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″ Enterococcal surface protein (Esp) – epidemiology and transfer between enterococcal strains″

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Abbreviations

aa	amino acids
AFLP	Amplified fragment length polymorphism
AMP	Ampicillin
AS	Aggregation substance
Bap	Biofilm-associated protein
BHI	Brain heart infusion
bp (kbp)	base pairs (kilobase pairs)
CLI	Clindamycin
CMP	Chloramphenicol
Cyl	Cytolysin
D-Ala	D-Alanine
D-Lac	D-Lactate
DNA	Deoxyribonucleic acid
EfaA	*Enterococcus faecalis* endocarditis antigen
Ent	Enterocin
ERY	Erythromycin
Esp*(fs; fm)*	Enterococcal surface protein (*faecalis*; *faecium*)
FUS	Fusidic acid
Gel	Gelatinase
GEN	Gentamicin
GRE	Glycopeptide-resistant enterococci
Hyl	Hyaluronidase
ICUs	Intensive care units
LMP-Agarose	Low-melting-point agarose
M	Molar
MIC	Minimal inhibitory concentration
min	minutes
MLST	Multilocus sequence typing
ORF	Open reading frame
OTE	Oxytetracycline
PBPs*(fm)*	Penicillin-binding proteins*(faecium)*
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMNs	Polymorphonuclear leukocytes
RAPD	Random amplification of polymorphic DNA
RIF	Rifampicin
STR	Streptomycin
TEI	Teicoplanin
TMP/SMX	Trimethoprim-sulphamethoxasole
U	Units (enzymatic activity)
UTIs	Urinary tract infections
$VAN^{R\ (S)}$	Vancomycin resistant (susceptible)
VRE	Vancomycin-resistant enterococci
VREF	Vancomycin-resistant *Enterococcus faecium*
VSEF	Vancomycin-susceptible *Enterococcus faecium*

Figures and Tables

1. Introduction

Enterococcus was first described in 1899 as a new streptococcus of enteric origin. The likely intestinal source of a similar gram-positive coccus recovered from a patient with endocarditis in 1906 determined the choice of name "*Streptococcus faecalis"*. In the mid 1930s, streptococci were classified into four divisions: pyogenic, viridans, lactic, and enterococcus, based on biochemical and physiological properties. Based on the serological scheme originated by Lancefield in the early 1930s, most enterococci were found to react with group D antiserum. The classification of enterococci as "group D streptococci" continued until the mid 1980s, when nucleic acid relatedness studies showed that enterococci were sufficiently distant from streptococci to be included in a new genus, named *Enterococcus* (Murray, 1990; Murray & Weinstock, 1999)

Enterococci are gram-positive cocci, facultatively anaerobic organisms, that grow in short chains, in pairs, or as single cells. Particular characteristics of enterococci used in their identification include growth in media containing 6.5% sodium chloride, survival at extreme pH values (up to 9.6) and growth at temperatures of 10-45°C, and for the most part, survival at 60°C for 30 minutes. Their ability to hydrolyze esculin in the presence of bile salts was also used in their identification (Murray, 1990; Facklam et al., 2002).

1.1 Natural habitats of enterococci

Enterococci are minority members of the bacterial community inhabiting the large bowel of humans, making up no more than 1% of the intestinal microflora of an adult. They are found in the intestine of almost all animals. Among the 24 species of enterococci that have been described, *Enterococcus faecalis* and *Enterococcus faecium* appear to be the most common species (Facklam et al., 2002). Although the oral cavity and vaginal tract can become colonized, enterococci are recovered from these sites in less than 20% of cases, which is important in order to differentiate between colonization and real infection when enterococci are isolated from related specimens (Klare et al., 2001; Tannock & Cook, 2002).

Enterococci can be readily recovered from vegetation and surface water, probably because of contamination by animal excrements or untreated sewage (Huycke et al., 1998). *E. faecium* is rather frequent in chicken and pigs. The pigmented species *E. casseliflavus* and *E. mundtii* can also be associated with plants. *E. durans* occurs both in humans and poultry. *E. gallinarum* and *E. avium* seem to be specific to poultry, but *E. avium* can be isolated from other farm animals as well (Klare et al., 2001).

1.2 Clinical infections

Enterococci have been recognized as being potentially pathogenic for humans since the early 1900s, when they were well established as a cause of endocarditis and urinary tract infections. Members of the species *E. faecalis* were known to be a common cause of nosocomial infections by the early 1980s (Murray, 2000). Enterococci are now the second most common cause of hospital-acquired infection after *Escherichia coli*. They primarly infect patients who have been hospitalized for prolonged periods and have received multiple courses of antibiotics. The increase in enterococcal infections in the hospital setting coincides with enhanced use of the third-generation cephalosporins and other broad-spectrum antibiotics (e.g., quinolones) with little or no activity against enterococci (Klare et al., 2001; Murray & Weinstock, 1999).

Of the 24 enterococcal species, only *E. faecalis* and *E. faecium* commonly infect humans in detectable numbers. *E. faecalis* is isolated from approximatively 80-90% of human enterococcal infections, and *E. faecium* from most of the rest. Infections with other enterococcal species are rare. Urinary tract infection (UTI) is the most common enterococcal infection in humans. Most of these infections are nosocomial or associated with structural abnormality or instrumentation of the urinary tract. The next most common sites from which enterococci are isolated are wounds (predominantly intraabdominal and pelvic), where they are usually part of mixed aerobic and anaerobic flora (Murray, 1998; Ostrowski & Eliopoulos, 1999). Enterococci cause an estimated 5 to 15% of bacterial endocarditis. As with other enterococcal infections, most isolates are *E. faecalis*. Enterococcal bacteremia is commonly encountered among hospitalized patients, and the organisms usually arise from UTIs, intraabdominal or pelvic sepsis, or are associated with indwelling intravascular catheters (Ostrowski & Eliopoulos, 1999; Hancock & Gilmore, 2000). Other enterococcal infections include infections in neonates (meningitis and bacteremia), central nervous system infections in adults and rarely osteomyelitis and pulmonary infections (Murray, 1998).

1.3 Resistance of enterococci to antimicrobial agents

The enterococci have become a focus of attention because of their increasing role in nosocomial infections and because of their increasing resistance to commonly used antibiotics. Resistance allow enterococci to survive in the hospital setting where antibiotics are used, providing a selective advantage for resistant organisms (Murray, 1990). Enterococci have naturally occuring or intrinsic resistance to many clinically useful antibacterial drugs, including

cephalosporins, penicillinase-stable penicillins, low levels of aminoglycosides and lincosamides, polymyxins, quinolones such as ciprofloxacin, partly to the glycopeptide vancomycin (*E. gallinarum, E. casseliflavus/E. flavescens)*, and in the case of *E. faecalis*, resistance to quinupristin/dalfopristin (Murray, 1990; Tannock & Cook, 2002). Unlike acquired resistance and virulence traits which are usually transposon or plasmid encoded, intrinsic resistance genes appear to reside on the chromosome. These genes are typically non-transferable (Huycke et al., 1998).

The low-level resistance to growth inhibition by β-lactams, particularly cephalosporins and semisynthetic penicillinase-resistant penicillins (e.g., oxacillin, methicillin) appears to be due to low affinity of the penicillin-binding proteins for β-lactams. *E. faecalis* is 10 to 100 times less susceptible to penicillin than are most streptococci (MICs for *E. faecalis* 2-8 μg/ml) whereas *E. faecium* is even more resistant than *E. faecalis* (MICs between 16-32 μg/ml or higher), due to the lower affinity of PBPs (Huycke et al., 1998; Klare et al., 2001). In strains of *E. faecium*, the low affinity PBP is reffered to as $PBP5_{fm}$ (Tannock & Cook, 2002; Shepard & Gilmore, 2002). In addition to the high MICs, enterococci are typically tolerant to all β-lactams; that is, they are not killed by concentrations many fold higher than the MICs (Murray, 1990; Murray, 1998). Because of this tolerance, combinations of cell-wall active agents (penicillin and vancomycin) and aminoglycosides (streptomycin and later gentamicin) were used to obtain the bactericidal synergy required to treat more serious enterococcal infections (Straut, 1997; Ostrowski & Eliopoulos, 1999; Shepard & Gilmore, 2002). All enterococci have intrinsic low-level resistance to aminoglycosides. The mechanism of low-level aminoglycoside resistance among *E. faecalis* strains appears to be due to low uptake of these agents. Addition of either a glycopeptide (e.g., vancomycin) or a β-lactam (e.g., ampicilin) in combination with an aminoglycoside greatly enhances the intracellular uptake of the aminoglycoside. In *E. faecium,* the MICs of some aminoglycosides (tobramycin, kanamycin) are even higher than those of *E. faecalis*. These strains produce low-levels of an aminoglycoside 6'-acetyltransferase (6'-AAC, encoded by a chromosomal gene) and are therefore resistant to synergistic combination of a cell-wall active agent and these aminoglycosides (Straut, 1997; Klare et al., 2001). The genus can also acquire genes that encode enzymes for high-level resistance to aminoglycosides.

Another characteristic feature of enterococci is their resistance to lincosamides (clindamycin and lincomycin). In addition, while the in vitro testing would suggest that many enterococci are susceptible to trimethoprim-sulfamethoxazole (TMP/SMX), this agent has no effect in animal models of infection. Thymidine and *p*-amino-benzoic acid are antagonists of this antibiotic combination and enterococci can use them when supplied (Klare et al., 2001).

Streptogramin antibiotics (e.g., quinupristin/dalphopristin) have good activities against *E. faecium*, but *E. faecalis* is naturally resistant (Thal & Zervos, 1999).

Low-level vancomycin resistance (MICs between 4 and 32µg/ml) but teicoplanin suscetibillity is an intrinsic property of most isolates of *E. gallinarum, E. casseliflavus/E. flavescens* (VanC phenotype) (Murray, 1998; Tannock & Cook, 2002).

In addition to natural low-level resistance to many agents, enterococci have evolved increased resistance either by the acquisition of antibiotic resistance genes on plasmids or transposons from other organisms, or by spontaneous mutations in the preexisting DNA that give the enterococci an enhanced level of resistance (Klare et al., 2001; Kak & Chow, 2002). Acquisition of new DNA may occur by transformation, transduction, but most often by conjugation involving broad- and narrow- host range plasmids, and conjugative transposons (Clewell, 1990; Murray, 1998).

High-level resistance to β-lactams has been associated with production of the enzyme β-lactamase in a few cases reported from U.S. involving *E. faecalis* strains. The vast majority of *E. faecium* with high or very high levels of resistance produce, respectively, either increased levels of an alternate PBP5 or PBP5 with decreased affinity for β-lactams, as a result of a gene mutation (Murray, 1990; Straut, 1997; Shepard & Gilmore, 2002).

Aminoglycosides are inhibitors of bacterial protein biosynthesis. High level resistance is due to acquisition of genes that encode aminoglycoside-modifying enzymes (aminoglycoside nucleotidyl transferases, ANT; aminoglycoside phosphotransferases, APH; aminoglycoside acetyltransferases, AAC). High-level resistance to streptomycin is due to ribosomal resistance to the drug or enzymatic modification of the drug by an ANT. High level resistance to kanamycin without high level resistance to gentamicin is due to production of a 3'-APH. The enzyme eliminates also the synergism between a cell-wall active agent and amikacin. Additionally, high level resistance to gentamicin, kanamycin, amikacin, tobramycin and to all aminoglycosides, except streptomycin, results from a bifunctional enzyme AAC(6')/APH(2") that possesses both acetylating and phosphorilating activity (Straut, 1997; Murray, 1998; Ostrowski & Eliopoulos, 1999; Klare et al., 2001). High-level resistance to aminoglycosides and/or resistance to penicillins leads to a failure of the synergism between these two groups of agents.

