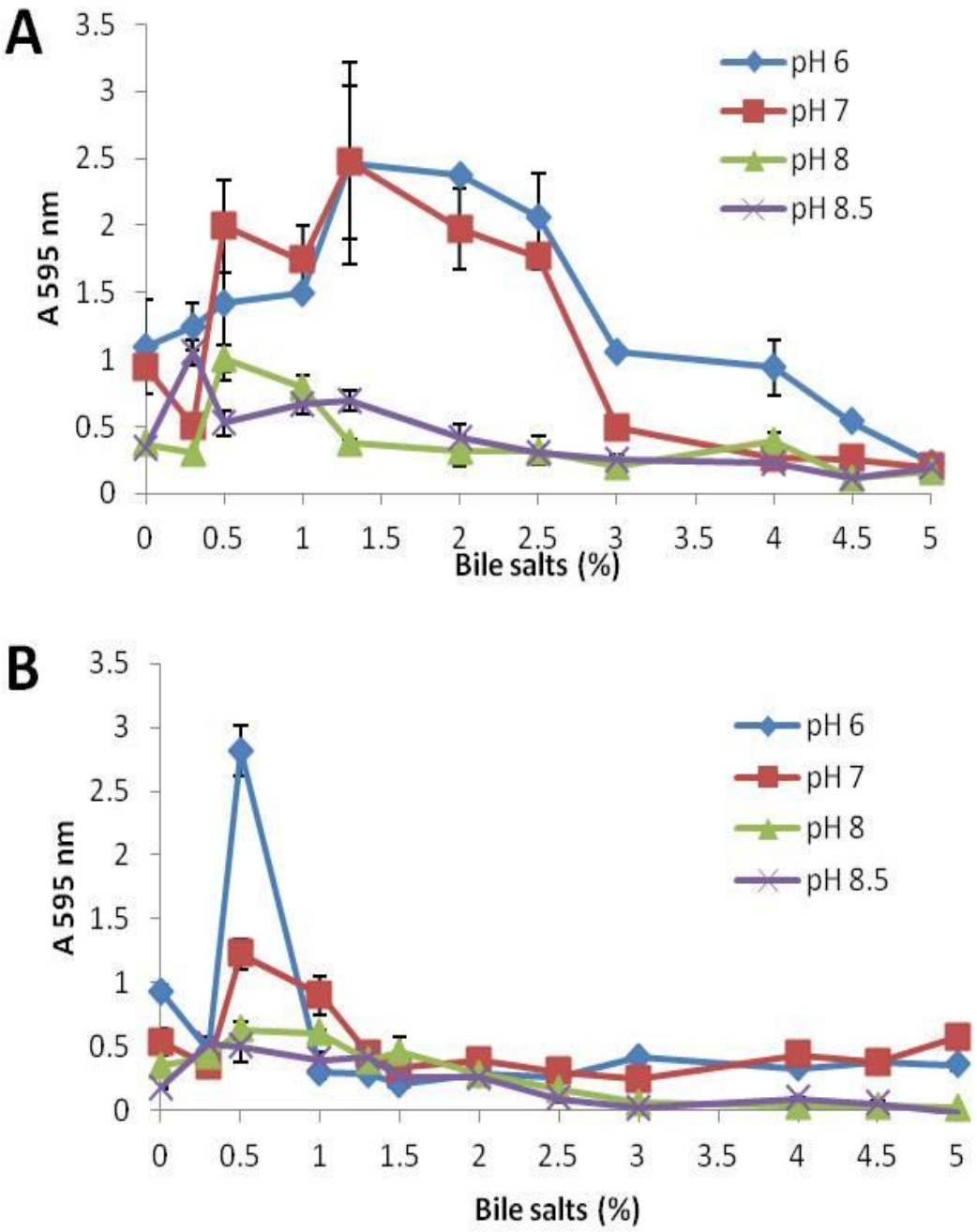



**Figure 5.8. Biofilm formation at different pH and in the presence of bile salts. (A) *E. faecium* E1162 and (B) E1162Δ*esp*. The error bars represent the mean ± the standard error.**

## *5.12. Ebr biofilm assay in the presence of bile salts.*

The expression of the *esp* gene is regulated by the activator *ebrB*, a enterococcal biofilm regulator gene that also controls other genes, including those encoding NADH oxidase Nox, a putative muramidase, a hypothetical protein, and a drug resistance transporter (EmrB/QacA). The *ebrB* gene is located adjacent to the *esp* gene in the genome of *E. faecium* E1162 on ICEEfm1, a integrative conjugative element (See figure 5.9; Janetta Top, personal communication). *E. faecium* E1162Δ*ebrB*, as well as E1162Δ*ebrB* containing a vector expressing *ebrB* (pAT-ebrB) and E1162Δ*ebrB* containing the empty vector (pAT18) were kindly provided by Janetta Top for biofilm testing in the presence and absence of bile salts.

As shown in Figure 5.10, *E. faecium* E1162 produces significantly more biofilm compared to E1162Δ*ebrB*, both with and without bile salts, similar to what has been observed with E1162Δ*esp*. In *E. faecium* E1162Δ*ebrB* (pAT-ebrB) biofilm formation was fully restored while, as expected, this was not the case with the strain containing the empty control plasmid (pAT18). In E1162Δ*ebrB* (pAT-ebrB) biofilm formation even appeared to be higher than the wild-type strain, which was possibly due to increased levels of EbrB when its gene is expressed from the plasmid.

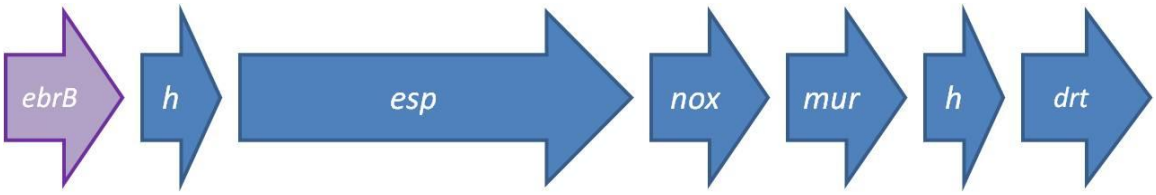



**Figure 5.9. Schematic of the section of the ICE*Efm1* containing the *ebrB* gene and the other genes it controls. h- represents hypothetical protein. drt- drug resistance transporter.**

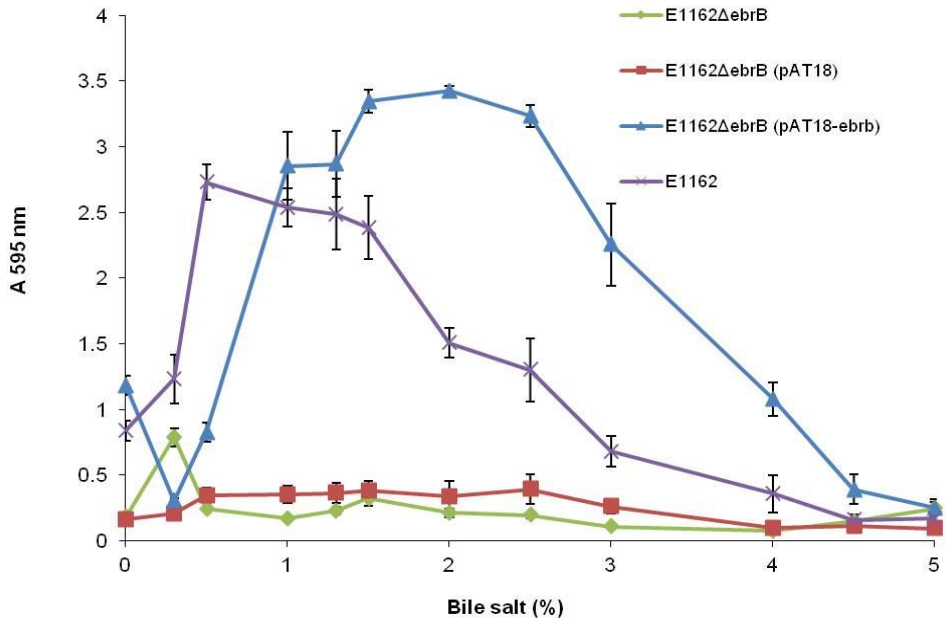



**Figure 5.10. Biofilm formation in the presence of bile salts for *E. faecium* E1162, E1162Δ*ebrB*, E1162Δ*ebrB* (pAT18) and E1162Δ*ebrB* (pAT-*ebrB)*. The error bars represent the mean ± the standard error.**

## *5.13. Confocal Laser Scanning Microscopy (CLSM) images of biofilms in the presence of bile salts for E1162.*

To visualise the biofilms formed in the presence or absence of bile salt, biofilms were grown on polyvinyl coverslips and analysed with Confocal Laser Scanning Microscopy. Polyvinyl coverslips were used as they have no autofluorescence, unlike polystyrene coverslips. Importantly, biofilms form equally well on polystyrene and polyvinyl (data not shown). Bacteria were stained with BacLight or acridine orange. BacLight shows living bacteria as green and dead bacteria as red and acridine orange only stains all cells green.

As shown in Figure 5.11, in the presence of 0.5% or 1.5% bile salts there are considerably more cells in the biofilms when compared those grown in the absence of bile salts, while also the maximum thickness of the biofilms increased in the

presence of bile salts. The images also confirmed that in the presence of 5% bile salts barely any bacteria adhered to the polyvinyl surface.

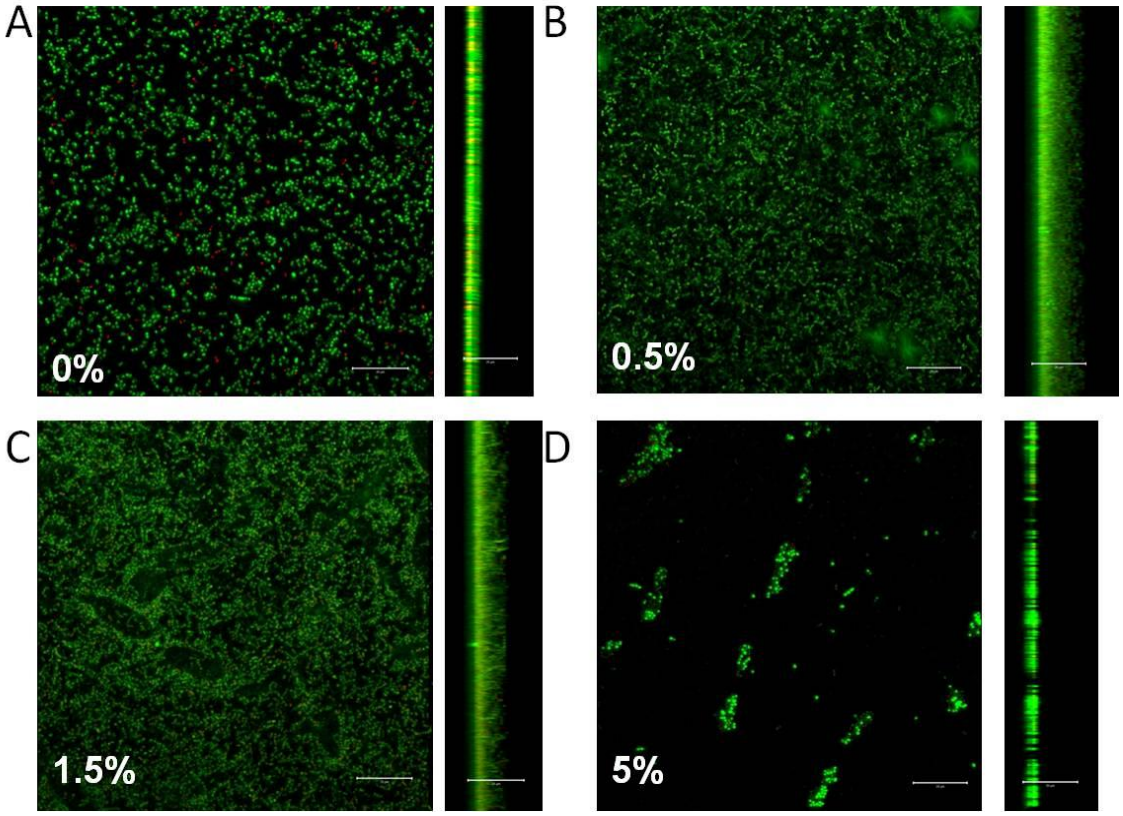



**Figure 5.11. CLSM images of E1162 stained with BacLight. Biofilms were grown in TSB-G over 72 hours (with fresh media every 24 hours) containing 0% (A), 0.5% (B), 1.5% (C) or 5% (D) bile salts. Scale bar represents 20$\mu$m.**

***Table 5.3. Maximum thickness and biomass ± standard error for E. faecium E1162, defined using COMSTAT2 software (Heydorn et al., 2000).***

| E1162 | TSB-G only | TSB-G containing 0.5% bile salts | TSB-G containing 1.5% bile salts. |
|---|---|---|---|
| Maximum thickness | 6.3µm ±1.3 | 17.3µm±1.5 ** | 18µm±1.6 * |
| Biomass ($\mu m^3/\mu m^2$) | 0.5 ± 0.2 | 3.0 ± 0.3 * | 3.9 ± 0.1 * |

**** P <0.005, * P<0.05.**

Frequently observed in biofilms grown in the presence of 0.5% or 1.5% bile salts (but not with 5%) were voids in the image (examples are shown in Figure 5.12). These were most likely aggregates of bile salt micelles, which have been observed previously in many different shapes such as circles, discs, rods, worm-like or even irregular. These shapes were shown to depend on the size of the bile salt molecule, the mixture of bile involved and the other components in the media (Jenkins, 2008, Partay et al., 2007, Fini et al., 2002).

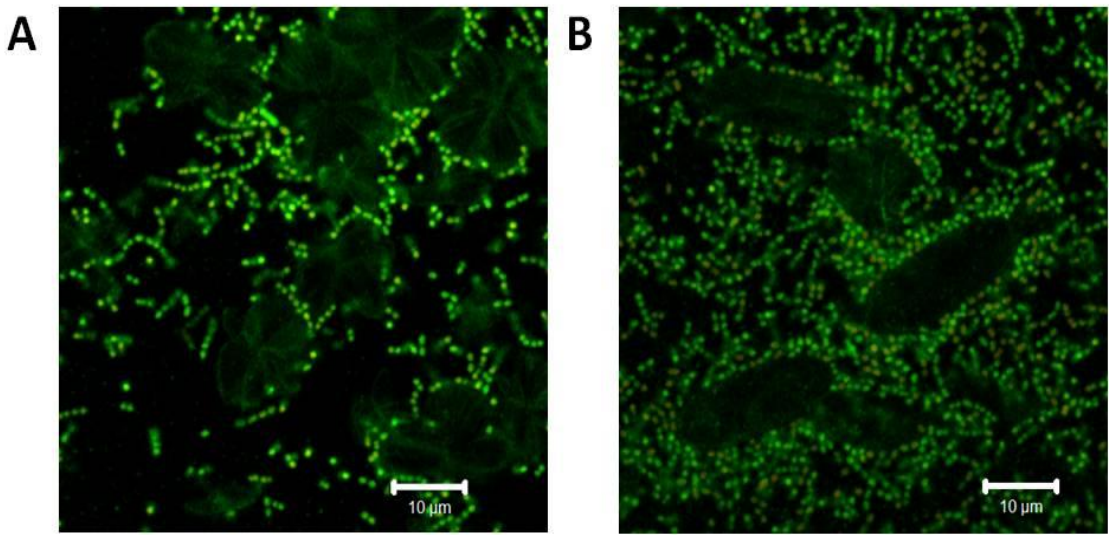



**Figure 5.12. Examples of CLSM images showing the different types of bile salt aggregates observed during imaging. Cells were stained with Baclight. A. Circular shaped, B. Rod shaped. Scale bar represents 20*µ*m.**

## *5.14. Confocal Laser Scanning microscopy images of biofilms in the presence of bile salts for E. faecium E1162Δesp and TX1330.*

A number of biofilm images were also taken for *E. faecium* E1162Δ*esp* and TX1330 (Figure 5.13). In the conditions used (24 hour biofilms), E1162Δ*esp* showed no increase in biofilm formation in the presence of 0.5% bile salts, which was in contrast to what was observed with the crystal violet assay at this concentration. Conversely, *E. faecium* TX1330 showed a clear increase in biofilm formation in the presence of 0.5% bile salts using this method, as evident by the images (compare Figure 5.13C and D) and also the maximum thickness, increasing from 4 µm in the absence of bile to 26 µm in the presence of bile. Although the maximum thickness is larger than seen with the *E. faecium* E1162 (Section 5.11) under the same condition, it is important to note that this is a single data point and not an average form the total biofilm in the image. The biofilm for *E. faecium* E1162 appears denser and of a more even thickness compared to the biofilms formed by *E. faecium* TX1330.

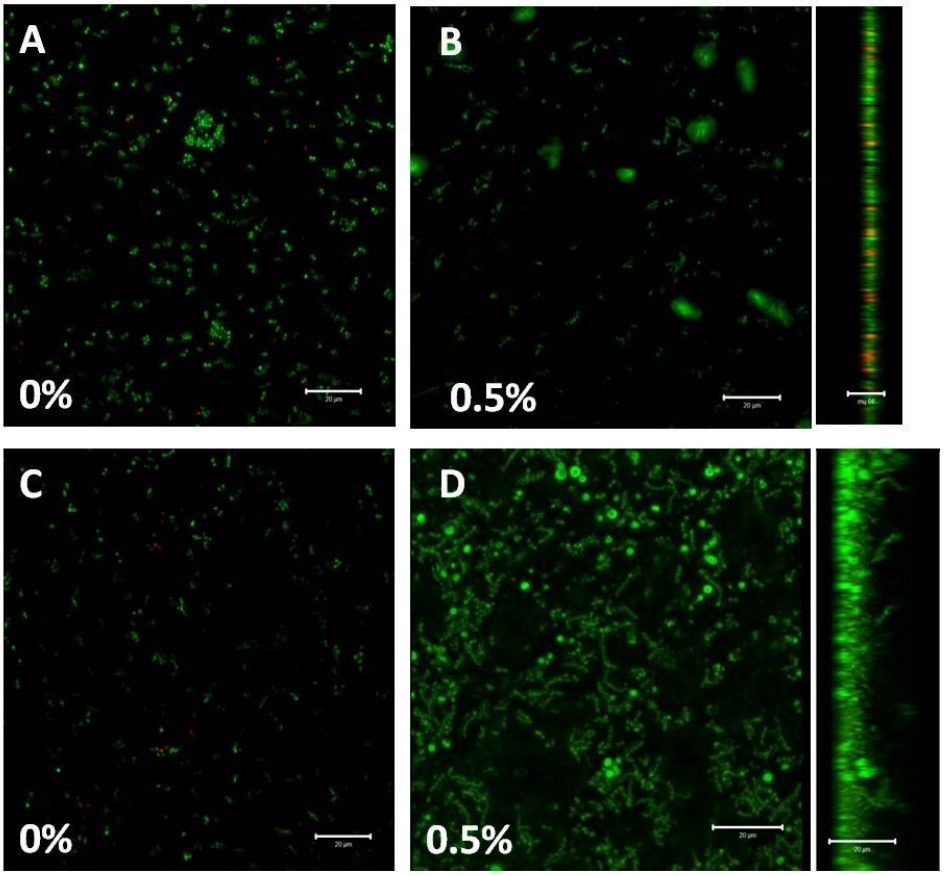



**Figure 5.13. CLSM biofilm formation images for *E. faecium* E1162Δ*esp*: (A) TSB-G; and (B) TSB-G containing 0.5% bile salts with side view. Biofilm images for *E. faecium* TX1330: (C) TSB-G and (D) TSB-G containing 0.5% bile salts with side view. Cells were stained with BacLight. Scale bar represents 20$\mu$m.**

## *5.15. CLSM images using calcofluor white staining of E1162 biofilms.*

Extracellular polymeric substances (EPS) surrounds the bacteria that have attached to the surface, aiding stability and structural scaffolding; it usually contains many polysaccharides, lipids, nucleic acids and proteins that help in further attachment to the surface and cell-cell attachment (Abee et al., 2011). Calcofluor white stain can be used detect EPS in biofilms by attaching to the β-1,4 and β-1,3 polysaccharides present (Chen et al., 2007). As shown in Figure 5.14, biofilms of *E. faecium* E1162 were stained with both calcofluor white (blue) and acridine orange to stain bacterial cells present. Comparing the images it is observed that in the presence of bile salts

there is more calcofluor white staining (blue; figure 5.14E) than seen in the absence of bile salts (Figure 5.14B), suggesting the presence of bile is stimulating EPS production. In the presence of bile salts also some blue-staining patches are observed (see e.g. Figure 5.14 E and F). These could be micelle aggregates of bile salts but that needs further confirmation. These patches were not observed in the absence of biofilm (data not shown).

