Chapter 1

General introduction

General

Enterococci are widespread in nature and commonly found in alimentary tracts of humans and other animals as well as in soil, water and food. In human adults, enterococci account for 1% of the intestinal microflora (90). Enterococci are facultative anaerobic, catalase-negative gram-positive cocci that occur singly, in pairs or as short chains. The optimum growth temperature is at 35°C, but the growth temperature can range from 10 to 45°C. All enterococci grow in broth containing 6.5% NaCl and hydrolyze esculin in the presence of 40% bile salts (bile-esculin medium) (32). Among the enterococcal species, *Enterococcus faecalis* and *Enterococcus faecium* are the most commonly encountered species in human faeces (67,74). Although enterococci were for years considered as harmless inhabitants of the gut flora, they are now among the leading causes of nosocomial infections of humans. Originally, the majority of clinical infections like bacteraemia, endocarditis, urinary tract and surgical wound infections were caused by *E. faecalis* (80-90%), while *E. faecium* was found much less frequently (isolated in almost 10% of the infections) (40,45,58,76,85). However, the ratio *E. faecalis* to *E. faecium* infections changed in favor of *E. faecium* in the US in late 1990s (66,75,103). Other enterococcal species which occasionally cause infections in humans are *Enterococcus durans*, *Enterococcus avium*, *Enterococcus casseliflavus*, *Enterococcus hirae*, *Enterococcus gallinarum*, *Enterococcus raffinosus* and *Enterococcus muntdii* (40,67,85).

Antimicrobial resistance in enterococci

Antimicrobial resistance in enterococci can be divided in two classes, intrinsic resistance and acquired resistance (Table 1). Intrinsic resistance is due to either lack of target sites for the antibiotic drug or insufficient penetration of the drug to the intracellular target site. For example, enterococci don't posses penicillin binding proteins (PBPs), which bind cephalosporins with high affinity (37,73). Furthermore, as a result of poor permeability of the enterococcal cell wall, aminoglycosides are unable to reach their target site (64). More important in the emergence of resistance is the ability of enterococci to acquire resistance through either chromosomal mutations or genetic exchange of mobile elements like transposons or plasmids (123). For example, mutations in the DNA gyrase or topisomerase genes reduce the affinity of quinolones for these genes (96). In *E. faecalis* and *E. faecium* many different transposons and plasmids have been identified conferring resistance to a wide variety of antimicrobial drugs, including vancomycin, streptomycin, kanamycin, tetracycline, gentamycin and erythromycin (113). These resistance genes are present in combinations on large

composite elements or as single genes. It has been hypothesized that *E. faecium* plays a central role in the acquisition, conservation and transfer of antimicrobial resistance genes among bacteria (116).

Ampicillin resistance

Enterococci are intrinsic resistant to ß-lactam antibiotics due to low affinity of their penicillin binding proteins (PBP) to ß-lactam agents (Table 1) (34,48,122). They possess at least five and sometimes more than nine different PBPs (121). The level of intrinsic resistance differs among the ß-lactam antibiotics. Generally, penicillins (e.g. ampicillin) have the highest activity, carbapenems slightly lower and cephalosporins have the lowest activity (48,67). Except for a few ß-lactamase producing *E. faecalis* isolates identified in the US (67,69,70), high level ampicillin resistance is mainly found in *E. faecium* isolates derived from clinical specimens. High level ampicillin resistance in *E. faecium* is due to either alterations by mutations in PBP5 resulting in even lower affinity for ampicillin (5,56,86,126) or by overproduction of PBP5 (33,51,126). In 2000, a novel mechanism of ß-lactam resistance has been described in a laboratory mutant of *E. faecium* not involving PBPs (61). In this strain cross-linking during cell wall elongation occurred by a LD-transpeptidation, which by-passes the usual ß-lactam-susceptible DD-transpeptidation (61,62). So far, no clinical isolates with this type of resistance have been reported.