Resistance to glycopeptides is phenotypically and genotypically heterogenous. The biochemical mechanism of glycopeptide resistance in enterococci involves modification of the target via a switch in the cell wall composition from pentapeptide ending in D-alanyl-D-alanine (D-Ala-D-Ala) to one ending in D-Ala-D-lactate (D-Lac) or a D-Ala-D-serine (D-Ser). Six different gene clusters mediating glycopeptide resistance have been described in enterococci:

vanA, vanB, vanC, vanD, vanE, vanG (Gholizadeh & Courvalin, 2000; McKessar et al., 2000). Five of these are known to be acquired traits, while one, *vanC*, is an intrinsic property of motile enterococci (*E. gallinarum, E. casseliflavus/E. flavescens*). The *vanA*-type is characterized by inducible high level resistance to both vancomycin and teicoplanin. The *vanA* cluster of nine genes is found on the transposable genetic element Tn*1546* in *E. faecium*. VanB-type strains have variable levels of inducibile resistance to vancomycin, but are susceptible to teicoplanin. VanB resistance determinants are located on Tn*1547* transposon, which can be part of larger mobile elements on the chromosome, but can also be located on plasmids. VanD resistance was found only in *E. faecium* strains, is expressed constitutively and is not self-transferable to other enterococci. VanC-type resistance is a characteristic of motile enterococci, and three species-specific *vanC* genes have been described: *vanC*-1 in *E. gallinarum*, *vanC*-2/*vanC*-3 in *E. casseliflavus/E. flavescens* (Straut, 1997; Gholizadeh & Courvalin, 2000; Shepard & Gilmore, 2002). The *vanE* gene has recently been described in an *E. faecalis* clinical isolate, which is resistant to low levels of vancomycin and susceptible to teicoplanin (Fines et al., 1999). Similarly, the *vanG* locus consisting of seven open reading frames, confers a moderate level of resistance to vancomycin and full susceptibility to teicoplanin. The seven genes of the *vanG* operon are organized differently from those of all other enterococcal *van* loci (McKessar et al., 2000).

Resistance to macrolide (erythromycin), lincosamide (clindamycin) and streptogramin B antibiotics (MLS_B phenotype) is encoded by the *ermB* gene cluster (as part of Tn*917*). The gene is often encoded on conjugative plasmids together with the *vanA* gene cluster and *vatD* gene (Hammerum et al., 2001; Kak & Chow, 2002) and recently a linkage of *ermB* gene and *vatE* gene on a conjugative plasmid was demonstrated (Werner et al., 2000). Streptogramin antibiotics are inhibitors of protein synthesis, and the new streptogramin combination of quinupristin and dalfopristin (QD) is used to treat multidrug-resistant enterococci. *E. faecalis* is intrinsically resistant to streptogramins, whereas in *E. faecium* resistance to streptogamin A is mediated by the streptogramin acetyltransferases SatA (VatD) and SatG (VatE). Resistance to QD requires resistance mechanism to both streptogramin A and streptogramin B (Klare et al., 2001; Kak & Chow, 2002).

1.4 Epidemiology of vancomycin-resistant enterococci (VRE)

The glycopeptide antibiotics are used to treat serious infections caused by multidrug resistant enterococci, including resistance to ampicillin and/or to aminoglycosides, or if the

patient has an allergy to penicillins. The first reports on vancomycin-resistant enterococci (later classified as VanA type of resistance) involved strains of *E. faecium* isolated from patients in France and England in 1986 (Murray, 2000) and soon after a VanB *E. faecalis* clinical isolate was reported from St. Louis in the United States (Mundy et al., 2000). Since their initial recovery, VRE have been found in many other countries, including Australia, Canada, Belgium, Denmark, Germany, The Netherlands, Spain and Sweden (Cetinkaya et al., 2000). Different European hospitals have reported VRE prevalence rates of 2-3.5% (Endtz et al., 1997; Gordts et al., 1995; Wendt et al., 1999). In Germany, vancomycin-resistant *E. faecium* was first isolated from peritoneal fluid in 1987 (Lutticken & Kunstmann, 1988). In 1992, Klare et al. have reported on the identification of four VanA-*E. faecium* strains from 52 *E. faecium* isolated from infected patients in different intensive care units (ICUs) in Berlin. In the U.S., VRE have been predominately a nosocomial problem and a number of hospital outbreaks of infection or colonization have been reported with both VanA and VanB isolates (Livornese et al., 1992; Handwerger et al., 1993; Boyle et al., 1993; Boyce et al., 1994). In the U.S., the percentage of the enterococcal isolates that were resistant to vancomycin in ICUs increased from 0.3% in 1989 to 25.2% in 1999 (Harbarth et al., 2002). In Germany, the first report of a hospital outbreak with VREF (vancomycin-resistant *E. faecium*) mainly in nephrologic patients has been mentioned from the City Hospital of N.(Bavaria). There has also been a preliminary report on the transmission of 9 VREF at another specialized German hospital. In 2000, an outbreak of VREF was reported at a Children's Hospital in Hamburg (Elsner et al., 2000a). Risk factors associated with colonization or infection by VRE include advanced age, abdominal surgery, dialysis, severe underlying illness and prior antibiotic use, including vancomycin, cephalosporins, fluoroquinolones and anti-anaerobic agents (Cetinkaya et al., 2000; Harbarth et al., 2002). Aditionally, longer duration of hospitalization, proximity to a patient colonized with VRE, care by hospital staff assigned to patients with VRE, and factors such as diarrhea, that increase the contamination of the environment or medical instruments, may also favor nosocomial transmission of VRE (Shepard & Gilmore, 2002).

The epidemiological parameters that contributed to VRE dissemination seem distinct for the U.S. and Europe. In Europe, suspected reservoirs related to animal husbandry and now community ecologies appear to be primary sources of VRE (Bates et al., 1994). The use of the glycopeptide avoparcin as a growth promoter in animal feeds was indicated as the major contributor to vancomycin resistance (Klare et al., 1995a/b; Murray, 2000). In Europe, VRE have been isolated from sewage, from various animal sources, including farm animals or pets, from food of animal origin (frozen poultry, raw minced pork) and from feces of healthy

European subjects (Klare et al., 1993; Jordens et al., 1994; Klare et al., 1995a/b; Devriese et al., 1996; Murray, 1998). After discontinuation of avoparcin usage in commercial animal husbandry in Germany, a decrease in the incidence of VRE in animals, foodstuffs, and healthy humans in the community was observed (Klare et al., 1999), with a frequency of 1-2% in healthy subjects and colonization rates of 1.5-1.77% in the community hospital (Reinert et al., 1999; Wendt et al., 1999). Thus, this favorable situation regarding the low rate of vancomycin resistance among enterococci in Germany must be, however, preserved by carefully monitoring the prevalence of VRE in the hospital settings and outside hospitals (Wallrauch et al., 1997; Reinert et al., 1999).

In the U.S., glycopeptides were never aproved for use in animal feeds, and VRE have not been found in the initial studies in the fecal flora of healthy persons in the community or in animals (Murray, 2000; Aarestrup et al., 2002). In the U.S., the healthcare settings are the primary reservoir of resistant enterococci, particularly VRE, which arised under antibiotic selective pressure, and VRE seem to have spread clonally within and between hospitals (Handwerger et al., 1993, Montecalvo et al., 1994; Chow et al., 1993; Moreno et al., 1995; Coque et al., 1996). In Europe, VRE show, in general, a higher degree of genetic diversity. Similarly, in Europe, VRE are often polyclonal, implying frequent horizontal transfer of resistance determinants among different enterococcal strains of distinct ecological origin (Klare et al., 1995a; Descheemaeker et al., 1999; Morrison et al., 1999; Aarestrup et al., 2002).

A recent study applying AFLP methodology to glycopeptide-resistant enterococci has shown that particular VREF genogroups are associated with particular hosts and environments (the so-called ecovars) (Willems et al., 2000). The study identified four clusters. The strains from hospitalized persons (recovered from Europe and U.S.) were genetically distinct from those of non-hospitalized persons, which supports the proposition that many clinical isolates belong to a more virulent subset of the species, distinct from those that colonize the GI tract of healthy humans, and that are endemic in hospitals. Existence of particular clones of *E. faecium* adapted to the hospital environment, that have subsequently acquired vancomycin resistance, was proposed by earlier studies (Suppola et al., 1999; Reinert et al., 1999), and evidence that hospital outbreaks are mainly caused by a specific subpopulation of VRE was further supported by the identification of a variant of the *esp* gene in epidemic GRE isolates (Willems et al., 2000). These results were confirmed by recent studies using alternative typing methods (MLST) (Homan et al., 2002).

1.5 Virulence of enterococci

Prior to the introduction and widespread use of antibiotics, enterococci were a recognized cause of endocarditis, urinary tract infections, and intra-abdominal infections. The heavy use of antimicrobial agents correlated with increased recognition of the problem of enterococcal infection and with increased incidence of enterococci in hospital-acquired infections (Gilmore et al., 2002). The degree to which antimicrobial usage has affected enterococcal infection is unknown. However, antibiotic resistance alone can not explain the high prevalence of enterococci in nosocomial infections, since *E. faecium*, a species less susceptible to commonly used antimicrobial agents is responsible for only 10 to 20% of hospital-acquired enterococcal infections, whereas *E. faecalis* is responsible for most of the remaining (Mundy et al., 2000; Toledo-Arana et al., 2001). This observation strongly supports the existence of additional virulence properties that may facilitate or enhance virulence of enterococci associated with infections. Several potential virulence factors have been identified in enterococci in recent years, and these have been found primarly in *E. faecalis*. These include cytolysin (Cyl), aggregation substance (AS), extracellular superoxide, surface carbohydrates and surface proteins, such as EfaA and Esp (Mundy et al., 2000; Franz et al., 1999; Eaton & Gasson, 2000; Gilmore et al., 2002).

Despite the increasing significance of *E. faecium* in human infections, the genetic determinants coding for virulence factors of *E. faecium* remain poorly characterized. Virulence traits that are best established as virulence factors in *E. faecalis* (Cyl, AS, gelatinase) are less common in *E. faecium* (Table 1.1). However, the Esp protein has been found to be common among infection-derived *E. faecium* strains (Willems et al., 2001; Woodford et al., 2001; Eaton & Gasson, 2002).

The aggregation substance is a surface-localized protein encoded by pheromone-responsive, self-transferable plasmids, which leads to clumping (aggregation) of donor and recipient cells followed by conjugative transfer of a plasmid. In addition, AS mediates adhesion to a variety of eukaryotic cell surfaces, such as cultured pig renal tubular cells, and promotes adherence and internalization by cultured human intestinal epithelial cells. Integrins seem to be the putative receptors (Sartingen et al., 1998; Murray & Weinstock, 1999; Mundy et al., 2000). AS may contribute to colonization and adherence, but other adherence factors might be involved in the pathogenesis of endocarditis or bacteremia (Coque et al., 1995; Huycke & Gilmore, 1995; Elsner et al., 2000b).

The *E. faecalis* adhesin, EfaA, is a serum-inducible surface protein, with significant homology to the adhesins of oral streptococci (Huycke & Gilmore, 1995; Klare et al., 2001). An *efaA* determinant was found at similar frequencies in *E. faecalis* and *E. faecium*, medical and food strains from U.K., in the study of Eaton & Gasson (2001), whereas Duprè et al. (2003) have not found the *efaA* gene in any of the clinical *E. faecium* strains recovered from Italy.

The group D antigen of enterococci is a membrane-associated lipoteichoic acid which may bind reversibly to human erythrocytes, and this could be relevant for a local inflammatory process (Jett et al., 1994). It seems also that the lipoteichoic acid is the binding substance of pheromone-producing cells, which recognizes the AS produced by donor cells, thus facillitating the plasmid transfer (Franz et al., 1999; Hancock & Gilmore, 2000).