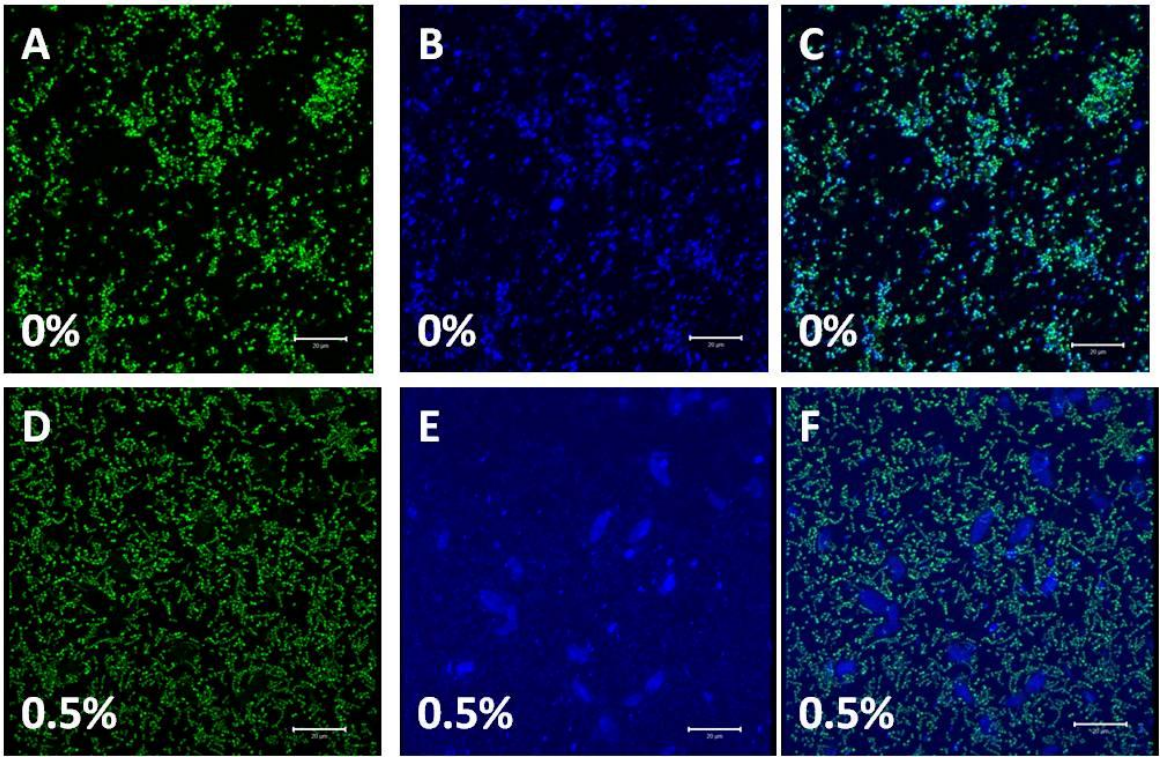



**Figure 5.14. Representative CLSM images of E1162 biofilms grown in the absence (A, B, C) and presence of bile salts (D, E, F). Acridine orange staining of bacteria shown in green (A and D), calcofluor white staining of EPS shown in blue (B and E), and an overlay of both (C and F). Scale bar represents 20$\mu$m.**

## *5.16. Analysis of microcolony formation*

Microcolonies are aggregates of cells that can mature into large biofilms (Davey and O'Toole G, 2000). Initial attachment assays (see section 5.8) showed a sharp increase in attachment at 6 hours. To analyse whether it was at this stage that microcolonies

of *E. faecium* E1162 were forming, the initial attachment test was performed on vinyl coverslips in order to visualise the attached cells. It can be observed that when no bile salts are present (Figure 5.15A), bacteria are attaching to the surface in pairs and that these pairs are starting to clump together. When bile salts are present (Figure 5.15B) we can see attachment of chains of bacteria on the surface and, that some of these chains are starting to clump.

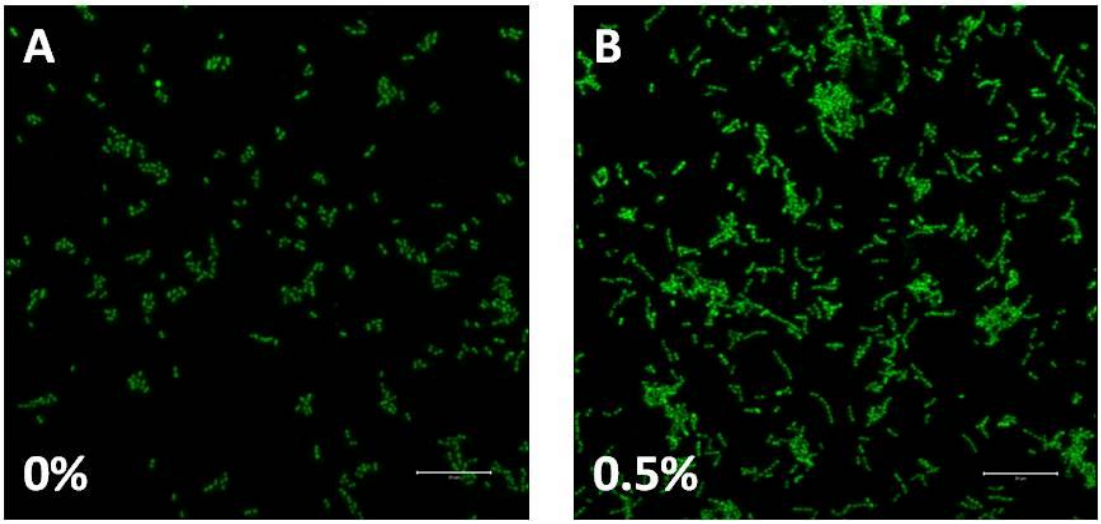



**Figure 5.15. *E. faecium* E1162 attachment CLSM images after 6 hour incubation, stained with acridine orange. (A) Cells grown in TSB-G and (B) cells grown in TSB-G containing 0.5% bile salts. Scale bar represents 20$\mu$m.**

## *5.17. Effect of iron limitation on biofilm formation*

Iron limitation is another stress factor that enterococci will encounter in the GI tract and so it was also studied to see if it affected biofilm formation on the *E. faecium* strains. In previous studies on *E. coli* the iron chelator used was 2,2-dipyridyl (at 0.5mM) to decrease iron presence in the media, and a positive control restoring iron depletion in the media was also tested by adding 40mM ammonium ferric sulphate (Alves et al., 2010, Wise et al., 2002). As a control, biofilms of *E.coli* strains JM109 and DH5α were grown, which showed the expected decrease observed in the presence of the iron chelator (Figure 5.16A; Wu and Outten, 2009, Alves et al., 2010). The *E. faecium* strains were then tested, and the results are shown in figure 5.16B *E. faecium* E1162Δ*esp* and TX1330 a small decrease was observed, but this

was statistically not significant ($p>0.5$ and $p>0.1$ respectively). In contrast, biofilms of *E. faecium* E1162 significantly decrease in the presence of the chelator does show a significant decrease ($p<0.006$). All strains show biofilm restoration when ammonium ferric sulphate is added to the media, to restore iron levels. In fact, *E. faecium* E1162Δ*esp* and TX1330 show slightly better biofilm formation, but this increase is not significantly.

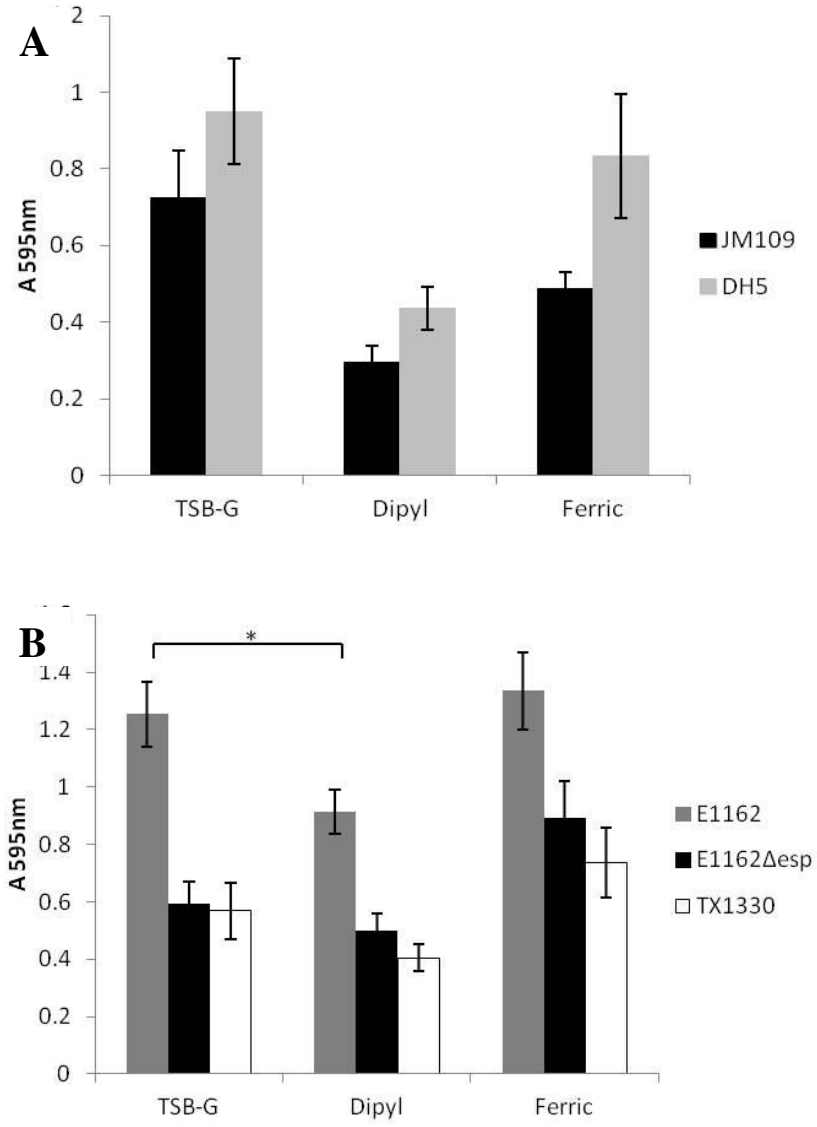



**Figure 5.16. Effect of iron limitation on *E. coli* biofilm formation (A) and *E. faecium* (B). Conditions include: TSB-G, TSB-G containing 0.5mM 2,2-dipyridyl (Dipyl) and TSB-G containing Dipyl and 40mM ammonium ferric sulphate (Ferric). The error bars represent the mean ± the standard error. * P<0.006.**

## *5.18. Discussion*

In the present study we demonstrate that biofilm formation by *E. faecium* is induced by the presence of bile. Induction of biofilm formation by bile has also been found for several other bacteria, such as with the gastrointestinal commensals *Bacteroides fragilis* (Pumbwe et al., 2007) and *Lactobacillus rhamnosus* (Lebeer et al., 2007), and the pathogens *Salmonella typhimurium* (Prouty et al., 2002) and *Listeria monocytogenes* (Begley et al., 2009). It was interesting to note that induction of biofilm formation by *E. faecium* was optimal at the concentrations of bile that are found in the small intestine. This indicates that, similar to other bacteria (Gunn, 2000), *E. faecium* may use bile as an environmental trigger to determine the location in the body and maximise the ability to colonise the gut. It is important to note that this response to bile salts was not due to the detergent nature of bile salts, as neither Triton X100 nor SDS had any effect on biofilm formation. This is reminiscent of findings that the proteome of *E. faecalis* was very dissimilar when grown in bile salts or SDS, showing that this closely related organism responds differently to these detergents (Flahaut et al., 1996).

The range of bile salt concentrations that stimulate biofilm formation was narrower for *E. faecium* E1162Δ*esp* than for its parental strain, suggesting that the presence of Esp influences this triggered response. Interestingly, biofilm formation by *E. faecium* E1162 was induced by sodium taurocholate but not by glycocholate, which was also observed in *Lactococcus lactis* (Zaidi et al., 2011). However, both taurocholate and glycocholate were required to induce biofilm formation to a level similar to that achieved with crude bile salts, indicating that the two salts act synergistically. Strikingly, neither taurocholate/glycocholate, nor a mixture of the two, induced biofilm formation in strains lacking Esp (*E. faecium* E1162Δ*esp* and TX1330). This indicates firstly that other components than tauro- or glycocholate in the Ox bile extract stimulates biofilm formation in these strains. Secondly, it suggests that Esp plays a role, either directly or indirectly, in the response to these salts. This role is at present not clear and requires further detailed analysis.

Our studies excluded a number of potential explanations as to why physiological concentrations of bile salts stimulate biofilm formation in *E. faecium*. The rate of growth is unlikely to play a role as we did not observe a significant difference in the doubling time when comparing 0.5% and 5% bile salts, whereas the difference in biofilm formation at these concentrations was very large. Cells lacking Esp are clearly less hydrophobic, an observation that was also made in *E. faecalis* (Tendolkar et al., 2004), as well as being less efficient in forming biofilms. It could be speculated that bile salts, due to their detergent nature, would increase the hydrophobicity of cells which, in turn, would increase biofilm formation; however, bile salts and its individual components had no or only a minor effect on hydrophobicity; with bile salts there was actually a reduction in hydrophobicity, albeit insignificantly. It can thus be concluded that, despite their detergent nature, bile salts do not stimulate biofilm formation through a change in hydrophobicity of *E. faecium* cells.

Tests were performed to establish whether there were other changes in *E. faecium* caused by the presence of bile salts. For instance, secretory proteins produced by *E. faecium* E1162 and E1162Δ*esp* were analysed using SDS-PAGE, but no obvious differences were observed when the strains were grown with or without bile salts (data not shown). Note that this was only tested for planktonic cells and not for biofilm cells, and it cannot be excluded that some proteins are only secreted while growing in biofilms.

Another reason for induction of biofilm formation might be through morphological changes caused by bile salts. Such changes induced by bile have been observed with *L. monocytogenes*, and it was suggested that this change increased initial attachment, which in turn enhanced biofilm formation (Begley et al., 2009). Better attachment was indeed observed of *E. faecium* in the presence of bile salts, which was most notable after 6 hours of growth, a stage at which microcolonies could already be observed. There was also an increase in chain length in the presence of bile salts that may have contributed to increased attachment or formation of microcolonies which, in turn, stimulated biofilm formation. Notably, for reasons unknown bile salts led to

longer chains in *E. faecium* E1162Δ*esp* compared to its parental strain or *E. faecium* TX1330, but this did not result in a significantly larger increase in biofilm formation. Thus, chain length is unlikely to be the only contributing factor to the induction of biofilm formation by bile salts, and our future studies will be aimed at analysing this process in more detail.

What was clearly observed was that in the presence of bile salts more EPS was produced. A similar observation was made in *L. lactis*, biofilms of which produced more EPS in the presence of cholate (Zaidi et al., 2011). This observation links in well with the increase attachment observed at 6 hours, a stage at which microcolonies start to form and EPS production increases (Hall-Stoodley and Stoodley, 2002), in turn EPS then provides key components for maturation of the biofilm (Flemming and Wingender, 2010). Therefore, the most likely explanation for our observations is that bile salts induce the production of EPS and that that, in turn, stimulates biofilm formation. How bile salts stimulate EPS production will be investigated by us in more detail in future studies.

Other environmental stresses were also studied to establish their effect on biofilm formation. Enterococci have been shown to grow in high alkaline conditions (Flahaut et al., 1997), but observed here was that a media change to around pH 8 significantly decreased biofilm formation, both in the presence and absence of bile salts, this decrease was not due to reduced growth (data not shown), while at pH 6 *E. faecium* formed similar biofilms as at pH 7. An increase in temperature to 42$^{o}$C showed no effect on biofilm formation in any of the *E. faecium* strains (data not shown), but in *E. faecalis* it increases collagen attachment, which was also observed for alkaline conditions (Kayaoglu et al., 2005). Iron limitation tests were performed on biofilms by adding an iron chelator. *E. faecium* E1162 was the only strain to show a significant decrease in biofilm formation, therefore suggesting that in enterococci iron limitation is not a biofilm formation trigger. Iron limitation in other bacteria have shown to decrease biofilm formation in *E. coli* (Alves et al., 2010), and increase biofilm formation in *S. aureus* (Johnson et al., 2005).

It is important to note that when producing a mutant such as *E. faecium* E1162Δ*esp,* there maybe pleiotropic effects on downstream genes. For instance, it was suggested that the muramidase gene in the *E. faecium* E1162Δ*esp* was upregulated due to the insertion of the chloramphenicol gene into the Esp gene (J. Top, personal communication). A study by Waters *et al* suggests that muramidase has a role in dechaining of cells in *E. faecalis* (Waters et al., 2003). Thus, one would expect an up-regulation of this gene leading to shorter chains of *E. faecium* E1162Δ*esp*, whereas the opposite is seen here. It is still possible that other unknown effects may have occurred due to making the deletion, but it is unlikely that this muramidase up regulation has a role in the increased chain length as observed for *E. faecium* E1162Δ*esp* here. There are thus still unanswered questions on the role of Esp, and the next logical step is to look at Esp expression under different environmental conditions, which leads us on to Chapter 6.

# Chapter 6: Results Section IV

# The effect of bile salts on Esp production in *E. faecium*.

## *6.1. Background and objectives*

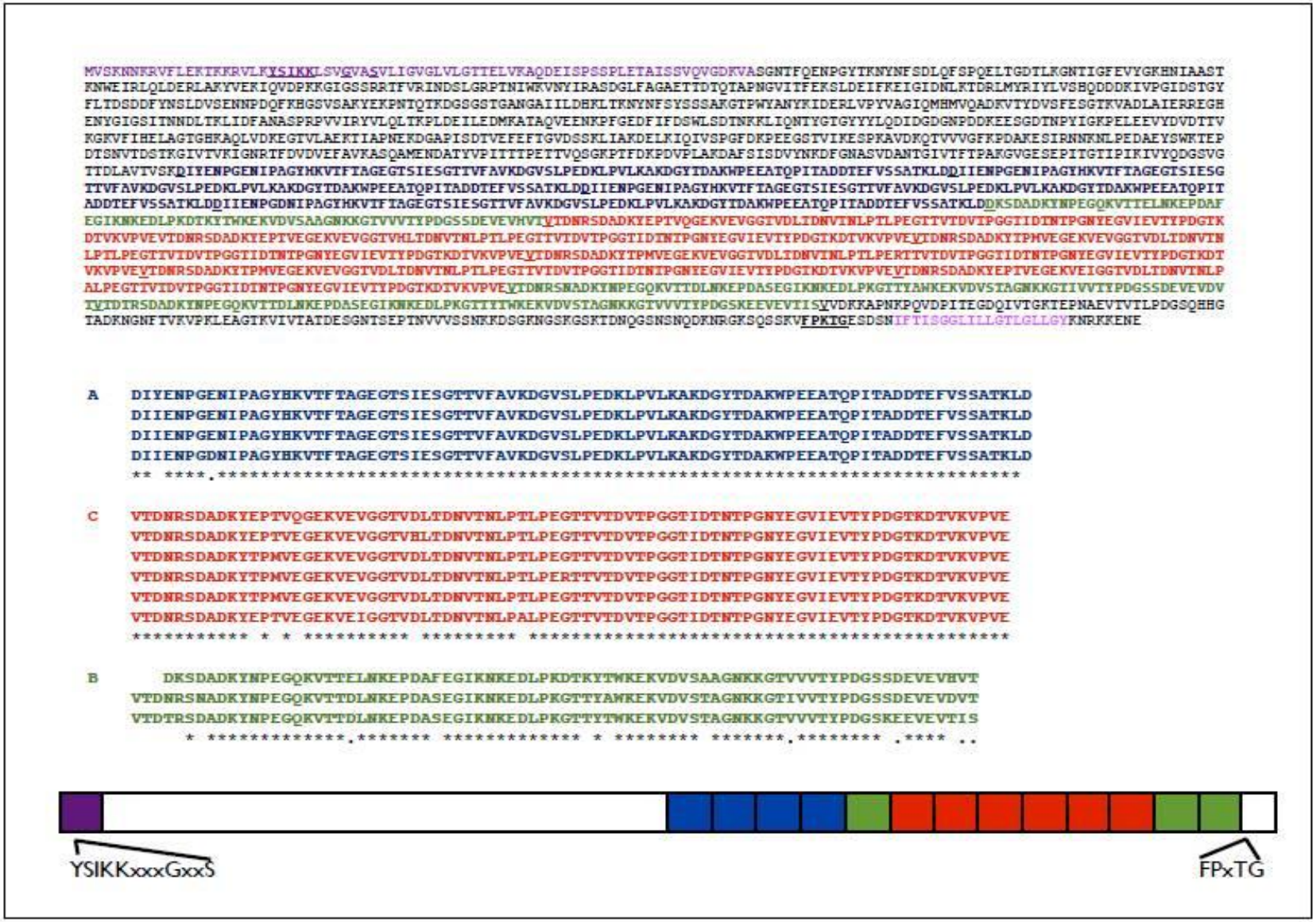
It has been demonstrated that biofilm formation by *E. faecium* E1162 is depends on the presence of Esp. Also, as shown in previous chapters, it was observed that *E. faecium* E1162 and *E. faecium* E1162Δ*esp* respond differently to the presence of bile. For these reasons it was decided to analyse the expression of the *esp* gene under various growth conditions, including the presence of bile salts.