In the US, the first reports on increase of infections and outbreaks due to ampicillin resistant *E. faecium* (AREfm) were published in the early 1980s (22,41,45,67). In several European countries a similar increase of AREfm has been observed, but with a 10 year delay (27,35,53,102). No data are available whether a similar increase of AREfm infections has occurred in the Netherlands. Such an increase will have clinical implications for the treatment of infections and will lead to increased use of vancomycin with the threat of increased selection of vancomycin resistant *E. faecium* (VREF).

Glycopeptide resistance

The first clinical isolates of vancomycin-resistant enterococci (VRE, both *E. faecium* and *E. faecalis*) were detected in Europe in 1986 (54,104). Since then, VRE have rapidly spread all over the world. Especially in the US, VRE prevalence rates increased from 0% in 1989 to 28.5% in 2003 (1,3). Consequently, in the early 1990s VRE were already the second most common nosocomial pathogen in

Table 1. Antimicrobial susceptibility of enterococci

	Antibiotic	Species	Mechanism of resistance
Intrinsic resistance	ß-lactams: - penicillins (low level) - carbepenems (moderate level) - cephalosporins (high level)	all enterococci	Low afinity penicillin binding proteins (PBP)
	Aminoglycosides (low level)	all enterococci	Inefficient uptake
	Aminoglycosides (moderate level)	*E. faecium*	Production of chromosomal AAC(6')Ii enzyme
	Trimethoprim-sulfamethoxazole	all enterococci	Inefficacy in vivo due to assimilation of exogenous folates
	Lincosamides and streptogramins A	*E. faecalis, E. avium, E. gallinarum, E. casseliflavus*	Putative efflux
	Glycopeptides (low level)	*E. gallinarum, E. casseliflavus*	Production of D-Ala-D-Ser ending peptidoglycan precursors
Acquired resistance	Ampicillin (high level)	*E. faecium, E. hirae*	Overproduction or alterations of PBP5
		E. faecalis	ß-lactamase (rare)
	Aminoglycosides (high level)	*E. faecalis, E. faecium, E. gallinarum, E. casseliflavus*	Aminoglycosides modifying enzymes e.g. AAC(6')-APH (2")
	Macrolides	most enterococci	Ribosomal methylation
	Chloramphenicol	*E. faecium, E. faecalis*	CAT encoding enzymes
	Tetracycline	*E. faecium, E. faecalis*	Modification of ribosome protein
	Quinolones	*E. faecium, E. faecalis*	Alterations in DNA gyrase and Topoisomerase IV
	Glycopeptides (high level)	*E. faecium, E. faecalis*	Precursor modification

the US (36) and became endemic in many hospitals (68). In Europe, VRE prevalence rates in hospitals are rising since the year 2000 (2,88).

In 1988, French researchers discovered that glycopeptide resistance was plasmid-mediated (54). A few years later, the same group identified that vancomycin resistance was located on a small mobile genetic element labeled transposon Tn*1546*, encoding the VanA phenotype (8). Furthermore, the same year a second phenotype, VanB, was identified on a different mobile element, labeled transposon Tn*1547* (79). Due to these self-transferable transposons and plasmids, dissemination of vancomycin resistance is not only the result of clonal expansion of resistant strains, but also of horizontal gene transfer between strains and even species. Already at that time the potential transfer of these easily movable resistance genes to more pathogenic gram-positive bacteria like methicillin resistant *Staphylococcus aureus* (MRSA) was feared. At that time vancomycin was the last antibiotic to treat patients with MRSA infections. The first high-level vancomycin resistant *S. aureus* (VRSA) was identified in Michigan (US) in 2002 (17). Up till now, five additional VRSA isolates have been identified (6). In one case a vancomycin-resistant *E. faecalis* was a likely source of the *vanA* gene cluster (114), while VRSA may have acquired the *vanA* gene cluster from an *E. faecium* isolate in another case (115).