Resistance of some *E. faecium* strains to neutrophil-mediated phagocytosis was reported, and the cellular component responsible for this resistance appears to be a carbohydrate moiety (Murray & Weinstock, 1999; Hancock & Gilmore, 2000). Sex pheromones and their inhibitors are chemotactic for PMNs (polymorphonuclear cells) from humans and rats, and induce the production of superoxide by neutrophils (Johnson, 1994; Klare et al., 2001). Extracellular superoxide production (0_2^-) is another trait associated with enterococcal virulence in bacteremia. Most *E. faecalis* and some *E. faecium* generate substantial extracellular superoxide, with a significantly greater production by invasive strains than by commensal isolates. It was shown to induce cell-DNA damage of human intestinal epithelial cells in culture, produce tissue damage, thus facillitating the access of enterococci to the bloodstream (Gilmore et al., 2002; Huycke et al., 2002).

The cytolysin is a bacterial toxin expressed by many strains of *E. faecalis* and *E. faecium* (Klare et al., 2001) that causes lysis of human, horse, rabit, but not sheep blood cells, and has a bacteriocin activity against many gram-positive bacteria (Murray & Weinstock, 1999; Haas & Gilmore, 1999; Mundy et al., 2000). The production of this cytotoxin was associated with increased severity of disease in an endophtalmitis model, by induction of tissue damage, and with increased mortality in a rabbit endocarditis model, in combination with AS (Mundy et al., 1999; Murray & Weinstock, 1999)

A number of strains of *E. faecalis* have been found to produce gelatinase, an extracellular metalloendopeptidase that hydrolyzes gelatin, collagen, hemoglobin, casein, and other bioactive peptides. In other organisms, proteases have been associated with inflamatory processess (Murray & Weinstock, 1999). Gelatinase (Gel) has been shown to contribute to virulence of *E. faecalis* in an animal model of endocarditis. A recent study identified the *gelE* gene in one *E. faecium* clinical isolate (Eaton & Gasson, 2001).

Table 1.1 Virulence factors identified in *E. faecium*

Factor	Role in virulence	References
EfaA	Cell-wall adhesin expressed in serum by *E. faecalis* and *E. faecium*, respectively	Lowe et al., 1995 Eaton & Gasson, 2001
Lipoteichoic acid	Stimulation of cytokine production by cultured human monocytes	Jett et al., 1994
Superoxide	Associated with virulence in bacteremia; induction of cell-DNA damage in human intestinal epithelial cells in culture	Mundy et al., 2000 Huycke et al., 2002
Cytolysin	Lytic towards gram-positive bacteria and selected eukariotic cells; decreased LD_{50}[a] in a mouse peritonitis model; tissue damage in rabbit endophtalmitis; increased mortality, in combination with AS, in rabbit endocarditis	Jett et al., 1994 Haas & Gilmore, 1999
Gelatinase[b]	Hydrolyzes gelatin, collagen, hemoglobin, casein; increased production by *E. fc*. endocarditis isolates	Klare et al., 2001 Eaton & Gasson, 2001
Hyaluronidase	Cell-surface-associated enzyme probably contributing to dissemination in connective tissues	Hynes & Walton, 2000 Rice et al., 2002
Esp	Enterococcal surface protein; role in adhesion to urinary tract epithelial cells *in vivo*, and to abiotic surfaces; role in colonization and spread; possible role in immune evasion	Shankar et al., 1999 Shankar et al., 2001 Toledo-Arana et al.,2001 Waar et al., 2002a/b

[a]LD_{50}, 50% lethal dose (quantity of organisms lethal for 50% of inoculated animals)

[b]The *gelE* gene was recently identified in an *E. faecium* clinical isolate, but the gelatinase activity was lost during subculture

Recently, the hyaluronidase enzyme was identified in *E. faecium* strains recovered from hospitalized persons (Rice et al., 2002). The protein displays 42% identity and 60% similarity to hyaluronidases from *Streptococcus pyogenes* (Rice et al., 2002). The hyaluronidase enzyme probably contributes to dissemination in connective tissues, allowing for bacterial spread. Some studies provide evidence that the hyaluronidase has a role in pneumonia, probably contributing to adhesion or colonization (Hynes & Walton, 2000; Rice et al., 2002). Strains of enterococci, including *E. faecalis* and *E. faecium*, are known to produce bacteriocins. In *E. faecalis*, the best characterized bacteriocin is the pAD1-enoded bacteriocin-hemolysin (cytolysin). Other bacteriocins, called enterocins (e.g., enterocin A, enterocin P), are membrane-active nonlantibiotic peptides, ribosomally synthesised, with antimicrobial activity usually against other enterococci as well as against pathogenic bacteria (*Listeria*, *Clostridium*, *Staphylococcus*) (Franz

et al., 1999; Foulquié Moreno et al., 2003). The ability to produce bacteriocins has been shown to confer an ecological advantage to the producing strains (Del Campo et al., 2001).

Among the virulence traits identified in enterococci, Esp, a novel surface protein, occur frequently among strains of both *E. faecalis* and *E. faecium*. The presence of the *esp* gene was first reported in *E. faecalis* by Shankar et al. (1999), in a clinical strain that caused multiple infections within a hospital ward, and they described the structure and the localization of the gene product to the surface of the organism. Soon after, the *esp* gene was detected also in *E. faecium* strains (Willems et al., 2001; Woodford et al., 2001; Baldasari et al., 2001) and a variant of the gene was characterized in an *E. faecium* medical isolate (Eaton & Gasson, 2002). The *esp* gene from *E. faecalis* (esp_{fs}) shows an unusually large structural gene consisting of 5622 nucleotides (nt) encoding a primary translation product of 1873 amino acids (aa) (Figure 1.1), while in *E. faecium* the open reading frame of 5718 bp encodes an 1975-aa residue, and it seems that the predicted Esp proteins are currently the largest ORFs identified in *E. faecalis* and *E. faecium*, respectively. The Esp protein described by Shankar et al. (1999) consists of an N-terminal domain followed by the core of the protein made up of two distinct tandem repeating units, and a C-terminus domain. The first 49 aa of the N-terminus could serve as a signal sequence directing the export of the mature protein outside the cytoplasm. Downstream, a 2082 nt putative signal sequence specifyes a 694-aa residue. The central part of the gene consists of 3-nearly identical 252-nt tandem repeats (A repeats) followed by a 207-nt spacer region (B region) and 7 nearly identical 246-nt tandem repeating units (C repeats). A partial C repeat is identical to the first 30 nt of repeat units C2 to C7. Immediately downstream of the partial C repeat is a second B repeat region of 207-nt, that share 74% aa sequence identity to the first B repeat. The C-terminus of Esp consists of 156 aa.

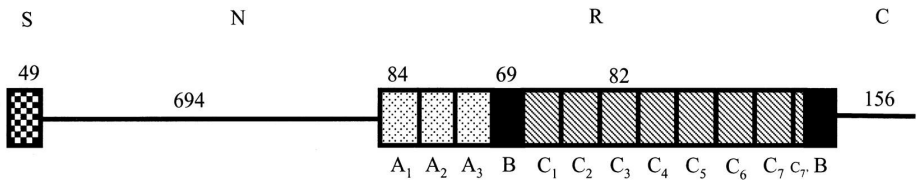


Figure 1.1 Deduced Esp_{fs} protein with inferred signal (S), N-terminal (N), repeat (R), and C-terminus (C), regions. Numbers above denote the number of amino acid residues in each segment. A, B, C – repeat blocks; C_7 denotes the partial C repeat (Shankar et al., 1999)

The reported sequence for the variant Esp in *E. faecium* (Esp_{fm}) is not globally significantly different from that of Esp_{fs} (Eaton & Gasson, 2002). The central part of Esp_{fm} consists of 4 nearly identical A repeats, followed by a B repeat and 6 nearly identical C repeats. Immediately downstream, a partial C repeat and a B repeat are located, followed by a second partial C repeat and a B repeat. The C-terminus region includes a membrane-spanning hydrophobic domain, a (F/Y)PKTGE cell wall anchor motif and a charged tail that are common to both *E. faecium* and *E. faecalis esp* genes. Overall, both genes and proteins share 89% identity. For both Esp_{fm} and Esp_{fs} variation in the number of A and C repeats was reported, with Esp_{fm} tending to have more A repeats and less C repeats than Esp_{fs} proteins (Shankar et al. 1999; Eaton & Gasson, 2002; Duprè et al., 2003). The number of A repeats varied from 1 to 3 for Esp_{fs}, and 2 to 6 A repeats in Esp_{fm}. The C repeat number reported for Esp_{fs} varied between 3 and 9, whereas 5 to 6 C repeats were reported for Esp_{fm}. The variations in the repeat number seem to be a result of homologous recombination within identical repeat units, resulting in expansions and contractions of the repeats (Toledo-Arana et al., 2001). Esp_{fs} was shown to be anchored by its carboxy-terminus, and it is possible that the N-terminus participate in interactions with the host.

The role of the Esp protein with regard to pathogenicity is not clear yet. Both Esp_{fs} and Esp_{fm} show global structural similarities to group B streptococcal proteins Rib and C alpha, that appear to contribute to immune system evasion, and to *S. aureus* biofilm-associated protein Bap. Shankar et al. (1999) have suggested that Esp could be involved in immune evasion, and that the repeat region might serve to retract the protein from the surface, hiding the protein from the immune system. The role of Esp_{fs} in adherence to urinary tract epithelial cells, but not to renal epithelial cells, was demonstrated in an animal model of infection (Shankar et al., 2001). Other studies have found no significant association between the presence of Esp and in vitro adherence of *E. faecalis* and *E. faecium* strains to cardiac, intestinal and renal cells (Archimbaud et al., 2002; Duprè et al., 2003). A correlation between the presence of *esp* and biofilm formation capacity was found for *E. faecalis* strains (Toledo-Arana et al., 2001), but this correlation could not be established for *E. faecium* strains in the study of Duprè et al. (2003).

Different epidemiologic studies in clinical and non-clinical enterococcal isolates have confirmed that *esp* is frequently found among isolates from the hospital environment, and occurs only rarely in stool isolates from community persons, animal or environmental isolates (Shankar et al., 1999; Willems et al., 2001; Waar et al., 2002a; Eaton & Gasson, 2002; Hammerum et al., 2002). These, together with the almost uniformly detection of *esp* among outbreak isolates, suggest a role for *esp* in the spread of epidemic strains, and possibly in colonization (Willems et

al., 2001; Waar et al., 2002a; Coque et al., 2002; this study). Thus, strains harbouring *esp* seem to have a higher potential for spreading in the hospital environment.

The questions adressed in the present thesis are the following:

1. Can *esp* be considered as an epidemiological marker in *E. faecium* for recognition of a clonal lineage of this bacterium which is especially qualified for dissemination in the nosocomial environment?
2. Is *esp* especially associated with glycopeptide resistance in *E. faecium*?
3. Where is the *esp* gene localized within the genome of *E. faecium* strains?
4. Can *esp* be transferred by conjugation and is the transfer coupled with the transfer of a particular resistance determinant?

2.1 Bacterial strains

The bacterial strains analysed with respect to the presence of the *esp* gene, were devided in two main groups:

(1) One group of strains referred to as *Enterococcus faecium* strains from different sources includes only *Enterococcus faecium* isolates of different ecological origins:

- Clinical strains recovered from patients in different hospitals in Germany, from clinical specimens and from stool of hospitalized patients (Table 2.1). Among this set of strains were included 27 representative isolates from outbreaks in different German hospitals (Table 2.2) and 8 representative isolates from outbreaks in the United States, United Kingdom, The Netherlands, and Australia (Table 2.2). From the stool isolates of hospitalized patients (n=45), 24 are recovered from one hospital in H. (Lower Saxony). The clinical isolates were collected from 1993-2001
- Commensal isolates from persons in the community, isolated between 1994-1999 (Table 2.1)
- Animal isolates recovered from food of animal origin and from manure (Table 2.3)
- Environmental isolates recovered from sewage treatment plants (Table 2.3)

(2) The second group of enterococcal strains includes clinical *Enterococcus faecalis* and *Enterococcus faecium* strains collected over a period of two years (2000-2001) in five intensive care units at Charité University Hospital in Berlin (Table 2.1). These strains are referred to as the strains from the SIR study. The goal of the SIR study was to investigate the clonal transmission of isolates between patients in each unit. The strains from the SIR study have been included in this thesis as a prospective investigation of genogroups in association with *esp* and vancomycin resistance.

