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Plasmids, Resistance and Hospital adaptation in Enterococci

-an epidemiological approach

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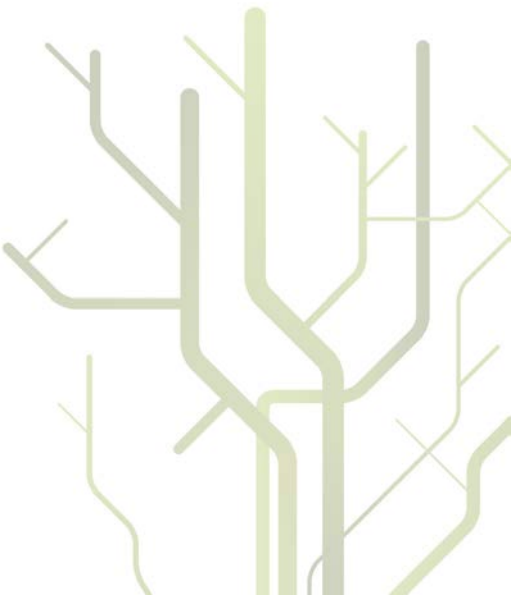

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1. Introduction

1.1 Enterococci

Enterococci have been known for over a century to be capable of causing infections in humans [1, 2]. For a long time, these infections were limited in numbers and mostly caused by *Enterococcus faecalis*. In the last decades enterococci have emerged as important nosocomial pathogens, largely due to their intrinsic antimicrobial resistance and their vast capacity to acquire antimicrobial resistance [3, 4]. Their genomic plasticity has also contributed to their adaptation to the hospital environment. In the early 1980s *E. faecalis* accounted for 90% of enterococcal infections [5]. Subsequently, ampicillin resistant *Enterococcus faecium* started to emerge [6], and in 1986 transferable high-level vancomycin resistant enterococci (VRE) was discovered [7, 8]. In addition, *E. faecium* was shown to easily acquire resistance to other antimicrobials [9]. Since then, a gradual increase in enterococcal infections has been seen. *E. faecium* infections have increased relative to *E. faecalis* and have partially replaced *E. faecalis* as a cause of hospital associated infections. Now the prevalence of infections caused by *E. faecium* is close to that of *E. faecalis* [10-12]. *E. faecalis* have also been shown to acquire antimicrobial resistance, high-level gentamicin resistance (HLGR) in particular, but resistance to ampicillin and vancomycin is infrequent [13]. Enterococcal infections are now the 3rd and 4th most frequent microorganism isolated from hospital associated infections in the US and Europe, respectively [10, 14].

1.1.1 General characteristics of enterococci

Enterococci are commensals of the human and animal intestinal flora [15-17]. They are also commonly used in food fermentation [18-20] and easily detectable in environmental sources such as in water, plants and soil [21-23]. Until the 1980s, species that today belong to the *Enterococcus* genus were classified as streptococci. In 1984 DNA homology studies showed that *Streptococcus faecalis* and *Streptococcus faecium* were so distantly related to streptococci that they were transferred to another genus; *Enterococcus faecalis* and *Enterococcus faecium*, respectively [24]. In the beginning of the 19th century, *S. faecalis* and *S. faecium* were considered the same species [1], but during the 1940s and 1950 studies showed that the two organisms had different biochemical characteristics and by the mid-1960s they were accepted as two distinct species [25]. A number of other enterococci have

been isolated [5, 26], and by 01.02.2012 there were 47 species in the *Enterococcus* genus registered in the Taxonomy browser in GenBank (http://www.ncbi.nlm.nih.gov/taxonomy/?term=enterococcus). Enterococci belong to the phylum *Firmicutes* and the family of *Enterococcacae*. They are Gram positive facultative anaerobic organisms that are catalase negative, with the ability to hydrolyse esculin in the presence of bile. They can grow under harsh conditions, including both 10°C and 45°C , in the presence of 6,5% NaCl, and at pH 9,6. In addition, enterococci survive for 30 minutes at 60°C [27]. The GC-content in the enterococci is low (36-40%), but can vary within the genome [28-30]. Sequencing of *E. faecium* and *E. faecalis* genomes have shown that both have an open pan genome, which means there is no limit to the number of genes that can be part of the joint genome of all bacteria within the species. It also revealed that the genomes are very flexible, with a large ability to recombine, that are at least in part due to the high numbers of IS- and other mobile genetic elements present in these genomes [28, 29, 31-34].

1.1.2 Clinical significance of enterococci

1.1.2.1 Hospital associated infections

Hospital acquired infections (HAI) are described as an infection occurring during hospitalization. Definition criteria often include that the infection was neither present nor incubating at the time of hospital admission. As a consequence, in many epidemiological surveillance systems, these infections are required to appear no earlier than 48 hours after hospital admission to be defined as HAI [14, 35, 36]. The European Centre for Disease prevention and Control (ECDC) have estimated the prevalence of HAI in European acute care hospitals to range from 3,5%-10,5% with an average of 7,1% among admitted patients. From this prevalence, the cumulative incidence have been estimated to approximately 5,1% [14]. This means that for every 100 persons who are admitted to the hospital, 5 persons will get a hospital acquired infection. The economic burden of HAI is a comprehensive and complex calculation, and the transferability between different studies have proven low [37]. To give an idea of the increased cost attributable directly to HAI, we can calculate the cost of the lengthened hospital stay as a result of HAI. A prudent valuation has estimated that HAI lengthen the hospital stay with an average of 4 days [38]. The average hospital stay has been calculated to cost EUR 435 per day [39], which means that for every 100 persons admitted to

the hospital, HAI will increase the costs with EUR 8700. This is only estimating the direct cost of the lengthen stay, not considering any indirect costs such as cost related to the need for additional medical procedures, the need for isolation, loss of income, increased morbidity or increased mortality. The share of deaths contributable to HAI is substantial. The US CDC estimated the direct attributable mortality of HAI to be 0,9%, in addition it contributed to 2,7% of deaths [38]. Combined with antimicrobial resistance, the consequences of HAI are even greater: higher costs, more morbidity and more mortality. Carmeli *et al.* shoved that for VRE infections the multiplicative effect for lengthened hospital stay was 1,73 and for hospital cost 1,4. Morbidity was also significantly increased and the risk of death was doubled [40].

1.1.2.2 Epidemiology

Enterococci are a common cause of HAI worldwide. In Europe, the prevalence of enterococcal HAI is around 8%, and enterococci are only outnumbered by *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* [14]. Although enterococci do not reach the top-ten list of nosocomial outbreak pathogens [41-43], ECDC has placed them on the list of pathogens posing a major threat to healthcare systems [14]. This is in large part a result of the increasing antimicrobial resistance in enterococci. In the US, 80% of *E. faecium* isolates are vancomycin resistant [10]. In Europe the prevalence of VRE has traditionally been low, and in the Scandinavian countries prevalence is still below 1%. However, increasing rates of VRE have been reported from many European countries, and in Greece and Ireland the prevalence is even >30% [44].

In Norway, as in the rest of the world, the prevalence of enterococcal infections is increasing, and *E. faecium* isolated from blood cultures have increased nearly a 4 –fold, while the number of *E. faecalis* isolates have doubled (Figure 1). The success of *E. faecium* has been tributed to the success of hospital adapted lineages of this species (see later). In Norway enterococci are the 5th most common aetiological agent causing bacteraemia [45]. In parallel to the increase in enterococcal infections in Norway, an increase of high-level gentamicin resistance (HLGR) have been observed (Figure 2;[45, 46]. This seems to be part of an international trend occurring in both European, Asian and South American countries [47-53].

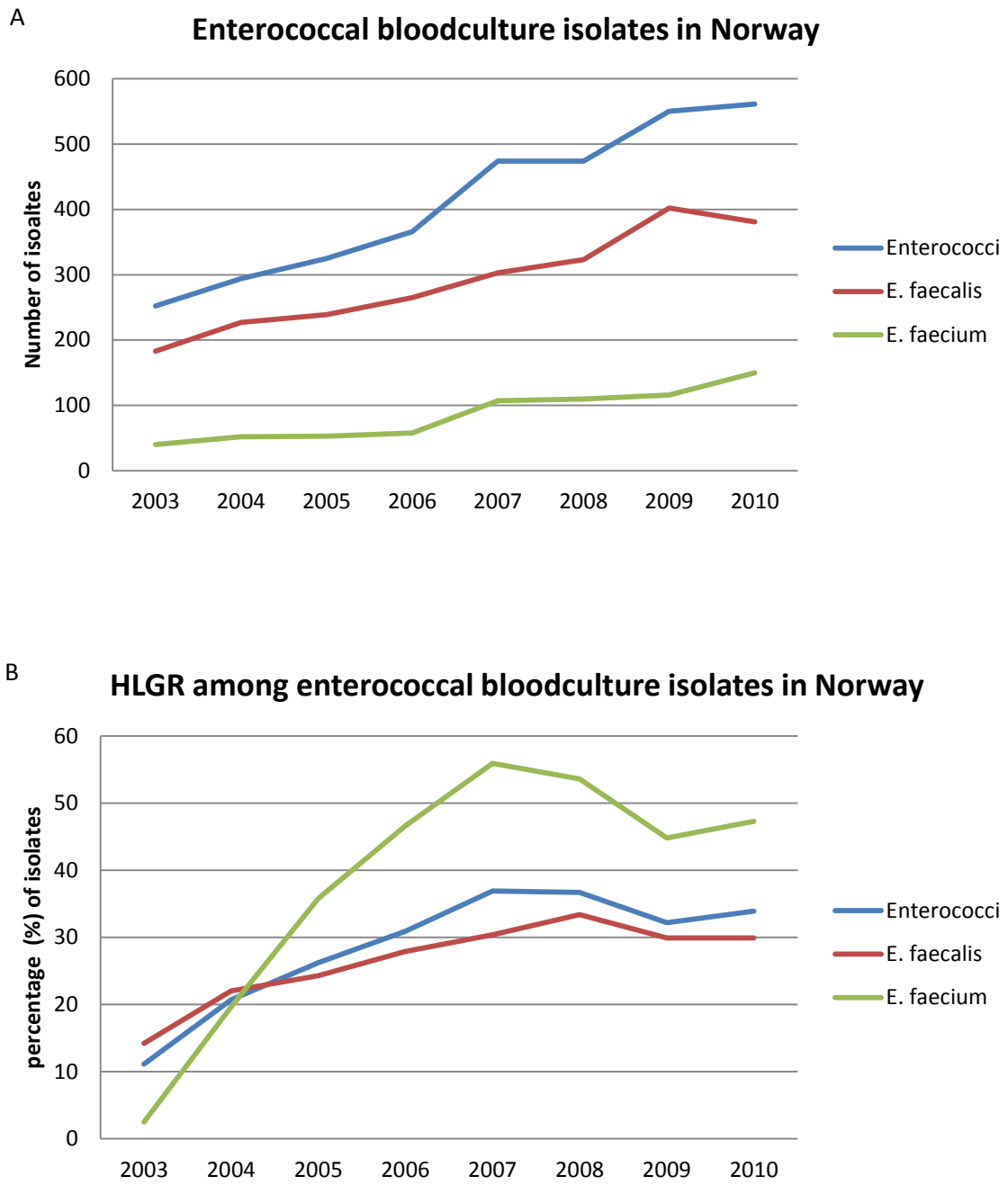

Figure 1: A) Number of blood culture isolates of *E. faecalis*, *E. faecium* and all enterococci combined in Norway during 2003-2010. B) Prevalence of high-level gentamicin resistance (HLGR) in blood culture isolates of *E. faecalis*, *E. faecium* and all enterococci combined in Norway during 2003-2010. Numbers collected from NORM (http://www.unn.no/rapporter/category10270.html)

1.1.2.3 Disease and treatment

Enterococci are considered opportunistic pathogens. As commensals of the human gut flora they do not normally cause infections in healthy people, with the exception of occasional urinary tract infections. However, enterococci have proven very competent in causing opportunistic infections in hospitalized patients, particularly in debilitated hosts [54-57]. Several studies have shown that exposure to antimicrobials facilitates changes in the intestinal microbiota, which promote colonisation by enterococci [58-62]. It has also been shown that increased density of colonizing enterococci in the intestine precedes bloodstream infections [62]. Other risk factors for colonization and subsequent infections with enterococci include admission to a critical care unit, co-morbidity, exposure to other patients with hospital adapted enterococci, long period of hospitalization, haemodialysis and solid organ and bone marrow transplantation [40, 63-67]. Most studies investigating risk factors focus on vancomycin resistant enterococci, but the crucial determinant giving enterococci the ability to colonize and infect a host is not only vancomycin resistance. Hence one could assume the risk factors for acquiring enterococcal infection should be somewhat similar between vancomycin resistant (VR) and vancomycin susceptible enterococci (VRE).