Vancomycin, as well as teicoplanin, belong to the group of glycopeptide antibiotics. These antibiotics bind with high affinity to the D-alanyl-D-alanine (D-Ala-D-Ala) C-terminus of peptidoglycan pentapeptide precursors and block the addition of pentapeptide precursors by transglycosylation to the nascent peptidoglycan chain, thereby preventing subsequent cross-linking catalyzed by transpeptidation (11,80).

Nowadays, six types of vancomycin resistance have been described in enterococci (Table 2, adapted from Courvalin (23)). Of the six phenotypes, the

VanA and VanB type of glycopeptide resistance are most frequently reported (19,82,87). Sequencing and functional analysis of the genes encoded by *vanA* and *vanB* gene clusters revealed that glycopeptide resistance is due to enzymes that encodes for (i) synthesis of low-affinity precursors, in which the C-terminal D-Ala residue is replaced by D-lactate (D-Lac) or D-serine (D-Ser), thus modifying the vancomycin-binding target and (ii) for elimination of the high-affinity precursors that are normally produced by the host, thus removing the vancomycin-binding target (8,9,15,81).

Clinical epidemiology of vancomycin-resistant enterococci

Although the first clinical VRE were detected in Europe, a remarkable difference exists in the epidemiology of VRE between Europe and the US. In the US colonization of hospitalized patients with VRE rapidly increased in the 1990s, up to the current endemic levels in many hospitals. In parallel, nosocomial VRE infection rates increased as well, while colonization in healthy people appeared to be absent. In Europe, prevalence rates in hospitals have remained much lower and only started to rise since the year 2000 (2,88). It has been suggested that the rapid increase of VRE in the US was due to 5-10 fold higher use of vancomycin in the US compared to five European countries, including France, Italy, Germany, United Kingdom and the Netherlands, which have, in total, a similar number of inhabitants (13,49).

In the Netherlands, VREF outbreaks have been reported in three different hospitals. In all cases, intervention measurements were successful in controlling the outbreak (63,100,112). In contrast to the US where VRE is restricted to hospitals, a large community reservoir of VRE among healthy people and farm animals exists in Europe, which is most probably linked to massive use of avoparcin in animal husbandry (95,105,107-111). Avoparcin is a glycopeptide antibiotic, like vancomycin, and has been used as growth promoter in the agricultural industry since the 1970s in most European countries. Since the presence of a large community reservoir of VRE was thought to pose a threat for VRE transmission into hospitals either by enterococcal strains harboring the vancomycin resistance genes or by horizontal transfer of Tn*1546* from animal strains to human strains, the European Union banned the use of avoparcin in April 1997. Since then, prevalence rates of VRE colonization among farm animals and human volunteers have decreased (4,50,106).

Table 2. Level and type of resistance to vancomycin in enterococci

Resistance	Acquired					Intrinsic
	High level	Variable level	Moderate level	Low level		Low level
Type	VanA	VanB1/B2/B3	VanD	VanE	VanG	VanC1/C2/C3
MIC (mg/L)						
Vancomycin	64-1000	4-1000	64-128	8-32	16	2-32
Teicoplanin	16-512	0.5-1	4-64	0.5	0.5	0.5-1
Conjugation	Positive	Positive	Negative	Negative	Positive	Negative
Mobile element	Tn*1546*	Tn*1547* Tn*1549* -Tn*5382*	Unknown	Unknown	Unknown	Not applicable
Species	*E. faecium* *E. faecalis* *E. gallinarum* *E. casseliflavus* *E. avium* *E. durans* *E. mundtii* *E. raffinosus* *S. aureus*	*E. faecium* *E. faecalis* *S. bovis*	*E. faecium* *E. faecalis*	*E. faecalis*	*E. faecalis*	*E. gallinarum* *E. casseliflavus* *E. flavescens*
Expression	Inducible	Inducible	Constitutive	Inducible	Inducible	Constitutive Inducible
Location	Plasmid Chromosome	Plasmid Chromosome	Chromosome	Chromosome	Chromosome	Chromosome
Modified target	D-Ala-D-Lac	D-Ala-D-Lac	D-Ala-D-Lac	D-Ala-D-Ser	D-Ala-D-Ser	D-Ala-D-Ser