As previously stated in the introduction, Esp is an approximately 202 kDa large cell wall protein found in both *E. faecium* and *E. faecalis* strains. The proteins in both organisms are very similar and have a sequence identity of around 90% (Heikens et al., 2007). The protein contains some key features shown in figure 6.1, which include a signal peptide, an N-terminal domain, A, B & C repeats, a cell wall anchor motif (FPKTGE). The repeat units mentioned (areas of repeat amino acids) have been separated and aligned, and it can be observed that the repeats are highly conserved (Figure 6.1).

The signal peptide contains a motif that is similar to the so-called YSIRK signal peptides, which are found in many surface proteins of staphylococci, streptococci and related organisms. Proteins containing this type of signal peptide have been shown in *S. aureus* to be distributed along the peptidoglycan cross wall that is formed when cells are dividing, while proteins without the YSIRK motif are predominately found at the poles (DeDent et al., 2008). It suggests that the motif has a role in trafficking the surface proteins to the correct destination on the cell wall. It has been shown that it is required for efficient secretion (Bae and Schneewind, 2003), switching signal peptides i.e. YSIRK present and absent signal peptides did cause the *S. aureus* surface proteins to switch locations, but that mutations in the motif did not cause the surface proteins to reach an incorrect location, they were still attached to the cell wall in the required destination (DeDent et al., 2008).

Previous studies on *E. faecalis* Esp had shown similarity with *S. aureus* Bap protein (Biofilm associated protein), the N-terminal domain of which showed a 33% identity with the N-terminal domain of Esp, whereas the C repeats of these proteins also have a similar level of identity (Toledo-Arana et al., 2001). As of yet Bap has only been isolated from bovine mastitis isolates and when deleted there is a decrease in biofilm formation (Valle et al., 2012). There are also similarities in the C repeat region of Esp to Rib and C alpha proteins in group B streptococci (GBS), although Esp also has additional repeats and an N-terminal region which is unrelated (Shankar et al., 1999). C alpha protein and Rib in GBS are surface-expressed antigens, which may confer resistance against the antibody-mediated immunity (Madoff et al., 1996, Wastfelt et al., 1996). Studies on Esp have also shown that the number of repeat units varies between isolates as a result of homologous recombination, but a complete loss of the repeat units have not been seen, suggesting that they may have an important role in stability (Shankar et al., 1999). Variation in the number of A and C repeats in Esp have shown no effect on the isolates ability to form biofilms (Toledo-Arana et al., 2001). This shuffling of repeat units has been suggested as a possible immune evasion technique which has been observed in C alpha proteins (Madoff et al., 1991, Madoff et al., 1996).

Tendolakar *et al* showed by making mutant forms of Esp lacking various domains and expressing them in a Esp negative isolate that the N-terminal domain was sufficient to mediate biofilm formation in *E. faecalis* (Tendolkar et al., 2005). Esp also consists of a cell wall-anchoring sequence, which for Gram-positive bacteria is usually LPXTGX (Schneewind et al., 1993), here *E. faecium* has the sequence FPKTGE, therefore the leucine in position 1 has been replaced with phenylalanine. The sortase enzyme cleaves between the threonine and glycine residues in this motif and the protein is then covalently immobilized to peptidoglycan in the cell wall (Hendrickx et al., 2009b). In spite of the rare residue change Esp is found on the surface of the cell wall, which was confirmed experimentally (Shankar et al., 1999, Heikens et al., 2007).


MVSKNNKRVFLEKTKRVLKYSIKKLSVGVASVLIGVGLVLGTTELVKAQDEISPSSPLETAISSVQVGDKVASGNTFQENPGYTKNYNFSDLQFSPQELTGDTLKGNTIGFEVYGKHNIAAST
KNWEIRLQLDERLAKYVEKIQVDPKKGIGSSRRTFVRINDSLGRPTNIWKVNYIRASDGLFAGAETTDTQTAPNGVITFEKSLDEIFKEIGIDNLKTDRLMYRIYLVSHQDDDKIVPGIDSTGY
FLTDSDDFYNSLDVSENNPDQFKHGSVSAKYEKPNTQTKDGSGSTGANGAIILDHKLTKNYNFSYSSSAKGTPWYANYKIDERLVPYVAGIQMHMVQADKVTYDVSFESGTKVADLAIERREGH
ENYGIGSITNNDLTKLIDFANASPRPVVIRYVLQLTKPLDEILEDMKATAQVEENKPFGEDFIFDSWLSDTNKKLIQNTYGTGYYYLQDIDGDGNPDDKEESGDTNPYIGKPELEEVYDVDTTV
KGKVFIHELAGTGHKAQLVDKEGTVLAEKTIAPNEKDGAPISDTVEFEFTGVDSSKLIAKDELKIQIVSPGFDKPEEGSTVIKESPKAVDKQTVVVGFKPDAKESIRNNKNLPEDAEYSWKTEP
DTSNVTDSTKGIVTVKIGNRTFDVDVEFAVKASQAMENDATYVPITTTPETTVQSGKPTFDKPDVPLAKDAFSISDVYNKDFGNASVDANTGIVTFTPAKGVGESEPITGTIPIKIVYQDGSVG
TTDLAVTVSKQIYENPGENIPAGYHKVTFTAGEGTSIESGTTVFAVKDGVSLPEDKLPVLKAKDGYIDAKWPEEATQPITADDTEFVSSATKLDQIIENPGENIPAGYHKVTFTAGEGTSIESG
TTVFAVKDGVSLPEDKLPVLKAKDGYTDAKWPEEATQPITADDTEFVSSATKLDQIIENPGENIPAGYHKVTFTAGEGTSIESGTTVFAVKDGVSLPEDKLPVLKAKDGYTDAKWPEEATQPIT
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EGIKNKEDLPKDTKYTWKEKVDVSAAGNKKGTVVVTYPDGSSDEVEVHVTVTDNRSDADKYEPTVQGEKVEVGGIVDLIDNVINLPTLPEGTTVTDVIPGGIIDINIPGNYEGVIEVTYPDGIK
DTVKVPVEVTDNRSDADKYEPTVEGEKVEVGGTVHLTDNVTNLPTLPEGITVIDVTPGGTIDINIPGNYEGVIEVIYPDGIKDTVKVPVEVTDNRSDADKYIPMVEGEKVEVGGTVDLIDNVIN
LPTLPEGTIVIDVTPGGTIDINTPGNYEGVIEVIYPDGTKDTVKVPVEVTDNRSDADKYTPMVEGEKVEVGGIVDLIDNVINLPTLPERTTVTDVIPGGIIDTNIPGNYEGVIEVTYPDGTKDT
VKVPVEVTDNRSDADKYTPMVEGEKVEVGGTVDLTDNVTNLPTLPEGTIVTDVTPGGTIDINIPGNIEGVIEVTYPDGIKDTVKVPVEVTDNRSDADKYEPTVEGEKVEIGGTVDLTDNVINLP
ALPEGTTVIDVIPGGTIDINTPGNYEGVIEVTYPDGTKDTVKVPVEVTDNRSNADKYNPEGQKVTTDLNKEPDASEGIKNKEDLPKGTTYAWKEKVDVSTAGNKKGTIVVTYPDGSSDEVEVDV
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TADKNGNFTVKVPKLEAGTKVIVTATDESGNTSEPTNVVVSSNKKDSGKNGSKGSKIDNQGSNSNQDKNRGKSQSSKVFPKIGESDSNIFTISGGLILLGTLGLLGYKNRKKENE

A DIYENPGENIPAGYHKVTFTAGEGTSIESGTTVFAVKDGVSLPEDKLPVLKAKDGYTDAKWPEEATQPITADDTEFVSSATKLD
DIIENPGENIPAGYHKVTFTAGEGTSIESGTTVFAVKDGVSLPEDKLPVLKAKDGYTDAKWPEEATQPITADDTEFVSSATKLD
DIIENPGENIPAGYHKVTFTAGEGTSIESGTTVFAVKDGVSLPEDKLPVLKAKDGYTDAKWPEEATQPITADDTEFVSSATKLD
DIIENPGDNIPAGYHKVTFTAGEGTSIESGTTVFAVKDGVSLPEDKLPVLKAKDGYTDAKWPEEATQPITADDTEFVSSATKLD
** ****.*********************************************************************************

C VTDNRSDADKYEPTVQGEKVEVGGTVDLTDNVTNLPTLPEGTTVTDVTPGGTIDTNTPGNYEGVIEVTYPDGTKDTVKVPVE
VTDNRSDADKYEPTVEGEKVEVGGTVHLTDNVTNLPTLPEGTTVTDVTPGGTIDTNTPGNYEGVIEVTYPDGTKDTVKVPVE
VTDNRSDADKYTPMVEGEKVEVGGTVDLTDNVTNLPTLPEGTTVTDVTPGGTIDTNTPGNYEGVIEVTYPDGTKDTVKVPVE
VTDNRSDADKYTPMVEGEKVEVGGTVDLTDNVTNLPTLPERTTVTDVTPGGTIDTNTPGNYEGVIEVTYPDGTKDTVKVPVE
VTDNRSDADKYTPMVEGEKVEVGGTVDLTDNVTNLPTLPEGTTVTDVTPGGTIDTNTPGNYEGVIEVTYPDGTKDTVKVPVE
VTDNRSDADKYEPTVEGEKVEIGGTVDLTDNVTNLPALPEGTTVTDVTPGGTIDTNTPGNYEGVIEVTYPDGTKDTVKVPVE
********** * * ********** ********* ***********************************************

B DKSDADKYNPEGQKVTTELNKEPDAFEGIKNKEDLPKDTKYTWKEKVDVSAAGNKKGTVVVTYPDGSSDEVEVHVT
VTDNRSNADKYNPEGQKVTTDLNKEPDASEGIKNKEDLPKGTTYAWKEKVDVSTAGNKKGTIVVTYPDGSSDEVEVDVT
VTDTRSDADKYNPEGQKVTTDLNKEPDASEGIKNKEDLPKGTTYTWKEKVDVSTAGNKKGTVVVTYPDGSKEEVEVTIS
* ************.******* ************* * ******** *******.******** .**** ..

YSIKKxxxGxxS
FPxTG

**Figure 6.1. *E. faecium* E1162 Esp structure. Amino acid sequence for Esp is shown, important sections of the sequence are highlighted and repeat sections have been separated out and alignments have been performed. The signal peptide is represented by purple and also contains the YSIRK motif. A, B and C repeats are represented by blue, red and green, respectively. Cell wall-anchoring sequence is in bold and underlined and the pink amino acids. See text for further details.**

## *6.2. Esp expression analysis using SDS-PAGE*

Firstly a fragment of the N-terminal domain of Esp was expressed and purified using the pN-tEsp vector, which was supplied by Dr W.J. van Wamel (Van Wamel et al., 2007). Purified Esp was used to raise polyclonal antibodies in rabbits as outlined in the Methods and Material chapter.

Various conditions were tested to get an understanding of Esp expression. *E. faecium* E1162 and E1162Δ*esp* were incubated under aerobic or anaerobic conditions in either TSA-G-B or TSB-G-B containing various concentrations of bile salts. Unfortunately, it was difficult to find the right conditions for western blotting. A number of conditions (transfer buffers and blotting time) were tested, but results were not consistent; this was likely to be due to the large size of the protein, which makes transfer to a membrane inefficient. Nevertheless, the protein was detectable in *E. faecium* E1162 (Figure 6.2), while it was absent in E1162Δ*esp* (compare lanes B and F). In the presence of bile, the amount of Esp appears reduced, although this is less obvious with 5% bile. In anaerobic conditions there were clearly increased levels of Esp, corroborating earlier findings (Van Wamel et al., 2007). Due to the poor quality of the western blots it was decided to analysis Esp expression using flow cytometry.

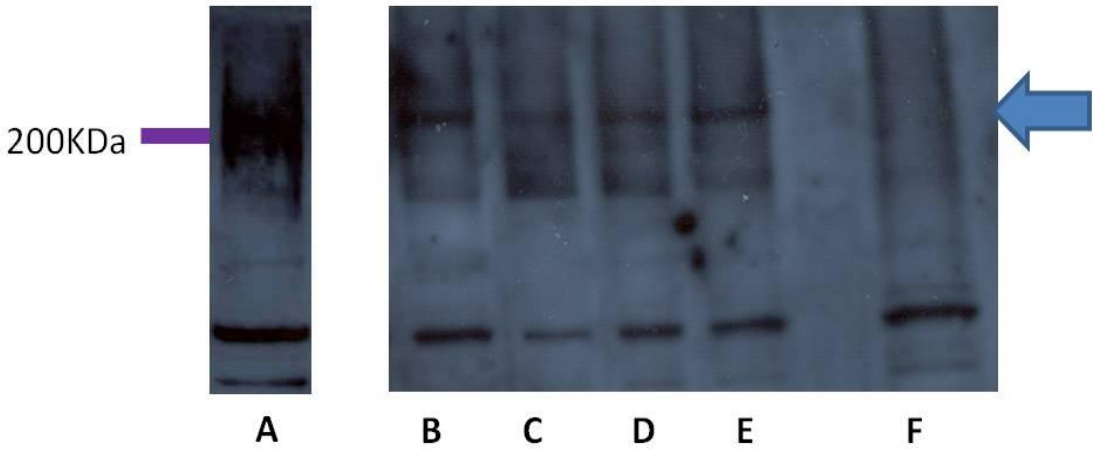


**Figure 6.2. Western blot analysis of Esp expression in TSB-G-B: *E. faecium* E1162 under anaerobic (A) aerobic (B) containing no bile salts and aerobically containing 0.5% (C), 1.5% (D) and 5% (E) bile salts. As a negative control *E. faecium* E1162Δ*esp* under aerobic conditions with no bile salts (F).**

## *6.3. Esp production analysis using flow cytometry.*

To test flow cytometry for the cell surface expression of Esp, both exponential growing cells and stationary phase cells were examined in the absence of bile salts. Interestingly, two peaks were observed in these cells; one peak with low, and one peak with high fluorescence intensity. This suggests that only part of the population produces the Esp protein on the cell surface. Strikingly, the percentage of cells producing Esp was considerable higher, at 40.4%, when in stationary phase (18 hours of growth; Figure 6.3B), while this was only 21.8% for exponentially growing cells (4 hours of growth; Figure 6.3A). This indicates that Esp is mostly produced in the stationary phase of growth. In 24-hour biofilm cells, 83.4% of cells produced Esp (Figure 6.3C), showing that in biofilms there is a further increase in Esp production. Note that in planktonic cells harvested from the wells after biofilm growth the production of Esp was significantly lower than in the biofilm cells (33.6%; Figure 6.5A).

We then tested the effect of bile salts on Esp production, in both stationary phase and biofilm cells. Interestingly, in the presence of 0.5% bile salts the percentage of cells producing Esp was significantly lower in both conditions: 23.9% for stationary grown cells (40.4% without bile, see above), and 31.7% Esp producing cells in biofilm grown cells (83.4% without bile; Figure 6.4). Thus, induction of biofilm formation by bile salts is not due to an increase in production of Esp. Planktonic cells that were not attached to the surface were also tested in the presence of 0.5% bile salts (Figure 6.4B); these cells show similar amounts of Esp production as seen when no bile was present in the growth media, approximately 34%.

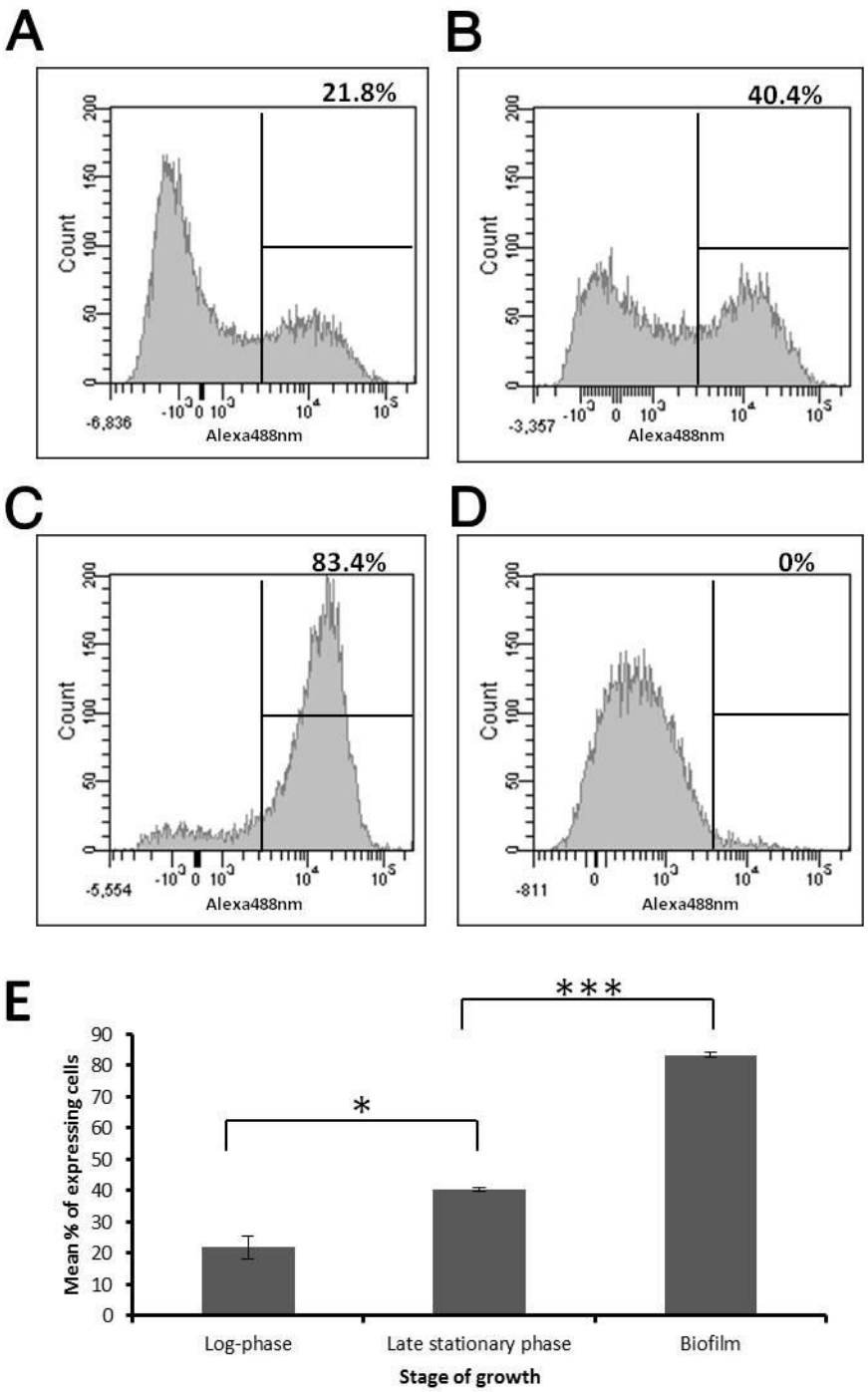


**Figure 6.3. Flow cytometry histograms, showing cell surface expression of *E. faecium* E1162 cells in the exponential growth phase (A), stationary growth phase (B) and biofilm cells (C). *E. faecium* E1162Δ*esp* grown under stationary phase growth was used as a negative control (D). Percentages of cells expressing Esp is indicated in each panel and in E. The error bars represent the mean ±the standard error. *** $p<0.0001$ and * $p<0.01$.**

