Characterisation of the Enterococcus faecalis EfaA gene product in infective endocarditis

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CHARACTERISATION OF THE *ENTEROCOCCUS FAECALIS EfaA* GENE PRODUCT IN INFECTIVE ENDOCARDITIS

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ABSTRACT

Enterococci are responsible for 12% of hospital infections. They represent a growing treatment problem, because of their antibiotic resistance- both intrinsic and acquired.

The aim of this project was to determine the role of the *Enterococcus faecalis efaA* gene product in infective endocarditis. The nucleotide sequence for the entire operon was obtained and analysed. Using RT-PCR it was possible to establish that *efaA* is transcribed under manganese-limited conditions. This confirmed that *efaA* belongs to a group of extracellular receptors called the Cluster 9 protein family. Sequence analysis of the region upstream of the operon identified a sequence showing homology to a DtxR box (first isolated in *Corynebacterium diphtheriae*), suggesting that *efaA* is upregulated as part of a global response to environmental metal ion concentration by *E. faecalis*. Attempts were made to construct a knockout mutant of *efaA*, but these were unsuccessful. Analysis of preliminary sequence data from TIGR allowed identification of a second Cluster 9 operon in *E. faecalis* (*adc*), predicted to respond to zinc. Additionally, three Fur protein homologues, a DtxR homologue and a thiol peroxidase gene were identified.

The *E. faecium efm* operon, which shows homology to *efa,* was sequenced. Analysis of the operon identified a region showing homology to the SirR box of *Staphylococcus epidermidis*, encoded upstream of the operon. This again suggested that the operon was under metal ion control, but this could not be confirmed by RT-PCR.

Aggregometry was used to study platelet-aggregating abilties in two *E. faecalis* strains. The strains tested were unable to aggregate platelets. The heterogeneity of several *E. faecalis* clinical isolates was examined to determine the extent of sequence conservation in the *efaA* gene. PCR-RFLP studies found that the gene was present in all strains tested and well conserved.

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1 Introduction

It has been estimated that approximately 5% of patients admitted to hospitals acquire infections (known as nosocomial infections) during their stay (reviewed by Swartz, 1994). A number of factors contribute to this problem, including the increasing age of patients and treatment of formerly untreatable diseases with invasive surgery or intensive medical therapies.

During the 1980s a new group of bacteria, mostly gram-positive, emerged as dominant pathogens in the hospital environment, amongst which were *Enterococcus* spp. The enterococci are the second most common cause of nosocomial infections, accounting for 12% of the total. Of this 12%, *E. faecalis* has been found to account for 80% and *E. faecium* 5-15% (reviewed by Murray, 1990).

1.1 Diseases Caused by Enterococci

Enterococci can be isolated from many polymicrobial infections but are also capable of causing disease on their own (Moellering, 1992). The most common infections caused are urinary tract infections (UTIs), particularly following catheterisation. Indeed catheterisation, together with broad-spectrum antibiotic use is thought to account for the significant increase in enterococcal UTIs seen since 1971 (Felmingham et al, 1992). Most infections are asymptomatic and are better thought of as colonisation, but enterococci can spread from the urinary tract to produce a generalised bacteraemia.

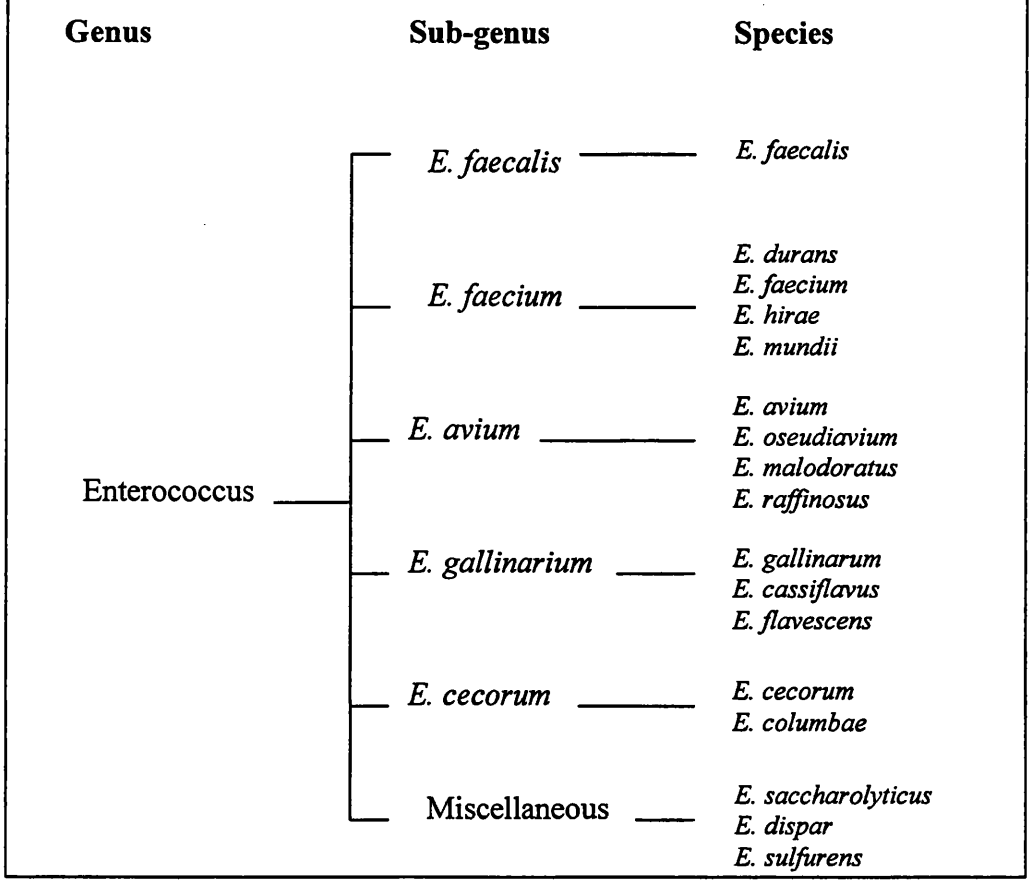
Enterococci can also be isolated from wound infections, mostly as part of a polymicrobial infection. Bacteraemias are the third most common enterococcal infection. Infective endocarditis (IE) is often seen as a result of enterococcal bacteraemia and in rare cases meningitis may occur. Endocarditis is an infection on the endocardial surface of the heart (reviewed by Scheld and Sande, 1994). It usually affects the heart valves, but can be found on septal defects or the mural endocardium (reviewed by Scheld and Sande, 1994). Enterococcal IE is a virulent disease which can have an associated mortality rate as high as 30% (Working Party, 1985). It causes 200 deaths per year in England and Wales (Working Party, 1985). The antibiotic resistance of enterococci combined with the difficulty in targeting antibiotic therapy to heart valves makes the disease very awkward to treat. This thesis will focus on enterococcal endocarditis in an effort to find novel strategies for tackling such infections. The pathogenicity of *E. faecalis* and *E. faecium* will be studied in detail, because these are thought to account for the majority of cases.

1.2 Taxonomy of Enterococcus Spp

Enterococci are gram-positive, non-spore-forming facultatively-anaerobic, catalase-negative bacteria. Until recently they were regarded as streptococci, but are now recognised to be a separate genus and to belong, with streptococci and lactococci, to the family of lactic acid bacteria (reviewed by Hardie and Whiley, 1997; Morrison et al, 1997). There are currently 17 species of enterococcus recognised (reviewed by Hardie and Whiley, 1997).

In the past two decades, 15 new species of bacteria have been proposed to be members of the genus Enterococcus.

Figure 1.1 Taxonomy of Enterococcus



Enterococci are found in many ecological niches, including the gastrointestinal tracts of man, mammals, birds, reptiles, insects and in plants. Although the lower gastrointestinal tract is thought to be the main habitat of enterococci they can be found in other places such as the upper GI tract, the genital tract and the oral cavity (reviewed by Morrison et al, 1997). They are able to survive for long periods on inanimate objects and in direct sunlight. Although they are not native to soil or water, they can live there, and their presence is regarded as a positive indicator of faecal pollution in the water supply (reviewed by Morrison et al, 1997).

Because of their complex nutritional requirements they have always been difficult to cultivate and manipulate *in vitro*: many strains are still considered non-transformable. However with advances in laboratory techniques, particularly in molecular biology and ribotyping, our understanding of these organisms has improved dramatically. Perhaps the most significant advance has come from ribotyping, which established the enterococci as a distinct genus (reviewed by Hardie and Whiley, 1997; Morrison et al, 1997).

Enterococci have a natural, intrinsic resistance to many antibiotics, which makes them difficult to treat. In addition they are able to acquire and express antibiotic resistance genes from other organisms. Serious enterococcal infections now carry a significant risk of mortality. Therapeutic options for such diseases are decreasing rapidly and it is highly probable that such infections will be impossible to treat in the near future.

Enterococci have become significant nosocomial pathogens partly because they can acquire genes by conjugation. For instance, *E. faecalis* strains can carry several types of mobile genetic elements harbouring genes for antibiotic resistance and haemolysins or other virulence factors. These conjugative elements can be divided into two classes: a) transposons, and b) sex pheromone plasmids.

1.2.1 Conjugative Transposons

A conjugative transposon may be thought of as a transposable element with the capacity to promote its own transfer in the absence of any plasmid or phage. They are capable of insertion into the chromosome and can therefore be stably maintained. In gram-positive bacteria, conjugative transposons are large and all encode tetracycline resistance of the Tet (M) class (which encodes resistance to tetracycline and minocycline) (reviewed by Scott and Churchward, 1995). Many will also encode additional resistance genes. Examples include Tn916 (16.4Kb) which encodes Tet(M) resistance only and Tn1545 (23Kb) which encodes resistance to kanamycin (*aphA-3* gene) and erythromycin (*ermAM* gene) as well (reviewed by Scott and Churchward, 1995; Caparon and Scott, 1989).

Transposons have a broad host range among gram-positive and some gram-negative species. They represent a highly efficient method of transfer of antibiotic resistance genes between unrelated bacterial genera.

1.2.2 Sex Pheromone Plasmids

Sex pheromone plasmids are almost totally exclusive to *E. faecalis* strains. Their uptake is induced by pheromones secreted by plasmid-free strains to cause clumping and conjugation with strains containing plasmids. Transfer of these elements is much more effective than with transposons (frequencies of up to 10^{-1} in sex pheromone plasmids compared with 10^{-6} in transposons)

due to the clumping mechanism (reviewed by Wirth et al, 1996). They will be discussed in more detail in section 1.7

The ability to acquire so many different genes has undoubtedly increased the pathogenicity of enterococcal strains. It has also caused concern, as these strains are capable of passing on their acquired antibiotic resistance to other gram-positive species. In this respect they should be thought of as a 'genetic reservoir' of resistance genes. Transfer of these genes to other more virulent gram-positive species must be considered a real threat.

1.3 Pathogenesis of Enterococcal Endocarditis

1.3.1 Predisposing factors for Infective Endocarditis

Any surgical or dental procedure that damages a mucosal surface colonised with bacteria can enable them to enter the bloodstream (reviewed by Scheld and Sande, 1994). Such bacteraemias can persist for up to 15 min, which is sufficient to allow access to the endocardium and initiate endocarditis.

Many dental procedures carry a high risk of inducing IE and patients deemed to be at risk of developing the disease are often treated with prophylactic antibiotics. However such treatment will never prevent all cases of IE and a proportion of cases can be attributed to dental treatment (Dajani et al, 1997).

Intravascular devices, such as IV catheters, hyperalimentation lines and intracardiac pacemaker devices can all become infected and introduce

bacteria into the bloodstream. Infections from prosthetic valves are also an increasingly common source of infection as these devices become more widely used (reviewed by Vongapatanasin et al, 1996).

IV drug abuse is a significant risk factor for IE and accounts for approximately 60% hospital admissions amongst this group of people. Cocaine use carries a particularly high risk (reviewed by Scheld and Sande, 1994). In addition to inducing bacteraemia, IV drug users traumatise the endothelial surface of the valve by frequently injecting foreign particles (reviewed by Kaye, 1985). Most cases of this type tend to affect the right side of the heart (the tricuspid valve) rather than the more common left side (the mitral valve) and are usually caused by *Staphylococcus aureus* rather than enterococci. However as IV drug abuse accounts for so many cases of IE it is significant (reviewed by Lukes and Durack, 1993).

In general immunosuppression is not a major risk factor for IE. Neutropoenic, T-cell deficient and humorally immunodeficient patients do suffer from IE but other immunosuppressed patients do not. Pre-existing antibiotic therapy in such patients may, however, cause superinfection with enterococci.

While all the above factors introduce bacteria into the bloodstream, a structural abnormality of the heart may lead to the formation of a platelet-fibrin clot to which bacteria adhere. Some abnormalities carry a greater risk than others; mitral valve prolapse has been implicated in a many IE cases.

Congenital heart disease (e.g. patent ductus arteriosis) is responsible for 6-24% of IE cases whilst degenerative cardiac lesions (e.g. post-myocardial infarction thrombus) contribute to 30-40% of cases where no other lesions are seen (reviewed by Scheld and Sande, 1994).

Although endocarditis used to be more commonly seen in younger patients it is now more often seen in the elderly - perhaps because of pre-existing cardiac lesions and increased hospitalisation or surgery in this patient group.

1.3.2 Adhesion

In order for a circulating bacterium to persist on the endocardium and cause infection, it must adhere to the tissue. This will prevent removal by the local high-velocity blood circulation and circumvent killing by host defence mechanisms (reviewed by Doig and Trust, 1993; Ford and Douglas, 1997). Adhesion is therefore a virulence factor, since a successful infection depends on its efficiency (reviewed by Baddour, 1994; Doig and Trust, 1993).

Attachment is believed to occur via a combination of high cell surface hydrophobicity and specific receptor interactions (Zareba et al, 1997). Most successful infections occur where there has been trauma to the endothelium. This denudes the upper endothelial cell layers and exposes the underlying extracellular matrix. Platelets and fibrin are deposited at the site of trauma to aid healing and both these and the extracellular matrix become targets for specific bacterial adhesins (reviewed by Baddour, 1994; Patti and Hook,

1994). Infective endocardial vegetations are therefore largely composed of fibrin, platelets, and bacterial colonies. Fibrin affords the bacteria some degree of protection against phagocytosis (reviewed by Ford and Douglas, 1997).

The extracellular matrix gives a basic structure for tissues, separating the epithelial cells from the underlying connective tissue. It mediates many biological signalling processes (reviewed by Patti and Hook, 1994). Because of its complex nature the extracellular matrix offers many targets for bacteria, including proteins, glycoproteins and carbohydrates. The most commonly cited targets for adherence are the integrins laminin, fibrinogen, collagens and vitronectin. They are involved in the adhesion of eukaryotic cells to each other and hence have a complex structure with multiple binding sites. Equally often microbial adhesins (e.g. pili) show a lectin-like activity. They bind to host carbohydrate structures on glycolipids and glycoproteins, in some cases recognising galactosyl-containing carbohydrates (reviewed by Jenkinson, 1994a).

There is very little understood of the microbial structures that bind extracellular matrix components, or their receptor targets in the extracellular matrix (reviewed by Doig and Trust, 1993). Studying the extracellular targets for enterococci *in vitro* has proved complex, as it appears that the expression of appropriate adhesins is highly dependent on growth conditions. Many studies show conflicting results and it is difficult to ascertain the true picture.

There are many structures on the cell surface of enterococci which could interact with host tissues, including polysaccharides and polypeptides. The polypeptides involved in adherence may be multifunctional. They can also be involved in nutrient transport, enzymatic activities such as synthesis and degradation or may be structural components of cell surface layers. Polysaccharides are adhesins and may have additional protective functions (reviewed by Jenkinson, 1995).

A study of enterococcal binding by Zareba et al (1997) showed that both *E. faecalis* and *E. faecium* bound poorly to fibronectin and collagen types I-IV. Both species were able to bind to vitronectin, lactoferrin (an iron source for bacteria) and thrombospondin (a glycoprotein found in platelets). However these results contrasted with Shorrock and Lambert (1989) who were able to demonstrate fibronectin binding by *E. faecalis*. This is probably due to the different binding assays used.

The adhesins expressed on the surface of an enterococcus are vitally important as this seems to determine their site of infection. Guzman et al (1991) found that *E. faecalis* strains isolated from endocarditis adhered better to endocardial cells than those isolated from infections of the urinary tract. However growth of urinary tract isolates in human serum greatly improved their binding to endocardial cells because it increased the expression of D-galactose and D-fucose-containing residues on the surface of *E. faecalis*. The D-galactose residues were also found to mediate internalisation of

enterococcus into human culture cells, which is a phenotype more often seen in endocarditis isolates. Lipoteichoic acid is thought to contain the sugar moiety involved in adherence. Interestingly, Xiao et al (1998) carried out all their binding studies after growth at 46°C and found that under these conditions *E. faecalis* was able to adhere to laminin and collagen types I-IV but not to fibronectin, fibrinogen or collagen type V. They postulated that increasing the growth temperature induces a stress response in *E. faecalis*, causing it to express different cell surface proteins.

1.3.3 Platelet Adhesion and Aggregation

The adhesion of bacteria to platelets has been closely studied since adhesion is thought to be the initial step in endocardial infection (reviewed by Baddour, 1994; Bayer et al, 1995). It is also possible for bacteria to bind to platelets in the blood circulation and then be deposited at a site of endocardial damage (reviewed by Murphy and Steckelberg, 1995). It is important to differentiate between the initial binding of bacteria to platelets and the subsequent platelet aggregation, which may be induced by the bacteria, leading to larger vegetations. Adhesion has no effect on the aggregation reaction, indeed some bacteria are able to adhere strongly to platelets but are unable to induce platelet aggregation (Bayer et al, 1995).

Once bacteria have adhered to platelets they must endure a range of processes designed to remove them. These include phagocytosis and antimicrobial factors released by thrombin-stimulated platelets. All of the

platelet factors have low molecular weight, are cationic and heat stable. They include β-lysins, platelet bactericidal protein, thrombodefensins and platelet microbicidal protein (reviewed by Murphy and Steckelberg, 1995; Dankert et al, 1995). Following bacterial adhesion, more platelets and fibrin will be deposited on the vegetation and the deposition of fibrin is a good indicator of thrombin activity. Therefore as well as having efficient adhesion a bacterium must be resistant to these thrombin-induced agents in order to cause a successful endocardial infection (Dankert et al, 1995). The endocardial vegetation is not static. Vegetation growth occurs by a complex, dynamic process, involving platelet aggregation and release, fibrin deposition, bacterial removal and then reseeding with circulating micro-organisms and loss of material via embolisation (reviewed by Ford and Douglas, 1997). Eventually the vegetation may grow and the bacteria may be held within the platelet-rich thrombus where they can reproduce, effectively protected from the host immune system.

1.3.4 Progression of Disease

An infected vegetation will have multiple consequences for the patient. Cardiac valves will be distorted or destroyed by ulceration, resulting in regurgitation of the blood flow. Even after antibiotic treatment valves may be so badly damaged that they require surgical repair (reviewed by Murphy and Steckleberg, 1985).

A persistent bacteraemia will cause the clinical signs of fever, rigours, night sweats, lethargy, anorexia and anaemia. The resulting bacteraemia will activate the host defence mechanisms resulting in immune complexes formed from associations of bacterial antigens with antibodies. The complexes may be deposited in any vessel causing inflammation (vasculitis) or vascular lesions. These can be seen in the skin as 'Osler's nodes' (small, painful, nodular lesions found on the pads of fingers or toes) or 'Roth spots' (seen in the eye where they are deposited in the nerve fibre layer of the retina). Immune complexes may also accumulate at joints where they will cause arthritis (Kumar and Clark, 1990; Stamboulian and Carbone, 1997). Parts of the vegetation can break up and be deposited in any organ (embolisms). In the skin, 'Janeway lesions' are seen on the palms of the hands or soles of the feet. Myocardial infarction or splenic and renal infarcts are commonly seen. The brain and lungs may also be affected. If left untreated the disease will progress to septicaemia. It is always fatal without antibiotic therapy.

1.4 Enterococcal Antibiotic Resistance

The treatment of enterococci has always been a problem as they are intrinsically resistant to many agents. These include cephalosporins, aminoglycosides, quinolones, lincosamides, semi-synthetic penicillinase resistant penicillins (e.g. oxacillin) and clindamycin (reviewed by Cormican and Jones, 1996; Murray, 1998, Leclerq, 1997). In comparison with streptococci they are also relatively resistant to penicillin, ampicillin and ureidopenicillins (Murray et al, 1998).

Treatment of endocarditis should be bactericidal not bacteriostatic to ensure sterilisation of the cardiac valve. This usually means combination therapy for four to six weeks (Gavalda et al, 1996; Stamboulian and Carbonne, 1997). For resistant organisms the duration of treatment may have to be extended to eight to twelve weeks (Stamboulian and Carbonne, 1997). Antibiotic penetration into vegetations is typically poor, especially with agents such as teicoplanin, making therapy additionally difficult (Aslangul et al., 1997; Murphy and Steckelberg, 1995). Infections associated with prosthetic devices, cannulae and other foreign bodies also show poor antibiotic penetration, presenting an additional treatment problem (Morrison et al, 1997).

In addition to intrinsic resistance, enterococci have acquired resistance to many other antibiotics. The species now has documented cases of resistance to chloramphenicol, tetracyclines, macrolides, streptogramins, lincosamides, glycopeptides and high level resistance to aminoglycosides and penicillins (Morrison et al, 1997).

The enterococci are an important reservoir of resistance genes (Leclerq, 1997) and they can pass their resistance on to other species. Staphylococci and enterococci can exchange genetic material and one of the biggest issues facing medicine today is the prospect that the enterococcal vancomycin resistance gene cluster will be passed on to methicillin resistant *Staphylococcus aureus* (MRSA). This has been shown to be possible *in vitro* (Noble et al, 1992). Isolated cases of vancomycin-resistant MRSA have been

documented in Japan and the USA (Tabaqchali, 1997; Hiramutsak et al, 1997; Smith et al, 1999), but these were due to cell wall thickening and increases in penicillin binding protein (PBP) 2 and murein monomer precursor, rather than the altered peptidoglycan precursors seen in enterococcal vancomycin resistance. The emergence of VanA resistance in MRSA would usher in a post-antibiotic era where infections can no longer be treated with any known antibiotics.

Conventional treatment of enterococcal endocarditis involves combination therapy with a penicillin and an aminoglycoside. Penicillins inhibit cell wall synthesis to allow the aminoglycoside to reach its ribosomal target more efficiently (reviewed by Swartz, 1994). Some controversy exists over the timing of doses, but it is generally accepted that divided daily dosings give the best results (Gavalda et al, 1996; Marangos et al, 1997).

1.4.1 Aminoglycoside Resistance

During the 1950s the aminoglycoside of choice was streptomycin but by the early 1970s 25-50% of clinical isolates showed high level resistance to streptomycin and kanamycin. In consequence gentamicin was substituted and worked well until 1979 when a strain with high level gentamicin resistance was isolated in Paris. Today greater than 50% of enterococci worldwide show resistance to penicillin-gentamicin therapy (reviewed by Swartz, 1994).

All enterococci show an intrinsic moderate level resistance to aminoglycosides (minimum inhibitory concentration, MIC 62-500 μg/ml), but this is a permeability problem normally overcome by combination therapy with a cell wall active agent. High-level resistance to streptomycin and gentamicin occurs by different mechanisms and hence it is important to test for both in the clinic.

Streptomycin resistance occurs either by a plasmid-encoded adenyl-transferase (which causes nucleotidylation of the hydroxyl group) or through a chromosomal mutation which alters the affinity of the ribosome for streptomycin (reviewed by Swartz, 1994; Leclerq, 1997; Gin and Zhanel, 1996). It does not, however confer resistance to gentamicin.

Gentamicin resistance can occur by two mechanisms but is usually via a plasmid-encoded enzyme which has both 2"-phosphotransferase and 6'-acetyltransferase activity (reviewed by Swartz, 1994). This phosphorylates the hydroxyl group on the aminoglycoside, using phosphate from ATP and acetylates the amino group using acetyl from acetyl-CoA (reviewed by Leclerq, 1997). The enzyme confers resistance to gentamicin, tobramycin, netilimicin, amikacin and cell-wall active agents, but not to streptomycin. Recently a second enzyme has been isolated which confers resistance by 2"-acetylphosphotransferase activity (reviewed by Murray, 1998). Fortunately 46% of high-level gentamicin-resistant *E. faecalis* isolates are susceptible to streptomycin.

E. faecium tends to be more resistant to aminoglycosides because it carries a 6'-acetyltransferase enzyme on its chromosome which renders all aminoglycosides except streptomycin and gentamicin inactive (reviewed by Swartz, 1994; Leclerq, 1997). In the early 1990s 70% of *E. faecium* isolates showed high-level resistance to gentamicin (reviewed by Swartz, 1994).

1.4.2 Penicillin Resistance

Enterococci are intrinsically more resistant to penicillins (MIC 2.5-5 μg/ml) than streptococci (MIC 0.02-0.08 μg/ml) (Swartz, 1994; Murray, 1998). For example, *E. faecalis* is 10-100 times less susceptible to penicillin than most streptococci, whilst *E. faecium* is 4-10 times less susceptible than *E. faecalis* (Gin and Zhanel, 1996). High-level penicillin resistance (MIC 8-64 μg/ml) has begun to emerge. β-lactamase-producing enterococci were first seen in the early 1980s but have remained at a low level with only a few isolated cases seen since then (Murray, 1998; Landman and Quale, 1997).

A far more significant cause of high-level penicillin resistance is the increased production of low affinity PBPs. These were first described in *E. hirae*, but have since been isolated in *E. faecalis* and *E. faecium* (Zorzi et al, 1996). PBPs are situated in the cell wall and are involved in cell wall peptidoglycan synthesis through transpeptidase and carboxypeptidase activities (Michel and Gutmann, 1997). They are two-domain proteins with a C terminal penicillin-binding domain and an N-terminal catalytic domain. Penicillins inactivate high molecular weight PBPs (i.e. PBPs 1, 2 and 3) but

they are unable to bind to the chromosomally-encoded PBP5 of *E hirae*, 56R of *E. faecalis*, PBP5fm of *E. faecium* or the plasmid-borne PBP3r of *E. hirae*. All these proteins show strong homology suggesting a common ancestor. They are induced in the presence of penicillin and are able to carry out all the functions of the other PBPs when these are saturated or inhibited by penicillins. Their transcription is negatively controlled by the PBP5 synthesis repressor (*psr*) gene, located 1kb upstream of PBP5, which controls expression of several other cell-wall related properties (e.g. autolysis and sugar expression in the cell wall) (Massida et al, 1996; Massida et al, 1998).

1.4.3 Glycopeptide Resistance

Vancomycin resistance was first reported in the UK by Uttley et al (1988). It has since spread to become a significant problem worldwide. It is a particular problem in intensive care units where it can create havoc with the treatment of severely ill patients. In one estimate, 13.6% of enterococci from intensive care patients were vancomycin resistant (Cars, 1997). The mortality associated with vancomycin-resistant enterococci (VRE) can approach 70%. Many isolates also show multiple resistance profiles (Gin and Zhanel, 1996).

Two forms of transferable vancomycin resistance have been described. The VanA type confers high level resistance to both vancomycin (MIC $\geq$ 64 μg/ml) and teicoplanin (MIC $\geq$ 16 μg/ml); whilst the VanB type confers variable resistance to vancomycin only (MIC 4 - > 1024 μg/ml). The low

level of resistance it confers makes it difficult to detect and it may be more prevalent than was first thought. Although both systems are homologous they are not identical and differ particularly in their induction. VanA resistance is switched on in the presence of vancomycin and teicoplanin but VanB resistance is induced only by vancomycin.

Figure 1.2 VanA Gene Cluster (after Arthur et al, 1998)

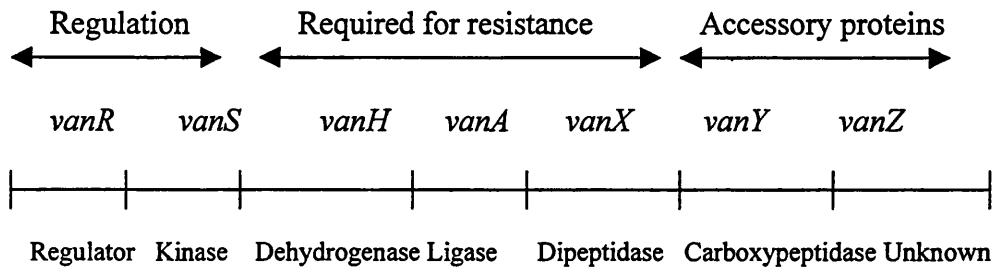
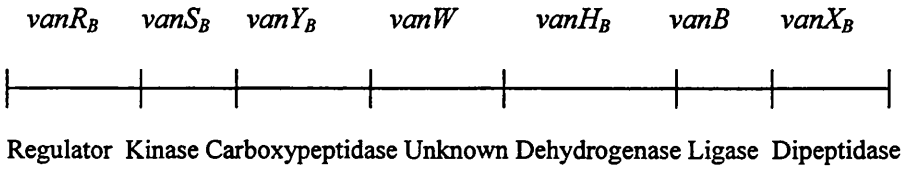


Figure 1.3 VanB Cluster (After Evers and Courvalin, 1996)



The *vanA* and *vanB genes* and the *vanH* and *vanX* genes show approximately 70% homology to each other, whilst *vanS* and *vanR* show 30% homology (Evers and Courvalin, 1996).

Glycopeptides have a bracelet configuration and inhibit cell wall synthesis by binding the D-alanine-D-alanine portion of peptidoglycan peptide precursors in the pocket formed by its structure. This prevents subsequent

transglycosylation and transpeptidation reactions which cross link peptidoglycan (Reynolds et al, 1994).

The VanA and VanB resistance operons contain a transmembrane receptor protein (VanS or $VanS_B$) which binds glycopeptides directly. Following activation of VanS the response regulator VanR or $VanR_B$ is phosphorylated and can then direct the transcription of *vanA* (*vanB*) and *vanH* (*vanH$_B$*) (Evers and Courvalin, 1996). VanH and $VanH_B$ are α-ketoacid reductases which generate D-lactate from pyruvate (Reynolds et al, 1994; Swartz, 1994). VanA and VanB are ligases which incorporate the D-lactate into the terminal portion of the peptidoglycan precursors to create D-ala-D-lac-containing residues (Michel and Gutman, 1997). This ester linkage replaces the D-ala-D-ala amide linkage that forms a crucial hydrogen bond with a carbonyl group on the vancomycin skeleton (reviewed by Nicas et al, 1997). As a result vancomycin cannot bind to D-ala-D-lac residues and the bacterium becomes resistant to vancomycin (Swartz, 1994; Reynolds et al, 1994). VanX ($vanX_B$) and VanY ($VanY_B$) proteins form a fail-safe mechanism. VanX and $VanX_B$ are dipeptidases which break down D-ala-D-ala-containing residues in the cytoplasm so that only D-ala-D-lact residues are incorporated into the cell wall (Reynolds et al, 1994). VanY ($VanY_B$) are also carboxypeptidases but cleave D-ala-D-ala peptides from peptidoglycan precursor molecules (N-acetylmuramic acid linked to the pentapeptide L-ala-D-glu-L-lys-D-ala-D-ala) (Chu et al, 1996). The *vanZ* gene of the VanA cluster confers low-level resistance to teicoplanin and is required for full virulence, although the mechanism of action is unknown (Chu et al, 1996;

Evers and Courvalin, 1996; Murray 1998). Similarly, the function of the VanW gene product of the VanB operon is unknown (Murray, 1998; Evers and Courvalin, 1996).

Intrinsic low level resistance to glycopeptides can also occur in some enterococci. This is chromosomally-encoded and is designated VanC. *E. gallinarum* ($VanC_1$), *E. casselflavus* ($VanC_2$) and *E. flavescens* ($VanC_3$) all express this phenotype. Peptidoglycan precursors terminating in D-ala-D-ser are produced, which vancomycin is unable to bind to (Murray, 1998). A fourth type of resistance, VanD has also been described, but is poorly characterised (Chu et al, 1996).

1.5 Treatment Of Enterococcal Infections

1.5.1 Prophylaxis

Appropriate antibacterial prophylaxis can prevent 49-94% of infective endocarditis cases in patients undergoing procedures with a risk of bacteraemia (Stamboulian and Carbonne, 1997). However prophylaxis should be targeted to patients who are most at risk to prevent the development of resistance. Conditions which confer a risk of endocarditis are listed in Table 1.1.

Table 1.1 Risk Factors for Endocarditis (after Stamboulian and Carbonne, 1997)

Assessment of risk	Underlying heart condition
High Risk	prosthetic valves congenital heart disease causing cyanosis previous bacterial endocarditis surgical systemic pulmonary shunts
Moderate Risk	rheumatic and other acquired valvular dysfunction mitral valve prolapse with regurgitation most congenital cardiac malformations hypertrophic cardiomyopathy
Negligible risk (no prophylaxis necessary)	aortocoronary bypass cardiac pacemaker peripheral vascular disease

Any procedures which cause bleeding can result in a bacteraemia that will cause endocarditis. Therefore even catheterisation could be considered a risky process. Appropriate hygiene measures should be taken to ensure that patients are not exposed to bacteria. However antibiotics are not usually considered necessary for most procedures. It is widely acknowledged that dental procedures carry the greatest risk of causing infective endocarditis (Stamboulian and Carbonne, 1997). Good oral health should be encouraged in all at-risk patients to prevent bacterial seeding during teeth cleaning. Antibiotic prophylaxis should be used for all dental procedures where there is a risk of significant bleeding. A one-off dose of amoxycillin 3g one hour before the procedure is the recommended regimen in Britain. Clindamycin 600mg or occasionally clarithromycin or azithromycin can be used if the

patient is allergic to penicillin (Dajani et al, 1997; Stamboulian and Carbonne, 1997; Murphy and Steckelberg, 1995).

1.5.2 Treatment Of Aminoglycoside-Resistant Enterococci

High-level gentamicin resistance represents a problem in therapy as it stops bactericidal therapy and bacteriostatic therapy may only just be achieved (Landman and Quale, 1997). In some cases high-dose ampicillin treatment can be sufficient, but unusual combinations of agents may be needed (e.g. ciprofloxacin + ampicillin) (Landman and Quale, 1997; Tripodi et al, 1996). A glycopeptide may be needed if the patient is allergic to penicillin but caution must be exercised to avoid inducing vancomycin resistance.

1.5.3 Treatment Of Penicillin-Resistant Enterococci

β-lactamase-producing enterococci can be treated with β-lactamase inhibitors (e.g. sulbactam, tazobactam). Resistance caused by low affinity PBPs can be treated with a glycopeptide + aminoglycoside. In some cases, simply increasing the dose of β-lactam may be sufficient (Rybak and McGrath 1996).

1.5.4 Treatment Of Vancomycin-Resistant Enterococci

Vancomycin-resistant enterococci represent a major therapeutic problem. As a reflection of the complex nature of this problem there is no generally

accepted 'ideal therapy.' Treatment will usually depend on local resistance patterns.

VanB resistance may be treated with teicoplanin. However care should be exercised as these bacteria can be induced to express VanA resistance on longer therapy due to the selection of more resistant mutants (Aslangul et al, 1997; Nicolau et al, 1996). Bactericidal therapy is again difficult to achieve. Combination therapy is useful because it usually shortens the treatment period and therefore reduces the risk of inducing new resistance (Nicolau et al, 1996; Aslangul et al, 1997). If vanB resistance confers only low level vancomycin resistance, a combination of vancomycin or teicoplanin with streptomycin can produce very good results (Nicolau et al, 1996).

VanA and amoxycillin resistance has been treated successfully with triple therapies. A β-lactam, a glycopeptide and gentamicin, or ciprofloxacin + rifampicin + gentamicin have both been used effectively (Michel and Gutmann, 1997).

The new streptogamin antibiotic quinupristin/dalfopristin (Synercid) has also been used to treat VRE. It shows 8-16 fold greater activity against *E. faecium* than *E. faecalis* (Cormican and Jones, 1996). Streptogramins can take two forms, A and B, and each form binds to a different position on the bacterial ribosome. Synercid exploits this by using both forms of streptogamin to produce synergism. Dalfopristin binds to the ribosome and alters the shape to enable quinupristin to bind more efficiently. Thus the

combination of the two agents is much more effective than either agent alone (reviewed by Nicolau et al, 1997; Rybak and McGrath, 1996). The combination can also enter human phagocytes which is vital as *E. faecium* has been shown to survive intracellular killing in phagosomes (reviewed by Rakita, 1998).

Nosocomial VRE infections mostly affect the severely ill who have already received extensive antibiotic therapy. Patients with leukaemia, bone marrow transplant patients and those undergoing haemodialysis or in intensive care are particularly at risk. To avoid the problem occurring in the first place it is important to avoid the overuse of vancomycin or agents which can encourage superinfection with resistant organisms (Cars, 1997). Previous treatment with vancomycin is nearly always found in VRE outbreaks and it has been estimated that 50-60% of vancomycin usage is inappropriate (Gin and Zhanel, 1996). Glycopeptides should only be used to treat enterococci when β-lactam resistant bacteria are known to be present, or are strongly suspected (Gin and Zhanel, 1997). Oral vancomycin should be a second line treatment after metronidazole in cases of *Clostridium difficile* (Gin and Zhanel, 1997; Rao et al, 1997). The use of broad-spectrum antibiotics should be avoided as this can lead to superinfection with enterococci that show intrinsic resistance to many antibiotics. Ciprofloxacin, metronidazole and third generation cephalosporins have all been implicated in this problem (Rybak and McGrath 1996; Cars, 1997). During a VRE outbreak it is important to isolate and treat all carriers of VRE, partly to prevent the spread of the outbreak and partly

because colonisation with VRE can often lead to a VRE infection if left untreated (McDonald et al, 1997).

Although most cases of VRE are nosocomially acquired, VRE colonisation is seen in the community. This may have been due at least in part to the unrestricted use of glycopeptides in animal husbandry. Vancomycin has never been used to treat animals, but the glycopeptide avoparcin has been used as a growth promoter in animal feedstuffs. This led to animals becoming colonised with VRE and the resistance was passed on through the food chain to humans. Avoparcin has now been banned in foodstuffs, but it is worth noting that many antibiotics are still used in agriculture. These include cell wall synthesis inhibitors, protein and DNA synthesis inhibitors and substances that moderate cation permeability (ionophores). Most of these substances are active against gram-positive bacteria and it is vital that constant surveillance occurs to avoid resistance being passed through the food chain again (Bates et al, 1994; Devriese et al, 1996; Helmuth and Protz, 1997; Michel and Gutmann, 1997; Murray 1998;).

1.6 Future Targets for Therapy

With the ever-decreasing treatment options for enterococcal endocarditis it is important to look for new antibiotics to treat this infection. Modifications of existing drug structures may provide short-term relief from resistance but a long-term solution to the problem should be found. This may involve identifying new targets for antibiotics. We may also need to find more efficient ways of targeting drugs to cells; to reduce the treatment duration

and the number of drugs needed for bactericidal therapy. It may even be necessary to find a vaccine against enterococci, to be given to patients considered to be at risk of developing endocarditis. Whatever course of action is taken, improved understanding of enterococcal processes is needed in order to define the virulence factors which make them pathogenic.

There are two areas of research which have been cited as possible targets for therapy: the sex pheromone plasmids (especially cytolysin-containing plasmids) and EfaA, a member of the Cluster 9 protein family.

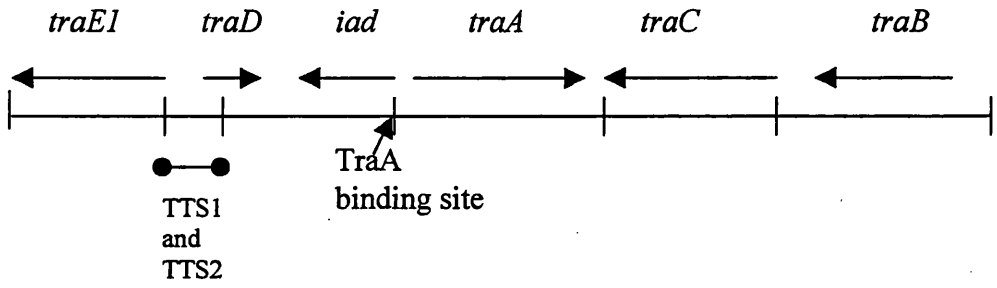
1.7 Virulence Factors on Sex Pheromone Plasmids

Pheromones can be defined as 'Substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific action, e.g. a definite behaviour or developmental process. The principal of minute amounts being effective holds' (reviewed by Wirth et al, 1996). Pheromones are one of the few ways a single bacterium can obtain information from others of the same species (Wirth et al, 1996). A plasmid-free cell (recipient cell) excretes a sex pheromone. It is detected by a plasmid-containing cell (donor cell) in the proximity, which then initiates conjugation (reviewed by Dunny and Leonard, 1997).

The *E. faecalis* sex pheromones are chromosomally-encoded, short, hydrophobic peptides and are specific for a particular plasmid. They are excreted in low amounts (usually 10^{-8} to 10^{-9} M) but it has been estimated

that only 1 – 10 molecules per cell (about 10^{-11} M) are needed for induction (reviewed by Dunny et al, 1995; and Wirth et al, 1996). It is not easy to distinguish between sex pheromone plasmids, except by the pheromone that induces them. Over 20 different restriction profiles exist (Wirth et al, 1996). A strain carrying one sex pheromone plasmid is still able to act as a recipient for others and many clinical isolates will contain several different plasmids.

Figure 1.4 The Plasmid pAD1 Regulation Locus (After Fujimoto and Clewell, 1998)



Plasmid pAD1 is arguably the best-studied sex pheromone plasmid. It is induced by the pheromone cAD1, which attaches to the cell via a receptor protein (TraC). TraC exploits the chromosomally encoded *opp* (oligopeptide permease) operon to transport the pheromone into the cell. The *opp* operon codes for an ABC transporter (see section 1.8). The internalised pheromone binds to the negative regulator traA, which is located at the *iad* promoter site. Bound TraA causes a conformational shift in the DNA that blocks complete transcription of the positive regulator TraE1 (instead it terminates at the transcription terminators TTS1 and TTS2). When cAD1 binds to TraA it releases TraA from its binding site and allows complete transcription of TraE1 (Fujimoto and Clewell, 1998).

TraE1 can also induce its own production through the promoter located within TTS2. Negative regulation occurs via the peptide iAD1, which reduces the sensitivity of the system so that it is only switched on when recipient cells are very close to donors. The inhibitor iAD1 is transcribed from the *iad* promoter site (Bastos et al, 1997). The region contained between the *iad* promoter and the first transcription termination site TTS1 is known to be very complex and has not been well studied. There is evidence for significant secondary structure in the potential transcripts and at least three RNA species could be involved in promoting or repressing expression (Bastos et al, 1997). TraB is believed to be co-transcribed with the extracellular receptor TraC and it sits in the membrane to prevent self-induction of conjugation (Dunny and Leonard, 1997). A 23 amino acid peptide TraD has also been detected. It has an important, but unknown, role in conjugation (Fujimoto and Clewell, 1998).

TraE1 promotes the transcription of 'aggregation substance' (Asa10). Aggregation substance causes the characteristic 'clumping' mechanism, by binding to enterococcal binding substance produced by recipient cells. Donor and recipient cells thus form aggregates to facilitate efficient conjugation (reviewed by Clewell, 1993). Asa10 is a 142kD signal peptide. It is anchored to the cell membrane by its C terminus, leaving a 74kD protein visible on western blots. The molecule has a random coil structure and an amorphous appearance on the cell surface. It appears in hair-like structures mostly on the 'old' cell wall (reviewed by Wirth et al, 1996; Dunny et al,

such as lanthionine and methyllanthionine. Class II bacteriocins are non-lantibiotics. They, too, are small (<13kD) proteins which are heat stable, cationic and hydrophobic peptides containing no modified amino acids (Cintas et al, 1998). Both types of bacteriocins are ribosomally synthesised as precursor peptides containing an N-terminal leader sequence which is cleaved as the bacteriocin is exported out of the cell. The leader sequences are often glycine-based and act as a recognition signal for the processing and secretion proteins (Cintas et al, 1998).