Enterococci can cause a variety of infections, most of them facilitated by indwelling devices or structural anatomic abnormalities. Urinary tract infections (UTI) are the most common enterococcal infection, and often associated with urinary catheters [68]. If not accompanied by bacteraemia, it generally only requires single-drug therapy, although seriously ill patients with pyelonephritis may benefit from combination therapy [68, 69]. Intra-abdominal and pelvic infections are often polymicrobial in origin. Although enterococci are detected in 20% of these [70], it is debatable to what extent they contribute to the infections [71]. However, these infections are common sources of bacteraemia [72, 73], hence antimicrobial therapy active against enterococci is regularly recommended [70]. Bacteraemia is not necessarily accompanied by an infection, but is none the less a bacterial invasion of the body. The source of the bacteraemia is often an infection or an indwelling device, but translocation of enterococci across intact intestinal epithelial cells may also lead to bacteraemia [72, 74]. The percentage of patients were endocarditis is the cause of enterococcal bacteraemia varies from about 1% to 32% in different studies [75]. Enterococci account for 5-20% of cases of endocarditis and are thus the 2nd -3rd most common cause of endocarditis. Enterococcal

meningitis is rare accounting for about 0.3% to 4% of meningitis cases [76, 77]. Severe enterococcal infection generally requires combination therapy [75, 78-80].

1.1.3.5 Antimicrobials used to treat enterococcal infections

Enterococci are traditionally treated with a combination of cell wall active antimicrobials such as β-lactams or glycopeptides, and aminoglycosides [80]. However, the increased rates of β-lactam and glycopeptide resistance in *E. faecium* and aminoglycoside resistance in both *E. faecium* and *E. faecalis* have called for the use of other and perhaps less efficient drugs.

Aminoglycoside antibiotics were one of the early discovered antibiotics and have been in use for over 60 years. They bind to the 30S ribosomal subunit, which plays a crucial role in providing high-fidelity translation of genetic material [81], rendering the ribosome unavailable for translation and thereby resulting in cell death [82]. Aminoglycosides have a broad antimicrobial spectrum covering a wide variety of aerobe Gram negatives and some Gram positives [83]. They display concentration-dependent bactericidal activity and is effective even when the bacterial inoculum is large [84]. The aminoglycosides are seldom drugs of first choice for monotherapy of infections, except for some cases of uncomplicated urinary tract infections [85]. Because of their synergism with cell wall synthesis inhibitors, they are recommended as part of an empirical combination therapy for severe infections such as septicaemia, nosocomial respiratory tract infections, complicated intra-abdominal infections and enterococcal endocarditis [80, 86-93]. Synergism presumably arises as the result of enhanced intracellular uptake of aminoglycosides caused by the increased permeability of bacteria after incubation with cell wall synthesis inhibitors such as β-lactams and glycopeptides [91, 94, 95]. Resistance rarely develops during the course of treatment [96, 97]. Gentamicin is the aminoglycoside most often used, because of its low cost and reliable activity against Gram negative aerobes [98]. The major limitations of aminoglycosides is a relatively low therapeutic index with both nephrotoxicity and ototoxicity, and that they are not absorbed orally due to their cationic nature and thus must be given parentally by either an intravenous or intramuscular route [96, 98].

Cell wall active antimicrobials such as β-lactams and glycopeptides act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls [99, 100]. Penicillins are

considered bacteriostatic against enterococci, and are the most widely used antimicrobials in the world [101]. Glycopeptides only work on Gram positive bacteria and is considered bacteriostatic against enterococci [3, 102]. In the last decade several antimicrobials with effect on enterococci have emerged. They all exhibit less than 100% clinical and microbiological success, usually around 70% [80, 103]. To improve their efficacy and reduce the development of resistance, it is preferable to employ them as part of a combination regimen [80, 103]. Linezolid inhibits protein synthesis and is active against all clinically important Gram positive bacteria, although it only displays a bacteriostatic effect [104, 105]. Daptomycin interferes with the cytoplasmic membrane causing depolarization and cessation of protein-, DNA and RNA-synthesis [106, 107]. It has concentration-dependent bactericidal activity against enterococci [108, 109]. Quinupristin-dalfopristin (Q/D) is a streptogramin antibiotic that is only active against *E. faecium*. It inhibits protein synthesis and is considered bacteriostatic against enterococci [110]. Tigecyclin is a broad-spectrum antibiotic that inhibits the protein synthesis. A recent review showed that it was more effective against enterococci than other Gram positive bacteria, but infections included were mostly skin and soft tissue infections and intra-abdominal infections [111, 112].

1.1.3 Antimicrobial resistance in enterococci

The discovery of antibiotics is considered one of the most significant health related events of modern times and antibiotic therapy is one of the cornerstones in modern medicine. Use and misuse of antimicrobials in human medicine and animal husbandry over the past 70 years have caused an unremitting selection pressure that has given rise to innumerable microorganisms resistant to these medicines. The use of antimicrobials are positively correlated to the emergence of resistant bacteria [113, 114]. Several bacteria in the hospital setting in many countries worldwide are now multiresistant [10, 14], leaving few treatment options. Hence, the development of antimicrobial resistance by bacteria constitutes a major threat to human health (http://www.who.int/drugresistance/en/).

1.1.3.1 Intrinsic resistance

Intrinsic resistance is a species characteristic, and thus present in all members of the species. Enterococci are resistant to most β-lactam antibiotics due to a penicillin-binding protein (PBP) that has a low affinity for beta-lactam agents [115, 116]. For ampicillin, ureidopenicillins, penicillin and imipenem the resistance is only low level. *E. faecium* generally display higher MICs than *E. faecalis* [5]. Enterococci display low level resistance to aminoglycosides (se later) and lincosamides [5]. *E. faecalis* also possesses an efflux pump conferring resistance to lincosamides and dalfopristin [117]. In addition, many wild-type enterococci possess endogenous efflux pumps that excrete chloramphenicol making them low level resistant [118]. Most enterococci are susceptible to co-trimoxazole *in vitro*, but this combination does not work *in vivo*, because enterococci are able to incorporate exogenous folic acid which enables them to bypass the inhibition of folate synthesis caused by co-trimoxazole [5].

1.1.3.2 Acquired resistance

A diversity of antimicrobial resistance genes have been demonstrated in the human gut microflora [119]. As inhabitants of the human intestinal flora, enterococci are in a position to acquire resistance genes from this community, thus making them notoriously difficult to treat and enabling them to transfer resistance genes to even more pathogenic bacteria, such as *vanA* to *S. aureus* [120, 121].

Aminoglycoside resistance

All enterococci and other facultative anaerobes have intrinsic low-level resistance to aminoglycosides because of impaired uptake (Figure 2) [122]. Minimal inhibitory concentrations (MICs) range from 4 μg/mL to as high as 256 μg/mL, and the MIC of gentamicin typically range from 6 to 48 μg/mL. In general these strains are assumed to be susceptible to ampicillin-gentamicin or vancomycin-gentamicin synergism, provided that they are not highly resistant to ampicillin or vancomycin [123]. In addition to the intrinsic resistance, all *E. faecium* strains produce a chromosomally encoded aminoglycoside acetyltransferace, AAC(6')-Ii, which eliminates synergism between cell wall-active antimicrobial and the aminoglycosides tobramycin, kanamycin, netilmicin and sisomicin

[124, 125]. It is also proposed that the EfmM metyltransferase, reducing susceptibility against kanamycin and tobramycin is encoded by all *E. faecium* [126].

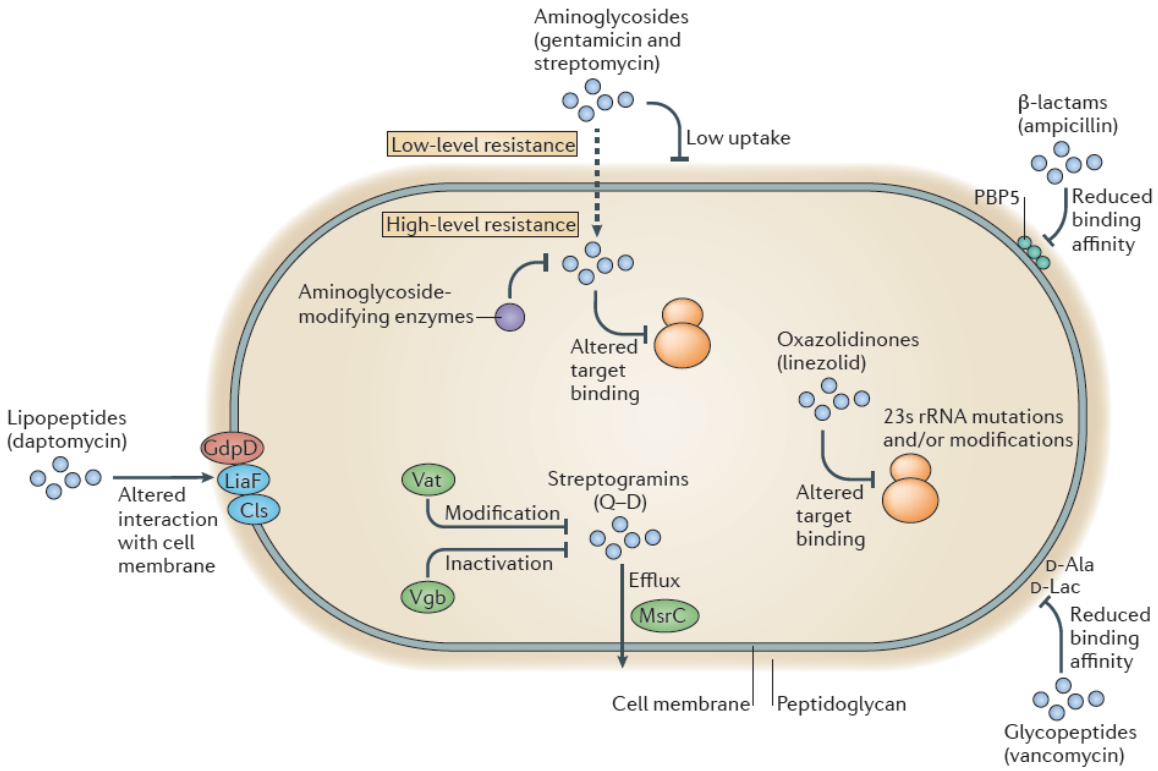

Figure 2: **Main mechanisms of antibiotic resistance in enterococci**: Resistance to ampicillin occurs through alterations of penicillin-binding protein 5 (PBP5), which leads to low affinity for β-lactams. Enterococci exhibit intrinsic low-level resistance to aminoglycosides such as streptomycin or gentamicin owing to low uptake of these highly polar molecules. High-level resistance results from the acquisition of aminoglycoside-modifying enzymes or, for streptomycin, can result from ribosomal mutations that result in altered target binding. Resistance to the glycopeptide vancomycin occurs through a well-characterized mechanism of reduced vancomycin-binding affinity, involving alterations in the peptidoglycan synthesis pathway. Resistance of Enterococcus spp. to the streptogramin quinupristin–dalfopristin (Q–D) involves several pathways, including drug modification (by virginiamycin acetyltransferase (Vat)), drug inactivation (through virginiamycin B lysase (Vgb)) and drug efflux (via the ATP-binding cassette protein macrolide–streptogramin resistance protein (MsrC)). Resistance to linezolid is rare, but the most common pathway involves mutation in the 23S ribosomal RNA ribosome-binding site. Resistance of *E. faecalis* to the lipopetide daptomycin has been shown to involve altered interactions with the cell membrane and requires the membrane protein LiaF and enzymes involved in phospholipid metabolism, such as a member of the glycerophosphoryl diester phosphodiesterase family (GdpD) and cardiolipin synthase (Cls).

