UNIVERSITY OF TROMSØ UIT

FACULTY OF HEALTH SCIENCES
DEPARTMENT OF MEDICAL BIOLOGY

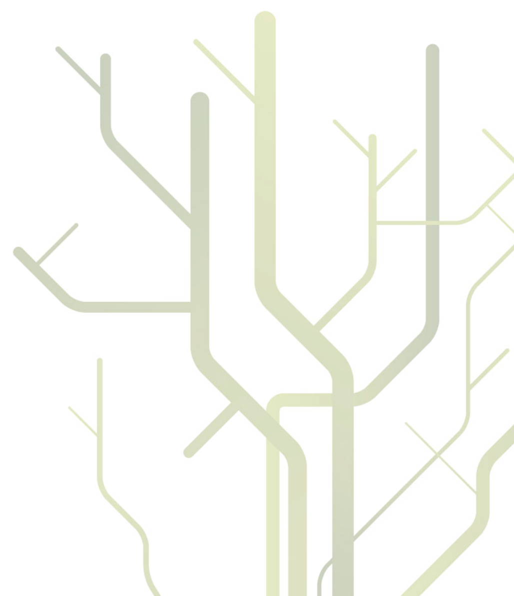
On Mobile Genetic Elements in Enterococci;

Adding More Facets to the Complexity

Eva K. Bjørkeng

A dissertation for the degree of
Philosophiae Doctor

July 2010



On Mobile Genetic Elements in Enterococci;

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By

Eva K. Bjørkeng

A thesis submitted according to the requirements for the degree of Philosophiae Doctor

Research group for Host-Microbe interactions
Department of Medical Biology
University of Tromsø

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The history of antibiotics

- *2000 B.C. – Here, eat this root*
- *1000 A.D. – That root is heathen. Here, say this prayer.*
- *1850 A.D. – That prayer is superstition. Here, drink this potion.*
- *1920 A.D. – That potion is snake oil. Here, swallow this pill.*
- *1945 A.D. – That pill is ineffective. Here, take this penicillin.*
- *1955 A.D. – Oops....bugs mutated. Here, take this tetracycline.*
- *1960-1999 – 39 more "oops"...Here, take this more powerful antibiotic.*
- *2000 A.D. – The bugs have won! Here, eat this root.*

— Anonymous

October 2010

Eva Bjørkeng

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Presented papers

Paper I

Eva Bjørkeng, Gunlög Rasmussen, Arnfinn Sundsfjord, Lennart Sjöberg, Kristin Hegstad, and Bo Söderquist. Clustering of polyclonal VanB-type vancomycin resistant *Enterococcus faecium* in a low-endemic area was associated with CC17-genogroup strains harbouring transferable *vanB2*-Tn*5382* containing pRUM-like plasmids with *axe-txe* plasmid addiction systems. ***Submitted***

Paper II

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Paper III

Bjørkeng E. K., Hjerde E., Lundblad E. W., Sollid J. E., Sundsfjord A., and Hegstad K. Sequence analyses of an approximately 100-kb chromosomal element supporting transfer of *vanB2*-Tn*5382*/Tn*1549* from *Streptococcus lutetiensis* to *Enterococcus faecalis* and *E. faecium*. ***In manuscript.***

Abbreviations

ccr	chromosomal cassette recombinase
GAS	Group A *Streptococcus*
GBS	Group B *Streptococcus*
GCS	Group C *Streptococcus*
GEI	Genomic Island
GGS	Group G *Streptococcus*
GI	gastrointestinal
GRE	Glycopeptide Resistant Enterococci
HGT	Horizontal Gene Transfer
ICE	Integrative Conjugative Elements
IS	Insertion Sequence
MGE	Mobile Genetic Element
MIC	Minimum Inhibitory Concentration
MLST	Multi Locus Sequence Typing
MRSA	Methicillin Resistant *Staphylococcus aureus*
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
SCC	Staphylococcal Cassette Chromosome
ST	Sequence Type
TA	Toxin-Antitoxin
VRE	Vancomycin Resistant Enterococci
VRSA	Vancomycin Resistant *Staphylococcus aureus*

1. Introduction

1.1 *Enterococcus*

1.1.1 Taxonomy:

Enterococci are Gram positive bacteria occurring as single cocci or in chains. They are lactic acid producers and many also produce bacteriocin. Enterococci are non-spore forming, facultative anaerobic organisms, catalase negative and have a remarkable ability to withstand extreme environments which includes high pH, temperature and salt concentrations (75,164). In addition they are relatively resistant to chemical disinfectants such as chlorine, gluteraldehyde and alcohol. These qualities are important for survival and spread in the hospital environment (28,80,127).

In 1899 Thiercelin first described the name "entérocoque" referring to its intestinal origin and its spherical shape (214). However the enterococci were until 1984 subgrouped into different streptococcus groups. The name was revised when *Streptococcus faecalis* and *Streptococcus faecium* was transferred from the genus *Streptococcus* to *Enterococcus* due to new molecular information (121,132). The enterococci share phylogenetic relationship with the streptococci. Both genera are also related to the *Lactococcus sp* (103). The phylogenetic relationship between enterococci is showed in Figure 1.

According to List of Prokaryotic names with Standing in Nomenclature (LPSN) there are at least 40 species included in the *Enterococcus* genus (http://www.bacterio.cict.fr/e/enterococcus.html. 2010.25.02). The two most clinical relevant species are the *Enterococcus faecium* and *Enterococcus faecalis* which cause the major part of human enterococcal infections (228).

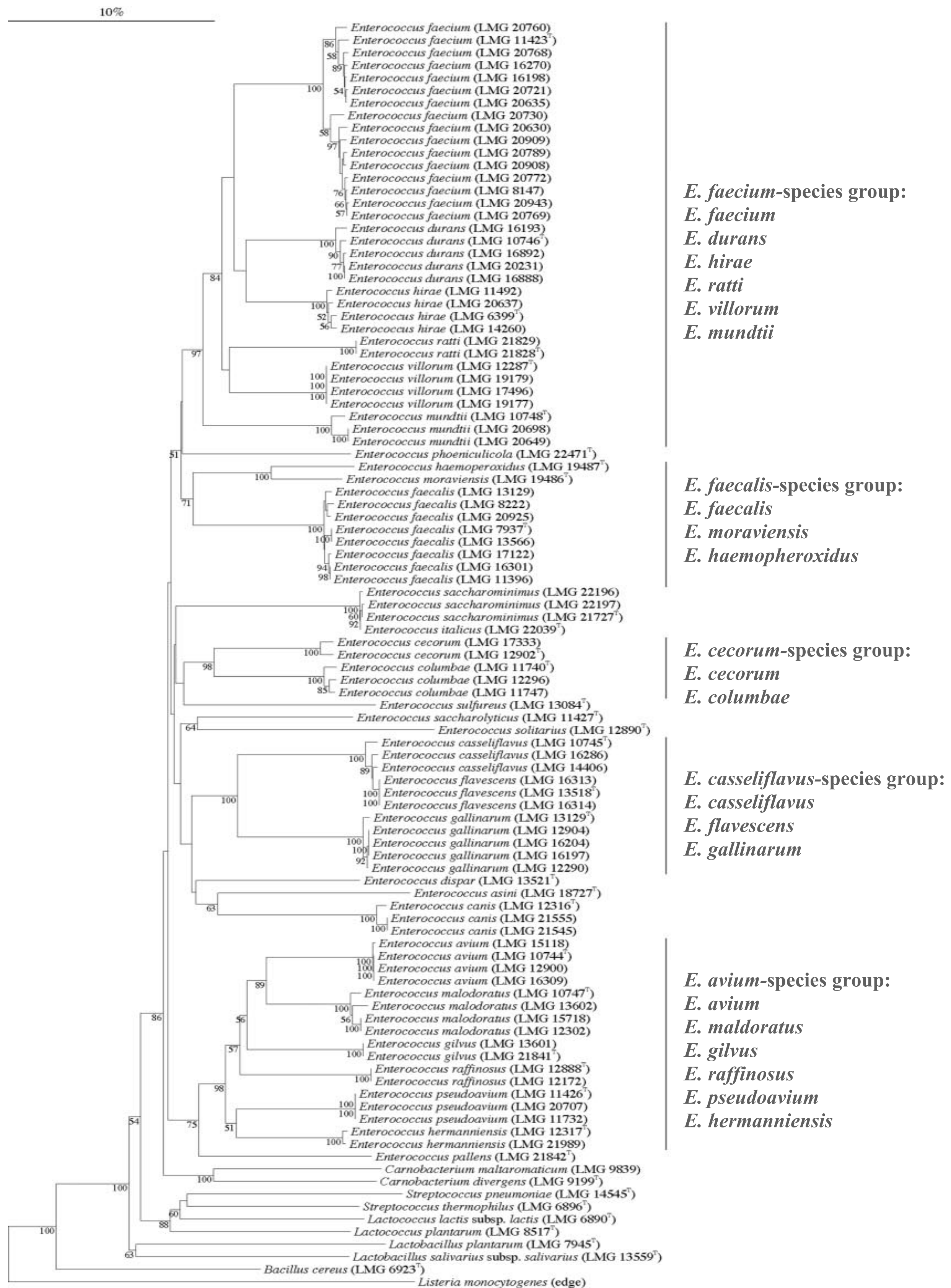

Figure 1. Phylogenetic tree showing the relationships among *atpA* sequences from different enterococcal strains. Bootstrap values after 1,000 repetitions are indicated. Reprinted from (172) with permission from the publisher.

1.1.2 Habitat

Enterococci are part of the normal intestinal flora in birds, humans, and animals. They can also colonize the oral cavity and vagina in humans (131). In addition these bacteria are found in the soil, on plants, surface water, and other environments exposed to human or animal faeces (75,136). In the last two decades enterococci have gained increased awareness as important nosocomial pathogens. The main enterococcal infections include urinary tract infections, infections in the intra-abdominal cavity, and endocarditis (24).

1.1.3 Clinical significance

The majority of clinical enterococcal infections are, as mentioned, caused by *E. faecalis* and *E. faecium*. *E. faecalis* is considered more virulent however, *E. faecium* is more likely to be resistant to antibiotics, even those of last resort (241). Twenty years ago only 10% of the nosocomial enterococcal infections was caused by *E. faecium* (241). Now around 40% of the enterococcal nosocomial infections worldwide are caused by *E. faecium* (145,228). This ratio started to change in favour of *E. faecium* in the US during the late 1990s and in Europe around the year 2000 (130,162,218,219). In the last two decades the emergence of enterococci as an important nosocomial pathogen has been increasingly documented (140,188). The relative proportions of *E. faecalis* and *E. faecium* in Norwegian blood culture isolates in 2008 was 4% and 1.4% respectively (NORM 2009. http://www.unn.no/getfile.php/UNN-Internett /Fagfolk/www.antibiotikaresistens.no/NORM-09/NORM%20NORM-VET%202008.pdf).

The pathogenesis of enterococcal infections is only partly understood. However, several adhesins, hemolysin, hyaluronidase, aggregation substances, gelatinase, and genes encoding pili are now considered possible virulence factors (75,79,108,110,199). So far, at least 22 different genes, collectively called *fms* (*E. faecium* surface protein-encoding genes) are considered putative virulence factors in *E. faecium* (202). Virulence factors encoded by acm_{fm} (*fms8*), *hyl*, esp_{fm}, *sgrA* and *ecbA*, are most strongly associated with clinical lineages in *E. faecium* (23,45,81,110,171,204).

Putative *E. faecium* virulence genes and products, their epidemiology and potential role in pathogenesis are listed in Table 1. Resistance in enterococci has increasingly become a problem. This issue will be discussed in section 1.3.