Cytolysin is a lantibiotic as shown by the presence of lanthionine and methyllanthionine on both peptide subunits (Booth et al, 1996). However cytolysin differs from other lantibiotics in many ways. It is able to lyse eukaryotic cells, it requires the interaction of two subunits for its activity and it has been implicated in virulence (Booth et al, 1996). The cytolysin determinant is composed of two regions encoded on five open reading frames. The first region (region A) is composed of *cylA*. CylA activates cytolysin and provides the bacterial cell with immunity to its effects. The second region (termed region L) encodes the cytolysin subunits and processing and secretion apparatus (Tomita et al, 1997). The two cytolysin peptide subunits $CylL_L$ and $CylL_S$ are synthesised ribosomally. They have a hydrophilic amino terminal and a hydrophobic carboxy terminal (Gilmore et al, 1994). They show no homology to each other except for a 26 amino acid region on both subunits, which acts as a recognition sequence for export. After synthesis the two subunits are modified in the cytoplasm by CylM. This may be the stage at which lanthionine and its derivatives are formed

(Gilmore et al, 1994). The two subunits are now exported through CylB, which is an ABC transporter. Further modification is believed to occur during transport. The two subunits are externalised with an amino terminal truncation ($CylL_L$' and $CylL_S$') (Booth et al, 1996).

The fifth protein in the cytolysin operon is transcribed separately from the others (Gilmore et al, 1994). CylA is involved in the final activation of the two subunits to give mature cytolysin. It is a serine protease and is thought to cleave the two subunits again ($CylL_L$'' and $CylL_S$'') before they are assembled and become active (Segarra et al, 1991). CylA is also thought to confer immunity to cytolysin. As immunity is a function of CylA concentration, it is thought most likely that the high concentration of component A in the microenvironment around the cell protects it from lysis (Segarra et al, 1991).

Haemolysins are needed by bacteria *in vivo* to inhibit leukocyte function and lyse erythrocytes to provide iron and other nutrients to bacterial cells (Chow et al, 1993). The role of cytolysin in virulence has remained controversial and is often blurred with the role of aggregation substance. The binding of aggregation substance to eukaryotic cells may bring cytolysin closer to its target and thus aid its activity. Cytolysin is believed to help enterococci in invasive diseases by altering leukocyte function to aid the early and uncontrolled release of inflammatory agents from damaged tissue of phagocytes (Jett et al, 1992). It may initiate endocarditis by injuring endocardial cells (Huycke and Gilmore, 1995).

In a mouse peritonitis model, transposon insertion mutants of pAD1 were significantly less virulent than wild-type strains exhibiting full cytolysin activity (Chow et al, 1993). Similarly in an endophthalmitis model of enterococcal infection, cytolysin-producing strains caused a much more serious disease. Cytolysin was found to penetrate and destroy cells deep within the retina to cause faster and more serious, devastating damage to vision than cytolysin-free strains (Jett et al, 1992).

It would therefore seem feasible to target the pAD1 plasmid for therapy. Inhibition of cytolysin and aggregation substance would reduce the virulence of enterococci and perhaps even render them incapable of causing disease. However these promising findings have been hard to reproduce in models of endocarditis infection. One study showed that 84% of endocarditis isolates and 68% of blood isolates had no haemolytic activity (Coque et al, 1995). More hopefully, a rabbit endocarditis model showed aggregation substance could mediate adhesion to cardiac cells and hence increase vegetation weight. Cells containing both aggregation substance and cytolysin were found to be more virulent. However mutants containing cytolysin but no aggregation substance were found to have no greater virulence than plasmid-free cells, leading to speculation that cytolysin may not be essential for virulence (Chow et al, 1993).

Therefore whilst aggregation substance- and cytolysin-producing strains do seem to cause a more virulent infection when present, neither plasmid-

encoded factor appears to be essential for virulence. About 45% of isolates contain neither factor (Coque et al, 1995), suggesting that eukaryotic cell damage and adherence must arise through other mechanisms. Although targeting sex pheromone plasmids would be an attractive and specific mechanism for controlling diseases caused by enterococci, it appears that it will not be an effective treatment for endocarditis.

1.8 Cluster 9 Proteins

Aitchison et al (1987) identified a 37/40 kD *E. faecalis* antigen that is only expressed during endocarditis. Since that time the protein has been cloned and sequenced (Lowe et al, 1995) and has been designated EfaA (for *Enterococcus faecalis* antigen A). Work by Xu et al (1997) confirmed the importance of EfaA in virulence. Singh et al (1998) created a knockout mutation of EfaA, which showed reduced virulence in a mouse model of peritonitis.

EfaA shows homology to streptococcal surface proteins that have been implicated in adhesion. These include FimA from *Streptococcus parasanguis*, ScaA from *Streptococcus gordonii*, SsaB from *Streptococcus sanguis* and AdcA from *Streptococcus pneumoniae*. All of these proteins are believed to be ATP binding cassette (ABC) transporters. They form a family of proteins that do not correspond to any of the eight groups of extracellular receptors defined by Tam and Saier (1993). Dintilhac and Claverys (1997) therefore designated this group 'Cluster 9 proteins.' They have previously

been called the lipoprotein receptor antigen 1 (Lra1) family (reviewed by Jenkinson, 1994a ,b).

Table 1.2 Cell Surface Transport Proteins (After Tam and Saier, 1993).

Group	Transport Function
Cluster 1	Oligosaccharides, glycerol-3-phosphate
Cluster 2	pentoses and hexoses
Cluster 3	polar amino acids and opines
Cluster 4	alipathic, hydrophobic amino acids
Cluster 5	peptides and nickel
Cluster 6	multivalent inorganic anion binding
Cluster 7	divergent, multivalent organic polyanion binding
Cluster 8	organic iron complexes

Table 1.3 Cluster 9 Proteins (After Dintilhac et al, 1997)

Name	Species	Substrate
PsaAv	*Streptococcus pneumoniae*	Manganese
SsaB	*S. sanguis*	(Manganese)
ScbA	*S. crista*	(Manganese)
ScaA	*S. gordonii*	(Manganese)
FimA	*S. parasanguis*	(Manganese)
PsaA	*S. pneumoniae ?*	(Manganese)
SpyP	*S. pyogenes*	(Manganese)
EfaA	*E. faecalis*	(Manganese)
Step	*Staphylococcus epidermidis*	Iron ?
H10362	*Haemophilus influenzae*	Iron ?
YfeA	*Yersinia pestis*	Iron
MntC	*Synechocystis*	Manganese
Spy9	*Staph. epidermidis*	(Zinc ?)
AdcA	*S. pneumoniae*	zinc
YcdH	*Bacillus subtilis*	zinc
ZnuA (YebL)	*Escherichia coli*	zinc
H10119	*H. influenzae*	(zinc)
Syn9	*synechocystis*	(zinc)
Ew1A	*Erysipelothrix rhusiopathiae*	?
Trompl	*Treponema pallidum*	?

Key
(not yet proven)
? = Classification unclear

The function of these proteins is only just beginning to be characterised. Whilst some members of the family have been shown to be involved in adhesion it has become clear that this is not their only role.

1.8.1 Structure Of Cluster 9 Protein Transport Operons

All ABC-like proteins have common components: one or two cytoplasmic ATP-binding proteins (ATPases), one or two hydrophobic membrane proteins and one extracellular solute binding protein. In gram-negative bacteria the solute binding protein is situated in the periplasm whereas gram-positive extracellular proteins are lipid-modified at their N-terminus to enable them to be anchored to the cell membrane (Reviewed by Jenkinson, 1994a, b).

The lipoprotein moiety of the gram-positive ABC transporter has four regions. The first 32 amino acids including the 19-or 20-residue 'leader sequence' are the least well conserved. This sequence is believed to direct transport of the protein across the cytoplasmic membrane. At the end of the leader peptide is the signal L S/A, A/G,↓, C, S, G which is the cleavage site for the enzyme signal peptidase II. The N terminal cysteine of the mature protein is anchored to palmatic acid of the cell membrane via an acyl-glyceride bond. Signal peptidase then cleaves the hydrophobic tail of the leader sequence. This maintains the binding protein close to the cell membrane components of the transport system (Tam and Saier, 1993; Kolenbrander et al, 1994). The lipid modification of the N terminus blocks

specific channel with the ATP binding proteins providing the energy for transport (Tam and Saier, 1993).

The cytoplasmic ATPases are evolutionally the most conserved proteins among the ABC transporters. Again, it is thought that there are two ATPases for each receptor protein. They are probably anchored to the membrane via a single transmembrane helix (Tam and Saier, 1993). The proteins have two conserved domains, known as Walker motifs A and B, which are thought to be the binding site for the nucleotide ATP.

Figure 1.6 shows the basic structure of all ATPases. The 'linker peptide' is the signature sequence and is unique to the ABC family. It is involved in ATP binding whilst the rest of the helical domain is not. Instead it is thought to interact with the membrane protein. The aspartate residues in the Walker A and B sites play a role in directing Mg^{2+} which is needed for ATP activity and in the correct positioning of ATP for catalysis. The 'switch region' is thought to be involved in signal transport following ATP hydrolysis (Schneider and Hunke, 1998).

1996). More recently it became clear that these receptors are also closely connected with metal ion transport.

1.8.2 Metal Ion Transport

Dintilhac et al (1997) concluded that the Adc operon was an ABC-type zinc permease; whilst the Psa operon was an ABC-type manganese permease. From phylogeny studies they grouped the cluster 9 family into transporters of zinc, manganese or iron (see table 1.3). Work by other groups has confirmed the metal binding properties of some of these lipoproteins (Kolenbrander et al, 1998; Bearden et al, 1998; Bartsevich and Pakrasi, 1995). EfaA was presumed to transport manganese in these reports.

1.8.2.1 Manganese

Manganese is an important element for bacteria. It is used in the synthesis of secondary metabolites, bacterial sporulation and in the production of many antigens and toxins. It is implicated in the adhesion of some mutans group Streptococci (Kolenbrander et al, 1998). Several enzymes use manganese as a cofactor in their reactions. These include redox enzymes (Mn-superoxide dismutase and Mn catalase) and metabolic enzymes (pyruvate carboxylase and phosphoenolpyruvate carboxylase) (Bartsevich and Pakrasi, 1995). Manganese is most needed at elevated oxygen concentrations where manganese superoxide dismutase is most active.

1.8.2.2 Zinc

Zinc (and to a lesser extent manganese) affects competence. *S. pneumoniae* cells which are starved of zinc do not respond to the pheromone competence stimulating peptide. Zinc is most likely required during the transcription of competence genes (Dintilhac et al, 1997).

1.8.2.3 Iron

Although iron is the fourth most abundant element in the earth's crust it is easily oxidised to insoluble ferric hydroxide and thereby becomes unavailable to bacteria. Many iron-scavenging agents have been identified, particularly in gram-negative bacteria. Siderophores (iron chelators), extracellular lactoferrin and transferrin and haemolysins (which release iron from haem and haemoglobin) are all well documented methods of obtaining iron (Litwin and Calderwood, 1993; Smith, 1998). The iron supply inside a human host is even scarcer. An inducible iron transport system, such as the *Yfe* operon in *Yersinia pestis* is therefore vitally important for the survival of the bacterium within the host (Bearden et al, 1998; Hill et al, 1998).

It is not yet clear whether the receptor proteins bind metal ions directly; however their expression is increased when the metal ion concentration is decreased. For example, ScaA is induced by decreasing the available manganese, or by increasing zinc. Zinc is believed to compete with manganese for uptake (Kolenbrander et al, 1998). It is also clear that the

The thiol peroxidase shows homology with *Upp* identified by Cha et al (1996) in *E. coli*. It metabolises H_2O_2 and other damaging oxygen radicals to inert substances. As superoxide dismutase activity requires manganese in Streptococci, it is highly likely that increased manganese transport coupled with thiol peroxidase action will greatly aid the bacterium's stress response (Kolenbrander et al, 1998). However a thiol peroxidase is not found downstream of *efaA*, suggesting that another form of regulation occurs in enterococci.

It is perhaps more appropriate to consider how metal ions are able to promote or repress the transcription of cluster 9 proteins. Iron dependant repressors regulate two recently sequenced ABC transporters, the *sit* operon of *S. epidermidis* and the *yfe* operon from *Y. pestis*.

The *sit ABC* operon is induced in low iron concentrations (Cockayne et al, 1998). Regulation occurs via *sirR*, which is located upstream of the *sit ABC* operon. Both iron and manganese can bind to SirR and regulate *sit* operon transcription. SirR binds to the *sit* operon promoter (in a 19bp palindromic 'Sir' box) and prevents transcription (Hill et al, 1998). SirR belongs to the DtxR family. DtxR represses diptheria toxin synthesis in *Corynebacterium diptheriae*. Therefore although all these repressors bind to DNA when the iron concentration is high, they may control the expression of many virulence genes, not just those involved in metal ion transport. It is therefore possible that SirR may regulate additional genes; perhaps ones involved in adhesion, or other virulence factors. As iron is severely limited in the host

environment the metal ion concentration could be a valuable environmental sensor for bacteria; enabling them to detect and produce a co-ordinated response to their environment.

A similar regulatory system is seen in the *yfe* operon. Here gene expression is thought to be controlled by a 'Fur homologue'; responding both to iron and manganese (Bearden et al, 1998). The 'ferric uptake regulator' or *fur* gene was first identified in *E. coli*. Its function is similar to the DtxR repressor, but the two are not interchangeable (Litwin et al, 1992). Both DtxR and Fur homologues are found in *S. epidermidis*, suggesting that the two systems can co-exist (Heidrich et al, 1996).

Some of the Cluster 9 lipoproteins have been investigated as possible target vaccinogens. A specific antibody to one of these proteins may confer immunity by increasing the clearance of bacteria and preventing adherence. An anti-FimA antibody was found to confer immunity against *S. parasanguis* endocarditis (Viscount et al, 1997). This immunity was thought to be caused by inhibiting adhesion of bacteria to platelets and fibrin. Recent work with PsaA has called into question the wisdom of using these proteins (Novak et al, 1998). PsaA was found to be a penicilllin tolerance gene. Tolerance, which should be distinguished from resistance, occurs when a strain stops growing in the presence of an antibiotic, but does not go on to die rapidly. In pneumococci this is dependant on autolysin (which lyses the cell wall), that is either not triggered or is not present. As previously mentioned the Psa operon affects the expression of choline binding proteins on the cell surface.

LytA, the major autolysin, is a choline binding protein and is absent in *psa* mutants, imparting penicillin tolerance to such strains (Novak et al, 1998). The use of these lipoproteins as vaccine targets should be treated with caution, as it is vital not to introduce further antibiotic tolerance into organisms that are already difficult to treat.

Whilst it is evident that the cluster 9 proteins are important virulence factors their precise function still needs to be clarified. The induction of all cluster 9 proteins merits further study. If they were able to regulate the production of other virulence factors, they would be a very useful drug target. It is particularly significant that EfaA is found in all enterococcal IE isolates tested (Aitchison et al, 1987). It would provide a very specific drug target for this disease. However further work on all these antigens is needed to clarify their precise function in cells and their role in pathogenesis.

1.9 Aims of This Project

This project aimed to study the role of *E. faecalis* EfaA in infective endocarditis. The effect of metal ions on the transcription of *efaA* would be studied. The entire *efa* operon needed to be sequenced and, additionally, the homologous operon in *E. faecium* needed to be identified and sequenced. Detailed studies on EfaA required inactivation of this gene, so attempts were made to construct a mutant by insertional inactivation.

In addition to these main aims, the project also tried to study the occurrence of EfaA in several clinical strains of *E. faecalis*. To aid laboratory work,

EfaA was purified by ammonium sulphate precipitation and was used to generate antibodies in a rabbit. These antibodies could then be used in immunoblotting experiments.

2 METHODS

2.1 Bacterial Strains and Media

E. faecalis EBH1 was isolated from a patient (GP) with severe enterococcal endocarditis at Birmingham Heartlands Hospital, Birmingham, UK (Aitchison et al, 1986). *E. faecium* UB1 was isolated from the same hospital, but there were no details available on the type or severity of the infection it caused.

Enterococcal strains were grown in Brain Heart Infusion (BHI-Oxoid, Basingstoke, UK) broth, or M9-YE medium (M9 salts, 5 x 0.64 % $Na_2HPO_4.7H_2O$, 0.15 % KH_2PO_4, 0.025 % NaCl, 0.05 % NH_4Cl, 0.3 % (Sigma Chemical Co., Poole, Dorset), yeast extract (Oxoid) with 0.2 % glucose (Sigma) and 0.1 % casamino acids (Difco, Detroit, MI)). When an especially rich medium was required, cells were grown in BHI supplemented with 1 % yeast extract (BHY broth). For experiments requiring a minimal medium, cells were grown in 1 % yeast extract. Unless otherwise stated all enterococcal strains were grown anaerobically at 37 °C.

When a chemically defined medium was required for *E. faecalis* a formulae given by Shockman et al (1961) was used. This contained, per litre, 400 mg KH_2PO_4, 300 mg K_2HPO_4, 26.65 g Na_2HPO_4, 16.45 g NaH_2PO_4, 20 g glucose, 6 g sodium acetate, 600 mg ammonium sulphate, 200 mg magnesium sulphate, 10 mg NaCl, 10 mg ferrous sulphate, 10 mg manganese sulphate.

2.5 Minimum Inhibitory Concentration (MIC) of an Antibiotic

To each of six test tubes was added 0.1 ml of an overnight bacterial culture and 1.9 ml double strength BHI broth. A solution of antibiotic was made to a concentration of 100 mg/ml. A series of two-fold dilutions was made to give six 2 ml antibiotic solutions. These solutions were added to each of the six test tubes to give the final concentrations of 25 μg/ml, 12.5 μg/ml, 6.25 μg/ml, 3.125 μg/ml, 1.56 μg/ml and 0.78 μg/ml. Tubes were grown at 37 °C overnight and the lowest concentration of antibiotic which inhibited bacterial growth was designated the MIC.

2.6 Preparation of Plasmid DNA from Bacterial Cells by Alkaline Lysis (Mini-Prep).

The method used was that described by Sambrook et al (1989). A single bacterial colony was transferred to 10 ml LB medium containing the appropriate antibiotic. This was incubated overnight at 37 °C in a shaking incubator.

Following incubation 1.5 ml culture was transferred to a micro-centrifuge tube and centrifuged at 14,000-x g for 2 min. The pellet was resuspended by vigorous vortexing in 100 μl solution I (50 mM glucose, 25 mM tris, 10 mM EDTA). Two hundred microlitres of freshly prepared solution II (0.2 M NaOH, 1 % SDS) were added and the contents of the tube were mixed by inverting gently. The tubes were placed on ice for 10 min, then 150 μl Solution III (3 M potassium acetate, 1.9 M glacial acetic acid) was added.

The sample was incubated at room temperature for 5 min. Neutralisation solution (350 µl) was added and the tube was mixed by inversion.

The bacterial lysate was centrifuged at 14,000-x g for 10 min at room temperature. The cleared lysate was transferred to a spin column in a 2 ml collection tube and was then centrifuged at 14,000-x g for 1 min at room temperature. The column was washed with 750 µl column wash solution and was centrifuged at 14,000-x g for 1 min. A further 250 µl column wash solution was added to the spin column and the tube was centrifuged at 14,000-x g for 2 min. The plasmid DNA was eluted in 100 µl Milli-Q water by centrifugation at 14,000-x g for 1 min.

2.8 Maxi Prep of Plasmid DNA

Large scale plasmid DNA extractions were carried out with the Qiagen Maxi Prep kit (Qiagen, GmbH, Hilden, Germany). A single colony of *E coli* XL1 containing the desired plasmid was selected and inoculated into 10 ml LB broth containing the appropriate antibiotic. The culture was grown for 8 h then subcultured 1:100 into 500 ml LB broth with antibiotic, and was grown overnight with shaking at 37 °C. The bacteria were harvested by centrifugation at 6,000-x g for 10 min at 4 °C. The cell pellet was resuspended in 10 ml Buffer P1 (4 °C). Buffer P2 (10 ml) was added and mixed gently. The suspension was incubated at room temperature for 5 min. Chilled Buffer P3 (10 ml) was added and the suspension was mixed immediately but gently, then incubated on ice for 20 min. The suspension

repeatedly until no precipitate formed at the interface. The solution was extracted once with chloroform: iso-amylalcohol.

Chromosomal DNA was precipitated by adding sodium acetate (pH 5.2) to 0.3 M, followed by 2 vol ice-cold ethanol. The DNA was allowed to precipitate for 5 min at room temperature and then recovered from the solution by winding the thread-like precipitate onto a sterile pasteur pipette. The DNA was washed in 70 % ethanol, dried under vacuum and suspended in TE.

2.11 Chromosome Extraction with the Igi Genie Genomic Extraction Kit (Helena Bioscience)

Small-scale chromosome extraction was carried out using the Igi Genie Genomic kit from Helena Bioscience (Beaumont, Texas).

An overnight culture of bacterial cells (1.5 ml) was centrifuged at 14,000-x g for 2 min. The pellet was resuspended in 300 µl Gen I. Gen II (300 µl) was added and the suspension was vortexed vigorously. The tubes were incubated at 55 °C for 30 min. Gen III (150 µl) was added and the lysate was mixed fairly vigorously. The tubes were left to stand at room temperature for 10 min, with occasional mixing. After centrifuging at 14,000-x g for 5 min the supernatant was transferred to a fresh tube. Chromosomal DNA was precipitated with 450 µl isopropanol (Sigma). The tubes were centrifuged at 14,000-x g for 5 min and the supernatant was discarded. The DNA pellet was washed with 200 µl 70 % ethanol, centrifuged for 5 min at 14,000-x g.

The pellet was air-dried for 5 min at room temperature then dissolved in 20 µl of TE buffer (pH 8.0).

2.12 Estimation of DNA Concentration by Ethidium Bromide Dot Quantification

The following DNA standard solutions were prepared in TE buffer (pH 8.0): 0 µg/ml, 1 µg/ml, 2.5 µg/ml, 5 µg/ml, 7.5 µg/ml, 10 µg/ml, 20 µg/ml. DNA was obtained from the pBR322 plasmid (Sigma)

Each standard DNA solution (4 µl) was added to 4 µl ethidium bromide 1 µg/ml. The unknown DNA solutions were prepared in the same way. A piece of Saran wrap was placed on the transilluminator and the standard and the unknown DNA solutions were spotted side by side on the plastic wrap. The fluorescence of the standard and unknown DNA solutions was compared under UV light and this was used to estimate DNA concentration of the unknown solutions.

2.13 Ethanol Precipitation of DNA

A 100 µl DNA sample was taken and 50 µl of 7.5M ammonium acetate with 375 µl 95 % ethanol was added. The reaction was centrifuged at 14,000-x g for 15 min at room temperature. The supernatant was discarded. The pellet was rinsed briefly in 250 µl 70 % ethanol then was centrifuged at 14,000-x g for 5 min. The ethanol was removed and the pellet was air-dried for 3-5 min. The DNA was resuspended in

10-25 µl Milli-Q water. The procedure could be scaled up or down as required.

2.14 Restriction of Plasmid DNA

DNA restrictions were carried out using New England Biolabs (Beverly, MA) enzymes. The sample, enzyme and appropriate buffer were added to a final volume of 20 µl. Plasmid restrictions were incubated at 37 °C for 1 h, whilst chromosomal restrictions were incubated overnight. The reaction was stopped by heating the solution to 65 °C for 20 min, or by cleaning with the Qiaquick PCR purification kit (Qiagen, see section 2.18).

Partial digestions were performed on chromosomal DNA. Four successive two-fold dilutions of the enzyme were prepared, DNA and buffer were added and the reaction was incubated overnight.

2.15 Preparing a Horizontal Agarose Gel.

Agarose gel electrophoresis was performed in Bio-Rad DNA sub-cells (Bio-Rad, Richmond, Ca). Electrophoresis grade agarose (1 %) was dissolved in 0.5 X TBE (0.045 M Tris.borate, 0.001 M EDTA) or 1x TAE (0.04 M Tris acetate, 0.001 M EDTA) at high temperature in a microwave oven. The solution was allowed to cool to approximately 60 °C then ethidium bromide was added to 0.5 µg/ml. The gel was poured into the casting tray and allowed to set at room temperature. DNA samples were mixed with 0.2 vol 6x DNA loading buffer (0.5 % SDS, 25 % glycerol, 0.25 % bromophenol

blue, 0.25 % xylene cyanol, 0.05 M EDTA) and electrophoresis was carried out at 80 V for 1.5 h. The DNA was visualised using a UV light box.

2.16 Qiaquick Gel Extraction Protocol

The Qiaquick kits from Qiagen were used to recover DNA from agarose gels, PCR reactions, restrictions etc (see section 2.18 for the Qiaquick PCR purification protocol). For both of these kits, the DNA was dissolved in a buffer then placed in a spin column from the kit, which contained a silica membrane. Under acidic or neutral pH conditions, the DNA binds to the silica membrane and impurities are washed through. The pH conditions are then changed to alkaline to elute the DNA.

The DNA fragment was extracted from the agarose gel with a scalpel. The weight of the agarose slice was obtained, then 3 vol of Buffer QG were added to 1 vol of gel. The reaction was incubated at 50 °C for 10 min with occasional mixing to melt the agarose sample. Isopropanol (1 vol) was added to the sample and the tube was mixed. The sample was applied to a Qiaquick column in a 2 ml collection tube and was centrifuged for 1 min at 14,000-x g. An extra 0.5 ml of Buffer QG was added to the column and centrifuged for 1 min at 14,000-x g to remove all traces of agarose. The column was washed with 0.75 ml Buffer PE, followed by centrifugation for 1 min at 14,000-x g. The column was centrifuged empty for 1 min at 14,000-x g to remove all traces of wash buffer. The DNA was eluted in 50 µl buffer EB (10 mM Tris.Cl, pH 8.5) by centrifugation for 1 min at 14,000-x g.

2.17 Polymerase Chain Reaction.

2.17.1 Synthesis and De-Protection of Oligonucleotides

Primers JF1 were synthesised by Dr A. Wolstenholme (School of Biochemistry, University of Bath). They were recovered from their synthesis columns by a concentrated ammonia solution. One ml of concentrated ammonia (0.88 M) was gradually drawn through the synthesis column with a 5 ml syringe (0.2 ml every 20 min). The eluted oligonucleotide was transferred to a screw-capped microfuge tube and was placed in a glass universal bottle. The top was sealed with parafilm and incubated overnight at 55 °C. This removed the amino-protecting groups.

The tubes were spun in a vacuum rotor to remove the ammonia. Milli-Q water (200 µl) was added, together with 20 µl sodium acetate and 400 µl ethanol, and was the tubes were left at -20 °C overnight. The ethanol precipitation was then repeated. The pellet was washed in 70 % ethanol, vaporised dry and resuspended in 300 µl water.

2.17.2 Primers for *E. faecalis* and *E. faecium*

For amplification of a 405 bp internal region of *efaA* (H3 region): -

JF1F GCG AAA GCT TCT GAA GCG GAC

JF1R GCA TTT AAA TCA TAA GCT TTG

Primers JCF2 to JCF6 were synthesised by Pharmacia (Uppsala, Sweden) and were diluted before use to 10 mM.

Degenerate primers to identify *efaA* homologue in *E. faecium*: -

JCF2F GGA AGC TTT AYG ARC CNY TNC CNG A

JCF2R GGA AGC TTA RRT TVY ANT TCA TCA T

Where Y = C or T, R = A or G, N = A, C, T or G and V = A, C or G

Primer for JF1 405 bp fragment with extension of three bases past Hind111 site: -

JCF3R GCA TTT AAA TCA TAA GCT TTG TGA

Primers for protein work from amino acid 20 to end of *efaA* gene: -

JCF4F TGC GGG AAT CAA GCC GCT G

JCF4R CGG AAT TCA TTT ACT CAT TAA GCC ATC

Reverse primer to enable more specific PCR of *efaA* analogue in *E. faecium*

Used internal amino acid sequence YIWEINTE: -

JCF5R ATR TAD ACC TYT ADT TRT G

Where R = A or G, D = A, G or T and Y = C or T

Specific primers for *E. faecium* homologue. JCF6F taken from ORF 122 bp in, JCF6R taken from ORF 432-449 bp.

Gives product of 327 bp. Product used for dig-PCR: -

JCF6F TAT GAA CCG TTT CCG GAA

JCF6R TTT TCC ACA TAT GTT CTG

JCF14F: TCT TGT ACT GGG ACC GGA AGC G Concentration 450.88 μg/ml

JCF14R: GAT GGG ATG TAC TCG CTC GCG T Concentration 383.31 μg/ml

2.17.3 Reaction Mix for Amplification of DNA by the Polymerase Chain Reaction.

PCR experiments were carried out according to the methodology of Perkin Elmer Cetus. Reaction mixtures (100 μl) were prepared with 200 μM each of dideoxynucleotides (Pharmacia), 2 mM magnesium chloride, 10 μM primers and 10 μl 10 x reaction buffer. Template DNA (up to 10ng plasmid DNA, or up to 500 ng genomic) was added and 1 unit of Taq polymerase (Helena Bioscience) was used in each reaction. Each reaction was overlaid with 50 μl mineral oil (Sigma).

Dig-labelling PCR was used to generate dig –labelled probes with reagents from Boerhinger-Mannheim. Reaction mixtures (100 μl) were prepared with 5 mM magnesium chloride, 10 μM primers, 10 μl 10 x reaction buffer, 10 μl template DNA and 0.5 μl Taq polymerase. Nucleotides dATP, dCTP and dGTP were added to a final concentration of 200 μM, along with 180 μM dTTP and 20 μM Digoxigenin-UTP.

Plasmid amplifications were carried out in a the Crocodile II thermocycler (Appligene, Watford, UK) with a temperature profile consisting of 94 °C for

2 min followed by three 1 min steps of 94 °C, 55 °C and 72 °C for 30 cycles. This was finished with one cycle of 72 °C for 5 min.

Amplification of chromosomal DNA required longer at 94 °C. The temperature profile for this consisted of one cycle of 94 °C for 3 min followed by three steps of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 2 min for 30 cycles. The final stage was 72 °C for 5 min.

2.17.4 Inverse PCR

This was carried out according to the method of Collins and Weissman (1984) and McNab et al (1994).

Templates were prepared by extracting chromosomal DNA using the Igi genie extraction kit (see section 2.11). DNA was digested overnight at 37 °C using the appropriate restriction enzyme (NEB). The digest was cleaned with the Qiaquick PCR purification kit (see section 2.18) and diluted to a concentration of 5 ng/µl in Milli-Q water. The diluted digest (4 µl) was ligated in a total reaction volume of 20 µl with 0.01 Weiss units µl-1 T4 DNA ligase (Boerhinger) for 24 hours at 10°C. These conditions favour the recircularisation of 1.9 kb fragments. A portion of the ligation mix (5 µl) was used in the PCR reaction.

PCR was performed with the Expand High fidelity PCR kit from Boerhinger Mannheim. The reaction was prepared in two separate tubes to reduce non-

specific priming. The template DNA was placed in the first tube and 200 μM dNTPs and 300 nM primers were added. The second tube was prepared with 5 μl Expand high-fidelity buffer (10 x) containing 15 mM $MgCl_2$ (to give a working concentration of 1.75 mM $MgCl_2$) and 2.6 U Expand high fidelity PCR system enzyme mix (containing Taq polymerase and pwo polymerase). The two tubes were vortexed and the contents of tube 2 were added to tube 1. The reaction mix was overlaid with mineral oil (Sigma). Thermocycling was performed in the Crocodile II thermocycler (Appligene) with the following temperature profiles: 2 min at 94 °C, then 30 cycles of 94 °C (10 sec), 50 °C (30 sec), 68 °C (2 min), followed by a final cycle of 7 min at 68 °C.

Some PCR reactions were carried out using the Expand Long Template PCR kit (Boerhinger Mannheim). Again, two 0.5 ml microfuge tubes were prepared. In the first tube the template DNA was added to 350 μM dNTPs and 300 nM primers. The second tube contained 5 μl 10 x Expand PCR buffer mix (to give a working concentration of 1.75 mM $MgCl_2$) and 2.5 U Expand enzyme mix. The two tubes were vortexed, the contents of tube 2 were added to tube 1 and the mix was overlaid with mineral oil. The same thermocycling conditions as above were used.

2.18 Qiaquick PCR Purification Protocol (Qiagen)

The Qiaquick PCR purification kit was used to clean up DNA after PCR, restriction digests and ligation or labelling reactions.

Buffer PB (5 vol) was added to 1 vol of the reaction mix to be cleaned and the solution was mixed. The sample was added to a spin column in a 2 ml collection tube and the tubes were centrifuged for 1 min at 14,000-x g. The flow-through was discarded and the column was washed with 0.75 ml buffer PE, then centrifuged for 2 min at 14,000-x g. DNA was eluted into a clean microfuge tube with 50 µl buffer EB by centrifuging for 1 min at 14,000-x g.

2.19 Ligation of DNA Using T4 DNA Ligase

Ligation of vector and foreign DNA was carried out according to the protocol described by Sambrook et al (1989).

Plasmid and insert DNAs were digested with the appropriate restriction enzyme. The fragments were isolated by gel electrophoresis (section 2.15) and purified using the Qiaquick gel extraction kit (section 2.16). The plasmid DNA was treated with alkaline phosphatase (New England Biolabs) for 1 h at 37 °C to prevent re-annealing of the sticky ends.

DNA was concentrated by ethanol precipitation. It was dissolved in TE at a concentration of 100 µg/ml.

The ligation reaction (10 µl) was set up by transferring 0.1 µg of vector DNA to a microcentrifuge tube. An equimolar amount of foreign DNA was added. Water was added to 7.5 µl. The solution was warmed to 45 °C for 5 min to

melt any cohesive termini that had reannealed. The mixture was then chilled to 0 °C again before addition of 1 µl DNA Ligase buffer (10 x) and 0.1 Weiss unit T4 DNA ligase.

The reaction was incubated at 16 °C for 1- 16 h, 1-2 µl of each of the ligation reactions was used to transform *E. coli* cells.

2.20 Preparation of Competent *E. coli* XL1 Blue Cells for Heat Shock.

A single fresh colony of *E. coli* XL1 blue or DH5α was inoculated into 5 ml LB broth and grown overnight at 37 °C. This primary culture was diluted 1:100 and grown until the OD_{550} reached 0.48.

The suspension was chilled on ice for 5 min, then centrifuged at 4,000-x g for 5 min. The cells were suspended in 2/5th volume transforming buffer I (Tfb1, 30 mM KCl, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15 % v/v glycerol, pH 5.8). After chilling on ice for 5 min the cells were centrifuged at 4,000-x g for 5 min at 4 °C.

The cells were then suspended in 1/25th volume of transforming buffer II (TfbII, 10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15 % v/v glycerol pH 6.5). They were left on ice for 15 min.

The cell suspension was then divided into 200 µl aliquots and snap frozen on dry ice/ethanol and stored at –70 °C.

2.21 Transformation of *E. coli* by Heat Shock.

The competent cells were thawed at room temperature. After chilling on ice for 10 min, the DNA was added and the suspension was mixed gently. The suspensions were left on ice for 30-45 min.

Heat shock was carried out at 37 °C for 2 min, or 42 °C for 90 sec. The cells were returned to the ice for 2 min immediately afterwards. Four volumes of LB broth were added and the suspensions were incubated at 37 °C for 1h.

The cells were then pelleted and suspended in 100 µl LB broth. All of the mixture was plated out onto LB agar plates containing appropriate antibiotic and seeded with 40 µl X-GAL (Sigma, 20 mg/ml in dimethyl-formamide) and 4 µl 0.84 M IPTG (Sigma) when the plasmid contained the *lacZ* gene at its multiple cloning site. After growth at 37 °C and incubation at 4 °C overnight, bacterial colonies containing the recombinant plasmid appear white, whilst those which still have the intact *lacZ* gene appear blue, enabling easy selection of recombinants.

2.22 Ligation and Transformation with the InVitrogen TA Cloning Kit

PCR was performed on *E. faecium* chromosomal DNA using the following cycles, 3 min at 94 °C followed by 30 cycles of 94 °C (1 min), 40 °C (1 min), 72 °C (1 min) then 60 min at 72 °C. Fresh PCR product (1 µl) was then added to 50 ng pCR2.1 vector in InVitrogen ligation buffer (InVitrogen

Corp., Carlsbad, Ca), with 4 Weiss units T4 DNA ligase. The reaction was incubated overnight at 14 °C.

Transformation was performed with competent cells provided in the kit. Competent cells were thawed on ice. β-mercaptoethanol (1.0 μM) was added to the cells. The ligation reaction (2 μl) was placed into the competent cells and mixed with a pipette tip. The cells were incubated on ice for 30 min.

The cells were subjected to heat shock for 30 sec at 42 °C followed by 2 min on ice. SOC medium (450 μl, InVitrogen) was added to each tube. The tubes were incubated at 37 °C with shaking for 1 hour. The vials were then placed on ice before plating out 50 μl and 200 μl onto LB plates containing 100 μg/ml ampicillin and 1.6 mg X-Gal. The plates were incubated for 18 h at 37 °C, after which they were placed at 4 °C for 2-3 h to allow the colour to develop.

2.23 Electrotransformation of *E. faecalis* EBH1 or OG1RF Cells

Electroporation of *E. faecalis* strains EBH1 and OG1RF was carried out according to the protocol of Dunny et al (1991). A single colony of *E. faecalis* EBH1 or OG1RF was grown in 10 ml BHI broth overnight. This was used to inoculate 1:100 into M9-YE broth containing glycine from 0-10 % in 0.5 % increments.

These cultures were grown overnight. All cultures where the OD_{660} had been reduced by more than 60 % judged against the control tube (0 % glycine) were used to inoculate 300 ml M9-YE media containing the same glycine concentration. This was grown until the OD_{660} reached 0.1-0.2. The culture was chilled on ice and the cells were harvested by centrifugation at 8,000-x g for 10 min.

The ionic strength of the cell suspension was reduced by washing in 1/3 original volume of chilled EB buffer (0.625 M sucrose, 1 mM $MgCl_2$, pH 4.0). The cells were then resuspended in 1/50 original volume EB buffer, divided into 40 µl aliquots and stored at –70 °C until needed.

The cell suspensions prepared above were thawed slowly on ice. The cells were washed three times in 40 µl sterile water and resuspended in 40 µl water. Chilled recombinant DNA was added and the mix was transferred to a pre-chilled 0.2 cm gap electroporation cuvette (Bio-Rad Laboratories). Electroporation was carried out in a Bio-Rad Gene Pulser apparatus using a 2.5 kV pulse (i.e. a capacitance of 25 µF, a resistance of 200 Ω and a voltage of 2500 V).

Following electroporation, cells were transferred into 1 ml BHI broth containing 0.25 M sucrose in a sterile 30 ml universal bottle. The cells were incubated at 30°C in a shaker for 1 h to allow plasmid-mediated resistance to develop. They were then plated out onto BHI agar plates containing erythromycin 10 µg/ml and grown overnight at 30°C.

2.24 Electrotransformation of *E. faecalis* JH2-2.

E. faecalis JH2-2 was electroporated according to the method of Cruz-Rodz and Gilmore (1990).

E. faecalis JH2-2 inoculated into 10 ml M17-glucose broth (M17 broth, Oxoid with 0.5 % glucose) and incubated overnight at 37 °C. After 20 h, 1 ml was subcultured into 100 ml SGM17-glucose media (M17-glucose + 0.5 M sucrose and 5 % glycine). Cells were grown overnight at 37 °C.

Cells were harvested by centrifugation at 1000-x g for 15 min at 4 °C and washed twice with 100 ml ice-cold electroporation buffer (EPB, 0.5 M sucrose with 10 % glucose with 10 % glycerol) by resuspension followed by centrifugation. Finally the cells were resuspended in 1 ml ice-cold EPB and stored frozen as 50 µl aliquots.

For electrotransformation cell suspensions were thawed on ice. Up to 8 µl DNA was added to an ice-cold 0.2 cm electrode-gap cuvette (Bio-Rad) and 40 µl of cell suspension was pipetted in. Cells were electroporated at 25 µF, 200 Ω and 2.5 kV in a Bio-Rad Gene Pulser apparatus. Immediately after electroporation 960 µl ice-cold SGM17-glucose + 20 mM $MgCl_2$ and 2 mM $CaCl_2$ was added, cells were resuspended and transferred to a microfuge tube. After resting on ice for 5 min the tubes were incubated for 2 hour at 37 °C. Serial dilutions were performed to 10^2 dilution factor and portions (0.1

ml) were plated onto selective BHY agar. Serial dilutions up to a dilution factor of 10^6 were plated onto non-selective BHY agar to estimate the total viable count.

Plates were incubated anaerobically for 24-48 h at 37 °C

2.25 Transformation of *S. gordonii* (Challis).

Transformation was carried out with a method supplied by H F Jenkinson (personal communication). *S. gordonii* was grown overnight in 10 ml BHY broth. It was subcultured 1:200 into pre-warmed BHY with 1 % foetal calf serum and 0.1 % glucose (BHY/FCS/Glc). The culture was grown anaerobically for 2 h at 37 °C. It was subcultured 1:100 into fresh pre-warmed BHY/FCS/Glc and grown anaerobically for 60 min at 37 °C.

To transform the cells, 1 ml of cells was added to a sterile, warm 10 ml capacity tube with 1-3 μg DNA. The culture was grown anaerobically for 3-4 h at 37 °C. A portion of cells (100 μl) were plated onto selective media, also 100 μl of a 10-fold dilution. Serial dilutions of the culture were made to a dilution factor of 10^6 and these were plated onto non-selective media to estimate total cell numbers.

2.26 RNA Extraction with the RNeasy Extraction Kit (Qiagen)

An overnight culture of bacterial cells was inoculated 1:100 into fresh media and grown to $OD_{550} = 0.3$. A portion of the culture (3 ml) was harvested at

5000-x g for 3 min. The supernatant was discarded and the cells were resuspended in 100 µl TE buffer (pH 8.0) containing lysozyme 3 mg/ml. The suspension was incubated for 10 min at room temperature. Buffer RLT (350 µl) was added to the sample and the tube was vortexed vigorously. Ethanol (250 µl of 96 %) was added to the lysate and mixed by pipetting.

The sample was applied to a spin column in a 2 ml collection tube. It was centrifuged for 15 sec at ≥8000-x g. Buffer RW1 (700 µl) was pipetted onto the column and it was centrifuged at ≥8000-x g for 15 sec to wash. The column was transferred to a fresh collection tube. Buffer RPE (500 µl) was added and the sample was centrifuged for 15 sec at 8000 x g. A further 500 µl of buffer RPE was pipetted onto the column and it was centrifuged at 14,000-x g for 2 min to dry the membrane. RNA was eluted in 30 µl Milli-Q water by centrifuging for 1 min at 8000-x g.

2.27 Preparation of Gel Tanks for Formaldehyde-Agarose Gel Electrophoresis

Bio-Rad DNA sub cells were washed with 0.5 % SDS solution. They were rinsed with water and dried with ethanol. Tanks were then filled with 3 % $H_2O_2$and left for 10 min at room temperature. They were rinsed thoroughly with Milli-Q water and allowed to dry.

2.28 Formaldehyde-Agarose (FA) Gel Electrophoresis

A 1.2 % FA gel was prepared by dissolving agarose in FA gel buffer (10 x FA gel buffer, 200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0). After cooling the solution to 65 °C, 0.54 ml 37 % formaldehyde and 3 µl 1 mg/ml ethidium bromide was added. The gel was poured into the gel support and allowed to set. The gel was equilibrated in 1 x FA gel running buffer (10 % 10 x FA gel buffer, 2 % 37 % formaldehyde).

RNA was mixed with an appropriate volume of 5 x RNA loading buffer (0.16 % saturated bromophenol blue, 0.4 mM EDTA, pH 8.0, 720 µl 37 % formaldehyde, 20 % glycerol, 30.84 % formamide, 40 % 10 x FA gel buffer). It was heated to 65 °C for 3 min then loaded onto the gel and run at 80 V for 1 h. RNA was visualised using a UV light box.

2.29 Reverse Transcriptase PCR

Reverse transcriptase PCR (RT-PCR) was performed using the Titan One-Step RT-PCR kit (Boehringer Mannheim).

The reaction was prepared in two separate tubes. In the first tube 1 µg RNA was added to 200 µM dNTP's, 0.4 µM primers, 5 mM DTT and 5 U RNase inhibitor (Promega). The second tube was prepared with 1xTitan RT-PCR buffer containing 1.5 mM $MgCl_2$ and 1 µl Titan enzyme mix (containing AMV and Expand High Fidelity PCR system). The contents of the second tube were added to the first and the reaction was overlaid with mineral oil.

Thermocycling was carried out in a Crocodile II thermocycler using the following temperature profiles: 30 min at 50 °C, 2 min at 94 °C followed by 10 cycles of 94 °C (30 sec), 50 °C (30 sec), 68 °C (45 sec). A further 20 cycles were performed at 94 °C (1 min), 50 °C (1 min), 68 °C (1 min) followed by a final stage at 68 °C for 7 min.

2.30 Random Hexamer Labelling with Digoxigenin

Random hexamer labelling was performed with the Dig DNA Labelling Kit from Boerhinger Mannheim.