The major mechanism of high level aminoglycoside resistance in clinical isolates of both Gram negative and Gram positive bacteria is enzymatic modification of the aminoglycosides (Figure 2). Three families of enzymes that perform co-factor dependent drug modification in the bacterial cytoplasm have been recognized: aminoglycoside phosphotransferases (APHs, the only one that produces high-level resistance), aminoglycoside acetyltransferases (AACs) and aminoglycoside nucleotidyltransferases (ANTs).

The bifunctional enzyme AAC (6')-Ie-APH (2")-Ia found in enterococcal, streptococcal and staphylococcal isolates renders them high level resistant (MIC >2000g/mL) to virtually all clinically available aminoglycoside antibiotics, except streptomycin and to some extent, arbekacin [9, 123, 127-129]. Genes encoding aminoglycoside-modifying enzymes are often located on plasmids, which permit cell-to-cell dissemination of the aminoglycoside resistance trait. The *aac(6')-Ie-aph(2")-Ia* gene is generally flanked by inverted repeats of IS256, making up composit transposons such as Tn*5281* in *E. faecalis* [130], Tn*4001* in *S. aureus* [131] and Tn*4031* in *Staphylococcus epidermidis* [132], which promote rapid dissemination at a molecular level [133].

Other types of resistance

Glycopetide resistance can be mediated by 9 different *van*-type gene clusters *(vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM* or *vanN*) [134-137] of which VanA- and VanB-type vancomycin resistance are considered the most clinically relevant. Their gene loci are most often located on transposons either on plasmids or on the chromosome [138, 139]. Vancomycin resistance is caused by replacing a glycopeptide-binding precursor in the peptidoglycan synthesis with a precursor that does not bind glycopeptides [138]. Hence, the cell wall synthesis will not be inhibited by vancomycin.

High level of resistance to β-lactams has most commonly been associated with point mutations in PBP5 (penicillin binding protein 5), that lowers the affinity for β-lactams [116]. In rare cases, the β-lactam resistance is due to production of a β-lactamase [140].

In addition, enterococci can acquire resistance to other antimicrobials such as chloramphenicol, tetracyclines, macrolides, lincosamides, streptogramins, and fluoroquinolones [5, 141] as well as for all the most recently introduces antibiotics such as linezolid and daptomycin [4, 142-145].

1.1.4 Population structure and hospital adaptation of enterococci

1.1.4.1 *E. faecium*

In the last two decades, *E. faecium* have evolved as a common nosocomial pathogen, increasing the total burden of enterococcal infections and partially replacing *E. faecalis* as a cause of HAI [11]. In the beginning of the millennium, genotypic population studies [146, 147] showed distinct genetic lineages spreading in the hospital, suggesting the existence of a specific subpopulation of *E. faecium* associated with hospital acquired infections, different from the community and animal population. Ampicillin resistance and *esp* (enterococcal surface protein- a putative virulence gene) were the early markers associated with this subpopulation [146, 148]. Later a pathogenicity island (PAI) containing *esp* [149], IS*16* [29, 150] and quinolone resistance was also linked to these strains [151, 152]. In addition, putative virulence genes [153, 154], and several surface proteins are enriched in this hospital associated subpopulation [155, 156]. A large genotypic study of population structure, typing over 400 isolates by Multi locus sequence typing (MLST) and analyzing it with eBURST, confirmed the existence of a subpopulation of *E. faecium* representing clinical and hospital outbreak strains [157]. It demonstrated genetic clustering of hospital associated strains, named clonal complex 17 (CC17) that was strongly associated with ampicillin resistance and the *esp* containing PAI. ST17 was presumed to be the founder of this clonal complex. A microarray-based comparative genomic hybridization of mixed whole genomes, hybridized against 97 isolates also supported the presence of a distinct phylogenetic group of hospital associated strains [29]. Many publications worldwide have acknowledged CC17 as by far the most prevalent genetic subcluster causing hospital acquired infections [51, 153, 158-160]. The seven major hospital associated STs (ST16, ST17, ST18, ST78, ST192, ST202 and ST203) accounts for 56% of the hospital associated isolates [161]. Later it has been reported that eBURST based clustering of MLST data to determine evolutionary decent is inaccurate in species with high levels of recombination such as *E. faecium* [162]. By using other approaches such as ClonalFrame [163] based phylogenetic trees, constructed from the concatenation of the seven MLST housekeeping genes [164], or a Bayesian modeling approach using BAPS software [161, 165], it has been showed that the CC17 subpopulation has not recently evolved from a single common ancestor; the hospital associated subpopulation is not clonal (ST17 is not the founder), but rather polyclonal. This polyclonal subpopulation constitutes several lineages that seem to have co-evolved into the clade now

commonly known as hospital associated *E. faecium*. A recent study that inferred phylogeny from 21 publically available *E. faecium* genomes by aligning 100 orthologs, showed a distinct separation of community-associated and hospital associated strains. They estimated the two lineages to have diverged over 300 000 years ago [33].

The hospital adapted subpopulation of *E. faecium* seems to have exploited a novel ecological niche- the hospital setting. They seem to be less fit when living outside the healthcare boundaries as the seven major hospital associated STs (ST16, ST17, ST18, ST78, ST192, ST202 and ST203) are only sporadically (41/513) found among non-hospital isolates [161]. This type of niche-exploitation often starts with adaptive changes [166]. Exactly which traits have given these strains the upper hand in the hospital setting is not known, but several properties have been suggested. Ampicillin resistance is one of the markers strongest associated with this subpopulation, thus it is suggested that the acquisition of ampicillin resistance was one of the vital traits enabling the strains to enter the hospitals and evolve into successful nosocomial pathogens [157]. This type of adaptive change may give rise to an amplifying selective process where isolates with the adaptive change (e.g. ampicillin resistance), more easily can acquire additional adaptive changes (e.g. changes in metabolism and other cellular processes) improving their relative fitness [167, 168]. The flexibility of the *E. faecium* genome is believed to significantly contribute to the hospital adaptation. Mobile genetic elements (MGE), particularly IS elements are believed to increase the genome plasticity and facilitate adaptive changes, thus enhancing the genetic variability in the hospital adapted strains [29, 32]. In the last years it has become apparent that megaplasmids are abundant among *E. faecium*, suggesting they play a role in the adaptation of *E. faecium* to particular hosts [49, 169-171]. Considering that megaplasmids had not been recognized among enterococci before the 1990s [172], and have been shown to play a role in both colonization, virulence and resistance in hospital associated *E. faecium* (se later) [173-175] they may have played an important role in the recent success of these strains.

1.1.4.2 *E. faecalis*

The available data indicates that *E. faecalis* has an epidemic population structure dominated by a limited number of genetic lineages with an overrepresentation of clonal complexes CC2, CC9, CC10, CC16, CC21, CC30,CC40 and CC87 [13, 176-178]. CC2, CC9 and CC87 are considered high risk CCs, as they are enriched in multidrug resistant isolates causing infections in hospitalized patients [13, 168, 176]. CC2 is a globally dispersed hospital associated lineage highly capable of causing

infections [13, 178-180]. Solheim et al. showed that over 250 genes were significantly enriched in CC2 isolates. Most of these genes have not been characterized, but some genes were shown to be located within mobile elements such as phage03, a putative integrative conjugative element and a *vanB* associated genomic island [179].
CC87 is particularly dominating in Poland [181], but are also found in other European countries as well as in the US [13, 176]. CC9 is spread globally, but high rates have especially been reported in Spain [176, 177, 182].

The seven most prevalent STs among clinical and outbreak-associated *E. faecalis* (ST6, ST9, ST16, ST21, ST28 ST40 and ST87), account for only 37% of the hospital associated isolates [161]. In contrast this is 56% for the seven most prevalent hospital associated *E. faecium* STs. Some *E. faecalis* STs (ST16, ST21, ST28 and ST40) are also found frequently in the community, including farm animals and food products isolates [13, 176], indicative of a reduced host specificity. It has been shown that near 60% of patients diagnosed with Vancomycin resistant (VR) *E. faecalis* bacteraemia in an US hospital, where infected prior to hospitalisation, and that bacteraemia caused by VR *E. faecalis* was significantly more likely to be present on admission than bacteraemia caused by VR *E. faecium* [183]. A recent study showed that CC21, CC16 and CC40 showed better *in vitro* fitness than those linked to nosocomial infections (CC2, CC9, CC87) [184]. This indicates that hospital associated CCs have acquired genetic elements, encoding specific traits (antibiotic resistance, virulence genes) making them successful in the hospital environment, but less fit in the environment. The most recent study on *E. faecalis* population structure of human isolates [13] showed that CC2 and CC87 were found exclusively in hospitals. It also showed that the six most commonly detected CCs (CC2, CC16, CC21, CC30, CC40 and CC87) accounted for 57% of the hospital isolates. Comparison of gene tree topologies of individual MLST genes indicates that recombination rates in *E. faecalis* are even higher than in *E. faecium* [185]. Hence, recombination seems to be the driving force in diversification and evolution of this species [168, 176, 177, 186]. Thus it is may be more accurate to consider CCs rather than STs as genetic lineages in *E. faecalis*.

1.2 Horizontal gene transfer in enterococci

Horizontal gene transfer (HGT) allows bacteria to rapidly acquire complex new traits, and it is a key driving force in bacterial evolution [187-189]. The ability to acquire mobile genetic element (MGE) encoding traits such as antibiotic resistance, has contributed to the emergence of enterococci, particularly *E. faecium* and *E. faecalis*, as leading hospital pathogens [29, 186]. Sequencing of the enterococcal genome have revealed a large accessory genome, especially in *E. faecium*; up to 38% [30]. The frequency and the diverse origin of mobile genetic elements seen in enterococci [28, 30, 32] suggests that the barriers to acquire foreign DNA is low. The clinical isolate *E. faecalis* V583 possesses over 600 kilobases more, than the non-clinical *E. faecalis* strain OG1RF, and most of this come from MGE [28, 31]. One of the proposed reasons for this is the lack of a complete CRISPR (clustered, regularly interspaced short palindromic repeats) element in V583. The CRISPR element is proposed to operate as a defence mechanism against invading DNA such as plasmids and phages [190, 191]. It has been shown in *E. faecalis* that there was a significant association between the absence of a complete CRISPR elements and the presence of antimicrobial resistance [192]. CRISPR elements seem to be rare in *E. faecium* [192, 193].