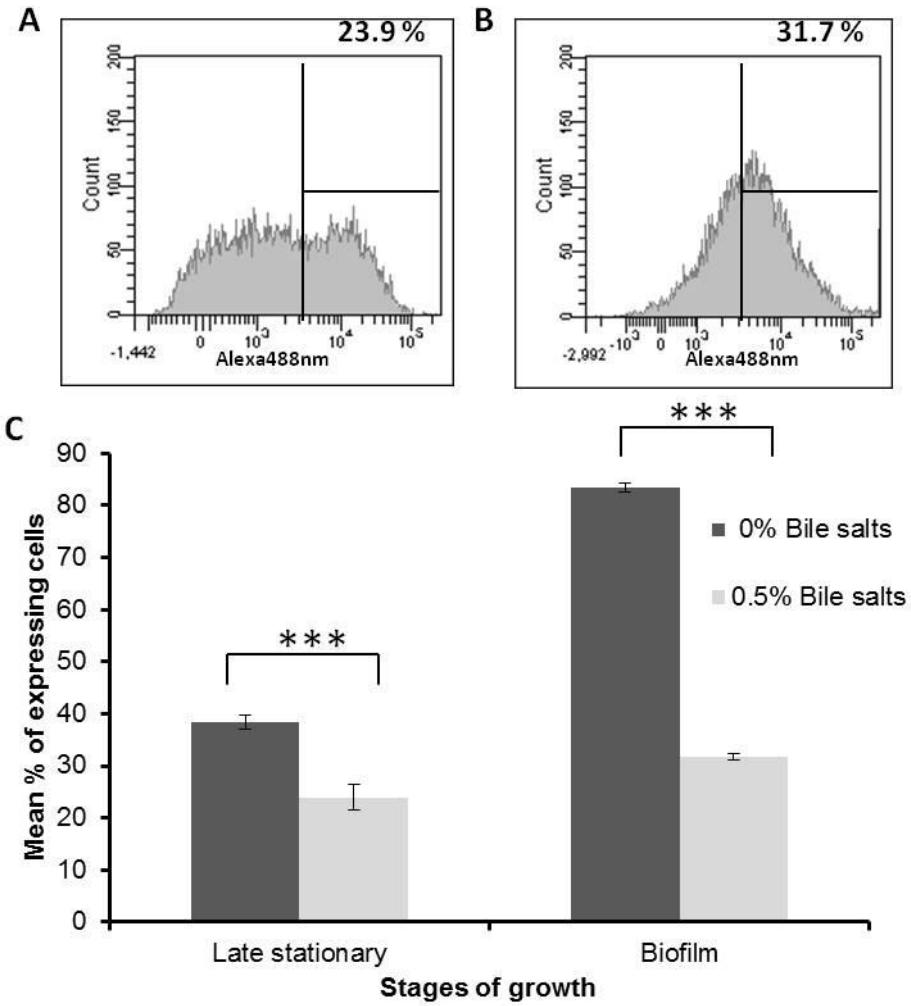


**Figure 6.4 Representative flow cytometry histograms, showing cell surface expression of Esp in *E. faecium* E1162 cells, grown in the presence of 0.5% bile salts, in the stationary phase (A) in biofilm cells (B). Percentages of cells expressing Esp is indicated in each panel and in C. The error bars represent the mean ± the standard error. *** $p<0.0001$.**

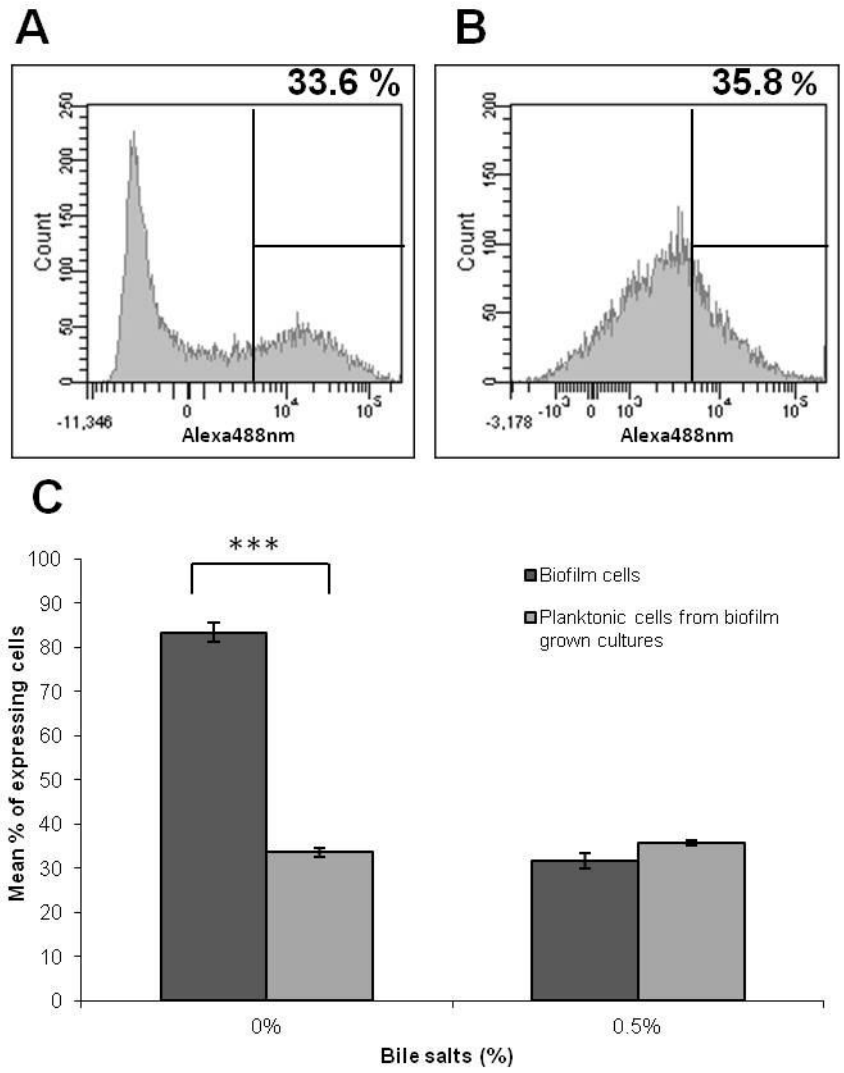


**Figure 6.5. Representative flow cytometry histograms, showing cell surface expression of Esp in *E. faecium* E1162 planktonic cells from biofilm grown cultures, in the absence of bile salts (A) and in presence of 0.5% bile salts (B). Percentages of cells expressing Esp is indicated in each panel and in C. The error bars represent the mean ±the standard error. *** $p<0.0001$.**

## *6.4. Discussion*

Flow cytometry experiments did reveal the interesting observation that in *E. faecium* E1162 cultures there are two populations of cells, one population producing Esp on the cell surface, and the other without Esp. The ratio between the two populations was clearly dependent on growth conditions: ~20% of cells producing Esp on the cell surface in exponentially growing cultures, while this increased to about 40% in stationary phase and nearly 80% in biofilm cells, reaffirming the clear link of Esp with biofilm formation. One previous study (Van Wamel et al., 2007) observed that Esp production in *E. faecium* was higher when grown at 37$^{o}$C compared to lower temperatures, while it also showed (as confirmed here) that there is an increase in production during anaerobic growth. This suggested that this increase in production of Esp was caused by a switch between environmental reservoirs, going from 21$^{o}$C and an aerobic condition on a hospital surfaces to 37$^{o}$C and an anaerobic conditions once inside the body, which helps *E. faecium* in early infection. Anaerobic conditions have also seen up-regulation in of genes involved in virulence in *E. faecalis* (Day et al., 2003). Glucose concentrations have also been shown to affect biofilm formation in Esp-positive strains of *E. faecalis*, but the actual production of Esp was not significantly different (Tendolkar et al., 2004).

It is at present not clear how bile salts induce biofilm formation in *E. faecium,* in the previous chapter (chapter 5) there was an increase in EPS production and initial attachment in the presence of bile salts. As shown here, bile salts reduced the cell surface expression of Esp in stationary and biofilm grown cells. It is thus clear that stimulation of biofilm formation by bile is not caused by an increase in the production of Esp. Interestingly, the presence of Esp was shown not to have an effect on colonisation of mice intestines (Heikens et al., 2009), and one might speculate that this was because of a reduced expression of Esp in the mice colon.

Research on *S. aureus* Bap protein has shown that the protein promotes adhesion but that it also interferes with host cell entry. The interference with host entry is caused

by Bap interacting with Gp96, a chaperone protein on the host cell surface. This interaction interferes with the fibronectin binding pathway that the bacteria would use to invade the cells (Valle et al., 2012). The authors also tested other Bap homologues to see if Gp96 interact with these: Esp from *E. faecalis* and BapA from *Salmonella enderitidis*, using the pull down and ligand overlay assays, they came up negative (Valle et al., 2012). Nevertheless, showing that *S. aureus* Bap can interact with proteins on the surface of the cell suggests that homologues of this protein, including Esp, may interact with host cell surface proteins. It would thus be interesting to test this. In addition, it needs to be tested whether the presence of bile may lead to, for instance, increased expression of other cell surface proteins in *E. faecium* that in turn leads improved biofilm formation and stimulation of colonisation in the GI tract.

# Chapter 7: Results Section V

# The effect of bile salts on biofilm formation in *E. faecalis*

## *7.1. Background and objectives*

As previously mentioned in Chapter 5, bile salts are toxic and enterococci have to cope with these conditions in order to survive and/or colonise the gut. Here we use isolates of *E. faecalis* that have been isolated form biliary stents and a faecal isolate (*E. faecalis* ATCC19433 (Jones and Shattock, 1960) and analyse their biofilm formation in the presence of bile salts. Biliary stents are used to alleviate obstructions caused by malignant or benign conditions in the biliary tract. Often blockages occur due to the build up of biliary sludge which consists of bacteria, protein and bile salt constituents 3 months after placement (Di Rosa et al., 1999). Bacteria infect this area via the Oddi sphincters (Sung et al., 1992) or via the portal venous system (Sung et al., 1991). The bile concentration in biliary stents is high (9±4%) (Donelli et al., 2007) and, if infected, *E. faecalis* is one of the more prominent bacteria present (Di Rosa et al., 1999, Dowidar et al., 1991).

The isolates used in this chapter, and some of the virulence genes they contain, are listed in Table 7.1.

***Table 7.1. E. faecalis isolate virulence factors[a]***

| *E. faecalis* | *esp* | *agg* | *gelE* |
|---|---|---|---|
| ATCC19433 | + | unknown | + |
| BS12297 | + | + | - |
| BS11297 | + | - | + |
| BS385 | - | - | - |

[a]Data from *E. faecalis* BS12297, BS11297, and BS385 from van Merode, van der Mei *et al*, (2006a, 2006b). In *E. faecalis* ATCC19433 the presence of *esp* confirmed by PCR, and the presence of GelE was confirmed on milk-agar plates (data not shown).

As mentioned previously in the introduction, for bacteria to attach to a surface they must overcome repulsive forces. There have been a number of studies on cell surface heterogeneity in *E. faecalis* and its influence on biofilm formation (van Merode et al., 2006a, van Merode et al., 2006b). When studying bacterial hydrophobicity and cell-surface charge, usually a pure culture is used. Within this culture it is presumed that the cells are the same, but in fact there may be subpopulations that differ in certain factors such as their production of flagella or cell-surface charge, and so calculations of cell-surface properties are usually an average. In these studies microelectrophoresis were used which can analyse cell surface charge for individual cells that is expressed as a zeta potential (charge on the cell surface taking into account ionizable groups exposed on the surface, pH and ionic strength). Using this method cultures of bacteria could be identified as heterogeneous (with many subpopulations, therefore more than one zeta potential) or homogeneous (no subpopulation, one zeta potential). It was found that strains that were heterogeneous were better at adhering to polystyrene and producing biofilms than homogeneous strains, and it was concluded that heterogeneity in cell surface charge gives bacteria an advantage. Interestingly, subculturing one of the subpopulations of a heterogeneous strain showed that this subpopulation alone was less efficient at biofilm formation (van Merode et al., 2006b). The cells from the biofilms were also compared to planktonic cells and it was found that *E. faecalis* strains that were heterogeneous when planktonic became homogeneous when in biofilm formation. (van Merode et al., 2006a)

## *7.2. Biofilm assays for the E. faecalis isolates*

Firstly, biofilm assays were performed under standard conditions for all the *E. faecalis* isolates. It was observed that *E. faecalis* ATCC19433 and BS12297 are the best biofilm formers, followed by *E. faecalis* BS385. *E. faecalis* BS11297 appears to be a poor biofilm former (Figure 7.1A).

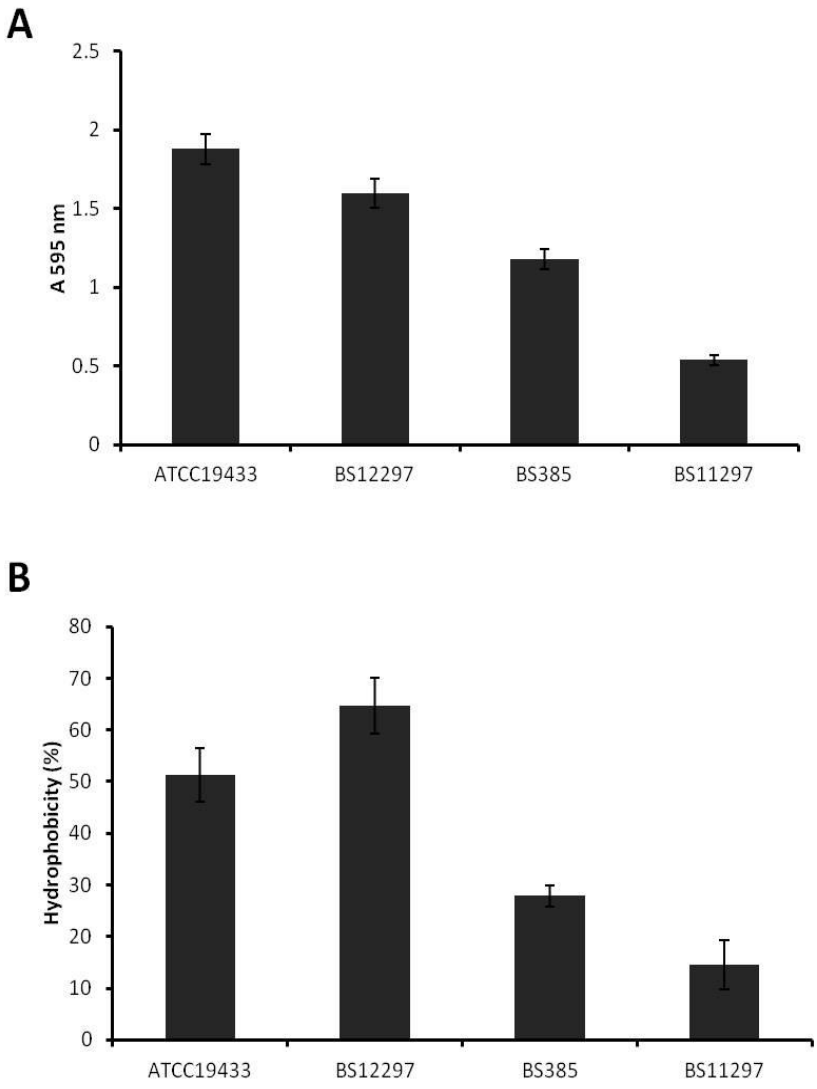


**Figure 7.1. Biofilm assay and hydrophobicity results for the *E. faecalis* isolates. (A) Biofilm formation and (B) Hydrophobicity.**

## *7.3. Hydrophobicity testing for the E. faecalis isolates*

Hydrophobicity tests were also performed (Figure 7.1B) on the *E. faecalis* isolates. As shown before for *E. faecium* (chapter 5), the trend in hydrophobicity followed the trend in biofilm formation, with the strains forming the best biofilms, *E. faecalis* ATCC19433 and BS12297, also having the highest hydrophobicity. These were then followed again by *E. faecalis* BS385 and *E. faecalis* BS11297, the latter of which had the lowest hydrophobicity.

## *7.4. CLSM images of E. faecalis biofilms.*

CLSM imaging was also performed (Figure 7.2) to visualise the structure of the biofilms present. All *E. faecalis* isolates were tested except for the poor biofilm-former *E. faecalis* BS11297. It can be observed that all the isolates produce good biofilm; this is also obvious from their maximum thickness and biomass (Table 7.2). *E. faecalis* BS12297 (Figure 7.2A) has a more uniform biofilm formation across the surface, were as the biofilm structures formed by *E. faecalis* ATTC19433 (Figure 7.2B) and BS385 (Figure 7.2C) appear to be more uneven (Figure 7.2C).

***Table 7.2. Maximum thickness and biomass for E. faecalis isolates ± standard error, defined using COMSTAT2 software (Heydorn et al., 2000).***

| *E. faecalis* | BS12297 | ATCC19433 | BS385 |
|---|---|---|---|
| Maximum thickness | 12.6µm ± 1.9 | 15.2µm± 1.0 | 9.29µm± 1.7 |
| Biomass ($\mu m^3/\mu m^2$) | 2.25 ± 0.5 | 1.98 ± 0.48 | 0.84 ± 0.2 |

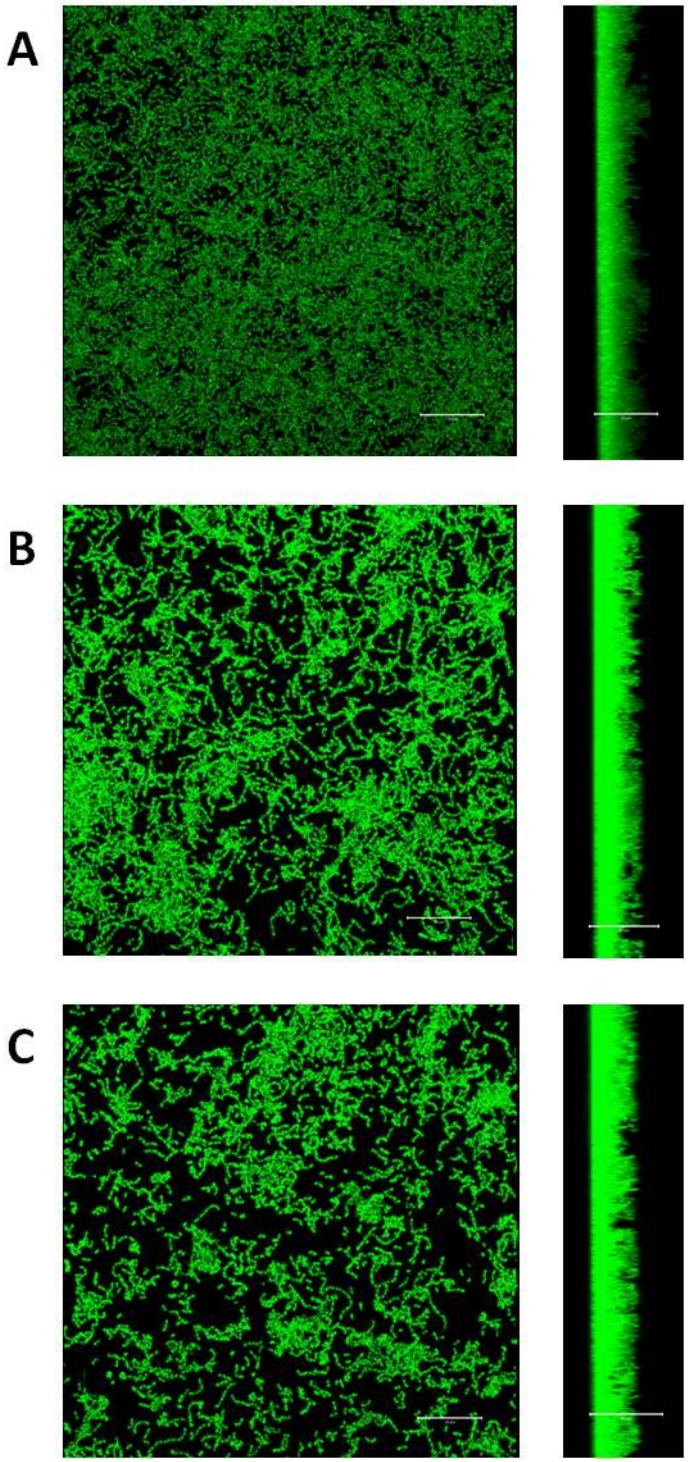


**Figure 7.2. Representative CLSM biofilm formation images and side views of *E. faecalis* isolates: (A) BS12297 (B) ATCC19433 and (C) BS385. Biofilms were grown in TSB-G over 72 hours (with fresh media every 24 hours); cells were stained with Acridine orange. Scale bar represents 20 $\mu$m.**

## *7.5. The effect of bile salts on E. faecalis biofilm formation.*

Biofilm formation was studied from the range of 0-5% bile salts for each of the *E. faecalis* isolates (Figure 7.3). When biofilms were grown in the absence of bile salts it can be observed that there is more biofilm growth after 48 hours growth compared to 24 hours growth (approx. 2 fold). All the isolates except for *E. faecalis* BS11297 showed an increase (approx. 2 fold) in biofilm formation in the presence of bile salts for 24 hour, and maximum biofilm formation is observed at 1.5% bile salts (Figure 7.3A). At 48 hours biofilm formation is similar in the presence or absence of bile salts (Figure 7.3B) for the isolates, *E. faecalis* ATCC19433 48h-biofilms have irregular dips and peaks in biofilm formation over the concentrations of bile salts that were not seen at 24 hours. The main observation is that *E. faecalis* ATCC19433 and BS12297 produced the most biofilm over the range of bile salts concentrations tested for 24 hours They show a consistent reduction in biofilm formation at low concentrations, followed by an increase and this increase is then followed by a reduction at the higher concentrations (3-4%) of bile salts, but this reduction is not as strong as observed with *E. faecium* (Chapter 5).