Template DNA (10 ng-3 μg) was denatured by heating at 95 °C for 10 min. The tubes were placed on ice and a 20 μl reaction was prepared with 2 μl hexanucleotide mix, 2 μl dNTP mix and 1 μl Klenow enzyme.

The reaction was incubated overnight at 37 °C. It was cleaned with the Qiaquick PCR Purification Kit (Qiagen) and eluted in 30 μl TE buffer.

2.31 Estimation of Labelling Yield in a Spot Test with a Dig-Labelled Control.

Estimation of dig-labelling yield was carried out by comparison of the dig-labelled sample with a dig-labelled control (Boehringer Mannheim).

Serial dilutions were carried out to a dilution factor of 10^5 on both sample and control DNA. A piece of nylon membrane (Boehringer Mannheim) was

taken and 2 µl of each dilution was spotted onto the membrane. The membrane was orientated then baked for 30 min at 120 °C. Chemiluminescent detection was then carried out (see section 2.34).

2.32 Southern Blotting.

This was carried out according to the method of Southern (1975).

An agarose gel was prepared and run at 80 V. Before the blot was set up the DNA was depurinated in 0.25 M HCl for 30 min. The gel was then transferred to denaturing solution (0.6 M NaCl, 0.2 M NaOH) and left for 30 min. Neutral pH was restored with 1.5 M NaCl, 0.5 M Tris HCl (pH 7.5) for 30 min.

DNA fragments were blotted onto a nylon membrane (Boehringer Mannheim) by transfer of 20 x SSC (3 M NaCl, 300mM sodium citrate, pH 7.0) overnight (Southern, 1975). The DNA was fixed onto the membrane by baking at 120°C for 30 min. The membranes were then hybridised with digoxigenin, as described in section 2.34.

2.33 Colony Blotting Gram Positive Bacteria

Colonies on agar were pre-cooled to 4 °C for approximately 30 min. A piece of nylon membrane was cut to the size of the agar plate to be blotted. It was placed on to the agar and left for 1 min, then was removed and blotted onto dry Whatmann 3 MM paper. The membrane was placed colony side up onto

Whatmann 3 MM paper soaked with TE saline (10 mM Tris, 1 mM EDTA, 0.15 M NaCl) containing 1 mg/ml lysozyme. After incubating for 1 min at room temperature the membrane was blotted briefly on Whatmann 3 MM paper then placed on 3 MM paper soaked with denaturation solution (0.5 M NaOH, 1.5 M NaCl), for 15 min. After blotting briefly it was placed on 3 MM paper soaked with neutralisation solution (1.0 M Tris, pH 7.5, 1.5 M NaCl) and left for 15 min. The membrane was blotted briefly then placed on paper soaked with 2 x SSC (diluted from a 20 x stock) for 10 min. The transferred DNA was fixed by baking for 30 min at 120°C.

Proteinase K (Sigma) treatment was then carried out to digest proteins which might interfere with subsequent hybridisation. The membranes were placed on clean aluminium foil and 0.5 ml of 2 mg/ml proteinase K was pipetted onto each membrane. The membranes were incubated for 1 h at 37 °C. The membranes were placed between two wetted sheets of 3 MM paper and pressure was applied with a ruler to allow the cellular debris to stick to the top sheet of filter paper which was then removed. The membranes were then subjected to hybridisation as described in section 2.34

2.34 Hybridisation with Digoxigenin-Labelled Probe.

The nylon membranes were prepared and fixed as described in the previous sections. They were taken and soaked in high SDS buffer (7 % SDS, 50 mM Sodium phosphate, 2 % blocking reagent (Boehringer), 5 x SSC (diluted from a 20 x stock), 0.1 % N-lauryl sarcosine (Sigma), 50 % formamide) for 2 h at 55°C. Membranes from colony blotting were hybridised in standard

hybridisation buffer (5 x SSC, 50 % formamide, 0.1 % sodium lauryl sarcosine, 0.02 % SDS, 2 % blocking reagent).

The digoxigenin probe was prepared by mixing 7.5 µl of digoxigenin-labelled stock with 42.5 µl water. This was heated at 95°C for 5 min then added to 1.5 ml high SDS buffer. The membranes were incubated in this solution overnight at 55 °C.

Following incubation the membrane was washed twice in 2 x SSC, 0.1 % SDS for 5 min at 55 °C. Then the membrane was washed twice in 0.1 x SSC, 0.1 % SDS for 5 min at 55 °C.

The membrane was placed in wash buffer (0.3 % Tween-20 in Buffer 1 (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 5 min at room temperature, then blocked in buffer 2 (10 % Blocking Reagent in Buffer 1) for 30 min at room temperature.

Anti-Digoxigenin-AP antibody (Boehringer Mannheim) was diluted 1:10,000 in buffer 2 and incubated with the membrane for 1 h. The membrane was then washed three times in wash buffer for 10 min. It was then washed in buffer 3 (100 mM Tris.HCl, pH 9.5, 100 mM NaCl) for 2-5 min at room temperature.

The lumigen (CSPD-Boehringer Mannheim) was diluted 1:100 in buffer 3 and spread evenly over the membrane. This was then covered and left for 5 min in the dark, followed by incubation at 37 °C for 30 min.

Kodak X-Omat AR film was exposed to the membrane for 30 min and then developed according to the manufacturer's instructions.

2.35 Stripping and Reprobing Nylon Membranes

Membranes were washed in Milli-Q water for 1 min. They were then incubated twice for 10 min at 37 °C in 0.2 M sodium hydroxide with 0.1 % SDS.

They were rinsed thoroughly in 2 x SSC and either stored in 2 x SSC or used immediately.

2.36 Induction Of *E. coli* XL1:pGP19

A single colony of *E. coli* XL1:pGP19 was inoculated into 10 ml LB broth containing ampicillin and tetracycline, and grown overnight. The culture was then diluted 1:100 in LB and grown for a further 2.5 h.

A sample (1.5 ml) was removed and placed on ice. IPTG was added to 10 mM. The suspension was then grown for 4 h and a sample was removed every hour. After 4 h the samples were spun down and resuspended in 500 µl saline.

The samples were run on an SDS-PAGE gel which was either stained directly or used for immunoblotting.

2.37 Induction of *E. faecalis* EBH1 with Serum.

A single colony of *E. faecalis* EBH1 was used to inoculate 10 ml of BHI, containing 1 % foetal calf serum and grown overnight. Cells from 1.5 ml of the culture were harvested by centrifugation at 14,000-x g for 2 min, followed by suspension in 0.5 ml saline. An appropriate volume of sample buffer was added and the samples were boiled for 10 min before loading onto an SDS-polyacrylamide gel.

For cell wall preparations, cells were grown overnight in 10 ml BHI broth without foetal calf serum. They were subcultured 1:100 the following morning and grown to log phase in BHI broth containing 1 % foetal calf serum. They were then prepared as described in section 2.40.

2.38 Lowry Protein Assay.

The Lowry protein assay was used to deter mine the amount of protein in the bacterial sample and hence to determine the volumes to be loaded on an SDS-PAGE gel. A calibration curve was prepared using various concentrations of bovine serum albumin (BSA, Sigma).

A 1 mg/ml stock of BSA was prepared, and used to make a series of dilutions from 1 mg/ml to 100 μg/ml. A 500 μl aliquot of each of the BSA samples was used to construct a standard curve. The experimental samples were treated in the same manner. NaOH (500 μl, 0.5 M) was added to the samples, which were then heated to 100°C for 10 min. The samples were allowed to cool to room temperature, then 2.5 ml Lowry C reagent (0.5 % w/v $CuSO_4$ / 1 % w/v NaK tartrate solution with 48 ml Lowry A (5 % w/v Na_2CO_3)) was added. The samples were mixed and left for 10 min.

Folin and Ciocalteau's phenol reagent (Sigma, 0.5 ml) was added and the samples were vortexed. The samples were left for 30 min then the OD_{750} was measured.

2.39 Protein Separation Using SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out using the Bio-Rad mini-protean system. A 12 % denaturing gel and sample buffer were prepared as shown in the table below:

Table 2.1 Solutions for Preparation of SDS-PAGE Gels

Constituent	*Running Gel*	*Stacking Gel*	*Sample Buffer*
acrylamide/bis stock (Sigma)	3.0 ml	1.25 ml	
10 % SDS	0.1 ml	0.125 ml	5 ml
1.5 M tris (pH 8.8)	2.5 ml		
0.5 M Tris(pH 6.8)		3.15 ml	2.5 ml
water	4.35 ml	7.95 ml	5 ml
TEMED (Sigma)	5 µl	12.5 µl	
10 % AMPS (Sigma)	50 µl	62.5 µl	
glycerol			2.5 ml
2 mercaptoethanol (Sigma)			0.25 ml
5 % bromophenol blue (Sigma)			0.2 ml

In some cases a 5:1 protein: SDS sample buffer was used to enable more protein to be loaded onto the gel. This consisted of 0.5 M Tris.Cl, 0.4 % SDS, pH 6.8, 3 ml glycerol, 1 g SDS, 0.9 g DTT, 1.2 mg bromophenol blue.

The tank was filled with SDS tank buffer (SDS 0.1 %, Tris 0.3 %, glycine 1.43 %, pH 8.3).

The samples were prepared by boiling an equal volume of cell suspension in sample buffer for 10 min. Samples (5-15 µl) were loaded into the wells and electrophoresed at 80 V until the dye front had run through the stacking gel and at 180 V until the dye front had reached the bottom of the separating gel. Cell wall preparations were also used and were prepared as described below.

2.40 Extraction of Gram Positive Cell Walls for Protein Gels

Enterococcal cell walls were extracted with glass beads, using a method supplied by H F Jenkinson (personal communication).

An overnight culture (0.5 ml) of enterococcal cells in BHY broth was inoculated into 10 ml of fresh medium. This was grown until OD_{550} = 1.0. The cells were harvested by centrifugation at 8000-x g for 10 min. Milli-Q water (5 ml) was used to wash the cells. The cell pellet was suspended in 0.4 ml TE buffer containing 0.2 mM phenylmethylsuphonyl flouride (PMSF, Sigma). Lysozyme (0.2 mg/ml) was added and the suspension was incubated for 10 min at 37 °C.

The suspension was transferred to a 1.5 ml microfuge tube and 0.2 g 0.1 mM glass beads (Sigma) were added. The mixture was vortexed vigorously for 1 min. The tube was placed on ice to cool for 1 min, then the vortexing was repeated twice with a 1 min rest on ice in between.

The tube was rested on ice so that the beads could settle, then the top suspension was pipetted into a fresh 1.5 ml microfuge tube. TE buffer 0.3 ml was added to the beads mixed briefly and the top suspension was added to the first in a fresh tube. The tubes were spun at 8,000-x g for 1 min to pellet unbroken cells. The supernatant was extracted and centrifuged at 14,000-x g for 30 min at 4 °C. Both the pellet (envelope fragments) and the supernatant (cytoplasmic fraction) were retained.

The pellet was resuspended in 50 µl Milli-Q water. High SDS buffer (10 µl) was added and the supernatant was heated at 95 °C for 10 min. The resulting solution was loaded onto an SDS-PAGE gel.

2.41 Coomassie Blue Staining.

The gels were stained by gentle agitation in 0.1 % Coomassie Brilliant Blue R-250 (w/v) in 50 % methanol/10 % acetic acid for 30-60 min, or overnight. The gel was destained with an aqueous solution of 10 % methanol/ 20 % acetic acid.

2.42 Western (Immuno-) Blotting

Proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes using the western blotting method of Towbin et al (1979).

Immunoblotting was carried out at 100 V for 1 h using the mini Trans-blot system (Bio-Rad) in ice-cold Transblot buffer (25 mM Tris, 192 mM glycine, 20 % methanol, pH 8.3). The kit was then disassembled and the nitrocellulose was removed and washed for 1 h in TBS (0.9 % NaCl in 10 mM Tris, pH 7.4) containing 1 % BSA. The procedure was carried out at room temperature and gentle agitation was used. The blot was probed for at least 3 h with serum (GP serum, diluted 1:500 in TBS). Gentle agitation at 4 °C was required. The blot was then washed three times in TBS, then soaked with gentle agitation in TBS/1 % BSA containing 0.25 µg/ml protein A-conjugated to horse radish peroxidase for 3 h at 4 °C.

The blot was visualised with freshly-prepared developing solution (0.01 % H_2O_2 w/v, 25 μg/ml 4-chloronaphthol in 10 mM Tris pH 7.4). The reaction was warmed gently and stopped with distilled water.

2.43 Protein Purification by Ammonium Sulphate Precipitation

The EfaA protein was purified from *E coli* XL1: pGP19 by ammonium sulphate precipitation (P.A. Lambert, personal communication).

An overnight culture (50 ml) of *E coli* XL1:pGP19 in LB (100 μg/ml ampicillin and 12.5 μg/ml tetracycline) broth was centrifuged at 6,000-x g for 10 min. The cells were suspended in the same volume of TE buffer containing 4 mg/ml lysozyme (Sigma). The suspension was placed on ice and sonicated for 3 x 40 sec bursts with a 20 sec recovery in between.

The suspension was centrifuged at 20,000-x g for 10 min at room temperature to remove the cell walls and membranes. The cytoplasmic supernatant was extracted and used in subsequent steps.

Nucleic acids were removed by precipitation with 20 mg/ml streptomycin (Sigma). After 30 min at room temperature the suspension was centrifuged at 15,000-x g for 10 min at room temperature and the supernatant was retained.

Table 2.2 Nomogram for Ammonium Sulphate

AMMONIUM SULPHATE. GRAMS TO BE ADDED TO 1 LITRE

From ↓ To →	5 %	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0 %	27	55	84	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	27	56	85	115	146	179	212	246	282	319	357	397	439	481	526	572	621	671	723	
10	28	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685		
15	28	58	88	119	151	185	219	255	292	331	371	413	456	501	548	596	647			
20	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609				
25	29	60	91	123	157	191	227	265	304	344	386	429	475	522	571					
30	30	61	92	126	160	195	232	270	309	351	393	438	485	533						
35	30	62	94	128	163	199	236	275	316	358	402	447	495							
40	31	63	96	130	166	202	241	281	322	365	410	457								
45	31	64	97	132	169	206	245	286	329	373	419									
50	32	65	99	135	172	210	250	292	335	381										
55	33	66	101	138	175	215	256	298	343											
60	33	67	103	140	179	219	261	305												
65	34	69	105	143	183	224	266													
70	34	70	107	146	186	228														
75	35	72	110	149	190															
80	36	73	112	152																
85	37	75	114																	
90	37	76																		
95	38																			

Using the nomogram above, ammonium sulphate was added to produce a 30 % saturated solution. The suspension was stirred for 30 min at room temperature, then centrifuged at 15,000-x g for 10 min. The pellet produced was suspended in the same volume of Milli-Q water, dialysed against distilled water and stored frozen.

The supernatant from the 30 % precipitate was used to produce a 60 % saturated solution in the same method as above. The spin and dialysis steps were repeated. A 90 % precipitate was obtained in the same way. The final supernatant was dialysed and stored frozen.

2.44 Platelet Aggregation Assays

Platelet aggregation was studied using a turbidimetric method supplied by C. Murphy (personal communication). Light transmission was studied through a platelet suspension when it was exposed to an agonist. Platelet shape changes caused a decrease in light transmission whilst aggregation caused an increase.

Rabbit blood was used in all experiments. After collection, the blood was centrifuged at 185-x g at room temperature for 10 min. The top layer (platelet rich plasma, PRP) was removed from the lower layer of red blood cells. Prostacyclin (PGI_2, from Dr Whittle, Wellcome Laboratories, Kent) was added to the PRP to produce a final concentration of 300 ng/ml. The PRP was mixed gently then centrifuged at 1256-x g for 15 min at room temperature to precipitate the platelet pellet. The plasma supernatant was removed and the platelets were resuspended in HEPES buffered Tyrode's solution (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_2HPO_4, pH 7.4 with 5.5 mM glucose and 0.25 % BSA). The platelets were allowed to recover for 90 min to allow the increased cAMP levels to return to normal. PGI_2 (100 ng/ml) was then added to the suspension and it was centrifuged at 1256-x g for 15 min at room temperature. This process was repeated once more to wash the platelets before use. Following the final centrifugation step the platelets were resuspended to give a final concentration of 2×10^8/ml.

Bacteria were grown for 20 hours at 37 °C in 5 ml Todd-Hewitt broth (Difco). After harvesting by centrifugation at 6,000-x g for 5 min at 4 °C, cells were washed three times in saline (0.9 % NaCl). They were finally resuspended in 5 ml phosphate buffered saline (PBS, 0.8 % NaCl, 0.02 % KCl, 0.144 % Na_2HPO_4, KH_2PO_4).

Aggregation assays were carried out in a Chronolog dual channel ionised Ca^{2+} aggregometer. To calibrate the light transmission a 10-fold dilution (2 x 10^7) of washed platelets was taken to measure maximum light transmission (100 %). Basal level light transmission (0 %) was taken to be 2 x 10^8 cells. Platelets were stirred at 900 rpm for the duration of the experiment. Changes in light transmission were recorded on a potentiometric chart recorder.

2.45 Sequencing and Sequence Analysis

DNA sequencing was carried out at the automated DNA sequencing facility in the Department of Biology and Biochemistry at the University of Bath. The sequences were analysed on the University of Bath GNOME Unix computer, using the Wisconsin GCG package. BLAST searches were carried out at the NCBI database (http://www.ncbi.nlm.nih/gov). Preliminary sequence data for *E. faecalis* was obtained from The Institute for Genome Research (http://www.tigr.org). The PEDANT database (http://pedant.mips.biochem.mpg.de) was used for molecular weight calculations, and the GENEQUIZ programme (http://columba.ebi.ac.uk:8765/ext-genequiz/) was used for secondary structure predictions.

3 Heterogeneity of the *efaA* gene Amongst Strains of *E. faecalis*

This work was carried out to determine whether *efaA* was present in a number of *E. faecalis* strains, and to study the degree of sequence conservation amongst those strains.

Previous work by Aitchison et al (1987) showed that antibodies to EfaA are only produced by enterococcal IE patients and not by those with enterococcal infections at other sites. This suggests that *efaA* is only expressed in endocarditis, although clinical isolates from other sites (e.g. UTI) can be induced to express EfaA by the addition of 1% foetal calf serum to the growth medium (Lambert et al, 1990). This reflects work by Guzman et al (1991) who showed that *E. faecalis* strains from UTIs adhered better to urinary tract cells, whilst those from IE adhered better to endocardial cells. By growing strains in serum, the expression of polysaccharide adhesins could be altered so that the UTI isolates expressed increased levels of D-galactose and L-fucose-containing adhesins, which are implicated in adhesion to heart cells.

One of the objectives of this present study was to study a panel of strains from a variety of sources, to confirm that the EfaA protein can be induced by growth in serum. The other purpose was to examine the heterogeneity of the *efaA* gene amongst these isolates.

Sequence conservation is usually characterised by observing the extent of genetic polymorphism. Polymorphisms are either point mutations or rearrangements caused by insertions or deletions in DNA. Such differences are detected by the presence or absence of DNA bands produced by PCR, or by the banding patterns produced from restriction enzyme digestion (known as restriction fragment length polymorphism, RFLP). Strains showing a similar banding pattern are assumed to have a close genetic relationship. In this work two approaches were used to study polymorphism, PCR-RFLP and nested PCR.

Firstly, a combined PCR-RFLP technique was carried out. A portion of the gene was amplified by PCR and the resulting product was restricted with *Hin*dIII. The banding patterns produced were studied on a 0.8% agarose/TAE gel. This method is commonly used to study genetic polymorphism (Laguerre et al, 1996; Denning et al, 1997; Shiroza et al, 1998).

Secondly, a nested PCR approach was used, similar to that described by Joosten et al (1997) and Aslanzadeh et al (1996). PCR was performed using primers for a region close to the N and C terminals of *efaA*. The products were analysed on a 0.8% agarose/TAE gel. A second PCR was performed, using the product of the first amplification and primers for an internal portion of the gene. This method permits study of the degree of conservation at both the ends of the gene and the internal region. Sequencing was not performed on these PCR products because some proof reading errors are inevitable

during DNA amplification and therefore it would be incorrect to attribute any base changes seen on sequencing to polymorphism.

Table 3.1 Origins of *E. faecalis* strains

Species	Strain Number	Hospital Source	Site of Infection
E. faecalis	OG1RF	Laboratory strain	
E. faecalis	EBH1	East Birmingham Heartlands Hospital	Endocarditis
E. coli	XL1:pSK+GP19	Laboratory clone	
E. faecalis	RUH3	Royal United Hospital, Bath	Urinary Tract Infection
E. faecalis	RUH4	Royal United Hospital, Bath	Urinary Tract Infection
E. faecalis	RUH5	Royal United Hospital, Bath	Urinary Tract Infection
E. faecalis	F64843	John Radcliffe Hospital, Oxford	Bloodstream Isolate
E. faecalis	M63307	John Radcliffe Hospital, Oxford	Bloodstream Isolate
E. faecalis	S22247	John Radcliffe Hospital, Oxford	Bloodstream Isolate
E. faecalis	X9058	John Radcliffe Hospital, Oxford	Bloodstream Isolate
E. faecalis	H039508	John Radcliffe Hospital, Oxford	Bloodstream Isolate
E. faecalis	S222588	John Radcliffe Hospital, Oxford	Bloodstream Isolate
E. faecalis	S19494	John Radcliffe Hospital, Oxford	Bloodstream Isolate
E. faecalis	H54428	John Radcliffe Hospital, Oxford	Bloodstream Isolate
E. faecalis	T66002	John Radcliffe Hospital, Oxford	Bloodstream Isolate

Table 3.1 details the strains of *E. faecalis* used in these experiments The identities of new strains were confirmed with the Rapid ID32 Strep kit (see Table 3.2). Many classifications in a hospital setting are made out of clinical convenience and they may not reflect the true taxonomy of the organism. This can be seen with strain T66002, which was classified and treated as *E.*

faecalis in hospital but was shown to be *Streptococcus uberis* on the test strips. Because of this re-classification it was excluded from further experiments. All other strains were confirmed to be *E. faecalis.*

Table 3.2 Results of API Rapid ID32 Strep Tests

Strain #	ADH	RAF	MEL	βGLU	VP	MLZ	βGAR	APPA	SAC	βGUR	βGAL	LARA	αGAL	PYRA	DARL	PAL	βNAG
OGIRF	+	-	-	+	+	+	-	-	+	-	-	-	-	+	-	-	-
RUH3	+	-	-	+	+	+	-	-	+	-	-	-	-	+	-	-	-
RUH4	+	-	-	+	+	+	-	-	+	-	-	-	-	+	-	-	-
RUH5	+	-	-	+	+	+	-	-	+	-	-	-	-	+	-	-	-
F64843	+	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-
M63307	+	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-
S22247	+	-	-	+	+	-	-	-	+	-	-	-	-	+	-	-	-
X9058	+	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-
H039508	-	-	-	+	+	+	-	-	+	-	-	-	-	+	-	-	-
S222588	+	-	-	+	+	-	-	-	+	-	-	-	-	+	-	-	-
S19494	+	-	-	+	+	+	-	-	+	-	-	-	-	+	-	-	-
H54428	+	-	-	+	+	+	-	-	+	-	-	-	-	+	-	-	-
T66002	+	-	-	+	+	+	-	-	+	-	-	-	-	+	-	-	-

Strain #	MBDG	RIB	GTA	TAG	MAN	HIP	βMAN	SOR	GLYG	CDEX	LAC	PUL	URE	TRE	MAL	Strain Identification (% Probability)
OGIRF	+	+	-	+	+	+	-	+	-	-	+	-	-	+	+	*E. faecalis* (95.2)
RUH3	+	+	-	+	+	+	+	+	-	-	+	-	-	+	+	*E. faecalis* (97.8)
RUH4	+	+	-	+	+	+	+	+	-	-	+	-	-	+	+	*E. faecalis* (97.8)
RUH5	+	+	-	+	+	-	+	+	-	+	+	-	-	+	+	*E. faecalis* (99.9)
F64843	+	+	-	+	+	-	-	+	-	+	-	-	-	+	+	*E. faecalis* (99.9)
M63307	+	+	-	+	+	-	-	+	-	+	+	-	-	+	+	*E. faecalis* (99.5)
S22247	+	+	-	+	+	-	-	+	-	+	+	-	-	+	+	*E. faecalis* (99.9)
X9058	+	+	-	+	+	-	-	+	-	+	+	-	-	+	+	*E. faecalis* (99.9)
H039508	+	+	-	+	+	+	-	+	-	-	+	-	-	+	+	*E. faecalis* (97.3)
S222588	+	+	-	+	+	-	-	+	-	+	+	-	-	+	+	*E. faecalis* (99.9)
S19494	+	+	-	+	+	-	-	+	-	+	+	-	-	+	+	*E. faecalis* (99.9)
H54428	+	+	-	+	+	+	-	+	-	+	-	-	-	+	+	*E. faecalis* (99.9)
T66002	+	+	-	+	+	-	-	+	-	-	+	-	-	+	+	*S. uberis* (93.9)

Key

ADH = Arginine dihydrolase
βGLU = β glucosidase
βGAR = β galactosidase
βGUR = β glucuronidase
α GAL = α galactosidase
PAL = alkaline phosphatase
RIB = ribose (acidification)
MAN = mannitol (acidification)
SOR = sorbitol (acidification)
LAC = lactose (acidification)
TRE = trehalose (acidification)
RAF = raffinose (acidification)
SAC = saccharose/ sucrose (acidification)
LARA = L-arabinose (acidification)
DARL = D-arabitol (acidification)
CDEX = cyclodextrin (acidification)
VP = acetoin production
APPA = alanine-phenylalanine-proline arylamidase
βGAL = β galactosidase
PYRA = pyroglutamic acid arylamidase
βNAG = N-acetyl-beta-glucosaminidase
GTA = glycyl-tryptophane arylaminidase
HIP = hydrolysis of hipurate
GLYG = glycogen (acidification)
PUL = puliulan (acidification)
MAL = maltose (acidification)
MEL = melibiose (acidificaton)
MLZ = melezitose (acidification)
MBDG = methyl-B-D glucopyranosidase (acidification)
TAG = tagatose (acidification)
βMAN = βmannosidase
URE = urease

The strains were grown overnight in BHY broth. They were subcultured the following morning into BHY containing 1% foetal calf serum and grown to log phase. The cells were harvested and wall proteins were separated on an

SDS-PAGE gel. Figure 3.1 shows the results of western blotting these gels, using serum from an endocarditis patient (GP).

Figure 3.1 Western Blot Analysis of Wall Proteins from *E. faecalis* Strains, *probed with patient serum (GP) diluted 1:100 in TBS. Blots were developed with protein A conjugated to horseradish peroxidase.*

Figure 3.1a *lane 1, E. faecalis X9058, lane 2, E. faecalis M63307, lane 3, E. faecalis S22247, lane 4, E. faecalis F64843, lane 5, E. faecalis S222588, lane 6, E. faecalis EBH1, lane 7, E. coli XL1 pSK+: GP19 (efa clone). Position of EfaA marked by arrow.*

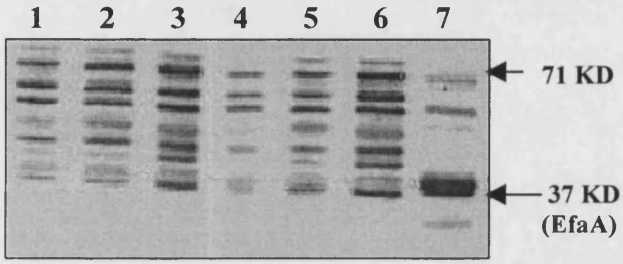
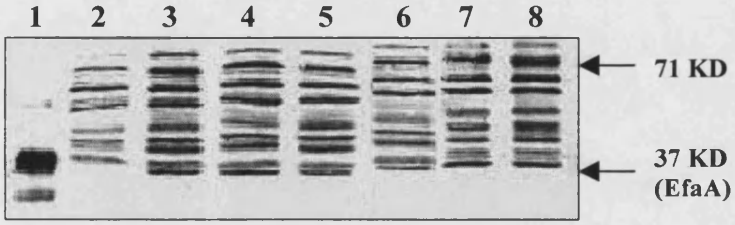


Figure 3.1b, *lane 1, E coli XL1 pSK+: GP19, lane 2, E. faecalis H54428, lane 3, E. faecalis H039508, lane 4, E. faecalis RUH5, lane 5 E. faecalis RUH4, lane 6, E. faecalis RUH3, lane 7, E. faecalis OG1RF, lane 8, E. faecalis EBH1. Position of EfaA marked by arrow.*



EfaA was visible in all strains tested (Figure 3.1). Thus the protein may be induced in strains from a variety of clinical sources, including UTI isolates and laboratory strains. It is not clear which component of serum is able to induce EfaA. Lowe et al (1995) dialysed serum against water and found that no *efaA* was transcribed suggesting that the inducing component is less than 10 kD. However, if *efaA* transcription is induced by Mn depletion (see

chapter 8) then it would seem most likely that albumin in serum is able to bind Mn and so decrease the amount available to *E. faecalis* cells, thereby inducing *efaA*.

Figure 3.2 Sequence of *efaA* *showing primers used in the PCR reaction (marked in bold). JCF4 primer pair used to amplify the fragment of efaA corresponding to the mature protein. Primer pair JCF1 used to amplify an internal portion of the gene. The product of the JCF4 amplification was cleaved with* Hin*dIII at the sites shown, to produce three fragments of 168, 427, 292 bases, respectively.*

```
    GAATTCCGGCCGGAATTCCGGCTTCTGGTGCGACGATTGTTTTAACCGCCGCCTTATTCT
  1 ---------+---------+---------+---------+---------+---------+ 60
    CTTAAGGCCGGCCTTAAGGCCGAAGACCACGCTGCTAACAAAATTGGCGGCGGAATAAGA

    TTTTATTGGCTTTCTTTTTCTCACCAAAGAAAGGCCTAGTATTTGTAAACCGTGAGAAAG
 61 ---------+---------+---------+---------+---------+---------+ 120
    AAAATAACCGAAAGAAAAAGAGTGGTTTCTTTCCGGATCATAAACATTTGGCACTCTTTC
                      EfaA
    AAATGGAGGAATCAACGAATGAAAAAATTTAGTTTATTTTTTTTAACACTTTTAGCAGGG
121 ---------+---------+---------+---------+---------+---------+ 180
    TTTACCTCCTTAGTTGCTTACTTTTTTAAATCAAATAAAAAAAATTGTGAAAATCGTCCC

                          JCF4F ──►
    TTAACGTTAGCTGCTTGCGGGAATCAAGCCGCTGAAAAGAAAGAAAAATTAGCAATTGTG
181 ---------+---------+---------+---------+---------+---------+ 240
    AATTGCAATCGACGAACGCCCTTAGTTCGGCGACTTTTCTTTCTTTTTAATCGTTAACAC

    ACAACGAACTCGATCTTATCCGATTTAGTGAAAAATGTTGGGCAAGACAAAATTGAGCTG
241 ---------+---------+---------+---------+---------+---------+ 300
    TGTTGCTTGAGCTAGAATAGGCTAAATCACTTTTTACAACCCGTTCTGTTTTAACTCGAC

    CATAGTATTGTGCCAATTGGGACAGACCCTCACGAATATGAACCGTTACCAGAAGACATT
301 ---------+---------+---------+---------+---------+---------+ 360
    GTATCATAACACGGTTAACCCTGTCTGGGAGTGCTTATACTTGGCAATGGTCTTCTGTAA

      HindIII
        ▼      JCF1F ──►
    GCGAAAGCTTCTGAAGCGGACATTTTATTCTTTAACGGCTTGAACTTAGAAACAGGCGGA
361 ---------+---------+---------+---------+---------+---------+ 420
    CGCTTTCGAAGACTTCGCCTGTAAAATAAGAAATTGCCGAACTTGAATCTTTGTCCGCCT

    AATGGCTGGTTTAACAAATTAATGAAAACGGCCAAAAAAGTTGAGAATAAAGATTACTTT
421 ---------+---------+---------+---------+---------+---------+ 480
    TTACCGACCAAATTGTTTAATTACTTTTGCCGGTTTTTTCAACTCTTATTTCTAATGAAA

    TCTACAAGCAAAAATGTTACGCCACAATATTTAACAAGTGCCGGTCAAGAACAAACAGAA
481 ---------+---------+---------+---------+---------+---------+ 540
    AGATGTTCGTTTTTACAATGCGGTGTTATAAATTGTTCACGGCCAGTTCTTGTTTGTCTT

    GATCCGCATGCTTGGTTAGACATTGAAAATGGCATCAAATATGTAGAAAACATTCGTGAC
541 ---------+---------+---------+---------+---------+---------+ 600
    CTAGGCGTACGAACCAATCTGTAACTTTTACCGTAGTTTATACATCTTTTGTAAGCACTG

    GTGTTAGTAGAAAAAGATCCAAAAAATAAAGATTTCTATACAGAAAACGCGAAAAATTAT
601 ---------+---------+---------+---------+---------+---------+ 660
    CACAATCATCTTTTTCTAGGTTTTTTATTTCTAAAGATATGTCTTTTGCGCTTTTTAATA
```

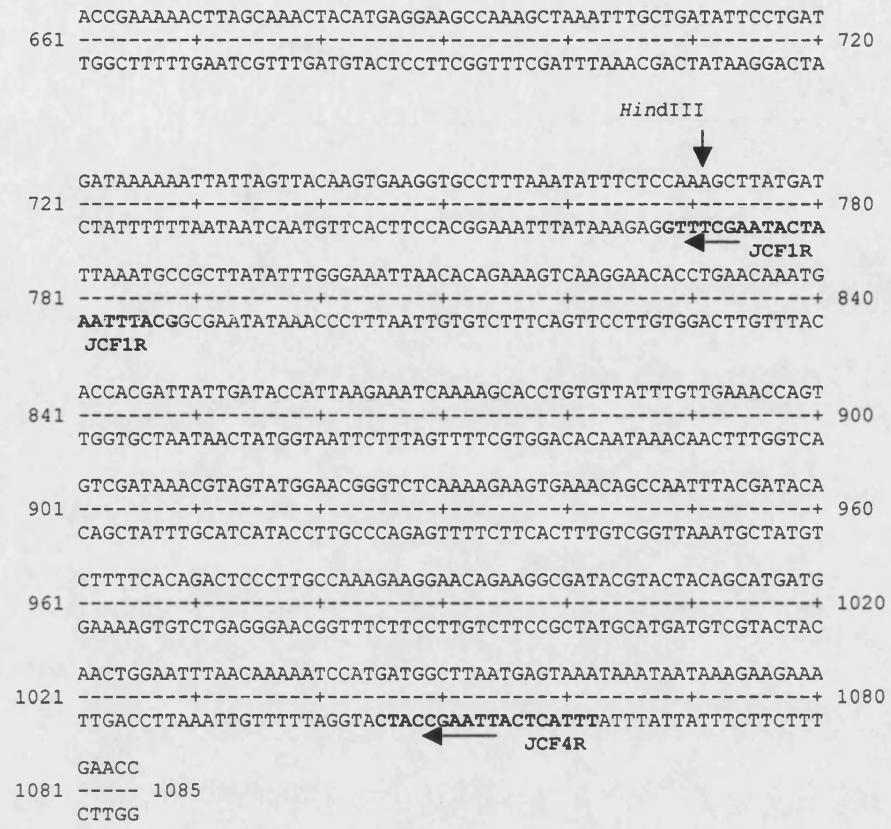


Figure 3.2 details the PCR strategy used for both nested PCR and PCR-RFLP. Primers JCF4 were designed to cover the mature portion of the protein after transport to the cell membrane and lipid modification. Thus the leader sequence was not included in PCR design. For nested PCR, primers JCF1 were used to amplify the internal portion of the gene. For PCR-RFLP, JCF4 primers were used and the resulting product was cut with *Hin*dIII to produce three fragments of 168, 427 and 292 bases, respectively.

Figure 3.3 PCR Analysis of *efaA* from Several *E. faecalis* Strains, Using Primer Pair JCF4, *to amplify a the region corresponding to the mature protein. Lane 1, E. faecalis EBH1, lane 2, E. faecalis X9058, lane 3, E. faecalis M63307, lane 4 E. faecalis S22247, lane 5 E. faecalis F64843, lane 6, E. faecalis S22588, lane 7, E. faecalis H54428, lane 8, E. faecalis H039508, lane 9, E. faecalis RUH5, lane 10, E. faecalis RUH4, lane 11, E. faecalis RUH3, lane 12 E. faecalis OG1RF, lane 13 E. faecalis EBH1, Lane 14, E. faecalis EBH1, Lane 15, molecular weight marker.*

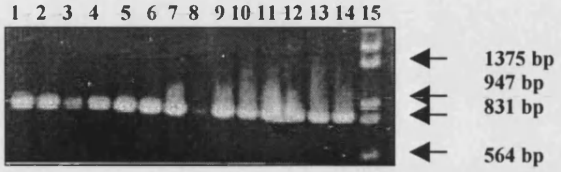
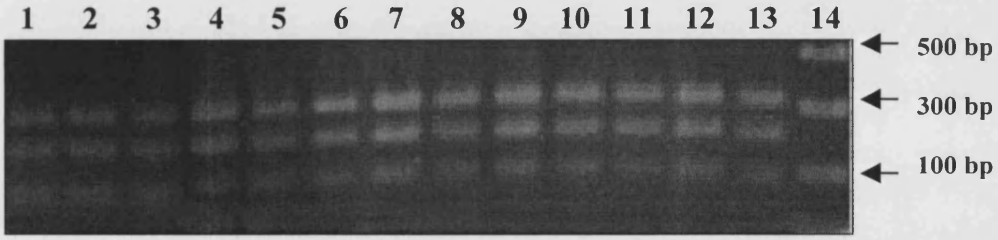
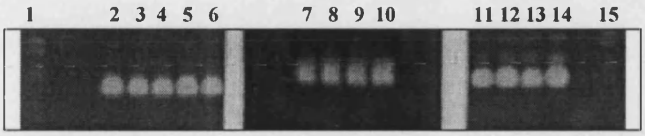


Figure 3.4 PCR-RFLP Analysis of *efaA* from several *E. faecalis* strains Using Primer Pair JCF4, *a portion of efaA corresponding to the mature protein was amplified by PCR and cut with HindIII to give three fragments of 168, 406 and 292 bases. Lane 1, E. faecalis EBH1, lane 2, E. faecalis X9058, lane 3, E. faecalis M63307, lane 4, E. faecalis S22247, lane 5, E. faecalis F64843, lane 6, E. faecalis S22588, lane 7, E. faecalis H54428, lane 8, E. faecalis H039508, lane 9, E. faecalis RUH5, lane 10, E. faecalis RUH4, lane 11, E. faecalis RUH3, lane 12, E. faecalis OG1RF, lane 13, E. faecalis EBH1, lane 14, molecular weight marker.*



The results of the PCR-RFLP work are shown in figure 3.4. All strains produced an identical banding pattern when cut with *Hin*dIII, indicating that the gene is very closely conserved.

Figure 3.5 PCR Analysis of *efaA* from Several *E. faecalis* Strains, Using Primer pair JCF1. *The PCR product obtained in figure 3.4 was used to amplify and internal portion of efaA with primer pair JCF1. Lane 1, molecular weight marker, lane 2, E. faecalis EBH1, lane 3, E. faecalis X9058, lane 4, E. faecalis M63307, lane 5, E. faecalis S22247, lane 6, E. faecalis F64843, lane 7, E. faecalis S22588, lane 8, E. faecalis H54428, lane 9, E. faecalis H039508, lane 10, E. faecalis RUH5, lane 11, E. faecalis RUH4, lane 12, E. faecalis RUH3, lane 13, E. faecalis OG1RF, lane 14, E. faecalis EBH1, lane 15, molecular weight marker.*



The nested PCR results shown in figures 3.3 and 3.5 support the results obtained from PCR-RFLP. All strains were amplified with the primers. The poor yield of product obtained with strains H039508 and M63307 could be attributed to low yields from the chromosome preparations (both strains were very slow-growing). Both strains produced good yields on the second PCR.

In conclusion, it would seem that all strains were able to express EfaA, regardless of clinical origin. In addition the *efaA* gene appears to have a low degree of polymorphism amongst the strains tested. This implies that *efaA* could be an important gene for *E. faecalis* and has hence been closely conserved during the evolution of this organism.

Similar results were seen when Sampson et al (1997) studied the heterogeneity of *psaA*. All 23 *S. pneumoniae* serotypes used in the vaccine and two additional serotypes (6A and 25) were found to express PsaA. PCR-RFLP studies were carried out, using ten different enzymes. Nine out of 10 enzymes produced identical banding patterns in all strains. One enzyme produced a slightly different pattern with some strains but as other strains

from the same serotype did not show these patterns when tested it was assumed that any differences were due to random point mutations. Sequencing of *psaA* genes from serotypes 6B and 2 showed that the two *psaA* genes were 99% identical, with only eight single base pair changes detected. However, several different groups observed that the original *psaA* sequence identified from strain R36A was not representative of the *psaA* sequence identified in other *S. pneumoniae* strains (Sampson et al , 1997, Berry and Paton, 1996, Dintilhac et al, 1997). The *psaA* gene from R36A possesses only about 80% homology to other strains (Sampson et al, 1997, Berry and Paton, 1996). This has led to speculation that strain R36A is either an atypical *S. pneumoniae* strain, or has been wrongly classified as *S. pneumoniae* (Sampson, et al, 1997, Berry and Paton, 1996, Dintilhac et al, 1997). These speculations underline the homology observed with these genes, since it is so rare to see any differences between the cluster 9 proteins of the same species, classification could be confirmed on the basis of identifying the cluster 9 genes. Such a hypothesis was proposed by Aitchison et al (1987) who found that the 37kD antigen later identified as EfaA was so specific to *E. faecalis* endocarditis that they suggested EfaA as a marker for this disease, to give positive identification of *E. faecalis* infection. Singh et al (1998) used DNA from *efaA* and *efmA* (see chapter 5) to probe other enterococcal species and found that the *efaA* probe only reacted with *E. faecalis* and the *efmA* probe only reacted with *E. faecium.*

Streptococcal cluster 9 proteins do not always show such specificity. Kolenbrander et al (1994) looked for *scaA* homologues in 23 streptococcal

species. They carried out southern blotting at high stringency with three 30-mer DNA probes, probe 1 corresponding to the N terminus of *scaA*, probe 2 corresponding to the C terminus of *ssaB* and probe 3 to the C terminus of *scaA*. The probes did react with several other streptococcal species (although they did not react with an *E. faecalis* strain). The workers found the C terminals to be more closely conserved than N terminals, a fact confirmed by Sampson et al (1997) and Berry and Paton (1996) who found that variations in *psaA* from R36A were mostly situated at the N terminus and corresponded to the leader sequence. Interestingly one strain of *S. gordonii* (ATTC 10558) gave two hybridisation bands with probe 1 but only the smaller fragment reacted with probes 2 and 3. Whilst this suggests that the smaller fragment corresponded to *scaA*, it is interesting to speculate whether all strains carry a second related gene of the cluster 9 family. This is known to be the case in *S. pneumoniae* where two cluster 9 proteins have been identified: the *adc* operon which responds to environmental zinc concentrations and *psaA* which responds to manganese. It would be interesting to ascertain whether two such systems operate in *E. faecalis*.

4 Platelet Aggregation Assays

Platelets are smooth, disc-shaped cells that circulate in the bloodstream, each having a life of about 10 days. In normal circumstances, they do not adhere to each other, or to the normal vascular endothelium. If the endothelium becomes damaged, the sub-endothelial layer is exposed. Platelets can then adhere to each other (aggregate) and to collagen, basement membrane and the microfibrils associated with elastin. This action is known as haemostasis and results in the formation of a blood clot, which is vital to the healing process. Following aggregation, platelets release substances from their organelles, such as adenosine diphosphate (ADP), adenosine triphosphate (ATP), 5- hydroxytryptamine (5-HT) and calcium ions.

Recent data have shown that aggregated platelets also release bactericidal proteins such as β-lysins and platelet microbicidal proteins, which contribute to host defence mechanisms (reviewed by Yeaman, 1997). Historically, the contribution of platelets to host defence mechanisms has been underestimated. In addition to releasing antimicrobial peptides, platelets can react with complement proteins, leucocytes and other components of the humoral immune defence system. They are capable of binding and internalising micro-organisms and are even able to release oxidative molecules and free radicals (e.g. superoxide anions) to aid bacterial killing (reviewed by Yeaman, 1997).

The ability of some species to aggregate platelets has been considered a virulence factor in IE. Many species have been shown to induce platelet aggregation, including enterococci (Usui et al, 1991) and oral streptococci (Douglas et al, 1990). Whilst these strains may aggregate platelets it is not clear whether such a reaction would help or hinder bacterial invasion. Sullam et al (1993a) showed that thrombocytopoenic rabbits (i.e. rabbits with an abnormally low platelet count) had smaller vegetations in IE, but these vegetations had a significantly higher bacterial count. Similarly streptococci with increased exopolysaccharide on their cell surfaces show increased virulence, due at least in part to the inhibition of platelet aggregation (Sullam et al, 1993b).