Table 1. Overview of putative virulence genes in *E. faecium*, their virulence and epidemiology

Virulence gene	Pathophysiology/Virulence	Epidemiology	References
Esp	Biofilm formation Pathogenesis of rat endocarditis Pathogenesis of mouse urinary tract infections Antigenic in humans during endocarditis and bacteremia	Specifically linked to hospital-associated *Enterococcus faecium* (*Efm*) on pathogenicity island	(107) (143) General Meeting of the American Society of Microbiology, Posters B-167 and B-211 (141) (139)
Acm (Fms8)	MSCRAMM[1] Binding to collagen type I, IV Pathogenesis of rat endocarditis Antigenic in humans during endocarditis	Widespread among hospital related *Efm*	(171) (170) (169)
Pili PilA (orf1904/fms21) PilB (orf2569/ebpC$_{fm}$)	MSCRAMM Predicted to be involved in pilus biogenesis.	Widespread among clinical isolates of *Efm*	(109) (129)
Scm (orf418/fms10)	MSCRAMM Binding to collagen type V and to a lesser extent binding to collagen type I and fibrinogen.	Widespread among *Efm*	(203)
EcbA (orf2430)	MSCRAMM Binding to collagen type V Binding to fibrinogen	Specifically linked to HA[3] *Efm*	(108) (110)
Hyl	Not known (putative glycoside hydrolase) Promotes colonization of mouse gastrointestinal tract	Specifically linked to hospital-associated *Efm*	(190) (225) (187) (82)
Orf903 (fms11)	[2]CWAP/MSCRAMM	High incidence in clinical strains	(108) (204)
Orf2010 (fms14)	CWAP/MSCRAMM	High incidence in clinical strains	(204) (108)
efaAfm	Cell wall adhesisn	High incidence in clinical strains	(67)
SgrA (orf2351)	MSCRAMM Binding to nidogen and fibrinogen. May play a role in biofilm formation	High incidence in clinical strains	(110)

[1]MSCRAMM – microbial surface component recognizing adhesive matrix molecules

[2]CWAP - cell wall-anchored proteins

[3]HA – hospital adapted. Table modified from (199) with permission from the publisher

1.1.4 Population structure and global dissemination

The spread of hospital adapted clonal complexes of *E. faecium* is considered the major cause for the spread of vancomycin resistant enterococci (VRE) globally (240). These lineages are most often resistant to ampicillin and ciprofloxacin, and contain a large transferable genomic island (140,229,235) and genes for certain virulence markers such as enterococcal surface protein (encoded by *esp*) and a putative glycoside hydrolase (encoded by hyl_{Efm}) is often

present (82,130). Adhesion to extracellular matrix proteins and formation of biofilms on medical devices such as catheters and stents are important properties of enterococci and add up to their pathogenicity (239). It seems that the enrichment of virulence genes may have added to the success of *E. faecium* and opened for newer hospital adapted clones (202). The clonal complexes have most likely evolved as a result of multiple recombination events rather than mutations and possibly stress-inducing conditions in the hospitals favouring selection of these clones (13,240). By subsequent acquirement of transposons containing the vancomycin resistance clusters *vanA* or *vanB* these VRE have become a basis of pandemic potential (240). Until recently, all *E. faecium* clonal complexes were considered to have a common ancestor and they all clustered into a single common clonal complex, CC17 (Figure 2). However it may seem that this single complex in fact are several hospital adapted clonal complexes that have distinct founders (e.g. ST17, ST18, ST22 and ST78 and more) and has developed independently in the same niche. The sequence-types ST17, ST18 and ST78 seem to be the predominant CC17-genotypes worldwide containing *vanA* and *vanB* (22,23,45,93,128,130,133,134,140,145,149,181,207,216,225,236,240). *E. faecium* of animal origin has been considered host specific and not particularly related to human lineages. However, ampicillin resistant *E. faecium* belonging to the CC17-genogroup has recently been isolated from pigs and dogs (20,56,57,60) indicating that the hospital lineages are not restricted to the hospital environment anymore. Putative virulence genes were also detected in isolates from dogs (57) The development of these hospital adapted lineages appears to be largely associated with horizontal gene transfer and the ability to readily acquire resistance genes and virulence determinants (85). In addition a higher number of accessory plasmids, such as Inc18 and pRUM, linked to vancomycin resistance, have been detected in CC17-related strains (106,195). A study performed on isolates of *E. faecium* from US hospital patients at different time periods clearly indicates that the evolution of successful hospital adapted clones have been linked to successive horizontal gene transfer of virulence markers and resistance determinants (85).

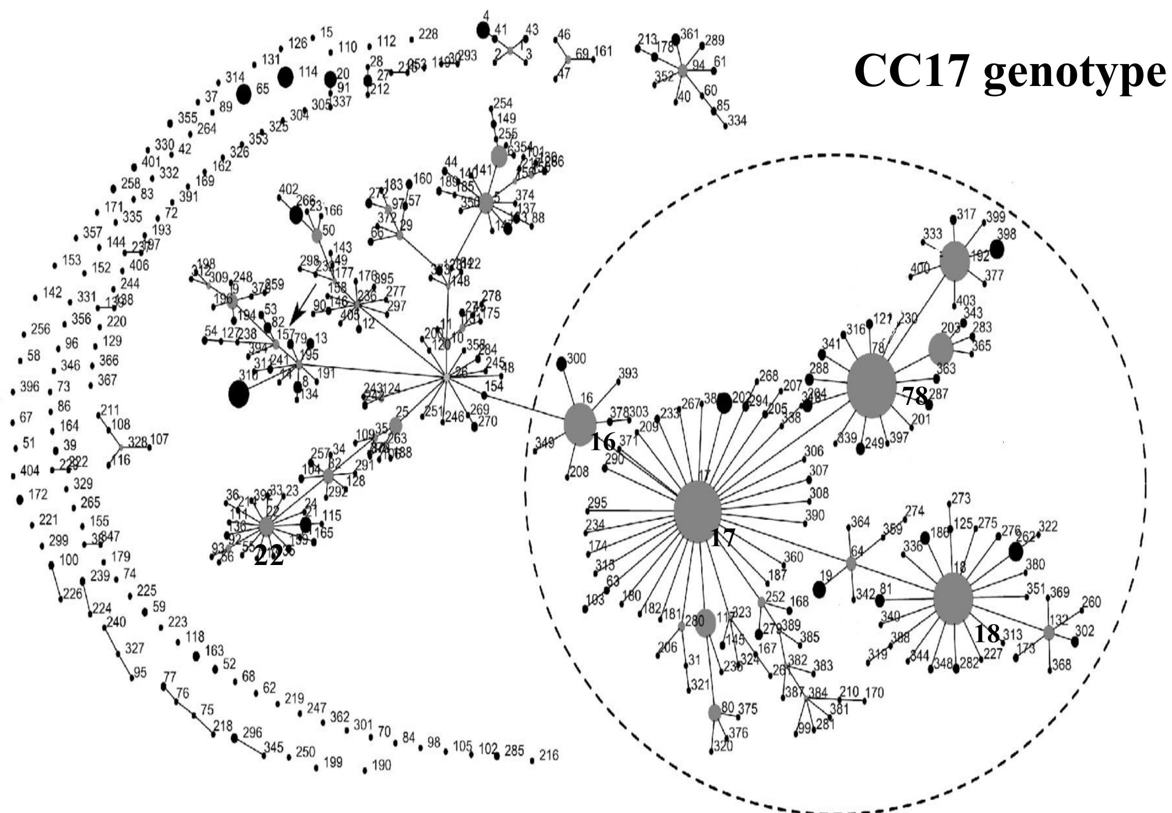

Figure 2. ***E. faecium*** **Clonal Complex 17 genotype, shown in circle, is the common way of classifying the typical VRE in hospital outbreaks. Reprinted from (57) with permission from the publisher.**

1.1.5 The *E. faecium* genome

The recent developments in pyrosequencing techniques have created a new era for extensive sequencing and characterization of the gene collection of bacterial isolates. The technology allows thorough analysis of diversity and population structure of clinically important bacteria such as *E. faecium* (228,238). In 2000 the first draft genome sequence of *E. faecium* DO was published (77) but the whole genome sequence for this strain has not been finished yet and there is still sequences missing (228). Recently genome sequencing of fifteen *E. faecium* has been performed due to the new technology (179,229). A study of the sequences of seven additional *E. faecium* from various sources revealed that there is a large difference in the genome size between the strains and a variable number of large plasmids present (229). Data showed that hospital strains were multi resistant whereas the commensal strains were not. The resistance genes appeared to be mostly located on plasmids and up to 30% of the *E. faecium* genome indicated to be non-core (228). Phylogenetic analysis revealed a relative small evolutionary distance between six of the seven strains. However there were considerable differences in gene content indicating both gene gain and loss as contributors in the evolution of *E. faecium*. Investigation of genes potentially involved in virulence revealed 26 conserved

proteins in the infectious strains and none in the non-infectious. Seven of these proteins were IS elements which may contribute to the genome flexibility in *E. faecium*. Presence of the known virulence genes *esp*, *hyl*, and *acm* were present in clinical strains however not in all. The *esp* gene was verified to be located on a large transferable putative pathogenicity island (PAI) indicating that the gene is acquired by horizontal gene transfer (HGT). Comparative genomic hybridization studies have also showed that the majority of *E. faecium* strains of clinical origin could be grouped into specific phylogenetic groups (a hospital clade) related to the Clonal Complex 17-genogroup. However a variable content of MGE between isolates shows that MGE may be important factors contributing to different phenotypic characteristics between CC17-related strains. Genes for IS elements and transposases were shown to be more enriched in the clinical isolates (228,229).

1.2 *Streptococcus*

Streptococci are commensal bacteria on the mucus membranes of the upper respiratory tract, gastrointestinal tract, genitourinary tract and the skin (103). The streptococci tend to be more susceptible to antibiotics than the enterococci. Streptococci are often classified into three main groups in their ability to haemolyse erythrocytes on a blood agar plate; the beta-haemolytic group, the non-beta haemolytic streptococci and the nutritionally variant streptococci, two of them are highlighted in Figure 3 (163). The beta-haemolytic streptococci are divided into several subgroups based on differences in specific carbohydrate antigens - group A (contains *Streptococcus pyogenes*) (GAS), group B (*Streptococcus agalacticae*) (GBS), group C (includes *Streptococcus dysgalacticae*, *Streptococcus equi*, *Streptococcus zooepidemicus*, and *Streptococcus constellatus*) (GCS), and group G (contains *Streptococcus equisimilis*) (GGS) are the most common ones. In addition there are group E, F, P, U and V (72). The non-beta haemolytic streptococci include *Streptococcus pneumoniae, Streptococcus bovis*, *Streptococcus lutetiensis*, and the viridans streptococci. Most of these belong to antigenic group D (72). The phylogenetic relationship between *Streptococcus* species are shown in Figure 3. This thesis will focus only on the *S. lutetiensis* (Group D_*Streptococcus*) (163). Normally the streptococci are susceptible for vancomycin. *S. lutetiensis* has been found both in animals and isolated from clinical isolates (72,183). In 1996 a *S. lutetiensis* isolate was shown to hold an approximately 100 kb genomic element containing a vancomycin resistance gene cluster highly homologous to the prototype *vanB* of *E. faecalis* V583.

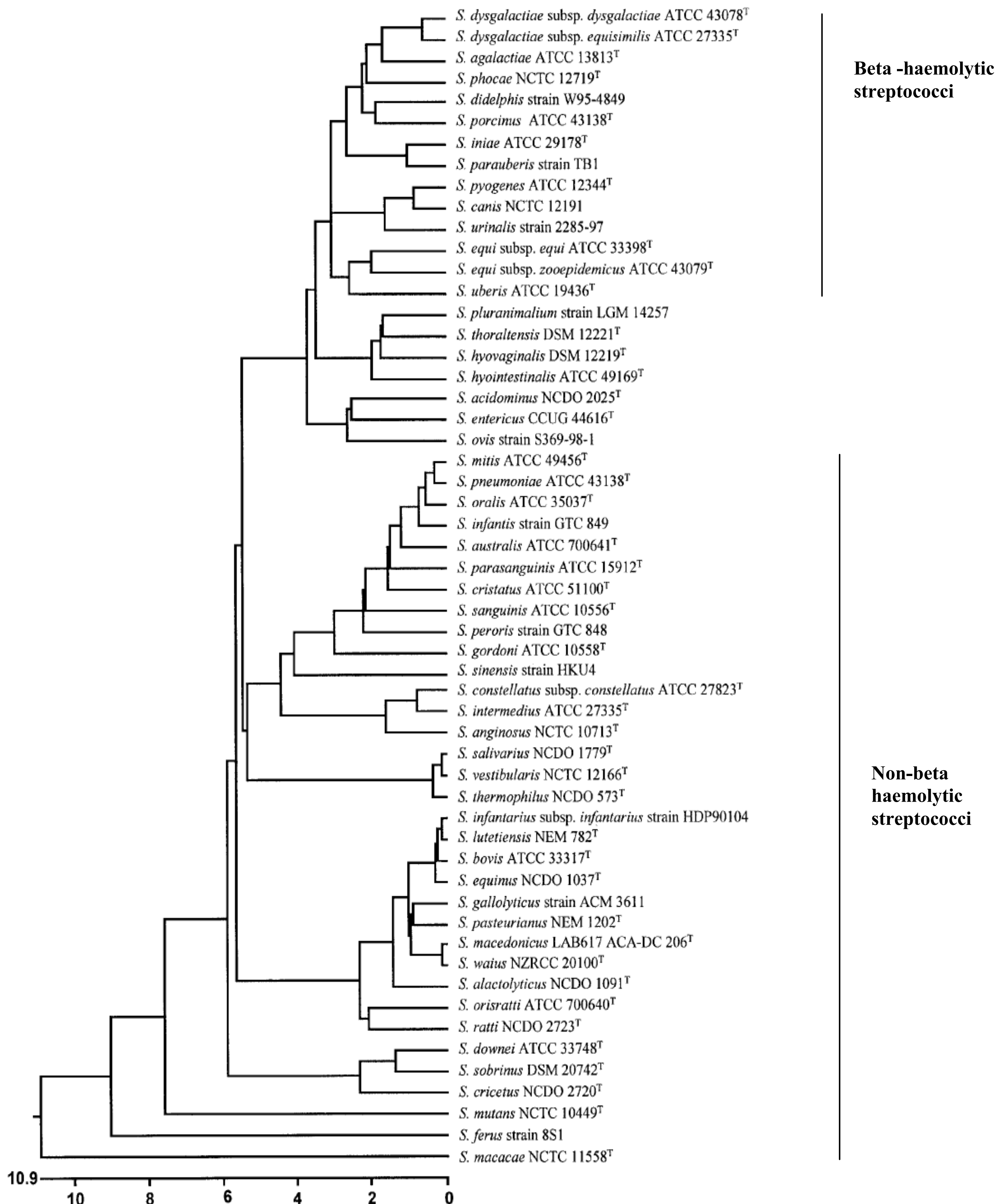

Figure 3. The phylogenetic relationship among 55 *Streptococcus* species and the classification of different streptococcal strains based on 16S rRNA sequences. One exception in this group is *S. dysgalactiae* subsp. *dysgalactiae* which is not beta-haemolytic but is included for taxonomical reasons. Reprinted from (72) with permission from the publisher.