1.2.1 Mechanisms for HGT

There are generally 3 ways DNA can be transferred horizontally between bacterial cells, namely by conjugation, transformation or transduction.

Transduction is bacteriophage (bacterial virus) mediated transfer of host DNA from one bacteria to another. It does not require cell to cell contact [194, 195]. Sequencing of enterococci have shown integration of phage DNA in the enterococcal genome [28, 30, 32] and transduction have been shown to transfer antibiotic resistance both intra- and interspecies [196, 197], but the role of bacteriophages in the genome plasticity of enterococci needs to be further explored.

Transformation is the process where a cell takes up naked DNA from the extracellular environment. Bacteria that have the ability to undergo transformation is said to be competent, and this is not a trait occurring naturally in the enterococci [198]. In both transduction and transformation the DNA sequences are usually rescued by a RecA-dependent homologous recombination [199]. Hence transduction and transformation commonly result in DNA

replacement rather than addition, and requires DNA sequences that have significant homology.

Conjugation is a process where a conjugative element such as a transposon or a plasmid is transferred between bacteria via a contact dependent semiconservative replication process. Plasmid mediated conjugative transfer is by far the most common mechanism of horizontal gene transfer [200, 201]. Conjugation requires two sets of genes, the mobility (MOB) genes and the mating pair formation (MPF) genes, in addition it will need an origin of transfer (*oriT*) [202]. The MOB genes code for a relaxase and DNA processing proteins, responsible for the relaxosome, and for the coupling protein that links the relaxosome to the mating channel. MPF genes encode for the membrane–associated mating pair formation complex, a form of type 4 secretion system (T4SS) that provides the mating channel [202]. The initial step in the conjugation process is the mating pair formation, where the donor and recipient connect physically. The second step involves relaxase-mediated nicking of the plasmid at *oriT* and formation of the relaxosome (coupling of single-stranded DNA and a protein complex). The relaxosome docks to the coupling protein which helps mediate transport through the T4SS into the recipient cell, followed by establishment and replication of the plasmid in the recipient [203], or in case of a transposon, incorporation into the recipient chromosome [204]. Mobilizable plasmids lack the genes that encode the functions that enable cells to couple (MPF) prior to DNA transfer, but usually encode the MOB functions needed specifically for transfer of their own DNA. Transfer can happen if a conjugative element supplies the MPF genes in trans [202].The simplest mobilizable plasmids only contain the *oriT* and will need both MOB and MPF genes supplied in trans in order to be mobilized [205].

1.2.2 Host range of HGT

Enterococci are considered notorious regarding acquisition and transfer of resistance genes. They can acquire resistance determinants from several species [206-208] and even more disturbing, transfer resistance genes to other potentially pathogenic bacteria. Enterococci have been shown to transfer resistance genes to clinically important bacteria such a *Clostridium difficile*, *E. coli*, *S. aureus*, streptococci and *Listeria* spp. [121, 209-213]. In addition, the presence of the same gene or plasmid, in several genus and species further indicates that intergenic transfer is not uncommon. Tn*916*, first discovered in *E. faecalis* [214], have been detected in, or transferred to over 35 different bacterial genera[215-217]. According to the new plasmid classification system [218], several plasmid families (1- rep_{pIP501}, 7- rep_{pUSA02},

13-rep_{pC194}) are detected in both enterococci, staphylococci and streptococci, indicating transfer among these species.

It has been shown both *in vitro* and *in vivo*, that antimicrobials can enhance gene transfer between bacteria [219-221]. Due to the selective pressure antimicrobials exert on the environment, the dissemination of resistance genes is closely associated with the use of antimicrobials [222]. Several studies have shown that conjugative transfer in nature is much more frequent than under laboratory conditions, and that it readily occurs in the digestive tract [223-227]. Considering the abundance of both antimicrobials and microbes in a hospital setting the intestine of a patient treated with antimicrobials can thus be considered a perfect place for transfer of antimicrobial resistance determinants. Hence, the enterococci are placed right in a hotspot for genetic transfer.

1.2.3 Plasmids and other mobile genetic elements

Mobile genetic elements (MGEs) can generally be divided into two major types; those elements that can move from one bacterial cell to another, such as plasmids and conjugative transposons, and elements that can move from one genetic location to another in the same cell (transposable elements), such as IS elements, transposons and integron cassettes. The transposable elements may move on to a plasmids or conjugative transposon (Figure 3) and thus facilitate its movement between bacterial cells [228]. All these MGEs may carry genes that are beneficial to their host and improve their ability to survive, but in addition, the MGE itself offer a number of opportunities regarding genetic diversity. Transposon and integron cassettes require some form of recombination (such as homologous, illegitimate or site-specific) to transfer from one DNA site to another. Hence, if transferred to a plasmid, this may result in rearrangements of plasmid DNA and further increases the plasmid diversity [139, 228, 229]. Moreover transposable elements may alter the gene expression when they insert into a plasmid either by disrupting a coding region or by insertion into the promoter region causing either a disruption of the existing promoter or creating a more efficient promoter [139]. Often when a gene moves on to a plasmid, its copy number in the cell will rise above one unit per cell and thus the overall mutation rate for that gene will increase. In addition, many plasmids are self-transmissible or mobilizable, so when a gene moves on to a plasmid, it increases its chance of moving between bacteria. Relocating to a plasmid after HGT also

increases the chance of being established in a new bacterium, as there is no need to integrate into the bacterial chromosome and thereby risk interrupting an essential gene [230]. Thus MGEs contribute to both bacterial adaptability and diversity, and in this context plasmids are considered key players.

1.2.3.1 MGE

Recent multigenome analysis of *E. faecalis* and *E. faecium* showed that both organisms can efficiently acquire and integrate foreign DNA in their gene pool [28, 30, 185, 231]. Mobile genetic elements hence play a crucial role in the diversification of these species.
As mentioned, mobile genetic elements (Figure 3) other than plasmids and phages include transposable elements (IS elements, composite transposons and complex transposons), integron cassettes and genomic islands, and these elements may or may not be conjugative, depending on if they code for, or are part of an element that code for transfer [232].
Transposable elements can translocate to new sites in the genome without requiring extensive DNA homology. They encode a protein or protein complex called a transposase which mediates the transposition. In addition they usually have short inverted repeats (IR) sequences of both ends of the element that are binding sites for the transposase [232, 233].

Elements that only code for the functions needed for transposition is known as an insertion sequence (IS) element [234]. IS elements are widespread and over 2000 different ISs have been identified [235]. Transposition mechanisms for most IS elements are known to be conservative, which means that the element is cut out from the donor in a double stranded form and inserted as a whole into the recipient ("cut and paste"), leading to degradation of the donor DNA molecule. Replicative transposition is also known to occur, leaving both the donor and the recipient with a copy of the gene [234, 236]. IS elements are abundant in enterococci [28-30], and seem to play a particular role in hospital adaptation of *E. faecium*.

Transposable elements that codes for other genes in addition, e.g., antibiotic resistance, are called transposons. A transposon can be classified either as a composite transposon or a complex transposon [232]. When IS elements function in a pair to move the DNA segment caught between them, the element is called as composite transposon [232, 237]. The IS elements form either inverted repeats (for example Tn*5281/4001)* [130] or direct repeats (for

example Tn*4003*) [238], and the central gene(s) usually encode a function (such as antimicrobial resistance) giving the composite transposon an identifiable phenotype. Different composite transposons may also merge together to form a large composite transposon. Hence composite transposon can differ much in size such as Tn*9* only existing of 2,5 kb DNA [239] while Tn*5385* holds approximately 65 kb [240]. Transposition mechanisms for composite transposons generally follow that of their IS elements. Composite transposons in enterococci have mostly been associated with aminoglycoside resistance [130, 240-242], but some carry resistance to glycopeptides [243, 244].

Complex transposons [139, 232] have a more complicated structure than other transposable elements, but the name only implies that it is not a composite transposon or a transposing phage. Tn*3*-related transposons [245] are considered classical complex transposons. The genes that do not hold transposition functions are integrated into the body of the element, rather than being flanked by genes coding for transposition. Complex transposons vary in size and may carry several different genes in addition to those encoding the transposition, including composite transposons and integrons. If complex transposons carry genes enabling cell to cell transfer, it is described as a conjugative transposon or an integrative conjugative element (ICE) [204]. Mobilizable and conjugative complex transposons are widespread in enterococci, and they are often major contributors to antibiotic resistance in this genus [139]. Most important is the complex transposon Tn*1546* [246], largely responsible for the rapid emergence of vancomycin resistance in *E. faecium* [137].

Genomic islands are distinct units within the chromosome, with a different G+C content than the core genome. The difference in G+C content indicate a different evolutionary descent and thus these elements have most likely been acquired through horizontal gene transfer [247]. Genomic islands may encode genes for their own conjugative transfer [248]. A subset of genomic islands are the pathogenicity islands (PAIs) that encode virulence determinants (often in addition to other genes), and is present more frequently in pathogenic strains compared to less pathogenic strains (of the same or related species) [232, 247]. PAIs are found in both *E. faecium* [149] and *E. faecalis* [149], and have been shown to transfer both intra- and interspecies [249, 250].

Integrons are site-specific recombination systems that can capture small mobile genes called gene cassettes. These genes contain a specific site (*attC*) that can recombine with the integron,

and they often encode antimicrobial resistance. Several gene cassettes can be captured by one integron. To further facilitate their spread, integrons may be part of a transposon (Figure 3) [251]. Integrons are not well studied in enterococci, but a recent study found that integrons commonly detected in both Gram negative and Gram positive species, were present in enterococci [252]. This rather exciting finding needs to be confirmed.

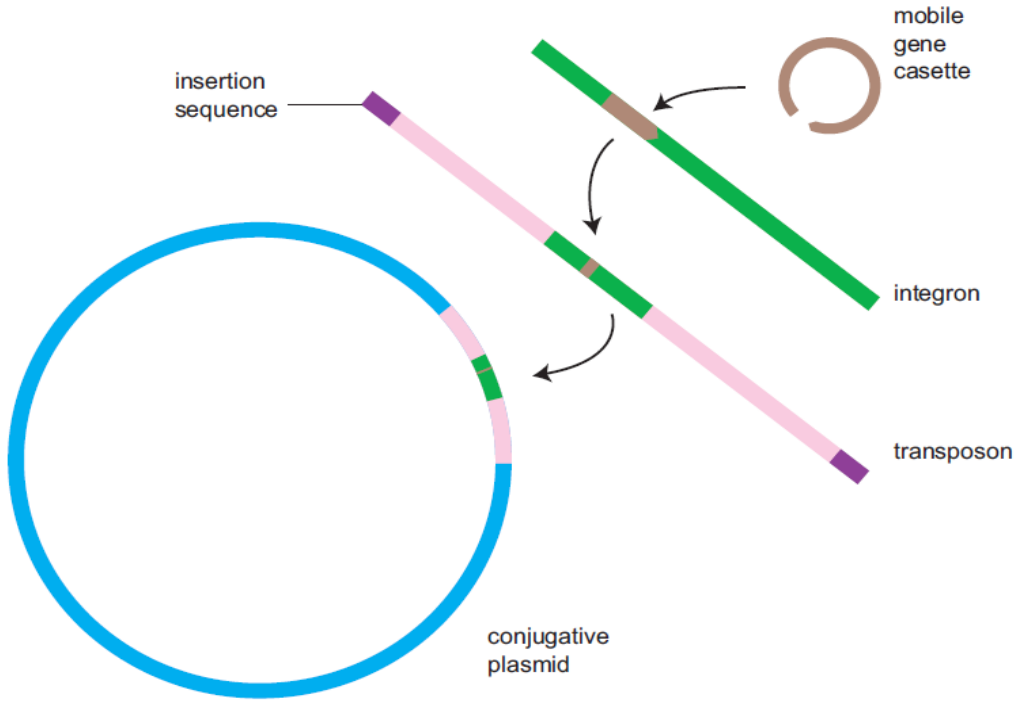

Figure 3. The modular and hierarchical composition of MGEs. Gene cassettes are inserted into integrons by integrase mediated site-specific recombination. Integrons may be flanked by IS elements of the same family making composite transposons, which in turn may be inserted into a dispersive element like a conjugative plasmid. The plasmid thus becomes a vessel for the transportation of other MGEs. Reprinted by permission from Royal society publishing. Philos Trans R Soc Lond B Biol Sci. 2009 Aug 12;364(1527):2275-89. Conjugative plasmids: vessels of the communal gene pool. Norman A, Hansen LH, Sørensen SJ.