It is important therefore to distinguish between the effect on virulence of platelet-bacteria binding and platelet aggregation. Platelet binding is important in the pathogenesis of IE, as shown by Sullam et al (1996) in *Staphylococcus aureus*: a mutant strain with decreased ability to bind platelets showed reduced virulence in an animal model of the disease. However it is becoming clear that platelet aggregation is also likely to be detrimental to bacteria.

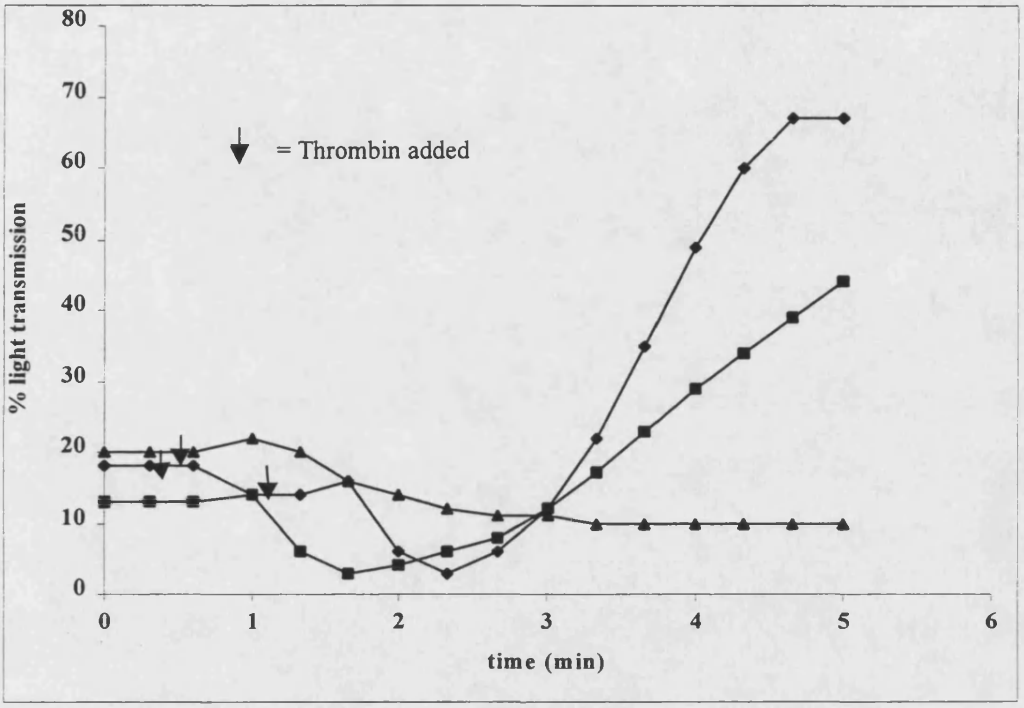
The aim of this part of the work was to study platelet aggregation in two *E. faecalis* strains, EBH1 (a clinical isolate) and OG1RF (a laboratory strain) and ultimately to see if EfaA had any effect on this property.

Platelet aggregation can be induced *in vitro* by adding aggregating agents (e.g. bacteria or thrombin) to platelet-rich plasma (PRP), which is being continually stirred. The rate and degree of aggregation is studied by measuring light transmission through the suspension using a spectrophotometer.

When an aggregating agent is added to the suspension, there is an initial lag phase, followed by a decrease in light transmission as the platelets begin to swell and change shape. When platelet aggregation occurs there is an increase in light transmission as the cells clump together.

Thrombin is used as a positive control in the reaction as it should cause all platelets in the sample to clot. This results in a maximal release of ADP and ATP from platelets (which can also be measured) and a maximum increase in light transmission.

Figure 4.1 Platelet Aggregation with Varying Thrombin Concentrations. *Rabbit platelet rich plasma (PRP) at a concentration of 2 x 10^8 cells/ml was added to an aggregometer and stirred at 900 rpm for the duration of the experiment. Thrombin was added to the cells at concentrations of 10 U/µl (♦), 5 U/µl (■) and 1 U/µl (▲).*



Platelet aggregation was induced by thrombin (Figure 4.1). As a working concentration of 10 U/µl produced the maximum aggregation this was used in subsequent experiments as a positive control.

Figure 4.2 Effect Of Adding ~ 5 X 10^6 Cells Of *E. faecalis* To Rabbit Platelets.

Bacteria were grown for 20 h at 37℃ in Todd-Hewitt broth. After washing in saline, ~ 5 x 10^6 cells were added to 2 x 10^8 cells of PRP. The reaction was stirred at 900 rpm for the duration of the experiment. Thrombin was added after 2 min and the reaction was observed. The experiment was carried out with two strains, E. faecalis EBH1 (♦) and E. faecalis OG1RF (▲).

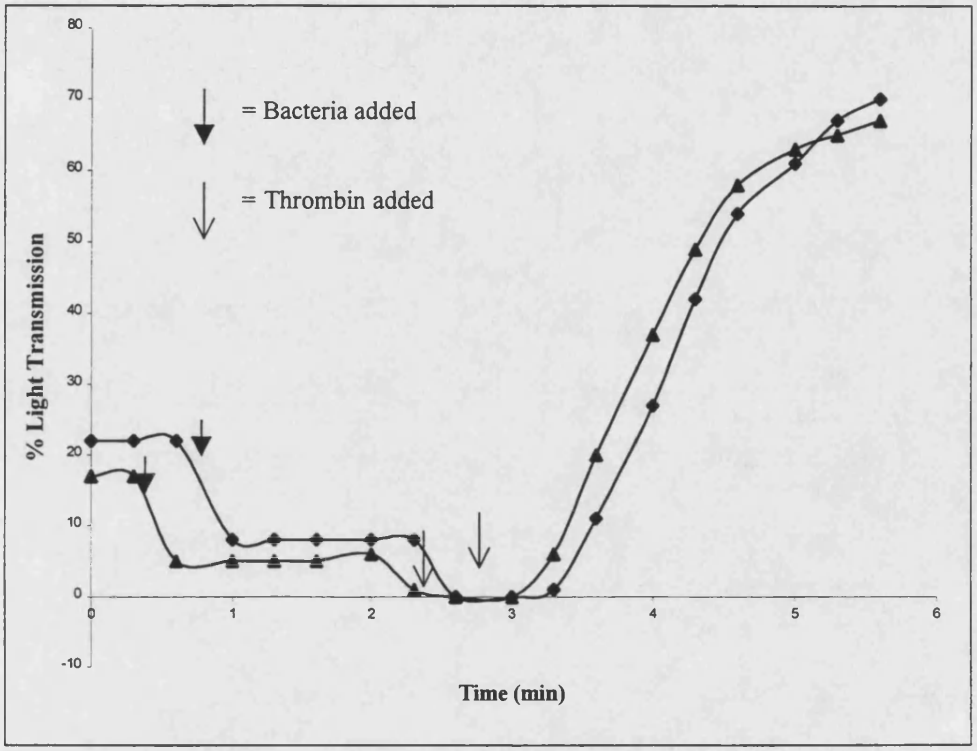


Figure 4.2 shows the results of adding a bacterial suspension containing ~ 5 x 10^6 cells. No aggregation was seen by either strain, although the suspension became denser following the addition of the bacteria. This probably accounts for the observed decrease in light transmission, it is unlikely to be caused by platelets changing shape. The addition of thrombin produced a reaction, showing that the platelets were capable of aggregation.

In case the bacterial suspension was too dense and was therefore making any changes in light transmission difficult to see, the bacterial suspension was diluted several times. Each dilution was tested with PRP to see if it was capable of causing aggregation or platelet shape change (see Figures 4.3 and 4.4). No response was seen from the addition of bacteria, but the platelets could still be aggregated by thrombin, indicating that they were still viable.

Figure 4.3 Effect Of Adding ~1 X 10^6 Cells Of *E. faecalis* EBH1 To Rabbit Platelets. *Bacterial cells were grown for 20 h at 37°C in TH broth. After washing in saline, ~ 1 x 10^6 cells were added to the PRP. The suspension was stirred at 900 rpm and any reaction was observed. Thrombin was added at approximately 2 min.*

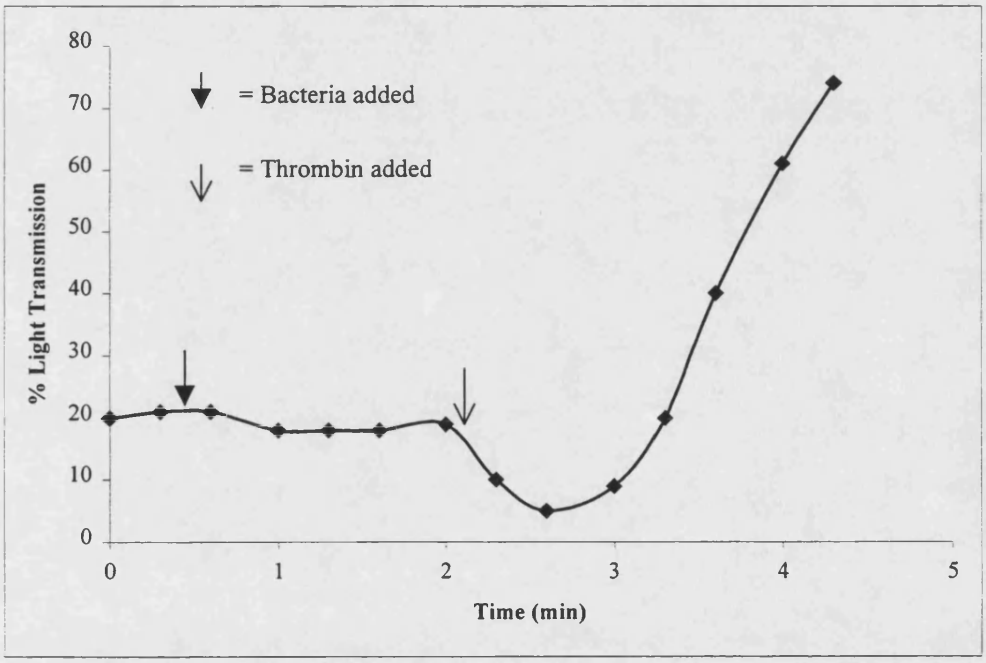
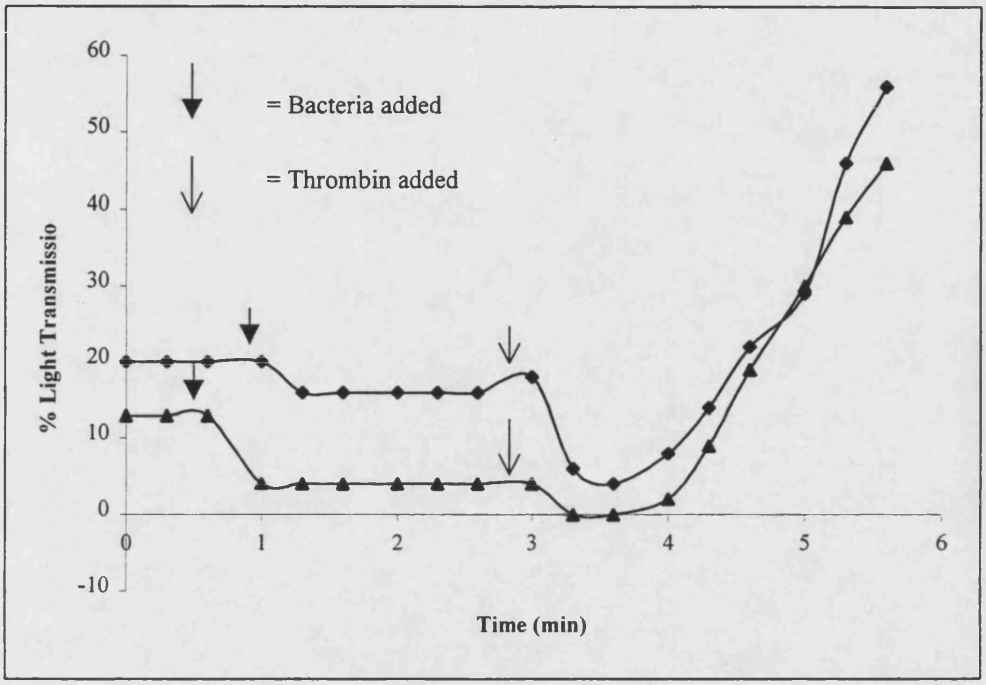


Figure 4.4 Effect Of Adding ~ 5 X 10^3 And ~ 1 X 10^4 Cells Of *E. faecalis* To Rabbit Platelets. *Bacterial cells were grown for 20 h at 37 ℃ in TH broth. After washing in saline, ~5 x 10^3 cells (♦) and ~ 1 x 10^4 cells (▲) were added to PRP and the suspension was stirred at 900 rpm. Thrombin was added after approximately 3 min.*



Bacteria were also grown in medium containing 1% foetal calf serum to see if this had any effect on their aggregating properties. The addition of foetal calf serum to *E. faecalis* growth medium is known to affect the production of several proteins (Lambert et al, 1990), so it was thought that this may have some effect on aggregation properties.

Figure 4.5 Effect on Aggregation of Rabbit Platelets after Growing Bacteria in Media Containing 1% Foetal Calf Serum. *Bacteria were grown for 20 h at 37 °C in TH broth with 1 % foetal calf serum (♦) and in media containing no serum (▲). After washing in saline, ~ 5 x 10^6 cells were added to PRP and the suspension was stirred at 900 rpm.*

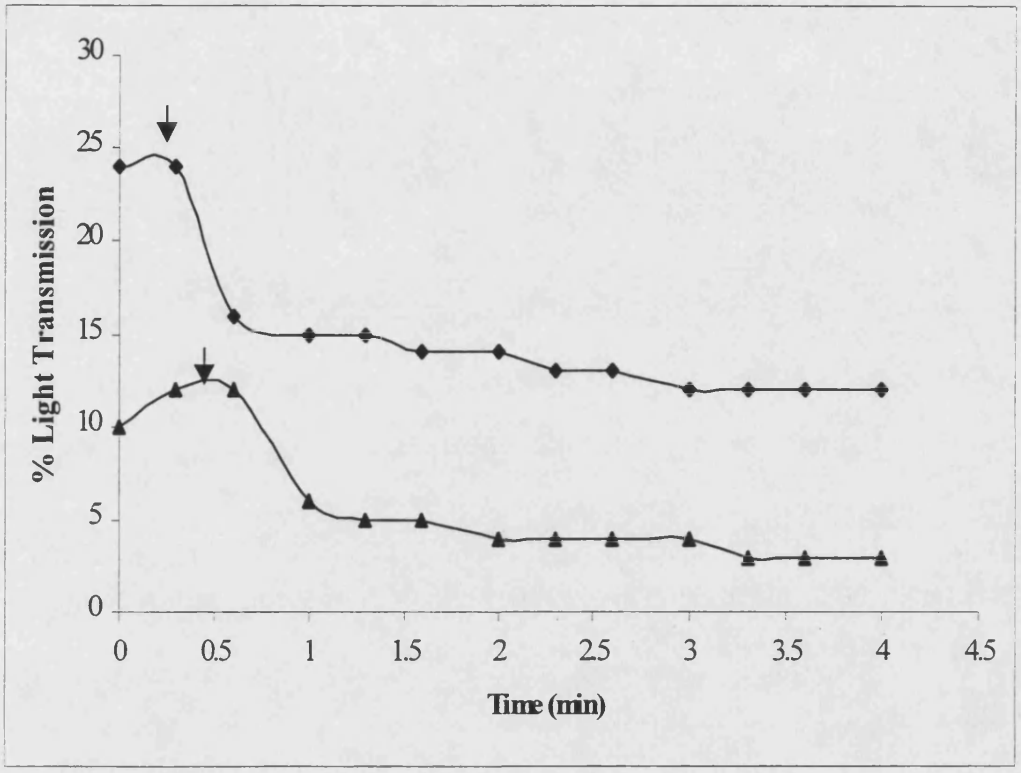


Figure 4.5 shows the results of the experiment: again no response was seen and growth in serum therefore had no effect on aggregation properties of *E. faecalis*.

In conclusion, it appears that the strains tested in these experiments are unable to cause platelet aggregation. Enterococcal isolates have been shown to aggregate platelets, although the reaction was not as vigorous as that seen in *S. aureus* or *S. pyogenes* (reviewed by Yeaman, 1997). Usui et al (1991) showed that their clinical enterococcal isolates could aggregate platelets after a lag time of 1 minute. However this aggregation was not seen in the strains

tested in this work. Not all pathogenic bacteria will cause platelet aggregation. Douglas et al (1990) found that only half of their IE-causing viridans streptococci strains were able to do so.

Therefore platelet aggregation *per se* does not appear to be a virulence factor and indeed strains that are unable to cause platelet aggregation may be more pathogenic as they are able to avoid inducing the platelet antimicrobial defences. Both OG1RF and EBH1 have been shown to express EfaA (see chapter 3) and as neither strain aggregated platelets it is unlikely that EfaA has any effect on this property. However, it would be interesting to test an *efaA* mutant of *E. faecalis* to see if this characteristic was altered in any way.

Most aggregation experiments are carried out using recent clinical isolates (Usui et al, 1993; Douglas et al, 1990). It is acknowledged that repeated subculturing of strains could alter their surface characteristics. The strains used in this experiment were repeatedly subcultured and were maintained on Todd-Hewitt broth or BHI as opposed to blood agar, which is used in many clinical microbiology laboratories for enterococci. This may have had an effect on the surface properties of the strains tested and could possibly have had an effect on aggregation. There were no fresh clinical isolates available at the time for testing in this work.

5 Sequencing of the *E. faecium* Efm Operon

Although *E. faecium* only accounts for 5 – 15 % of enterococcal infections, it causes a considerable problem in a nosocomial setting. This is due to its high level of antibiotic resistance (both intrinsic and acquired). New targets for drug therapy are urgently needed, and as *efaA* has been identified as a significant protein in *E. faecalis* endocarditis, it was decided to look for a homologue in *E. faecium*. Identification of such a protein would enable further studies on the role of this homologue in *E. faecium* endocarditis.

To isolate a portion of the *efaA*-homologous gene, PCR was performed with degenerate primers. The product was cloned into the InVitrogen cloning vector pCR2.1 and was sequenced. Once the first portion of the gene had been characterised, an inverse PCR strategy was used to sequence the remainder of the operon.