Interestingly unlike *E. faecium*, the *E. faecalis* strains have increased biofilm formation at concentrations above the usual physiological concentrations found in the small intestine. Bile salt concentrations are likely to be higher in bile stents, as concentration of bile occurs here prior to release into the small intestine and this therefore could explain this occurrence. This is also surprisingly true for *E. faecalis* ATCC19433, a faecal isolate, but interestingly biofilm formation is effected at 48 hours growth, when concentrations of bile salt are above physiological concentrations found in the small intestine.

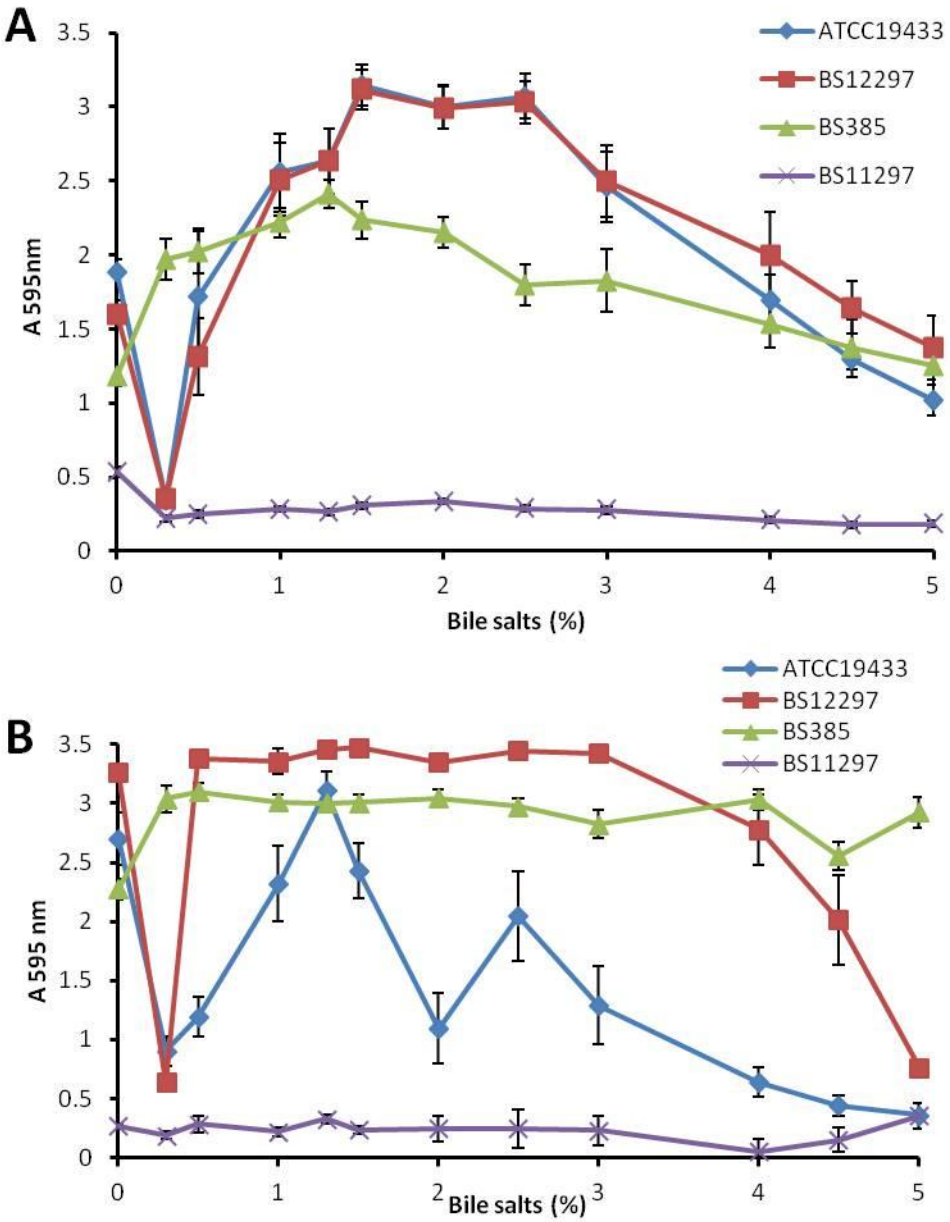


**Figure 7.3. Biofilm formation in the presence of bile salts for 24 hours (A) or 48 hours (B). With the latter fresh media was provided after 24 hours. The error bars represent the mean ± the standard error.**

## *7.6. Biofilm formation in the presence of sodium glycocholate, sodium taurocholate and a 1:1 mix of the two.*

The two main components of bile salts are sodium taurocholate and sodium glycocholate. To investigate whether either of these components is important in the stimulation of biofilm formation, assays were performed in the presence sodium taurocholate, sodium glycocholate and a 1:1 mixture of the two; concentrations tested were between 0-2 percent.

Sodium glycocholate stimulated more biofilm formation in *E. faecalis* isolates ATCC19433, BS12297 and BS385. The first two strains were the best biofilm formers when in the presence of sodium taurocholate also, while *E. faecalis* BS385 remained the same in this condition. *E. faecalis* BS11297 showed no increase in biofilm formation in any of the conditions tested (Figure 7.4 & Table 7.2). The maximum biofilm observed for the other three isolates is at 0.5% sodium glycocholate or sodium taurocholate, with approximately a 2-fold increase compared to biofilm formation in the absence of bile salts (Figure 7.4A and B). When using a 1:1 mix of sodium taurocholate and glycocholate, again *E. faecalis* ATCC19433 and BS12297 showed an increase in biofilm formation, which is in this case was observed at a higher concentration of approximately 1-1.5% (Figure 7.4C). For *E faecalis* BS385 there was no obvious stimulation of biofilm formation in the presence of a mix of sodium glycocholate and taurocholate, suggesting other components in the crude bile salts had stimulated the biofilm formation seen previously.

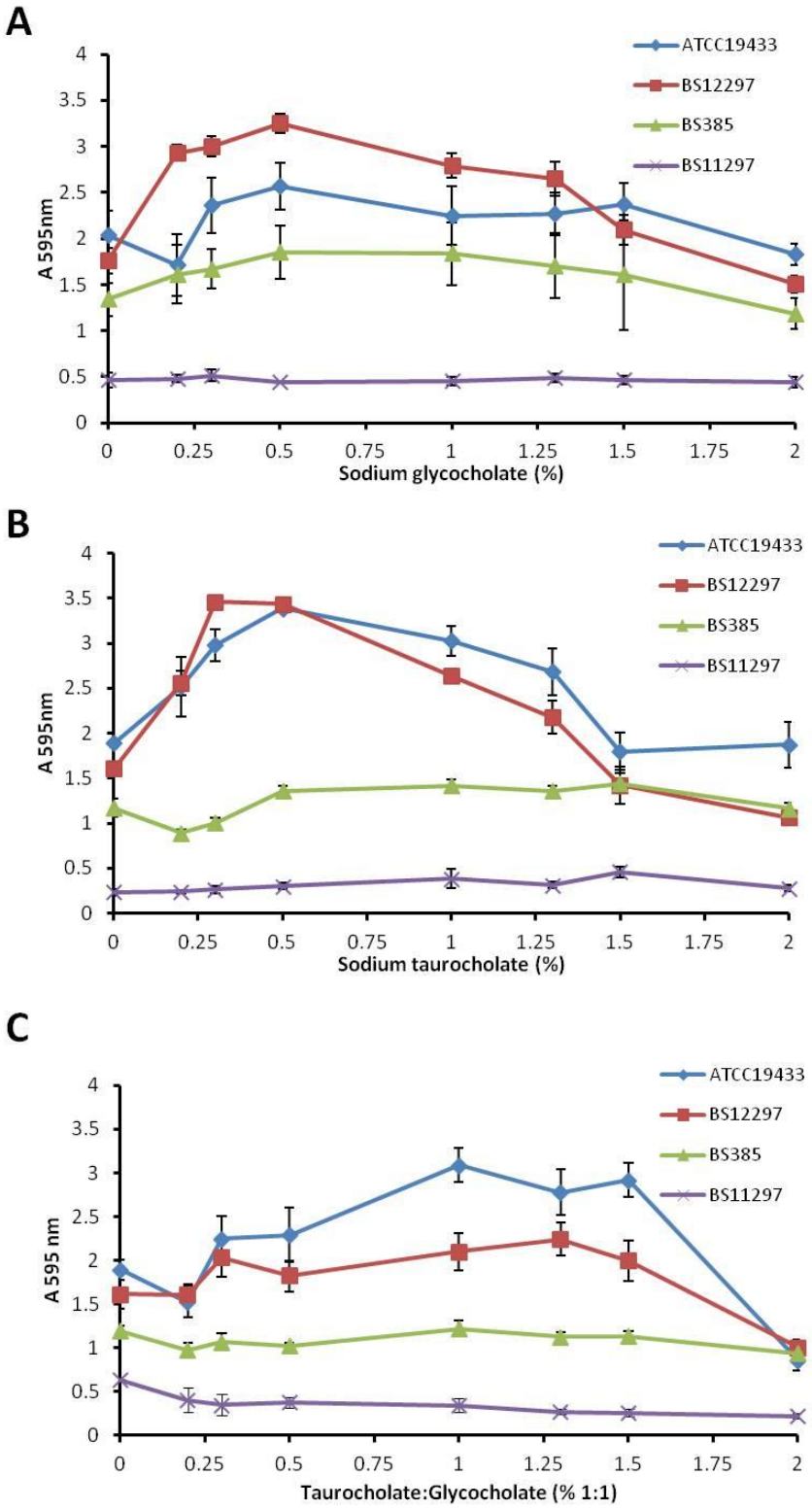


**Figure 7.4. Biofilm assays in the presence of (A) sodium glycocholate, (B) sodium taurocholate, and (C) Sodium taurocholate: Sodium glycocholate (1:1). The error bars represent the mean ± the standard error.**

***Table 7.3. Summary of biofilm formation results from Figure 7.3***

| | Sodium taurocholate | Sodium glycocholate | Sodium glycocholate: sodium taurocholate (1:1) |
|---|---|---|---|
| **ATCC19443** | ↑ | ↑ | ↑ |
| **BS12297** | ↑ | ↑ | ↑ |
| **BS385** | ↔ | ↑ | ↔ |
| **BS11297** | ↔ | ↔ | ↔ |

↑ - increase biofilm formation when the bile component is present at some concentration
↔ - remains unaffected by the presence of the bile component.

## *7.7. Hydrophobicity in the presence of bile salts.*

It was decided to test hydrophobicity when cells were grown in the presence of bile salts (Fig 7.5). In the presence of bile salts, hydrophobicity *E. faecalis* BS12297, BS11292 or BS385 did not change significantly. The only strain that in which the hydrophobicity altered significantly due to bile salts was *E. faecalis* ATCC19433.

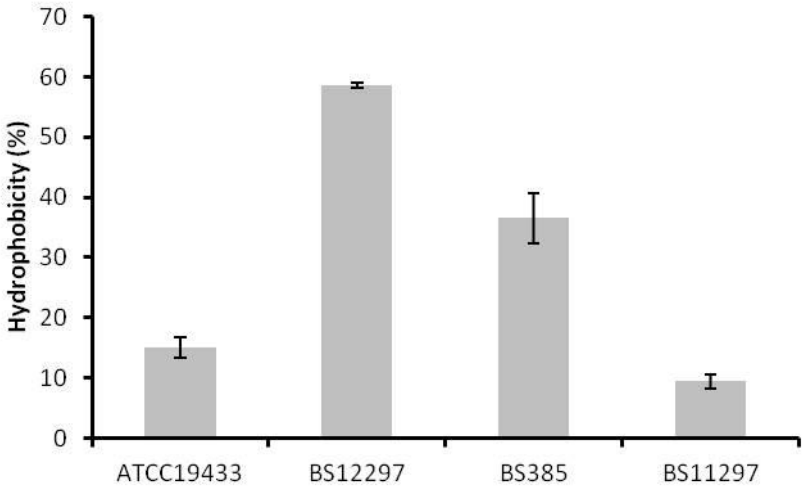


**Figure 7.5. Hydrophobicity in the presence of 1.5% bile salts for the *E. faecalis* isolates.**

## *7.8. Effect of bile salts on initial attachment.*

Initial attachment to a surface is important step in the formation of biofilms, and here we study if bile salts have an effect on this process. Usually, initial attachment is determined during the first four hours, while microcolonies start to form at longer time-periods (~6 hours (Mohamed et al., 2006). *E.faecalis* BS11297 is clearly extremely poor in initial attachment compared to the other *E. faecalis* isolates tested (Figure 7.6D). For the other three isolates, initial attachment after 4 hours is either decreased (ATCC19433 and BS12297) or unchanged (BS385) in the presence of bile salts (Fig 7.6 A-C). In contrast, when measuring the number of attached cells after 6 hours (when microcolonies are formed), an increase is seen for *E. faecalis* ATCC19433 and BS385, while no difference was observed for *E. faecalis* BS12297. In all cases, high concentrations of bile salts (5%) reduce initial attachment of cells.

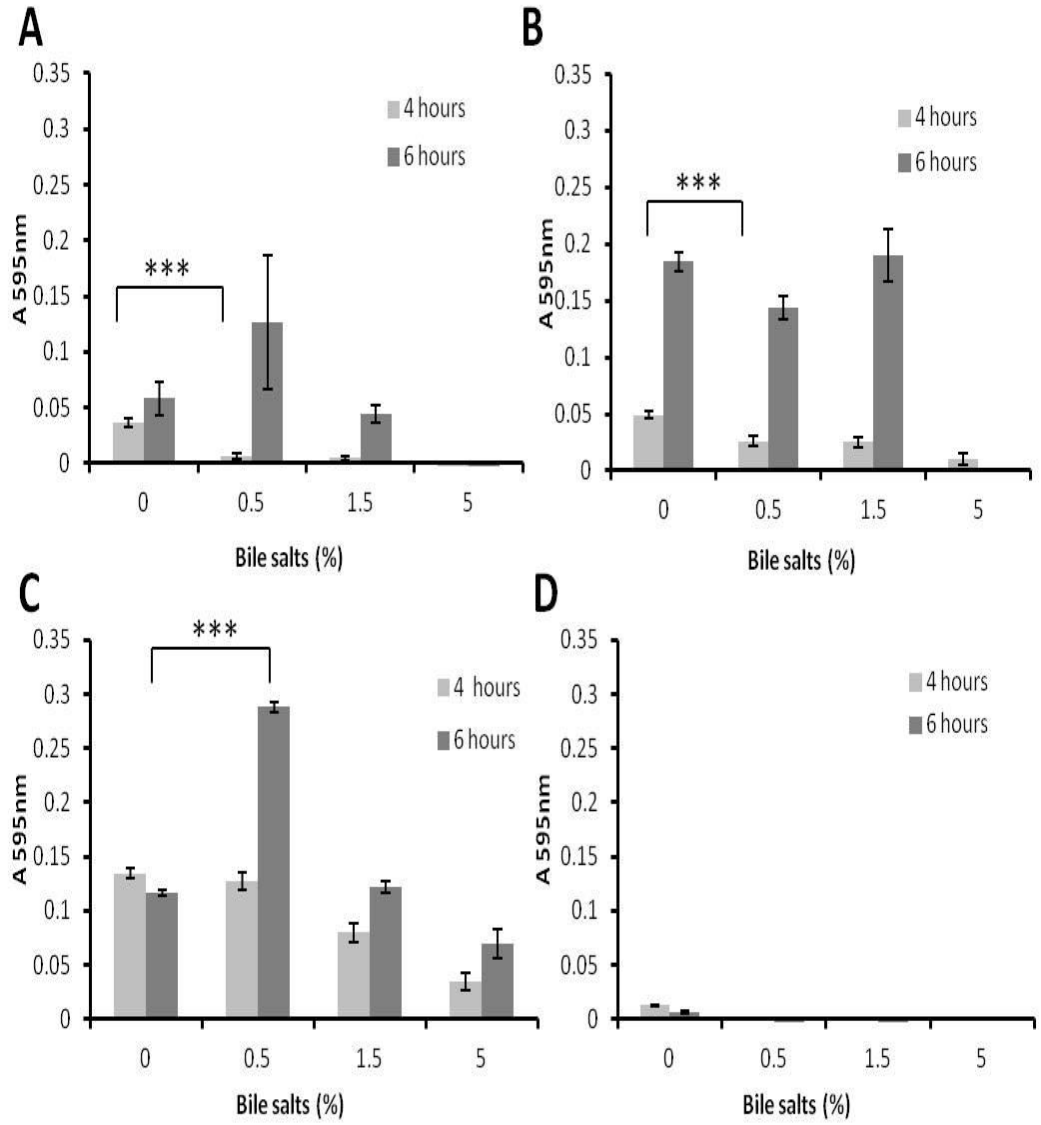


**Figure 7.6. Initial attachment assays in the presence of bile salts for *E. faecalis* isolates (A) ATTC19433, (B) BS12297, (C) BS385 and (D) BS11297. The error bars represent the mean ± the standard error. *** P<0.0001.**

## *7.9. Analysis using flow cytometry of Esp production.*

Esp from *E. faecalis* and *E. faecium* are very similar proteins with approximately 90% sequence identity. We therefore tested whether the antibodies raised against *E. faecium* Esp would also recognise Esp on the cell surface of *E. faecalis* cells. All isolates were grown to log phase and were then tested for Esp production using flow cytometry (Figure 7.7). It can be observed that *E. faecalis* BS12297 and ATCC19733 produce Esp, and indeed both strains also contain the *esp* gene. As expected, *E. faecalis* BS385, which does not contain the *esp* gene, shows no Esp production. Interestingly, BS11297 does not show any production of Esp, while it does carry the gene. However, it should be noted that this strain is a very poor biofilm former and is relatively low in hydrophobicity; this could suggest that the *esp* gene is expressed either at a very low level or not at all in this strain. Very similar results were obtained when the isolates were grown to stationary phase (Figure 7.8).

We also analysed the effect of bile salts on the production of Esp of two of the *E. faecalis* isolate which contain the *esp* gene: BS12297 and BS11297 (which previously showed no Esp production). It was observed in *E. faecalis* BS12297 (Figure 7.9) that in log phase cells, in the presence of bile salts, production of Esp is significantly decrease ($p=0.03$), while in stationary phase cells there appeared to be an increase in Esp production, although this was not significant ($p=0.15$). Interestingly, a statistically significant increase in Esp production was observed in the presence of bile when comparing stationary phase to exponential phase cells, a difference not observed in the absence of bile salts (Figure 7.9). *E. faecalis* BS11297 shows no differences in Esp production when bile is present in either growth phases (data not shown).