1.3 Antimicrobial resistance

The impact of antimicrobial resistant bacteria in terms of cost, morbidity and mortality is increasing worldwide (21,46,88). In this regard it is of importance to reveal factors and mechanisms which are involved in the evolution and spread of such bacteria. Enterococci are considered difficult-to-treat pathogens with inherent clinical resistance to all cephalosporins and sulphonamides as well as low-level resistances to aminoglycosides, lincosamides and trimethoprim. In addition *E. faecium* have the unique capacity of acquiring high-level resistance to aminoglycosides, ampicillin and vancomycin, the most efficient anti-enterococcal drugs (5,164). Being part of the gastrointestinal flora the enterococci are in a unique situation to receive resistance genes from other commensals, but also transfer these to other and more pathogenic bacteria located in the gastrointestinal tract (70,233). This emphasizes the clinical importance of enterococci as a reservoir for antimicrobial resistance determinants.

1.3.1 Development of antimicrobial resistance

An antibiotic is defined as a substance having a biological, semi synthetic or synthetic origin which shows selective activity against bacteria and may thus be used in treatment of bacterial infections. Antimicrobial susceptibility testing of a clinical isolate is usually based on Minimum Inhibitory Concentration (MIC). The MIC value is the lowest concentration of antimicrobial agent which inhibits the growth of the bacteria. Clinical MIC breakpoints are designed to guide therapy and do not necessarily distinguish between bacteria with and without resistance mechanisms. Thus, epidemiological cut off values (the highest MIC value of the wild-type population) are designed to detect low level resistance mechanisms and monitor resistance development. The clinical MIC breakpoints generally divide bacteria into three categories of susceptibility: susceptible, intermediate, or resistant (123). A clinical resistant microorganism is unlikely to respond to even maximum doses of a given antibiotic (71). Antibiotic resistance was described early after the introduction of the first “real” antibiotic – penicillin (147). Later on, this has been the general pattern of observations. Within few years after introduction of new antibiotics in clinical practice, resistance tend to emerge (157).

The major part of antibiotics used today and the resistance-genes that gives rise of the resistance in several human pathogens have originated from the environment, especially from the soil, and have existed for a long time in nature. For example genes for penicillin resistance (beta-lactamases) have been estimated to have evolved about 2 billion years ago and genes for

vancomycin resistance more than 200 million years ago (231,245). Antibiotic resistance in human pathogens is primarily derived from mutation or horizontal gene transfer (4). The major driving force has been the selective pressure of antibiotics use, in medical therapy, veterinary practice, agriculture and animal farming. However some bacteria do contain intrinsic resistance or reduced susceptibility to some antibiotics. Often these genes coding for resistance may have as a role to inhibit growth of bacterial competitors in the soil (157,231). The species responsible for infections are not producers of antibiotics themselves (157).

1.3.2 Biochemical mechanisms for antimicrobial resistance

The biochemical mechanisms of resistance are divided into four classes where the bacteria can utilize one or more of these in their defence against antimicrobial agents: *i)* inactivation/ modification of the antimicrobial target which includes degradation and/or chemical modification, *ii)* reduced access due to altered penetration and or efflux that actively get rid of the antimicrobial agent before it reaches its target site, *iii)* altered target or protection of target site in which the antibacterial agent can not bind, *iv)* metabolic bypass by overproduction or alternative pathway.

Antimicrobial resistance in enterococci can be divided into two classes; intrinsic and acquired resistance.

1.3.3 Intrinsic antimicrobial resistance in enterococci

Intrinsic resistance is due to the lack of target sites for the antibiotic in question or insufficient access of the target site within the cell. This resistance is an intrinsic property encoded by the host chromosome and is a species-related trait (4,217).

Enterococcus is generally considered naturally resistant to cephalosporins due to their low affinity for enterococcal penicillin binding proteins (PBPs). The level of susceptibility other β-lactam drugs varies between enterococcal species (217). In addition, enterococci express resistance to sulphonamides due to their ability to use environmental folate (98,164). Low level resistance of aminoglycosides is due to low uptake of the drugs, and resistance to lincosamides is due to putative efflux mechanisms (14,166,217). *E. gallinarum* and *E. casseliflavus* are in addition intrinsically resistant to low levels of vancomycin due to production of D-Ala–D-Ser ending peptidoglycan side chain precursors for which vancomycin has a lower binding affinity compared to the normal D-Ala-D-Ala side chains (131,217).

1.3.4 Acquired antimicrobial resistance in enterococci

There are two ways for a bacterium to acquire resistance to antibacterial agents, either through chromosomal mutations or by horizontal gene transfer (HGT), where HGT is considered the major mechanism for spread of antimicrobial resistance (59,196).

The intrinsic resistance to several commonly used antibiotics may have given enterococci an advantage for acquiring new resistance phenotypes (162). High levels of resistance to penicillins associated with β-lactamase activity have been reported in *E. faecalis*, but is considered to be rare (9). Resistance to ampicillin is most often due to modifications in the expression of or mutations in essential PBP proteins. Resistance to ampicillin is often seen in *E. faecium* but occurs rarely in *E. faecalis* (131). Resistance to high levels of aminoglycosides (e. g. gentamicin, streptomycin and kanamycin) is due to three mechanisms: reduced uptake (due to increased efflux and/or decreased cell permeability), alterations at the ribosomal binding sites, or production of aminoglycoside modifying enzymes. Streptomycin resistance is acquired mainly via ribosomal resistance by mutations of the 16S or 23S ribosome or enzymatic modification by aminoglycoside adenylyl transferases (ANT) of the antibiotic. High-level resistance to kanamycin and gentamicin is obtained by aminoclycoside modifying enzymes such as phosphotransferases (APH) and adenyltransferases (AAC). The most common found in enterocooci are the bifunctional enzyme AAC(6´)-Ie-APH(2´´)-Ic which give resistance to most aminoglycosides except streptomycin. The *ermB* gene is the major contributor to the development of resistance to macrolides, lincosamides, and streptogramins. The gene encode a methylase modifying an adenosine residue in the bacterial 23S rRNA. A plasmid encoded chloramphenicol acetyltransferase gene, *cat*, has been shown in 20-42% of enterococci. Resistance to tetracycline is encoded by *tetM* and *tetN* genes among others and is seen in up to 80% of enterococci. Resistance is due to production of a protein interacting with ribosomes in such a way that the ribosome is protected and unaffected by the antibiotic. High-level quinolone resistance is gained trough mutations in the subunit for gyrase (GyrA) and in the ParC subunit of topoisomerase IV. Enterococci are now also commonly resistant to quinolones (8,131).

1.3.5 Glycopeptide/vancomycin resistance in *Enterococcus* – GRE/VRE

The first reports of vancomycin resistant enterococci (VRE) appeared in 1987 in France (142) and the United Kingdom (224). In 1989 North America reported their first incidence. Within few years New York experienced several major outbreaks of VRE in hospitals (83). Within

two decades VRE became the third- to fourth-most important hospital-acquired pathogen (234).

The epidemiology of VRE infections is somewhat different in Europe and the US. In Europe VRE are often isolated from domestic animals due to the use of avoparcin, an antimicrobial drug used in husbandry giving co-resistance to vancomycin in the enterococcal flora of the farmers. Avoparcin was forbidden in 1997 however vancomycin resistance genes continuing to persist in the environment function as a source for VRE in the community (24,240). In the US avoparcin was not used. Still, transmission of VRE and nosocomial infections has been more frequent due to higher use of vancomycin in the hospitals (24).

The emergence of glycopeptide resistant enterococci has become a major problem because it leaves few options for treatment. The vancomycin resistance genes are transferable to other species, including *S. aureus*, and selection pressure for the VRE may give rapid expansion of resistant populations. In addition, once the problem with VRE has established it is difficult to limit (162). Despite the global problem it is important to emphasize that vancomycin resistance is not the only challenge we are facing; the enterococci are increasingly acquiring resistance to macrolides, amphenicols, fluoroquinolones, aminoglycosides, and even new antibiotics such as oritavancin have been selected (8,14,95,144).

1.3.6 Glycopeptide resistance mode of action

The peptidoglycan layer of Gram positive bacteria consist of sugar derivatives which are cross linked and a group of amino acids. The peptidoglycan is multilayered where the sugars are connected by crosslinking. In *E. faecalis* and *E. faecium* the crosslink consist of two alanine molecules; D-Ala – D-Ala which serve as targets of glycopeptides (47) (Figure 4). Binding of vancomycin to the D-Ala-D-Ala inhibit two steps in the formation of the new cell wall. It inhibits the addition of new sugar derivatives and the interaction interferes with cross-linking of the peptidoglycan chains to each other. Consequently, the strength of the cell wall is weakened and the bacteria are now likely to lyse.

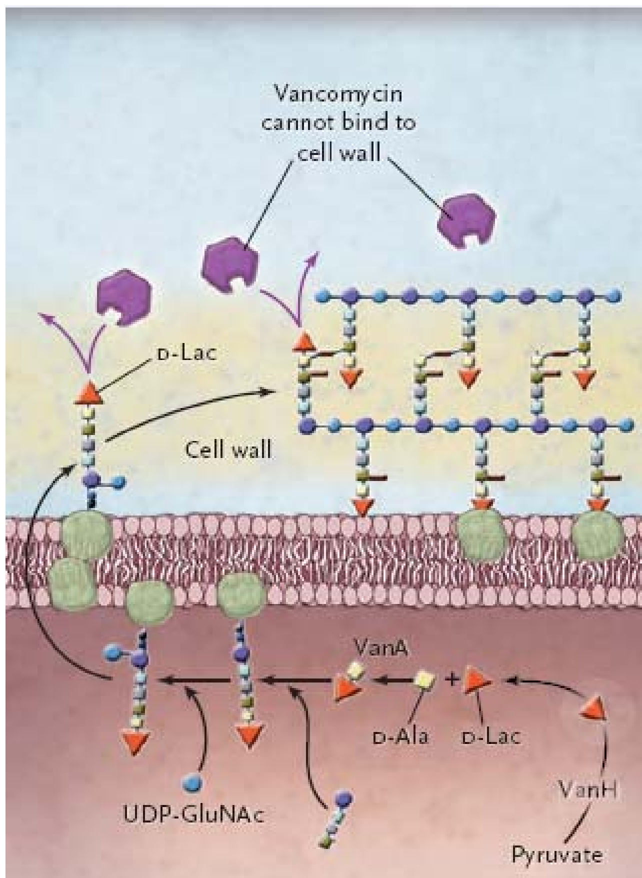

Figure 4; Resistance to glycopeptides caused by the replacement of D-Ala peptidoglycan precursor with a D-Lac precursor makes the glycopeptide unable to bind to the cell wall. Reprinted from (9) with the permission from NEJM.
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Glycopeptide resistance is currently divided into nine different classes (*vanA, vanB, vanC*, *vanD*, *vanE*, *vanG*, *vanL,* vanM, and *vanN*) (106,246) (Table 2). The most prevalent acquired gene clusters are the *vanA* and *vanB*.

vanA is the most common form of acquired glycopeptide resistance found among the enterococci. This is also the only type detected in *S. aureus* so far (47). It is characterized by resistance to high levels of both vancomycin and teicoplanin and is often plasmid mediated. It is carried on transposon Tn*1546* or closely related elements (200).
vanB type of resistance is associated with inducible variable levels of resistance to vancomycin and susceptibility to teicoplanin. The *vanB* genotypes can be divided into three subgroups; *vanB1-B3* due to sequence diversity (53). The *vanB* operon can be located chromosomally or on plasmids and is transferable. The *vanB* cluster consists of seven *van* genes mostly as part of a larger conjugative element (185).

Table 2. Vancomycin resistance in clinical relevant bacteria caused by acquired *van*-type gene clusters. Adapted and modified from (106) with permission from publisher.

Resistance level:	**AQUIRED** **High**	**Variable**	**Moderate**	**Low**					**INTRINSIC** **Low**
Type:	**VanA**	**VanB**	**VanD**	**VanE**	**VanG**	**VanL**	**VanM**	**VanN**	**VanC**
MIC in mg/L: Vancomycin Teicoplanin	 ≥ 16 > 8	 ≥ 4 0,5-1	 ≥ 64 4-64	 6-32 0,5	 12-16 0,5	 8		 8	 2-32 0,5-1
Expression	Inducible	Inducible	Constitutive/ Inducible (*vanD2*)	Inducible/ (Constitutive)	Inducible				Constitutive/ Inducible
van ligase gene	*vanA*	*vanB1-B3*	*vanD1-5*	*vanE*	*vanG1-2*	*vanL*	*vanM*	*vanN*	*vanC1-C3*
Modified target	D-alanine- D-lactate	D-alanine- D-lactate	D-alanine- D-lactate	D-alanine- D-serine	D-alanine- D-serine	D-alanine- D-serine	D-alanine- D-lactate	D-alanine- D-serine?	D-alanine- D-serine
Conjugative transfer	Yes	Yes	No	No	Yes	No	Yes	Yes	No
Location	Plasmid/ chromosome on transposon(s)	Plasmid/ chromosome ±transposon/ICE[a]	Chromosome	Chromosome	Chromosome on possible ICE	Chromosome?			Chromosome
Distribution	*E. faecium* *E. faecalis* *E. avium* *E. casseliflavus* *E. durans* *E. gallinarum* *E. hirae* *E. mundtii* *E. raffinosus* *S. aureus* *B. circulans* *O. turbata* *A. haemolyticum* *Paenibacillus* *Rhodococcus*	*E. faecium* *E. faecalis* *E. casseliflavus* *E. durans* *E. gallinarum* *E. hirae* *S. epidermidis* *Streptococcus* *Clostrdium* *Ruminococcus* *Eggerthella*	*E. faecium* *E. faecalis* *E. avium* *E. gallinarum* *E.raffinosus* Non-enterococcal fecal flora	*E. faecalis*	*E. faecalis* Non-enterococcal fecal flora	*E. faecalis*	*E. faecium*	*E. faecium*	*E. gallinarum* - *vanC1* *E. casseliflavus* - *vanC2/3*

a Integrative and conjugative elements

The *vanB* genotype has mainly been seen in hospital outbreaks in Scandinavia and Australia. (16,17,93,104,218,237). The *vanB2* subtype seems to be the dominant *vanB* genotype in most studies. This dominance is presumably related to its integral location in the conjugative transposon Tn*5382*-like (51,55,61,100,150-152,159,164,223,234).