Table 2.1 Clinical and commensal isolates investigated

Material / *Strains*	*Blood* catheter	*Blood* culture	*Urine* catheter	*Urine*	*Wound* Swab	*Stool*	*Others*	*Unknown*	*Total*
(1) ***Enterococcus faecium**** **clinical isolates**	3	15	-	19	21	45	10	43	156
Enterococcus faecium **community**	-		-	-	-	36	-	-	36
(2) SIR study ***Enterococcus faecium***	4	1	3	-	30	-	2	-	40
Enterococcus faecalis	18	6	19	11	26	-	11	2	93

***** only clinical isolates shown**,** including the outbreak isolates from Germany and from other countries

Table 2.2 ***Enterococcus faecium*** **outbreak isolates resistant to vancomycin (VanA-type) from Germany and from other countries**

Types of strains	*Strain*	*Origin of the strain*	
		Town/County/State	*Material*
VAN^R outbreak isolates from Germany	UW 805	N (Bavaria)	blood
	UW 901	D1 (Hesse)	n.d.
	UW931	G (Lower Saxony)	bronchoalveolar lavage fluid
	UW1505	L (North Rhine-Westphalia)	wound
	UW1806	Berlin1	bronchoalveolar lavage fluid
	UW2306	Berlin2	bronchoalveolar lavage fluid
	UW2320	Berlin3	wound
	UW2321	Berlin4	Tumor-drainage
	UW2322	Berlin5	urine
	UW2383	Berlin6	blood-catheter
	UW2390	Berlin7	urine
	UW2413	Berlin8	wound
	UW2442	Berlin9	wound
	UW2384	E. (Brandenburg)	urine
	UW2368	H. (Lower Saxony)	n.d.
	UW2441	L. (Schleswig-Holstein)	n.d.
	UW2467	Hamburg	blood
	UW2683	S. (Mecklenburg-Vorpommern)	urine
	UW1987	M.(North Rhine-Westfalia)	wound
	UW1983	K. (Hesse)	blood
	UW1978	K. (Hesse)	wound
	UW2444	C. (Saxony)	wound
	UW2319	F. (Hesse)	wound
	UW2389	I.O. (Rhine Land Pfalz)	urine
	UW1984	B. (Baden-Württemberg)	Trachea
	UW2490	H. (Baden-Württemberg)	blood
	UW2486	M. (Bavaria)	urine
VAN^R outbreak isolates[a] from other countries	UW 3314	The Netherlands 1-1[b]	n.d.
	UW 3315	The Netherlands 2-2[b]	n.d.
	UW 3316	The Netherlands 3-1[b]	n.d.
	UW 3317	U.K.	n.d.
	UW 3318	Australia 1[b]	n.d.
	UW 3319	U.S.A. 1[b]	n.d.
	UW 3320	U.S.A. 2-2[b]	n.d.
	UW 3321	The Netherlands 2-3[b]	n.d.

[a] isolates provided by R. J. L. Willems from The National Institute for Public Health and the Environment, The Netherlands

[b] number of the epidemic clone, as denoted by Willems et al., 2001

n.d. = no data

Table 2.3 Non-human isolates and other used strains

Types of strains	*Source of isolation (material)*	*No. of strains*	*Remarks*
Animal isolates	Pig manure	19	Isolates collected in 1993 and 1999
	Poultry manure	16	Isolated between 1993-1999
	Raw minced pork	2	UW 261 and UW 262, isolated in 1994
	Poultry meat	1	UW 53 – isolated from broiler in a hospital kitchen from Saxony-Anhalt county in 1993
Environmental isolates	Waste water samples from sewage treatment plants	24	Collected between 1993-1998
	Species	***Strain***	
Other isolates	*E. fm.*	64/3	Recipient in conjugation experiments
	E. fc.	JH2-2	Recipient in conjugation experiments
	Staph. aureus	NCTC 8325	Molecular-weight standard for PFGE (Tenover et al., 1995)
	E. fm.	UW 1983	Positive control for bacteriocin production
	E. fm.	UW 2677	Negative control for bacteriocin production

2.2 Media and chemicals

The ammounts are given for one liter solution (add. Aqua dest.). The liquid media have the same composition, except for agar.

2.2.1 Nutritive media

Enterococcosel™ Agar (BBL Becton Dickinson)

Enterococcosel agar/broth is used for rapid, selective detection of group D streptococci. This group of streptococci (including enterococci) hydrolyze the glycoside esculin to esculetin and dextrose. Esculetin reacts with ferric ammonium citrate, to form a dark brown complex. Oxgall is used to inhibit gram-positive bacteria (other than enterococci) which are sensitive to bile acids. Sodium azide is inhibitory for gram-negative microorganisms.

Pancreatic digest of casein	17 g	
Peptidic digest of animal tissue	3 g	
Yeast extract	5 g	
Oxgall	10 g	
NaCl	5 g	
Esculin	1 g	
Sodium azide	0.25 g	
Ferric(III)-ammonium citrate	0.5 g	
Sodium citrate	1 g	
Agar	13.5 g	pH 7.1

GCG-Agar (Galle-Chrysoidin-Glycerol-Agar, Heipha Diagnostika, Biotest AG)

This nutrient agar is applied for isolation and differentiation of facultative pathogenic *Enterobacteriaceae* and various other aerobic gram-negative bacteria. Enterococci grow on this medium forming small yellow-grey colonies with a yellow zone around the colonies.

Peptone	10 g	Albumin hydrolysate	2 g
Yeast extract	5 g	NaCl	5 g
Oxgall	8 g	Sodium thiosulfate	1 g
Bromothymol blue	120 mg	Ferric(III)-ammonium citrate	2 g
Urea	1 g	Glycerol	20 ml
Chrysoidine	12.5 mg	Agar	15 g

pH 7.3

Blood-Agar

OXOID Nutrient Agar	28 g
Bacto-Tryptone	12 g
Sheep blood	3%
pH 7.3	

BHI-Agar (Brain heart infusion, Difco Laboratories, Michigan, USA)

Calf brain, infusion from 200 g	200 g
Beef brain, infusion from 250 g	250 g
Proteose peptone (Difco)	10 g
Glucose	2 g
NaCl	5 g
Disodium phosphate	2.5 g
Agar	15 g
pH 7.4	

2.2.2 Standard solutions

TBE buffer

Tris-base	45 mM
Boric acid	45 mM
EDTA	1.25 mM
pH 8.0	

Loading buffer for agarose gels

Na_2-EDTA	40 mg
Sucrose	4 g
Bromophenol blue	3 mg
add. 10 ml Aq. dest.	

Ethidium bromide solution
Final concentration 0.5 µg/ml
in Aq. dest.

Saline solution
NaCl 0.85%

2.3 Methods

2.3.1 Antimicrobial susceptibility test by microbroth dilution method

The method involves the use of small volumes of broth dispensed in sterile plastic microdilution trays, to which the antibiotics solubilized in the corresponding solution and diluted in broth, are added. The antibiotic concentrations of each tray depend on the antimicrobial agent tested and cover a range of concentrations bellow and beyond a threeshold value. A standardized inoculum suspension is diluted in saline and then dispensed with a specific dispensing device. The microtiter plates are incubated at 37°C for 24h. The minimal inhibitory concentration (MIC) represents the lowest concentration of antimicrobial agent that completely inhibits growth of the organisms. If growth is detected in wells with an antibiotic concentration beyond the threeshold value, the microorganisms are considered resistant, and susceptible if growth is observed in wells bellow the threeshold value. For certain antibiotics, to which enterococci are intrinsically resistant (e.g., aminoglycosides) the high-level resistances are also tested.

2.3.2 Identification of bacterial species

A preselection of enterococci was performed by growth on Enterococcosel-, GCG- and blood agar. Identification at the species level was further performed according to Devriese et al. 1993, on the basis of acid formation in different sugars, and from other characteristics such as generation of pigment and motility.

2.3.3 PCR

The PCR reactions have been performed in Perkin-Elmer Thermal Cyclers, using Ready·To·Go™ PCR Beads (Amersham-Pharmacia Biotech, Freiburg, Germany). They are provided as dried beads, and each bead contains Taq DNA polymerase, nucleotides, and buffer. The primers and the template DNA must be added when performing the reaction. When brought to a final volume of 25 µl, each reaction will contain 1.5 U Taq DNA polymerase, 200µM each dNTP, 0.1 µM each primer and 2.5 µl template DNA.
The primer pair esp5/6 was used to amplify an internal fragment of the *esp* gene covering the A repeat region (Figure 1.1), between nucleotides 1973-3254 of the *E. fc*. *esp* gene entry AF 034779. The primers hyl1 and hyl2 amplified a fragment of 661 bp from the *hyaluronidase* gene of *E. faecium* isolates (Rice et al., 2003).

The PCR-products were resolved in 1.4% agarose gel, stained with ethidium bromide for 15 min. and visualized in UV light.

The sequence of the primers are:	Programme:
esp5: 5′ ACGTGGATGTAGAGTTTGC	$T_a \rightarrow 55°C$, $t_p \rightarrow 30$ sec, 30 cycles
esp6: 5′ GAATATGTCACTACAACCGTAC	
hyl1: 5′ GAGTAGAGGAATATCTTAGC	$T_a \rightarrow 50°C$, $t_p \rightarrow 30$ sec, 30 cycles
hyl2: 5′ AAGCTCCAATTCTGT	

(T_a – annealing temperature; t_p – polymerisation time; no. of amplification cycles)

DNA isolation

The isolation of the DNA was performed as described in Klare et al. 1997. The cell wall of enterococci is not easily broken, but short steps at high temperature and a high concentration of Chelex 100 Resin will lead to its disruption.

- ✓ Centrifugation of 1.5 ml from an overnight culture in BHI broth
- ✓ Wash the pellets in 0.85% saline
- ✓ Add 5% Chelex 100 Resin® (BIO-RAD Labs., California, USA) dissolved in water to a final concentration of 50 mg/ml
- ✓ Incubation at 56°C for 60 min with gentle shaking
- ✓ Incubation at 95°C for 15 min
- ✓ Keep at 4°C until use
- ✓ Before using, centrifugation of the tube for precipitation of cell components/Chelex-Resin-particles, the DNA remainig in the aqueous phase at the surface
  (Centrifugations at 13,000 rpm for 5 min. at 4°C)

Preparation of a digoxigenin-labeled probe:

a) Labeling of the probe by PCR reaction, with a final reaction volume of 100 µl; the concentration of the enzyme, buffer and deoxyribonucleotides as described above, with the addition of dTTP to a final conc. of 190 µM and digoxigenin-labeled DIG-dUTP (Roche Mannheim, Germany) to 10 µM.

b) Labeling of the probe using the DIG High-Prime System (digoxigenin random-primed DNA labeling). Before labeling, the normal-PCR product is purified using the Qiaquick-PCR purification kit, as described in the manufacturer instructions. The purified DNA (16 µl) is denaturated for 10 min at 96°C and cooled on ice. Four µl of the Dig High-Prime solution (containing diferent combinations of short digoxigenin-labeled oligonucleotides and the

polymerase) are added and the tube is incubated at 37°C overnight. The reaction is stopped by heating at 65°C for 10 min.