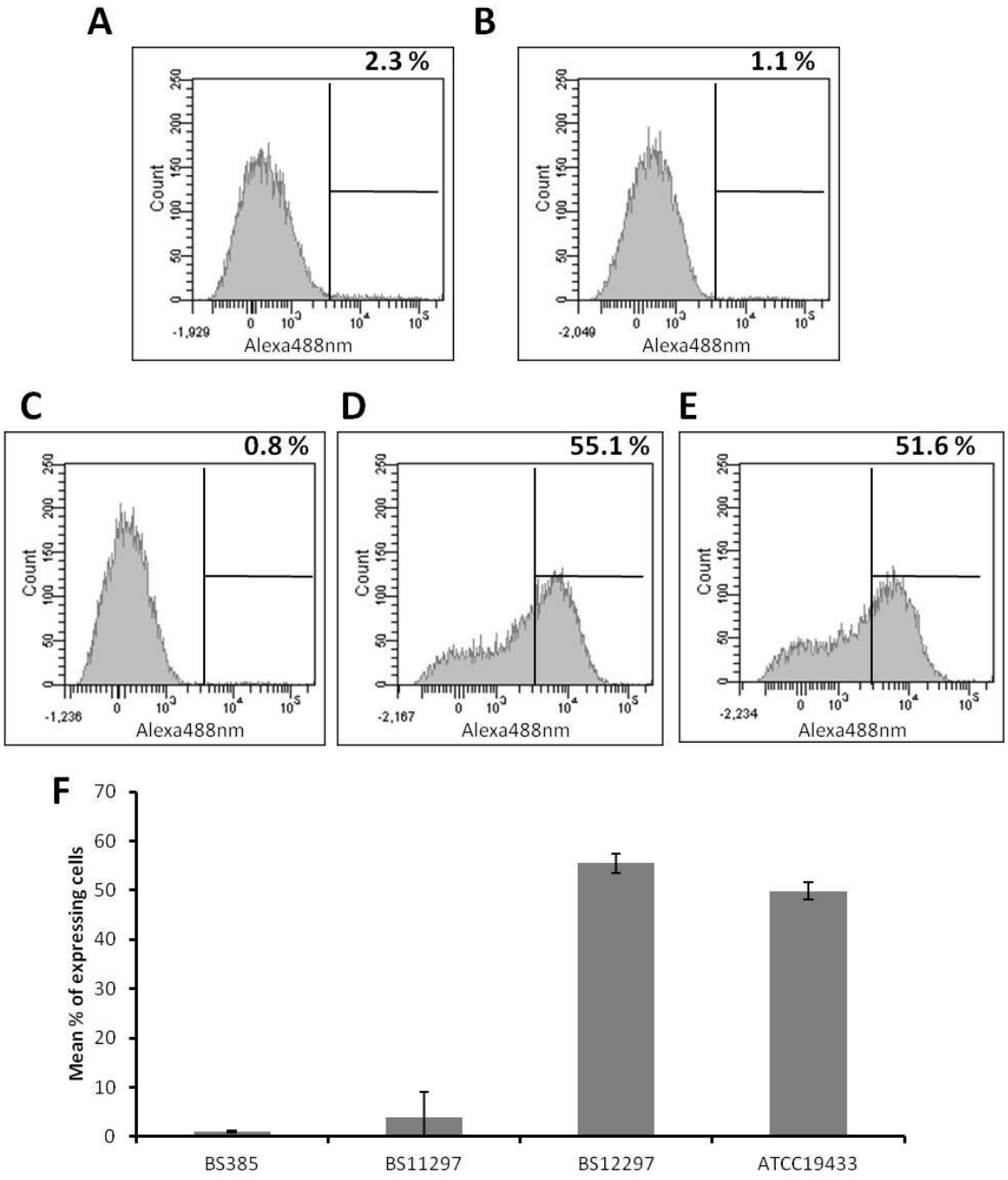


**Figure 7.7. Flow cytometry histograms, showing cell surface expression of *E. faecalis* isolates. Representative histograms are shown for: (A) negative control (pre-immune sera tested on the isolates), (B) BS385, (C) BS11297, (D) BS12297 and (E) ATCC19433. All the isolates were grown to log phase. Percentages of cells expressing Esp is indicated in F. The error bars represent the mean ±the standard error.**

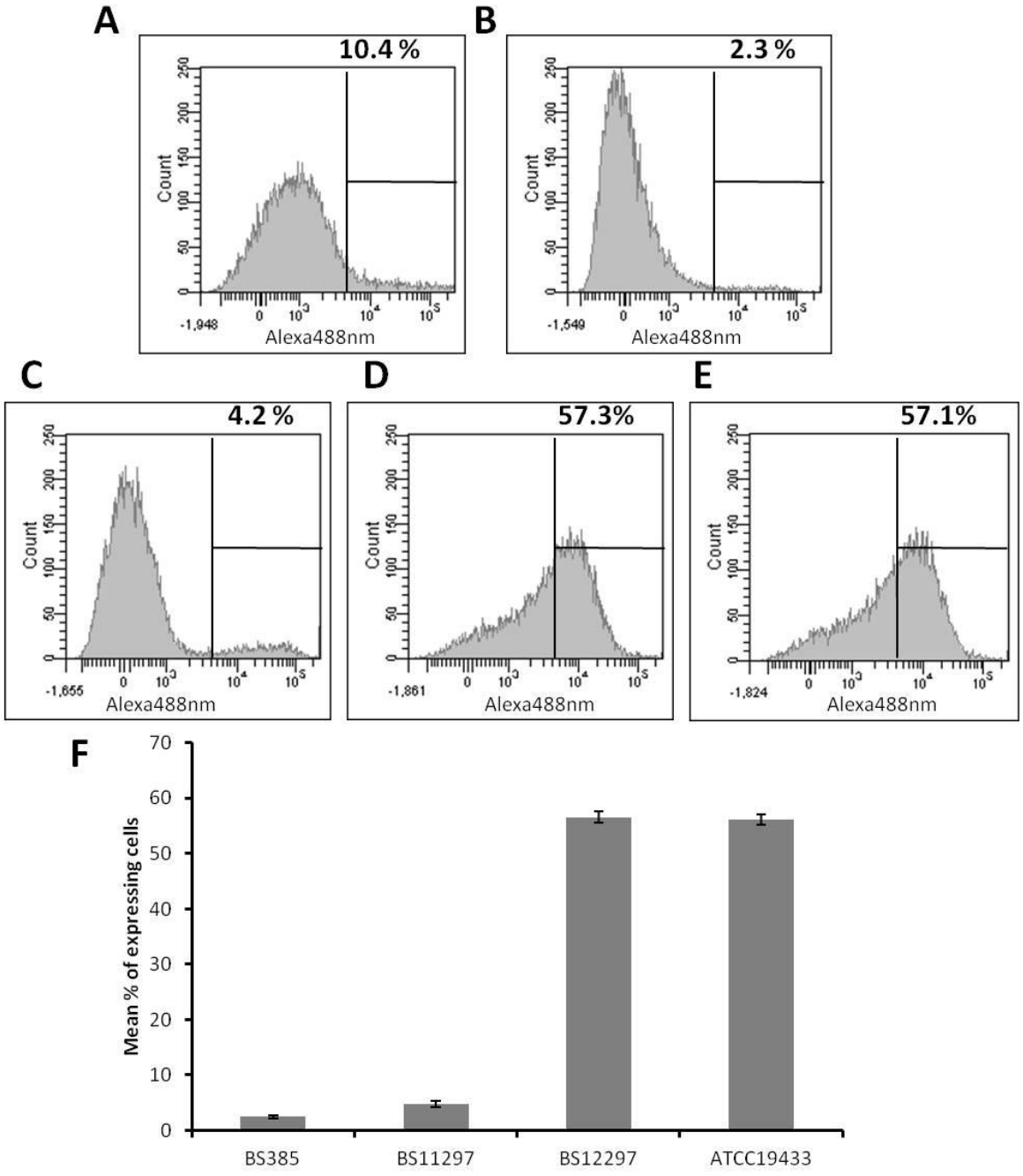


**Figure 7.8. Flow cytometry histograms, showing cell surface expression of *E. faecalis* isolates. Representative histograms for (A) negative control (pre-immune sera tested on the isolates), (B) BS385, (C) BS11297, (D) BS12297 and (E) ATCC19433. All the isolates were grown to stationary phase. Percentages of cells expressing Esp is indicated in F. The error bars represent the mean ±the standard error.**

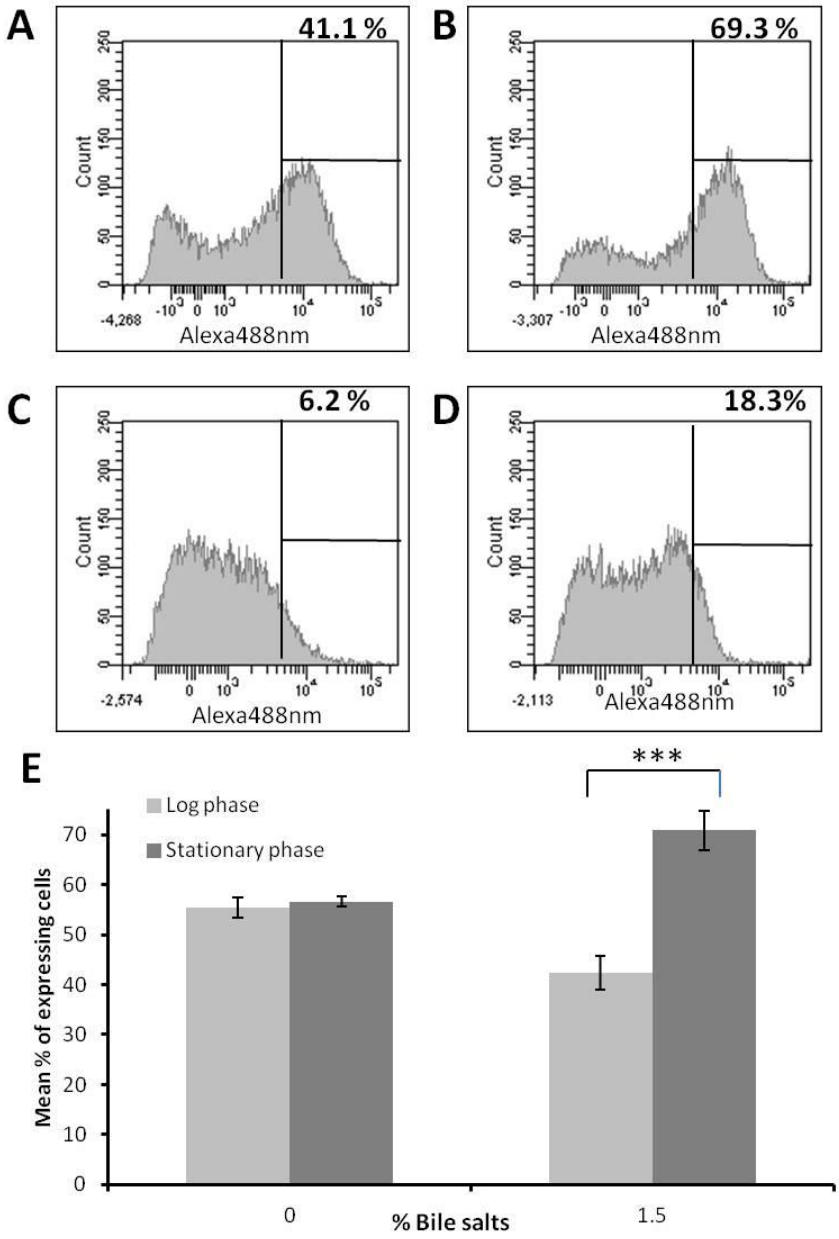


**Figure 7.9. Flow cytometry histograms, showing cell surface expression of *E. faecalis isolate* BS12297 grown in log (A) and stationary (B) phase in the presence of 1.5 % bile salts. Negative controls using pre-immune sera are shown for log phase in the presence of bile (C) and stationary phase grown cells in the presence of bile (D). Percentages of cells expressing Esp is indicated in (E) alongside data when no bile salts were present in the media when grown. The error bars represent the mean ±the standard error.**

## *7.10. Discussion*

Variations in biofilm formation between isolates are obvious in the *E. faecalis* isolates tested. As mentioned in chapter 1 and this chapter, more virulence factors have been identified in *E. faecalis* than *E. faecium*, and several have shown a role in biofilm formation. Furthermore, research has shown conflicting evidence on the role of Esp in biofilm formation, which can be Esp dependent (Tendolkar et al., 2004) or independent (Kristich et al., 2004) in various *E. faecalis* isolates. Other factors important in biofilm formation include the protease GelE (Hancock and Perego, 2004) and the aggregation substance Agg, both of which also have a role in attachment and conjugation between cells (Olmsted et al., 1991).

A previous study also performed biofilm assays on the bile stents isolates used here and we both obtain similar biofilm formation results, with isolates showing the same trends in biofilm formation (van Merode et al., 2006a). This chapter also confirms results in other papers that have shown that there can be biofilm production independent of the presence of Esp, suggesting that there are other factors in biofilm formation involved here that are yet to be identified (Kristich et al., 2004, Di Rosa et al., 2006). The trend in hydrophobicity is also observed, isolates better at biofilm formation are also more hydrophobic.

A surprising result was observed for *E. faecalis* BS11297. This strain contains both Esp and GelE, but biofilm formation was consistently poor in any condition tested. This could be explained in part by the low hydrophobicity of this isolate and its poor initial attachment. In chapter 5 we have shown that hydrophobicity is in part determined by the presence of Esp, and together with the flow cytometry data it seems likely that the *esp* gene is actually not expressed. This is not very unusual; for example, the *gelE* gene has also been found to be present in some isolates while not being expressed (Biavasco et al., 2007), and perhaps this is also occurring here with *esp*. However, this still does not fully explain the poor biofilm formation, as for instance *E. faecalis* BS385 (which lacks both Esp and GelE) still forms reasonable biofilms. A previous study on heterogeneity of surface charge in cell cultures showed that *E. faecalis* BS11297 is significantly more homogeneous (the same

surface charge on all cells) compared to *E. faecalis* BS385, and it was suggested that this cell surface heterogeneity is important in surface attachment and biofilm formation (van Merode et al., 2006a).

In the *E. faecalis* strains, stimulation of biofilm formation by bile salts was seen in all strains except *E. faecalis* BS11297. Different responses were observed when assays were performed in the presence of sodium taurocholate or glycocholate; *E. faecalis* BS835 increased in the presence of sodium glycocholate but not with sodium taurocholate and the opposite was seen with *E. faecalis* ATCC14933. These strains vary in their genetic virulence determinants and therefore this variation is probably due to genetic differences causing biofilm formation to be effected by different bile components. This was also seen in Chapter 5, were *E. faecium* TX1330 was shown to have stimulated biofilm formation from different bile components than *E. faecium* E1162.

A previous study by van Merode *et al* also studied the effect of ox-bile on biofilm formation and cell heterogeneity in *E. faecalis* strains. They found *E. faecalis* BS12297 and BS385 decreased in biofilm formation, but they only tested 5% bile salts (van Merode et al., 2006a). Our tests ranged from 0% to 5% and we observed that at lower concentrations biofilm formation increased, while at a high concentration (5%), biofilm formation remained the same or decreased (compared to conditions without bile). With this in mind the paper suggests that high levels of bile may stop adherence and therefore biofilm formation, they suggest this is probably due to surface charge. Initial adherence tests in this chapter confirm this suggestion as all isolates show a decrease in initial attachment and microcolony formation at 5% bile salts, but whether this is due to surface charge would need further study.

Hydrophobicity testing was also performed in the presence of bile salts (1.5%); varying results were obtained with all but one isolate (*E. faecalis* BS385) decreasing in hydrophobicity. This variation in results is also observed in other research testing at 5% ox-bile on these *E. faecalis* isolates (van Merode et al., 2006b).

In *E. faecium*, Esp production is clearly dependent on the growth phase (chapter 6). Similar to *E. faecium*, cultures of *E. faecalis* BS12297 and ATCC19433 contained mixed populations, with approximately 50-60% of cells expressing Esp on the cell surface. In contrast to what was found for *E. faecium*, no clear differences between log phase and stationary phase cells were found.

There have been a few studies on how stress effects gene expression in *E. faecalis*, in particular with concerns to virulence. One study observed that there are many sub-lethal environments, including the presence of bile salts that cause an increase in gene expression of virulence associated genes, including *ace* (collagen binding MSCRAMM, involved in adhesion to host cells), *efaA* (endocardial adherence factor antigen, involved in adhesion to host cells) and *cylB* (cytolysin, lyses of cells). Unfortunately Esp was not included in that study (Lenz et al., 2010). However, it was noted that the gene expression response to general stresses, such as salt and temperature, had a different pattern of gene expression than observed with bile salts, suggesting that bile leads to regulation of different networks as compared to the general stress response (Lenz et al., 2010). This is also observed when the proteome in the presence of bile was compared to the proteome of different detergents (Flahaut et al., 1996). This would be expected as *E. faecalis* is a GI tract commensal and is therefore more likely to encounter bile than other stress factors, such as increased temperature. *E. faecalis* ATCC19433 was analysed in a different study in which it was observed that different proteins were produced with differing lengths of exposure to bile (Rince et al., 2003). There has also been a study on bile salts-sensitive mutants; genes inactivated included homologues to genes involved in: fatty acid biosynthesis, a transcription regulator, an exonuclease, DNA mismatch repair, cell wall synthesis and some with unknown function. In this study a mutation in a *E. faecium* SagA homologue was also identified as a bile-sensitive mutant; this protein is a major secreted antigen with a role in physiochemical stresses, cell wall metabolism and extracellular matrix (ECM) binding (Breton et al., 2002). We found that the production of Esp in *E. faecalis* BS12297 was induced in cells in the stationary phase compared to log phase cells, but only in the presence of bile. The above illustrates that when put under stress *E. faecalis* does increase its expression of

virulence factors, some of which are involved in initial adherence step in biofilm formation.

The presence of bile has shown varying effects on different strains, microcolony formation and therefore biofilm formation. Esp expression is shown to be consistent during growth stages without bile, but there was an increase in expression in the presence of bile in stationary phase. Taken together that data shown in this chapter and chapter 5, it is clear that bile stimulated biofilm formation and thus increases virulence.

# Chapter 8: Results Section VI

# *E. faecium* virulence in *C. elegans*.

## *8.1. Background and objectives*

*C. elegans* is a nematode found in soil that is approximately 1 mm in length when adult (Figure 8.1). It has a simple structure but also shares some features with higher animals, features include the gut, epidermis, neurons, muscles and the innate immune system. The nematodes basically are comprised of two tubes: firstly, the outer tube containing the hypodermis, cuticle, excretory system, neurons and muscles; and secondly, the inner tube that contains the pharynx, intestine and if the worm is adult the gonad (Altun and Hall, 2009, Sifri et al., 2005).

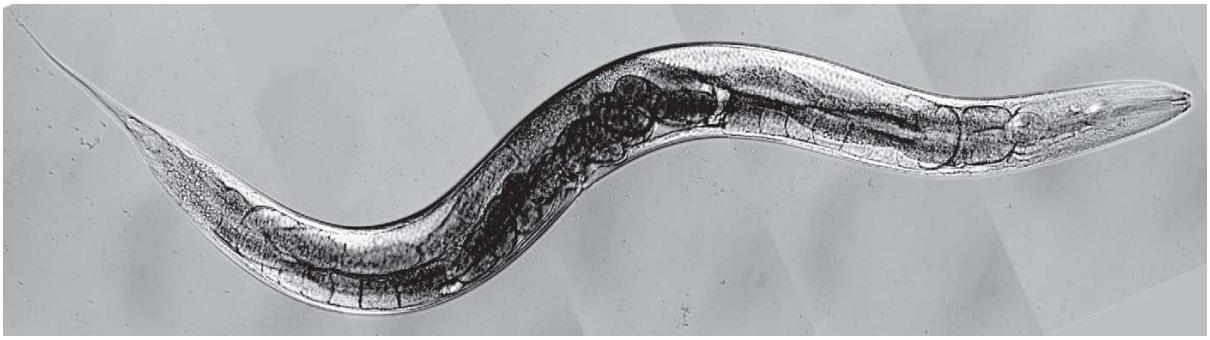

**Figure 8.1. Adult *C. elegans*. (Sifri et al., 2005)**

*C. elegans* became an experimental genetic model in the 1960s (Sifri et al., 2005) and the interest in this organism increased in 1998 when it was the first multicellular organism with a completed genome sequence (Hodgkin, 2005). It has been the genetic model for studies in aging, development and neurobiology (Sifri et al., 2005), but in this chapter we are more interested in *C. elegans* as a microbial virulence model. *C. elegans* is a good model organism for several reasons: it has a quick generation time, it can self fertilise, making genetic tractability easier, it is simple to maintain and is inexpensive, has a defined cell lineage map and most importantly, as mentioned above a fully sequenced genome which has revealed a large number of genes that are vertebrate orthologues (Alegado et al., 2003, Gravato-Nobre and Hodgkin, 2005).

*C. elegans* is a bacterivore that in the lab is maintained on agar plates and feeds on lawns of non-pathogenic *E. coli*. However, when its supply of food is switched to a human pathogen its lifespan is often drastically reduced. Research has revealed that mammalian pathogens can infect and kill *C. elegans* in one of five methods: invasion, persistent infection, intestinal infection with colonisation, toxins and biofilm formation (Gravato-Nobre and Hodgkin, 2005, Sifri et al., 2005). These occurrences have encouraged more detailed studies of how the pathogens infect *C. elegans*. Initially bacteria encounter the physical barriers and need to get past the cuticle, which is usually achieved via the mouth and anus. Once in the mouth the microbes reach the pharynx (which contains the grinder), which breaks up the microbes preventing them getting any further alive (Gravato-Nobre and Hodgkin, 2005, Alegado et al., 2003). If microbes do pass the pharynx they are able to enter the intestine, where they can proliferate, cause cell damage and even death.