1.2.3.2 Plasmids

Plasmids represent a vast reservoir of genetic variability that are shared among many bacterial species, and they exhibit a rich diversity of form, function and utility [200]. They are defined as extrachromosomal genetic elements that replicate independently of the bacterial chromosome [253], although the majority of replication functions are provided by the host

cell [254]. Typically plasmids are circular molecules of double stranded DNA, although some bacteria have linear plasmids [255].

Plasmids was first described in 1952 by Lederberg, who used the term to describe extrachromosomal hereditary determinants [256]. They have been reported in a wide range of host, including prokaryotes, archaea and eukaryotic fungi [257, 258]. Plasmids do not encode essential cellular functions in a non-selective environment, but may confer a selective advantage under certain conditions, such as enzymes for the utilization of unusual carbon sources [259, 260], resistance to substances such as heavy metals [261] and antibiotics [228, 262], and synthesis of toxins and other proteins that allow the successful infections of higher organisms [263]. These non-essential genes are often referred to as adaptive or accessory genes.

Plasmids generally replicate by one of three different mechanisms, the theta mechanism, the strand displacement replication or the rolling-circle replication [264].
Plasmids depend on having an origin of DNA synthesis of its own (*oriV*). In addition, most plasmids encode for specific replication initiator proteins (*rep*) that binds to this *oriV* [254, 264]. To ensure their stable maintenance in the host cell plasmids employ a variety of mechanisms; If it is not a high-copy number plasmid (over 5-10 copies) it will need an active partitioning (*par*) mechanism to direct better than random segregation and thus secure stable plasmid inheritance over many generations [265]. Another problem plasmids need to solve is dimerization due to the tendency of identical gene copies to recombine. To prevent any subsequent impediment of segregation of the plasmid into the daughter cells, plasmids have developed multimer resolution systems (*mrs*) to resolve dimerization [266]. Further securing their stable inheritance, many plasmids have acquired toxin-antitoxin (TA) systems (se later), that will kill or impair growth of bacteria that do not inherit the plasmid [266]. In addition, if plasmids encode functions for mobilization or conjugative transfer they further increase their chances for survival. These different genes or gene loci are often organized into functional modules and clustered together with relatively few interruptions from insertions of accessory genes (Figure 4). Together these modules constitute the plasmid backbone [267]. It seems that being clustered together reduces the chance of any gene being disrupted by an insertion of any kind. Accessory genes are frequently acquired at about the same place in the plasmid, avoiding backbone genes. This suggests that events disrupting backbone functions may lead to reduced fitness of the plasmid and eventually such plasmids will be lost [200, 268].

Clustering of the backbone modules seems to be favoured by evolution: optimizing the reproductive capability and increasing the efficiency of spread, minimizing the burden on the host and maximizing the benefit are major driving forces. The organization of the plasmid maintenance genes will therefore continue to improve until the refinement potential is exhausted [268].

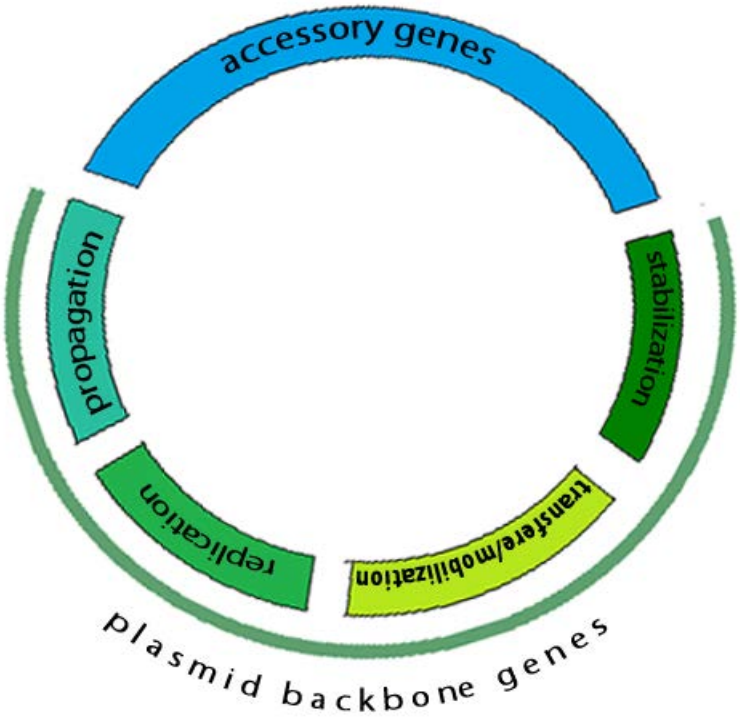

Figure 3: Plasmid modularity. Schematic view of plasmid modularity

1.2.3.3 Toxin Antitoxin systems

One way for the plasmid to increase its chances of stable inheritance is by harbouring a functional toxin antitoxin (TA) system that kills or impair growth of cells who fail to receive the plasmid [269]. TA systems consist as the name imply of a toxin and an antitoxin. The toxin in its free form attacks cellular targets, causing reduced growth or cell death, but it is inhibited by binding of the antitoxin. As long as the plasmid is present, both the toxin and the antitoxin will be expressed, rendering the toxin inactive. If the gene pair is lost (e.g. loss of the encoding plasmid), the more unstable antitoxin will be degraded before the toxin, permitting the toxin to attack its target. Four TA loci, *axe-txe, ω-ε-ζ, mazEF* and *relB* have

been detected in enterococci [270-273], but only *ω-ε-ζ* and *axe-txe* have been shown to promote plasmid stabilization in enterococci [270, 271, 274-276].

1.2.3.4 Plasmid typing

The ability to detect and group plasmids based on their phylogenetic relationship could give valuable information regarding their distribution in nature, their relationship with their host cell, and in the case of resistance -their role in dissemination of antimicrobial resistance. In a more clinical context the plasmid content in bacteria can be used as an additional marker (together with other characteristics such as MLST, resistance genes and virulence genes) for comparative analysis of strains during epidemiological investigations.

When plasmids first were discovered, there were no methods for physical detection of plasmids and the presence of plasmids were known by the phenotypes they conferred in the bacteria. Consequently many plasmids were named after the genes they carried or the phenotype they conferred, like the hemolysin-bacteriocin plasmid and the resistance plasmids described in *E. faecalis* [277], or the ColE1 plasmid in *Escherichia coli* [278] that encode the protein colicin E1. For a period during the sixties plasmid typing was based on the ability to inhibit F-fertility, but a desire to use more fundamental plasmid properties for classification impelled a scheme based on replication and partitioning systems, namely the incompatibility (Inc) grouping of plasmids [279-282]. Plasmid incompatibility relies on the fact that plasmids who have closely related replication control and/or partitioning systems are unable to be stably inherited in the same host in the absence of external selection [281]. Inc typing works by introducing a plasmid, by conjugation or transformation, into a strain carrying another plasmid. If the second plasmid destabilized the inheritance of the first, the two are said to be incompatible and are designated the same Inc group. There are a number of difficulties in creating pairwise combinations of plasmids from bacteria. For instance it is difficult to select for a plasmid with no suitable marker gene, or distinguish entry exclusion from incompatibility. Minor genetic divergences between closely related plasmid may be enough to weaken the incompatibility reaction and result in interpretation difficulties. In addition, multiple replicons may cause misleading conclusions and point mutations may change the plasmids incompatibility behaviour [283]. As a response to these concerns a classification scheme in Gram negatives based on replicon typing vas developed by Couturiere et al. [284].

This scheme is based on Southern blot hybridization with specific DNA probes that contain the genes involved in plasmid maintenance used on purified plasmid DNA. The classification of replicon groups could mostly be correlated with Inc groups. Both these methods are quite time consuming and not very suitable for typing plasmids in a large bacterial population. In recent years polymerase chain reaction (PCR) based replicon typing have been developed for both Gram positive and Gram negative bacteria [218, 285, 286] enabling large scale plasmid typing [171, 272, 287]. This typing system relies on the homology of genes dealing with replication. Due to the modularity of the functions for replication, propagation, stability and mobilization/transfer, replicon typing systems may not be congruent with the Inc typing or typing systems based on other survival functions such as mobilization or conjugation [288, 289].

1.2.3.5 Rep typing system in enterococci

The PCR based plasmid typing system for Gram positives developed by Jensen and co-workers is based on homology of conserved areas of the replication initiation genes (*rep*). An alignment of 111 published plasmid sequences derived from 100 Gram positive bacteria and two Gram negatives was used to define replicon families. A plasmid family was defined if two or more distinct sequences (alleles) from two or more plasmids clustered together, with a cut-off value above 80% for both DNA sequences and proteins. All together 19 plasmid families and 19 unique sequences were defined [218], and 12 of these plasmid families have been identified in *E. faecium* and *E. faecalis* (Table 1). Recently a megaplasmid name pLG1, with a novel replication initiation gene was fully sequenced [290], thus adding another *rep* gene to the rep typing system.

1.2.3.6 Plasmids in the enterococci

Plasmids are abundant in enterococci [171, 272], and seem to play a role in hospital adaptation, at least in *E. faecium* [170, 171]. As described above, there are many ways of grouping plasmids (replication mechanisms, Inc typing, transferability, resistance gene carriage, etc.).Traditionally enterococcal plasmids have been classified into 3 groups by a mixture of these methods; the Inc 18 group of plasmids (rep group 1, represented by pIP501 and partially rep family 2, represented by pRE25), the rolling circle replication (RCR)

plasmids (mostly rep family 4 and 6, represented by pMBB1 and pS86, respectively) and the pheromone responsive plasmids of *E. faecalis* (rep family 8 and 9, represented by pAM373 and pCF10) [172]. Mixing different plasmid classification methods when grouping plasmids is not very clarifying. Although the traditional grouping of enterococcal plasmids is relatively congruent with the new PCR replicon typing system, it misses some new and important plasmids such as pRUM (rep family 17) [270], pHTβ (unique rep family) [291] and megaplasmid pLG1[290]. In addition to these classifications, a plasmid family named RepA_N has been proposed [292]. RepA_N include rep family 8 and 9 as well as rep family 17 and the pLG1 plasmid, hence it is not congruent with previous classifications of enterococcal plasmids.