2.3.4 Enterocin production

Production of enterocin was detected as the ability of producing-strains to inhibit the growth of the indicator strain around the test colonies. As indicator strains, *Enterococcus faecalis* JH2-2 and *Enterococcus faecium* 64/3 were used.

- Tenfold dilution in NaCl from a fresh 3 h culture in BHI broth of the indicator strain
- Incubate BHI agar plates with the dilution of indicator strain (JH2-2 and 64/3, respectively) using a swab stick and let it dry
- Test strains are spotted onto the BHI agar plates and allowed to form colonies overnight at 37°C

The production of bacteriocin was indicated by the formation of clear zones of inhibition around the test colonies in the indicator lawn. Each strain was tested for production of bacteriocin against both indicator strains, and they were considered positive when inhibition of one or both indicator strains was observed and when the diameter of the inhibition zone around the test colonies was minimum 0.5 mm. Test colonies with an inhibition zone of 5-6 mm around them were considered as strong bacteriocin-producers (e.g. the VAN^R *E. faecium* outbreak strains).

2.3.5 RAPD (random amplification of polymorphic DNA) - typing PCR

(after a method described by van den Braak et al. 2000)

This PCR-mediated typing procedure generates complex mixtures of amplicons that can be translated into DNA fingerprints by simple electrophoresis in agarose gels. RAPD analysis was characterized by the authors as well-suited for epidemiological typing of enterococci and a 75% homology setting for interpretation of the data gives optimal overlap between RAPD and PFGE results.
One primer was used for amplification of the template DNA (RAPD 10) with the nucleotide sequence 5′ TGCTCTGCCC.

- The whole-cell DNA was isolated using the DNeasy Tissue kit from QIAGEN® (QIAGEN GmbH, Hilden, Germany), as given in the manufacterer instructions (see bellow)
- The concentration of the DNA was determined with the pico green kit, Molecular probes, as described in the instructions accompaning the product
- The PCR reaction was performed with the beads from Amersham-Pharmacia, with a final reaction volume of 25 µl containing 1 x polymerase buffer, 200 µM of each deoxyribonucleotide, 50 ng of template DNA, 1 µM primer and 1 U polymerase enzyme

- The programme used: 95°C for 4 min., 95°C for 30 sec., 34°C for 60 sec, 72°C for 1 min, 72°C for 7 min.; 35 cycles
- The bands were separated on 2% agarose gel

2.3.6 Pulsed-field gel electrophoresis (PFGE)

(Witte & Grimm, 1993)

The pulsed-field gel electrophoresis is a typing-method of the microorganisms at molecular level and a technique for resolving chromosome size DNAs. PFGE involves embedding of the organisms in agarose, lysing the organisms in situ and treatment with detergents to remove all of the cellular components from the DNA, and digesting the chromosomal DNA with restriction endonucleases that cleave infrequently (*Sma*I for enterococci). Slices of agarose containing the DNA fragments are inserted into the wells of an agarose gel, and the restriction fragments (10 to 20 for enterococci, with sizes between 5 and 400 kb for *E. faecium*, 600 kb for *E. faecalis*) are separated by alternating the electric field between spatially distinct pairs of electrodes arranged in a hexagonal contour. The variing electric field allow the separation of DNA fragments ranging in size from a few kb to some megabases.

1st day. Cell lysis

⇒ Centrifugation of 1 ml from a 5 ml overnight culture (BHI broth) in an Eppendorf-tube (15,000 rpm, 1 min, 4°C)
⇒ Wash the sediment with 1 ml ice-cooled PIV-solution, centrifugation, resuspend the pellets in 0.7 ml ice-cooled PIV-solution
⇒ Mix 50 µl from this suspension with 50 µl 1.2% LMP Agarose (Biometra, FMC BioProducts, Rockland, USA)
⇒ Mix in an Eppendorf tube and immediately pipette into the sample mold (BIO-RAD)
⇒ Allow the mold to cool at 4°C for 1h
⇒ Place the agarose plugs in new Eppendorf tubes and add 1 ml lysis solution
⇒ Incubate the tubes overnight at 37°C with gentle shaking (lysis of the cells)

2nd day. Deproteinization

⇒ Remove the lysis solution
⇒ Wash 3 x 1 ml ES-buffer
⇒ Add 1 ml ESP-solution and incubate at 50°C for ≥ 17 h with gentle shaking

3rd day. Restriction phase

⇒ Remove the ESP-solution

⇒ Wash the agarose plugs 2 x 14 ml TE-buffer, incubate each time for 30-60 min. at room temperature (RT)
⇒ Place the agarose plugs in new Eppendorf tubes and add in each tube 1ml 1x *Sma*I-buffer (Buffer A, Roche Mannheim, Germany), equilibrate (30 min.)
⇒ Remove the *Sma*I-buffer
⇒ Add freshly-prepared 1 x *Sma*I-buffer (from 10 x *Sma*I-buffer) + 40 U *Sma*I-enzyme in each tube
⇒ Incubate at 25°C overnight

4th day. Pulsed-field electrophoresis (CHEF-DR® II / III-System, Bio-Rad Laboratories, Richmond, California)

⇒ Prepare the agarose gel with agarose (Sigma, Type II Medium EEO) and 1 x TBE-buffer (pH 8.5) (concentration see bellow)
⇒ Stop the restriction reaction in the Eppendorf tubes by adding 50 µl loading buffer (bromophenol blue)
⇒ Bring the agarose plugs on a smooth clear surface and using a razor blade or a spatula, place the plugs into the sample wells of the agarose gel, after cutting them to appropriate size
⇒ Cover the wells with the rest of agarose from the gel preparation
⇒ Fill the electrophoresis chamber with 1 x TBE-buffer (running buffer; pH 8.5; ~ 2 l) and place the gel into the electrophoresis chamber
⇒ Running the gel:

(a) Buffer temperature in the chamber → 14°C, mantained by the recirculating pump
(b) Set the Pulsewave parameters as follows:

Table 2.4 PFGE – running parameters

	E. faecalis (1% agarose gel)	*E. faecium* (1% agarose gel)
[1] Mode: Initial time: Final time: Start ratio: Running time:	1 1 sec. 10 sec 1 15 h	1 1 sec. 13 sec. 1 13 h
[2] Mode: Initial time: Final time: Start ratio: Running time:	10 10 sec. 40 sec. 1 10 h	10 13 sec. 30 sec. 1 5 h
[3] Mode:	11	11

(c) Set the voltage (200V or 6V/cm)

5th day. Removing and staining the gel

⇒ Remove the gel from the electrophoresis chamber and stain in ethidium bromide for 15 min, wash shortly in Aq. dest. and visualize the gel under UV light

⇒ Analysis of the molecular weights and similarity levels according to the guidelines of Tenover et al. 1995 and of Claus et al. 1996

Tenover et al. 1995 have proposed a set of guidelines for interpreting DNA restriction patterns, which are intended to be used to examine relatively small sets of isolates, related to putative outbreaks spanning relatively short periods of time (1 to 3 months; see Figure 2.1). The DNA restriction patterns of the isolates are compaired with one another to determine their relatedness. Conform with these guidelines, the PFGE patterns of isolates representing the outbreak strain would be indistinguishable from each other. After identifying the common or outbreak pattern, the size and number of fragments in the outbreak pattern are compaired with the fragments that make up the patterns of the other isolates. Random genetic events, including point mutations, and deletions and insertions of DNA alter the PFGE patterns. Thus, the pattern of each isolate is assigned to one of four categories (Tenover et al., 1995):

I. Indistinguishable from the outbreak pattern, when the restriction patterns have the same number of bands and the corresponding bands are of the same apparent size.

II. Closely related to the outbreak strain, when the PFGE pattern differs from the outbreak pattern by changes consistent with a single genetic event, i.e. a point mutation or an insertion or deletion of DNA (Figure 2.1), which will lead to 2 or 3 bands difference. A spontaneous mutation can create a restriction site, determine the loss of the original large fragment (one band difference) and the appearance of two smaller fragments (two bands difference); thus, there is a three-bands difference between the outbreak pattern and that of the test isolate. Insertion or deletion of DNA from the outbreak pattern will be translated into two bands difference.

III. Possibly related to the outbreak strain, if the PFGE pattern differ from that of the outbreak strain by changes consistent with two independent genetic events (four to six bands difference). Such variation has been observed among isolates collected over long periods (≥ 6 months) or taken from large numbers of patients involved in extended outbreaks.

IV. Unrelated to the outbreak pattern if the PFGE pattern differs from the outbreak pattern by changes consistent with three or more independent genetic events (generally, seven or more bands differences).

When a larger number of isolates investigated, which are collected over a period longer than three months, up to six bands difference can be admitted for isolates of the same strain. Thus, Morrison et al. 1999 have proposed an 82% similarity level between isolates of one cluster when the *Sma*I patterns of 30 VREF (vancomycin-resistant *Enterococcus faecium*) from a clinical

setting were analysed by PFGE. Van den Braak et al. 2000 have clustered the enterococcal isolates analysed upon an 86% similarity breakpoint, for strains of different ecological origins and from different countries.

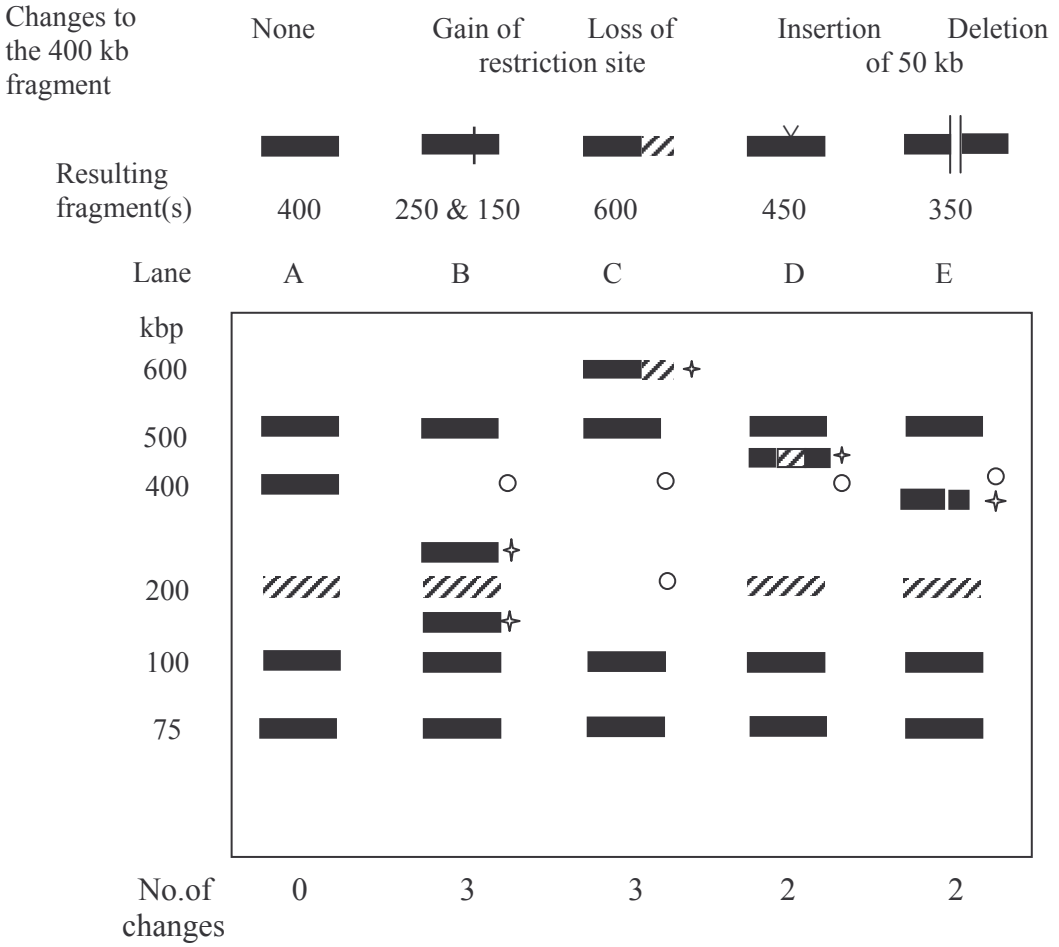


Figure 2.1 Schematic diagram showing the changes in the PFGE pattern of an isolate as a result of various genetic events. Lane A, outbreak pattern; lane B, gain of a restriction site; lane C, loss of a restriction site; lane D, insertion of DNA in a preexisting fragment; lane E, deletion of DNA from an existing fragment. The open circles indicate fragments present in the outbreak isolate but missing from the test isolate as a result of a genetic event; little stars indicate fragments absent from the outbreak pattern but present in the test isolate after a genetic event (Tenover et al., 1995)