*C. elegans* also has an innate immune responses controlled by signalling pathways that include Transforming growth factor (TGF-β), insulin-like growth factor (IFG)-1, p38 MAP kinase pathway, programmed cell death and the Toll pathway (Gravato-Nobre and Hodgkin, 2005). The mammalian immune responses are more complicated than that of *C. elegans*, as they also have an adaptive immune response, while there are also aspects of the innate response that are absent or not used in the same way as in *C. elegans*. For instance, *C. elegans* does not have phagocytes like mammalians. However, they do contain a type of scavenger cell, coelomocytes, but there is no evidence as yet that they phagocytise bacteria or have any role in immunity (Sifri et al., 2005). Interestingly, *C. elegans* does contain the Toll signalling pathway orthologues, but they do not play the same innate immune system role as in mammalians as they are more involved in development and avoidance of pathogens. This avoidance mechanism allows *C. elegans* to recognise the difference between a good food source and a bad one (made of pathogens) and therefore move from the bad food source to the good (Pujol et al., 2001). This mechanism is probably very important due to the lack of adaptive immunity.

Studies using various pathogens against *C. elegans* have shown that virulence factors involved in mammalian pathogenicity are also important in *C. elegans* infection and the level of virulence in *C. elegans* is predictive for the virulence in a mouse model (Styer et al., 2005). There are several advantages of this method over rodent infection model as it is cheaper, less laborious, far easier to scale up, and it does not suffer from an ethical burden (and therefore follows the 3Rs rules). Furthermore, it has also proven useful in antimicrobial drug discovery (Moy et al., 2006). This has allowed for high throughput studies of various pathogens and their virulence by, for instance, screening of libraries of mutant pathogens. There are various examples of virulence factors known to be important in human infection that are also involved in nematode infections, including genes in cell wall structures (*S. marcescens*, *wzm* (Kurz et al., 2003)), biofilms (*Y. pseudotuberculosis*, *hms*HFRS (Darby et al., 2002)), extoxin (*E. faecalis*, cyl (Garsin et al., 2001), exoenzymes (*E. faecalis*, *gelE* (Sifri et al., 2002)), two component regulators (*P. aeruginosa*, *gac*AS (Tan *et al*. 1999)) and quorum-sensing systems (*S. aureus* (*agr*) and *E. faecalis* (*fsr*) (Sifri et al., 2005, Garsin et al., 2001)).

Several studies using *C. elegans* to study virulence in enterococci have already been performed and have shown that *E. faecalis* is one of the bacteria that are not only able to kill *C. elegans* but also colonize and persist in the intestine of *C. elegans* (Garsin et al., 2001). As mentioned above cytolysin (*cyl*), sucrose-6-phosphate hydrolase (*scrB*), gelatinase (*gelE*), *fsr* quorum-sensing system, and serine protease (*sprR*; (Sifri et al., 2002)) are involved in this infection. Interestingly, the closely related *E. faecium* is also able to persist in the gut of *C. elegans*, but appears not to kill *C. elegans* when grown aerobically (Garsin et al., 2001). Surprisingly, however, *E. faecium* does has a "fast killing mode" (i.e. within a few hours rather than days for *E. faecalis*) when, before infection, the cells are grown anaerobically; this effect was shown to be due to the production hydrogen peroxide (Moy et al., 2004).

Published research has shown *E. faecium* to lack pathogenicity in *C. elegans,* with the occurrence of colonisation but no death, but this has only been shown for a few strains (Moy et al., 2006, Moy et al., 2004, Garsin et al., 2001), and for that reason

we tested several clinical isolates to test whether the lack of pathogenicity applies to all *E. faecium* strains. In addition, we tested whether there was any influence of the presence of Esp on virulence.

## *8.2. C. elegans killing assay.*

The *E. faecium* strains tested included strains mentioned in the previous chapters (*E. faecium* E1162, E1162Δesp, and TX1330) and the clinical vancomycin-resistant *E. faecium* isolates 1-5 (Figure 8.3A & B) received from the University of Southampton, details of which are in the Methods and Material chapter. They were tested alongside a negative control (*E. coli* HB101) and a positive control (*E. faecalis* BS11297). In brief, the *C. elegans* worms were age-synchronized and grown to the young adult stage on *E. coli*, and then transferred to agar plates with a lawn of the particular pathogen or the *E. coli* control. Over several days the plates were examined to establish the number of alive (moving) and dead nematodes (non-moving, straight and not responsive to gentle touch).

Results for *E. faecium* E1162, E1162Δesp, and TX1330 are shown in figure 8.2. It can be observed that with *E. coli* HB101, the percentage survival does not go under 94% even after 10 days; therefore the longevity of *C. elegans* worms is good when fed with this. When *C. elegans* were fed on the *E. faecium* strains as sole source of food, the longevity of the worms was unaffected, while there was also no difference between *E. faecium* E1162 and *E. faecium* E1162Δ*esp*. *E. faecalis* BS11297 is shown to be pathogenic to the worms as it has killed all worms by day 10.

The *E. faecium* isolates provided by the University of Southampton were obtained from a range of wards and source samples (See Methods and Materials), with all the isolates being resistant to vancomycin as well as other antibiotics. As only a few *E. faecium* strains were tested with *C. elegans*, it was speculated that perhaps some other isolates might be pathogenic due to, for instance, virulence factors found in only some *E. faecium* isolates. However, as before it can be observed that there are no significant differences in the levels of virulence of the isolates (Fig 8.3) with, in all cases, survival of 80% or more after 10 days.

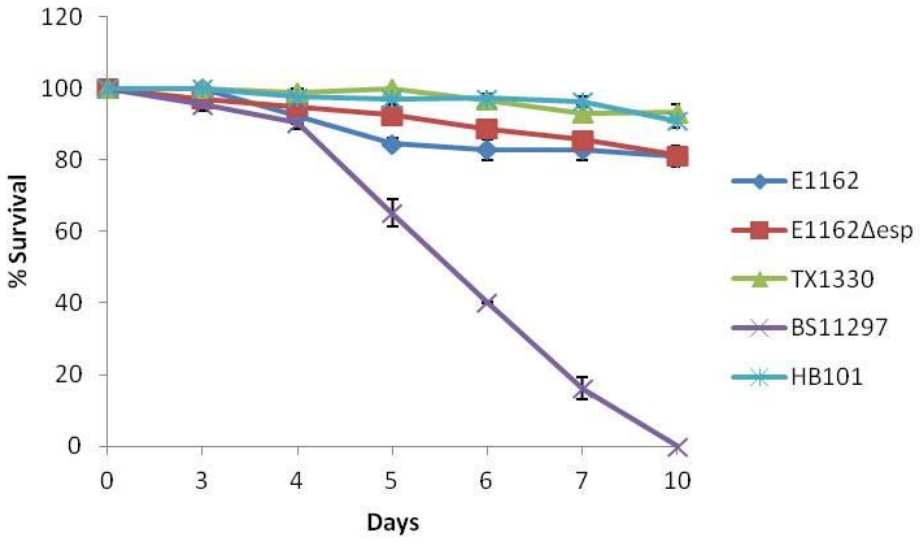


**Figure 8.2. *C. elegans* killing assay results for: *E. faecium* E1162, E1162Δesp and TX1330. *E. faecalis* BS11297 as positive control. *E. coli* HB101 as negative control. The error bars represent the mean ± the standard error.**

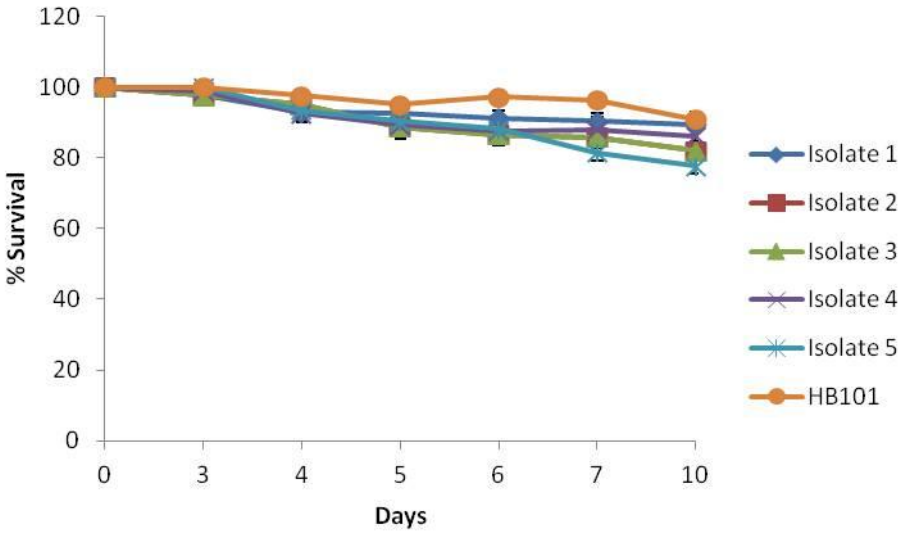


**Figure 8.3. *C. elegans* killing assay results for *E. faecium* hospital clinical isolates 1-5. *E. coli* HB101 as negative control. The error bars represent the mean ± the standard error.**

The *C. elegans* killing assay was also performed on *E. faecalis* isolates previously used in this thesis (Figure 8.4; data was kindly provided by Sarah Bukhari, a fellow PhD student in the Department of Pharmacy and Pharmacology at the University of Bath). All the *E. faecalis* strains showed significant pathogenicity to *C. elegans* as they all decreased survival below 80%. *E. faecalis* BS11297 showed the highest level of virulence against *C. elegans*, with 16% survival at day 7, and *E. faecalis* ATCC19433 shows the lowest level of virulence of the *E. faecalis* strains tested, with 51% survival at day 7. Interestingly, *E. faecalis* BS11297 and *E. faecalis* ATCC19433 both contain Esp and GelE virulence factors. However, it should be noted that *E. faecalis* ATCC19433 is a reference strain that was isolated in the 1940s (Shattock, 1949), which may have lost other virulence factors, whereas the *E. faecalis* BS strains are recent clinical isolates.

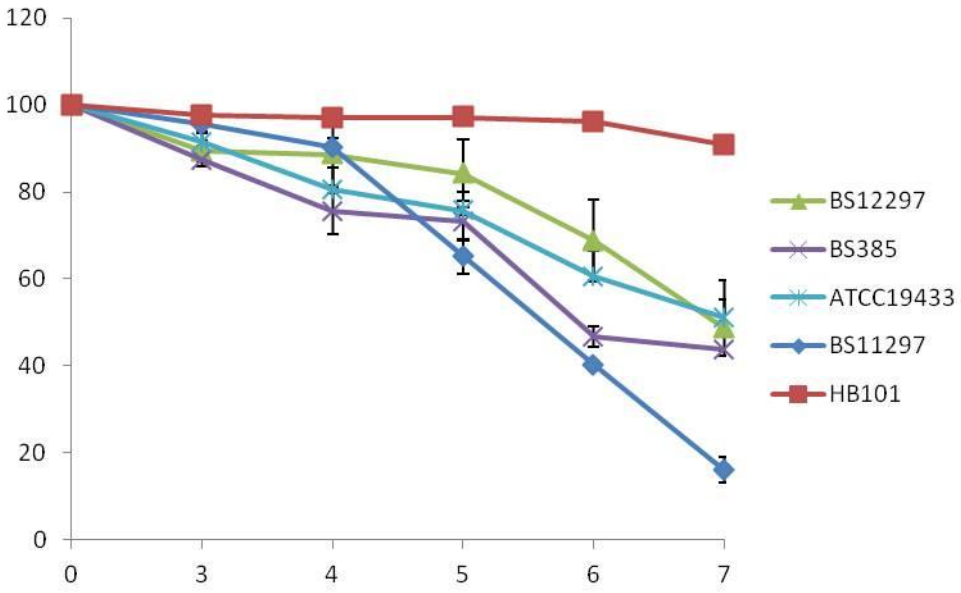


**Figure 8.4. *C. elegans* killing assay results for *E. faecalis* isolates. *E. coli* HB101 as negative control. The error bars represent the mean ± the standard error.**

## *8.3. C. elegans anaerobic killing assay*

As previously mentioned in the introduction, *E. faecium* has a 'fast killing mode' against *C. elegans* which takes only hours and is caused by the production of hydrogen peroxide (Moy et al., 2004). Here it was decided to test if *E. faecium* E1162 too can kill *C. elegans* in this manner. Bacteria were grown anaerobically on plates to form a lawn, after which *C. elegans* was deposited and then survival counts for the worms are performed (data kindly provided by undergraduate project students Patrick Siu and Doris Ng). It can be seen from the results (Figure 8.5) that when grown anaerobically *E. faecium* E1162 also has a 'fast killing mode' against *C. elegans*, which is not seen when *E. faecium* E1162 is grown aerobically.

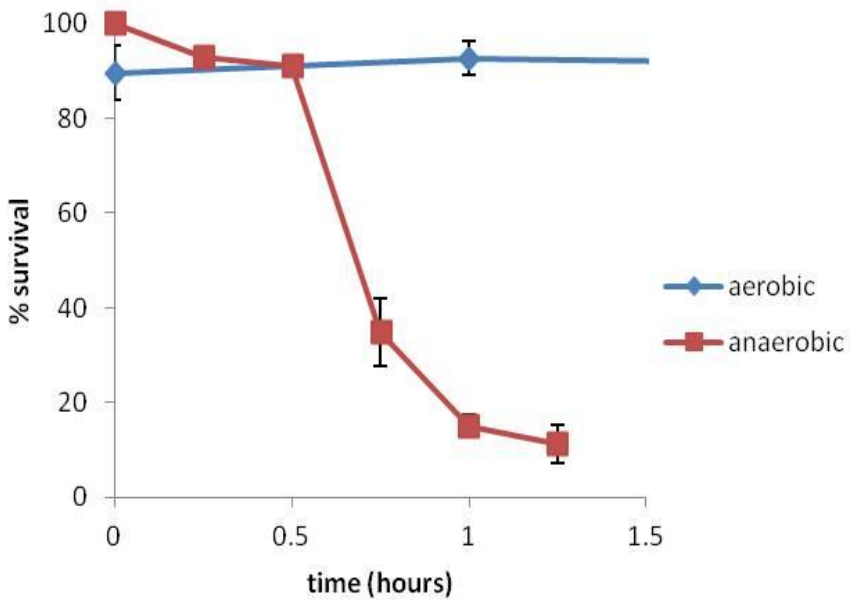


**Figure 8.5. *C. elegans* killing assay results for *E. faecium* E1162 when grown aerobically and anaerobically. The error bars represent the mean ± the standard error.**

## *8.4. Discussion.*

In this chapter we have confirmed that *E. faecium* isolates, including highly-resistant clinical strains, are non-pathogenic to *C. elegans* when grown aerobically. Other pathogens that do not kill *C. elegans* are not widely known. One such pathogen is *Streptococcus pyogenes* which also shows no significantly killing of *C. elegans* under aerobic conditions on BHI (Garsin et al., 2001); the author suggests that this is likely to be due to the media it has been grown on. Studies have shown the effect of media on *C. elegans* killing assays as the level of virulence does depend to some extend on the composition of the growth medium (Alegado et al., 2003). Even *E. coli* OP50, the usual source of food for *C. elegans* in the laboratory, becomes somewhat virulent to *C. elegans* if it is grown on BHI (a rich media) (Garsin et al., 2001). Another example is *S. pyogenes*, mentioned above, which is pathogenic to *C. elegans* when grown on THB, but not when its grown BHI (Jansen et al., 2002). Thus, it is feasible that pathogenicity of *E. faecium* is also dependent on the medium, but due to time constraints that has not been tested further.

Interestingly, other research into streptococci have shown that their killing is due to hydrogen peroxide killing, which can occur under aerobic conditions (Bolm et al., 2004), *E. faecium* too is able to kill *C. elegans* using this method, but only under anaerobic conditions (Moy et al., 2004), and we have observed this also here with *E. faecium* E1162. As streptococci are close relatives of enterococci, they may have unspecialised evolutionarily conserved mechanisms of virulence, and perhaps it is therefore not surprising that they have a similar mode of killing in *C. elegans*.

It is also important to ask the question of why there is a difference between *E. faecalis* and *E. faecium* as they are very similar species. As mentioned in the introduction to this chapter there have been several virulence factors identified in *E. faecalis* that are involved in *C. elegans* infection (*cyl, scrB, gelE, fsr*, and *sprR*; (Sifri et al., 2002)) and this chapter has also observed the difference in virulence that can occur between the *E. faecalis* isolates which is probably due to the presence and absence of specific virulence factors. *E. faecium* has none of the above factors, except for *scrB*, and this may well be the reason for its apparent reduced level of virulence. *E. faecalis* also contains Esp, and it was observed that *E. faecalis* BS385,

the only isolate without Esp, showed similar levels of virulence to *E. faecalis* BS12297, which does contain Esp, suggesting that Esp does not play a part in virulence in *C. elegans*. However, it is important to note that these strains come from different backgrounds and other factors may play a role here. Here using the *C. elegans* killing assay no difference between *E. faecium* E1162 and *E. faecium* E1162Δ*esp* was shown, suggesting that Esp alone is not a determining factor in virulence. It is also interesting that virulence factors identified involved in *C. elegans* infections in bacteria do not currently include surface proteins involved in adhesion (Sifri et al., 2005, Alegado et al., 2003).

Here we have further established that *E. faecium* does not cause *C. elegans* killing under aerobic conditions, but in the case of *E. faecium* E1162 does under anaerobic conditions and that the lack of Esp does not allow increase survival. Future tests would include testing the pathogenicity of *E. faecium* on different media, such as THB, to see if virulence differs. It would also be interesting to analyse whether biofilm-associated surface proteins in enterococci to test if any are involved in virulence in *C. elegans*.

# Chapter 9: Final discussion

## *9.1. Final discussion*

Initially, as described in chapter 3, three SPases were identified in *E. faecium*. Only a few other Gram-positive bacteria have more than one SPase, which includes *B. subtilis*, *C. perfringens, S. aureus* and *S. epidermidis* (van Roosmalen et al., 2004). Many proteins are secreted via the Sec-dependent pathway of which SPases are an important component. For this reason a bioinformatic analysis was performed to identify secretory proteins in *E. faecium* E1162. In *E. faecium* E1162 2.3% of proteins contained a Sec-type signal peptide, which is relatively low compared to other Gram-positive bacteria that contain approximately 4% secretory proteins (Tjalsma et al., 2000a), and 1.8% contained a lipoprotein signal peptide. The amount of lipoproteins identified here is also low, especially if compared to *E. faecalis* V385 genome which has 2.7% lipoproteins (Reffuveille et al., 2011). It is possible that it is these extra lipoproteins that make *E. faecalis* more prevalent in hospitals. The secreted proteins identified included many virulence factors, such as Esp, autolysins, PilB subunit protein, two Bee3 proteins and collagen adhesion proteins. It was speculated that some SPases may be important either in specific conditions or in the processing of a specific subset of secretory proteins. The aim was to delete the individual SPases in *E. faecium* and then to study the phenotypic effects (e.g. on virulence) and analyse whether SPases would be suitable antimicrobial targets in enterococci. Inhibitors of SPases have already been identified (Harris et al., 2009) and so further research on SPases could encouraged additional research into inhibitors.

Unfortunately, deletion mutants of the SPases in *E. faecium* were not obtained inferring the genes could be essential. However, there are only a small number of genetic manipulation tools available for enterococci, and in order to investigate if the genes were essential, an inducible integration vector was constructed. The vector was constructed, but not tested due to time constraints.

Virulence in enterococci was then studied investigating antibiotic resistance and biofilm formation in enterococci and the role of Esp. Firstly antimicrobial resistance was investigated using calorimetry. Calorimetry has been used previously to differentiate between growing cultures of meticillin-susceptible and meticillin-resistant *S. aureus*; it was able to do this in 4-5 hours (von Ah et al., 2008). Here in chapter 4 it was also shown to be a rapid and sensitive method when investigating antimicrobial resistance in bacteria. This was particularly shown when a difference in ampicillin resistance was observed between *E. faecium* E1162 and *E. faecium* E1162Δ*esp*, whereas this difference was not observed with classical antibiotic-sensitivity tests. The *esp* mutant was shown to be more sensitive to ampicillin and this was the first evidence of Esp's role in antibiotic resistance.

Biofilm formation was tested for all the isolates studied. In *E. faecium* the presence of Esp enabled higher levels of biofilm formation, which was shown by comparing *E. faecium* E1162 to *E. faecium* E1162Δ*esp* and *E. faecium* TX1330. The presence of Esp in *E. faecium* was also demonstrated to increase hydrophobicity, which is very clearly linked to the levels of biofilm formation and this has also been shown to be the case in *E. faecalis* (Tendolkar et al., 2004). Indeed, *E. faecalis* isolates also demonstrated that good biofilm formers were more hydrophobic in nature. Esp expression in *E. faecium* was also studied under different growth conditions (Chapter 6). The results indicated that Esp expression in *E. faecium* E1162 and *E. faecalis* cultures contained two populations, only one of which was expressing Esp. It was also observed that Esp expression in *E. faecium* E1162 was growth-dependent illustrated by low Esp expression at exponential growth phase with increasing amounts in stationary phase, and then as many as 80% of cells expressing Esp during biofilm formation. Interestingly, this growth-dependent expression was not observed in *E. faecalis* BS12297, as no difference was seen for Esp expression between cells in exponential and stationary phase. Expression of Esp in *E. faecalis* BS12297 cells in biofilm cells was not tested.