1.4 Gene transfer mechanisms and their contributions to the development of antimicrobial resistance in enterococci

1.4.1 Mechanisms

Transfer of genes in bacteria is performed in two ways; vertical and horizontal gene transfer. **Vertical gene transfer** is genes inherited from parent cells to their offspring. All successful antibiotics targets single gene products encoding essential, functions in the bacteria. A mutation which only slightly changes the protein to which the antibiotic is directed may well make the organism resistant to that drug. These bacteria will persist in an environment containing the actual antibiotic. However in an environment where exposure to lethal levels of antibiotics is not continuous it is not likely that a single mutation leading to the resistance would become an epidemic problem (210). One exception to this notion seems to be the acquisition of stable high-level fluoroquinolone resistance which is associated with mutations in chromosomal genes involved in DNA-replication. Thus, the acquisition of new DNA by horizontal gene transfer have a relative higher impact on the development of antimicrobial resistance compared to mutations.

Horizontal gene transfer (HGT). Horizontal gene transfer (HGT) is the process of which an organism take up genetic material from a different organism which is not an offspring of that particular organism. This way of acquiring new genetic material has had a great impact on the evolution and genome plasticity of prokaryotic organisms, and is the reason for a great extent of the spread of resistance genes (180,193,206).

Resistance genes can be transferred not only within related species but across major taxonomic bacterial divisions. The genetic information may be transferred intercellular in three major ways; transduction, transformation and conjugation. Not all bacteria are able to perform all three processes, but several free living bacteria is able to use at least two of them (210). Integration of a foreign element is often limited by defence mechanisms exerted by the

recipient (111,156,173) so gene transfer is only successful if the element is able to replicate in the host by itself or through insertion in the host genome, expressed and an advantage for the recipient.

Transduction. Transduction is a process carried out by temperate bacteriophages which insert into the bacterial chromosome as a prophage. This prophage replicates and may at low frequency pack the host DNA or some of the host DNA with its own into the new prophage. When the cell lyses the bacteriophage may infect a new cell and the new prophage DNA integrates into the new recipient chromosome. Normally a transduction is only successful in transferring alleles of homologous genes among bacteria which are closely related. Bacteriophages often transfer toxins and virulence factors and do rarely carry antibiotic resistance (84,210). Bacteriophages in enterococci have been shown, although transduction of resistance genes to enterococci trough bacteriophages *in vivo* has not yet been revealed it is believed that phages may play a role in the spread of antibiotic resistance in enterococci (29,131,247).

Transformation. Transformation is a process in which naked, exogenous DNA is taken up and recombined into the genome of a competent bacterial cell. This process may facilitate evasion of host defences by mediating pilin switch and transfer of resistance genes or might be a process for acquiring nutrient sources for the cell. Transformation may also be a mechanism in the repair of damaged DNA (205). Enterococci are not known to be naturally competent (131), thus this process is not considered an important factor in the evolution of this genus.

Conjugation. Conjugation is carried out by Mobile Genetic Elements (MGE) such as conjugative plasmids and Integrative Conjugative Elements (ICE) that encode a mating apparatus which facilitate transfer of the cellular DNA from a donor cell to a recipient. This process requires close cell-to-cell contact between donor and recipient and a pore through which the DNA can be transferred and then recombined into the recipient genome or circularized as a plasmid (84,210). Conjugative plasmids can also transfer non-conjugative plasmids by mobilization. MGEs may carry virulence factors and they often contain genes encoding antibiotic resistance. Gram positive bacteria and especially enterococci use conjugation as a system for genetic exchange (43).
Figure 5 shows an overview of the three mechanisms of horizontal gene transfer.

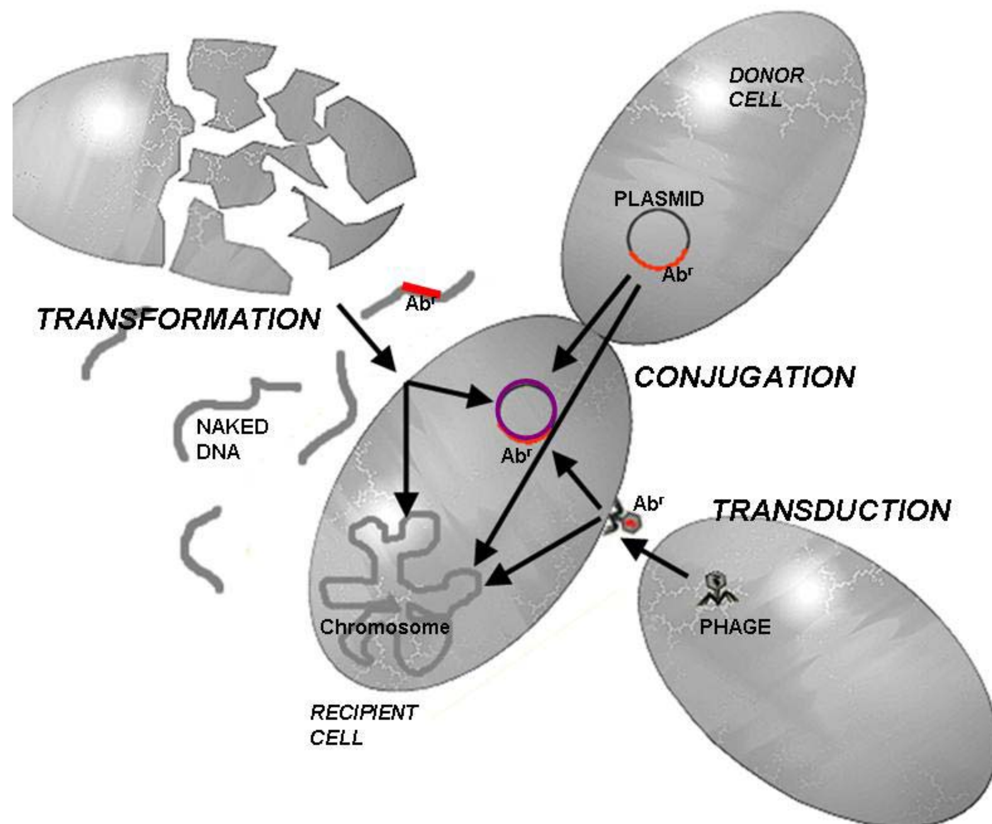


Figure 5; Acquisition of antibiotic resistance genes. Resistance genes (Ab^r) can be transferred to a recipient trough three mechanisms; Transformation (uptake of free, naked DNA), transduction (infection of a bacteriophage) or conjugation (plasmids, ICE) (Modified from Kenneth Todar, Lectures in Microbiology, University of Wisconsin-Madison. (http://textbookofbacteriology.net/themicrobialworld/bactresanti.html)

1.4.2 Mobile genetic elements

Mobile genetic elements (MGEs) are DNA fragments that mediate their own mobilization within or between cells (84). A vast variety of mobile genetic elements has been identified and new elements are continuously being found. The conjugative plasmids and transposable elements will be highlighted below.

Conjugative plasmids

In the bacterial cell the plasmids are replicating autonomously and controlled trough a negative control system; however, they may sometimes integrate into the bacterial chromosome. Several plasmids are self-transmissible or may be mobilized by other plasmids

making them able to spread to other hosts and provide them additional traits such as virulence and antibiotic resistance.

In enterococci a plasmid classification system based on the replication (*rep*) genes has been developed. Currently 19 *rep* families has been identified (119)
Conjugative plasmids encode proteins necessary for their own transfer from a donor to a recipient cell. These proteins involve the pili or the aggregation factors which promote cell-to-cell contact. Before plasmid transfer, a nick is engaged at *oriT* (origin of transfer) by a relaxase, followed by transfer of a single DNA strand by a rolling-circle or theta-replication, trough a type IV-like secretion system, and synthesis of the second strand in both donor and recipient. Several genes are required for transfer of a plasmid. These includes genes for relaxase, the formation of a mating channel (transglycosylase), genes for energetics of transfer (ATPases), and genes for production of coupling proteins (34,94). Figure 6 shows the organization of the transfer region in the broad host-range plasmid pIP501 as an example.

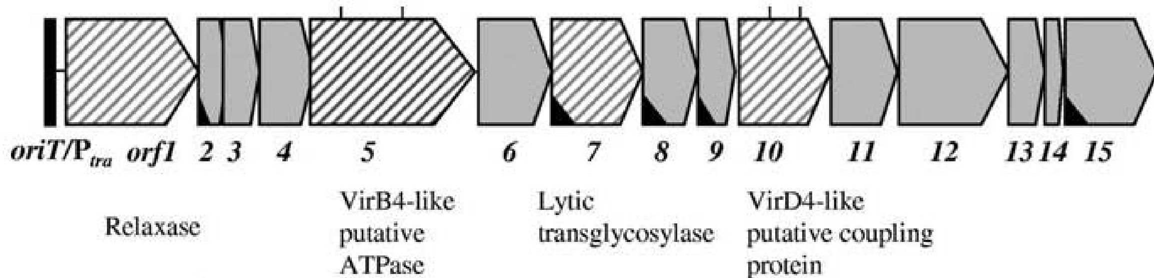


Figure 6; The pIP501 transfer region. Reprinted from (1) with permission from the publisher

The conjugative transfer of plasmids in enterococci is often divided into two major groups; pheromone responsive plasmids and broad host range plasmids;

Pheromone responsive plasmids – The communication process of pheromone responsive plasmids seems restricted to *E. faecalis* (rarely described in *E. faecium*) and involves the excretion of different small hydrophobic peptide sex pheromones which are specific for different types of plasmids and act as interbacterial signals. The pheromones are produced by a recipient and in close contact with a potential donor the transcription of a gene on the donor

plasmid is switched on. The result is production of an aggregation substance on the donor-cell surface mediating cell-cell contact between donor and recipient. The donor can now transfer the pheromone responsive plasmid to the recipient. Once the plasmid is transferred expression of this particular sex pheromone will be turned off in the recipient. Other bacterial species do also produce peptides similar to pheromones. For example *S. aureus* may induce enterococci to enter the aggregative state which may start intergenetic spread of plasmids. However transfer of vancomycin resistance by this mechanism has not yet been shown (44,94,165,178).

Broad host range plasmids – are plasmids that are able to transfer between enterococci and other Gram positive organisms including streptococci and staphylococci. The frequency of transfer is generally much lower than with the pheromone responsive plasmids. Since staphylococci, streptococci, and enterococci share several resistance genes, the broad host-range plasmids may well be a vehicle through which some of these resistance genes have spread among these different genera of bacteria (165).
Most broad host range plasmids are identified in streptococci and enterococci and often hold resistance genes to a wide spectrum of antibiotics. The lower limits in size for these types of plasmids are in the range of 15-20 kb which is the size of the transfer region with no additional genes (94).

Transposable elements

Transposable elements are defined as "specific DNA segments that can repeatedly insert into one or more sites in one or more genomes" (Roberts *et al*., 2008). It is a diverse group of mobile genetic elements of which some are capable of excising from the bacterial chromosome, transfer to a recipient by conjugation and insert into the recipient chromosome. The simplest transposable elements are the Insertion Sequences (IS) which are small < 2.5kb DNA elements. They encode a transposase which is an enzyme that cuts the transposable element out and unite it into the DNA where it will be inserted by homologous- or non-homologous recombination between short repeats (48).

Transposable elements may be mobilized by other conjugative elements; in some cases this includes elements in the host chromosome (194). However transposable elements do not hold the phenomena of incompatibility, so conjugative transposons may interact with each other and with other replicative and non-replicative elements. Different variations and rearrangement of mobile and non-mobile elements may be formed resulting in the formation

of a mosaic of larger and more complex elements giving several benefits to the host (194). After the sequencing era started new and more complex variants of elements are frequently discovered and described. These complex variants make it difficult to classify the transposable elements and several attempts have been proposed during the years. One way of classifying these elements is shown in Table 3. The composite transposons, unit transposons, integrative and conjugative elements and mobile genomic islands will be highlighted below.