Figure 5.1 Nucleotide Sequence of *E. faecalis efaA*, *showing the degenerate primers used to amplify the homologous cluster 9 protein gene in E. faecium UB1. Primer pair JCF2 (shown in bold) was used to amplify the initial fragment. Primer JCF5R was then used in a semi-nested strategy to amplify a portion of the JCF2 fragment. The full degenerate primer sequences are listed in section 2.17.2.*

```
   GAATTCCGGCCGGAATTCCGGCTTCTGGTGCGACGATTGTTTTAACCGCCGCCTTATTCT
 1 ---------+---------+---------+---------+---------+---------+ 60
   CTTAAGGCCGGCCTTAAGGCCGAAGACCACGCTGCTAACAAAATTGGCGGCGGAATAAGA

   TTTTATTGGCTTTCTTTTTCTCACCAAAGAAAGGCCTAGTATTTGTAAACCGTGAGAAAG
61 ---------+---------+---------+---------+---------+---------+ 120
   AAAATAACCGAAAGAAAAAGAGTGGTTTCTTTCCGGATCATAAACATTTGGCACTCTTTC

   AAATGGAGGAATCAACGAATGAAAAAATTTAGTTTATTTTTTTTAACACTTTTAGCAGGG
121 ---------+---------+---------+---------+---------+---------+ 180
   TTTACCTCCTTAGTTGCTTACTTTTTTAAATCAAATAAAAAAAATTGTGAAAATCGTCCC

   TTAACGTTAGCTGCTTGCGGGAATCAAGCCGCTGAAAAGAAAGAAAAATTAGCAATTGTG
181 ---------+---------+---------+---------+---------+---------+ 240
   AATTGCAATCGACGAACGCCCTTAGTTCGGCGACTTTTCTTTCTTTTTAATCGTTAACAC
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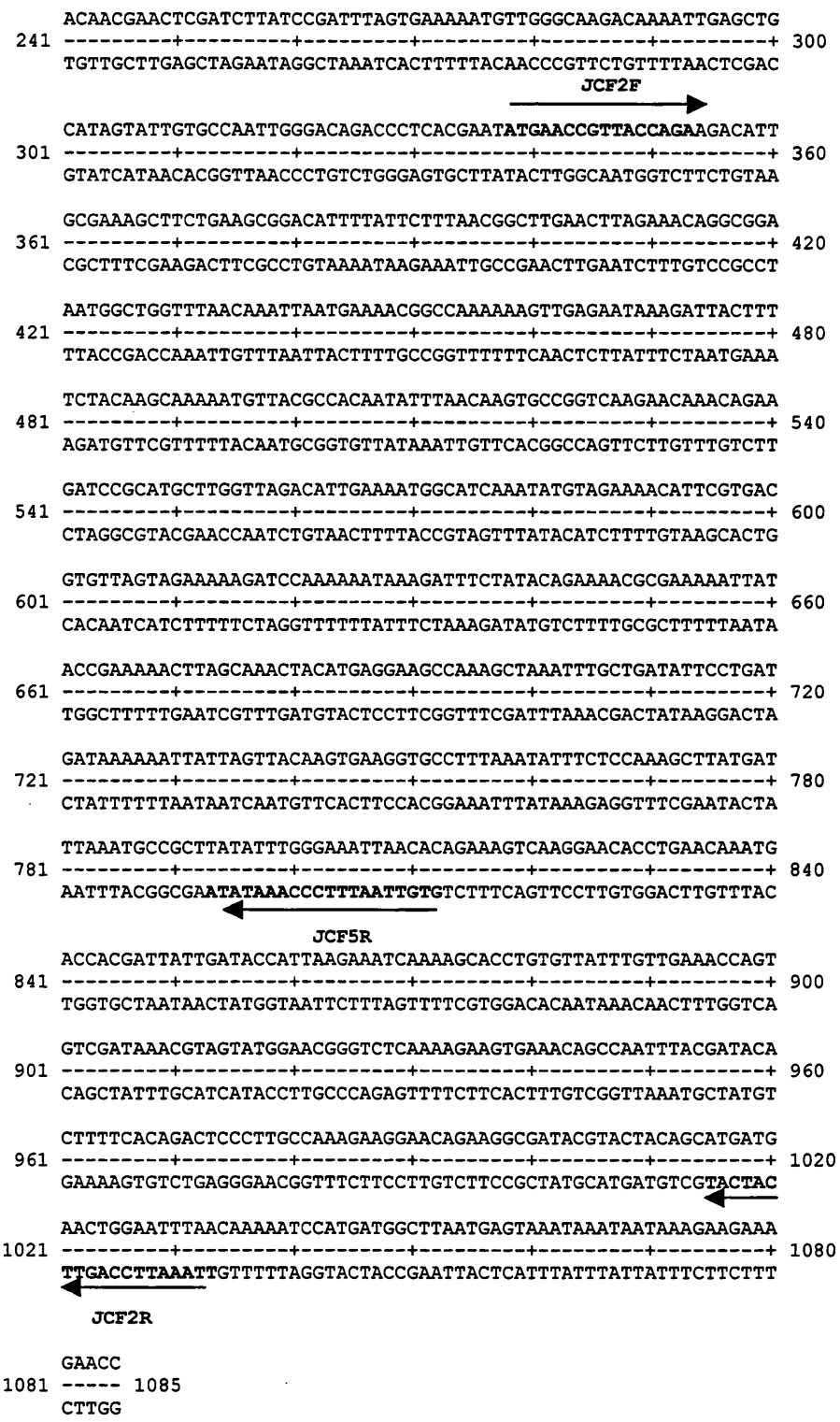
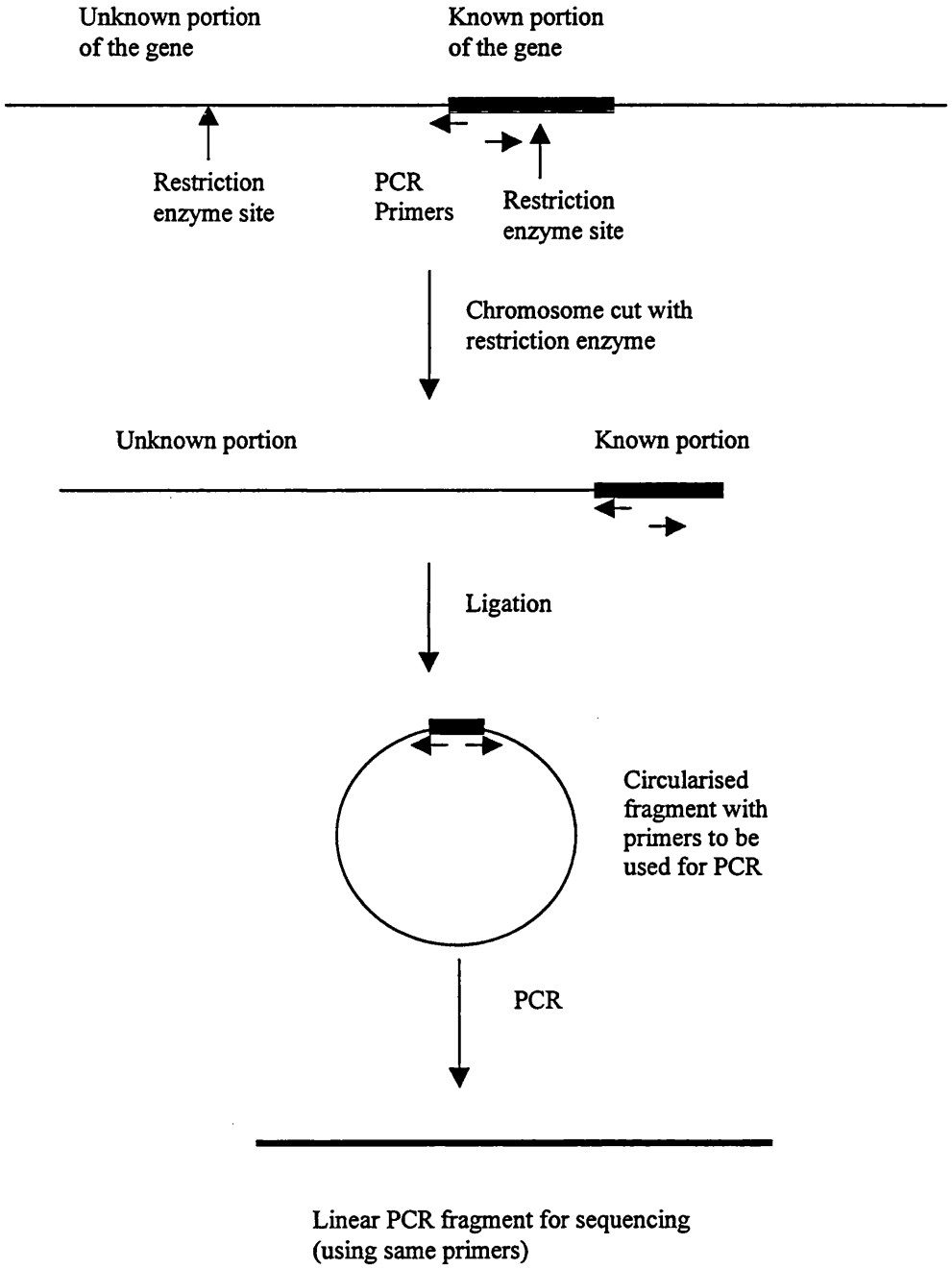


Figure 5.1 shows the portion of the *efaA* gene used to design degenerate primers. Primers JCF2F and JCF2R were used in isolation at first but they showed very non-specific binding. Primer JCF5R was designed to enable more specific PCR of the *efaA* homologue. A semi-nested PCR approach

was used and a second PCR was performed on the product of the first PCR using primers JCF2F and JCF5R.

This strategy allowed the first portion of the *efmA* gene to be sequenced. Inverse PCR was used for subsequent sequencing (Figure 5.2).

Figure 5.2 Strategy of Inverse PCR. *A known portion of the gene to be sequenced is used to design PCR primers, as shown. The chromosome is restricted overnight at 37 °C with an appropriate restriction enzyme. The fragments are ligated at 14 °C for 24 h. PCR is performed on the circularised fragments to produce a linear PCR product, containing a portion of known sequence and a large portion of unknown sequence. Sequencing is then carried out on the PCR product.*



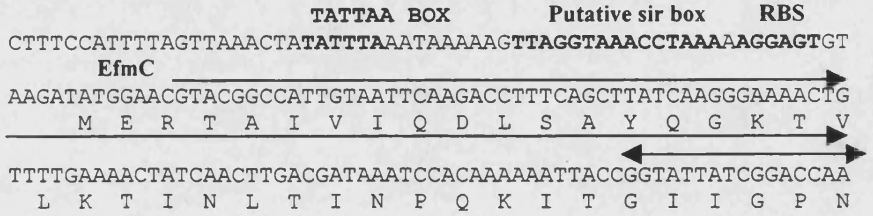
A known portion of the gene is used to design PCR primers, which are orientated the opposite way to normal. A restriction enzyme is selected to

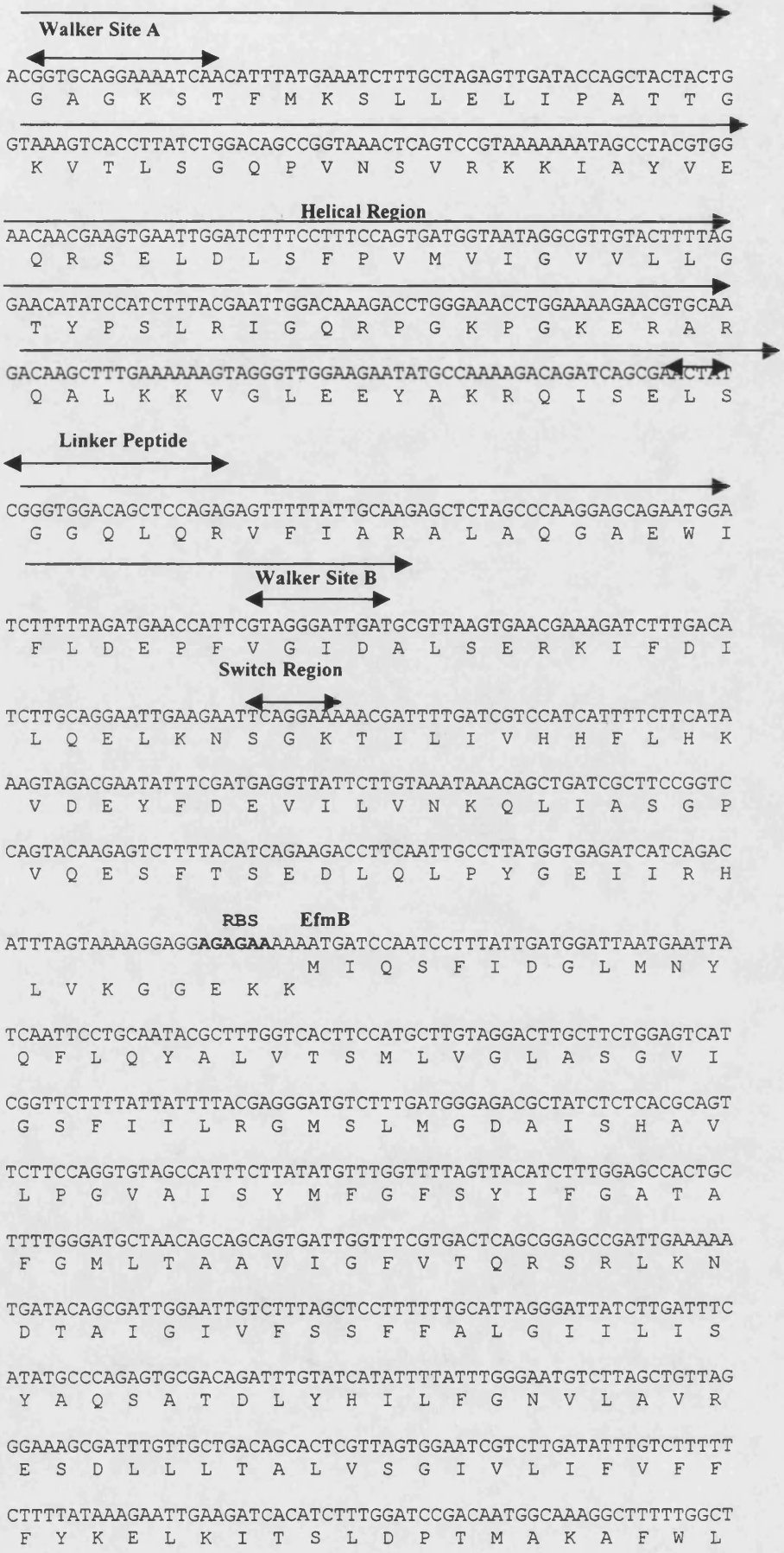
cut in the known portion of the gene. A chromosomal digest is performed with the enzyme to produce a multitude of fragments, some of which will contain the known sequence. A ligation reaction is performed for 24 h to circularise the fragments and thus enable PCR to be performed. The primers are used for PCR and the linear DNA fragment produced is sequenced.

Inverse PCR is a useful tool for sequencing as it enables sequences to be obtained quickly without the need for cloning. Although cloning has traditionally been thought to give more accurate sequencing results this may not always be the case. There are large differences between gram-positive and gram-negative bacteria and some gram-positive proteins can be toxic to the gram-negative bacterium *E. coli*. *E. coli* may make mistakes in processing gram-positive DNA and may even delete portions which are particularly toxic. A proof-reading polymerase was used to minimise mistakes from PCR.

Using the above techniques, the *efm* operon was sequenced and an additional gene upstream of the operon was partially sequenced.

Figure 5.3 Outline of the *efm* Operon. *Putative promoters are marked in bold at the start of efmC. Ribosome binding sites (RBS) are marked in bold at the start of the gene. The secondary structure, where known, is indicated on the map.*

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G A G K S T F M K S L L E L I P A T T G
GTAAAGTCACCTTATCTGGACAGCCGGTAAACTCAGTCCGTAAAAAAATAGCCTACGTGG
K V T L S G Q P V N S V R K K I A Y V E
Helical Region
AACAACGAAGTGAATTGGATCTTTCCTTTCCAGTGATGGTAATAGGCGTTGTACTTTTAG
Q R S E L D L S F P V M V I G V V L L G
GAACATATCCATCTTTACGAATTGGACAAAGACCTGGGAAACCTGGAAAAGAACGTGCAA
T Y P S L R I G Q R P G K P G K E R A R
GACAAGCTTTGAAAAAAGTAGGGTTGGAAGAATATGCCAAAAGACAGATCAGCGAACTAT
Q A L K K V G L E E Y A K R Q I S E L S
Linker Peptide
CGGGTGGACAGCTCCAGAGAGTTTTTATTGCAAGAGCTCTAGCCCAAGGAGCAGAATGGA
G G Q L Q R V F I A R A L A Q G A E W I
Walker Site B
TCTTTTTAGATGAACCATTCGTAGGGATTGATGCGTTAAGTGAACGAAAGATCTTTGACA
F L D E P F V G I D A L S E R K I F D I
Switch Region
TCTTGCAGGAATTGAAGAATTCAGGAAAAACGATTTTGATCGTCCATCATTTTCTTCATA
L Q E L K N S G K T I L I V H H F L H K
AAGTAGACGAATATTTCGATGAGGTTATTCTTGTAAATAAACAGCTGATCGCTTCCGGTC
V D E Y F D E V I L V N K Q L I A S G P
CAGTACAAGAGTCTTTTACATCAGAAGACCTTCAATTGCCTTATGGTGAGATCATCAGAC
V Q E S F T S E D L Q L P Y G E I I R H
RBS
EfmB
ATTTAGTAAAAGGAGGAGAGAAAAAATGATCCAATCCTTTATTGATGGATTAATGAATTA
M I Q S F I D G L M N Y
L V K G G E K K
TCAATTCCTGCAATACGCTTTGGTCACTTCCATGCTTGTAGGACTTGCTTCTGGAGTCAT
Q F L Q Y A L V T S M L V G L A S G V I
CGGTTCTTTTATTATTTTACGAGGGATGTCTTTGATGGGAGACGCTATCTCTCACGCAGT
G S F I I L R G M S L M G D A I S H A V
TCTTCCAGGTGTAGCCATTTCTTATATGTTTGGTTTTAGTTACATCTTTGGAGCCACTGC
L P G V A I S Y M F G F S Y I F G A T A
TTTTGGGATGCTAACAGCAGCAGTGATTGGTTTCGTGACTCAGCGGAGCCGATTGAAAAA
F G M L T A A V I G F V T Q R S R L K N
TGATACAGCGATTGGAATTGTCTTTAGCTCCTTTTTTGCATTAGGGATTATCTTGATTTC
D T A I G I V F S S F F A L G I I L I S
ATATGCCCAGAGTGCGACAGATTTGTATCATATTTTATTTGGGAATGTCTTAGCTGTTAG
Y A Q S A T D L Y H I L F G N V L A V R
GGAAAGCGATTTGTTGCTGACAGCACTCGTTAGTGGAATCGTCTTGATATTTGTCTTTTT
E S D L L L T A L V S G I V L I F V F F
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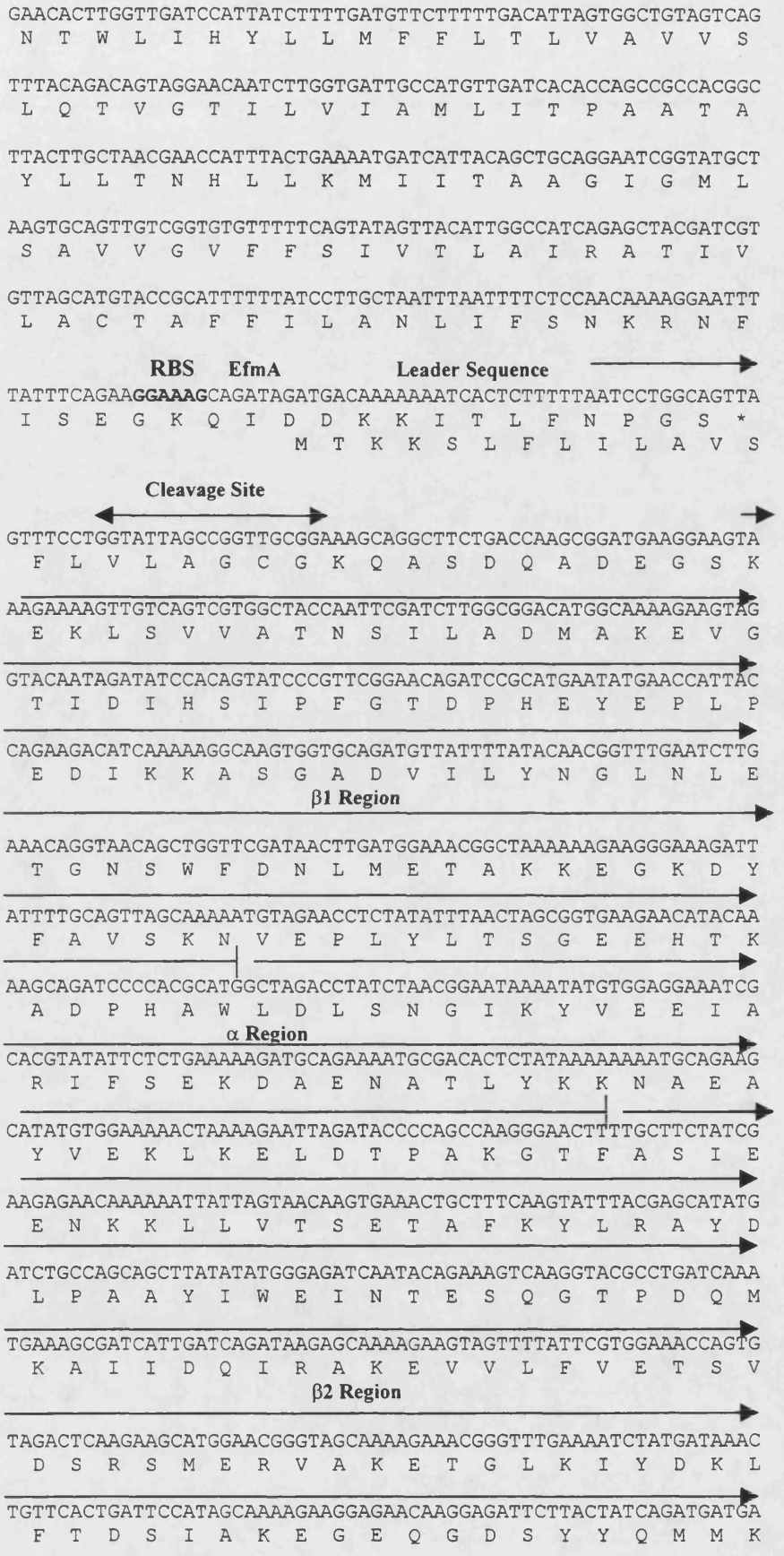
F Y K E L K I T S L D P T M A K A F W L


GAACACTTGGTTGATCCATTATCTTTTGATGTTCTTTTTGACATTAGTGGCTGTAGTCAG
N T W L I H Y L L M F F L T L V A V V S
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L Q T V G T I L V I A M L I T P A A T A
TTACTTGCTAACGAACCATTTACTGAAAATGATCATTACAGCTGCAGGAATCGGTATGCT
Y L L T N H L L K M I I T A A G I G M L
AAGTGCAGTTGTCGGTGTGTTTTTCAGTATAGTTACATTGGCCATCAGAGCTACGATCGT
S A V V G V F F S I V T L A I R A T I V
GTTAGCATGTACCGCATTTTTTATCCTTGCTAATTTAATTTTCTCCAACAAAAGGAATTT
L A C T A F F I L A N L I F S N K R N F
RBS
EfmA
Leader Sequence
TATTTCAGAAGGAAAGCAGATAGATGACAAAAAAATCACTCTTTTTAATCCTGGCAGTTA
I S E G K Q I D D K K I T L F N P G S *
M T K K S L F L I L A V S
Cleavage Site
GTTTCCTGGTATTAGCCGGTTGCGGAAAGCAGGCTTCTGACCAAGCGGATGAAGGAAGTA
F L V L A G C G K Q A S D Q A D E G S K
AAGAAAAGTTGTCAGTCGTGGCTACCAATTCGATCTTGGCGGACATGGCAAAAGAAGTAG
E K L S V V A T N S I L A D M A K E V G
GTACAATAGATATCCACAGTATCCCGTTCGGAACAGATCCGCATGAATATGAACCATTAC
T I D I H S I P F G T D P H E Y E P L P
CAGAAGACATCAAAAAGGCAAGTGGTGCAGATGTTATTTTATACAACGGTTTGAATCTTG
E D I K K A S G A D V I L Y N G L N L E
β1 Region
AAACAGGTAACAGCTGGTTCGATAACTTGATGGAAACGGCTAAAAAAGAAGGGAAAGATT
T G N S W F D N L M E T A K K E G K D Y
ATTTTGCAGTTAGCAAAAATGTAGAACCTCTATATTTAACTAGCGGTGAAGAACATACAA
F A V S K N V E P L Y L T S G E E H T K
AAGCAGATCCCCACGCATGGCTAGACCTATCTAACGGAATAAAATATGTGGAGGAAATCG
A D P H A W L D L S N G I K Y V E E I A
α Region
CACGTATATTCTCTGAAAAAGATGCAGAAAATGCGACACTCTATAAAAAAAAATGCAGAAG
R I F S E K D A E N A T L Y K K N A E A
CATATGTGGAAAAACTAAAAGAATTAGATACCCCAGCCAAGGGAACTTTTGCTTCTATCG
Y V E K L K E L D T P A K G T F A S I E
AAGAGAACAAAAAATTATTAGTAACAAGTGAAACTGCTTTCAAGTATTTACGAGCATATG
E N K K L L V T S E T A F K Y L R A Y D
ATCTGCCAGCAGCTTATATATGGGAGATCAATACAGAAAGTCAAGGTACGCCTGATCAAA
L P A A Y I W E I N T E S Q G T P D Q M
TGAAAGCGATCATTGATCAGATAAGAGCAAAAGAAGTAGTTTTATTCGTGGAAACCAGTG
K A I I D Q I R A K E V V L F V E T S V
β2 Region
TAGACTCAAGAAGCATGGAACGGGTAGCAAAAGAAACGGGTTTGAAAATCTATGATAAAC
D S R S M E R V A K E T G L K I Y D K L
TGTTCACTGATTCCATAGCAAAAGAAGGAGAACAAGGAGATTCTTACTATCAGATGATGA
F T D S I A K E G E Q G D S Y Y Q M M K

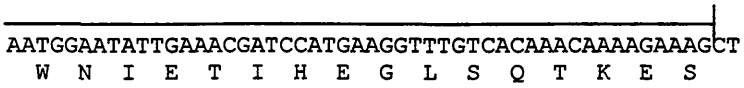


Figure 5.3 outlines the basic layout of the *efm* operon. As in other homologous operons the ATP binding protein is transcribed first, followed by the membrane protein and the receptor protein. Figure 5.3 also shows putative ribosome binding sites and promoters for the *efm* operon. The standard promoters for genes include the – 10 TATTAA box upstream of the ribosome binding site. This box is marked in figure 5.3b.

Perhaps the more interesting putative promoter is the Sir box, marked downstream of the TATTAA box. The Sir box is related to the DtxR family of metal ion-dependent repressors (Lee et al, 1997). In *Staphylococcus epidermidis*, the Sir protein binds to the Sir box under metal ion-replete conditions and prevents transcription of the gene. When metal ion concentration decreases, SirR no longer binds to the Sir box to allow transcription. SirR was found to regulate the production of the *sitABC* operon (which is a member of the Cluster 9 family), responding most strongly to Mn^{2+} and Fe^{2+} concentrations (Hill et al, 1998). The Sir box in the *efm* operon has only been assigned arbitrarily and would need to be proved experimentally. Preliminary studies of metal ion concentration on transcription are described in Chapter 8.

Figures 5.4, 5.5 and 5.6 show the comparison between homologous genes as determined by CLUSTA analysis. As can be seen, all the proteins show a high degree of homology.

Figure 5.4 CLUSTA Analysis of Cluster 9 ATP Binding Proteins

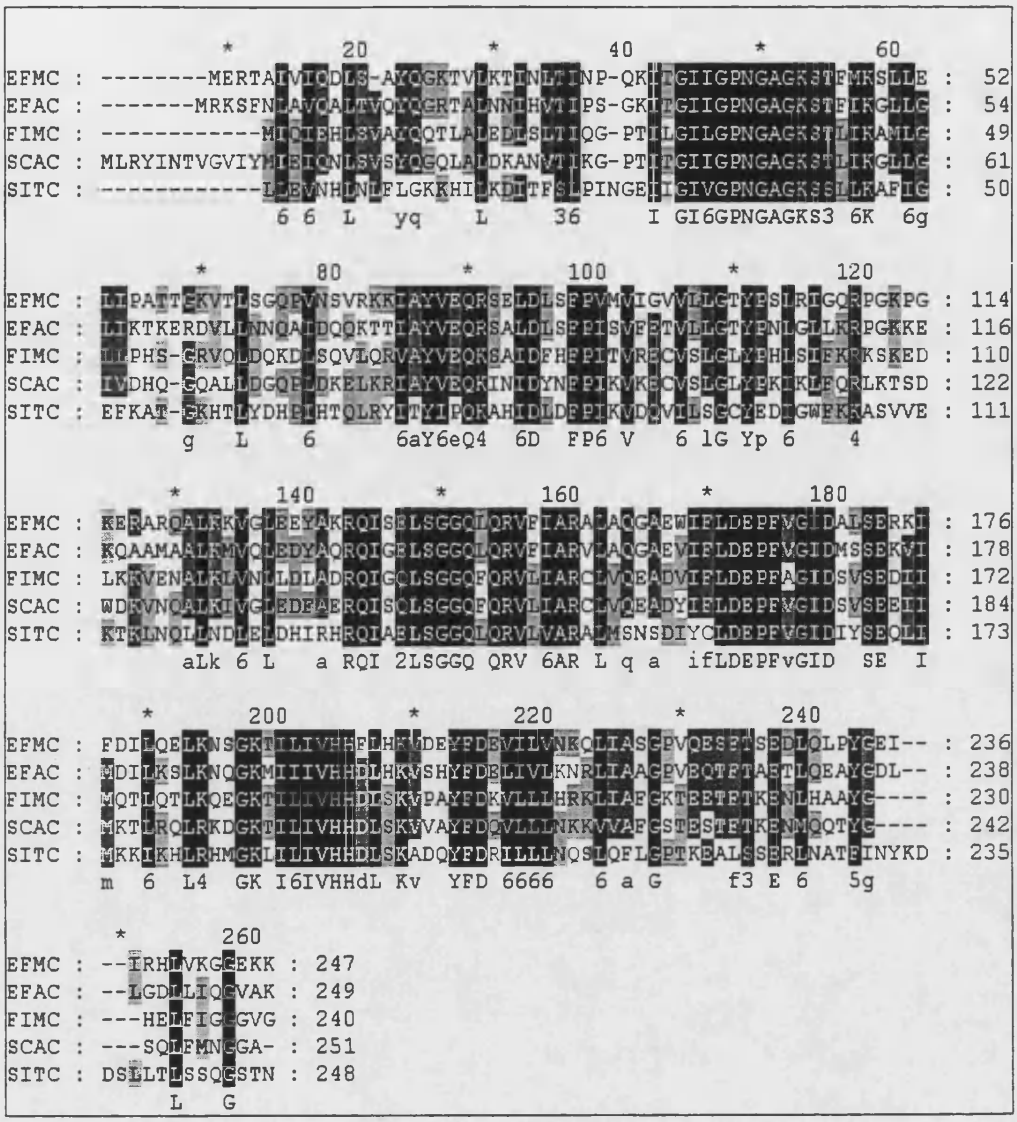


Figure 5.5 CLUSTA Analysis of Cluster 9 Membrane Proteins

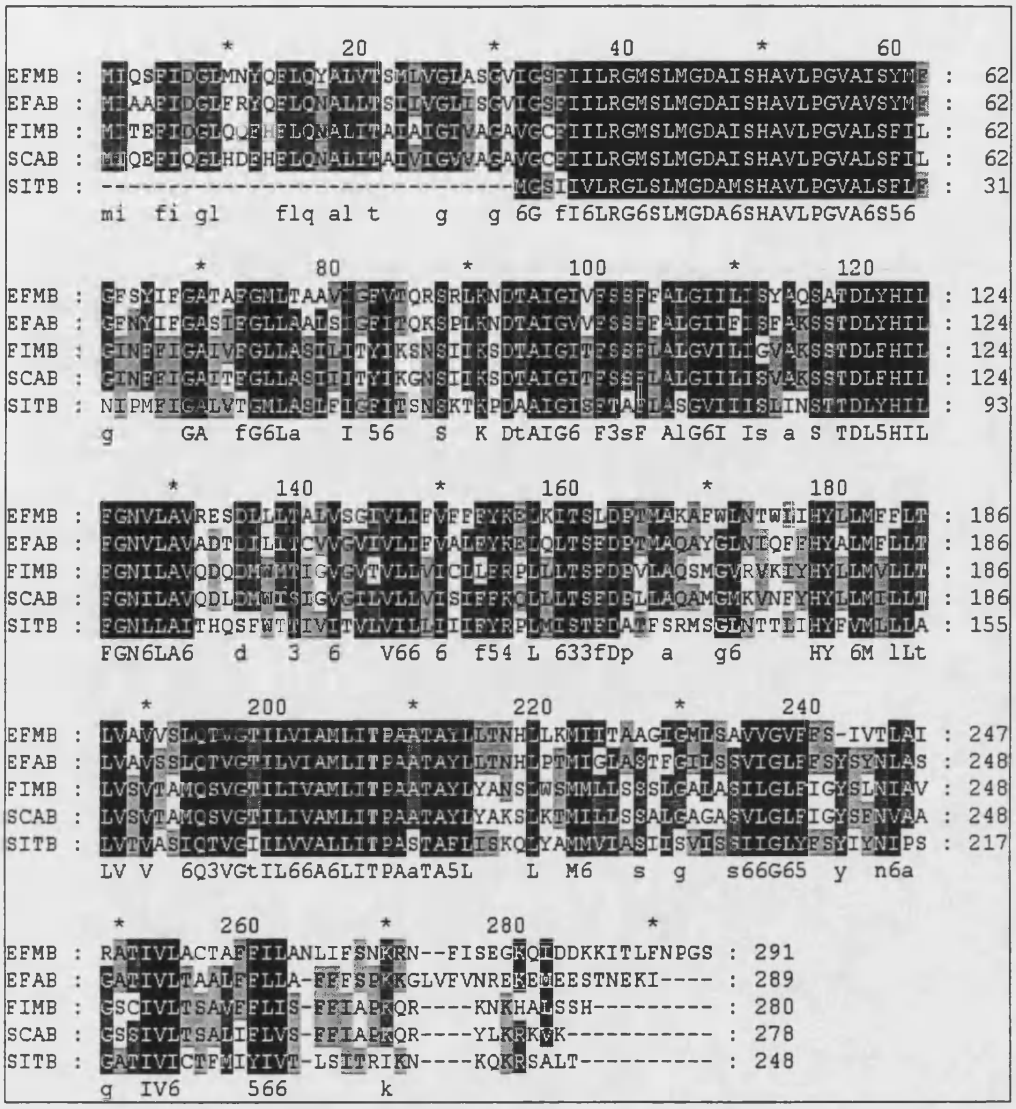
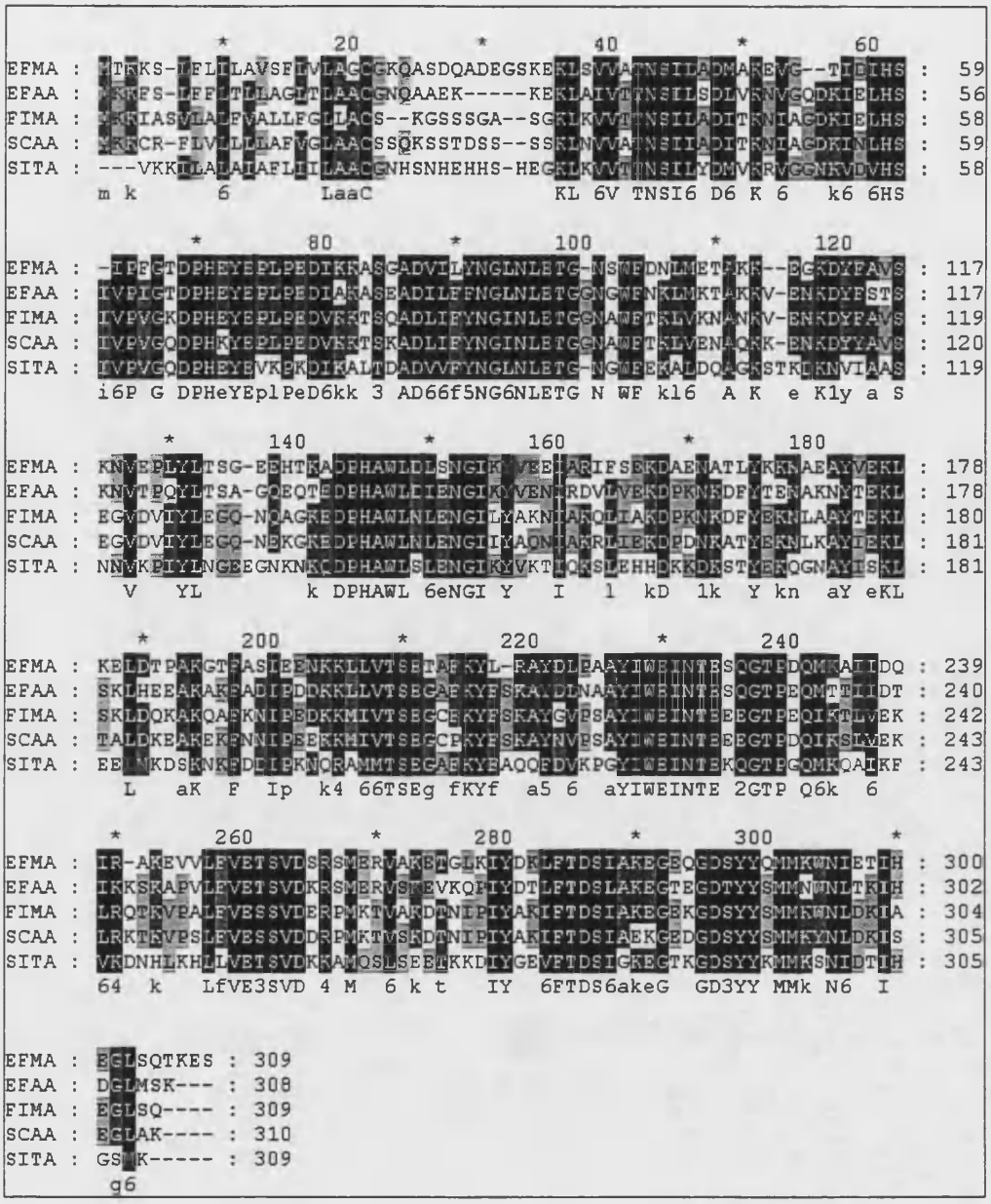


Figure 5.6 CLUSTA Analysis of Cluster 9 Receptor Proteins



A recently published paper by Singh et al (1998) included a sequence of *efaA*$_{fm}$ and an unknown gene located upstream. Figures 5.7 and 5.8 below show the CLUSTA analysis of the published results and the results obtained in this work.

Figure 5.7 CLUSTA Analysis to Compare *efaA$_{fm}$* and *efmA*.

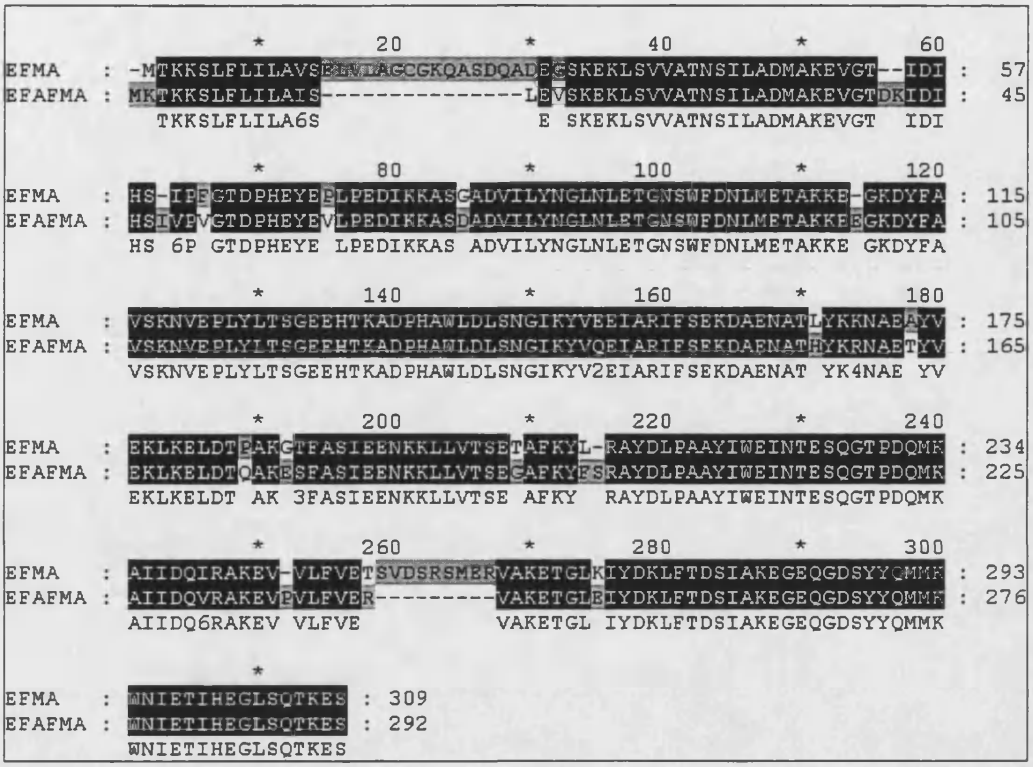
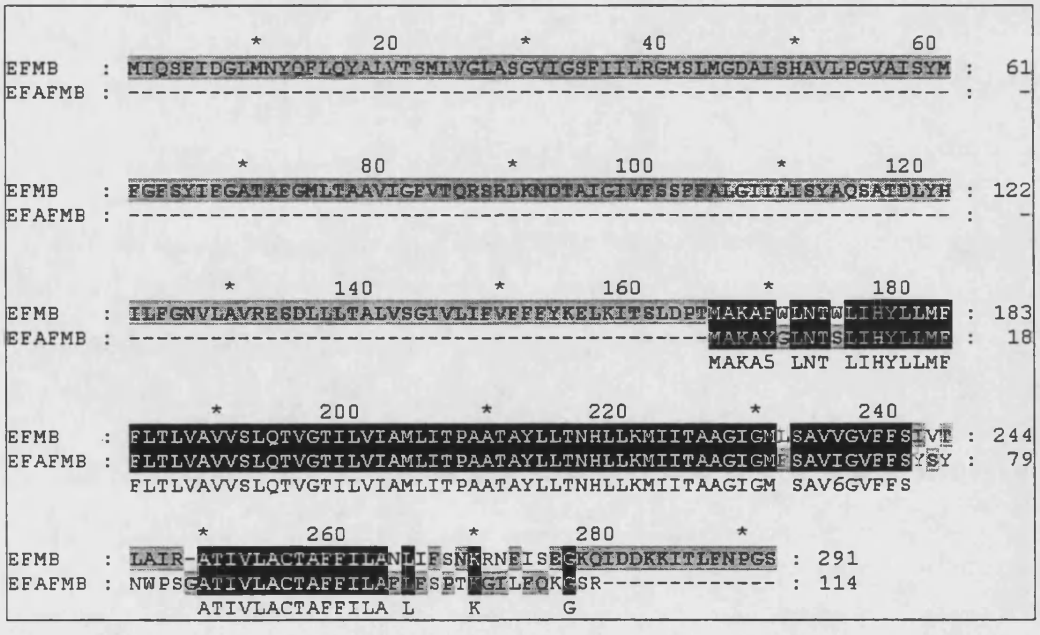


Figure 5.8 CLUSTA Analysis to Compare *efaB$_{fm}$* and *efmB*. *The gene situated upstream of efaA$_{fm}$ has been designated 'unknown gene' by Singh et al (1998), but corresponds to the 3' end of efmB, as illustrated below.*

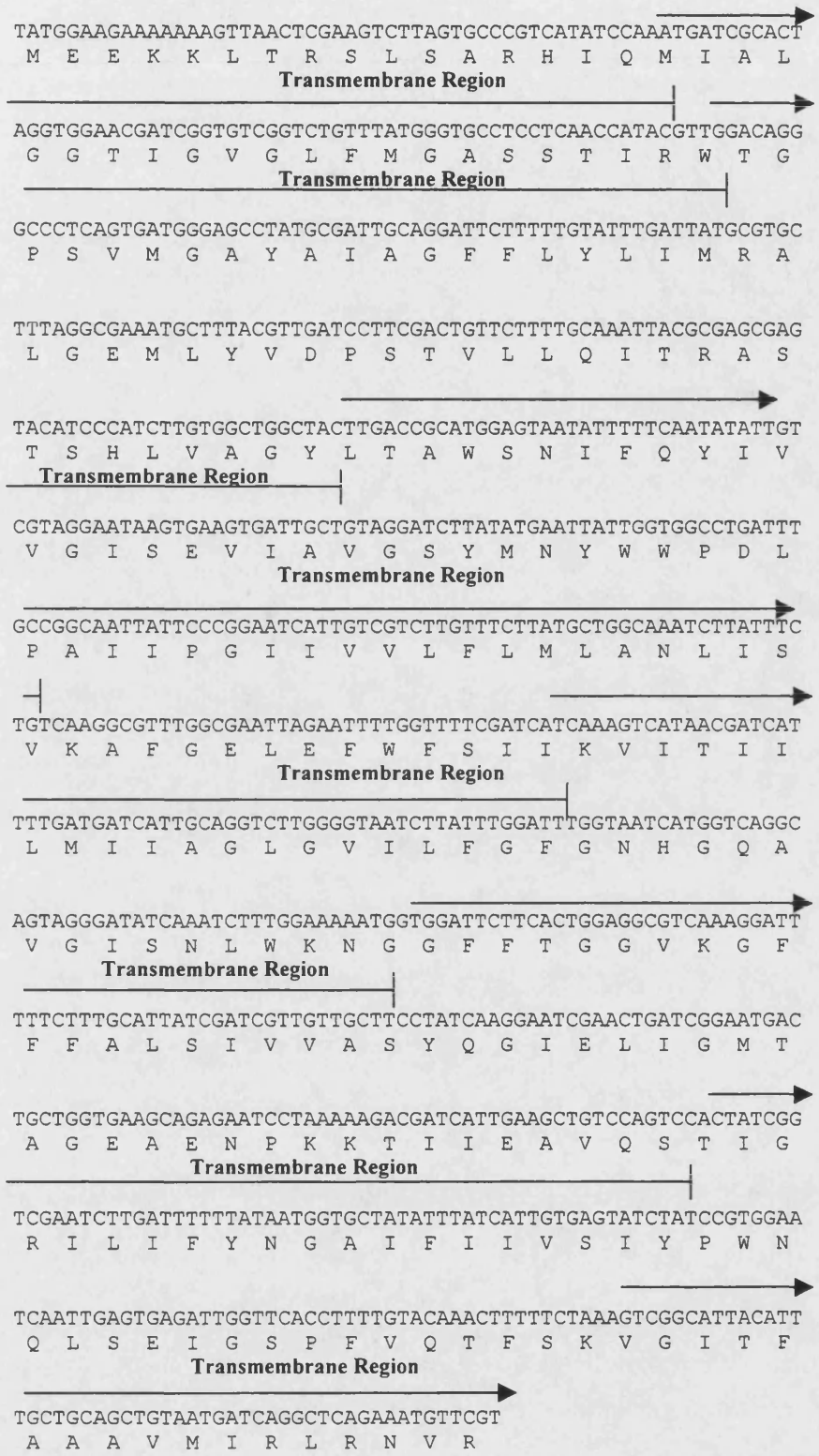


As can be seen the results are similar but the *efaA*$_{fm}$ gene is missing two sections of bases. This could be due to mistakes in processing. It should also be noted that this group obtained their sequence by cloning it into *E. coli*. One of the missing sections contains the lipoprotein cleavage site which is known to be very toxic to gram-negative bacteria, so was probably removed during processing of the foreign DNA. The unknown gene corresponds to a portion of the membrane protein.

Because of the differences obtained in this work, our version of the sequence was registered in GenBank under the accession number AF097414. The title *efm* operon was used to distinguish the two sequences.

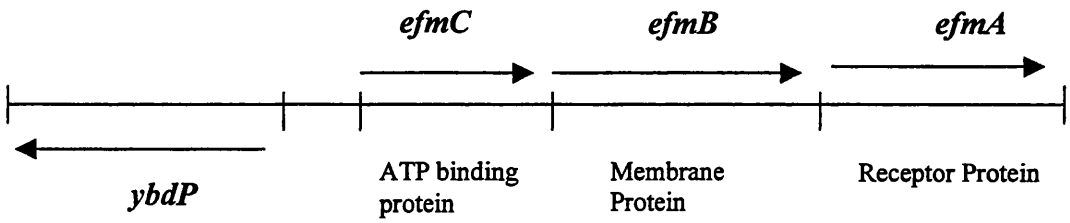
In addition to the *efm* operon, the gene upstream of the *efm* operon was partially sequenced. Figure 5.9 shows the sequence identified.

Figure 5.9 Map Of: Amino Acid Permease Sequence From: 1 To: 877 *the putative secondary structure is illustrated.*



From the homology studies it is possible to predict that the gene is an amino acid or metabolite transport protein which belongs to the amino acid permease family. The most homologous gene, *ybdP* from *Bacillus subtilis* was discovered during a project to sequence the entire *B. subtilis* genome (Kunst et al, 1997). Consequently there is no detailed information on the gene and it is only possible to speculate on its function and therefore the function of the *E. faecium* homologue. The SWISSPROT entry of YbdP was updated recently to include transmembrane domains and these have been shown on the *E. faecium* gene in figure 5.12. However it would be useful to examine the *E. faecium* gene more closely, as it may prove to have a role in endocarditis. A study by Coulter et al (1998) showed that peptide and amino acid transporters have a large impact on virulence in animal models of *S. aureus* disease. A further study by Bayer et al, (1999) showed that the PutP proline permease uptake system contributed towards *S. aureus* virulence in a rabbit endocarditis model. Proline is used by *S. aureus* to provide an inorganic nitrogen source and the bacterium is auxotrophic for this, and several other amino acids. In addition the PutP system helps to maintain osmotic stability by co-transporting sodium ions. Mutants in *putP* show decreased virulence in a rabbit endocarditis model, and produce a smaller vegetation size. It is thought that mutants in other amino acid transporters would show similar effects. It would be interesting to study whether the amino acid permease identified in *E. faecium* is able to affect the organism's virulence in an endocarditis model – as this may provide another target for drug therapy.

Figure 5.11 Outline of the *efm* Operon and Upstream Region in *E. faecium*. *The diagram summarises the sequence obtained during this project and indicates the direction of transcription of the genes identified.*

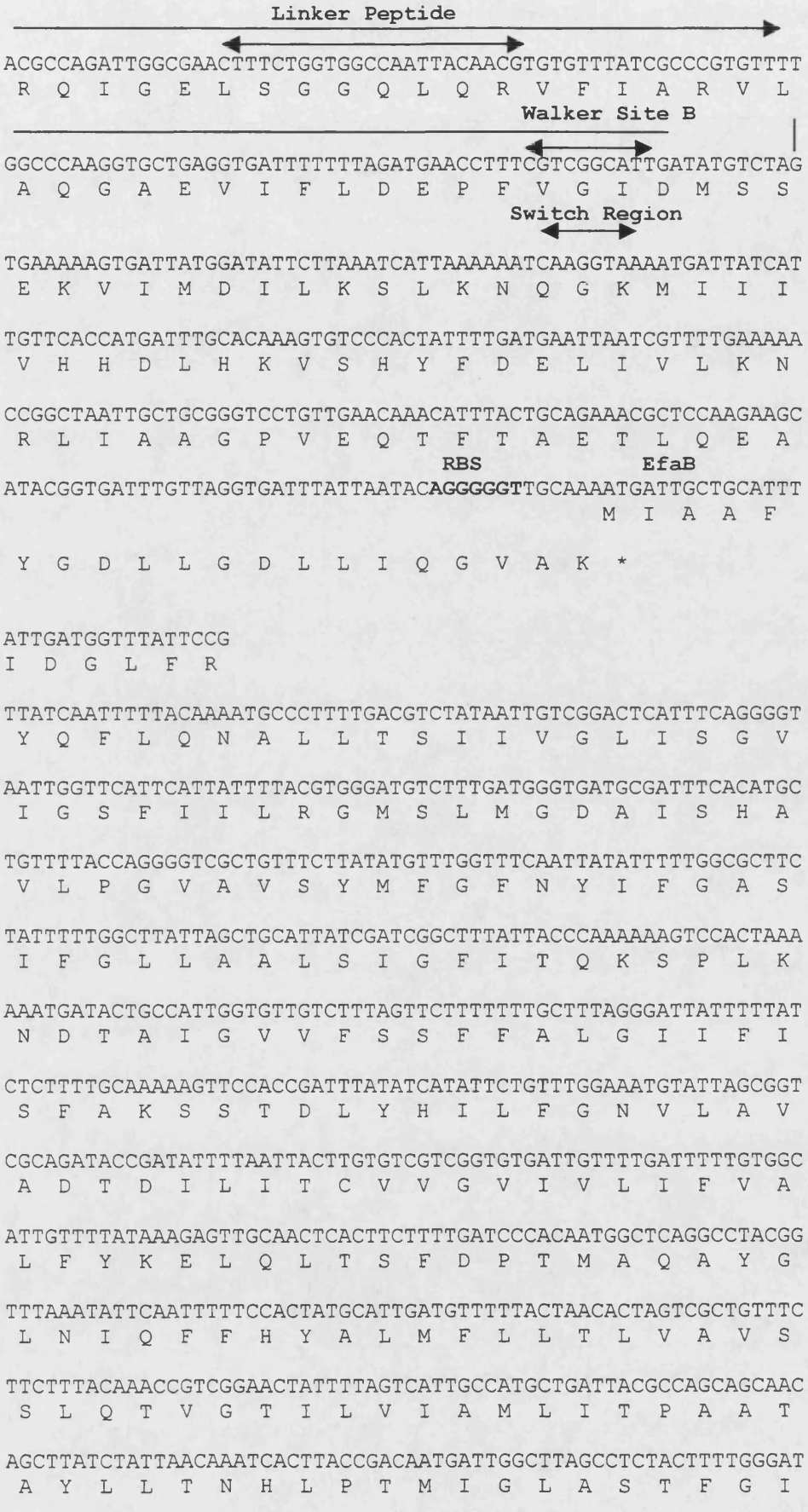


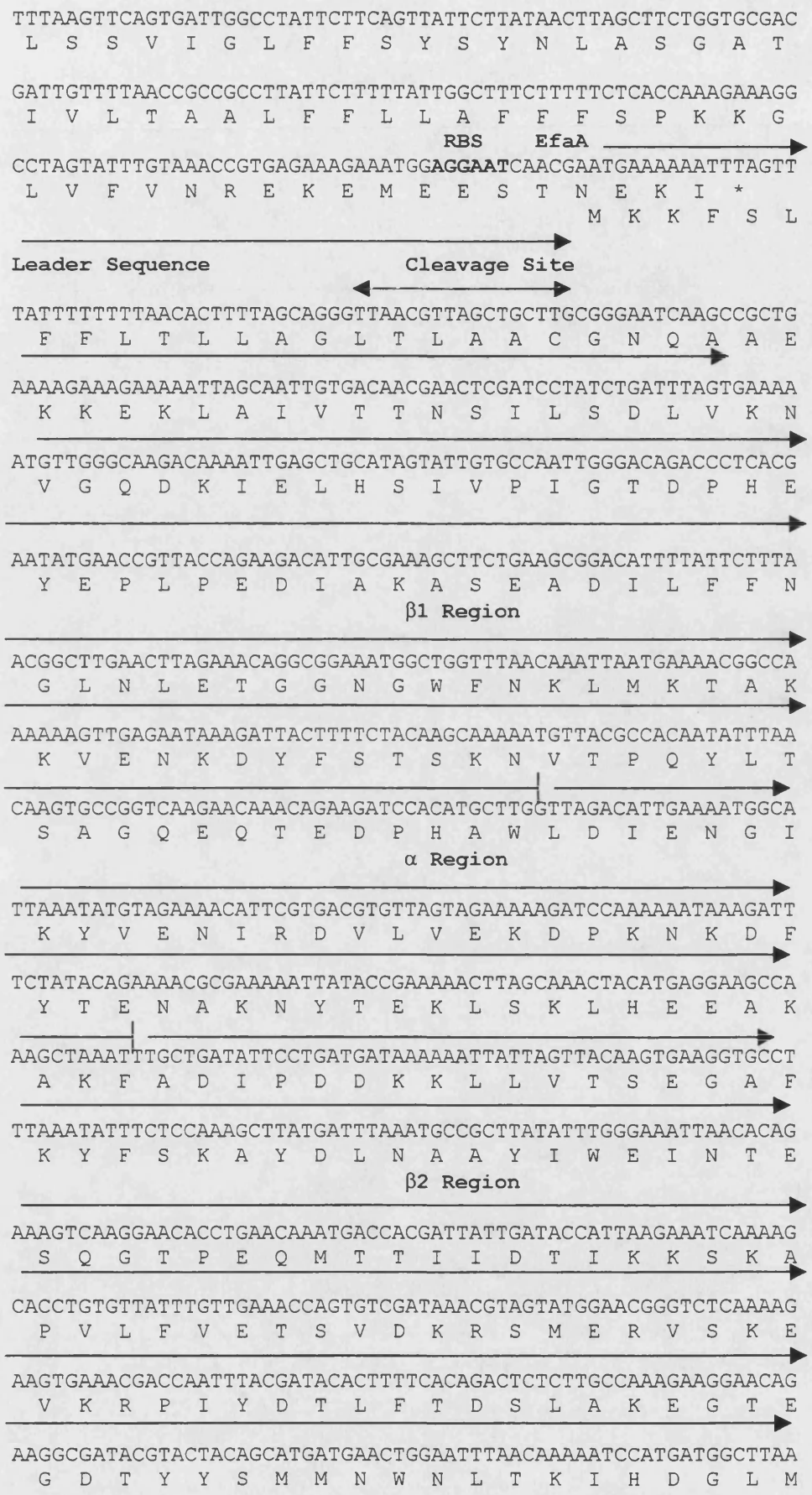
In summary therefore, this section of work presents the successful sequencing of the entire *efm* operon. Putative promoter sites were identified and the partial sequencing of the upstream amino acid permease gene was obtained. Further discussion of the *efm* promoter can be found in Chapters 6 and 8.

6 Sequence Analysis of the *E. faecalis efa* Operon

This section of work aimed to analyse the *efa* operon from *E. faecalis*. The initial sequencing was carried out by Lowe et al (1995). Subsequently the sequences of *efaB* and *efaC* were obtained from Dr Gil Choi of Human Genome Sciences (Maryland, USA). The entire sequence of the *efa* operon has been compiled and is shown here (figure 6.1). Additional sequence data were obtained from The Institute for Genome Research (TIGR) at http://www.tigr.org/.

Figure 6.1 Map of the *efa* Operon, *shows the nucleotide sequence of the operon with the protein sequence aligned underneath. Putative ribosome binding sites (RBS) are marked. The presumed -10 (TTATTA) and -35 (DtxR-homologue binding site) promoter regions of the efa operon are shown upstream of efaC in bold. Some predictions of secondary structure are included.*


Linker Peptide
ACGCCAGATTGGCGAACTTTCTGGTGGCCAATTACAACGTGTGTTTATCGCCCGTGTTTT
R Q I G E L S G G Q L Q R V F I A R V L
Walker Site B
GGCCCAAGGTGCTGAGGTGATTTTTTTAGATGAACCTTTCGTCGGCATTGATATGTCTAG
A Q G A E V I F L D E P F V G I D M S S
Switch Region
TGAAAAAGTGATTATGGATATTCTTAAATCATTAAAAAATCAAGGTAAAATGATTATCAT
E K V I M D I L K S L K N Q G K M I I I
TGTTCACCATGATTTGCACAAAGTGTCCCACTATTTTGATGAATTAATCGTTTTGAAAAA
V H H D L H K V S H Y F D E L I V L K N
CCGGCTAATTGCTGCGGGTCCTGTTGAACAAACATTTACTGCAGAAACGCTCCAAGAAGC
R L I A A G P V E Q T F T A E T L Q E A
RBS
EfaB
ATACGGTGATTTGTTAGGTGATTTATTAATACAGGGGGTTGCAAAATGATTGCTGCATTT
M I A A F
Y G D L L G D L L I Q G V A K *
ATTGATGGTTTATTCCG
I D G L F R
TTATCAATTTTTACAAAATGCCCTTTTGACGTCTATAATTGTCGGACTCATTTCAGGGGT
Y Q F L Q N A L L T S I I V G L I S G V
AATTGGTTCATTCATTATTTTACGTGGGATGTCTTTGATGGGTGATGCGATTTCACATGC
I G S F I I L R G M S L M G D A I S H A
TGTTTTACCAGGGGTCGCTGTTTCTTATATGTTTGGTTTCAATTATATTTTTGGCGCTTC
V L P G V A V S Y M F G F N Y I F G A S
TATTTTTGGCTTATTAGCTGCATTATCGATCGGCTTTATTACCCAAAAAAGTCCACTAAA
I F G L L A A L S I G F I T Q K S P L K
AAATGATACTGCCATTGGTGTTGTCTTTAGTTCTTTTTTTGCTTTAGGGATTATTTTTAT
N D T A I G V V F S S F F A L G I I F I
CTCTTTTGCAAAAAGTTCCACCGATTTATATCATATTCTGTTTGGAAATGTATTAGCGGT
S F A K S S T D L Y H I L F G N V L A V
CGCAGATACCGATATTTTAATTACTTGTGTCGTCGGTGTGATTGTTTTGATTTTTGTGGC
A D T D I L I T C V V G V I V L I F V A
ATTGTTTTATAAAGAGTTGCAACTCACTTCTTTTGATCCCACAATGGCTCAGGCCTACGG
L F Y K E L Q L T S F D P T M A Q A Y G
TTTAAATATTCAATTTTTCCACTATGCATTGATGTTTTTACTAACACTAGTCGCTGTTTC
L N I Q F F H Y A L M F L L T L V A V S
TTCTTTACAAACCGTCGGAACTATTTTAGTCATTGCCATGCTGATTACGCCAGCAGCAAC
S L Q T V G T I L V I A M L I T P A A T
AGCTTATCTATTAACAAATCACTTACCGACAATGATTGGCTTAGCCTCTACTTTTGGGAT
A Y L L T N H L P T M I G L A S T F G I


TTTAAGTTCAGTGATTGGCCTATTCTTCAGTTATTCTTATAACTTAGCTTCTGGTGCGAC
L S S V I G L F F S Y S Y N L A S G A T
GATTGTTTTAACCGCCGCCTTATTCTTTTTATTGGCTTTCTTTTTCTCACCAAAGAAAGG
I V L T A A L F F L L A F F F S P K K G
RBS
EfaA
CCTAGTATTTGTAAACCGTGAGAAAGAAATGGAGGAATCAACGAATGAAAAAATTTAGTT
L V F V N R E K E M E E S T N E K I *
M K K F S L
Leader Sequence
Cleavage Site
TATTTTTTTTAACACTTTTAGCAGGGTTAACGTTAGCTGCTTGCGGGAATCAAGCCGCTG
F F L T L L A G L T L A A C G N Q A A E
AAAAGAAAGAAAAATTAGCAATTGTGACAACGAACTCGATCCTATCTGATTTAGTGAAAA
K K E K L A I V T T N S I L S D L V K N
ATGTTGGGCAAGACAAAATTGAGCTGCATAGTATTGTGCCAATTGGGACAGACCCTCACG
V G Q D K I E L H S I V P I G T D P H E
AATATGAACCGTTACCAGAAGACATTGCGAAAGCTTCTGAAGCGGACATTTTATTCTTTA
Y E P L P E D I A K A S E A D I L F F N
β1 Region
ACGGCTTGAACTTAGAAACAGGCGGAAATGGCTGGTTTAACAAATTAATGAAAACGGCCA
G L N L E T G G N G W F N K L M K T A K
AAAAAGTTGAGAATAAAGATTACTTTTCTACAAGCAAAAATGTTACGCCACAATATTTAA
K V E N K D Y F S T S K N V T P Q Y L T
CAAGTGCCGGTCAAGAACAAACAGAAGATCCACATGCTTGGTTAGACATTGAAAATGGCA
S A G Q E Q T E D P H A W L D I E N G I
α Region
TTAAATATGTAGAAAACATTCGTGACGTGTTAGTAGAAAAAGATCCAAAAAATAAAGATT
K Y V E N I R D V L V E K D P K N K D F
TCTATACAGAAAACGCGAAAAATTATACCGAAAAACTTAGCAAACTACATGAGGAAGCCA
Y T E N A K N Y T E K L S K L H E E A K
AAGCTAAATTTGCTGATATTCCTGATGATAAAAAATTATTAGTTACAAGTGAAGGTGCCT
A K F A D I P D D K K L L V T S E G A F
TTAAATATTTCTCCAAAGCTTATGATTTAAATGCCGCTTATATTTGGGAAATTAACACAG
K Y F S K A Y D L N A A Y I W E I N T E
β2 Region
AAAGTCAAGGAACACCTGAACAAATGACCACGATTATTGATACCATTAAGAAATCAAAAG
S Q G T P E Q M T T I I D T I K K S K A
CACCTGTGTTATTTGTTGAAACCAGTGTCGATAAACGTAGTATGGAACGGGTCTCAAAAG
P V L F V E T S V D K R S M E R V S K E
AAGTGAAACGACCAATTTACGATACACTTTTCACAGACTCTCTTGCCAAAGAAGGAACAG
V K R P I Y D T L F T D S L A K E G T E
AAGGCGATACGTACTACAGCATGATGAACTGGAATTTAACAAAAATCCATGATGGCTTAA
G D T Y Y S M M N W N L T K I H D G L M

```
TGAGTAAATAAATAATAAAGAAGAAAGAACCAAGTTGACTCCCCCTCAACTTGGTTCTTT
  S   K   *
```

The putative promoter sites of the *efa* operon are shown in figure 6.1. The most interesting promoter site is the *dtxR* box seen around the -35 element. The DtxR promoter region was first identified in *Corynebacterium diphtheriae* where it represses the production of diphtheria toxin when iron concentrations are high (Boyd et al, 1990, Schmitt and Holmes, 1991). DtxR is presumed to act as a dimer. It binds to its recognition sequence which overlaps the -35 promoter and prevents RNA polymerase from binding and initiating transcription (Schmitt et al, 1992). Under iron-limiting conditions, DtxR releases its hold on the DtxR box and allows transcription to take place (Schmitt et al, 1992).

Since the discovery of DtxR, homologues have been found in many other species, including *Mycobacterium tuberculosis* and other mycobacteria, *Streptomyces lividans*, *Streptomyces pilosus* and *Brevibacterium lactofermentum* (see Table 6.1). They have been termed iron dependent regulators (IdeR). It is thought that they all have pleiotropic effects on gene expression. Insertional inactivation of *ideR* in *Mycobacterium smegmatis* caused de-repression of siderophore biosynthesis under high iron concentrations, decreased production of Mn-dependent superoxide dismutase and catalase/peroxidase and increased susceptibility to killing by hydrogen peroxide (Dussurget et al, 1996). In *C. diphtheriae* additional DtxR promoter/operators have been identified (termed IRP1-5), which have products varying from a 38 kD membrane-associated lipoprotein (*irp1*),

thought to be a receptor for ferric siderophore, to a haem oxygenase needed to utilise haem and haemoglobin (Schmitt and Holmes, 1994, Lee et al, 1997). In addition to responding to iron, DtxR can respond to Co^{2+} or Ni^{2+} concentrations (Schmitt et al, 1992).

The DtxR site shown in figure 6.1 is similar to three mitis group Streptococci analysed by Dr N. Jakubovics (personal communication, see Table 6.1). FimC (*S. parasanguis*), PsaC (*S. pneumoniae*) and ScaC (*S. gordonii*) all contain the sequence AAATTAACTTGACTTAATTT around the -35 element. This is very similar to the AAATTATTTGCATTTTCTT box found in *efaC* around the -35 site. Interestingly this contrasts with the putative promoter sequence found in the *efm* operon (see Chapter 5), which showed greater homology to the Sir box found upstream of the *sit* operon in *S. epidermidis*. Whilst SirR, the repressor in *S. epidermidis* is a DtxR homologue, it is not clear if the two respond in exactly the same way. Indeed SirR has been shown to respond to manganese but DtxR has not been studied in this respect (Hill et al, 1998). The slight base changes in the promoter sites may also affect the response of the repressors. Lee et al (1997) found that one site (IRP3) showed a weaker response to iron than other promoters and needed higher iron concentrations to be activated. This was thought to be due to a T-to-C substitution at the 3' end of the primary recognition sequence. The effect of metal ion concentration on the expression of the *efa* and *efm* operons will be discussed in Chapter 8.

Table 6.1 DtxR Boxes

Species	Name of box	Sequence
S. epidermidis	sir box	TTAGGTTAAC-CTAAACTTT
E. faecium	*efm* operon	TTAGGTAAAC-CTAAA---
C. diphtheriae	DtxR tox box	T-ATAATTAGGATAGC-TTTA-CCTAATTA-TT
C. diphtheriae	DtxR irp1 box	C-ATTTTTAGGTTAGC-CAAACCTTTGTTG-GTG
C. diphtheriae	DtxR irp2 box	C-CGCGC-AGGGTAGC-CTAACCTAAACCG-GCG
C. diphtheriae	DtxR irp3 box	GT-CTATTAGG-TGAGACGCACCC-ATCGGAATG
C. diphtheriae	DtxR irp4 box	TT-TCATTA-CTAACGCTAAACCTAAGTAGCATA
C. diphtheriae	DtxR irp5 box	TTAGCACTAGGATTGC-CTACACTT-ACTA-A
C. diphtheriae	HmuO box	T-G----AGGGGAAC-CTAACCTAA
S. lividans	DesA box	C-GACATTAGGTTAGG-CTCACCTAAGTT---CA
DtxR Consensus	Consensus box	TTAGGTTAGCCTAACCTAA
E. faecalis	*efa* operon	AAATTATTTGCATTTTCTT-
Mitis group Streptococci	*fim* operon, *sca* operon and *psa* operon	AAATTAACTTGACTTAATTT

The presence of a DtxR homologue in *E. faecalis* was confirmed by studying preliminary sequence data from TIGR. The sequence of this homologue is shown in Figure 6.2. The predicted protein is 222 amino acids in size and has a molecular weight of 25.486 kD. A BLAST search revealed that it has 39% identity to SirR from *S. epidermidis* and 27% identity to DtxR from *C. diphtheriae*. Unlike DtxR itself it does not appear to be auto-regulated: no obvious DtxR box was found upstream of the ORF. Its precise position on the chromosome cannot be determined yet, but a search of the 1000 bp either side of the sequence revealed that the only significant ORF in that area encodes for a glucose – 6 – phosphate – 1- dehydrogenase enzyme immediately downstream.

The sequence was studied with the Gene Quiz programme at EMBL in an effort to determine its secondary structure. The programme did not predict any significant secondary structure, but a search of the literature revealed that DtxR proteins are believed to act as dimers and consist of three domains. The first, N terminal domain, contains a helix-turn-helix DNA binding motif.

The central dimer interface domain is thought to contain two metal binding sites and the third, C terminal, domain is the dimerisation/stabilisation region (Lee et al, 1997, Hill et al, 1998).

It would be interesting to ascertain the metal binding specificity of the *E. faecalis* DtxR protein. The SirR protein was found to be capable of binding iron and manganese and Hill et al (1998) hypothesised that the protein functioned as a manganese- rather than an iron-dependent repressor. The *efa* operon was predicted by Dintilhac et al (1997) to be a manganese transporter (see also Chapter 8), so it would seem probable that the *E. faecalis* DtxR homologue is also a manganese-dependent repressor. The differences between DtxR homologues and their DNA recognition sequences may, in part, reflect their different metal binding preferences. However this hypothesis requires experimental support.

Figure 6.2 Map of the *E. faecalis dtxR* Homologue *The putative ribosome binding site (RBS) is marked in bold.*