Solutions

PIV-solution

10 mM Tris-HCl, pH 8.0
1 M NaCl
pH 7.6

LMP-Agarose

1.2% in PIV-solution

Lysis-buffer

6 mM Tris-HCl pH 7.6
1 M NaCl
100 mM Na_2-EDTA pH 7.6
0.5% Brij 58
0.2% sodium deoxycholate
0.5% sodium lauroyl sarcosine
⇒ storage at –20°C until use

add before use:

- RNase-solution to a final conc. of 60µg/ml (from a solution of 10 mg/ml RNase in 10 mM Tris-HCl and 15mM NaCl stored at –20°C)
- Lysozyme to a final conc. of 1 mg/ml

ES-buffer

0.5 M Na_2-EDTA pH 9.0
1% sodium lauroyl sarcosine
pH 9.0 controled with NaOH

ESP-buffer

Proteinase K (Sigma), 1mg/ml in ES-buffer

TE-buffer

10 mM Tris-HCl
1 mM Na_2-EDTA
pH 7.5

2.3.7 In vitro filter mating (filter conjugation)

Filter conjugation involves close cell-to-cell contact of donor and recipient cells by harvesting them on nitrocellulose filters. This was accomplished in a filtermating apparatus from Sartorius, Germany. The filters loaded with bacteria are then placed top up on agar plate without antibiotics and incubated as described below.

- Overnight culture of recipient and donor strains in BHI broth at 37°C
- Tenfold dilution in 5 ml BHI broth and growth again until exponential phase (3-5 h)
- Pipette 1 ml donor and 1 ml recipient culture in one tube and mix gently

- Apply the cells on the fixed filter and then apply vacuum
- Place the filter with the bacterial cells top up on a BHI agar plate
- Incubate overnight for mating
- Dilutions from the recipient culture in 5 ml NaCl as follows: 10^{-2}, 10^{-4}, 10^{-6}, 10^{-7}, 10^{-8}, inoculate 100 µl from the last three dilutions on BHI agar overnight at 37°C and the next day count the colonies
- Harvest the cells from the filter by diluting the bacteria on the filter in 1 ml NaCl using a glass tool and collect it in a 1.5 ml centrifuge tube
- Centrifuge the tube at 8,000 rpm for 5 min. at 4°C
- Wash the pellets with 500 µl NaCl
- Resuspend the pellets in 500 µl NaCl and mix gently
- Dilutions from the transconjugants in 5 ml NaCl: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}
- Inoculate 100 µl each dilution of transconjugants on selective plates and incubate at 37°C overnight

Table 2.5 Antibiotics used for selection of transconjugants

ANTIBIOTIC	*DISSOLVING SOLUTION*	*FINAL CONCENTRATION*
Erythromycin	Ethanol	20 µg/ml
Oxytetracycline	1 N HCl	15 µg/ml
Vancomycin	H_2O	30 µg/ml
Fusidic acid	Ethanol	20 µg/ml
Rifampicin	1 N HCl	30 µg/ml

2.3.8 Plasmid isolation

Enterococcal plasmids were isolated after modification of the method from Woodford et al. 1993.

- Overnight culture on BHI agar plate at 37°C
- Suspend the bacterial cells in 1 ml TES-buffer, pH 8.0
- Centrifugation at 8,000 rpm for 5 min
- Resuspend the pellets in 200 µl TES-buffer containing 10 mg/ml lysozyme and 5 µl/ml RNase
- Incubation at 37°C for 60 min
- Add 400 µl 0.2 N NaOH / 1% SDS (invert the tube gently)
- Incubation at 56°C for 60 min
- Add 300 µl 3M potassium acetate, pH 4.8; invert the tube and keep on ice for 20 min
- Centrifugation at 15,000 rpm for 15 min

- Transfer the supernatant in a new tube and extract 2-3 x with phenol/chloroform with centrifugation at 12,000 rpm for 10 min and collecting the upper (aqueous) phase
- Precipitate the DNA from the aqueous phase with 2 vol. absolute ethanol and keep at –20°C overnight
- Centrifugate at 15,000 rpm for 30 min at 4°C
- Wash the pellets with 500 µl 70% ethanol and centrifugate
- Let the pellets dry at 37°C for 30 min
- Dissolve the pellets in 20 µl Aq. dest. for ~ 20 min at room temperature
- Visualize the plasmid DNA after electrophoresis in 1% agarose gel

TES-buffer

10 mM Tris-HCl, pH 8.0
1 mM EDTA, pH 8.0
25% sucrose

2.3.9 Isolation of whole-cell DNA

(performed with DNeasy Tissue kit from QIAGEN® Hilden, Germany)

The genomic DNA was isolated after a method provided by the manufacterer, with the addition of the initial lysis step for a more efficient breakage of the cell wall. The procedure involves cell lysis followed by selective binding of DNA to the DNeasy-membrane and wash steps for removing the contaminants.

- Centrifugation of 1 ml from an overnight culture in BHI broth at 37°C
- Resuspend the pellets in 180 µl lysis-buffer
- Incubation at 37°C for 30 min
- Addition of 25 µl Proteinase K and 200 µl Buffer AL, short vortexing
- Incubation at 70°C for 60 min
- Addition of 200 µl ethanol (absolute), mix
- Load the content onto the DNeasy mini column
- Centrifugation at 8,000 rpm for 1 min
- Add 200 µl Buffer AW1 and centrifugate again
- Add 200 µl Buffer AW2 and centrifugate at 14,000 rpm for 3 min
- Add 50 µl elution buffer (Buffer AE)
- Incubation at RT for 1 min
- Centrifugation at 8,000 rpm for 1 min, collect the DNA and use immediately or store at –20°C

Lysis buffer

20 mM Tris-HCl, pH 8.0
2 mM EDTA
1.2% Triton X-100
store at –20°C until use

add before use

20 mg/ml lysozyme

Digestion of plasmid DNA with *Hind*III

10-20 µl plasmid DNA
3 µl Buffer B
H_2O
80 U *Hind*III
30 µl final volume

Digestion of genomic DNA with *Hind*III

5-10 µl DNA
3 µl Buffer B
H_2O
80 U *Hind*III
30 µl final volume

The tubes were incubated at 37°C overnight.
(The enzyme and buffer were provided by Roche Mannheim, Germany)

2.3.10 Southern-blot using capillary transfer and vacuum transfer

(according to the methods described in Sambrook et al. 1989)

Southern-blot is a method for transfering DNA fragments from agarose gels to solid supports (nitrocellulose filters or nylon membranes). The membranes are usually positively charged for a more efficient binding of the negatively-charged DNA to the membrane. The methods we used and which are described bellow are capillary transfer and vacuum transfer of DNA onto positively-charged nylon membranes from Roche Mannheim, Germany.

I. Capillary transfer

Capillary transfer involves the transfer of DNA fragments from the gel on the surface of a solid support, carried in a flow of liquid which is drawn through the gel by capillary action. The capillary force is established and mantained by a stack of dry, absorbent paper towels which are deposed on the top of the membrane. Prior to the transfer, the agarose gel must be washed in different buffers in order to render the DNA in smaller single-stranded fragments. These fragments are then more efficiently and faster eluted from the gel.

Washing the gel
- 0.25 M HCl 1 x 10 min
- Aq. dest. - shortly
- 0.5 M NaOH, 1.5 M NaCl 2 x 15 min
- Aq. dest. - shortly
- 0.5 M Tris, 20 mM NaCl 2 x 15 min

♦ Cut a piece of membrane about 1 mm larger than the gel in both dimensions

- Fill a dish with the transfer buffer (3 M NaCl, 0.3 M Sodium citrate), and bild the blot as described in Sambrook et al. 1989: the inverted gel is placed on the wet 3MM Whatman paper who's ends are imersed in the transfer buffer; over the gel place the nylon membrane and a stack of dry paper towels. This construction sets up a flow of liquid from the reservoir through the gel and the nylon membrane
- Allow the transfer of denatured DNA to proceed for 8-24 h
- To fix the DNA to the membrane, expose the side of the membrane carrying the DNA to a source of ultraviolet irradiation (crosslinking at 150 mJoule, 30 sec)
- The membrane can be used immediately in hybridization experiments or let it dry for a later use

II. Vacuum transfer

DNA can be transferred rapidly and quantitatively from gels under vacuum. This transfer method is more efficient than capillary transfer and it was used for the transfer of DNA from the pulsed-field gel (Vacuum blotter TDNA, Appligene-Oncor, Heidelberg, Germany).
The transfer method was performed according to the intructions accompaning the apparatus.

- Construct the blot as mentioned in the instructions
- Apply 20 mbar and add Buffer 1 to cover the gel completely
- Washing the gel as follows:

Buffer 1	50 mbar	30 min
Buffer 2	50 mbar	30 min
Buffer 3	50 mbar	30 min
Transfer buffer	50 mbar	120 min

- Crosslinking of the wet membrane at 150 mJoule for 30 sec.
- Wash 10 min in Aq. dest. and allow to dry at room temperature or immediately use

Buffer 1 → 0.25 M HCl

Buffer 2 → 0.5 M NaOH
1.5 M NaCl

Buffer 3 → 1 M Tris
2 M NaCl

Transfer buffer → 3 M NaCl
0.3 M Sodium citrate

Dot-blot experiments were also performed for rapid screening of genomic DNA. This method involves denaturation of the genomic DNA preparation which is then applied with a pipette directly onto the nylon membrane (5-10 µl), followed by crosslinking of the dry membrane under UV light (50 mJoule, 30 sec) and use in hybridization experiments.

2.3.11 Hybridization with digoxigenin-labeled probes

(after a method supplied by Roche Mannheim, Germany)

The membrane loaded with single-stranded DNA is further hybridized with a digoxigenin-labeled DNA probe at 68°C as recommended by the manufacturer.

- Prehybridization of the nylon membrane with at least 20 ml hybridization buffer at 68°C for 2-3 h
- Replace the solution with 12 ml hybridization buffer containing 5 µl freshly denatured probe DNA
- Incubate the membrane for 12 h at 68°C
- Wash the membrane - 2 x 5 min at room temperature with SSC-buffer 1
  - 2 x 15 min at 68°C with SSC-buffer 2
- Use the membrane directly for immunological detection of hybridized DNA or store air-dried for later detection

Hybridization buffer → 5 x SSC, 1 x blocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS

20 x SSC → 3 M NaCl, 0.3 M sodium citrate

SSC-buffer 1 → 2 x SSC, 0.1% SDS

SSC-buffer 2 → 0.1 x SSC, 0.1 % SDS

Blocking reagent (stock solution) → 10% blocking reagent (Roche Mannheim, Germany) in buffer 1 (see immunological detection)

2.3.12 Immunological detection

(DIG nucleic acid detection kit, Roche Mannheim, Germany)

DIG-labeled DNA is detected, after hybridization to target nucleic acids, using an antibody-conjugate (anti-digoxigenin alkaline-phosphatase conjugate, anti-DIG-AP) which binds selectively to the digoxigenin-labeled dUTP. The complex formed in this way is then visualized with ATTOPHOS™ (ATTHOPHOS Detection kit, Roche Mannheim, Germany). AttoPhos is a highly sensitive fluorimetric substrate, which generates a fluorochrome (fluorescent emitter) in the presence of an alkaline phosphatase-labeled antibody. This fluorochrome can be then visualized in a fluorimeter.