Composite transposons – These are DNA segments, often carrying one or more resistance genes to antibiotics, flanked by two separate IS elements of the same IS family which may or may not be exact replicas. Instead of each IS element transposing separately, the whole length of DNA spanning from one IS element to the other is moved in one unit. Composite transposons have been reported in enterococci. The most widespread is the Tn*5281* (identical to Tn*4001* in *Staphylococcus*) which hold resistance to high levels of aminoclycosides. The prototype Tn*5281* (Figure 7) is flanked by two copies of IS*256.* In *Enterococcus* the IS elements within the IS*256* and IS*1216* families often form composite transposons (106). Another example of a composite transposon found in enterococci is Tn*5385* which is a large (65 kb) complex element containing both Tn*5281* and Tn*5384,* holding resistance to erythromycin, aminoglycosides, mercuric chloride, streptomycin and penicillin (191)

Unit Transposons – Unit transposons encode often an enzyme which is engaged in the excision and integration of the element. These are often site-specific recombinases or resolvases. In addition the elements include one or more accessory genes in their genetic unit and are often flanked by inverted repeats (IR) (193). An example of a unit transposon is Tn*1546* encoding the VanA phenotype in *Enterococcus* (10,142).

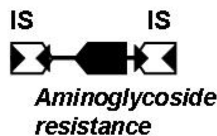
Table 3. Transposable elements in Gram positive bacteria: definitions and examples.

Type of transposable element*	Definition	Examples in Gram positive bacteria	Reference
Composite transposons	Flanked by IS elements. The transposase of the IS element is responsible for the catalysis of insertion and excision	Tn*5281*, Tn*5384*, Tn*1547*	(116,186,192)
Unit transposons	Typical unit elements encode an enzyme involved in excision and integration (DD(35)E or tyrosine) often a site-specific recombinase or resolvase and one or several accessory (e.g. resistance) genes in one genetic unit	Tn*3*, Tn*1546*	(10,146)
Conjugative Transposons (CTns) / Integrative Conjugative elements (ICEs)	The conjugative transposons (CTns) belong to a group known as integrative conjugative elements (ICEs) which carry genes for excision, conjugative transfer and integration within the new host genome. They carry a wide range of accessory genes, including antibiotic resistance	Tn*916*, Tn*1545* Tn*vamp* (Tn*5382*/Tn*1549*),	(36,48,78,86,189)
Mobilisable transposons (MTns)/integrative mobilisable elements (IMEs)	The mobilizable transposons (MTns), also known as integrative mobilizable elements (IMEs), can be mobilized between bacterial cells by other ''helper" elements that encode proteins involved in the formation of the conjugation pore or mating bridge. The MTns exploit these conjugation pores and generally provide their own DNA processing functions for intercellular transfer and subsequent transposition	Tn*4451*	(2)
Mobile genomic islands	Some chromosomally integrated genomic islands encode tyrosine or serine site-specific recombinases that catalyze their own excision and integration but do not harbor genes involved in transfer. They carry genes encoding for a range of phenotypes. The name of a genomic island reflects the phenotype it confers, e.g. pathogenicity islands encode virulence determinants (toxins, adhesins, etc.)	SCC*mec*	(126)
Integrated or	An integrated or transposable prophage is a phage genome inserted as part of the	φNM1–4	(11,227)

transposable prophage	linear structure of the chromosome of a bacterium which is able to excise and insert from and into the genome	PH15	
Integrated satellite prophage	Bacteriophage genome inserted into that of the host which requires gene products from ‘‘helper” phages to complete its replication cycle	IL1403	(39)
Group I intron	Small post-transcriptionally splicing (splicing occurs in the pre-mRNA), endonuclease encoding element. Will home to allelic site	Unnamed group I intron inserted the DNA polymerase gene of *Bacillus subtilis* phage SPO1	(90)
Group II intron	Small post-transcriptionally splicing (splicing occurs in the pre-mRNA), restriction endonuclease encoding element	Tn*5397* containing group II intron	(161)
Istron	Chimeric ribozyme consisting of a group I intron linked to an IS*605* like transposase	CdlSt1	(30)
Intein	Small post-translational splicing (splicing occurs in the polypeptide), endonuclease encoding element. Will home to allelic site	LLP-KSY1 PolA	The intein database http://www.neb.com/neb/inteins.html

* Not all reported elements have shown to be mobile. Adapted and modified from (193) with permission from the publisher.

Tn*5281* composite transposon



Tn*1546* transposon of the Tn*3* family

Tn*1549*-/Tn*5382*-like conjugative transposon

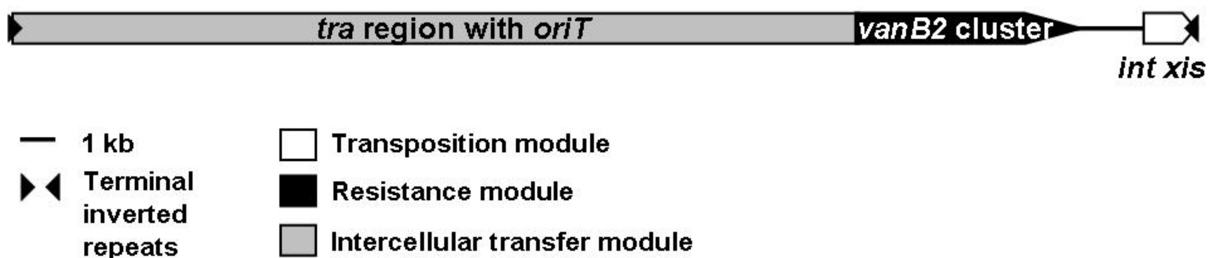


Figure 7. Schematic presentation of the three transposons representing the transposon groups transferring resistance genes in enterococci. Adapted from (106) with permission from publisher.

ICEs (Integrative and conjugative elements)

ICEs are elements which excise by site-specific recombination and integrate like prophages but transfer in a covalently closed circular intermediate by conjugation like plasmids. Before transposing to a recipient these elements needs to excise and form a circular intermediate. This is a process mediated by site-specific recombinases. At times they may also require an excisionase. The transfer mechanism is thought to be similar to that of plasmids, though with a specific *oriT* (origin of transfer) (194).

The circular intermediate is replicated during conjugation but replication is not required for maintenance of the element. However the intermediate is not self-replicating and will require replication factors from the host (78). Three types of recombinases have been found to be involved in insertion and integration of these elements; tyrosine, serine and DDE recombinases (32,35). Several of the tyrosine recombinases catalyse site-specific integration into genes encoding tRNA (35). Most of the ICE encodes a tyrosine integrase which catalyse the recombination between the attachment sites *attL* and *attR* which flanks the element. This

would lead to the excision of a circular intermediate containing an *attI* site which can transfer to a recipient by conjugation. The element will be able to integrate at an *attB* site (31). The conjugative elements may facilitate transfer of additional DNA by including genes or by integrating into other elements where they may take advantage of the self-transmissibility of the ICE (76,215).

One of the best known ICEs is the Tn*916*-Tn*1545* family. Tn*916* from *E. faecalis* was the first conjugative transposon to be identified (32,43,78). This 18kb transposon is widely distributed among Gram positive bacteria and especially in *Enterococcus* and *Streptococcus*. Tn*916* was shown to insert into various sites on a plasmid, pAD1 and at several sites in the chromosome of a recipient bacteria (43). Within the Tn*916*/Tn*1545* family there has been identified several different ICE like the Tn*5382*/Tn*1549*-like. Tn*5382/*Tn*1549* has shown to be approximately 34kb encoding *vanB2* in enterococci. It has been shown that Tn*5382*/Tn*1549* forms a circular intermediate; and transfer of the element has been shown (55,86,138). However it is not established whether this element is conjugative or mobilizable. An *oriT* and relaxase of the MOB_{p7} family has been identified. It has been proposed that Tn*1549* has been transferred from *Clostridium symbiosum* to *Enterococcus* by a helper element (221). A vast diversity of ICE elements has been shown. Several of these are shaped by extensive recombination and formation of mosaic genomes formed by inter-ICE recombination and tandem array structures (87,243).

The streptococcal ICE*Sde*3396 shares several open reading frames with a putative ICE from *S. agalacticae* (GBS), *S. dysgalacticae* (GGS), and several genes from *S. pyogenes* (GAS). In addition, ICE*de*3396 harbours genes from non-streptococcal bacteria such as *Enterococcus* and *Lactococcus*, giving the element a mosaic structure in an 18kb internal region. This MGE is able to transfer to other β-haemolytic streptococci and has been shown transferable to other GGS, GBS and GAS.This ICE element does not contain genes for antibiotic resistance rather it includes genes for cadmium and arsenate resistance and a virulence gene encoding agglutinin receptor precursor (58). This example illustrates the dynamics and spread of such elements.

Mobile genomic islands – Genomic islands (GEI) are part of the flexible bacterial gene pool and are typically discrete DNA elements between closely related bacterial strains. The nucleotide content of GEIs often differs from the rest of the chromosome; they are large in

size (10-200kb), are often inserted at tRNA genes, may be unstable, and may have a different codon usage and GC content than the core genome. GEIs are often flanked by 16-20bp perfect or almost perfect direct repeats (DR) arisen by site specific integration into the target site and do often act as recognition sites for excision. These elements contribute to a great extent to the diversification and adaptation of microorganisms and have a large impact on the genome plasticity and evolution, dissemination of antibiotic resistance and virulence genes, and formation of catabolic pathways. One hypothesis is that the GEIs embrace a family of elements including recognized mobile elements like ICE, conjugative transposons and some prophages (97,122). Genomic islands with functions that increase the fitness of the bacteria have probably directly or indirectly been positively selected trough evolution (97). One example of a mobile genomic island is the methicillin resistance (*mecA*) of MRSA isolates is located within the Staphylococcal Cassette Chromosome (SCC) region of the staphylococcal chromosome containing transposon within transposon features. The *mecA* gene is not originally found in *S. aureus* and may have been transferred from coagulase negative staphylococci (CoNS) which may be capable to infer resistance to methicillin. MRSA and other resistance phenotypes are often a result of integration of transposons containing resistance genes for example erythromycin (Tn*554*). The basic SCC*mec* is composed of the resistance genes, component of its regulatory region and the transposition genes *ccrA* and *ccrB* in combination or the single *ccrC*. The origin of SCC*mec* is not known however it is believed that the *ccr* and *mec* genes accumulated in CoNS from and unknown source followed by deletions in the regulatory part of the *mec* genes took place whereby the genes were transferred to *S. aureus* (7,112,167,212). Recently a SCC*mec*-like element was identified in a strain of *Macrococcus caseolyticus* (a commensal bacterium in food animals). This element has not previously been observed and has shown a potential mechanism for the generation of new SCC*mec*-like elements (220). This may give further knowledge of the origin of SCC*mec* and related elements.

There are eight different types SCC*mec* (type I-VIII) described so far and each type may vary, showing a diversity of this mobile genomic island (62,249). Even the *ccr* genes are varying up to 5% at nucleotide level within one type SCC*mec* (101,176). The whole region of SCC*mec* can excise from the chromosome through the gene products of the *ccr* genes making SCC*mec* a presumable mobile element (189). The detailed mechanism around transfer of SCC*mec* is not yet fully understood.

Pathogenicity islands (PAI) are genomic islands present on the genomes of pathogenic strains but are not found in the genomes of non-pathogenic members of the same or related species. These were first described in human pathogens of *E. coli* but have been found in the genomes of various pathogens of humans, animals, and plants, both Gram positive and Gram negative (97). An example is the *E. faecalis* PAI coding for virulence genes including the gene for cytolysin toxin and the enterococcal surface protein (*esp*). In addition there are traits suspected to contribute to pathogenicity or altering *E. faecalis* relationship with the host, including a bile acid hydrolase and carbohydrate utilization pathways (158). An interesting part of this PAI is the diversity of the island between closely related strains even between strains belonging to the same clonal complex. McBride and co-workers hypothesize that the pathogenicity island in *E. faecalis* has entered piece by piece and not as a whole, complete island which later rearranged into the diversity of islands we see today. The genome of *E. faecium* has also shown to contain PAIs and other related elements (229). Recently an *E. faecalis* PAI was shown to transfer via mechanisms independent of ICE functions. Transfer of this particular PAI only occurred via pheromone responsive type of conjugative plasmids and showed horizontal transfer of all chromosomal regions including a vancomycin resistance transposon and alleles included in MLST (155).

1.4.3 Host range of mobile elements

Mobile DNA disseminates both within a bacterial species but also between different species and genera dependent on the host range of the element. Some elements are even able to transfer from bacteria to eukaryotes (e.g. *Agrobacterium tumefaciens*).

Plasmids. As mentioned above, plasmids may have a broad host range making it able to spread to several species, but others are restrained to certain bacterial species. It has also been shown that the enterococcal plasmid pAM830 has facilitated transfer of a *vanA* Tn*1546*-like element to *S. aureus*. This plasmid was lost. Broad-host range plasmids in enterococci have shown to transfer between several Gram positive bacteria. Different *rep* families display different host ranges. An example is *rep* family 7 and 10 which has shown to transfer to staphylococci, streptococci and *Bacillus* (119).

Bacteriophages. Bacteriophages, like all viruses exhibit a defined host range. Some are viewed to have a narrow host range and highly specific, whereas others infect a range of

different bacterial species. Examples of a broad host-range bacteriophage is phage 812 which infect several staphylococcal species (124).

Little is known about the enterococcal phages and their ability to spread virulence traits or antibiotic resistance however they contribute to a large extent to the genome diversity in *E. faecium* (120,175,229).