Molecular epidemiology of *E. faecium*

In the late 1990s spread of multi resistant enterococci mainly considered *E. faecium* (19,66,75). Molecular typing methods are essential to determine, in detail, the epidemiology of *E. faecium* and its resistance traits, and to identify outbreaks in hospitals. Furthermore, the recognized presence of *E. faecium* in different ecological niches created an additional need to determine its population structure and genetic evolution.

The first molecular typing methods for enterococci were based on the analysis of plasmid profiles, including plasmid composition and restriction endonuclease analysis of specific plasmids (59,125). In the late 1980s, a new typing method was developed based on analysis of chromosomal DNA restriction endonuclease profiles by pulsed field gel electrophoresis (PFGE) (18), which was soon adapted for enterococci (39,71). Until recently, many laboratories considered PFGE as the "Gold Standard" typing method. However, this method is only suitable to trace transmission of strains in hospital outbreaks. Interlaboratory data exchange is problematic as there is a lack of standardized conditions for electrophoresis and criteria for interpreting PFGE banding patterns (31).

To study the genetic relatedness between epidemiologically nonrelated VREF, amplified-fragment length polymorphism analysis (AFLP) was developed, which allows analysis of polymorphisms among small restriction fragments (119). With this technique particular *E. faecium* genogroups appeared associated with particular hosts, like pigs, calves, poultry and humans. Most importantly though, there were genetic differences between VREF isolated from feces of nonhospitalized persons without infection (genogroup A) and hospital isolates from fecal origin or from infected body sites like blood (genogroup C). Other studies confirmed the existence of these genogroups among vancomycin susceptible *E. faecium* (VSEF) isolates originating from different sources (14,16,21,46,47). Furthermore, AFLP exhibited a discriminatory power comparable to PFGE and discriminated outbreak related isolates from other isolates (46).

Although AFLP appeared to be a robust and fast typing method generating reproducible data within a given laboratory, this method was less suitable for data exchange between different laboratories and for studying the global epidemiology and the evolution of *E. faecium*. For this, a typing method is required, which generates unambiguous data suitable for the development of web-based databases. In 1998, multi locus sequence typing (MLST) was proposed for *Neisseria meningitides* with the aforementioned properties (60). MLST is based on identifying alleles from DNA sequences of internal fragments of housekeeping genes resulting in a numeric allelic profile. Each profile is assigned a sequence type (ST). In addition, an Internet site with the possibility for data exchange was

developed (www.mlst.net), which currently, together with www.pubMLST.org, contains MLST schemes of 38 different bacterial species, including *E. faecium* (43) and *E. faecalis* (84). MLST of 123 isolates, including VREF and VSEF originating from human (nonhospitalized, clinical and hospital outbreak) and animal sources from various countries, confirmed the genogroups as determined by AFLP, including the hospital related genogroup C (43). MLST typing of the hospital related isolates revealed that the outbreak isolates clustered in a sub-population designated lineage C1, which was subsequently confirmed in many studies performed world wide (12,21,25,52,55,94,117).

A more detailed study on the population structure and evolution of *E. faecium* is needed for better understanding of the worldwide epidemiology of *E. faecium*. Furthermore, the recognition of hospital adapted *E. faecium* subpopulation created a need for rapid identification and typing of *E. faecium*, in order to better target infection control measures in hospitals.

Molecular characterization of DNA polymorphisms in the *vanA* gene cluster of Tn*1546* in isolates from humans and animals revealed high degrees of DNA polymorphisms due to point mutations, deletions and insertions of different insertion sequences e.g. IS*1216V* and IS*1251* (26,44,89,110,120,124). Identical Tn*1546* variants among VREF recovered from farm animals and humans were identified, which could be a result of either colonization of animal-derived VREF in humans or transfer of Tn*1546* from animal VREF to human enterococcal isolates.