In recent years it has become apparent that large plasmids (>100kb), also known as megaplasmids are widespread in the *E. faecium* population [49, 170, 171, 293]. They seem to influence the ability of *E. faecium* to cause infections by both enhancing the colonization capacity [173], and increase virulence [174, 175] [175]. In addition megaplasmids seem to both carry and disseminate antimicrobial resistance genes in an effective manner [174, 290] [170, 171, 272]. Recently it was shown that megaplasmids of *E. faecium* can harbor alternative carbon utilization mechanisms, though this trait was only found in isolates colonizing humans rather than causing infections [260]. We recently revealed that all megaplasmids in blood culture isolates from Norway in 2008 harbored the pLG1 *rep* gene [171], thus suggesting a particular megaplasmid is spreading in the *E. faecium* population. Interestingly, megaplasmids have not been described in enterococci as late as around 2000 [172].

Table 1. Overview of plasmid families detected in enterococci

Replicon family	Other known plasmids in the family	Information about the rep group
1 (pIP501)	pAMβ1, pBT233, pGB354, pIP680, pIP816, pMD101, pRE25, pSM19035, pTEF1	Belong to the Inc18 group of plasmids, broadly distributed among low G+C Gram positive bacteria such as enterococci, streptococci and staphylococci [172]. Transferable to Gram negative species [210]. Shown to confer vancomycin resistance transfer within and between species [272, 293-295].
2 (pRE25)	pEF1, pIP816, pVEF1, pVEF2	Enterococcal plasmids of both animal, healthy human and clinical origin. pRE25 is a multi-resistance plasmid [262, 296], while pIP816, pVEF1 and pVEF2 has been shown to confer vancomycin resistance [297, 298]. Commonly found in both *E. faecalis* and *E. faecium* [171, 272, 299, 300]. Assumed narrow host range [218], except for pIP816 and pRE25 that have a broad host range due to its additional rep_{pIP501} gene [262, 298].
4 (pMBB1)	pCRL291.1, pKC5b	Small cryptic plasmids found in both enterococci and lactobacilli. Broad host range [218].
6 (pS86)	p703/5, pAMα1, pEF47, pEFC1, pLCR255	Small theta replicating plasmids found in both enterococci and streptococci [218, 301, 302]. Broad host range [218].
8 (pAM373)	pEJ97-1	Pheromone-responsive plasmids found in *E. faecalis*. Confer antibiotic resistance and virulence traits such as aggregation substance [303]. Narrow host range [218, 292].
9 (pCF10)	pAD1, pPD1, pTEF1, pTEF2	Pheromone-responsive plasmids found in *E. faecalis*. Confer antibiotic resistance and virulence traits such as Aggregation substance [28, 304, 305]. pTEF1 and pTEF2 have been shown to promote chromosomal diversification in *E. faecalis* [250]. Narrow host range [218, 292].
11 (pEF1071)	pB82, pEFR	Plasmids from enterococci. Commonly found in *E. faecium* [171, 299]. Assumed narrow host range [218].
13 (pC194)	pSK89, pSSP1, pWBG1773	Small plasmids generally found in staphylococci. pC194 confers chloramphenicol resistance [306]. Broad host range [218, 307].
14 (pRI)	pEFNP1, pKQ10, Aus004_p2, Aus004_p3	Small mobilizable plasmids commonly found in *E. faecium* [171, 308]. Assumed narrow host range [218].

17 (pRUM)	Aus004_p1	Plasmids commonly found in *E. faecium*, [30, 218, 270, 272, 299]. Known to harbour the axe-txe TA system [270, 272]. Assumed narrow host range [218, 292].
18 (pEF418)	p200B, pEF415,	Plasmids from enterococci. Commonly found in Chinese VR *E. faecium* [299]. Assumed narrow host range [218].
U[4] **(**pMG1)	pHTβ	Plasmids found in *E. faecium* [171, 272, 291]. pHTβ has been shown to confer vancomycin resistance [291], while pMG1 like plasmids have been shown to carry gentamicin resistance determinants [309]. Unknown host range.
U (pLG1)		*Plasmids that are ubiquitous in *E. faecium* [49, 170, 171, 293]. Known to confer several antimicrobial resistance determinants such as vancomycin [290, 293], and HLGR [171]. Shown to encode determinants that enhance both colonization abilities and virulence in murine peritonitis [173, 174]. Assumed narrow host range [292].

* Here rep_{pPLG1} represent megaplasmids. The rep type has not always been investigated in megaplasmids, but later I will argue (se discussion) that megaplasmids generally belong to the rep_{pLG1} family.

2. Aims of study

The overall aim of this study was to investigate the prevalence and distribution of plasmids in hospital adapted enterococci, and to determine their contribution in the dissemination of antimicrobial resistance determinants.

Paper I

Plasmids play an important role in the spread of antibiotic resistance, but modest knowledge is currently available about enterococcal plasmids. We wanted to investigate the overall plasmid prevalence and distribution in an international collection of *E. faecium* strains using a newly developed *rep*-typing system in combination with a traditional S1 nuclease method and determine the genetic linkage of specific rep-types to vancomycin resistance and plasmid stabilising toxin-antitoxin systems.

Paper II

During 2003 to 2008 high level gentamicin resistance (HLGR) increased dramatically among blood culture isolates of *E. faecium* in Norway. The aim of this study was to determine the molecular mechanism(s) involved in the increased prevalence of HLGR in Norwegian invasive *E. faecium*, and to examine both population structure and plasmid epidemiology in order to elucidate their role in HLGR dissemination.

Paper III

An increase of HLGR was also reported among blood culture isolates of *E. faecalis* in Norway during 2003 to 2008. The aim of this study was to determine the molecular mechanism(s) involved in the increased prevalence of HLGR in Norwegian invasive *E. faecalis*, and to elucidate the population structure. In addition we wanted to determine if *E. faecalis* and *E. faecium* share a common gene-pool for HLGR determinants, and to what extent plasmids contributed in the spread of HLGR within the *E. faecalis* population as well as between *E. faecalis* and *E. faecium*.

3. Summary of main results

Paper I

- The study revealed that plasmids are common in *E. faecium*, with an average of 2,3 plasmids per isolate, and 0-4 replicon genes detected in the isolates.

- The average number of plasmids were significantly higher in isolates belonging to Clonal Complex (CC)17 (now more commonly named "hospital associated isolates) than non-CC17 isolates.

- The most common replicon types detected were pRE25 (60%), pRUM (40%), pIP501 (18%), and pHTβ (15%). Of these pIP501 and pHTβ were almost exclusively present in hospital associated isolates.

- Toxin- antitoxin loci were found in 61% of the isolates. *axe-txe* (n=42) were more frequently detected than ω-ε-ζ (n=18).

- Co-hybridization analyses showed that axe-txe was commonly linked to the pRUM replicon type, and ω-ε-ζ commonly linked to pRE25 replicon type.

- We most commonly detected co-hybridization between *van* and replicon type pIP501, but co-hybridization was also detected for pRE25, pRUM and pHTβ replicon type. In addition we detected co-hybridization of *vanA* to 150 kb plasmids of unidentified replicon types.

Paper II

- MLST revealed a polyclonal strain collection and detected 26 STs. The most common STs detected; 203 (*n*=28), ST17 (*n*=18), ST18 (*n*=10), ST202 (*n*=8) and ST192 (*n*=7) are typical hospital associated STs.

- High level gentamicin resistance (HLGR) was observed in 57% of the isolates, and most prevalent in ST203 (20/28), ST17 (15/18), ST202 (7/8) and ST192 (6/7).

- The 99 isolates harboured none to six plasmids ranging in size from <10 kb to >400 kb with an average of 3,1 plasmid per isolate.

- The most prevalent replicon types detected were rep_{pLG1} (90%), rep_{pRE25} (73%), rep_{pRUM} (66%) and rep_{pRI1} (62%), with an average of 3,2 *rep* genes per isolate.

- Nearly all HLGR-isolates (98%) were positive for the *aac(6')-Ie-aph(2'')-Ia* gene, and it was plasmid located in all but one isolate. All *aac(6')-Ie-aph(2'')-Ia* positive plasmids co-hybridized to rep_{pLG1}.

- The prevalence of ω-ε-ζ and *axe-txe* was 65 and 66 % respectively. 76% of the isolates had one or both TA loci. 57% of rep_{pLG1}hybridizing plasmids co-hybridized with *axe-txe.*

- Isolates belonging to major hospital associated STs had a significantly higher prevalence of plasmids, rep genes, putative virulence genes and TA-systems, compared to the remaining isolates.

- All donors were able to transfer the HLGR determinant into *E. faecium* (BM4105-RF and/or 64/3) with transfer frequencies ranging from 4×10^{-2} to 6×10^{-7} TC per recipient cell.

Paper III

- The HLGR population was dominated by ST6 (CC2) (21/30), and CC2 and CC87 accounted for 90% (27/30) of the HLGR isolates. PFGE showed that these genetic lineages were diverse and not consistent with any outbreaks. The non-HLGR isolates were mostly unrelated.

- All HLGR-isolates were positive for the *aac(6')-Ie-aph(2")-Ia* gene, and hybridization analyses revealed that it was present on the chromosome in all except one isolate.

- In *E. faecalis*, IS*256* flanked the *aac(6')-Ie-aph(2")-Ia* gene on both sides, while it was missing on the left side in *E. faecium*.

- The prevalence of the tested replicon types was higher in the HLGR isolates compared to the non-HLGR isolates: rep_{pCF10} (93%vs. 21%), rep_{pRE25} (50% vs. 29%),) and rep_{pMBB1} (47% vs. 14%), except for rep_{pS86} (13% vs. 21%), rep_{pAM373} (0 vs. 14%), and rep_{pRUM} (0 vs. 7%), respectively. The pCF10 replicon type was present in all ST6 isolates and in 78% of the remaining HLGR isolates.

- The ω-ε-ζ toxin- antitoxin system was present in 50% of all isolates.

- The HLGR determinant was transferable between *E. faecalis*, as well as between *E. faecalis* and *E. faecium*. Transfer of the HLGR determinant from both chromosome to chromosome, chromosome to plasmid, plasmid to chromosome and plasmid to plasmid were detected.

4. General discussion

Enterococci are known for their notorious capacity to acquire and disseminate resistance genes [3, 4]. They are a frequent cause of hospital acquired infections, and often multidrug resistant [10, 14]. In addition enterococci can transfer resistance genes to other and more pathogenic bacteria, such as transfer of vancomycin resistance to MRSA [120]. It is acknowledged that plasmids contribute significantly to the spread of resistance genes in enterococci. However, not much is known about which plasmid types confer resistance, and even less is known about their occurrence and distribution in enterococcal populations. In our studies we have examined the molecular epidemiology of enterococcal plasmids within defined enterococcal populations. Moreover, we have looked into their contribution in acquisition and dissemination of antimicrobial resistance.