The bacterial viruses are highly adaptive and continuously co-evolving with its hosts (230) and a particular bacteriophage may transfer a DNA element to different bacterial species. Certain genetic elements may be transduced by several different phages and thus disseminate to several different bacterial species (154).

Integrative and Conjugative Elements. The host-range of a self conjugating element will depend on the specificity of the recombinases/integrases responsible for the conjugation. Tn*916* from *E. faecalis* has shown to have a very broad host range (more than 50 different bacterial species from 24 genera) including both Gram positive and Gram negative bacteria (19,43).

ICE*St1* and ICE*St3* in *Streptococcus thermophilus* has shown to be able to transfer from *E. faecalis*. These elements have also been found in *S. pyogenes* showing the evidence that they may move between different species. The two ICE elements are also closely related to putative ICEs in *S. pyogenes*, *S. mutans*, and *S. agalacticae*. In addition one of the elements is carrying cadmium resistance genes closely related to plasmids from *Lactococcus lactis*, *Listeria innocua*, *S. aureus*, *Staphylococcus saprophyticus*, and ICEs of *Listeria monocytogenes*, *S. agalacticae*, *S. dysgalactigae*, and SCC*mec* genomic island from *Staphylococcus* (18,26,177).

The mixing of genes in ICEs and other genetic elements would seem to be a central mechanism for the exchange and spread of genes into different ecological niches (18).

1.4.4 Integration sites

The integration of a transposable element is not a random process. Enzymatic activity is required for integration of a transposable element. In prokaryotes these enzymes are mostly transposases, integrases and recombinases which recognize specific regions in the target DNA and promote homologous or non-homologous recombination between them (84). Most elements show some target specificity having preferences over some targets than others. The specificity may as well be the structure rather than the sequence of the target DNA, that is

bends in the DNA or the presence of other proteins bound to DNA (transcription factors) (49,135,242). However some transposable elements require a specific sequence in the target DNA for integration to occur. This may include a special attachment (*att*) site or a special order of nucleotides. Examples of this are the SCC*mec* in *Staphylococcus aureus* and phage λ which both integrates into an *att* site of the target. In SCC*mec* the *ccrA* gene are responsible for the site specificity of integration of the element. *ccrB* alone are able to initiate excision however at an alternative *att* site. Pathogenicity islands (PAIs) have a tendency to integrate into a tRNA gene. Some transposons create target site immunity when they integrate. This is a feature where the transposons do not integrate into a site close to another transposon in the same target DNA (49,113,176,193,208,232).

1.4.5 Putative host mechanisms for protection against phages and MGE

Bacteria may hold several mechanisms which function as immune systems against prophages. In *Lactococcus lactis* more than 50 phage resistance systems have been characterized. These include restriction-mechanisms which comprise phage genome uptake blocks, superinfection immunity, restriction modification, Clustered regulatory interspaced short palindromic repeats (CRISPR) and mutations (96,117) and abortive infection systems (abi). The section below will focus on the two latter mechanisms that recently have been discovered in *Enterococcus* sequences – abi and CRISPR

Some bacteria harbour resistance mechanism to phage infection denoted exclusion or abortive infection (Abi). This mechanism starts as a normal phage infection including adsorption and injection of DNA into the host. However the phage development is then interrupted leading to the release of no or a few progeny particles. The infected cell will die and further spread of the phage is prevented in the bacterial population. Several Abi systems are known, most of them from *Lactococcus* and most are plasmid encoded (40). A Tn*5382*-*vanB* containing MGE transferable from *Streptococcus* to *Enterococcus* have showed to contain an abiG system. This is the first report of such a sequence in enterococci (**Paper III**). The *abi* genes are also known to be involved in increased stress tolerance of the host. These genes are commonly found in *Streptococcus* (114).

CRISPR are found in the genome of about 40% of bacterial genomes and nearly all archaeal genomes (15). The CRISPR are short (24-48 nt) repeats split by unique spacer sequences with similar size as the repeats and most often flanked by CRISPR-associated (*cas*) genes. The

CRISPR spacer sequences and the associated repeat are transcribed into small RNAs that protect the organism from specific bacteriophage infection and horizontal gene transfer of specific MGE and plasmids by targeting the interfering DNA (156).

Interestingly, the *E. faecalis* OG1RF contains two CRISPR loci, however the genome of. *E. faecalis* V583 is absent of prophages, has no CRISPR loci and consist of several MGEs which include prophages or prophage remnants. It has not yet been proven but the hypothesis is that the CRISPR loci in OG1RF are the main reason for the lack of prophages and MGEs in this strain (25). CRISPRs has been located in *E. faecium*, however no cas genes has been linked to them so they are not considered functional (229).

1.4.6 Plasmid maintenance.

To ensure their survival in the bacteria during cell division, the plasmids have developed several systems. Low copy number plasmids associated with antibiotic resistance and pathogenicity is prone to contain core regions which are mandatory for the replication, segregation and transfer. The stability of the segregation of the plasmid may be maintained trough a specific system where cells failing in receiving a copy of the plasmid will not survive due to selective killing or growth impairment. These mechanisms are collectively known as Toxin-Antitoxin (TA) or plasmid addiction systems (Figure 8), and include a stable toxin and an unstable antitoxin produced within the cell. The TA-systems, in addition to promote selective killing promote active partitioning and resolution of multimers by site-specific recombination (91,105). Several TA-systems has been described, in *E. faecium* the *axe-txe*, the *maz*EF, *rel*BE and the ω-ε-ζ-system has been detected (105,160,195). The pneumococcal epsilon zeta Antitoxon Toxin (PezAT) system have recently been detected in an enterococcal sequence indicating that more TA-systems may be found in these species (**Paper III**).

Toxin –Antitoxin systems have also been shown located on the prokaryotic chromosome (168) and on MGEs (244). For instance the putative PezTA system in enterococci is located on a MGE transferred from *Streptococcus* (**Paper III**). The function of chromosomally encoded TA-systems is being discussed however it is hypothesised that they may be involved in growth control and stress response (41,42), stabilization of MGE (244).

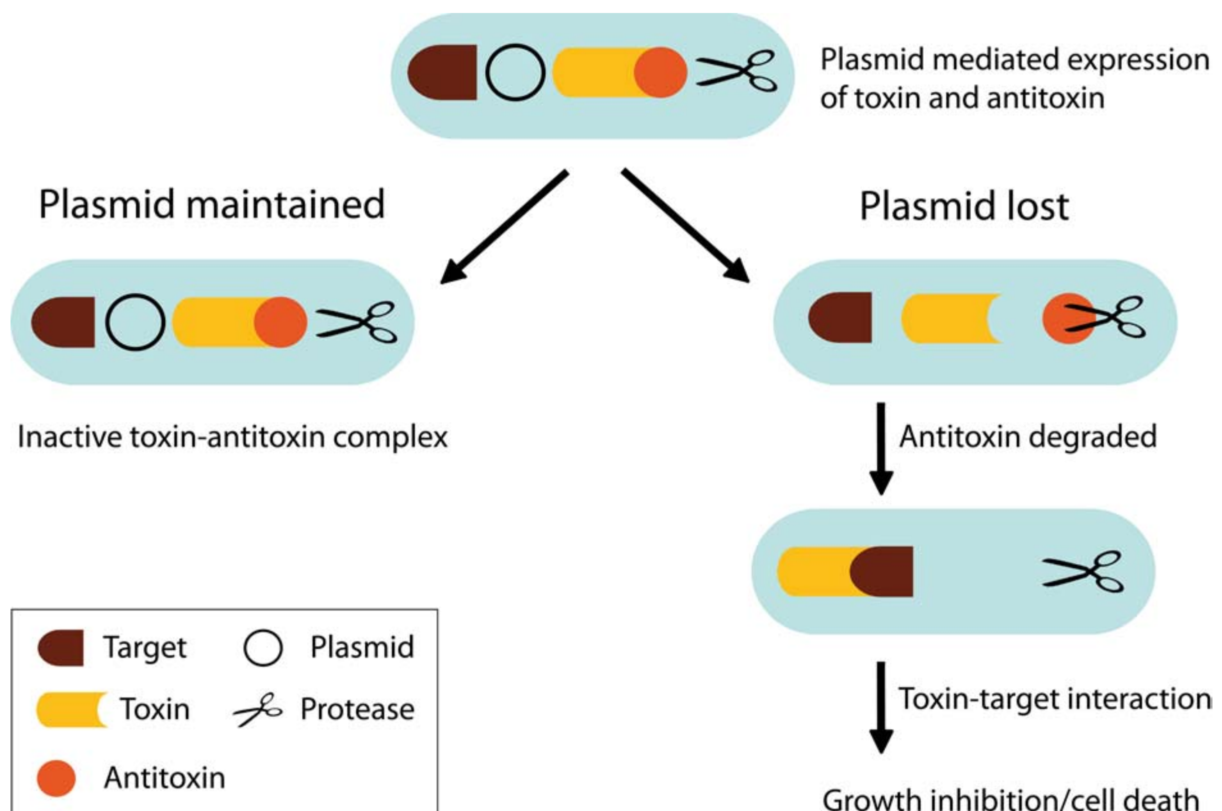


Figure 8; A general outline of cell death induced by plasmid-located TA modules. The toxin (yellow) and antitoxin (orange) proteins form a complex that cancels the harmful activity of the toxin. The antitoxin is degraded by a protease more rapidly than the toxin, but the toxin is constantly bound by fresh antitoxin. As long as the plasmid is maintained, the cell tolerates presence of the TA complex (left). If a missegregation event or replication defect produces a plasmid-free cell (right), the degraded antitoxin cannot be refilled so that the free toxin attacks an intracellular target to cause death or growth restriction of the plasmid-free cell. Reprinted with permission from publisher (106).

1.4.7 Horizontal gene transfer – *In vivo*

The community of microbes in the GI tract changes between and within adults and the bacterial community of an infant are different from an adult (137). A habitat will at all times be populated by resident species in addition to several transient species which are only filling an empty niche (73). The gastro intestinal environment has revealed to be important for gene-flux both within transient and resident species and between them. *In vivo* studies have shown that the condition in the mammal GI tract in fact favours HGT and that this has been difficult to reproduce under laboratory conditions. Transient bacteria may transfer resistance determinants to the resident flora even without colonizing (52). Spread of resistance genes

may come from ingested soil and water bacteria via contaminated food and drinks as well as from human pathogens (198).

The *E. faecium* colonize the GI tract of humans and animals at high levels (187). Studies have shown that Tn*5382*/Tn*1549*-like elements containing *vanB2* have been identified in other genera than *Enterococcus* belonging to the normal gastrointestinal flora such as *Clostridium, Ruminococcus, Eggerthella,* and *Streptococcus* (12,54,64). A study performed by Seville *et al.,* (201) showed that 5 of 6 faecal metagenomes contained the Tn*5382*/Tn*1549*-like integrase gene which suggests that this family of elements are common in the microbiota inhabiting this niche and are involved in horizontal gene transfer in the human intestinal flora. In addition the *vanB2* resistant conjugative transposon Tn*5382/*Tn*1549*-like has been shown to transfer from *Clostridium* to *Enterococcus* in the gut of gnotobiotic mice (138). Fecal carriage of *vanB* mainly of the *vanB2* subtype has been observed at high rates in the absence of cultivable VRE both for community and hospital specimens (64,92,248).

Although the GI tract is a well known environment for bacterial colonization and spread of antimicrobial resistance determinants the oral cavity may be even a better place for spread of resistance determinants. The oral cavity contains several ecological habitats and is in contact with a number of growth substrates via the food eaten. The number of bacterial species in the oral cavity is thought to be up to 1000 (194).

E. faecium has been found very rarely in the oral cavity. However there is a high prevalence of tetracycline resistance in the oral *E. faecalis* which may be a result of dissemination of a *tet*(M) containing Tn*916*-related element in the oral microbiota (211). The *tet*(M) tetracycline resistance gene and the Tn*916* integrase has shown to be most prevalent in the human oral metagenome (201).
Antibiotic pressure may not only affect the microbial flora, but also promote transfer of elements encoding resistance to the particular drug. In addition bacteria growing in biofilms have higher rates of transfer of genetic elements than planktonic cells due to both the closeness of the bacteria and the stability of the mating pairs in order for conjugation to be successful (148,194). For the non-motile enterococci biofilm formation will be an important factor for transfer (89).

The presence of enterococci in the GI tract of food-animals leads to a high risk of contamination of meat during slaughtering. Enterococci have been isolated from beef, pork cuts and cheese. *E. faecium* has even been isolated from pork meat and salami (79,209). Enterococci and VRE has been isolated from various environmental surfaces such as remote controls and daily newspapers within hospital environments revealing the ease of contaminating the exteriors which we all may be in contact with (93). It has been shown that the hospital environment play an evident role in the transmission of VRE between patients (69,174,182). The clothes of health care workers are also subjected for contamination and dissemination of VRE in addition these organisms survive for a long time in a hospital environment. In one study enterococci survived for more than 90 days on polyester, polyethylene, and on hospital clothing and drape (27,174).

Resistance determinants may also reach outside the hospital environment where enterococci may serve as a reservoir for the spread of MGE for example through the sewage (118) and even our domestic animals (57).