- Wash membrane briefly in Buffer 1 + 0.3% Tween® 20
- Incubate for 30 min with 100 ml Buffer 2
- Dilute anti-DIG-AP conjugate 1 : 10,000 in Buffer 2

- Incubate membrane for 30 min with 50 ml of diluted antibody conjugate solution
- Remove unbound antibody conjugate by washing 2 x 15 min with 100 ml Buffer 1
- Equilibrate membrane for 2 min with 20 ml of Buffer 3
- Incubate membrane with 2-4 ml ATTOPHOS for 5 min at 37°C
- Store the membrane in a plastic bag and visualize the hybridized fragments

Buffer 1

0.1 M maleic acid
0.15 M NaCl
pH 7.5

Buffer 2

1 x blocking reagent in Buffer 1

Buffer 3

0.1 M Tris-HCl
0.1 M NaCl
50 mM $MgCl_2$
pH 9.5

3.1 Prevalence of *esp* in *Enterococcus faecalis* and *Enterococcus faecium* of different clinical and environmental sources

A large number of *E. faecalis* and *E. faecium* strains were analysed by means of PCR for the presence of *esp*, using the primer pair esp5/6 designed upon a consensus sequence of different *esp* alleles from *E. faecium* strains (R. Willems, unpublished results), which amplifies the A repeat region of the gene. The analysed strains included clinical isolates of *E. faecalis* and *E. faecium* collected during a study in a five-unit-hospital from Berlin (SIR study), and a number of clinical *E. faecium* isolates recovered from different hospitals across Germany. The goal of the SIR study was to determine the intra-ward spread of clinical *E. faecalis* and *E. faecium* isolates from a five-unit-hospital (Berlin2). Additionally, the presence of *esp* was prospectively investigated, and the inter-ward spread of strains was analysed.

Non-clinical *E. faecium* isolates were also examined, including stool isolates from community people collected over a period of 4 years (1994-1997) and non-human isolates comprising animal isolates (food and manure) and sewage isolates (Table 3.1).

3.1.1 Occurrence of *esp* among *E. faecium* strains of different origins

The distribution of the *esp* gene was investigated in 156 hospital isolates of *E. faecium* recovered from different hospitals in Germany and 103 non-clinical isolates (Table 3.2, Appendix). The gene was detected in 46% (72/156) of hospital isolates, but none of the non-clinical isolates was found to be *esp*-positive. Most of the clinical isolates tested here were vancomycin-resistant VanA-type (142/156) and included also 35 clonally related outbreak strains and 45 stool isolates from hospitalized persons (41 isolates from stool samples, and 4 isolates from rectal swab), from which 25 are clonally related isolates. From the 35 VAN^R outbreak strains, 30 were identified as *esp*-positive, comprising 42% of the total *esp*-positive clinical isolates. Similarly, from the 45 stool isolates from hospitalized patients, 17 were harbouring the gene, accounting for 24% from the total number of clinical esp^+ isolates. The length of the PCR product was variable: fragments were found of 1000 bp, 1300 bp, 1500 bp, 1700 bp, 2000 bp and 2200 bp, according to the number of A repeats.

The medical isolates tested here included 19 urine isolates, 18 blood isolates, 21 wound swab isolates and 45 stool isolates, 10 isolates recovered from other sites (trachea, sputum) and 43 with unspecified site of isolation. A high incidence of the *esp* gene was found among blood

isolates (67%) and wound infections (67%), 47% for urine isolates and 38% for stool isolates. From the isolates recovered from other sites, 7 were found *esp*-positive. The *esp* gene was also present in 39.5% of the isolates of unknown site of isolation.

To confirm the presence of *esp* established by PCR, a small number of strains were analysed by dot blot hybridization. The strains tested included 3 pig manure isolates, 7 isolates from pork, 8 stool isolates from healthy individuals, 8 infection-derived isolates including *esp*-positive and *esp*-negative samples, and 4 control strains, *E. faecalis* JH2-2 and *E. faecium* 64/3 which are *esp*-negative, *E. faecalis* MMH594 and *E. faecium* UW 901, which are *esp*-positive, respectively. The hybridization results confirmed the PCR results, so that the gene was found only in the positive controls and in the infection-derived isolates already identified by PCR as *esp*-positive (data not shown).

3.1.2 Prevalence of *esp* among clinical *E. faecalis* and *E. faecium* isolates from SIR study

A set of 93 *Enterococcus faecalis* isolates and 40 *Enterococcus faecium* isolates from a five-unit-hospital in Berlin were documented for the presence of *esp.* All *E. faecalis* isolates were vancomycin-susceptible and only 4 *E. faecium* isolates were found to be resistant to vancomycin with VanA-type resistance.

A higher frequency of *esp* was found in *E. faecalis* than in *E. faecium*. The gene was found in 65% of the *E. faecalis* strains and in 55% of the *E. faecium* strains recovered from the same hospital. From the 4 VAN^R *E. faecium* isolates, only one was positive for *esp.*

The *E. faecalis* clinical isolates comprised 30 urine isolates, 19 of them involving catheter usage, 24 blood isolates (18 from a catheter and 6 from blood) from which 11 blood isolates were involved in 2 outbreaks. Twenty-six isolates were recovered from wound swabs and 13 isolates from other sites (Table 3.2, Appendix). The *esp* gene was found in 19 isolates from urine (63%) and in 15 wound swab isolates (58%). From the blood isolates, *esp* was found in 14 isolates from blood-catheter (78%), and in 4 from 6 blood isolates. From the 13 isolates from other sites, 8 were *esp* positive.

The *E. faecium* medical isolates are comprised mostly from wound swab isolates (n=30) from which 19 were *esp*-positive (63%); 3 urine-catheter isolates with 1 positive for *esp*; 5 blood isolates, 4 from catheter and 1 from blood, the last one being esp^+, and 2 isolates from other sites with one positive for *esp* (Table 3.2, Appendix).

Table 3.1. Detection of *esp* and enterocin production in strains of *E. faecium* and *E. faecalis* of different origins

Isolates origin	Total isolates	*esp* positive		Enterocin production		
		total (%)	[a]VAN^R	Total isolates tested / esp+	ent+ (%)	ent+/esp+
a) Hospital isolates	289					
Enterococcus faecalis						
SIR study	93	60 (65)	-	66/45	44 (67)	35
Enterococcus faecium						
➢ SIR study	40	22 (55)	1/4	40/22	8 (20)	5
➢ Strains recovered from other hospitals	156	72 (46)	70/142	97/46	38 (39)	29
b) Stool isolates from healthy individuals (E. faecium)	36	-	0/19	32/-	1	-
c) Animal isolates (E. faecium)	38					
Poultry meat	1	-	0/1	1/-	-	
Pork	2	-	0/2	2/-	-	
Poultry manure	16	-	0/1	7/-	-	
Pig manure	19	-	0/2	16/-	-	
d) *Sewage isolates (E. faecium)*	24	-	0/5	15/-	-	

[a] number of esp+ VAN^R isolates / total VAN^R isolates

Dot blot experiments were also performed for a small number of *E. faecium* and *E. faecalis* strains. For the 6 *E. faecalis* strains analysed, the hybridization experiments confirmed the PCR results (data not shown). Among the 6 *E. faecium* isolates tested, 3 of them showed after PCR a weaker band comparing to the positive control and to the isolates with a strong

signal, one isolate had 2 amplified-fragments, one showed a strong PCR-product and one isolate was negative for *esp*. After hybridization, all six isolates were found to be *esp*-positive (Figure 3.1).

Further hybridization experiments have shown that a larger number of isolates were *esp*-positive (22 isolates) compared with the PCR results (8 isolates).

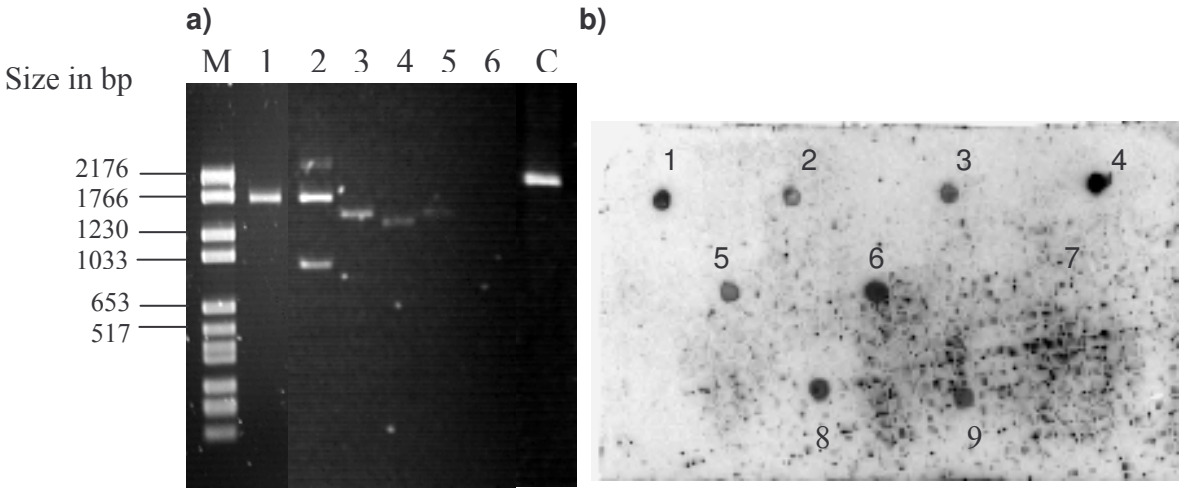


Figure 3.1 Detection of *esp* gene in *E. faecium* isolates by PCR and by dot blot hybridization.

a) PCR products resolved in 1.4% agarose gel

Lane M – Mol. Marker VI (Roche Mannheim, Germany);

lane 1 – UW 3058; lane 2 – UW 3056; lane 3 – UW 3050;

lane 4 – UW 3367; lane 5 – UW 3279; lane 6 – UW 3097 ; C- positive control UW 901

b) Dot blot hybridization with a labeled *esp* probe

Spots 1 to 6 corespond to the lanes 1 to 6 from **a)**

7 – negative control *E.fm.* 64/3 ; 8, 9 – positive controls UW 805 and UW 901

3.2 Enterocin production in *Enterococcus faecalis* and *Enterococcus faecium* of different origins

Bacteriocin (enterocin) production was investigated by a simple method and defined as the capacity of producing bacteria to kill the cells of indicator non-bacteriocin-producing strains *E. faecalis* JH2-2 and *E. faecium* 64/3. The ability to produce enterocin was detected by the formation of a clear zone of inhibition arround the test colonies in the indicator lawn.

3.2.1 Enterocin production by *E. faecium* strains from different sources

From the 97 clinical isolates tested, 38 (39%) were identified as bacteriocin producers. The strains tested included 21 clonally related outbreak strains, 36 isolates from feces of hospitalized patients and the remaining of 40 representing isolates from different other hospitals (Table 3.2, Appendix). Fourty-six *esp*-positive isolates were analysed, from which 29 (63%) produced also bacteriocin. On the other hand, 76% of the enterocin-positive strains were also *esp*-positive. The 29 isolates positive for both traits were vancomycin-resistant and included, however, 20 clonally related outbreak strains (from 21 ent+) and 9 isolates recovered from different other hospitals. The outbreak isolates were characterized as strong bacteriocin producers (production of an inhibition zone of 5-6 mm around the test colonies). None of the stool isolates from hospitalized patients tested included both traits. Finally, 42 (43%) clinical isolates tested for enterocin production did not harbour either *esp* or enterocin.