Virulence determinants in *E. faecium*

In contrast to *E. faecalis*, little is known about virulence of *E. faecium* (38). Many clinical isolates of *E. faecium* are resistant to phagocytosis by neutrophils (7), which might be considered a pathogenic property.

Other putative virulence factors are the secreted antigen SagA (99) and a surface exposed antigen designated Acm (72). Both antigens are able to bind to human extracellular matrix proteins. In contrast to the specific collagen-binding adhesin Acm, SagA has broad-spectrum binding to fibrinogen, collagen type I, collagen type IV, fibronectin and laminin. Although the exact role of both antigens in the pathogenesis of *E. faecium* infections is not well understood, adherence to extracellular matrix proteins might be the first step in colonization of the host.

In *Caenorhabditis elegans*, *E. faecium* produces hydrogen peroxide at levels that cause cellular damage (65). Additional studies are necessary to investigate the relevance of hydrogen peroxide production by *E. faecium* in the human host.

Gelatinase is an extracellular zinc mettaloprotease, which contributes to *E. faecalis* virulence in some animal models and is regulated through a cell-density-dependent manner by the *fsr* operon (77,78). Recently, dissemination of gelatinase was also described in *E. faecium* (57).

The identification of a hospital adapted *E. faecium* subpopulation raised the question whether this population contained specific traits, which contribute to increased abilities in spread and/or infections among hospitalized patients. Screening of human and animal isolates for the presence of the *esp* gene, which has been associated with increased virulence and biofilm formation in *E. faecalis* (92,93,97,98,101), revealed that in *E. faecium* the *esp* gene is restricted to hospital-derived isolates belonging to the hospital subpopulation (10,13,20,29,30,42,118). Interestingly, in *E. faecalis* this gene is contained on a pathogenicity island (91) and was identified among clinical and animal derived isolates (24,28,42,93). Analysis of the up- and downstream regions of the *E. faecium esp* gene are necessary to determine whether, as in *E. faecalis*, the *esp* gene in *E. faecium* is contained on a pathogenicity island.

In 2003, another putative virulence gene, hyaluronidase (hyl_{Efm}) with homology to the same gene in *Streptococcus pyogenes* and *Streptococcus pneumoniae* was described to be enriched among clinical *E. faecium* isolates (83). Although the presumed function of hyaluronidase in *E. faecium* is still unknown, in *S. pneumoniae* it is suggested that hyaluronidase may contribute to the invasion of the nasopharynx.

Conclusion and aims of the thesis

The recognition of a hospital adapted *E. faecium* subpopulation, which had apparently spread worldwide, and which was, amongst others, characterized by the presence of the *esp* gene, lead to the following research questions:

(i) Can we develop (and validate) rapid identification and typing schemes for *E. faecium*?

For this the accuracy to identify enterococci of current phenotypic tests, automated microbiology systems, API system and a newly developed identification method designated Raman spectroscopy were evaluated (chapter 2). In addition a rapid, robust and cheap typing method (MLVA) allowing the study of genetic relatedness and epidemiology of *E. faecium* with the possibility of interlaboratory data exchange via Internet was developed (chapter 3) and compared to the currently considered "Gold standard" for enterococcal genotyping Pulsed Field Gel electrophoresis (chapter 4).

(ii) Is the *esp* gene in *E. faecium* contained on a pathogenicity island as it is in *E. faecalis* (chapter 5)?

(iii) What is the population structure of *E. faecium* and can we determine evolutionary steps that have lead to the hospital adapted subpopulation (chapter 6)?

(iv) What is the epidemiology of ampicillin resistant *E. faecium* in our hospital (UMCU) (chapter 7) and in the Netherlands (chapter 8)?

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