```
TTTCTTACAACTTGAATATTATTAAATAGTCAAATGCGCCTTTTTCTGATAAACTTAGGA
                  RBS
TGATGTTTTTTTGAAAGGATGAGCCCCTATGACACCAAATCGCGAAGACTATTTAAAATT
                            M  T  P  N  R  E  D  Y  L  K  L

AATTTTTGAATTAGGTGGCGACGAAGTTAAAGTGAATAATAAACAAATTGTTTCTGGACT
 I  F  E  L  G  G  D  E  V  K  V  N  N  K  Q  I  V  S  G  L

CGATGTTTCGGCAGCTTCGGTTAGTGAGATGATTTCAAAGTTAGTAAAAGAAGATTTGGT
 D  V  S  A  A  S  V  S  E  M  I  S  K  L  V  K  E  D  L  V
```

```
TGAGCATTCTCCTTATCAAGGGGTACAATTAACTGAAAAAGGCTTAAAAAAAGCGAGTAC
 E  H  S  P  Y  Q  G  V  Q  L  T  E  K  G  L  K  K  A  S  T

GTTAATTCGCAAACACCGAATCTGGGAAGTCTTTTTAGTAGAGCACTTAAATTACACTTG
 L  I  R  K  H  R  I  W  E  V  F  L  V  E  H  L  N  Y  T  W

GAATGATGTGCACGAAGAGGCAGAAGTTTTAGAACATGTTACTTCACAGACGCTTGTGAA
 N  D  V  H  E  E  A  E  V  L  E  H  V  T  S  Q  T  L  V  N

CCGTTTAGCGGATTATTTAAATCATCCAGAATTTTGTCCACACGGTGGTGTTATTCCCGA
 R  L  A  D  Y  L  N  H  P  E  F  C  P  H  G  G  V  I  P  E

AGATAATCAACCCATTCATGAGGAGAAACGCCAAACGTTAACAGACTACCCTGTTGGCAC
 D  N  Q  P  I  H  E  E  K  R  Q  T  L  T  D  Y  P  V  G  T

AAAAATTCGGATTGCACGTGTCTTAGACGAAAAAGAATTACTGGATTATTTAGTTTCCAT
 K  I  R  I  A  R  V  L  D  E  K  E  L  L  D  Y  L  V  S  I

TGATTTAAATATTCAAGAAGAATATACGATTAAAGAAATTGCTGCATATGAAGGACCGAT
 D  L  N  I  Q  E  E  Y  T  I  K  E  I  A  A  Y  E  G  P  I

CACCATTTATAATGAAAACAAAGAATTATCCGTCAGCTTTAAAGCAGCAAACACAATTTT
 T  I  Y  N  E  N  K  E  L  S  V  S  F  K  A  A  N  T  I  F

TGTTGAGCCGTTGATTAGAGAAAGTGAGGAAAACTAATGAATGAAAAAGTAGCATTATTT
 V  E  P  L  I  R  E  S  E  E  N  *
```

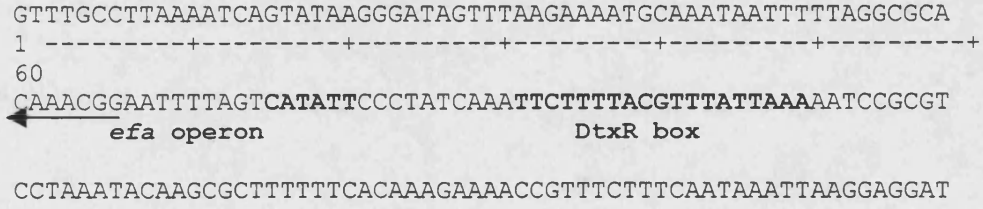
The preliminary *E. faecalis* sequence data from TIGR were also used to study the significant ORFs on either side of the *efa* operon.

Two open reading frames were detected upstream of *efaC* (Figure 6.3). Both ORFs are situated on the opposite strand and therefore run in the opposite direction to *efa*. The gene closest to *efaC* shows significant homology to genes encoding 5-phospho-D-ribosyl-α-1-pyrophosphate (PRPP) synthetase. Such genes are usually designated *prs*, so this term is used here. PRPP synthetase catalyses the production of PRPP from ribose-5-phosphate. ATP is used in the reaction. PRPP is then used to synthesise purines and pyrimidines, the amino acids tryptophan and histidine and NAD (Nilsson et al, 1989). The *E. faecalis* PRPP synthetase shows 53% identity to the PRPP

synthetase from *L. monocytogenes* and 51% identity with the *B. subtilis* protein.

The second gene, encoded upstream of *prs*, is a NifS protein homologue. The TIGR sequence appears to contain an error at the start of the gene. BLAST studies show that the correct start site is the one shown in Figure 6.3. A base change from T to A at position 1326 would mean that methionine is transcribed instead of leucine and the correct start site is obtained. The NifS protein was first identified as a component of the nitrogen-fixing operon in bacteria. It has subsequently been identified in non-nitrogen fixing bacteria, such as *B. subtilis* (Sun and Setlow, 1993). The *E. faecalis* NifS shows 53% identity to the *B. subtilis* protein. It is thought to catalyse the specific desulphurisation of L-cysteine to provide organic sulphide, which is then formed into Fe-S clusters. Fe-S clusters are used in the synthesis of enzymes for the production of NAD, and in tRNA processing and mitochondrial metabolism (Sun and Setlow, 1993). Neither of these upstream proteins appears to have any function linked to *efa* and they are unlikely to be co-transcribed with the *efa* operon.

Figure 6.3 Map of the Region upstream of the *efa* operon. *The map shows the two genes encoded upstream of efa operon on the opposite strand, therefore the map has been reversed and the position of the efa operon is shown by the arrow. The DtxR box and the TTATTC promoter are also indicated.*



RBS *prs*

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T E D I M T E N Y Q D D T L R I F S L N

ATGCAAATCGACCACTTGCTGAAAAAATCGCAGCATCAGTAGGAACAGAATTAGGAAAAA

A N R P L A E K I A A S V G T E L G K S

GCACCGTGCGCCAATTTAGTGATGGCGAAATTCAAATTAATATTGAAGAAAGTATTCGTG

T V R Q F S D G E I Q I N I E E S I R G

GCGATCATGTCTACATCATCCAAGCAACGAATGCTCCTGTAAATGATCATTTGATGGAAT

D H V Y I I Q A T N A P V N D H L M E L

TGCTTATTTTAATTGATGCATTAAAACGTGCTAGCGCAAAAACAGTTAATGTTATTTTGC

L I L I D A L K R A S A K T V N V I L P

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Y Y G Y A R Q D R T A K P R E P I T A K

AATTAGTTGCTAATATGTTGGTAGAGGCAGGCGCAACACGTTTGTTGACATTAGACTTGC

L V A N M L V E A G A T R L L T L D L H

ATACTGTTCAAGTCCAAGGATTTTTTGATATCCCAGTAGATAATTTATTTACAATGCCTT

T V Q V Q G F F D I P V D N L F T M P L

TGTTTGCTCATTATTATCGTCAACAAGCATTAGTAGGGGAAGAAATTGTGATTGTTTCGC

F A H Y Y R Q Q A L V G E E I V I V S P

CTAAAAACAGTGGCGTTCAACGCGCACGTAGTTTATCGGAGTACTTAGATGCGACCTTAG

K N S G V Q R A R S L S E Y L D A T L A

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I V D H E E I D G V R Q E G Y V I G N V

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A G K K C I L V D D I L N T G Q T L A T

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A A E V L M K N G A Q E V Y A C A S H G

GTTTGTTATCAGAAGGCGCAAAAGCAACCTTAGAAAATGCGCCAATTAAGGAAATTAGTA

L L S E G A K A T L E N A P I K E I S I

TTACTGACTCTGTTTATACGACAGCAGACCGCCAACCAGCAACCTTGAATATTATTTCTT

T D S V Y T T A D R Q P A T L N I I S C

GCGCTGAGTTAATGGGGGAAGCGTTACTTCGTATCCATGAAAATAAACCAATGAGTCCAT

A E L M G E A L L R I H E N K P M S P L

TATTCCGTTTAGAACCAAAAGGAGAATAAGTGATTCCTTAAACTAGAAATAGGTCTAACA

F R L E P K G E *

ACTTTAATAGTGTTGGGCCTATTTTTCTTATCTCTTTTATAAATAGAAGAGAAAGTATTC

RBS

TGATTTTGTGTTTTAAGCTTTTTATGCTAGAATAACGCCAGAACATAAGGAA**AGGGAC**GA

GTCATTTGGAACCCATTTATTTAGATCATGCGGCAACAACACCGCTTCATCCAACGGTCA

L E P I Y L D H A A T T P L H P T V I

```
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AAGAAACGATCTTGGTTTCAATGATGTATGGCAATAATGAAATTGGAAATCGATTACCGA
  E  T  I  L  V  S  M  M  Y  G  N  N  E  I  G  N  R  L  P  I

TTGCTGAAGTTGGTGCAATTCTTAAAAATCATTCGGCGATTTTTCATACAGATGCCGTCC
  A  E  V  G  A  I  L  K  N  H  S  A  I  F  H  T  D  A  V  Q

AAGCTTATGGAAGTGAAGTCATTTTACCTCATGAATTAGGGATCGACTTATTAAGTATTT
  A  Y  G  S  E  V  I  L  P  H  E  L  G  I  D  L  L  S  I  S

CCGCTCATAAAATCAATGGTCCAAAAGGCGTAGGTTTTTTATTTAAAAGCGATGCAATCC
  A  H  K  I  N  G  P  K  G  V  G  F  L  F  K  S  D  A  I  Q

AATTACCACCTCTTTTACATGGTGGGGAACAAGAAGAAAAACGACGTGCGGGCACGGAAA
  L  P  P  L  L  H  G  G  E  Q  E  E  K  R  R  A  G  T  E  N

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  L  A  G  I  I  G  M  G  T  A  V  S  L  L  T  S  A  E  K  Q

AAGCAAGAAAAACAGCCTATCAAAGTTTTCAAACGATTATTTTAAAAGCGTTAGAAGAAG
  A  R  K  T  A  Y  Q  S  F  Q  T  I  I  L  K  A  L  E  E  A

CCAATATTGATTTTTCAATTAATGGTGAACCAACCAATCGTTTAGCGCATGTCTTAAATC
  N  I  D  F  S  I  N  G  E  P  T  N  R  L  A  H  V  L  N  L

TTCATTTAAAAGGAATTCCCAGTGATTTGCTCTTAATGCATTTGGACTTAAGAGGTATTG
  H  L  K  G  I  P  S  D  L  L  L  M  H  L  D  L  R  G  I  A

CGATTTCAACAGGCTCGGCTTGTACAGCGGGAACTGTGGATCCTTCGCACGTGTTGACAG
  I  S  T  G  S  A  C  T  A  G  T  V  D  P  S  H  V  L  T  A

CGATGTATGGAGAAAATTCCTCAGCTATTAAAGAATCAATCCGGATTAGTTTTGGCTACG
  M  Y  G  E  N  S  S  A  I  K  E  S  I  R  I  S  F  G  Y  G

GGAACACACCAGAAGAAATTGCGACTTTTTCTGAAGTACTAGTTGCAGTGATTCAACAAT
  N  T  P  E  E  I  A  T  F  S  E  V  L  V  A  V  I  Q  Q  L

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  K  K  *

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TTTTACAAAGTACATCCTAGTGCAAAACGCTATACATTAAAAGATAACGGCTTTACTGAA
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ACGGCAAATGGTTTAAAACCAATGAATATTTTCAAGAATGAACAACATGAAATGAGTAAA
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TAATTTAGAGAAACCTGAAGAAACAAGCGAAAGTAGCTTGTTCCTTCAGGTTTTCTGTTT
ATTTGAAAGCAATAGCTTGTTGGCTGTGTCTTTGTTCCAAGTGTTTTCTCATTATACGCC
CGGCTTGCTTTA

A study of the region downstream of the *efa* operon revealed four ORFs situated on the complementary strand (see Figure 6.4). All four ORFs showed homology to proteins in the Genbank database that have been identified during sequencing projects. This makes it difficult to assign a definite function to these proteins. However a putative structure or function has been assigned where possible.

The gene encoded closest to *efaA* is a 502 amino acid proteins which shows 38% identity to YfmM from *B. subtilis*. (Yamamoto et al, 1997). YfmM was sequenced during the sequencing project of the *B. subtilis* genome. YfmM is an ATP binding protein and is believed to have another protein, YfmL as its translation initiation factor. It is similar to hypothetical ABC transporters in *H. influenzae*, *E. coli* and a positive effector in *Saccharomyces cerevisiae* (gcn20). Gcn20 is activated by Gcn2 to phosphorylate the α sub-unit of translation initiation factor 2 in yeast.

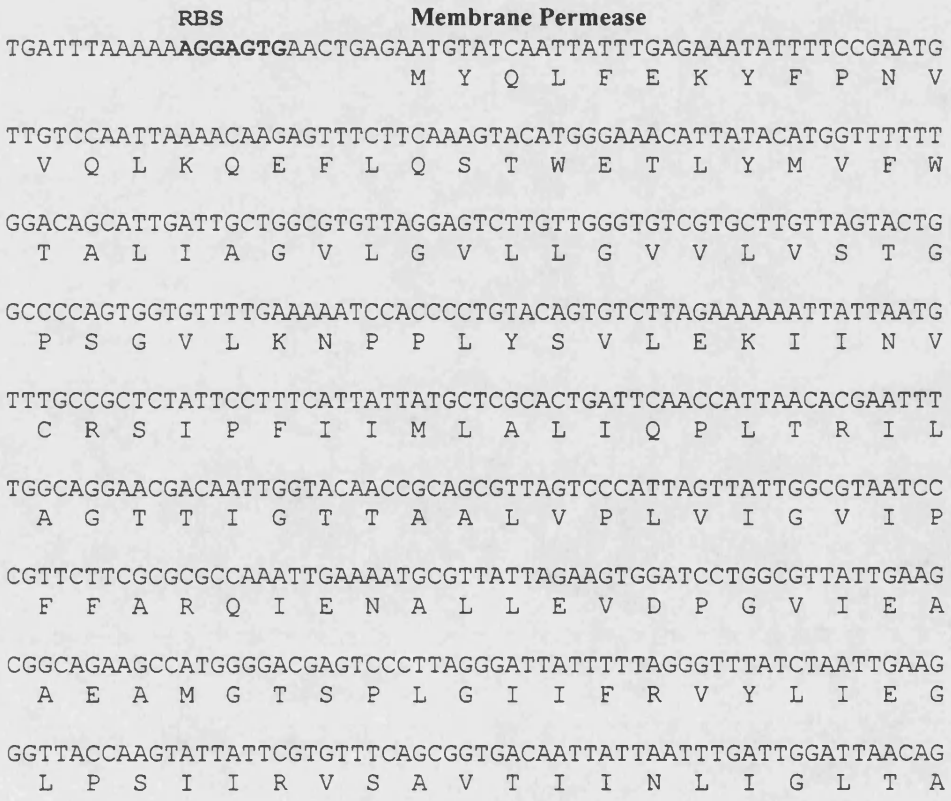
The gene encoded upstream of the YfmM homologue shows 35% identity to the *B. subtilis* hippurate hydrolase protein and 32% identity to an N-acyl-L-

amino acid hydrolase from *Synechocystis*. The Gene Quiz programme did not predict any secondary structure.

The next gene upstream is a lipoprotein of unknown function. A BLAST search revealed 39% identity to a *Pasturella haemolytica* outer membrane lipoprotein and 42% identity to YaeC, a hypothetical lipoprotein from *E. coli*.

The last gene to be identified in this work shows homology to membrane permeases from *B. subtilis* (35% identity) and *H. pylori* (29% identity).

Figure 6.4 Map of the Region Downstream of the *efa* Operon in *E. faecalis*. *As the genes are encoded on the opposite strand to efa, the reverse complement is shown. The end of efaA is shown at the end of the map to allow orientation. The ribosome binding sites are marked (RBS) and where possible, secondary structure has been illustrated.*



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ATCGGTTTCAAACCGATGTGACATTTATGGCCACGTTAATTATTTTAATTATGGTATTTA
  R  F  Q  T  D  V  T  F  M  A  T  L  I  I  L  I  M  V  F  I
                                                      RBS
TCAGTCAAGCCATTAGTAATCAATTAATCAAAAAAACATCACATTAGAAAAAAGGAGAAA
  S  Q  A  I  S  N  Q  L  I  K  K  T  S  H  *

          Lipoprotein                  Leader Sequence
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      M  K  K  F  S  K  L  I  G  L  I  G  V  L  A  F  T  I
Cleavage Site
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A  G  C  A  S  G  S  V  K  D  T  K  T  E  T  V  K  L  G  V

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GATTTACAATTGGTAGAATTTACAGACTATACGCAACCAAACGCAGCATTAGCAGAAAAA
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GAAATTGATTTAAATGCCTTTCAGCATCAAATCTTTTTAGACAATTACAATAAAGAGCAT
E  I  D  L  N  A  F  Q  H  Q  I  F  L  D  N  Y  N  K  E  H

GGAACGAAATTAGTATCAATTGGCAATACAGTCAATGCACCATTGGGAATTTACGCTAAT
G  T  K  L  V  S  I  G  N  T  V  N  A  P  L  G  I  Y  A  N

AAATTGAAAGATATCACGAAAATTAAAGACGGCGGAGAAATTGCTATTCCTAATGACCCA
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T  N  G  G  R  A  L  I  L  L  Q  T  A  G  L  I  K  V  D  P

GCGAAACAGCAACTACCGACTGTCAGTGATATTACTGAAAATAAACGCCAATTGAAAATA
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N  S  G  M  A  V  D  A  G  Y  T  P  D  K  D  A  I  F  L  E

CCTGTAAACGAAAAAGCGAAACCTTATGTGAACATTGTCGTGGCCCGAGAAGAAGATCAA
P  V  N  E  K  A  K  P  Y  V  N  I  V  V  A  R  E  E  D  Q

GAGAATAAACTTTATCAAAAAGTTGTAGAAGAATATCAACAAGAAGAAACGAAAAAGGTC
E  N  K  L  Y  Q  K  V  V  E  E  Y  Q  Q  E  E  T  K  K  V

ATTGCAGAAACATCAAAAGGCGCCAATGTTCCAGCCTGGGAAACATTTGGTAAAAAATAA
I  A  E  T  S  K  G  A  N  V  P  A  W  E  T  F  G  K  K  *
  RBS                Hippurate Hydrolase
AGGAGGCATTTATAATGAGTACAACAACGATTCAAACAATCCAAGAAGCTATTGCTACAG
               M  S  T  T  T  I  Q  T  I  Q  E  A  I  A  T  E

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  K  E  W  I  I  H  L  R  R  H  F  H  Q  Y  P  E  A  S  L  K

AAGAATATGAAACGATTAAGCGAATTAAAGAAGAACTACTAGCCTTAGCTATTCCTTTTG
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  A  F  Q  P  A  E  E  I  G  A  G  A  R  Q  F  V  E  G  N  Y

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  L  V  A  T  K  G  A  T  N  A  S  C  D  I  F  K  I  E  V  S

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TCGTAGTAGGAATTGGCGTTTTACAAGCAGGAACACGCTATAATATTGTAGCAAACCAGG
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  V  E  E  I  A  H  E  I  A  Q  S  N  R  T  E  I  A  D  F  S

CTGTTTATGCGGCAGCGAATCCATTAATTAATGAAGAACAAGCAACCAACCGTGCACAGC
  V  Y  A  A  A  N  P  L  I  N  E  E  Q  A  T  N  R  A  Q  Q

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  E  R  A  L  L  L  A  A  E  Y  H  V  R  Y  A  L  N  Y  L  S

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  E  *

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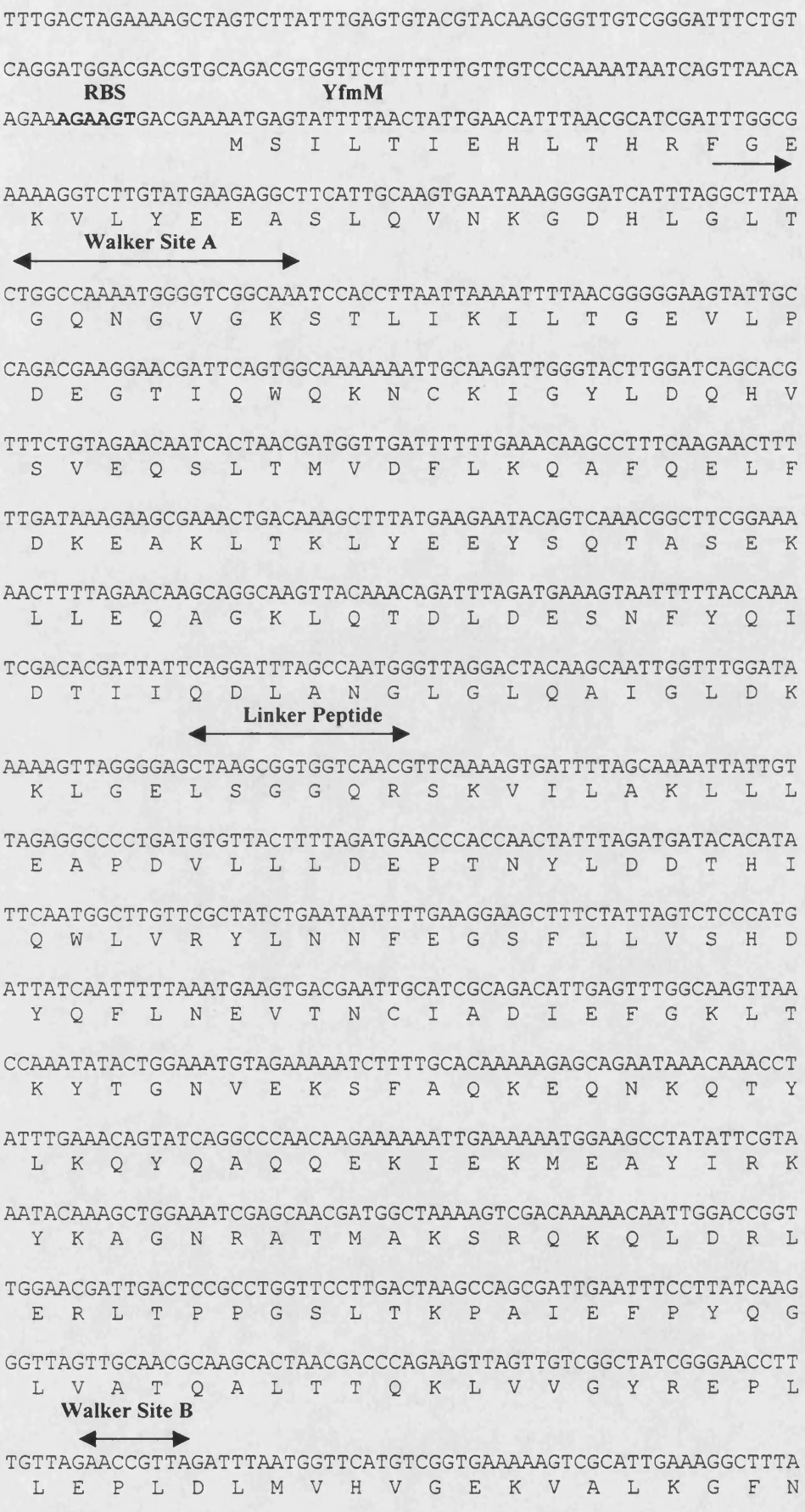
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D T I I Q D L A N G L G L Q A I G L D K
Linker Peptide
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K L G E L S G G Q R S K V I L A K L L L
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E A P D V L L L D E P T N Y L D D T H I
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Q W L V R Y L N N F E G S F L L V S H D
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Y K A G N R A T M A K S R Q K Q L D R L
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E R L T P P G S L T K P A I E F P Y Q G
GGTTAGTTGCAACGCAAGCACTAACGACCCAGAAGTTAGTTGTCGGCTATCGGGAACCTT
L V A T Q A L T T Q K L V V G Y R E P L
Walker Site B
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L E P L D L M V H V G E K V A L K G F N

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  G  I  G  K  S  T  L  I  K  T  L  T  K  V  I  P  S  L  D  G

GAGAATTTCATTATCCGCTGAATACAAAAATTGCTTATTTTACCCAAGACTTAGCGTGGC
  E  F  H  Y  P  L  N  T  K  I  A  Y  F  T  Q  D  L  A  W  P

CTAATGAGCAGTTAACGCCACTAGACTATTTATCAGATCGTTTTCCAGATACAACGATTA
  N  E  Q  L  T  P  L  D  Y  L  S  D  R  F  P  D  T  T  I  K
                          Switch Region
                          <------>
AAGAGCGAAGAAGTCATTTGGCTAGAGCAGGTTTACCAGATAAGTTAGCAATGCAGTCGC
  E  R  R  S  H  L  A  R  A  G  L  P  D  K  L  A  M  Q  S  L

TAGCCCTGTTAAGTGGTGGTGAGCAAACAAAAGTAAAACTAGCGGAACTAATGATGCAAA
  A  L  L  S  G  G  E  Q  T  K  V  K  L  A  E  L  M  M  Q  T

CAAGTAATTTGTTATTTTTAGATGAGCCAACCAATCATATTGATGAAGCGGCCAAAAAAA
  S  N  L  L  F  L  D  E  P  T  N  H  I  D  E  A  A  K  K  I

TTTACAAGAAGCAATTCACGTTTACCCAGGAACGGTTTTTCTGGwTTCCCATGAAGCAGA
  Y  K  K  Q  F  T  F  T  Q  E  R  F  F  W  ?  P  M  K  Q  I

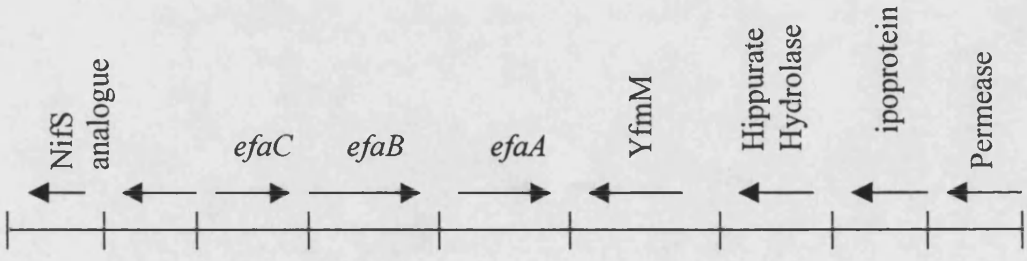
TTTTTATGAGGAAATTGTGGATCGAGTAATTGATATTGAGGAATTAGTTAAATAAAGAAG
  F  M  R  K  L  W  I  E  *

AAAGAACCAAGTTGAGGGGGAGTCAACTTGGTTCTTTCTTCTTTATTATTTATTTACTCA
---------+---------+---------+---------+---------+---------+
TTTCTTGGTTCAACTCCCCCTCAGTTGAACCAAGAAAGAAGAAATAATAAATAAATGAGT
                                                  *  K  S

TTAAGCCATCATGGATTTTTGTTAAATTCCAGTTCATCATGCTGTAGTACGTATCGCCTT
 ---------+---------+---------+---------+---------+---------+----------+
AATTCGGTAGTACCTAAAAACAATTTAAGGTCAAGTAGTACGACATCATGCATAGCGGAA
M  L  G  D  H  I  K  T  L  N  W  N  M  M  S  Y  Y  T  D  G
<------
EfaA
```

The overall layout of the *efa* operon and surrounding region is illustrated in Figure 6.5

Figure 6.5 Summary of *efa* Operon and Surrounding Region.



It is also worth noting that no other significant ORFs were detected downstream of *efaA*. The streptococcal cluster 9 proteins all encode a thiol peroxidase gene downstream of their receptor proteins and there has been some speculation as to whether there is transcriptional read-through to this gene from the cluster 9 proteins. The *S. gordonii* thiol peroxidase (tpx) encoded downstream of the *sca* operon was used in a BLAST search of the *E. faecalis* sequence in the TIGR database. A protein of 162 amino acids with a molecular weight of 59.94 kD was identified. It shows 44% identity to the *B. subtilis* hypothetical thiol peroxidase, 41% identity to PsaD and ScaD and 38% identity to the *S. parasanguis* thiol peroxidase. The region surrounding the thiol peroxidase was obtained and this showed that the gene is not located near *efaA*, or any other lipoprotein (data not shown). The sequence around the –35 promoter of the gene does show limited homology to the Fur boxes from other species (see Table 6.2). Fur (Ferric Uptake Regulator) has a homologous function to DtxR, and has been found in *E. coli* and *S. epidermidis* and many other bacteria. Until recently it was assumed that a bacterial species contained either a DtxR homologue or a Fur homologue. It is now recognised that both repressors can be found in the same species (Hill et al, 1998). Whilst DtxR and Fur have a similar function they are not homologous at either a nucleotide or amino acid level and are not functionally interchangeable. DtxR is unable to act on Fur sites and *vice versa* (Boyd et al, 1990). Fur boxes do seem to vary quite considerably, especially between gram-positive and gram-negative species (Heidrich et al, 1996) and it is difficult to ascertain whether the thiol peroxidase gene is regulated by Fur. It is also interesting to note that *B. subtilis* contains three

fur homologue genes: *yqkL* codes for the Fur protein and is sensitive to low iron and manganese concentrations. The *ygaG* product, PerR, is sensitive to low iron and manganese concentrations and to increased concentrations of H_2O_2 (Bsat et al, 1998). The third fur homologue, *yqfV*, responds to zinc (Gaballa and Helmann, 1998) and is similar to the *E. coli* Zur protein (Patzer and Hantke, 1998). These three proteins are produced in addition to the product of *yqhN*, a DtxR homologue. The thiol peroxidase gene illustrated here has not been proven to be under the control of a Fur protein. However it would seem logical that a gene involved in the stress response would be controlled by a repressor which responds to H_2O_2 levels (known to increase when a bacterium is in the aerobic environment of a host) and manganese or iron (which would decrease in a host environment). Table 6.2 includes details of the identified Zur box sequences and per box sequences found in *B. subtilis* and *L. seeligeri* (Chen et al, 1995, Haas et al, 1991, Gaballa and Helmann, 1998) to allow comparison with fur boxes. The per boxes identified so far have all been situated ~15 bp upstream of the –35 promoter.

It would perhaps be more correct to conclude that the *E. faecalis* thiol peroxidase gene is not controlled by DtxR and is not linked to *efa* transcription. If, however, the tpx gene is controlled by Fur, it will be transcribed in identical situations to the *efa* operon, i.e. when manganese or iron concentrations are low. It would certainly seem to be worth studying the effect of metal ion concentration on the *E. faecalis* thiol peroxidase.

Figure 6.6 Thiol Peroxidase Gene in *E. faecalis*, with putative ribosome binding site (RBS) and putative fur box marked.

Putative Fur Box

AAATAGCCCAAAGCAATAAAATTGTCTTCATAAAAATCCTC**TACTATACTTATTATTGA**A

RBS

AGTGGAAA**AGGAGGAT**TTCAGAATGAATGTTACAAGAAAAGGGCACGTATTAGAATTGAC

M N V T R K G H V L E L T

AGGTGAGCAGCCTAAAGTTGGCACAAAAGCTCCCGTCTTTTCTTTGAAAAATTTAAACAA

G E Q P K V G T K A P V F S L K N L N N

CCAAGAAATCAACTTGGCAGATTATAAAGGTAAAACGGTTTTAATTAGTGTGGTTCCTGA

Q E I N L A D Y K G K T V L I S V V P D

TATCGACACACGTGTTTGTTCACTGCAAACGAAACGTTTCAATCAAGAAGCGGCGAAATT

I D T R V C S L Q T K R F N Q E A A K L

AGACGGTGTTCAGATTATCACGATTTCCAATAACACAGTTGAAGAACAAGCAAATTGGTG

D G V Q I I T I S N N T V E E Q A N W C

TGCCGCTGAGGGTGTCGAAATGGAAATGCTTCATGATACTGAAGACTCATTTGGTGCAGC

A A E G V E M E M L H D T E D S F G A A

TTATGGTTTGTATATTCCAGAAATGGGCCGTTTAGCACGTGCTATTTTTGTGATTGACCC

Y G L Y I P E M G R L A R A I F V I D P

AGAAGGAACGTTAGTTTATGAAGAAATCGTCTCAGAAGTTTCGTCGGAACCCGATTATCA

E G T L V Y E E I V S E V S S E P D Y Q

ACAAGCGTTAGAATCTGCAAAAAAAGTGTAATCCCTCTAGATTACAAATTGACTTTGCTT

Q A L E S A K K V *

Table 6.2 Fur Boxes

(After Hardham et al, 1997; Chen et al, 1995; Haas et al, 1991; Bearden et al; 1998; Bsat et al, 1998; Litwin and Calderwood, 1993; Patzer and Hantke, 1998; Gaballa and Helman, 1998)

Species	Name of Fur box	Sequence
Consensus	consensus	GAT AAT GAT A ATC ATT ATC
E. coli	*iucA*, primary	GAT AAT GAG A ATC ATT ATT
E. coli	*iucA*, secondary	GAT AAT TGT T ATT ATT TTA
E. coli	*fepA*	TAT TAT GAT A ACT ATT TGC
E. coli	*slt1*	GAA TAT GAT T ATC ATT TTC
E. coli	*irgA*	GAA ATT AAG A ATA ATT ATC
E. coli	*fhuA*	CTT TAT AAT A ATC ATT CTC
E. coli	*fur*	TAT AAT GAT A CGC ATT ATC
Y. pestis	*yfeE*	CAT AAG TTA T AAC CTT TAC
Y. pestis	*yfeA*	TGG TAT TGA T AAT CAT TTT
T. pallidum	*Tro Operon*	TAC TTT GAT G CAT CAA AAT
E. faecalis	Thiol peroxidase homologue	TAC TAT ACT T TAT TAT TGA
E. faecalis	AdcA homologue	CAA TAA ACA A AAG AAA ATT
E. faecalis	Fur homologue	GAA ATA GTA T TAT TTA AGA
B. subtilis	Per box consensus	CTA t-T Tat AAT -ATTATAAattA
L. seeligeri	catalase gene, per box	GAT AAT GAA G ATT ATT TTT
B. subtilis	*yciC* gene, Zur box	TTT AAA TCG TAA TCA TTC TA
B. subtilis	*ycdH* gene, Zur box	ACA AAA TCG TTA TCA TTT TG

The TIGR database was used to identify Fur proteins from *E. faecalis* (Figure 6.7 – 6.9). Unfortunately it was only possible to identify the complete sequence of the *ygaG* gene homologue. The protein identified is 147 amino acids long and has a molecular weight of 16.945 kD. A putative Fur binding site was identified around the –35 element, suggesting that *fur* is auto-regulated (Figure 6.7 and Table 6.2). A BLAST search revealed 63% identity to the PerR homologue in *B. subtilis* and 43% identity to the *S. pyogenes* Fur. The Gene Quiz programme did not identify any significant secondary structure, but a study of the literature revealed that the Fur protein is thought to act as a dimer and can complex with manganese, iron and cobalt (reviewed by Litwin and Calderwood, 1993). The N terminal domain is thought to be involved in binding to DNA and contains a helix-turn-helix motif, whilst the C terminal is involved in dimerisation and binding metals

(Heidrich et al, 1996). As with DtxR it would be interesting to study whether this *E. faecalis* Fur would bind preferentially to iron or manganese, as this might give some indication of which metal is more important to *E. faecalis*. It would also be worth investigating whether it responds to raised H_2O_2 levels.

Figure 6.7 Map of the *ygaG* Fur Homologue of *E. faecalis*. *The putative Fur box is highlighted and the ribosome binding site is marked (RBS). This protein is believed to respond to increased environmental concentrations of H_2O_2 and to decreased levels of manganese and iron.*

```
                          Putative Fur Box                    RBS
TTCTTGTCAATCGTAATAAGAAATAGTATTATTTAAGAAAGATAGACAGGGGAGGGATAC

AAAGATGGACAACGTATTGGTTAAAAATGCACTTGCTGAATTAAAAGAAGCCAATATCCG
    M  D  N  V  L  V  K  N  A  L  A  E  L  K  E  A  N  I  R

AATTACTCCGCAACGTTATGCTATCTTGGAATATTTAATCGAAAATCATACACACCCAAC
 I  T  P  Q  R  Y  A  I  L  E  Y  L  I  E  N  H  T  H  P  T

AGCTGATGAAATTTATCGCGCACTAGAAGATCATTTTCCAAATATGAGTGTAGCAACGGT
 A  D  E  I  Y  R  A  L  E  D  H  F  P  N  M  S  V  A  T  V

TTACAACAATCTACGTTTATTTACTGAAATCGGTTTCGTTCAAGAAATGAGTTATGGCGA
 Y  N  N  L  R  L  F  T  E  I  G  F  V  Q  E  M  S  Y  G  D

TGCATCTAGTCGTTTTGATTTTAGTTCGAAGAAACATTATCACGTGATTTGTCAAAAATG
 A  S  S  R  F  D  F  S  S  K  K  H  Y  H  V  I  C  Q  K  C

TGGTAAAATCGTTGATTTTCATTATCCAGGGTTAGAGGACGTTGAAATGGCCGCTAGTAA
 G  K  I  V  D  F  H  Y  P  G  L  E  D  V  E  M  A  A  S  K

ATTAACAGGCTTCGAAATTAATGAACATCGTTTAGAATTATATGGATTATGTCCAGATTG
 L  T  G  F  E  I  N  E  H  R  L  E  L  Y  G  L  C  P  D  C

CCAACAAGCACAACAGGAGAATGTGTAAAAAATTACATACTGTTAAAAATTTATCAAAAG
 Q  Q  A  Q  Q  E  N  V  *
```

It was only possible to identify partial coding sequences of the other two *E. faecalis* Fur homologues. Figure 6.8 shows the start and end of the *yqkL* homologue, which responds to iron and manganese. The start of the protein shows 75% homology to *B. subtilis yqkL* product and the end of the protein

shows 45% homology in BLASTP studies. Unfortunately the sequence spans two contigs and it is not possible to speculate how they will join up.

Figure 6.8a Map of the Start of the *yqkL* Fur homologue in *E. faecalis*.
This is situated on the end of one contig, and is expected to join up to the end of the sequence, situated on the start of another contig (shown below). The predicted molecular weight of this protein is 7.7 kD. If the two halves of the protein were joined, they would form one protein with an approximate molecular weight of 16 kD, which corresponds to the size of most Fur proteins. This Fur protein is predicted to respond to decreased environmental concentrations of manganese and iron.

```
TTGATAAATTAAGTTTCTTAATTTGTAATCATTCTAAACAgGAGCTGTAATAATGGATTC
                                                    M  D  S

GACTGCTGCTTTAAAAAAGACACAAAAACAATTACATGAATCACGCTTTAAATTAACACC
 T  A  A  L  K  K  T  Q  K  Q  L  H  E  S  R  F  K  L  T  P

ACAACGGGAAGCAACCGTACTTGTATTATTGGAAAATGAAAAAGATCATTTATCAGCAGA
 Q  R  E  A  T  V  L  V  L  L  E  N  E  K  D  H  L  S  A  E

AGAAATTTACTTCCTGGTAAAACAAAAAAGTCCGGAAATTGGTCTAGCAACAGTCTATCG
 E  I  Y  F  L  V  K  Q  K  S  P  E  I  G  L  A  T  V  Y  R

AACGCTAGAAATTTAACAGACTT
 T  L  E  I  *
```

Figure 6.8b Map of the End of the *yqkL* Fur Homologue in *E. faecalis*
showing the end of the predicted yqkL homologue, situated at the start of one contig. This is expected to join up with the start of the protein, as shown above.

```
ATTGAGGAAGTGGAAGAAGACTTGCTTGGTGAAGTAGAGCAAATTGTTGAAAGTCGGTAT
I  E  E  V  E  E  D  L  L  G  E  V  E  Q  I  V  E  S  R  Y

CATTTTTTAGTCAAAGATCATCGCTTAACTTTTCATGGTATTTGTCAAAGTTGTCAAAGT
H  F  L  V  K  D  H  R  L  T  F  H  G  I  C  Q  S  C  Q  S

AAACATTAAAAAAACTGCCTTGCATTAATTTATGCAAGGCAGTTTTTTATTCATTGGAAT
K  H  *
```

The partial sequence of the zinc repressor (*yqfV*) homologue is shown in Figure 6.9. Again this is situated at the start of the contig and it is not possible to pinpoint the beginning of the gene accurately. The identified region shows 53% identity with YqfV on BLASTP studies.

The presence of three Fur homologues in *E. faecalis* raises interesting questions about the regulation of genes. It is clear that metal ion concentration is a common environmental sensor, but it is not clear how sensitive the *E. faecalis* Fur homologues are, or which metal ions they respond to. Further work is needed to answer these questions and to determine the consensus binding sequence for each of these proteins.

Figure 6.9 Map of End of *yqfV* homologue (Zinc repressor) in *E. faecalis*
This shows the end of the yqfV homologue (the product of yqfV, Zur, is a zinc repressor in B. subtilis). This is situated at the start of a contig and the beginning of the gene is not available yet.