4.1 The plasmid classification system

Plasmids play a significant role in the biology of enterococci. They represent an immense reservoir of genetic variability and contribute to genetic exchange between bacteria [200]. The ability to detect and classify plasmids based on their phylogenetic relationship would provide an essential tool for investigating their distribution among bacteria and to elucidate their significance in the host cell, such as their role in dissemination of antimicrobial resistance. A simple method for plasmid detection would be a very useful tool to trace resistance plasmids in a clinical setting, such as the hospital, for epidemiological surveillance. Hence, the new PCR based plasmid classification system for Gram positive bacteria, targeting replication initiation genes, can be of great value in the detection and identification of enterococcal plasmids [218]. The classification system is based on DNA and protein homology of replication initiation genes, and cut-off is sat at 80% identity for both. Although the new classification system has significant advantages, it also has some limitations; underling that it should be used in combination with other methods:

i) The classification system is not directly based on incompatibility; plasmids from the same replicon family may be compatible with each other and coexist in the same bacterium, but only one replicon is detectable by PCR. Thus, if we detect one rep type by PCR and see multiple

bands by S1 nuclease/PFGE typing, it can lead to misinterpreting plasmids as "unknown rep type". Hybridization assays may then result in multiple hybridizing bands, and it will not be known if the results are based on plasmid dimerization, or actually two different plasmids. If only PCR's are performed, it will lead to the under estimation of number of plasmids in the bacterium. ii) Available sequence information suggest that some plasmids, such as pRE25 [262] may have several *rep* genes, which makes it difficult to group the plasmid into a specific family. It may also lead to over estimating of the number of plasmids. S1nuclease/PFGE results can be difficult to interpret as there may be several reasons, in addition to multiple *rep* genes in one plasmid, for detecting fewer plasmids with S1 nuclease assays compared to rep typing. The plasmid could be very small and have run out of the gel, the plasmid could be very large and is still in the well, or the plasmid could be fragmentised due to physical sharing or unspecific S1 cutting and no clear band would be visible. Hybridization assays will usually clarify this problem. iii) When using the original primers and control strains (as defined by Jensen et al) to make probes, another problem may arise. When the classification system was developed, the number of plasmids analysed in each rep family was limited and primer sequences were in general targeting the supposed most conserved region. Newly detected plasmids that were not analysed in the classification scheme may in theory be detected by the PCR primers but differ so much in the region between the primers that the similarity threshold is below hybridization stringency. This can generally be solved by using different plasmids within a *rep* family as probe template, or to use the PCR positive, hybridization negative isolate as probe template. Lowering the hybridization stringency may also solve the problem. iv) This plasmid classification system is only based on plasmids with sequenced replication initiation genes. Hence, plasmids with a new *rep* gene will remain undetected by this system. This was the case in paper I, were the *rep* gene of pLG1 was not included yet. It resulted in a higher plasmid count by S1 nuclease/PFGE, than by rep typing. v) Another limitation of this classification system is due to the modularity of plasmids; two plasmids may in theory only share the *rep* gene, and still be classified as the same plasmid type, even if the rest of the gene content differs.

Nonetheless, being aware of these weaknesses and taking them into account when performing plasmid typing in combination with other methods such as S1nuclease/PFGE and hybridization assays – this plasmid classification system have proven very useful in both detecting plasmids and dedicating different traits to plasmid types. It has been of great value in plasmid

epidemiology surveys (paper I-III). The rep typing system could only account for 60% of the plasmids detected by the S1nuclease/PFGE assay (paper I). In paper II we supplemented with the *rep* gene from the newly sequenced plasmid pLG1, and this resulted in a very good agreement between the number of plasmids detected by S1 nuclease/PFGE, and number of *rep* genes detected. As both methods may over or underestimate the number of plasmids, we can assume the total number is generally correct when using both methods. Hence, we anticipate that the enterococcal plasmid classification system based on rep-typing now detects the major plasmid families, at least in *E. faecium*. However, the overall agreement in plasmid numbering between rep-typing and S1 nuclease/PFGE results, as shown in paper II, needs to be further explored in other *E. faecium* populations.

4.2 Plasmids in enterococci

The plasmid population in enterococci has not been extensively studied, and their contribution to important phenotypic traits such as the ability to colonize the host, enhance the virulence of bacteria and acquire and disseminate antimicrobial resistance is still poorly elucidated. In all three papers we aimed to investigate the plasmid distribution within *E. faecium* (paper I and II) and *E. faecalis* (paper III) populations, and elucidate to what extent the different plasmid types were linked to defined antimicrobial resistance determinants. We detected a high prevalence of plasmids, particularly in *E. faecium*, and several replicon types (up to 5), were identified in the same bacterium (paper I-III). The detectable plasmids varied greatly in size, from less than 10 kb to larger than 400 kb (paper I-III). Size diversity was also seen within each plasmid group, but this variation was less than in the total plasmid population. rep_{pRE25} -type plasmids varied between 25 and 80 kb, while rep_{pRUM} –type plasmids were below 100 kb. rep_{pLG1} -type plasmids were generally above 150 kb (paper I and II). The size variation within each plasmid group may reflect the flexibility of the plasmid scaffold, were genes easily can move in and out. Occasionally we detected a rep_{pRE25} or rep_{pRUM} -type plasmid above 100 kb, but then co-hybridization to another rep type was also seen, suggesting plasmid co-integrates (paper II-unpublished results).

It seems that each species has a distinct plasmid population that is not commonly shared between them (paper I-III), the exception being *rep*$_{pRE25}$-type plasmids that are commonly found in both species (paper I-III), and the broad host range *rep*$_{pIP501}$-type plasmids (Inc18 family) that are found in a variety of Gram-positive bacteria [172]. *E. faecium* and *E. faecalis* seem to have species specific plasmid groups (paper II and III). These plasmid groups seem to play a significant role in both genetic variability and genetic exchange between bacteria. In E. *faecalis* the pheromone sensitive plasmids (like pCF10, pAD1 and pAM373) transfer between *E. faecalis* at high rates [310, 311]. We disclosed that *rep*$_{pCF10}$ –type plasmids was involved in dissemination of both plasmid and chromosomal resistance determinants (paper III). This is in agreement with previous studies, which have shown that pheromone sensitive plasmids often confer antimicrobial resistance [303, 305, 312] and may play an important role in chromosomal recombination and diversification [250]. In *E. faecium*, megaplasmids have recently been brought to our attention as they may confer resistance to several antimicrobials (paper I) [290, 293], and have been commonly detected in *E. faecium* strains from both environmental, animal and human clinical and community samples worldwide (paper I and II) [49, 170, 290, 293, 313]. Megaplasmids have been shown to increase both the ability for gastrointestinal colonization and virulence in murine peritonitis [173, 174] as well as to confer alternative carbon utilization pathways [260] and resistance to copper and mercury [313]. These observations support the notion that megaplasmids may contribute significantly to the adaptability of *E. faecium*. Megaplasmids differ in size between bacteria, from about 150 kb to larger than 400 kb (paper II), and we have observed that they frequently alter size when being transferred into a new strain (unpublished results and paper II, data not shown). This suggests that megaplasmids provide significant genetic flexibility and that genetic elements readily move in and out of the plasmid. Due to the omnipresence of megaplasmids they probably provide *E. faecium* with increased adaptability in many different environments, not only in the hospital setting. Megaplasmids are a new term in enterococci. They have only been reported in isolates from 1995 and onwards in several recent publications [49, 170, 290, 293, 313] paper I. This may be a result of limitations in plasmid detection methods in previous studies, or that megaplasmids may be a more recent phenomenon resulting from “old” plasmid types gaining larger genetic elements, and maybe new plasmid backbone combinations making the plasmid particularly flexible. Another possibility is that it is a recently introduced rep type. Only a few studies have investigated the rep type of these

megaplasmids, but those who have, always found them to be *rep*$_{pLG1}$ [293, 313]. Re-examination of isolates from paper I showed that 85% of isolates with visible megaplasmids were rep$_{pLG1}$ PCR positive (unpublished results). In paper II, *rep*$_{pLG1}$ –type megaplasmids were present in over 90% of the isolates, and were the sole distributor of HLGR; further supporting the clinical significance of megaplasmids in *E. faecium*. Our results (unpublished results and paper II) combined with other studies [293, 313] and (Hegstad, personal communication), suggest that most *E. faecium* megaplasmids belong to the *rep*$_{pLG1}$ family.

Based on known *rep* genes, the general number of plasmids seems to be higher in *E. faecium* compared to *E. faecalis*. This might be caused by several compatible plasmids with the same rep type, but this seems less likely as our hybridization data (paper III) generally indicate one plasmid band per *rep* probe. Plasmids of unknown replicon type, not detected by PCR, may also be one explanation, though S1 nuclease/PFGE assays (paper I-III) suggest that there is a real difference in plasmid numbers between *E. faecium* and *E. faecalis*. An explanation for the relative lower plasmid number in *E. faecalis* may be the prominent role pheromone responsive plasmids play in genetic shuffling within the *E. faecalis* population, and hence a decreased need for other plasmids.

Resistance plasmids in enterococci

Epidemiological studies targeting the contribution of specific plasmid types in the dissemination of resistance genes in enterococci are limited, with a partial exception of pheromone responsive plasmids. Plasmid mediated dissemination of vancomycin resistance is perhaps the best studied antimicrobial resistance in enterococci [7, 272, 291, 293, 294, 297, 298, 314, 315]. In paper I we showed that plasmids with *rep*$_{pIP501}$, was the most common carrier of vancomycin resistance. Other plasmid types known to confer vancomycin resistance is pHTβ (paper I) [316] (paper I) , *rep*$_{pRE5}$- [293, 315] (paper I), *rep*$_{pRUM}$- [317] (paper I) and *rep*$_{pLG1}$-type [290, 293]. Plasmids of *rep*$_{pIP501}$ type are known to have a broad host range, and have been linked to the spread of *vanA* to *S. aureus* [294]. To what extent other plasmid types contribute to propagation of resistance determinants to other genus is largely unknown, although *rep*$_{pRUM}$, and *rep*$_{pLG1}$ are believed to have a narrow host range [290, 292].

High level gentamicin resistance (HLGR) determinants have mostly been detected on plasmids in enterococci [318-321], but the plasmids were not further characterized except for size. Hence, it is not known if there are particular plasmid types that confer gentamicin resistance. HLGR determinants have though been detected on pMG1 like plasmids [309], but the significance of this plasmid type in HLGR dissemination is unknown. In Norway (paper II) rep_{pLG1} type plasmids are major contributors to the spread of HLGR *in E. faecium*, but it is yet to be elucidated if this is a widespread trend. Interestingly, in paper III we were able to transfer the HLGR encoding gene residing on rep_{pLG1} –type plasmids in *E. faecium* to the chromosome of *E. faecalis* (OG1RF and JH2-2) No plasmids were detected in the *E. faecalis* TCs, suggesting that the plasmid is transferable to, but not maintained in *E. faecalis*. This indicates that rep_{pLG1} –type plasmids are not able to replicate in *E. faecalis* and support a narrow host range of rep_{pLG1} –type plasmids, consistent with their relatedness to the RepA_N family [292]. In paper II we were not able to document transfer of the HLGR determinant from *E. faecium* to *E. faecalis*. We did however observe growth on the TC plates, but none of the assumed TC's harboured the HLGR encoding gene (50 colonies for each mating were screened by PCR). Recently, we disclosed that the plates used in that transfer study most likely contained below 100 µg/ml gentamicin (EUCAST clinical breakpoint for HLGR is 128 µl/ml). Since enterococci generally are low level resistant to gentamicin, recipients could probably grow on the TC plates without the acquisition of HLGR. So even if transfer did happen, the TCs would be outnumbered by the recipients, and therefore could not be detected.

In two *E. faecalis* isolates the HLGR determinant resided on rep_{pCF10} –type plasmids (paper III), and observations in paper III indicate that rep_{pCF10} –type plasmids were involved in transfer of the chromosomal HLGR determinant. Moreover it was observed in over 90% of the HLGR encoding isolates compared to only 20% in the non-HLGR isolates. This suggests that this plasmid type contribute in the spread of HLGR, but its role in HLGR dissemination in *E. faecalis* needs to be further examined.