1.5 Molecular epidemiology and typing methods

Molecular epidemiology is a field in biology which quite recently emerged from integration of molecular biology into the traditional epidemiologic research. This has become an essential field for the development of medical diagnostics and public health prevention strategies and is important in the determination of physical sources of an outbreak, biological relationships, transmission routes and important virulence genes which the pathogenic organism holds. The requirement for suitable typing methods is particularly important in cases where pathogens increase in prevalence or acquire qualities increasing their capacity to cause disease. In these cases identification below the species is required (226).
Several different typing methods has been described, such as amplified fragment length polymorphism (AFLP), Multiple Loci VNTR Analysis (MLVA), PFGE and MLST, the two latter are described below. Guidelines have been given for interpreting the results of the different typing methods, but there is generally a lack of standardized criteria and running conditions. This makes it difficult to compare results between different laboratories. Which method to use is also a crucial question and the choice must be taken on the basis of good knowledge of the organism. Enterococci are a group which vary considerably in their genetic content due to extensive recombination amd horizontal gene transfer (140,197,222). This is a

factor which must be taken into account when choosing typing method for epidemiological research of enterococci.

Pulsed Field Gel Electrophoresis (PFGE) has been considered a gold standard of the typing methods for several medically important bacteria and is essentially the comparison of large genomic DNA fragments after digestion with restriction enzymes and the DNA fragments separated by agarose gel electrophoresis designed for separating large fragments and giving a fingerprint of the whole genome. The bacterial chromosome is typically a circular molecule and enzymatic digestion yields several linear DNA molecules at different lengths. By comparing two isolates that are the same strain (i.e. clonal), the sites at which the restriction enzymes act on the DNA and the length between these sites would be identical. If the DNA banding patterns between isolates is identical the isolates are considered the same strain. If two isolates are not the same strain, then the sites at which the restriction enzymes act on the DNA and the length between these sites would be different and their DNA banding patterns will be different. PFGE has been important for the identification of clonal relatedness between isolates that are epidemiologically associated in time and space (38). By comparing DNA-band patterns one may identify closely related or more distantly related samples (213). Transfer of genetic elements from a donor to a recipient will be visible as shift of bands on the gel. A disadvantage is then that several differences in bands will lead to the conclusion that the isolates compared are distantly or not related where they in fact may be closely related isolates with high frequency of horizontal gene transfer. The resolution power is a limiting factor which makes it difficult to compare gels and reproduce results (6). The advantage of this technique is that rearrangements in the genome such as insertions and deletions of genetic elements may be visible and the surveillance of for example an outbreak is possible such as in the case of enterococci. Very small changes may though not be visible. However the method is not suitable for global and long-term epidemiological studies (197).

Multi Locus Sequence Typing (MLST) is based on variations in mutations in the sequence of 450-500 bp from seven housekeeping genes (115). The method was developed to overcome the challenge of comparing the non standardized typing methods between laboratories. Nucleotide data from several alleles of the seven genes is stored in a database giving the advantage of a good resource for global epidemiology and high discriminatory power between isolates due to the quite large number of genes compared (153). Housekeeping genes are not target for extensive mutation over a short period of time; however a certain change over time

is expected. For each allele, the different sequences are assigned a distinct allelic profile number. The profile of all seven alleles is defined a sequence type (ST). The number of nucleotide differences is not related to the allele number given. The basis for this is that one genetic event that result in a new allele may occur by point mutation or recombinational replacement. Sequence types are grouped into clonal complexes by their similarity to a central allelic profile. These profiles are often identified by a database called eBURST which incorporates a simple model of bacterial evolution in which strains increasing in frequency diversify to form clusters of similar genotypes, descended from the founding strain. Isolates of an expanding founding strain initially have the same allelic profile, but diversification result in the appearance of variants in which one change in a MLST loci give a single locus variant (SLV). A clonal complex if defined in eBURST as a group of STs in a population that share six of seven alleles with at least one other ST in the group (222). MLST and eBURST has been used for typing of isolates belonging to CC17 genogroup, which is a large complex, however the algorithm was not optimal for investigating bacteria with such high recombination frequencies. Later new algorithms has been developed displaying that CC17 is not one complex developed from a single ancestor. It is more probable that the STs belonging to CC17 are not related but rather are several complexes evolved in parallel (85,99,241). This was also observed in a study of *S. agalacticae* where strain classification methods, like MLST; did not reflect the real genetic diversity which could be described by whole-genome analysis. MLST is thus a method that do not consider the variable genome of the species (33).

2. Aims of study

The general aim of this work was to investigate factors and mechanisms important for horizontal gene transfer in *E. faecium*.

Specific aims;

Paper I.

- Investigate the clustering of VanB-type vancomycin resistant *E. faecium* at the Örebro University Hospital between November 2002 and April 2004 using clinical and demographic data to identify potential risk factors.
- Characterize the strains with regard to clonal relatedness and possible mobile genetic elements involved.

Paper II.

- Identify $ccrAB_{Ent}$ recombinase-like genes in the *E. faecium* DO genome *in silico*, examine the distribution of $ccrAB_{Ent}$ genes in different enterococcal species and the expression of the $ccrAB_{Ent}$ in DO.

Paper III.

- Identify and characterize the conjugative 100-kb chromosomal element supporting transfer of *vanB2*-Tn*5382*/Tn*1549* from *S. lutetiensis* to *E. faecium* and *E. faecalis*.

3. Summary of papers

Paper I

Clustering of polyclonal VanB-type vancomycin resistant *Enterococcus faecium* in a low-endemic area was associated with CC17-genogroup strains harbouring transferable *vanB2*-Tn*5382* containing pRUM-like plasmids with *axe-txe* plasmid addiction systems.
Eva Bjørkeng, Gunlög Rasmussen, Arnfinn Sundsfjord, Lennart Sjöberg, Kristin Hegstad, and Bo Söderquist. ***Submitted***

Background

The Nordic countries have for a long time been considered a low-endemic area with respect to human VRE infections. A clustering of vancomycin resistant *Enterococcus faecium* was detected over a time period of 18 months in November 2002 - April 2004. Seventeen isolates obtained from 15 patients from the University Hospital in Örebro were analyzed. The average age of the patients was 60.7 years (range 37-89), and there were eight male and seven females. Most of the patients had underlying diseases and/or conditions such as diabetes mellitus, renal insufficiency, haematological malignancies, other active malignancies, organ transplant recipient, liver failure, recent surgery, stay in intensive care unit, and immunosuppressive therapy such as high-dose corticosteroids or chemotherapy. During the last three months all patients had been treated with antimicrobial agents such as vancomycin (n=5), cephalosporins (n=9), fluoroquinolones (n=7), aminoglycosides (n=3) or metronidazole (n=7). All of the 15 patients had received prior treatment with at least 2 of these antibiotics. Of all 15 patients 12 seemed to have an infection caused by VRE while the remaining three were only colonized. Positive cultures with VRE were yielded from blood, wounds or abscesses, urine, ascites, a tip from a urinary catheter, and faeces.

Summary of results

Fourteen of the isolates grouped in 3 different PFGE-types whereas the last 3 isolates held unique PFGE-patterns. The isolates had multi-locus sequence-types ST17 (n=5), ST18 (n=3), ST125 (n=7), ST262 (n=1), and ST460 (n=1) which all belong to the successful hospital adapted genogroup previously designated CC17. All 17 isolates contained the virulence genes *EfaAfm* and *sgrA* and most of the isolates harboured *ecbA* and *acm* all associated with CC17. Only a few of the isolates harbored the virulence genes *hyl* and *esp*.

All 17 isolates belonged to the *vanB2* subtype expressing various levels of vancomycin-resistance (MIC 8 to > 256 mg/L) and susceptibility to teicoplanin in accordance with the genotype. The first isolate had a unique PFGE-type and a chromosomally located transferable *vanB2*-Tn*5382* element whereas the other five PFGE-types had Tn*5382* located on pRUM-like plasmids containing the plasmid addiction system *axe-txe*. The resistance pRUM-plasmids were mainly of 120-kb and supported intraspecies *vanB*-transfer by filter mating. In one patient (patient 6) both PFGE type III ST17 and later PFGE-type I ST125 were isolated. Plasmid transfer in this patient seems to have resulted in a more successful clone (PFGE-type I ST125) also isolated from patients 9-11 and 13-15.

Paper II

***ccrAB*$_{\text{Ent}}$ serine recombinase genes are widely distributed in the *Enterococcus faecium* and *Enterococcus casseliflavus* species-groups and expressed in *E. faecium*.**

Eva Katrin Bjørkeng, Girum Tadesse Tessema, Patrick Butaye, Rob Willems, Johanna Ericsson Sollid, Arnfinn Sundsfjord, Eirik Wasmuth Lundblad, and Kristin Hegstad.

re-submitted Microbiology

Background

Recombinases are proteins facilitating the exchange of DNA fragments and are essential for bacterial genome plasticity. The staphylococcal cassette chromosome (SCC) element is a family of mobile genetic elements serving as vehicles for exchange of genetic information in staphylococci, with the support of the recombinase (*ccr*)-genes. The presence, distribution, phylogeny and expression of cassette chromosome recombinase (*ccrAB*$_{\text{Ent}}$) genes, homologus to the staphylococcal *ccrAB* genes, were examined in 421 enterococcal isolates distributed between 13 species from Europe, USA, and Australia.

Summary of results

A total of 118 (28 %) isolates were positive for *ccrAB* genes by PCR and some were confirmed by Southern hybridization and DNA sequencing. The *ccrAB*$_{\text{Ent}}$ genes were present in *Enterococcus faecium* (n=58/216, 27 %), *Enterococcus durans* (n=31/38, 82 %), *Enterococcus hirae* (n=27/52, 50 %), *Enterococcus casseliflavus* (n=1/4, 25 %), and *Enterococcus gallinarum* (n=1/2, 50 %). In eight other species tested, including *Enterococcus faecalis* (n=94), *ccrAB*$_{\text{Ent}}$ genes were not found. Analysis of *ccrAB*$_{\text{Ent}}$ gene sequences (n=38) from five different species showed 94-100 % identity between different enterococcal isolates at the nucleotide level. Both *ccrA*$_{\text{Ent}}$ and *ccrB*$_{\text{Ent}}$ genes formed two distinct clades. *ccrAB*$_{\text{Ent}}$ genes from the human isolates were clustered in clade I, except for *ccrA*$_{\text{Ent}}$ from *E. faecium* E1304. The two phylogenetic trees showed incongruence between the *ccrA*$_{\text{Ent}}$ and *ccrB*$_{\text{Ent}}$ genes for 11 of the animal isolates, which mean that the *ccrA*$_{\text{Ent}}$ and *ccrB*$_{\text{Ent}}$ belonged in different clades for these isolates. Phi test showed no statistical significant evidence for recombination within the sequenced regions of the *ccr* genes.

Expression analysis of *ccrAB*$_{\text{Ent}}$ genes from the *E. faecium* DO strain was performed under normal growth conditions and showed that *ccrAB*$_{\text{Ent}}$ genes were constitutively transcribed as a

bicistronic mRNA. The expression was lower during log- than stationary-phase in relation to total mRNA. *ccrAB*$_{Ent}$ genes were detected both in the hospital derived clonal complex (CC) 17 strains (n=10/25, 40 %) and non-hospital non-CC17 strains (n=6/16, 38 %) of *E. faecium*. Specific sequence types within CC17 were represented by both *ccrAB*$_{Ent}$ -positive and -negative isolates which suggests an introduction or deletion of *ccrAB*$_{Ent}$ in *E. faecium* through several distinct events.

Paper III

Sequence analyses of an approximately 100-kb chromosomal element supporting transfer of *vanB2*-Tn*5382*/Tn*1549* from *Streptococcus lutetiensis* to *Enterococcus faecalis* and *E. faecium*.

Eva K. Bjørkeng, Erik Hjerde, Eirik W. Lundblad, Johanna E. Sollid, Arnfinn Sundsfjord, and Kristin Hegstad. ***In manuscript.***

Background

A 100-kb *vanB2*-containing element has previously been identified in a human clinical isolate of *Streptococcus bovis* and in a veal calf faecal strain of *S. lutetiensis* designated 5-F9. The *vanB2* gene demonstrated to be part of a Tn*5382*/Tn*1549*-like transposon in *S. lutetiensis* 5-F9. The 100-kb element transferred from streptococci to *E. faecalis* and *E. faecium*. However, further transfer between enterococci required a helper plasmid indicating that the streptococcal host may harbour factors necessary for transfer which are not transferred with the 100-kb element or that these factors do are functional within enterococci.

Summary of results

The *E. faecium* transconjugant MM5-F9a constructed by transfer of a 100-kb *vanB2*-Tn*5382*/Tn*1549* element from *S. lutetiensis* 5-F9 to the *E. faecium* recipient BM4105-RF was used to generate a BAC library. An 82-kb contig of the element was sequenced and annotated and showed to contain 67 ORFs. This element was designated Slu*van*. A smaller 9-kb contig with 9 ORFs was also identified and a possible location within the 82-kb contig was found but not verified. The 82-kb contig of Slu*van* was shown to contain *vanB2*-Tn5382/Tn*1549* which had integrated into parts of a streptococcal ICE*Sde*3396-like element. The parts of ICE*Sde*3396-like that were located in Slu*van* included a non-functional abortive infection protein system, some components of a type IV secretion system, a putative mobilization protein of the MobC family and a relaxase. Several other putative streptococcal genes were found located in Slu*van* including a putative virulence gene, a PezTA (Toxin-Antitoxin) system, an IS*L3*-family transposase and a serine recombinase. The 9-kb contig differed from the rest of the Slu*van* element in which the origins of the genes, were most importantly, a transposase of the IS*30* family, a TnpX site-specific recombinase, two relaxases and hypothetical proteins from *Faecalibacterium, Lactobacillus* and *Clostridium*.