```
GCCAAGAGGTGGAACATCATCATCACTTTATTTGTACGGTTTGTGGCAAAACGGAAGAAA
  Q  E  V  E  H  H  H  H  F  I  C  T  V  C  G  K  T  E  E  I

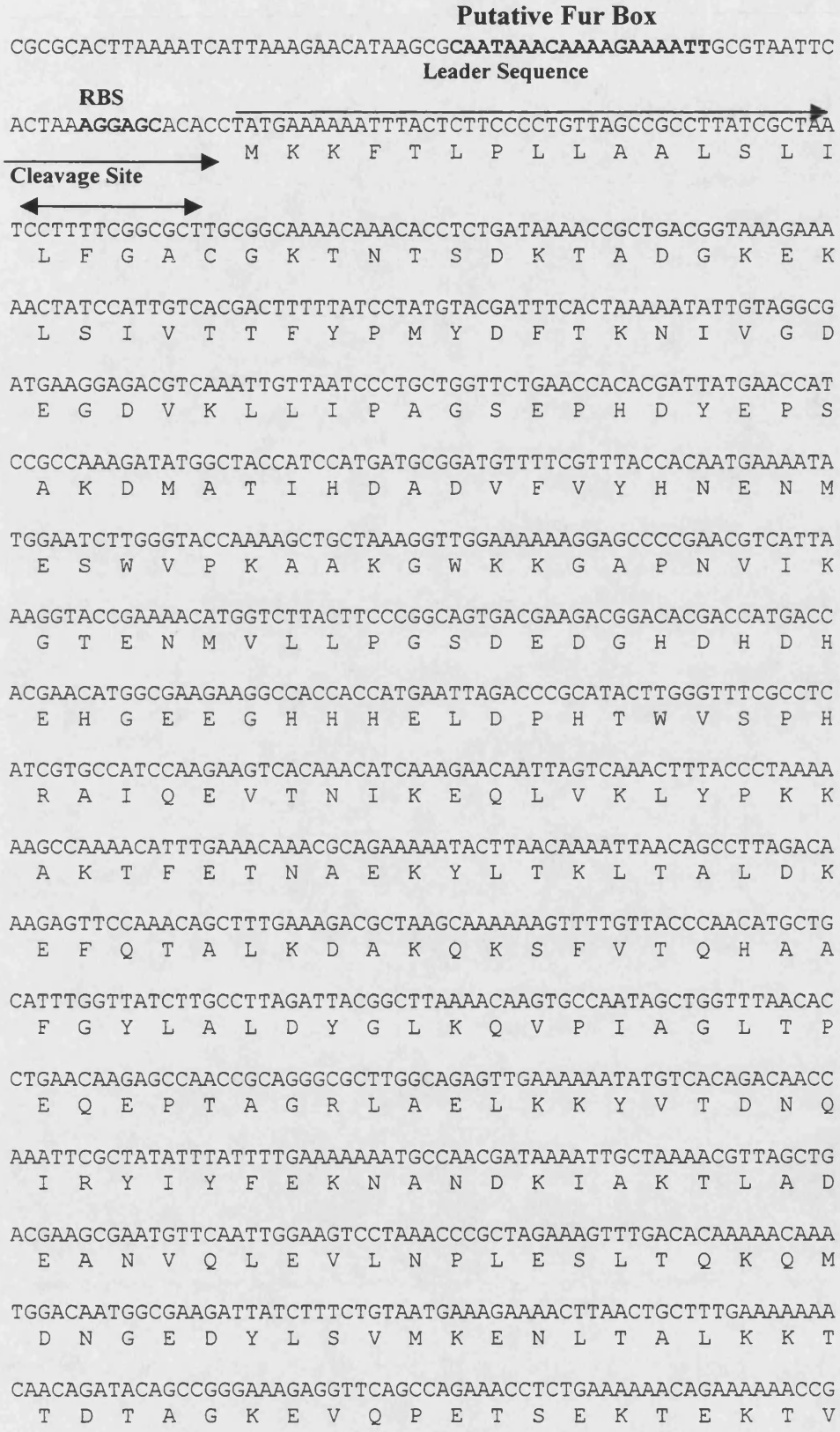
TTCACATGTGTCCGATGAATTTTTTTGAAGAACAGTTAAAAGGTTGTTCAATTGAAGGAC
  H  M  C  P  M  N  F  F  E  E  Q  L  K  G  C  S  I  E  G  H

ATCGCTTTGAAATCCTTGGTCGTTGTGCCGATTGTTGCGAAAAATAGCGAATAAGACGAA
  R  F  E  I  L  G  R  C  A  D  C  C  E  K  *
```

Studies by Dintilhac and Claverys (1997) have shown that there are two Cluster 9 proteins in *S. pneumoniae*; one, AdcA, is a zinc transporter and the other, PsaA, is a manganese transporter. Kolenbrander et al, (1994) also hypothesised from Southern blotting studies that there could be another homologous operon to *sca* in *S. gordonii*. It was therefore highly possible that there was another Cluster 9 operon in *E. faecalis*. A BLAST search of the TIGR *E. faecalis* sequence with AdcA revealed that there was indeed a protein with 56% identity to AdcA (see Figure 6.8a). The *E. faecalis* AdcA protein is 511 amino acids long and has an Mr of 57,663. There is a leader sequence at the start of the protein, with a cleavage site for signal peptidase II, suggesting that AdcA is a lipoprotein. The mature protein would have a molecular weight of 55,663.

Further searches found homologues to AdcB and AdcC (Figure 6.8b). However the complete *E. faecalis* sequence has not yet been compiled in TIGR and the *adcB* and *adcC* genes are encoded on a different contig from *adcA*. The start of *adcC* is also missing. However the sequence downstream of *adcB* is available and does not contain *adcA* (data not shown). This presumably means that the operon does not follow the C-B-A order seen most often in Cluster 9 operons. It is possible that AdcA is encoded first. Significantly, this does occur in both *B. subtilis* and *E. coli*, where operons homologous to *adc* have been identified. In both the *ycdH* (*B. subtilis*) and *znu* operons (*E. coli*), the receptor protein is encoded first followed by the ATP binding protein and the membrane protein (Gaballa and Helmann, 1998; Patzer and Hantke, 1998). Without viewing the complete genome sequence and the start of *adcC* it is not possible to clarify this. Interestingly, however, there is an area ~ 35 bases upstream of *adcA* which shows limited homology to Fur binding sites. If AdcA is coded first, this could be a Zur site, and it would raise an interesting possibility that the *E. faecalis* Zur homologue is controlling transcription. The *adc* operon in *S. pneumoniae* is controlled by its own repressor, AdcR, encoded upstream of AdcC. However a BLAST search revealed no homologues to AdcR in the TIGR *E. faecalis* sequence. The recent evidence that Zur controls the *ycdH* operon in *B. subtilis* and the *znu* operon in *E. coli* does add credence to the theory that the *E. faecalis adc* operon would be controlled in this way (Gaballa and Helmann, 1998, Patzer and Hantke, 1998).

Figure 6.7a Map of the *adcA* Gene Homologue in *E. faecalis*, *showing the ribosome binding site (RBS), putative Fur binding site and significant secondary structure predictions.*



```
TGGCTAACGGATATTTCAAAGACAGTGAGGTGGCTGAGAGAACACTGACAGATTACGCTG
  A  N  G  Y  F  K  D  S  E  V  A  E  R  T  L  T  D  Y  A  G

GAAATTGGCAATCCGTCTATCCTTTATTAAAAGATGGCACATTAGACCAAGTCTTCGATT
  N  W  Q  S  V  Y  P  L  L  K  D  G  T  L  D  Q  V  F  D  Y

ACAAAGCGAAACTGAAAAAAGATAAAACACCAGCCGAATACAAAACCTACTATGATGCCG
  K  A  K  L  K  K  D  K  T  P  A  E  Y  K  T  Y  Y  D  A  G

GCTATCAAACCGATGTCGACCACATCAACATCACTGATTCCACCATTGAATTTCTGGTCA
  Y  Q  T  D  V  D  H  I  N  I  T  D  S  T  I  E  F  L  V  N

ATGGCAAACCACAAAAATTCACCTATAAAGCAGCCGGTTATAAAATTTTAAACTATGCAA
  G  K  P  Q  K  F  T  Y  K  A  A  G  Y  K  I  L  N  Y  A  K

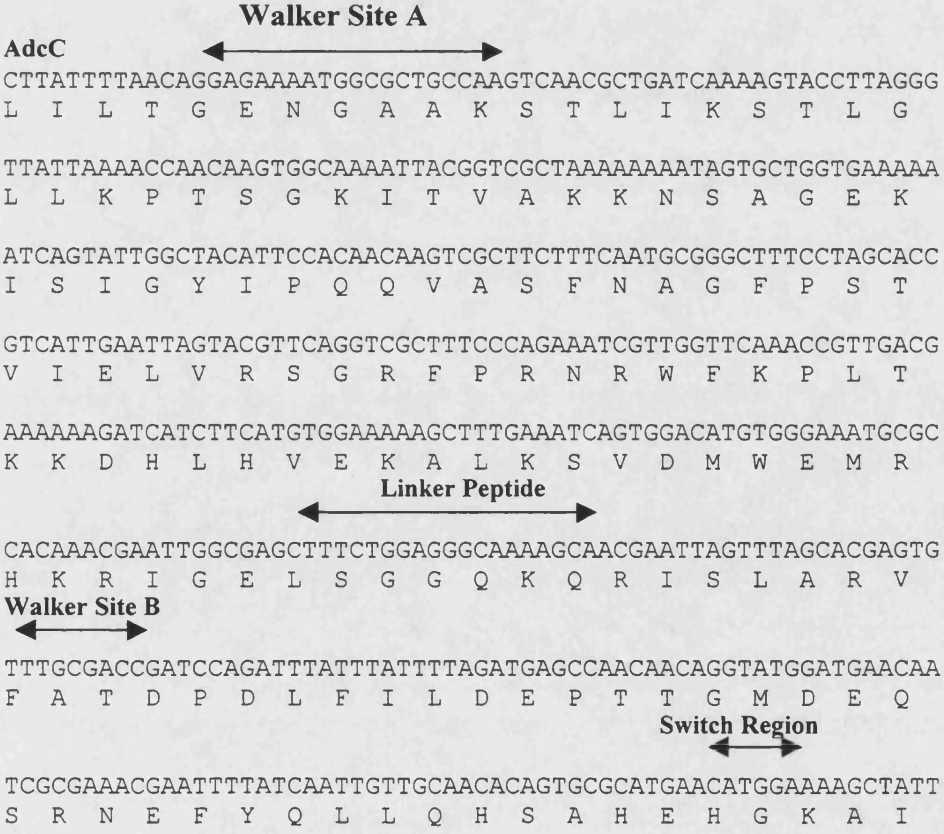
AAGGCAACCGTGGCGTCCGTTTCCTTTTTGAAACAGACGATGCCAATGCTGGGCGGTTTA
  G  N  R  G  V  R  F  L  F  E  T  D  D  A  N  A  G  R  F  K

AATACGTCCAATTTAGCGACCACAACATCGCACCAACGAAAGCCGCTCATTTCCACATCT
  Y  V  Q  F  S  D  H  N  I  A  P  T  K  A  A  H  F  H  I  F

TCTTCGGCGGCGATAGCCAAGAAAGTCTGTTCAATGAAATGGACAACTGGCCAACGTATT
  F  G  G  D  S  Q  E  S  L  F  N  E  M  D  N  W  P  T  Y  Y

ATCCAAGCGACTTAAGCAAACAAGAAATTGCCCAAGAAATGATTGCGCATTAAGCATTCA
  P  S  D  L  S  K  Q  E  I  A  Q  E  M  I  A  H  *
```

Figure 6.8b Map of *adcB* and *adcC* Analogues in *E. faecalis.* *showing significant secondary structure. The ribosome binding sites are marked (RBS).*



```
TTGATGATTACGCATGATCATGAAGATATCAAAACCTATGTGGATCGTCAAATTCGTCTT
L  M  I  T  H  D  H  E  D  I  K  T  Y  V  D  R  Q  I  R  L

GTCCGCAAAGAAGATTCGAAATGGCGTTGTTTCCATATGAGTGAAGAATCGTATACGTAA
V  R  K  E  D  S  K  W  R  C  F  H  M  S  E  E  S  Y  T  *

ATTTTCCAATAAAACGTTATCTGGCTCGATCATAACGAAAGCCTAGGAACGGCTTAAAGA

AATGTGGGAAAAATTTAAAAAATAAGCAGGAATTAAAAAAATAATTCGCTTAAACGTCGC
                                  RBS          AdcB
TACAATGGTAGTGTTCATTTTAGCGGTAAGGAGTGAATCGTATGGCAGAAATGCTTTCTT
                                              M  A  E  M  L  S  Y

ATGCATTTATGCAAAAGGCCTTTTTAGCAGCACTGTTTATCTCAGTGATTGCCCCAATGC
  A  F  M  Q  K  A  F  L  A  A  L  F  I  S  V  I  A  P  M  L

TCGGCGTCTTTCTAGTTATTCGCCGACAATCTTTAATGGCAGATACCCTTTCACATGTGT
  G  V  F  L  V  I  R  R  Q  S  L  M  A  D  T  L  S  H  V  S

CATTAGCCGGTGTGGCACTAGGCTTCTTTTTTAATTGGAATCCTAATTTAATGACCTTAA
  L  A  G  V  A  L  G  F  F  F  N  W  N  P  N  L  M  T  L  I

TTGTCGTGATTGTGGCTGCAATCATTCTAGAATATTTACGAATGATTTATAGCACCTATT
  V  V  I  V  A  A  I  I  L  E  Y  L  R  M  I  Y  S  T  Y  S

CAGAAATTTCGATTGCTATTTTAATGTCAGGCGGTTTGGCTTTGGCGTTAGTTTTGATGA
  E  I  S  I  A  I  L  M  S  G  G  L  A  L  A  L  V  L  M  N

ATTTAACAGGAGGCAATTCAGCTGCTAGTATTCAATCGTATTTATTTGGTTCCATCGTCA
  L  T  G  G  N  S  A  A  S  I  Q  S  Y  L  F  G  S  I  V  T

CGATTACGTGGGATCAAGTGGTTATGTTGGCAATTTTATTCGTAGTTTTAGTTCTATTGT
  I  T  W  D  Q  V  V  M  L  A  I  L  F  V  V  L  V  L  L  F

TTATGTTATTTAAACGTCCAATGTATGTTTTAACATTTGATGAAGATACTGCTCATGTTG
  M  L  F  K  R  P  M  Y  V  L  T  F  D  E  D  T  A  H  V  D

ATGGGCTACCTATTCATTGGATGTCGATGCTTTTTAATGTAATTACTGGTGTGGCGATTG
  G  L  P  I  H  W  M  S  M  L  F  N  V  I  T  G  V  A  I  A

CTGTGATGATTCCGATCGCGGGAGCCTTGTTAATTTCAGCAATTATGGTCTTACCAGCTG
  V  M  I  P  I  A  G  A  L  L  I  S  A  I  M  V  L  P  A  A

CAATAGGTATGCGAATTGGTAAAGGCTTTAACACGGTGATTATTATCAGTGTGTTTATGG
  I  G  M  R  I  G  K  G  F  N  T  V  I  I  I  S  V  F  M  G

GCTTGATTGGCATGCTAACAGGGTTGACTAGCTCGTATTATTTGGAAACACCACCGAGTG
  L  I  G  M  L  T  G  L  T  S  S  Y  Y  L  E  T  P  P  S  A

CAAGTATTACCCTAATTTTTATTGGTTTATTCTTATTAGTCAATATTTATCGCCGAGTGG
  S  I  T  L  I  F  I  G  L  F  L  L  V  N  I  Y  R  R  V  V