Toxin antitoxin systems

Plasmid carriage generally represents a fitness cost to the host bacterium, with a reduction in growth rate measured *in vitro* to be 1-6% [267, 322]. To prevent being lost in a non-selective environment, plasmids apply several maintenance strategies to overcome the fitness cost, such as

minimizing size, tight gene regulation and low copy number [323-326]. Another useful trait to promote plasmid stabilization is toxin-antitoxin (TA) systems [269]. In enterococci both *relB*, *mazEF*, *ω-ε-ζ* and *axe-txe* TA systems have been detected (paper I-III) [270, 273], but only *ω-ε-ζ* and *axe-txe* have been shown to promote plasmid stabilization in enterococci [270, 271, 274-276]. We have shown that TA-systems are commonly detected in enterococci, and associated with some of the most prevalent enterococcal plasmid types such as rep_{pIP501}, rep_{pRE25}, rep_{pRUM}, and rep_{pLG1} (paper I-III). A prevalent co-hybridization of *ω-ε-ζ* and rep_{pRE25} was detected in isolates from both Europe and the US (paper I and II). In paper I, *axe-txe* was localized to plasmids of rep_{pRUM} –type in the majority of *axe-txe* positive isolates investigated. Interestingly, in paper II we detected a prevalent co-hybridization of *axe-txe* and rep_{pLG1} –type megaplasmids. rep_{pRUM} and rep_{pLG1} both belong to the repA_N family [292], and the prevalent linkage of *axe-txe* to this family may suggests that the *axe-txe* - repA_N replicon linkage is particularly stable and beneficial.

4.3 High level gentamicin resistance

The prevalence of HLGR among enterococci has increased across the world [47-52], and has in general been associated with the *aac(6')-Ie-aph(2")-Ia* gene [318, 319, 321, 327-329]. Our results are in line with this, as we detected the *aac(6')-Ie-aph(2")-Ia* gene in 56/57 HLGR *E. faecium* and 30/30 *E. faecalis* isolates (paper II and II). In *E. faecium* we located the gene to rep_{pLG1} -type megaplasmids (paper II). The *aac(6')-Ie-aph(2")-Ia* gene has commonly been detected on plasmids [319, 321, 327, 328], but to my knowledge it has not been reported on plasmids above 100 kb. In *E. faecalis* the *aac(6')-Ie-aph(2")-Ia* gene was located on the chromosome (paper III). This has previously only been sporadically reported [321, 327], and more studies are needed to elucidate if this is a novel widespread trend in *E. faecalis*. Interestingly 70% of the *E. faecalis* HLGR isolates tested belonged to clonal complex 2 (CC2). The routes of propagation (clonal dissemination of bacteria or dissemination of plasmids or transposons) that are most common for HLGR dissemination in enterococci have mostly been unknown. Some studies have suggested plasmids of particular sizes (rep type was not investigated) as the mediator [318-321], but the potential contribution of clonal dissemination was not very well investigated in these studies.

The *aac(6')-Ie-aph(2")-Ia* gene is commonly flanked by inverted repeats of IS*256*, known as Tn*5281* in enterococci. Different flanking patterns have been detected and this pattern may be used to infer recent transfer of Tn*5281* between bacteria. Several studies have investigated the IS*256* flanking pattern of Tn*5281*. They showed great variation in the patterns both within and between studies [318, 319, 321, 328-331]. No common trend was seen in *E. faecium*, while IS*256* flanking both sides was slightly the most common detected pattern in *E. faecalis*. A few studies have investigated the flanking patterns in both *E. faecalis* and *E. faecium* isolates collected from single hospitals in Japan, Thailand and Tunisia, respectively [329-331]. None of the studies reported a consistent flanking pattern within one of the species, suggesting several propagation routes for the HLGR determinant, even within the same hospital. These observations are in contrasts to our findings in paper II and III where we detected one common flanking pattern for *E. faecalis* and another common flanking pattern for *E. faecium*. In *E. faecalis*, the *aac(6')-Ie-aph(2")-Ia* gene was flanked by IS*256* on both sides, in all but one isolate, while in *E. faecium* the left side IS*256* was missing in all isolates.

In *E. faecium* the HLGR determinant resided on rep_{pLG1} type plasmids (paper II). Together with a high transfer frequency of the HLGR determinant (paper II) and detection of a common IS*256* flanking pattern (paper III), this suggest that HLGR dissemination in *E. faecium* is promoted by rep_{pLG1} –type plasmids. In *E. faecalis* the HLGR dissemination was at least partly linked to the spread of CC2. *E. faecalis* transfer studies revealed a highly mobile HLGR determinant, which imply that propagation of the HLGR determinant itself may also be a cause of HLGR dissemination in *E. faecalis* (paper III). What the HLGR determinant includes has not been further elucidated. Tn*5281* have been shown to be part of larger composite elements such as Tn*5384*, Tn*5385* and Tn*924* [240-242]. PCR revealed that a Tn*5281* –like element is present in our isolates, but further analyses were not performed (paper III). We cannot exclude the possibility of a conjugative HLGR determinant, but our results indicate that plasmids are involved in the dissemination in both *E. faecalis* and *E. faecium* (paper III).

Further examination of the flanking elements of chromosomal Tn5281-elements need to be performed. Tn*5281* have been shown to be part of larger composite elements such as Tn*5384*,

Tn*5385* and Tn*924* [240-242]. PCR revealed that a Tn*5281*-like element was present in Norwegian *E. faecalis* isolates, but further analyses were not performed (paper III).

Our results suggest that Norwegian clinical isolates of *E. faecium* and *E. faecalis* do not readily share a common pool of Tn5281 elements (paper II and III). Whole genome sequencing of *E. faecium* and *E. faecalis* strains has shown that their genetic content is very different [28, 30, 32, 34, 161]. Even though interspecies transfer of genetic determinants have been shown in several studies [249, 332-336], the species barrier is maintained, suggesting that stable transfer events between the two species is not a very prevalent *in vivo*.

4.4 Population structure in *E. faecium* and *E. faecalis*

The general population structure in *E. faecium* is well investigated, and several recent publications have discussed the somewhat surprising subpopulation of hospital adapted *E. faecium* [33, 157, 161, 164, 168, 185]. According to a study by Galloway-Peña and co-workers, this subpopulation separated from the rest of the population over 300 000 years ago [33]. This was rather unexpected as the hospital adapted *E. faecium* population is a fairly new phenomenon [337]. This subpopulation is polyclonal and spans significant genetic diversity. Even so, hospital adapted *E. faecium* are less diverse and more closely related than the community associates strains [33]. They also share many properties making them more or less distinguishable from the community associated population [29, 146, 148-154]. Although it is a polyclonal population, it is generally dominated by a few genetic lineages [161]. Genotyping of the Norwegian blood culture population is in agreement with these findings (paper II). We found bacterial isolates comprising a diversity of STs. However, those isolates enriched in plasmids, resistance and virulence genes generally belonged to only a few STs, while more susceptible isolates, harboring fewer plasmids and putative virulence genes, belonged to a variety of STs.

Thus it appears that a few genetic lineages are thriving in the hospital, and seem to have acquired several traits making them more fit in that environment. One of the assumed reasons for this is a concept known as “genetic capitalism” or “the Mathew effect”; the rich tend to become richer [167, 168]. This means that by acquiring useful genetic traits, the bacterium will increase its

adaptability to the hospital environment and further increase its ability to acquire new genetic determinants. This was seen in our isolates (paper I and II). The ST's comprising the highest number of isolates (and thus could be considered more successful) harbored more plasmids, resistance genes, putative virulence genes and TA systems (paper II). We also observed a positive correlation between number of plasmids and number of putative virulence genes in all isolate (paper II, data not shown), further supporting "the richer tend to become richer" hypothesis. It is believed that mobile genetic elements (MGEs), particularly IS elements, have provided the hospital associated lineages with the genetic flexibility needed to adapt to the hospital environment [28, 29, 32]. The prominent position of rep_{pLG1} –type megaplasmids in the entire *E. faecium* population give reason to hypothesize that this plasmid provides further access to accessory DNA enhancing their adaptability. Whole plasmid sequence analyses of an extended representative number of pLG1-plasmids and linked functional genomics analysis are necessary to approach this hypothesis.

The population structure of *E. faecalis* is not as well characterized as for *E. faecium*, but several recent studies have shed some light on the subject [13, 34, 176, 178, 180-182, 186, 293]. Previous studies have showed a diverse *E. faecalis* population, dominated by particular genetic lineages [176]. It has been shown that the most common genetic lineages found in the hospital setting are also prevalent in the environment, indicating that the *E. faecalis* population is not very host specific [161, 178]. However, a recent large European study on contemporary human *E. faecalis* isolates revealed that the most commonly detected CCs in the hospitals accounted for nearly 60% of the infections and that several of these lineages were almost exclusively detected in the hospital [13]. These results indicate a more specialized population of *E. faecalis* thriving in the hospitals. Our results further support this notion, as the majority of isolates belonged to the most commonly detected CCs (paper III). The population structure seen in our study, where 6 CCs comprises over 90% of the HLGR isolates, is in contrasts to the genetic diversity observed in previous studies [13, 176, 178]. This epidemic population structure can result from some unknown Norwegian conditions that have caused a selective pressure promoting certain clones. Another possibility is that a particularly hospital adapted *E. faecalis* genetic lineage is emerging in the hospitals, outcompeting less specialized lineages. One could hypothesize that genetic capitalism is a driving force, also in this population as we observed a higher prevalence of

plasmids in ST6 isolates compared to other isolates, in addition to all ST6 isolates being HLGR positive. This enrichment of antimicrobial resistance in ST6/CC2 isolates has been reported in several publications [13, 178, 186, 338] and Solheim and co-workers found over 250 genes enriched in CC2 isolates [179]. A recent study further support hospital adaptation of isolates from specific CCs, as they showed a general *in vitro* fitness reduction in isolates belonging to CCs typically linked to hospital acquired infections (including CC2), compared to isolates of CCs frequently found in the community [184]. This suggests that isolates belonging to hospital linked CCs harbor traits particularly useful in the hospitals environment that evens out the reduced fitness.

5. Concluding remarks

Little research has been done regarding plasmid epidemiology in enterococci. Hence little is known about the significance of different plasmid types in conferring and disseminating antimicrobial resistance.

We have shown that plasmids are abundant in enterococci, particularly in *E. faecium*, and unravelled the most commonly detected plasmids groups in enterococci, including rep_{pPLG1} megaplasmids in *E. faecium*. We have also revealed plasmids that commonly confer vancomycin resistance and high level gentamicin resistance (HLGR), and demonstrated their transferability. We have linked plasmid stabilizing toxin-antitoxin systems to particular plasmid types, including those conferring antimicrobial resistance. In addition we have investigated the HLGR determinants in Norwegian invasive *E. faecium* and *E. faecalis* and disclosed a plasmid and chromosomal location respectively. Finally, we have elucidated the population structure of Norwegian invasive *E. faecium* and *E. faecalis,* revealing a polyclonal *E. faecium* population and a more epidemic *E. faecalis* population with a particular dominance of CC2.

In summary, these results have provided increased knowledge about the plasmid population in enterococci. Further studies of enterococcal plasmids epidemiology is needed to confirm if our findings are widespread. It is also important to monitor the plasmid population in enterococci to better understand changing trends in plasmid epidemiology, and to possibly intervene in the spread of antimicrobial resistance.

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Paper I

Paper II

Paper III