The effect of bile salts on biofilm formation was studied in both *E. faecium* (Chapter 5) and *E. faecalis* (Chapter 7) as enterococci have to withstand bile salts in the GI

tract, which is their natural habitat. Bile salts appeared to increase biofilm formation for both *E. faecium* and *E. faecalis*, especially around the physiological concentration (0.2-2%) of the small intestine in humans (Hofmann, 1998). *E. faecalis* not only showed an increase in biofilm formation at the physiological concentration, but also at somewhat higher concentrations of bile salts. This is not surprising as some of these isolates came from bile stents were bile concentrations are around 9±4% (Donelli et al., 2007). Increases in biofilm formation when bile salts are present have also been observed in other bacteria such as with the gastrointestinal commensals *B. fragilis* (Pumbwe et al., 2007) and *L. rhamnosus* (Lebeer et al., 2007), and the pathogens *S. typhimurium* (Prouty et al., 2002) and *L. monocytogenes* (Begley et al., 2009). It is also important to note that the increase in biofilm formation observed here was not due to the detergent nature of bile salts, as other detergents did not influence biofilm formation. Biofilm formation was also shown to vary in the presence of individual components of bile salts, *E. faecium* E1162 was induced by sodium taurocholate but not by sodium glycocholate, which was also observed in *Lactococcus lactis* (Zaidi et al., 2011). In *E. faecium* the presence of bile salts caused an increase in initial attachment, microcolony formation and EPS production. These increases were not due to hydrophobicity, cell growth or cell morphology. In the case of *E. faecalis*, isolates varied in the biofilm formation when bile salts were present; where there was an increase there was also an increase microcolony formation.

Interestingly *E. faecium* E1162 and E1162Δ*esp* showed differing biofilm formation when bile salts were present in the media, suggesting a possible role in sensing bile salts. *E. faecium* E1162Δ*esp* showed increased biofilm formation only at 0.5% bile salts, were as *E. faecium* E1162 showed increase of biofilm formation over a range of concentrations. It was also observed that when separate components of bile salts were tested, it was found that the stimulation in biofilm formation found in *E. faecium* E1162Δ*esp* was not caused by sodium glycocholate or taurocholate. The next step was to study Esp expression in the presence of bile salts for *E. faecium* E1162 biofilm cells, surprisingly tests indicated there was a decrease in expression (Chapter 6). Further research is require to understand this occurrence, perhaps the

lack of Esp causes other interactions to occur allowing more biofilm under conditions when bile is present.

Virulence in enterococci was also investigated using *C. elegans* infection models (Chapter 8). Various highly antibiotic-resistant *E. faecium* isolates were not pathogenic to *C. elegans*. However, *E. faecium* E1162 demonstrated pathogenicity within hours when grown anaerobically, which has also been shown in other *E. faecium* isolates (Moy et al., 2004). Interestingly *E. faecalis* does not have this fast killing mode, as for this organism it can take up to 5 days to kill *C. elegans* under anaerobic conditions (Moy et al., 2004), illustrating again the differences seen between *E. faecium* and *E. faecalis*. *E. faecalis* isolates showed varying pathogenicity to *C. elegans* depending on the virulence factors they contained.

One of the main aims of this thesis was to further understand the role of the Esp protein, as its role is still unclear. These tests were able to confirm its role in biofilm formation, its possible role in ampicillin resistance and bile salts response, and that it also does not appear to affect virulence in *C. elegans*. It was also observed that different growth phases effected the expression of Esp, but there still remains a lot to understand. Below are some ideas for future work.

## *9.2. Future work*

Future work on SPases is to continue searching for mutants and test the integration vector. This vector will not only be useful here for the study of SPases but also for other genes and, more importantly, essential genes that if deleted are lethal to enterococci. This extends the number of genetic tools that are available for further enterococcal research, which currently are quite limited (Kristich et al., 2007). It would also be interesting to further characterise some of the hypothetical secreted proteins identified in *E. faecium* E1162. All the factors involved in biofilm formation are not fully understood and additional factors involved in biofilm formation are

being identified every day with new genetic tools. With the use of genetic tools more proteins are being identified as virulence factors; this was the case when pili in *E. faecalis* were discovered (Sillanpaa et al., 2004), these were all originally hypothetical proteins. Much is to be discovered about the functions of existing proteins (e. g. Esp) and hypothetical proteins must also have roles to be discovered, they could interact with or be themselves virulence factors. It was also surprising to observe that *E. faecalis* V385 (Reffuveille et al., 2011) has more lipoproteins than *E. faecium* E1162; it would therefore be interesting to analyse more *E. faecium* genomes to see whether the percentages for lipoproteins and secreted proteins seen here are true for all the genomes.

As mentioned in the introduction, biofilm formation can increase antibiotic resistance in bacteria from 10-1000 fold (Hoiby et al., 2010). In this thesis calorimetry was demonstrated to be a sensitive method for studying antibiotic resistance therefore future research with the use of calorimetry could help in the discovery of effective drugs against enterococcal biofilms. Research has also been performed into adapting this method for biofilm analysis and this method has shown promising results when studying bactericidal and bacteriostatic treatments of biofilms (Buchholz et al., 2010a).

We and others have observed that *E. faecalis* appears to produce more biofilm than *E. faecium*. Many more virulence factors have been studied in detail in *E. faecalis* than in *E. faecium* and this includes the *frs*-system and GelE production which have a role in eDNA production in *E. faecalis*. This system has not yet been identified in *E. faecium* and the production of gelatinase is sporadic (Biavasco et al., 2007, Vankerckhoven et al., 2004, Billstrom et al., 2008). It would be interesting to establish if eDNA is important to *E. faecium* biofilm formation and, if so, what are the mechanisms of its release if GelE is not present.

Further understanding of Esp is still required; a study into *S. aureus* Bap protein, which has similarities to Esp, observed that the protein attaches to Gp96, a

chaperone protein on the host cell surface (Valle et al., 2012). That study also showed that Esp did not interact with Gp96 (Valle et al., 2012), but Esp may interact with other host cells surface proteins and therefore this would be interesting to establish this. To further extend information on Esp, crystallisation trials using purified N-terminal domain were performed (Bukhari and Bolhuis, unpublished data), but unfortunately no crystals were obtained. Further trials with the full protein are unlikely due to the size of the protein, but sections of the protein can be further studied and may give more information on the function of this protein. Currently analysis of the A repeat units with nuclear magnetic resonance (NMR) are being performed. Infecting *C. elegans* with *E. faecalis* suggested that Esp does not play a role in virulence. It would be interesting to establish if this is true for other surface proteins in other bacteria and so further testing is required.

The increase in biofilm formation due to bile salts also requires further studies. In the case of *E. faecium* there was a distinct difference in biofilm formation in the presence of bile between *E. faecium* E1162 and *E. faecium* E1162Δ*esp*, suggesting Esp had role in bile response. However, in the presence of bile the expression of Esp decreases, which seems counterintuitive. EPS production was shown to increase in biofilm formation when bile salts were present in *E. faecium*. This was not tested in *E. faecalis* therefore this requires confirmation, but also it would be interesting to study the composition of the EPS. Are there differences between *E. faecium* and *E. faecalis*? Does the presence of bile salts also effect composition? Enterococci have bile salt hydrolases and one theory is that these deconjugate the bile salts and that leads to insertion of the resulting components into cell membrane (Begley et al., 2005). It is conceivable that would result in increased amounts of EPS, either through direct flux of bile or the glycine/taurine amino acids into the EPS, or indirectly through increased expression of enzymes involved in EPS production. Studying how bile increases EPS would also be required, perhaps using proteomics or transcriptomic studies. Proteomics studies have been used to study mixed-species biofilms, biofilm-related proteins produced under stress (Klein et al., 2012) and for the analysis of the differences in secreted proteins in the planktonic and biofilm cells (Muthukrishnan et al., 2011). However, proteomics as of yet has not been fully used to study EPS (Seneviratne et al., 2012), and it would be therefore interesting to

utilise this more and test if regulatory proteins and/or other factors involved can be identified. Such studies do not need to be limited to EPS production only, but can be extended to include other factors involved in biofilm formation. For instance, in *B. fragilis* it was observed that fimbriae are overproduced in the presence of bile salts (Pumbwe et al., 2007), which could also be the case for enterococci.

In *B. fragilis* (Pumbwe et al., 2007) and *Bifidobacterium animalis* subsp. *lactis* (Ruas-Madiedo et al., 2009) observed the production of vesicles in the presence of bile salts was observed. Ruas-Madiedo *et al* (2009) suggest this is a method of detoxification, but other studies have shown that vesicles can carry factors involved in attachment (Grenier and Mayrand, 1987) and the release of quorum sensing signalling molecules (Mashburn and Whiteley, 2005), all of which are important for colonisation and virulence. Vesicle release has also been linked with the release of eDNA (Kadurugamuwa and Beveridge, 1995), which links well with other future research on the study of eDNA production in *E. faecium*. Therefore it would be interesting to examine by transmission electron microscopy (TEM) to see if vesicles are also observed in on enterococcal cells in the presence of bile salts for. As shown here there are many ideas to investigate why biofilm formation is increased in the presence of bile salt.

## *9.3. Summary of the main results from this thesis.*

- The *E. faecium* genome contains three SPases one of which is an ER-type SPase. This is unusual as they are usually found in sporulating bacteria which *E. faecium* is not.
- 2.3% of the secreted proteins have a Sec-type signal peptide and 1.8% have a Lipo-type signal peptide.
- Esp was shown to have a possible role in ampicillin resistance using calorimetry. This technique was also demonstrated to be a sensitive and rapid method to study antimicrobial resistance
- Esp in *E. faecium* increases hydrophobicity and thereby biofilm formation.

- *E. faecium* Esp is expressed in a growth-dependent manner, with most Esp expression occurring in biofilm cells. This growth-dependent manner was not observed in *E. faecalis* BS12297.
- In both *E. faecium* and *E. faecalis* the presence of physiological concentrations of bile salts induced biofilm formation. It was also observed that individual components of bile salts differed in their effect on biofilm formation depending on the isolate.
- Induction of biofilm due to bile salts was shown to occur independently of the presence of Esp, however the presence of Esp in *E. faecium* E1162 did allow for increased biofilm formation in a wider variety of concentrations than seen in *E. faecium* E1162Δesp, suggesting Esp's involvement in the increased biofilm formation under this growth condition.
- In *E. faecium* the presence of bile salts caused an increase in initial attachment, microcolony formation and EPS production.
- This increase in biofilm formation was not caused by an increase in Esp expression.
- Multi-drug resistant clinical isolates of *E. faecium* where shown not to kill *C. elegans*. *E. faecium* E1162 did kill *C. elegans* when grown anaerobically using the 'fast killing mode' and *E. faecalis* Esp was shown not to be a virulence factor in *C. elegans* infection.

# Appendix

## *Appendix A.*

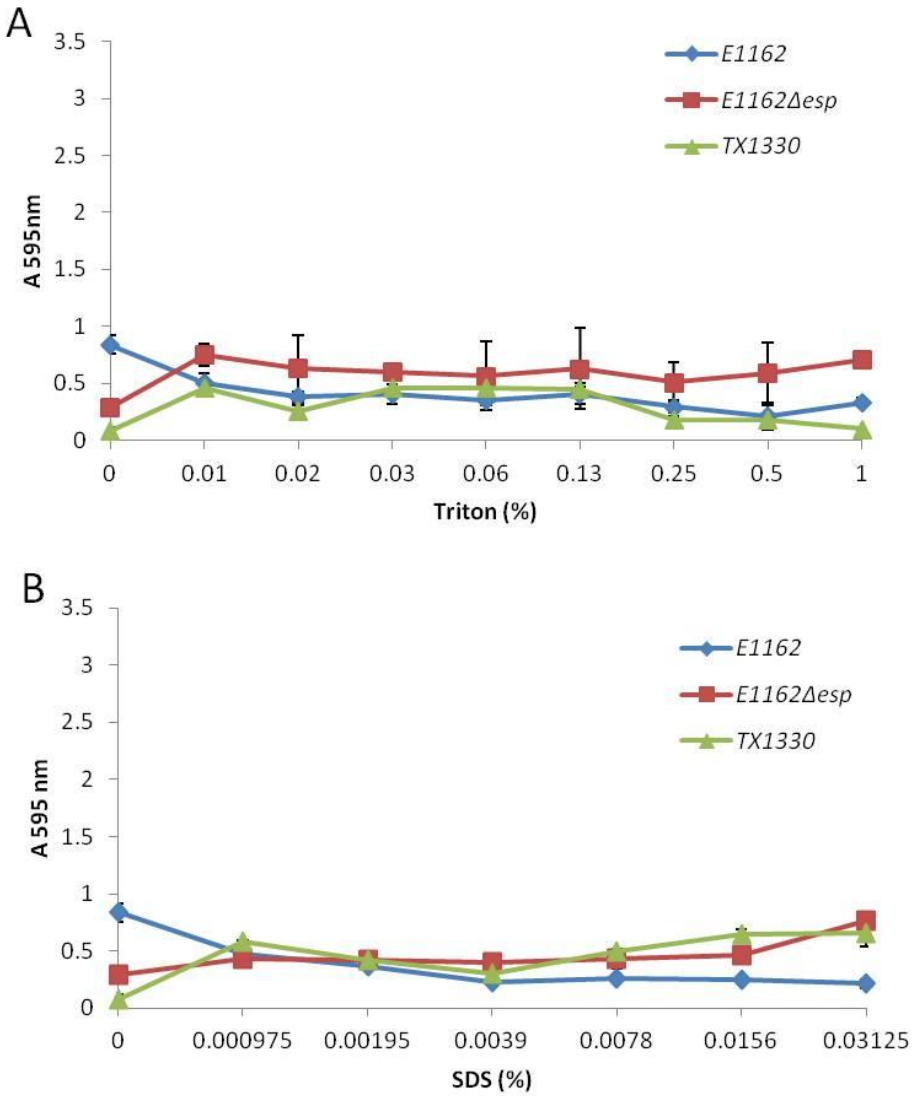


**Figure A.1. Biofilm formation for *E. faecium* isolates in the presence of Triton X100 (A) and SDS (B).**

## *Appendix B.*
## *Published work.*

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**Enterococcal surface protein Esp affects antibiotic sensitivity in *Enterococcus faecium***

*Keywords:*
Ampicillin
*Enterococcus faecium*
Calorimetry
Enterococcal surface protein
Antimicrobial susceptibility tests

*Sir,*

Enterococci are part of the natural intestinal flora. They are relevant clinically due to their emergence in recent years as opportunistic pathogens. This is worrying as many isolates are resistant to antibiotics such as penicillin or vancomycin [1], complicating treatment. Enterococci are also known to form biofilms, which are communities of microbes that, encased in an exopolysaccharide matrix, are attached to a biotic or abiotic surface. Biofilms are a large problem clinically as they are very difficult to eradicate and are involved in >60% of bacterial infections [2]. One of the proteins involved in enterococcal biofilm formation is the enterococcal surface protein (Esp) [3], but the exact role of Esp has not been firmly established.

The goal of this study was to test the use of isothermal calorimetry [4] as a tool to measure antibiotic resistance and to compare it with traditional methods such as disk susceptibility and minimal inhibitory concentration (MIC) measurements [5]. Calorimetry can be used to measure the heat released from growing cultures of cells as they metabolise nutrients and it provides real-time data. The resistance of three strains was analysed: *Enterococcus faecium* E1162 [3], an ampicillin-resistant clinical blood isolate; E1162Δ*esp*, an E1162 mutant that lacks Esp [3]; and the control strain TX1330, an ampicillin-sensitive and *esp*-negative strain [6].

Three standard susceptibility tests were performed, namely microbroth dilution [5], disk susceptibility (10 μg ampicillin disks; Oxoid, Basingstoke, UK) and MIC Evaluator strips (Oxoid). All three tests showed that *E. faecium* E1162 and E1162Δ*esp* have the same level of resistance. Microbroth dilution showed a MIC of 32 μg/mL for both E1162 and E1162Δ*esp*, whilst the MIC Evaluator strips showed a MIC of 16 μg/mL for both strains. As expected, the control strain *E. faecium* TX1330 was more sensitive, with a MIC of 4 μg/mL. Disk susceptibility confirmed these results, giving small 1.5 mm clearing zones on agar plates with E1162 and E1162Δ*esp* and a large 10 mm zone on plates with TX1330 cells.

Power–time curves were recorded with a Thermometric 2277 Thermal Activity Monitor (Thermometric AB, Järfälla, Sweden). The calorimeter was calibrated using the electrical substitution method at 37 °C. Cultures were started with $1.3 \times 10^4$ cells/mL in tryptic soy broth (Oxoid). This was incubated in an external water-bath at 37 °C and pumped continuously through the flow-through calorimeter using a peristaltic pump at a flow rate of 1 mL/min. The calorimetric output (power) was measured over time. Using calorimetry, power–time curves were recorded in the absence of antibiotics, which showed that the heat output from *E. faecium* E1162 was nearly identical to that of *E. faecium* E1162Δ*esp*; the profile of *E. faecium* TX1330 was also very similar (Fig. 1A). The peaks and troughs observed are probably due to the sequential use of nutrient sources in the complex culture medium [4]. With all three strains, distinct peaks were observed at similar times and with similar heat output, implying that they metabolise the medium in the same way and therefore showing that Esp is not connected to metabolic processes.

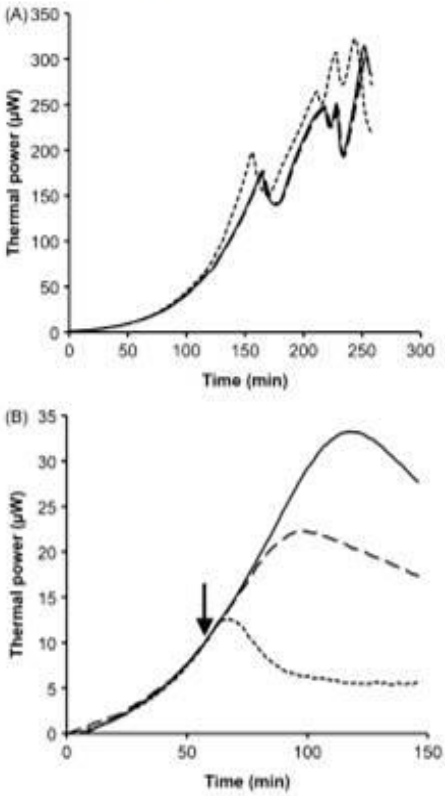


**Fig. 1.** Power–time curves (A) in the absence of ampicillin and (B) when 64 μg/mL ampicillin was added when the output reached 10 μW (indicated with black arrow). Solid lines, *Enterococcus faecium* E1162; long dashed lines, E1162Δ*esp*; short dashed lines, TX1330.

To test the effect of ampicillin on the three strains, a final concentration of 64 μg/mL was added when the cultures reached 10 μW (Fig. 1B), which is equivalent to ca. $1.2 \times 10^5$ cells/mL. The concentration chosen was double the MIC of the *E. faecium* E1162 and E1162Δ*esp* strains, but still one-half the value of their minimal bactericidal concentration (MBC) (128 μg/mL for both strains), thus ensuring a strong response without killing all cells immediately. As expected, *E. faecium* E1162 responded differently to the addition of ampicillin compared with the control strain *E. faecium* TX1330. Whereas the maximum output of the E1162 strain reached 36 μW, the heat output from the TX1330 strain (MBC = 8 μg/mL) stopped almost immediately after addition of ampicillin, reaching only a maximum of 12 μW.

The main surprise was the response of *E. faecium* E1162Δ*esp*, which showed a consistently lower output than E1162, with an average peak value of 24 μW. Thus, the output of the strain lacking Esp was only 66% of that of its parental strain. This was an unexpected result as the traditional antimicrobial susceptibility tests showed no difference in MIC between *E. faecium* E1162 and its *esp* mutant, indicating that calorimetry is more sensitive than traditional methods for determining resistance to antibiotics.

The exact role of Esp is currently not clear and it has been connected to several different processes such as biofilm formation [3] and colonisation [7]. Our findings suggest that Esp also has a role in ampicillin sensitivity. Ampicillin, like other antibiotics belonging to the penicillins, inhibits the formation of peptidoglycan, thereby weakening the cell wall structure. Esp is a large cell wall protein and we suggest that the absence of Esp leads to subtle changes in the cell wall structure that indirectly leads to changes in ampicillin resistance.

In conclusion, isothermal calorimetry is a far more sensitive method than traditional methods for analysing susceptibility to antibiotics. The method is also very rapid and would therefore be of real benefit if used clinically.

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*Competing interests*: None declared.

*Ethical approval*: Not required.

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