The transferable Slu*van* element seems to consist of a mosaic of genes originating from several streptococcal species and other faecal bacteria in addition to the *vanB2*-containing conjugative transposon Tn*5382*/Tn*1549* frequently found in enterococci.

4. General discussion

Enterococci are well known for their ability to acquire and transfer mobile genetic elements, including plasmids and transposons, both within the enterococcal species and to other bacteria such as *Staphylococcus aureus*. The unique ability of the enterococci to gain resistance determinants, in addition to their intrinsic antimicrobial resistances and their capacity to survive the harsh hospital environment, has enabled the enterococci to become a major nosocomial challenge worldwide. Despite the known high genetic transferability of the enterococci, very little is known about the mechanisms of gene transfer, the composition of relevant mobile genetic elements, and their relative contribution to the dissemination of antimicrobial resistance. However, spread of mobile genetic elements has been observed in the gastrointestinal tract from commensal bacteria to enterococci and further dissemination of genes from the enterococci to other more pathogenic bacteria in the bowel flora is a possibility (64). Transfer of resistance genes, especially vancomycin resistance, in hospital settings, and the spread of hospital adapted clones of enterococci has become a problem, even in low-antibiotic use areas such as Scandinavia. The observation that successful clones and selected plasmids may give rise to hospital outbreaks makes the challenge even more complex. To be able to limit outbreaks of VRE, rapid and good methods for detection and surveillance is crucial.

In **paper I** PFGE and MLST were used to investigate the clustering of a polyclonal VanB-type vancomycin resistant *Enterococcus faecium* in Sweden during a period of 18 months. The two methods were used in combination to increase the resolution power. In MLST only seven housekeeping genes are taken into consideration when typing isolates. This gives no opportunity to look at the whole bacterial genome. This is a disadvantage when investigating bacterial species with high recombination frequencies, such as *E. faecium,* and may give a contracted view of the evolution. Transfer of genomic elements, genomic rearrangements and the presence of plasmids will not be observed which may be a key point in understanding the actual epidemiology.

The Örebro clustering of VanB-type VRE (**paper I**) was a good example of the importance of combining MLST and PFGE to enable a better molecular epidemiological resolution. The presence of two major ST-types and single locus variants of these give the impression of less genomic variability than PFGE-typing. By using PFGE the genetic rearrangements may be visible as seen when comparing subtypes within the two major PFGE types (I and III) in this

VRE cluster. However, the combined use of PFGE typing and plasmid analyses indicated that spread may be due to unique clones, bur are more likely due to a prominent plasmid spread among several different clones. A pRUM plasmid, shown to contain both Tn*5382* and a plasmid addiction system (*axe-txe*), was the probable cause of *vanB* dissemination during the first 9 months of the clustering. However, PFGE type I ST125 became prominent during the last 6 months. Thus, a successful combination of a stably maintained pRUM-like plasmid containing *vanB2*-Tn*5382*-like has established in a PFGE type I ST125 background.

All VRE isolates were found to be *E. faecium* carrying the *vanB2* subtype as an integral part of the conjugative transposon Tn*5382*. This is typical for the *vanB2* subtype (51,54,223,250). High rates of faecal *vanB* carriage primarily of the *vanB2* subtype have been described in both community and hospital samples despite the absence of cultivable vancomycin resistant enterococci (65,74). Studies have also shown that both oral and subcutaneous distribution of several antibiotics including vancomycin, metronidazole and cephalosporins are associated with persistent high-density intestinal VRE colonization and colonization of other vancomycin resistant Gram positive microorganisms. However, vancomycin is only administrated orally to treat *Clostidium difficile* enterocolitis and thus play a limited role in the acquisition of VRE in the GI tract of the Örebro patients (3,66,68,102). Studies also report that use of fluoroquinolones may be associated with VRE colonization (37,102). Selective concentrations of intravenous administrated vancomycin in the GI tract has been indicated only when the drug was given over a period of more than 5 days (50). Thus, it may be more likely that other antimicrobial agents have more profound role in the Örebro setting. Most of the patients in this study had received vancomycin, metronidazole, cephalosporins, fluoroquinolones or combinations of two or three of these agents. The spread of the mobile genetic element *vanB2*-Tn*5382* may have been increased by this selective pressure. Tn*5382*-like elements containing *vanB2* have been identified in other bacterial species belonging to the normal gastro-intestinal flora (12,54,64). Tn*5382*-like has been shown to transfer from *Clostridium* to *Enterococcus* in the gut of germ free mice during vancomycin exposure (64). Thus, a selective antimicrobial environment may promote transfer and selection of seldom transfer events of Tn*5382*-like from *Clostridium* or another genus present in the normal gut flora to enterococci.

Transfer of mobile genetic elements are dependent on recombinases and integrases which serves as “scissors and glue” for the excision and integration of the element. In order to

understand the mechanisms for horizontal gene transfer in enterococci it is essential to survey these genes. In **paper II,** genes homologous to the staphylococcal recombinase genes *ccrA* and *ccrB* were identified in *Enterococcus*. In staphylococci these genes are responsible for the excision and integration of the staphylococcal cassette chromosome (SCC), which is generally known for carrying methicillin resistance (SCC*mec*). The enterococcal *ccrAB*$_{\text{Ent}}$ genes resembled the staphylococcal *ccrAB* proteins in several ways, in addition to nucleotide and protein sequence. The *ccrAB*$_{\text{Ent}}$ were linked and constitutively transcribed as a bicictronic mRNA. The genes were not found in all enterococcal species, which is also the case for staphylococci. The surrounding regions of the *ccrAB*$_{\text{Ent}}$ showed a variable pattern, but all tested isolates were positive for a transposase gene. The order of the surrounding genes was not conserved. These observations are consistent with the regions neighbouring the staphylococcal *ccrAB*. SCC may carry the genes conferring methicillin resistance but can also be a tool for genetic exchange of other genes among staphylococcal species (125). Our study showed 6 % and 4 % sequence variation in *ccrA*$_{\text{Ent}}$ and *ccrB*$_{\text{Ent}}$ genes, respectively. This is also in concordance with the *ccrAB* genes which has shown up to 4 % variation within the genes (101).

The *ccrAB*$_{\text{Ent}}$ genes both clustered into two major clades, clade I and II. The majority of the *E. faecium* were localized in clade I, whereas the other enterococcal species tested were mostly localized in clade II. *E. hirae* seems to be more dispersed between the two clades in *ccrA*$_{\text{Ent}}$. Except for *ccrA*$_{\text{Ent}}$ in one isolate of *E. faecium* the *ccrAB*$_{\text{Ent}}$ genes of the human isolates were clustered in clade I, whereas the animal isolates were found in both. The two phylogenetic trees were incongruent for some of the animal isolates, which suggest recombination of the *ccr*$_{\text{Ent}}$ genes. The results are in congruence with previous findings in staphylococci, where the *ccrAB* pair may also divide into different clades.

ccrAB$_{\text{Ent}}$ genes were found only in isolates belonging to the *E. faecium*- and *E. casseliflavus*-species groups. These belong to the same tree-branch in phylogenetic trees based on enterococcal 16S rDNA or *sodA* gene diversity (63,184). Absence of *ccrAB*$_{\text{Ent}}$ in the other species could be explained by the lack of integration sites or by a low number of isolates tested, except for *E. faecalis*. Optionally, the *ccrAB*$_{\text{Ent}}$ genes in these isolates may exhibit such a low sequence identity to the *ccrAB*$_{\text{Ent}}$ genes identified in this study that they are missed using our PCR and hybridisation conditions. The observation that the enterococcal species harbouring *ccrAB*$_{\text{Ent}}$ genes belong to the same branches in the *ccrAB*$_{\text{Ent}}$ phylogenetic tree as in

the 16S rDNA/*sodA* phylogenetic tree may also indicate that these genes are only present in the *E. faecium*- and *E. casseliflavus*-species groups.

The $ccrAB_{Ent}$ genes are located at the very end of a large contig (contig 655) in the sequenced *E. faecium* DO. We do not have the knowledge of the genes downstream of the $ccrAB_{Ent}$, thus it is difficult to predict whether the two genes are involved in the excision and integration of a genetic element as seen in staphylococci. However upstream of the $ccrAB_{Ent}$ genes there are several transposases and hypothetical genes which may be part of an integrative element. However, based on the function of the staphylococcal *ccr*, genes we may speculate that the $ccrAB_{Ent}$ may be involved in recombinations within the enterococcal cell and may be contributing to the enterococcal dynamics of the genome. If this is true we may further speculate that these genes may aid transfer of genes out of the cell when including a helper plasmid containing the factors needed.

If the $ccrAB_{Ent}$ are involved in genome plasticity then why do only some enterococci contain these genes, and why are we not able to see these genes in all isolates belonging to the same species. If these genes are achieved trough “gain” or “loss” we may speculate that there are factors such as enzymes in the genomes of some isolates which may contribute to such events.

In **Paper III** we set out to investigate a *vanB2*-Tn*5382*/Tn*1549* containing ~100-kb conjugative element from *S. lutetiensis*. This particular element has been shown to transfer by conjugation from *S. lutetiensis* to *E. faecium* and *E. faecalis*. However, retransfer of this element within enterococci has not been seen without a helper plasmid. We aimed to search for the content of this genetic element and the genes that may be involved in the transfer process. The sequenced 82 kb element which we designated Slu*van* was shown to contain a large parts homologous to an ICE element – ICE*Sde*3396 previously found in *S. dysgalacticae* (58), in addition to several other putative streptococcal genes, including a toxin-antitoxin system and components of a type IV secretion system. The latter is known to be involved in conjugative transfer in bacteria. Toxin-antitoxin (TA) systems have been known to be part of a stabilising system for plasmids. However there is evidence for these systems also being part of a stabilising system for chromosomal ICE elements in addition to being involved in stress response. We may hypothesize that the TA system is involved in stabilising the Slu*van* element. In addition to the 82 kb Slu*van* element we sequenced a 9 kb element which exact location was not found, however there are indications that this element should be located

within the Slu*van*. This smaller element contains highly repetitive sequences and A+T rich sequences which may present a problem for the assembly and attempts to find the localization. The 9 kb element contained several genes from other faecal bacteria. Slu*van* showed to be a mosaic element of genes from several different species which demonstrate a classical example of the evolution of mobile genetic elements and genomic plasticity. The species which have donated genes to the Slu*van* are typically bacteria colonizing the gastrointestinal tract where there is a high frequency of horizontal gene transfer compared to other localizations, and we may speculate that the Slu*van* element was formed in such an environment.

5. Concluding remarks

Enterococci, mainly *E. faecium* and *E. faecalis*, have during the last decades developed from being considered harmless into one of the most important causes of hospital acquired infections in the western world. MGEs have been seen as major contributors to the evolution of enterococci and especially in hospital adapted strains.

The presented studies are contributions to the process of finding some of the missing pieces needed for understanding this rapid development in enterococci.
We have **in paper I** investigated the molecular evolution of a clustering of vancomycin resistant *E. faecium* at a University Hospital in Sweden. This particular incidence demonstrated the complexity and dynamics of vancomycin resistant *E. faecium* and how MGEs play important roles in the dissemination of VRE in a hospital setting. We were able to investigate enterococcal evolution closely and see the factors involved. The conjugative transposon Tn*5382*/Tn*1549* carrying the *vanB* resistance genes integrated in a particular plasmid background (pRUM) containing a plasmid addiction system hypothesised to contribute to stability and maintenance of the plasmid when transferred into a new strain.

Several factors are needed for transfer of MGEs both inter- and intracellularly. **In paper II** putative *ccr* recombinase genes were found in *E. faecium*. The *ccr* genes have previously only been found in staphylococci where they are involved in the movement of the SCC elements. The enterococcal *ccr* genes (ccr_{Ent}) were shown to be expressed in *E. faecium* and were located in five enterococcal species belonging to the *E. faecium*- and *E. casseliflavus* species groups. Considering the role these genes play in staphylococci we hypothesize that these genes are involved in enterococcal chromosomal dynamics and that recombination may be a driving force for enterococci acquiring these genes.

In enterococci several MGEs have been discovered. One of them is a 100-kb element encoding vancomycin resistance as part of transposon Tn*5382*/Tn*1549.* This 100-kb element which we **in paper III** designated Slu*van*, transferred from *Streptococcus lutetiensis* to *E. faecium* and *E. faecalis*. The Slu*van* element showed a classical example of genetic dynamics by the structure of a *vanB2*-Tn*5382*/Tn*1549* transposon most often found in enterococci, inserted into parts of a streptococcal ICE region constituting about half of the Slu*van* element. The remaining parts of the Slu*van* were made of genes from several streptococcal species and

other bacteria normally located in the gastrointestinal tract of humans and animals. This may lead us to believe that transfer of genes to Slu*van* may have occurred in the gastrointestinal tract.

The enterococcal genome is quite complex and highly dynamic, and there are several factors contributing to the success of the hospital adapted enterococci. In the work presented in this thesis a few new facets has been added to the ever increasing complexity of mobile genetic elements found in the enterococcal genome.

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Paper I

Paper II

Paper III

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