TTGTCATGGTCCAACGAAAACAAAAAATGCAAAGAAACTAAGGAGAAAAACGAAAGTTTT
  V  M  V  Q  R  K  Q  K  M  Q  R  N  *
```

In conclusion, therefore, analysis of the preliminary *E. faecalis* sequence at TIGR has enabled identification of the entire *efa* operon and surrounding sequence. Furthermore the thiol peroxidase gene and DtxR and Fur protein homologues have also been identified. This should allow intensive study of metal ion regulation in this pathogen.

7 Purification of EfaA by Salt Fractionation

Prior to the present study, all western blotting experiments had been carried out using serum from an endocarditis patient (GP). However, due to the increasing age of this serum it became necessary to generate new antibodies to the EfaA protein. In order to do this, EfaA had to be extracted and purified and then used to generate antibodies in a New Zealand white rabbit. Salt fractionation by ammonium sulphate precipitation was used to purify EfaA from *E. coli* XL1 pSK+: GP19.

The *E. coli* clone containing *efaA* was used because this was engineered to over-express EfaA and hence would give a better yield than the same number of *E. faecalis* cells. EfaA would also be present in the cytoplasm not the membrane in these cells. Furthermore gram-negative cell walls are much easier to break down than gram-positive ones thus aiding extraction of the cytoplasm. Some extraction protocols recommend using IPTG in the growth medium to induce the expression of foreign proteins. However IPTG is toxic to cells and as the basal concentration of EfaA in *E. coli* XL1 pSK+:GP19 is already quite high, it was decided that this was unnecessary.

The first step in protein purification is to get the desired protein into a more or less soluble form. Cytoplasmic proteins are soluble in aqueous media, so in micro-organisms it is merely necessary to lyse the cell walls and release the cytoplasmic extract. Cells were spun down and resuspended in TE buffer prior to sonication and lysozyme treatment. TE was added partly for EDTA to augment the action of lysozyme and partly to provide liquid for the

extracted proteins. Some liquid needs to be added to prevent proteins being trapped in the cellular debris following extraction and centrifugation.

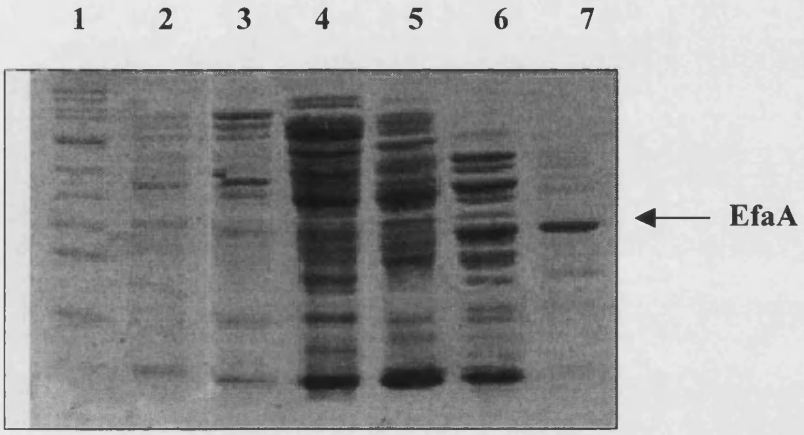
Following extraction streptomycin was added to the solubilised cell extract to precipitate the ribosomes and hence remove the nucleic acids. Because nucleic acids are polymers and carry a strong charge they may co-precipitate with proteins during salt fractionation and thus will not be purified. Furthermore they alter the solubility and adsorption properties of proteins.

As proteins will precipitate out over a range of concentrations and not at a particular point, collecting precipitate over a wide range (e.g. 25 %) will ensure that all the protein is collected. Collections over a narrow range (e.g. 5 %) will give a small quantity of pure protein. It may be necessary to compromise on purity in some extractions in order to extract a sufficient quantity of protein. The aim of the extraction procedure in this case was to obtain as much EfaA as possible. The purity of the preparation was not of paramount importance, since it was only to be used to generate antibodies and would not be used for any experiments where pure protein was required. Thus it was initially decided to collect protein precipitated over a 30 % range (i.e. to collect 30 %, 60 % and 90 % precipitates).

Initial experiments showed that all the proteins tended to precipitate out of solution at 30 % saturation, leaving little or no protein to precipitate out at the 60 % or 90 % levels. This could have been caused by inadequate stirring in the solution leading to localised regions of high ionic strength. To try and

remedy this the ammonium sulphate was ground in a mortar to break up large crystals or lumps and was added to the solution slowly (over 10 min) so that the desired concentration was reached slowly and evenly over time. The solution was stirred for a further 30 min after the ammonium sulphate had been added to allow the levels of precipitated and dissolved protein to equilibrate. However the proteins were still precipitating out together at 30 %. It was therefore decided to add the ammonium sulphate in smaller increments to effect a more gradual change in ionic strength. The experiment was repeated, adding the ammonium sulphate in 10 % increments from 20 % to 90 %. The results are shown in figure 7.1. The 80 % and 90 % fractions contained no protein, but a gradual precipitation had been achieved over all the other concentration points. The 70 % fraction was used to generate antibodies.

Figure 7.1 SDS-PAGE of Fractions obtained in EfaA Extraction Procedure,. *Fractions were analysed on a 12 % SDS-PAGE gel and were stained with coomassie blue. Lane 1, marker; Lane 2, 20 % saturation fraction; Lane 3, 30 % saturation fraction; Lane 4, 40 % saturation fraction; Lane 5, 50 % saturation fraction; Lane 6, 60 % saturation fraction; Lane 7, 70 % saturation fraction. The EfaA protein band (indicated by arrow) is most prominent in the 70 % fraction, so this fraction was used to generate antibodies in a rabbit.*



Time constraints prevented proper testing of the antibodies generated in this way. However figure 7.2 compares two western blots generated using GP serum and the rabbit serum. Although the human serum produced the strongest bands on a western blot, it can be seen that the rabbit serum was capable of producing a response.

Figure 7.2 Comparison of Immunoblotting with Human and Rabbit Serum, *figure 7.2a shows E. faecalis EBH1 (Lane 1) and E. coli XL1 pSK+: GP19 (Lane 2) probed for 12 hours with human serum (diluted 1:100 in TBS) obtained from patient GP. Figure 7.2b shows molecular weight markers (Lane 1) and E. faecalis EBH1 (Lane 2), probed with EfaA-antibody-containing serum (diluted 1:20 in TBS) generated from a New Zealand white rabbit injected with the 70 % saturation fraction obtained in the ammonium sulphate extraction procedure.*

Figure 7.2a

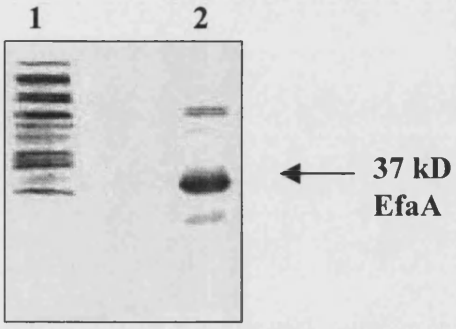
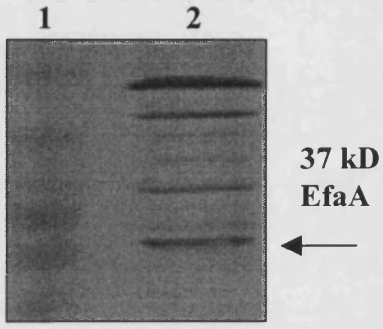


Figure 7.2b



8 Metal Ion Regulation of the *efa* and *efm* Operons

The recent work of Dintilhac et al (1997) and Kolenbrander et al (1998) has highlighted the role of metal ions in the transcription of Cluster 9 proteins. There is now growing interest in the metal ion regulation of Cluster 9 genes. It would appear from identification of DtxR-like promoter boxes upstream of both *efa* and *efm* operons (see chapters 5 and 6) that a similar regulation mechanism is likely to occur in enterococci. Dintilhac et al (1997) carried out some phylogeny studies on Cluster 9 proteins and predicted from this that *efaA* would act in the same way as *PsaAv* in *S. pneumoniae* and would be transcribed under manganese-limiting conditions (see table 1.3). In order to verify all of these predictions experimentally, a semi-quantitative RT-PCR strategy was designed to clarify the effects of some transition metal ion concentrations on transcription of the *efa* and *efm* operons.

RT-PCR is becoming an increasingly common tool to study RNA transcription. It can be performed on total RNA or mRNA. The first stage is the annealing of a primer to template RNA, followed by transcription to cDNA with a reverse transcriptase such as AMV. The reverse transcriptase is inactivated by heating the reaction to 94 °C, which will also melt the RNA-cDNA hybrid. PCR is then performed to amplify the cDNA.

RT-PCR can simply be used to detect the presence of an RNA species, or can be used in a more sophisticated manner to study the relative expression of RNA species under different experimental conditions. This latter approach

was used in this study, where the transcription of *efaA* and *efmA* were studied under varying metal ion concentrations.

RT-PCR is a sensitive technique, because of linear amplification during PCR, so even small variations between tubes can produce dramatic differences in total yields. Contamination can also be a significant problem, especially from DNA. All known RNA extraction techniques result in some DNA contamination. Therefore it is not enough to assume that any differences observed between samples in an experiment are due to the parameter being tested. Controls must be included to verify that the differences observed are genuine.

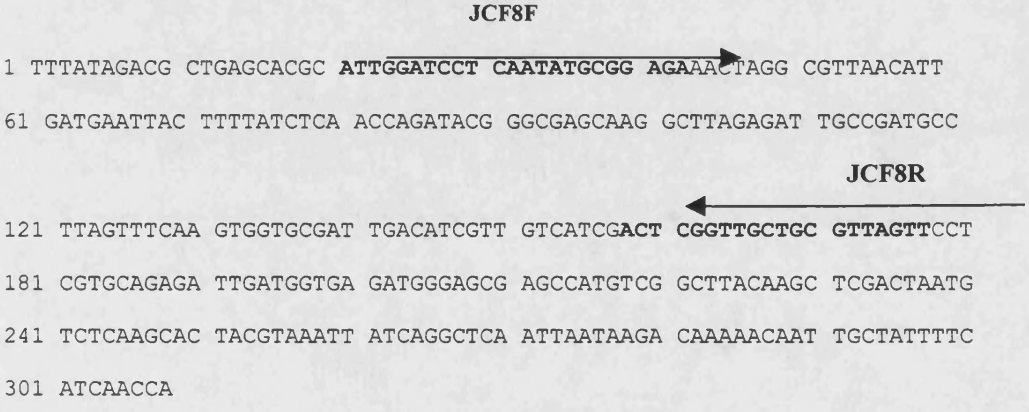
DNase treatment was used in these experiments to degrade DNA, but care was needed to avoid damaging the RNA. As most damage occurs during the inactivation step, inactivation was carried out at 75 °C for 5 min, which has been found to be the lowest temperature to cause most complete inactivation (Boerhinger, personal communication). A DNase with low RNase activity was selected. The removal of DNA contamination was ascertained by performing a PCR without the reverse transcription step for each sample (data not shown).

The most reliable method of identifying sample variation in RT-PCR is to use an 'internal' control by amplifying a control mRNA in addition to the experimental RNA. The relative quantities of each species are then compared. 'External' controls such as OD or absorbence measurements or

constant cell number can be easily made and would seem to eliminate the need for internal controls. However they would not account for errors introduced in the PCR reaction by pipetting, transfer to the reaction tube and unequal amplification due to variation in the thermal cycler.

The control genes used are usually constitutively expressed housekeeping genes. Ideally they should be unaffected by the experimental conditions, be essential cell products and be expressed at all times in the cell cycle at a constant level. However no single RNA species will have a constant level of expression and variation is inevitable between samples, not least due to the inherent errors in any RT-PCR technique. In these experiments the *recA* gene (involved in DNA replication and repair) was selected as the housekeeping gene. Figure 8.1 shows the portion of the gene used in the PCR reactions. The purpose of these experiments was to look for a trend in RNA production, not to determine the exact quantity of an RNA species present. Therefore a simple comparison between the target RNA and control RNA was sufficient.

Figure 8.1 The Partial Sequence of *recA* from *E. faecalis*, *showing the portion of the gene identified in the sequencing project of Dybvig et al (1992). The primers used in these experiments are shown in bold.*



The results obtained with *E. faecalis* were further confirmed by western blotting experiments using cell walls extracted from cells in early log phase grown in yeast extract containing the appropriate metal ion.

Figure 8.2 RT-PCR Results for *E. faecalis* EBH1 Cells Grown in 1 % YE Containing Variable Manganese Concentrations. *PCR products were analysed on a 0.8 % agarose gel and stained with ethidium bromide and viewed under UV light. Lane 1, marker, Lane 2, no template control, Lane 3, yeast extract medium, supplemented with 1 % foetal calf serum only (YE/FCS), Lane 4, YE/FCS with 0.1μM Mn, Lane 5, YE/FCS with 0.5 μM Mn, Lane 6, YE/FCS with 1 μM Mn, Lane 7, YE/FCS with 5 μM Mn, Lane 8, 10 μM Mn*

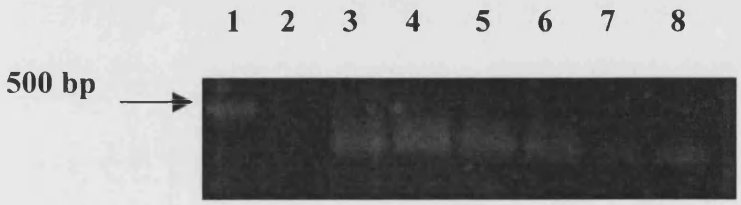


Figure 8.3 Western Blot Analysis of *E. faecalis* EBH1 Cell Walls After Growth in 1 % YE Containing Variable Concentrations of Manganese, *E. faecalis cell wall extracts were subjected to SDS-PAGE electrophoresis and then were probed with patient's serum (GP). Blots were visualised with protein A conjugated to horseradish peroxidase. Lane 1, E. coli XL1 pSK+ GP19 (EfaA Clone), lane 2, YE/FCS + 10 μM Mn, Lane 3, YE/FCS + 5 μM Mn, Lane 4, YE/FCS + 1 μM Mn, Lane 5, YE/FCS + 0.5 μM Mn, Lane 6, YE/FCS + 0.1 μM Mn, Lane 7, YE/FCS, Lane 8, YE.*

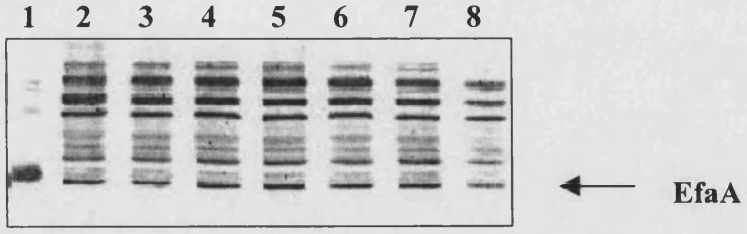


Figure 8.4 RT-PCR Results for *E. faecalis* EBH1 Cells Grown in Medium Containing Variable Zinc Concentrations. *PCR products were analysed on a 0.8 % agarose gel and stained with ethidium bromide and viewed under UV light. Lane 1,YE with 10 μM Zn, lane 2, YE with 5 μM Zn, lane 3, YE with 1 μM Zn, lane 4, YE with 0.5 μM Zn, Lane 5, YE with 0.1 μM Zn, Lane 6, YE/FCS, Lane 8, Molecular weight marker.*

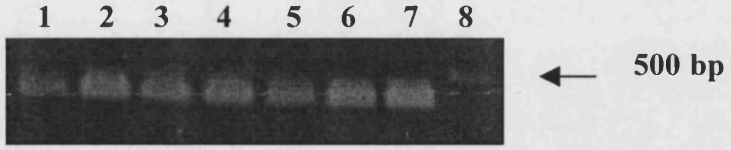


Figure 8.5 Western Blot Analysis of *E. faecalis* EBH1 Cell walls After Growth in 1 % YE Containing Variable Concentrations of Zinc. *E. faecalis cell wall extracts were subjected to SDS-PAGE electrophoresis and then were probed with patient's serum (GP). Blots were visualised with protein A conjugated to horseradish peroxidase. Lane 1, YE, lane 2, YE/FCS, lane 3, YE with 10 μM Zn, Lane 4, YE with 5 μM Zn, Lane 5, YE with 1 μM Zn, Lane 6, YE with 0.5 μM Zn, Lane 7, YE with 0.1 μM Zn, Lane 8, E. coli XL1 pSK+ GP19.*

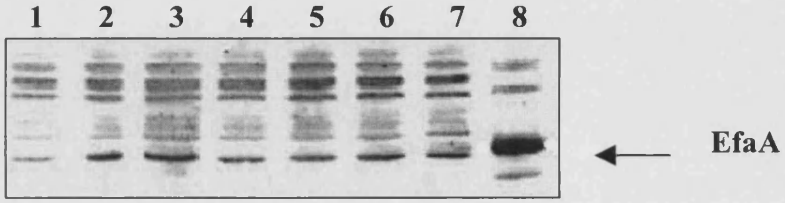


Figure 8.6 Analysis of RT-PCR Results from *E. faecalis* EBH1 Cells Grown in Medium Containing Manganese and Foetal Calf Serum. *PCR products were analysed on a 0.8 % agarose gel, stained with ethidium bromide and viewed under UV light. Densitometry was used to measure the relative transcription of efaA and recA genes. The results are shown here, expressed as the percentage transcription of efaA: recA.*

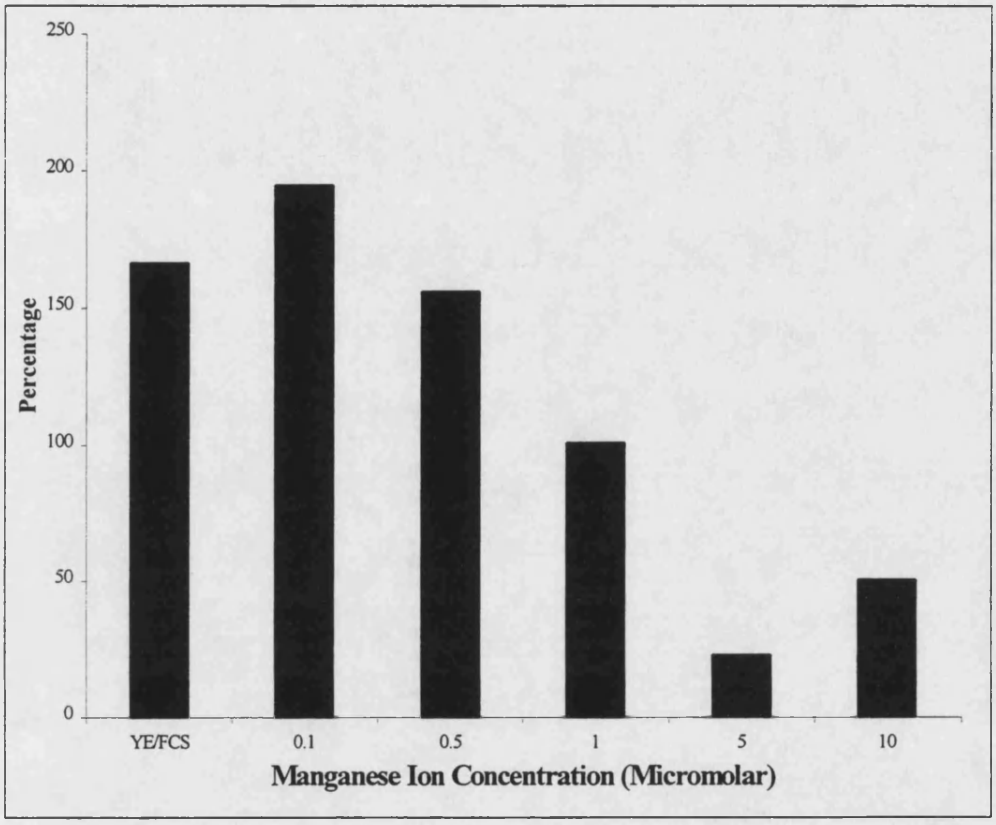
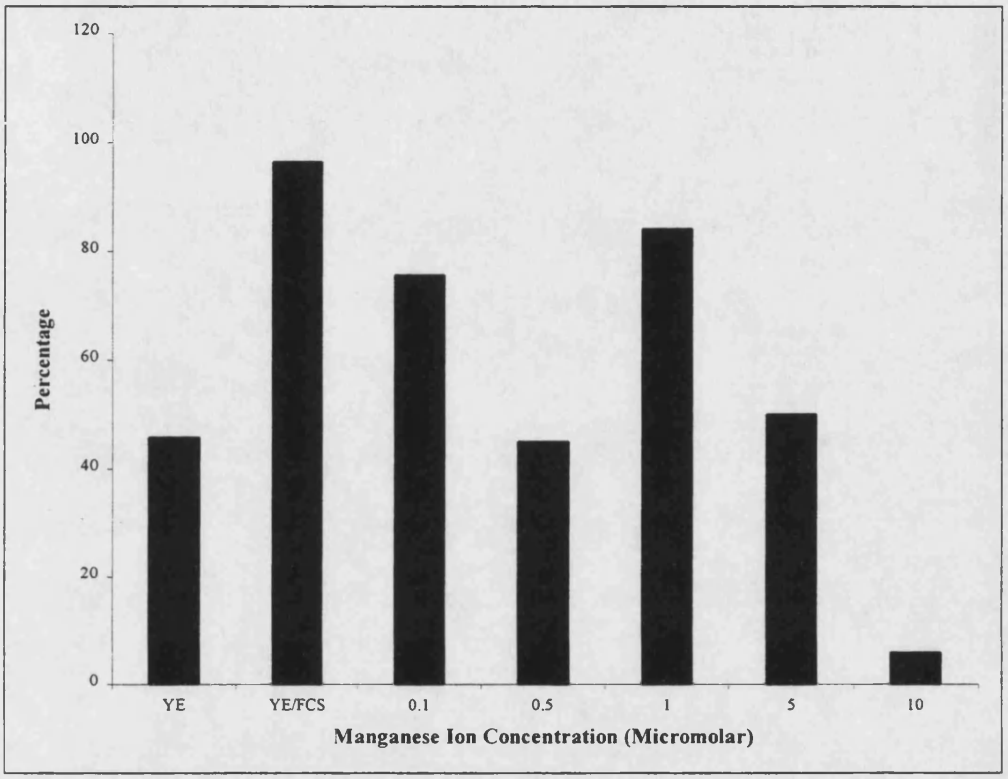


Figure 8.8 Analysis of RT-PCR With RNA From *E. faecalis* EBH1 Cells Grown in 1 % YE Containing Manganese Only. *Cells were grown in 1 % YE, which was not supplemented with 1 % FCS. PCR products were analysed on a 0.8 % agarose gel, stained with ethidium bromide and viewed under UV light. Densitometry was used to measure the relative transcription of efaA and recA genes. The results are shown here, expressed as the percentage transcription of efaA: recA.*



Figures 8.2-8.12 show the results of the RT-PCR experiments on *E. faecalis* EBH1. The agarose gels (figures 8.2 and 8.4) illustrate the differences in expression seen with the RT-PCR products and figures 8.6-8.12 show the results graphically. The effects of manganese are seen clearly in these experiments (figures 8.2, 8.3 and 8.6): the addition of manganese to the growth medium reverses the induction effect seen with serum. The gene must therefore be responsive to the environmental manganese concentration. If manganese is added to growth medium without foetal calf serum, *efaA* transcription remains at or below its basal level (figure 8.7).

Figure 8.8 Analysis of RT-PCR Results from *E. faecalis* EBH1 Cells Grown in 1 % YE Containing Zinc. *PCR products were analysed on a 0.8 % agarose gel, stained with ethidium bromide and viewed under UV light. Densitometry was used to measure the relative transcription of efaA and recA genes. The results are shown here, expressed as the percentage transcription of efaA: recA.*

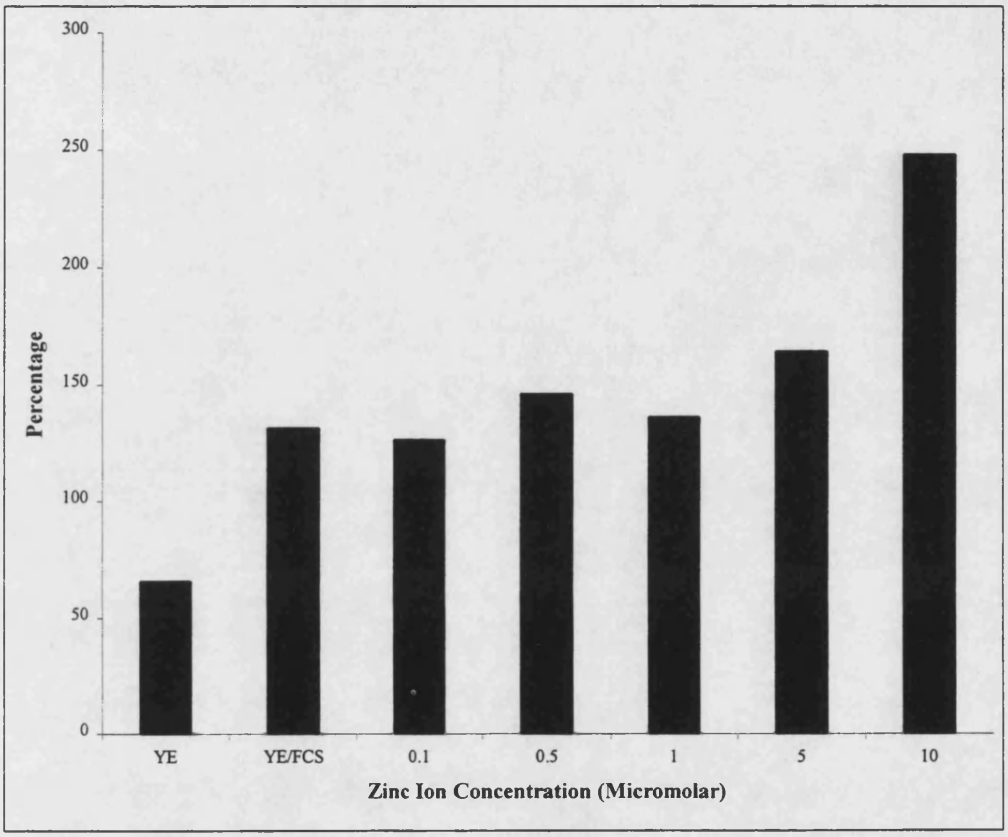
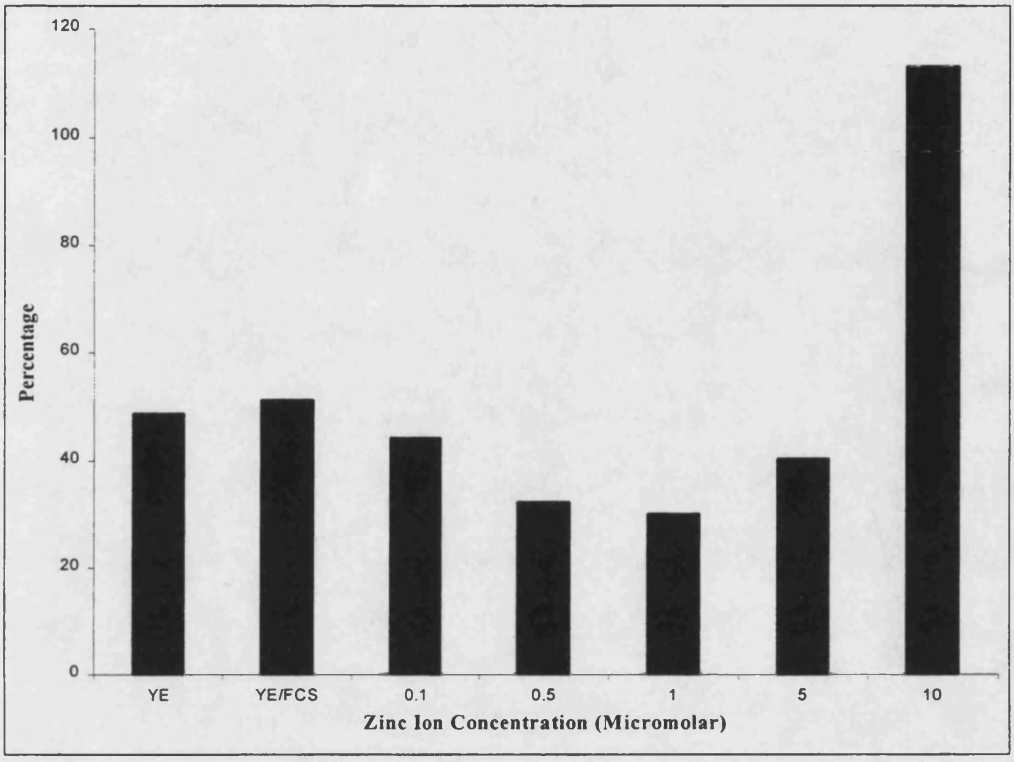
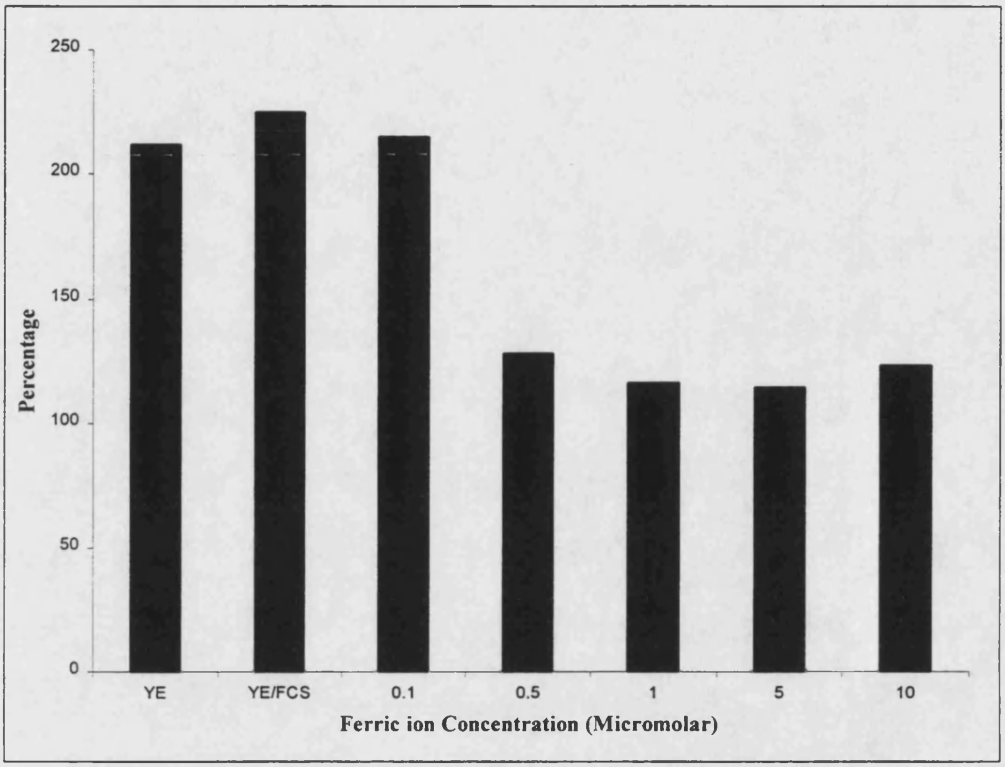


Figure 8.10 Analysis of RT-PCR of RNA from *E. faecalis* EBH1 Cells Grown in 1 % YE Containing Variable Zinc Concentrations and 40μM Manganese, *PCR products were analysed on a 0.8 % agarose gel, stained with ethidium bromide and viewed under UV light. Densitometry was used to measure the relative transcription of efaA and recA genes. The results are shown here, expressed as the percentage transcription of efaA: recA.*



The effects of zinc on *efaA* transcription are also interesting. Figures 8.4, 8.5 and 8.8 illustrate that zinc at a concentration of 5μM or higher is able to induce *efaA* transcription. Indeed 10 μM zinc is able to induce *efaA* to a greater extent than that seen with foetal calf serum. Intriguingly, figure 8.10 shows that adding 40 μM manganese as well as zinc can reverse the induction seen with 5 μM zinc or lower. These results taken together suggest that zinc is acting as a form of competitive inhibitor of manganese. It may be able to bind to the same receptor as manganese and therefore decrease the effective concentration of manganese available to the cell.

Figure 8.11 Analysis of RT-PCR Results from *E. faecalis* EBH1 Cells Grown in 1 % YE Containing Iron. *PCR products were analysed on a 0.8 % agarose gel, stained with ethidium bromide and viewed under UV light. Densitometry was used to measure the relative transcription of efaA and recA genes. The results are shown here, expressed as the percentage transcription of efaA: recA.*



Further experiments showed that magnesium and copper have no significant effect on *efa*A transcription (data not shown). However, it is interesting to note that iron causes a decrease in transcription compared to both YE and YE/FCS controls (Figure 8.11). The presence of a DtxR promoter box (see Chapter 6) upstream of the *efa* operon suggests that iron should have an effect on *efaA* transcription and this is seen here.

The results obtained in these experiments are in agreement with those of Kolenbrander et al (1998). They found that ScaA in *S. gordonii* was up-regulated under manganese-depleted conditions and that zinc could compete

with manganese to cause this up-regulation. Dintilhac et al (1997) also predicted that *efaA* would respond to manganese. Whilst Kolenbrander et al (1998) and Dintilhac et al (1997) proposed that their Cluster 9 proteins are manganese- or zinc- transporters, it is not possible to make such deductions about EfaA from these experiments. It was found that adding manganese repressed the induction observed in YE/FCS medium. This suggests that foetal calf serum is imposing manganese limitation, perhaps by the absorption of manganese onto albumin. However, no uptake studies have been carried out with *E. faecalis*. Such studies would require a knockout mutation of *efaA*. Many Cluster 9 proteins were originally identified as adhesins. FimA has been shown to mediate adhesion to the salivary pellicle (Fenno et al, 1995) and fibrin monolayers (Burnette-Curley et al, 1995). PsaA is involved in adhesion to mucosal or other cells (Berry and Paton, 1996). SsaB facilitates adhesion to salivary-coated hydroxyappatite (Ganeshkumar et al, 1988). The later hypothesis that these proteins are metal ion transporters does not sit well with the adhesion theory. It is difficult to see how the proteins could have a dual role as metal ion transporters and adhesins. It is feasible that a metal ion transporter could act as an adhesin by binding to a metal ion complexed to a host protein. If the ligand is firmly bound, it will not be transported and the cell will be adhered to the host protein. However most metal ions in human serum will be only loosely bound to albumin or transferrin. It would seem more likely that it is the metal transported by cluster 9 proteins that has the direct effect on adhesion or other virulence factors. Manganese is required for glucan-associated adhesion in some mutans group streptococci and both manganese and zinc

are required for pneumococcal transformation (Bauer et al, 1993, Dintilhac et al, 1997). Therefore even though these proteins may not be direct adhesins, they could still have an important role in virulence. Metal ions, and therefore metal ion transporters, could indirectly affect virulence: the metal may be a cofactor for a virulence protein, may be a growth-limiting element, or may regulate the expression of virulence proteins. This last possibility is looking increasingly likely with the identification of multiple Fur homologues and a DtxR protein in *E. faecalis*. It is likely that the metal ion response seen with *efaA* occurs as part of a global regulation of many virulence proteins by a DtxR homologue in *E. faecalis*. The bacteria may use the environmental metal ion concentration to sense when they are inside a host and therefore that they need to synthesise a different set of proteins in order to survive in this environment. It is conceivable that EfaA has a transport or adhesion function that is totally unrelated to metal ion transport, and it is necessary to determine its precise function carefully before drawing any conclusions about its role in *E. faecalis* endocarditis.

Some preliminary RT-PCR studies were also carried out on the *efm* operon. No antibodies are available for *efmA*, so no western blotting was possible. Similarly no details of an *E. faecium* housekeeping gene were available in the databases at this juncture, so it was possible only to look for a crude response and no quantification of this response was possible. Initial results showed problems with non-specific primer binding and primer-dimers so the one step RT-PCR strategy had to be modified. The initial reverse transcription step was carried out with only the reverse primer (JCF7R). Then the PCR reaction

was hot-started after the addition of the high fidelity enzyme mix and the forward primer (JCF7F). This action eliminated all of the extra bands seen previously, but it was still not possible to see any variation in response to the metal ion concentration. Figures 8.12-8.15 illustrate the results seen.

Figure 8.12 RT-PCR Results for E. *faecium* UB1 *efmA* Grown in 1 % YE supplemented with Manganese. *PCR products were analysed on a 0.8 % agarose gel and stained with ethidium bromide and viewed under UV light. Lane 1, marker, lane 2, 10μM Mn, lane 3, 5 μM Mn, lane 4, 1 μM Mn, lane 5, 0.5μM Mn, lane 6, 0.1 μM Mn, lane 7, yeast extract supplemented with 1 % foetal calf serum, lane 8, yeast extract grown cells, lane 9 no template control.*

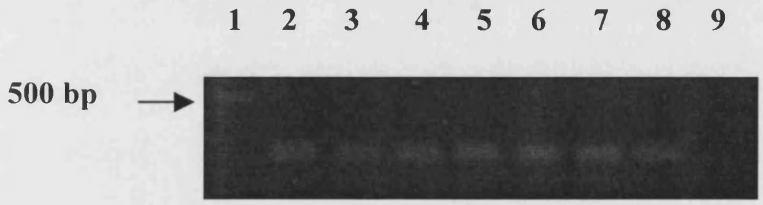


Figure 8.13 RT-PCR Results for *E Faecium* UB1 *EfmA* when Cells Grown in 1 % YE Supplemented with Zinc. *PCR products were analysed on a 0.8 % agarose gel and stained with ethidium bromide and viewed under UV light. Lane 1, marker, lane 2, 10 μM Zn, lane 3, 5μM Zn, lane 4, 1μM Zn, lane 5, 0.5μM Zn, lane 6, 0.1μM Zn, lane 7, yeast extract supplemented with 1% foetal calf serum, lane 8, yeast extract, lane 9, no template control.*

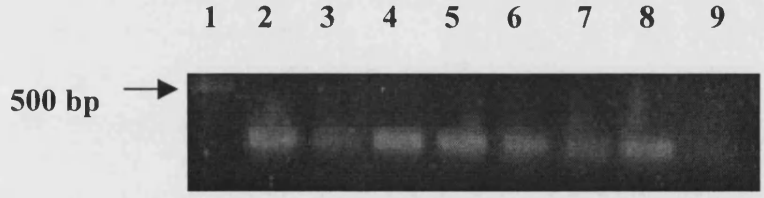


Figure 8.14 RT-PCR Results for *E. faecium* UB1 *efmA* when Cells are Grown in 1 % YE Containing Magnesium. *PCR products were analysed on a 0.8 % agarose gel and stained with ethidium bromide and viewed under UV light. Lane 1, marker, lane 2, 10 μM Mg, lane 3, 5 μM Mg, lane 4, 1 μM Mg, lane 5, 0.5μM Mg, lane 6, 0.1 μM Mg, lane 7, yeast extract supplemented with 1% foetal calf serum, lane 8, yeast extract grown cells, lane 9 no template control.*

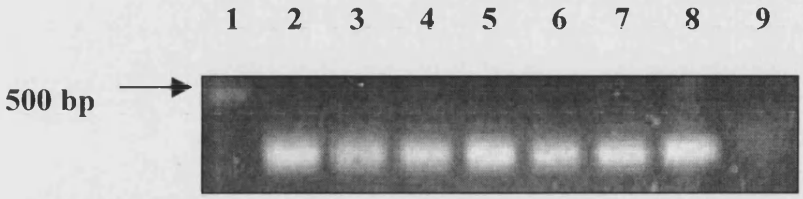
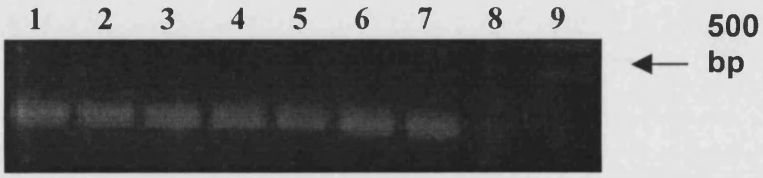


Figure 8.15 Agarose Gel showing Results of RT-PCR of *E. faecium* UB1 *efmA* Cells are Grown in Yeast Extract Medium Containing Variable Concentrations of Copper. *lane 1, 10 μM Cu, lane 2, 5 μM Cu, lane 3, 1 μM Cu, lane 4, 0.5 μM Cu, lane 5, 0.1 μM Cu, lane 6, yeast extract supplemented with 1% foetal calf serum, lane 7, yeast extract grown cells, lane 8 no template control, lane 9, marker.*



These results are surprising as there is clearly a SirR promoter box upstream of the *efm* operon and thus it would be predicted that *efm* would respond to both iron and manganese concentrations (see chapter 5). Although problems with non-specific binding were experienced, adapting the PCR technique eliminated these. Therefore it is unlikely that these would account for the results seen. Comparison with transcription of a housekeeping gene may have revealed more subtle variations in *efmA* transcription. However large variations were seen in *efaA* transcription, and it is probable that *efmA* would show equally dramatic results when tested under appropriate conditions.

It is possible that the yeast extract medium selected as the minimal medium in these experiments was inappropriate. *E. faecium* was very slow growing even in rich medium such as BHI, but in yeast extract it would take approximately six hours to reach early log phase growth. This strongly suggests that *E. faecium* requires a different medium to optimise growth. The long growth curve would indicate that the cells were under stress and possibly were expressing different proteins from normal, which might indicate different metal ion requirements. Further investigations of metal ion requirements in *E. faecium* might require a different growth medium.

It is possible that the metal ion concentrations used in these experiments were not high enough to elicit a response in *efmA* transcription. Hill et al (1998) used concentrations of 20 μM for all the metal ion species that they added to their staphylococcal cells. These experiments were carried out in different media (yeast extract as opposed to RPMI used by Hill et al) and with a different species of bacterium. However, it is still possible that the repressor binds to the SirR box with greater affinity than to the DtxR box (which occurs in *E. faecalis* and mitis-group streptococci), and needs higher concentrations of metal ions to release its hold. If this were the case it would imply that *E. faecium* has a higher requirement for manganese than *E. faecalis*. Perhaps this could be because *E. faecium* has a higher requirement for metal ions to synthesise enzymes to cope with oxidative stress. If this is the case it might explain why *E. faecium* is less pathogenic than *E. faecalis*. If *E. faecium* is less able to cope with oxidative stress, and hence has a higher requirement for metal ions, it will be less able to survive in the hostile

environment of the host. It would be interesting to test this hypothesis further. It would also be interesting to investigate the other metal ion dependent repressors in *E. faecium*, and to compare their relative sensitivities to metal ions.

In conclusion therefore, it has been shown that the *efa* operon is regulated by the concentration of manganese and zinc in the growth medium. This is in agreement with the predictions of Dintilhac et al (1997) and the identification of a metal ion promoter box found upstream of the *efa* operon which shows homology to the DtxR box (see chapter 6). However it was not possible to confirm that the *efm* operon is responsive to metal ions. Clarification of this unexpected finding, and further work on the function of these operons must still be carried out.

9 Insertional Inactivation of the *efaA* Gene in *E. faecalis*

In this section of work, efforts were made to inactivate *efaA* by insertional mutagenesis. The subsequent 'knockout mutation' was to be used in animal studies to determine whether *efaA* had an effect on virulence.

Until recently enterococci were regarded as non-transformable. Their tough cell walls are mostly impermeable to large molecules such as DNA. In addition the different restriction-modification systems in gram-positives were incompatible with many common *E. coli* plasmids, rendering them inactive. However advances in molecular biology in recent years and the advent of electroporation have enabled transformation of many 'non-transformable' species, including enterococci.

Electroporation involves applying a high transmembrane voltage across the cell, which overcomes the natural barrier function of the membrane, allowing pores to open up and the passage of ions and water soluble molecules across the membrane (Weaver, 1993). Electroporation does not heat up the cells, as dramatic membrane rearrangements occur instantaneously, however some cell death is inevitable as membranes will rupture and chemical imbalances occur across cells due to the uncontrolled influx and efflux through pores (Weaver, 1993). The amount of voltage needed to open up membranes is usually expressed as field strength and is defined as voltage applied over the distance between the two electrodes (kV/cm). The electric field required to transform a cell depends on the cell radius. Larger, mammalian cells require

lower field strengths than smaller, bacterial cells (Weaver, 1993). Usually bacteria require field strengths of between 5 and 20 kV/cm (Miller, 1994).

Even with such high field strengths enterococci can remain resistant to transformation, so agents which disrupt cell wall synthesis are often used (e.g. glycine, threonine, lysosyme, penicillin G) (Weaver, 1993; Miller, 1994; Dunny et al, 1991). The many different protocols for transforming enterococci depend on different combinations and concentrations of these cell-wall-active agents. The most effective transformation results in enterococci are seen with glycine (Dunny et al 1991). Sucrose (0.5 M-0.625 M) is then added to the recovery medium to stabilise the cells (Miller, 1994).

Several plasmids were used in an attempt to transform *E. faecalis* EBH1. They were all 'shuttle vectors,' that is plasmids which have been designed to exist in more than one species, e.g. *E. coli* and *Streptococcus* spp. They will usually contain an origin of replication for both the species that they inhabit. Plasmids that contain only one origin of replication, such as the *E. coli* one, are designated 'integrative vectors.' In order to survive in their other host, they must combine with the chromosome. The target sequence for such recombination is usually the gene sequence of interest, which has been inserted into the plasmid. When the plasmid combines with the chromosome at its designated target, it will disrupt transcription of the gene. Either a small portion of the plasmid may combine with the chromosome, or the entire plasmid may be incorporated. The latter plasmids are designated 'suicide vectors.'

Figure 9.1 Plasmid Maps of pVA838 and pVA891 *coordinates (kb) are shown on the inside of each map. The approximate location of the erythromycin resistance (Em^R) and the chloramphenicol resistance (Cm^R) are indicated. The streptococcal segments of each plasmid are shown in thick lines.*

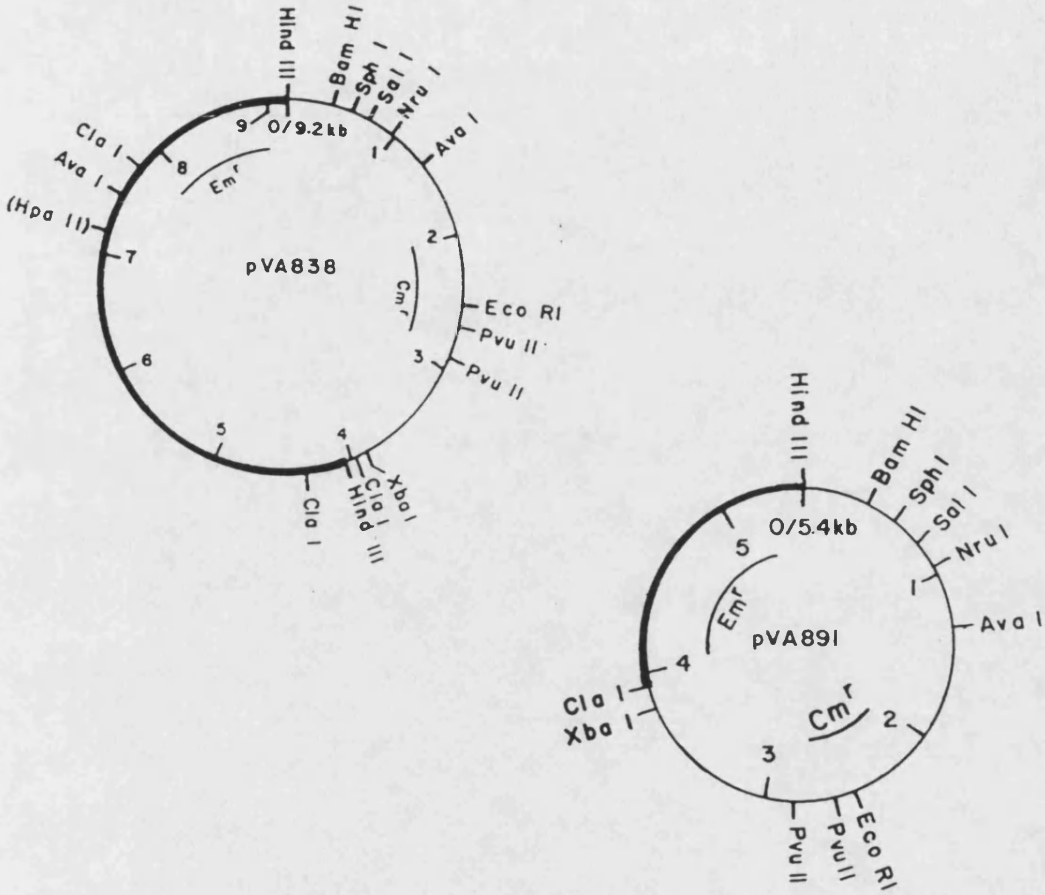
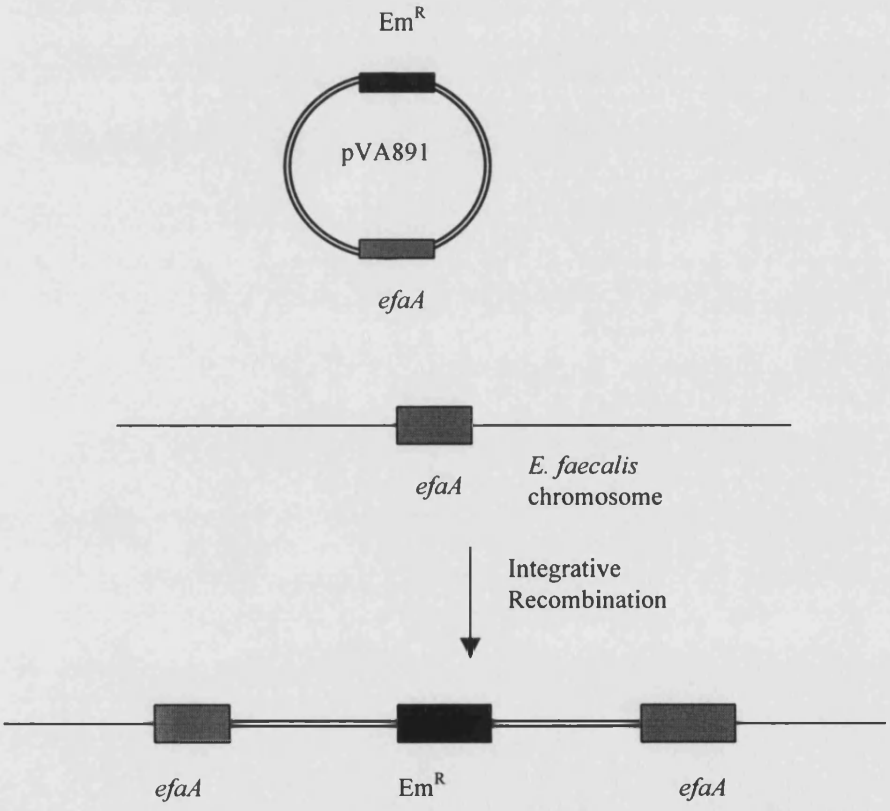


Figure 9.1 shows the plasmid maps of two shuttle vectors used in the course of these experiments. Plasmid pVA838 (Macrina et al, 1982) is a 9.2 Kb shuttle vector designed for use in *E. coli* and *S. sanguis*. It confers erythromycin resistance and chloramphenicol resistance in *E. coli*, but only erythromycin resistance in streptococci. It is able to replicate in both species. Its derivative, pVA891 (Macrina et al, 1983) is the product of *Cla*I cleavage and self-ligation of pVA838. The resulting plasmid is 5.4 Kb and contains only 1.5 Kb of streptococcal sequence (corresponding to the erythromycin gene). All other streptococcal genes, including the plasmid replication

machinery have been removed, so the plasmid must combine with the chromosome in order to survive. A 405 bp portion of *efaA* was ligated into pVA891 to enable the plasmid to pair with *efaA* on the chromosome and combine with the chromosome via a single crossover (Campbell-like recombination). The mechanism of this event is illustrated in figure 9.2

Figure 9.2 Single Crossover Recombination, *homologous recombination results in a 'single crossover' reaction in this case. The plasmid pVA891, containing a 405bp portion of efaA and erythromycin resistance (Em^R), is inserted into the E. faecalis chromosome, leaving it flanked by the duplicated target sequences, i.e. the 405 bp portion of efaA. The efaA gene is now inactivated and the mutant gene can be selected for by screening for erythromycin resistant transformants.*



G. M. Dunny (see 2.23) supplied the electroporation protocol. Cells were grown in glycine-containing medium to destabilise their cell walls. They were further washed in magnesium chloride, which has been found to

increase transformation efficiencies, probably by decreasing extracellular polysaccharide (Berthier et al, 1996). They were then subjected to electroporation with a field strength of 12.5 kV/cm. Initial attempts were unsuccessful, so some modifications were made to the protocol. *E. faecalis* EBH1 is a clinical isolate and these can prove difficult to transform. Failure of some clinical isolates to electroporate may be due to resident plasmids, which can interact with transformed plasmids and inactivate them (Berthier et al, 1996). The plasmid content of *E faecalis* EBH1 has never been studied, but it is highly probable that it contained both plasmids and transposons, all of which might interact unfavourably with pVA891. Some enterococcal strains are acknowledged to be untransformable by electroporation (Teng et al, 1998), even when they are naturally transformable. Interestingly a study with naturally competent *Haemophilus influenzae* cells showed that they were several orders of magnitude less efficient than non-competent cells for electroporation (Miller, 1994). Furthermore variations in the thickness of the peptidoglycan layer between different strains will likely affect the ability to transform them. The laboratory strain, OG1RF was accordingly substituted for EBH1, but attempts were still unsuccessful.

An electroporation apparatus was borrowed from Flowgen, which was able to apply field strengths up to 3.5 kV/cm. Electroporation using very high field strengths has proved successful in untransformable *Lactobacillus* spp (Flowgen, personal communication). The apparatus was also capable of a biphasic electrical pulse. The first pulse was high voltage, to open pores in the membrane, while the second was lower voltage to encourage the DNA to

enter the cells. Again this approach proved unsuccessful. There was a considerable problem with arcing at higher field strengths, resulting in increased cell death. Additionally a potential problem at these field strengths is the degradation of DNA, due to increased heat passed into solution from the high voltage. Both factors prompted a search for alternative protocols.

The poor results suggested that transformation efficiency was low. To investigate transformation further, plasmid pVA838 was utilised. Attempts were made to transform pVA838 into both *E. faecalis* OG1RF and *S. gordonii* Challis by electroporation. In both cases pVA838 was successfully transformed (albeit with a low efficiency, data not shown). Some work was carried out on optimising the electroporation, but it was found that the original protocol gave the optimum results (data not shown). As mentioned previously, restriction-modification systems can be a significant barrier to transformation. The efficiency of transformation with plasmid DNA into *Campylobacter jejuni* increases by at least four orders of magnitude when plasmid DNA is prepared from *Campylobacter* instead of *E. coli* (Miller, 1994). To clarify whether restriction-modification systems were causing an incompatibility problem, pVA838 was prepared from *E. coli* XL1, *S. gordonii* Challis and *E. faecalis* OG1RF. The plasmid preparations were transformed into *E. faecalis* OG1RF. The transformation frequencies are shown in Table 9.1. The plasmid preparation from *E. faecalis* transformed back more efficiently than plasmids prepared from *S. gordonii* or *E. coli*. The restriction-modification systems therefore present a significant problem

in mutagenesis and this may partly explain the low success rate with pVA891*efaA*, which had been extensively subcultured in *E. coli*.

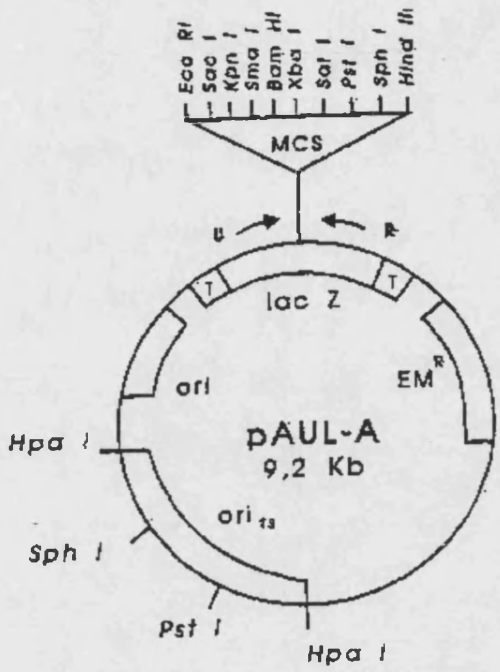
Table 9.1 Transformation efficiency of pVA838 into *E. faecalis* OG1RF, *plasmid pVA838 was prepared from E. coli XL1, S. gordonii Challis and E. faecalis OG1RF and was transformed into E. faecalis. Transformation efficiency was defined as the fraction of surviving cells transformed (reviewed by Miller, 1994)*

Source of Plasmid pVA838	Transformation Frequency
E. coli XL1	1.42×10^{-7}
S. gordonii Challis	9.64×10^{-7}
E. faecalis OG1RF	1.21×10^{-6}

As attempts with pVA891 were unsuccessful, a second plasmid, pAULA, was used. This 9.2 Kb shuttle vector was developed for use in *E. coli* and *Listeria* spp (Schaferkordt and Chakraborty, 1995). It contains an origin of replication from both *E. coli* and *S. aureus*, but the gram-positive replicon is temperature sensitive. At the permissive temperature (30°C) the plasmid may be transformed and will replicate in gram positives. If the temperature is then increased to 42°C, the plasmid is no longer able to replicate and must combine with the chromosome via a single crossover in order to survive. The 405 bp *efaA* fragment was inserted into the plasmid to act as a target for recombination. The advantage of this system is that the initial stage in the mutation process, transformation, is separated from later recombination with the chromosome, enabling troubleshooting at each stage. The results obtained, however, were not encouraging. Whilst a limited number of colonies were obtained at the permissive temperature, no colonies were seen when the temperature was raised to 37 °C, indicating that the colonies seen at the lower temperature were most likely to be spontaneous erythromycin

resistant mutants, and not transformants. This implies that pAULA is not stable in enterococci, but further testing would be required to confirm this.

Figure 9.3 Plasmid Map of pAULA *the positions of the lac α fragment, the erythromycin resistance gene (EmR) are marked. The origins of replication are also shown, where ori = E. coli origin of replication and* ori_{TS} *= temperature sensitive gram positive origin of replication.*



U = universal primer
R = reverse primer

As it was not possible to optimise the rate of transformation to any greater degree it was decided to look at more efficient methods of recombination, in order to compensate for poor transformation efficiency. Integrative plasmids may combine with the chromosome by two different mechanisms (see figure 9.4). Firstly a 'Campbell-like' single crossover can occur, which will result in the incorporation of the entire plasmid into the chromosome at the target site and duplication of the target sequence. Alternatively the chromosomal

target may be replaced by a double crossover with a portion of linear DNA. In order for this to occur the antibiotic resistance gene must be flanked by DNA homologous to the target gene.

Figure 9.4 Comparison of Double and Single Crossover Recombination *showing the two possible scenarios for integration of foreign DNA into a chromosome. Diagram a shows the results of a single crossover reaction, where both target DNA and plasmid are incorporated into the chromosome, leading to duplication of the target sequence. In diagram b a double crossover occurs resulting in replacement of the target sequence on the chromosome with the target sequence from the plasmid (containing the antibiotic resistance marker, Ab^R). After Teng et al, 1998.*

Figure 9.4a

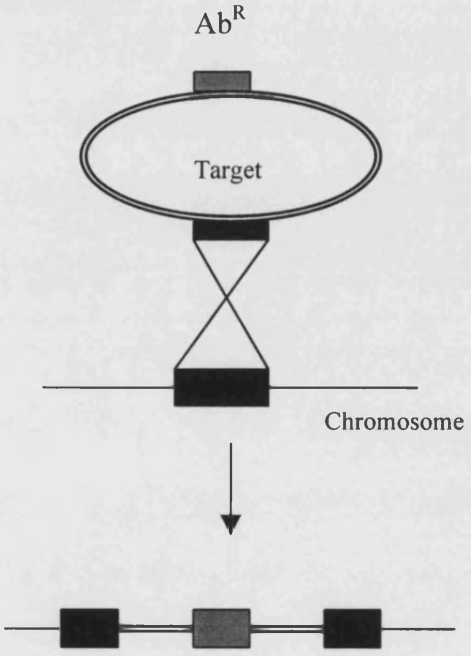
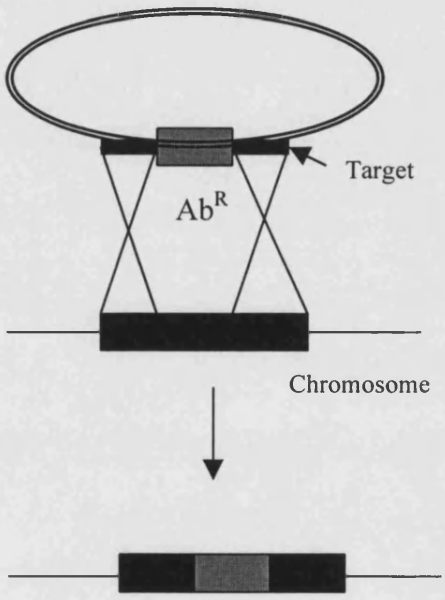


Figure 9.4b



It is acknowledged that bacterial strains tend to show a preference for one particular type of recombination (Fenno et al, 1993). This can depend on the method of transformation and the modification procedures that occur inside the strain. Transforming DNA can often be modified by the cell and may be nicked into a linear fragment. Campbell recombination requires a circular DNA molecule and so tends to occur in strains undergoing natural

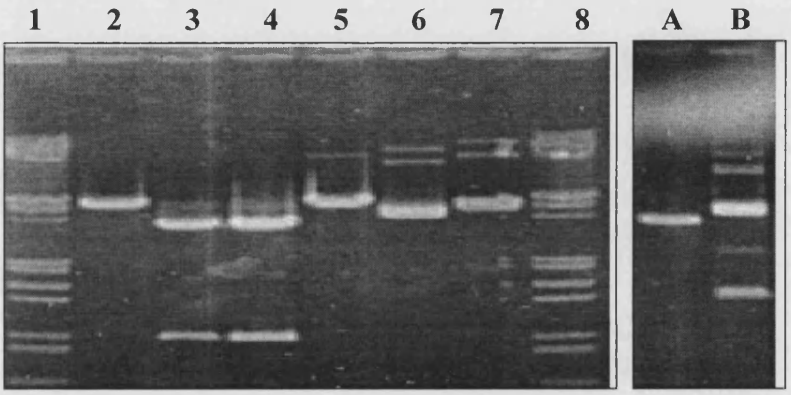
transformation (e.g. conjugation), where DNA is processed before transport then reformed and ligated inside the cell. Electroporation requires uptake of intact DNA without intermediate binding and processing and so double crossovers are often the preferred mechanism of integration (Fenno et al, 1993). Plasmid transformed into *S. gordonii* Challis has been shown to undergo Campbell-type recombination when it is naturally transformed and double crossover recombination when it is electroporated (Fenno et al, 1993). It would therefore seem advisable to use a construct that could integrate via a double crossover, to see if this was more successful.

The amount of homologous DNA needed to construct a mutant can be an important factor. Berry and Paton (1996) used only 254 bp homologous DNA to construct a *psaA* mutant. However work by Fenno et al (1993) showed that specific allellic replacement in the *fim* operon was five times more efficient with plasmids containing 6 Kb homologous DNA than with those containing only 3 Kb. The 405 bp fragment used in previous experiments may not have been big enough for targeting.

A new plasmid was therefore constructed, capable of integration into the chromosome via a double crossover. The pBluescript clone of the *efa* operon was used, which contains 1.9 Kb of the *efa* operon (named pSK+: GP19). It was cleaved at a unique Sph1 site located 560 bp into the clone. The ends were flushed with T4 polymerase. An erythromycin resistance gene (*erm*AM) was cleaved from a plasmid (pSLERY, provided by H. F. Jenkinson), using Nde1. The sticky ends were filled by Klenow enzyme. The

erythromycin gene was blunt-ligated into the pSK+: GP19 clone and the resulting construct was used in electroporation experiments.

Figure 9.5 Construction of the pBluescript:GP19 (*erm*AM) Integrative Plasmid. *Plasmid pSK+: GP19 was cut with Sph1 and then treated with T4 polymerase. Plasmid pSLERY was cut with Nde1 then was treated with Klenow enzyme. The ErmAM fragment from pSLERY (1 kb) was blunt ligated to pSK+: GP19/Sph1 overnight.. Lane 1, Molecular weight marker, Lane 2, pSK+ : GP19 cut with Sph1 and treated with T4 polymerase, Lane 3, pSLERY cut with Nde1 and treated with Klenow, Lane 4, pSLERY cut with Nde1, Lane 5, pSK+ : GP19 cut with Sph1, Lane 6, pSLERY cut with Nde1, Lane 7, pSK+ : GP19, uncut, Lane 8, Molecular weight marker. Lane A, pSK+: GP19 cleaved with Nde1, Lane B, pSK+: GP19ermAM cleaved with Nde1.*



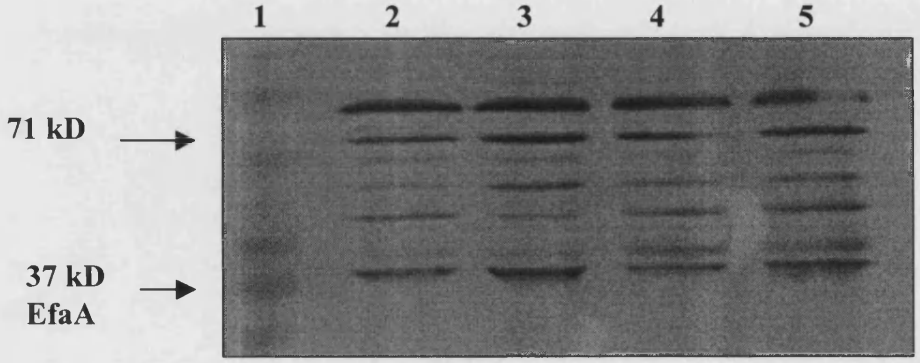
A new strain of *E. faecalis* was used in these experiments. *E. faecalis* JH2-2 is known to transform well with the protocol developed by Cruz-Rodz and Gilmore (1990, see section 2.24), so was used in subsequent experiments to ensure optimum transformation efficiencies. Transformation with this plasmid appeared to be much more successful (see Table 9.2), with initial results suggesting that this construct was allowing recombination.

Table 9.2 Time Constants and Viable Count (cfu/ml) *following electroporation of E. faecalis JH2-2 with pSK+:GP19 (ermAM). Cells were electroporated according to section 2.24. The time constants were noted. The samples were incubated for 48 h then the number of colony forming units on erythromycin-containing media was noted. The number of viable cells following electroporation was determined to be 6.2 x 10^7 cfu/ml.*

Volume Plasmid Used (μl)	Time Constant (ms)	No. cfu / ml
0	4.8	0
2	4.7	34
4	4.7	117
6	4.7	113
8	3.9	73
10	4.6	70

Initial colony blotting results were also promising, with several colonies showing a response to the *erm*AM probe. Five colonies were chosen for more complete study. However western blotting results were less successful. All five strains still showed a band corresponding to the *efaA* protein (Figure 9.6).

Figure 9.6 Western Analysis of Cell Wall Extracts from Potential *E. faecalis efaA* Mutants. *Cell wall extracts were subjected to SDS-PAGE gel electrophoresis then were probed with rabbit-anti-efaA serum diluted 1:20 in TBS. Blots were developed with protein A conjugated to horseradsh peroxidase. Lane 1, molecular weight marker, lane 2, E. faecalis EBH1 control, Lane 3, E. faecalis JH2-2 control, Lanes 4 and 5, E. faecalis JH2-2 potential mutant strains.*



It is worth considering whether the transformation itself was unsuccessful, or whether the screening process was ineffective. Time constraints prevented a repeat of this experiment, but any further attempts would have required a more foolproof screening process, as colonies that looked promising on colony blots did not contain the inactivated *efaA* gene. Chromosomal PCR might be a solution to this problem. It would be possible to perform two PCR reactions, the first with primers for the *ermAM* gene, where a product would only be visible in mutant strains. As a control a second PCR could be performed using primers for a region of the *efaA* gene spanning the Sph1 site where the *ermAM* gene was inserted. Mutant strains would show a PCR product 1 Kb larger than wild type strains. This technique should enable a large number of colonies to be screened, before further tests, such as western and southern blotting could be carried out on more promising strains to confirm results.

After the laboratory work for this project had finished, Singh et al (1998) published a report of the generation of an *efa* mutant in strain OG1RF. The vector used was a derivative of pBluescript (SK-), with ~730 bp of *efaA* inserted and an enterococcal ampicillin resistance gene inserted into the β-lactam resistance gene. The plasmid would have to be incorporated into the chromosome via single crossover recombination, resulting in duplication of the target sequence. The success of this contrasts with the conclusions drawn in this work about the efficacy of single crossover recombination in this species. It is also surprising that such a small region of homology was needed to target the plasmid to the *efaA* gene on the chromosome.

The method used for electroporation in this study is unclear. The text states that the competent cells were prepared using a protocol published by Friesenegger et al (1991). This technique involves growing *E. faecalis* cells to stationary phase in Todd-Hewitt broth, then carrying out sequential washes of the cells in decreasing volumes of 10% glycerol. However the text states that electroporation was carried out according to the protocol of G.M. Dunny (Dunny et al, 1991, see section 2.23). It is difficult to see how these two very different protocols could be combined. For example, the protocol used by Dunny et al (1991) requires the cells to be grown in media containing glycine. It is not evident if glycine was used by Singh et al (1998). When preparing competent cells, the method of Friesenegger et al (1991) does not use glycine or any other growth inhibitor. In addition, it is not clear which buffer was used for electroporation. The Friesenegger protocol uses 10% glycerol whilst the Dunny protocol requires sucrose and magnesium chloride. The two methods require completely different recovery media: Dunny et al (1991) used BHI broth containing sucrose, whilst Friesenegger et al (1991) used Todd Hewitt broth without sucrose. Without clarification of these discrepancies, it is not possible to ascertain the method of insertional mutagenesis of *efaA* used by Singh et al (1998).

Although the method adopted by Singh et al (1998) was successful, it is not easy to compare their results with those obtained in this work. The use of different plasmids and electroporation techniques is likely to have accounted for their success, but without further clarification of the exact methods used it

is not possible to come to any meaningful conclusions about the best technique to employ in further mutation experiments.

In conclusion therefore, the process of insertional mutagenesis in Enterococci is a fast-developing field. Many new techniques are being developed to improve transformation efficiency and the last few years have seen some dramatic improvements. However the optimum method for this process has not been clarified and it appears to vary depending on the strain of enterococcus. Some strains still remain untransformable and others are poorly transformable by electroporation. These will require other methods to mutate them and some recent research has looked at these other methods of transformation, particularly in other species of enterococcus. For example, electroporation of *E. faecium* strains has proved even more troublesome than *E. faecalis*. Conjugation has recently shown great promise as an alternative to electroporation in poorly transformable enterococci (Teng et al, 1998). Shuttle vectors containing an origin of conjugal transfer (*ori*T) have been used to transfer DNA from *E. coli* to gram-positive species. The transfer requires another plasmid (IncP) to be present in *E. coli* cells. Teng et al (1998) have shown that it is possible to knockout the autolysin gene in *E. faecalis* using this method, and have managed to transfer a conjugal plasmid from *E. coli* to *E. faecium* SE34, a strain which showed poor transformation efficiency by electroporation. Conjugation could therefore provide a realistic answer to mutation in *E. faecium*. Any further work on the *efm* operon may require insertional inactivation one or more genes, and it would certainly be worth using conjugation as a method of generating these mutants.

10 General Conclusions and Future Directions.

The work described in the previous chapters has allowed detailed study of the *efa* and *efm* operons in *E. faecalis* and *E. faecium,* respectively. The entire operons have been sequenced and analysed and have revealed several areas that would merit further study. Manganese has been shown to regulate the transcription of *efaA*, but it was not possible to establish a similar regulation mechanism with *efmA*. Three *E. faecalis* Fur homologues have been described, together with a DtxR homologue, raising interesting questions concerning metal ion regulation of enterococcal genes.

It is clear that any further work on Cluster 9 proteins should focus on their ability to respond to metal ions. In particular it is important to establish whether *efa* and *efm* are metal-ion-transporters, or whether they are simply upregulated under specific metal ion depleted conditions. This is important, since without this information it is not possible to determine whether these proteins are adhesins, or whether they indirectly affect adhesion. Without resolving this issue it will be difficult to define the role of these operons in IE.

It is important to establish the role of transition metals in enterococcal metabolism and in particular the role of manganese and zinc, which have been underestimated until now. The data presented in this project emphasise the importance of metal ions to enterococci, not only in specific reactions but also in regulation of cellular processes. Methods to restrict access of manganese and zinc to enterococcal cells may prove to be an effective

method of killing these organisms and therefore may provide a new target for therapy. Thus it is legitimate to study further metal ion dependence in these bacteria. The full sequences of the *E. faecalis* Fur homologues need to be identified, together with their consensus binding sites. Footprint analysis or gel shift mobility assays will be of use in identifying proteins that are controlled by Fur or DtxR. Studies like this will determine the extent of metal ion regulation in *E. faecalis*. The relative importance of iron and manganese to enterococci needs to be qualified. The gram-positive bacterium lactobacillus has been shown to require little or no iron in its metabolism (reviewed by Weinberg, 1997), but it is considered to be unusual. Enterococci are likely to require some iron, but whether or not they require more manganese than iron needs to be determined.

The *adc* operon in *E. faecalis* should be investigated for its ability to respond to zinc, and for any role it may have in IE. The predicted molecular weight of mature *E. faecalis* AdcA is 55 kD. The three prominent antigens previously identified in *E. faecalis* IE were 37, 40 and 73 kD (Aitchison et al, 1987). This suggests that AdcA is not strongly expressed during *E. faecalis* endocarditis, but this needs to be confirmed experimentally. However an area of investigation that is likely to show more promise is the study of competence. The *adc* operon in *S. pneumoniae* has been shown to have an indirect role in competence (Dintilhac and Claverys, 1997). Cells deficient in *adc* were unable to undergo transformation. Zinc was later shown to be required by competence stimulating peptide to induce expression of competence genes (Dintilhac et al, 1997). It would be very interesting to

determine whether zinc was required for transformation in enterococci, as this might enable us to understand further the mechanisms by which enterococci acquire antibiotic resistance.

Any further studies of the cluster 9 proteins in *E. faecalis* and *E. faecium* will require the insertional inactivation of these genes. The work carried out in this project has established the best method for carrying out mutation of genes in *E. faecalis*. However it has been shown that such inactivations are certainly not easy to carry out, and in some strains they are impossible. Further work on insertional inactivation is likely to require the use of transposons, especially in *E. faecium*. Whilst the double crossover experiments showed great promise in *E. faecalis*, it is acknowledged that *E. faecium* is almost impossible to transform by plasmid mutagenesis and it would be much more effective to use transposons in any work involving *E. faecium*.

In summary, therefore, the work carried out in this study has revealed many possible opportunities to study *E. faecalis* and *E. faecium* pathogenesis. In particular, the field of metal ion transport and regulation has shown great promise for further work. Future studies in this area should greatly increase our understanding of these highly problematic bacteria, and are likely to reveal many potential drug therapy targets.

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