The animal strains as well as the sewage isolates were not found to produce bacteriocin. Only one from 35 stool isolates from healthy individuals was detected to produce bacteriocin only against the *E. faecalis* indicator strain.

3.2.2 Enterocin production among *Enterococcus faecium* and *Enterococcus faecalis* from SIR study

The enterocin production was detected in 44 (67%) of *Enterococcus faecalis* isolates tested, 35 (80%) of which were also *esp*-positive (Table 3.2, Appendix). This set of isolates positive for both traits included, however, 22 clonally related isolates involved in two outbreaks.

Eight out of 40 (20%) *E. faecium* were identified as bacteriocin producers, from which 5 were also *esp*-positive (Table 3.2, Appendix). Thus, 23% of the *esp*-positive isolates were enterocin producers. One VAN^R strain was positive for both traits, the other three VAN^R isolates harbouring neither *esp* nor enterocin.

3.3 Pulsed-field gel electrophoresis from *Enterococcus faecalis* and *Enterococcus faecium* strains of different origins

To investigate the genetic relationship of the clinical strains and the frequency of *esp* in epidemiologically and genetically related strains, PFGE analysis was performed for the

Enterococcus faecalis strains and the *E. faecium* strains from SIR study, and for a subset of *E. faecium* strains from different German hospitals.

3.3.1 PFGE analysis of *E. faecium* strains from different sources

The PFGE patterns of the 141 clinical *E. faecium* isolates analysed were resolved into five clusters (A to E) (Table 3.3, Figure 3.2, see Appendix). The isolates also included representatives of 27 outbreak strains recovered from different German hospitals and a set of eight outbreak isolates which were kindly provided by Rob Willems from the National Institute for Public Health and the Environment, The Netherlands. Four of these isolates originated from the Netherlands, two from U.S., one isolate from U.K., and one from Australia.

Clusters A to C included mainly *esp*-positive isolates recovered from different hospitals in Germany and isolated from a clinical site, whereas isolates from cluster D and E represented mainly stool isolates from hospitalized patients.

Cluster A comprised 4 isolates, two *esp*-positive outbreak isolates from two different German hospitals and two clinical isolates, of which one is positive for *esp*. The isolates from cluster B are mostly isolates recovered from infections, and include also four outbreak isolates from different German hospitals and six outbreak isolates from other countries (Table 3.3, Figure 3.2). This cluster could be further devided in three subclusters: B_1, B_2 and B_3. Subcluster 1 includes 33 isolates that share about 91% similarity, 16 of which are *esp*-positive (48.5%). Among these isolates were found three outbreak isolates from Germany (two of which are *esp*-positive) and one esp^+ outbreak isolate from the U.K. Subcluster 2 includes five isolates negative for both *esp* and bacteriocin. Subcluster 3, formed at a similarity breakpoint of 91%, includes 14 isolates from which 9 are positive for *esp*. Five outbreak isolates from other countries (of which four are *esp*-positive) and one *esp*-positive outbreak isolate from Germany are also found in this subcluster. Isolates with indistinguishable or closely related genotypes were identified in cluster B, that were heterogenous with respect to the presence of *esp*. Bacteriocin production was only rarely detected in this cluster, but only a few isolates were tested.

Most of the German outbreak isolates (20 from 27) were found in cluster C, together with a small number of clinical isolates from other sources and with one one outbreak isolate from the U.S. The isolates in this cluster shared more than 93% similarity and may originate from a single clonal lineage. This cluster included also one isolate with VanB-type resistance, which was also positive for *esp* (UW 3069).

Cluster D and cluster E comprised mainly stool isolates from hospitalized patients, and cluster E included only isolates negative for both *esp* and bacteriocin. Cluster D was composed mainly from fecal isolates of hospitalized patients from H. (Saxony-Anhalt; n= 23/31) and included also one outbreak isolate from The Netherlands (*esp*-negative). The isolates in the latter cluster shared >90% homology and may have derived from the same parent strain. Interestingly, isolates from this cluster harbour either *esp* or enterocin, none of the isolates being positive for both traits.

Although a few strains tested (n=29), hyaluronidase (*hyl* gene) was not detected by PCR in any of the isolates (data not shown).

3.3.2 PFGE analysis of *Enterococcus faecalis* and *Enterococcus faecium* from SIR study

Enterococci from the SIR study are included in order to have a look at strains from a prospective investigation which covers both isolates from clonal transmission between different patients and also several isolates from sporadic infections.

When *E. faecalis* strains analysed, eight clusters were identified (Figure 3.3, see Appendix). Overall, the clusters comprised strains from different units except for cluster VII and cluster VIII which mostly included strains from one unit. Cluster VII included 5 strains from unit CCM1 with identical genotypes representing the outbreak strain, one strain from CVK43, another one from unit CVK8 and the last one from CCM1 which are closely related to the outbreak strain, representing outbreak 1. Cluster VIII included 14 strains, 12 strains with identical genotypes from unit CCM1 (identified as the outbreak strain in outbreak 2) and two other strains from CVK8 and CVK43, respectively, which are closely related, if not identical to the outbreak strain.

The *esp* gene has been detected among all clusters, except for cluster III (n=5 strains) and VI (n=8 strains) which included only *esp*-negative isolates. The size of the PCR-product was variable, even within one cluster. However, the *esp* fragment detected in strains from cluster VII was about 1300 bp, and 750 bp for strains in cluster VIII, respectively. The *esp*-positive strains that belong to both clusters are also enterocin producers. If we consider the isolates with identical genotypes, recovered from ≥ 3 patients as epidemic strains, 17 epidemic strains can be identified from which 11 (65%) were positive for *esp*. From the 27 non-epidemic strains identified (recovered from ≤ 2 patients), 13 (48%) were *esp*-positive. It has to be noted that some epidemic strains (e.g., UW 2881, 18 patients in one unit; UW 3026, 11 patients in one unit) are represented in the diagram by only one isolate.

Among *E. faecium* isolates, 9 clusters were obtained, each cluster including isolates from different units (Figure 3.4, see Appendix). The *esp* gene was detected mostly in clusters 1 through 5, with cluster 2 and 4 including only *esp*-positive strains, and clusters 3 and 5 comprising both *esp*-positive and *esp*-negative isolates. One outbreak strain was identified, represented by six isolates UW 3056-UW 3062 in cluster 4, which are *esp*-positive. Related to the outbreak strain, but with 6% heterogenity are the three strains from cluster 3, recovered from three different units, of which one is *esp*-positive.

Based on the criteria stated above for epidemic strains, the isolates depicted in the diagram from Figure 3.4 can be devided in 8 epidemic strains and 20 non-epidemic strains. All the epidemic strains are found in clusters 1 through 5, from which 6 were *esp*-positive (75%) and included also the outbreak strain. The *esp* gene was also detected in 6 non-epidemic strains (30%). However, a few epidemic strains (UW 3047, 4 patients in unit 43; UW 2901, 4 patients in unit 8) are depicted in the diagram by one representative isolate. A small number of isolates were tested for the presence of the *hyl* gene (n=8), from which three isolates gave positive reactions (UW 3047, UW 3096, UW 2901; data not shown).

3.3.3 Genotypic comparison of *E. faecium* isolates from SIR study and from different other hospitals

In order to explore the clonal relationship of the clinical isolates from both sets of strains, the PFGE patterns of 43 isolates were further compared, with the isolates from SIR study (n=12) being vancomycin-sensitive, except for one strain, which was VAN^R and *esp*-positive (UW 3048). The patterns could be devided in two groups (Figure 3.5, see Appendix).

Of the 15 isolates of genogroup 1, five were *esp*-positive; the isolates could be further grouped in four clusters with a 90% similarity breakpoint. The isolates of the second group formed five PFGE clusters, most of the isolates being *esp*-positive. Only two isolates from 28 belonging to the second group were *esp*-negative.

Cluster 1 included three isolates recovered from Berlin2, two of them were part of the SIR study. Cluster 2 includes three outbreak isolates recovered from U.S., Australia, and from The Netherlands, respectively. The stool isolates from H. (Saxony-Anhalt) were found mainly in cluster 3 together with one outbreak isolate from The Netherlands and one epidemic isolate from SIR study (UW 3370).

The outbreak isolates recovered from Germany were grouped in PFGE cluster 8. This cluster also included three outbreak isolates from U.S., U.K., and the Netherlands, respectively, and one VAN^S epidemic isolate from SIR study (UW 3097).

PFGE cluster 9 contained mainly isolates from SIR study (3 from 5 isolates), and one of them is the VAN^S outbreak strain (UW 3060). The VAN^R *esp*-positive isolate from SIR study was also included in this cluster (UW 3048). Generally, the isolates from SIR study were spread among clusters in both genogroups. Additionally, isolates susceptible and resistant to vancomycin, with closely related genotypes, were identified.

Bacteriocin production was detected among isolates from different clusters. However, isolates positive for both *esp* and bacteriocin were found mainly among outbreak isolates from cluster 8, and among isolates in cluster 5 and 9.

3.4 Conjugation experiments

In order to determine whether the *esp* gene is a transferable marker and whether the transfer can be correlated to a particular resistance determinant, conjugation experiments were further performed.

The strains were chosen on the basis of resistance phenotype and the presence of *esp*. The mating experiments were performed on nitrocelulose filters using a filter mating aparatus from Sartorius Germany.

3.4.1 Transfer of resistance determinants

A total of 14 strains (*E. faecalis* and *E. faecium*) with resistance to either vancomycin (VAN), oxytetracycline (OTE) or erythromycin (ERY) (the most easily transferable determinants) and susceptible to fusidic acid and/or rifampicin were mated with the recipients *E. faecium* 64/3 or *E. faecalis* JH2-2, which are resistant to fusidic acid and rifampicin, respectively (Table 3.4).

E. faecium strains UW 3185, UW 3181, UW 3311 did not give any detectable transconjugants. *E. faecalis* strains UW 2981, UW 2982, UW 2840 did not transfer any resistance determinant according to the resistance phenotype, and the mating rates obtained for the above mentioned crosses reflect probably associations of donor and recipient cells that have managed to grow on selective plates.

In the mating pair UW 3410 x JH2-2, ERY^R and OTE^R were transferred to 5 transconjugants from 18 tested. *E. faecium* UW 2384 transferred the resistance to ERY, OTE and VAN to 4 out of 6 transconjugants tested. Strain UW 3114 (*E. faecalis*), *E. faecium* strains UW 1978, UW 2834, UW 3308, UW 3183 and UW 3440 transferred the above mentioned resistance determinants to all the transconjugants tested. Strain UW 3440, which is resistant to ERY and VAN transferred only vancomycin resistance to the recipient cells after selection on VAN plates.

When both present, resistance determinants for erythromycin and vancomycin have transferred together, exception makes, however, the mating pair UW 3440 x 64/3, which gave rise to transconjugants with resistance only to vancomycin. OTE resistance has transferred in all cases when present, together with the other resistance determinants, but not exclusively. In this respect, after mating between UW 2384 and the recipient *E. faecium* 64/3, transconjugants were detected that acquired only OTE resistance, after selection on OTE. Selection on vancomycin determined transconjugants with acquired resistance to all three antibiotics.

Finally, resistance transfer was observed in 2 from 5 matings with *E. faecalis* donor strains and in 6 from 9 matings with *E. faecium* donor strains.

3.4.2 Detection of the *esp* gene in the transconjugants

A number of transconjugants were subsequently tested by means of PCR for the presence of *esp*, together with the donor strains. The primer pair esp 5/6 was used for the detection of the gene.

From the eight strains tested, only 3 (2 *E. faecalis* and one *E. faecium* strain) were identified with *esp*-positive transconjugants. The *E. faecium* strain UW 3308 and *E. faecalis* UW 3114 had a high frequency of *esp* transfer: 13 *esp*-positive transconjugants from 15 tested for UW 3308, and 3 *esp*-positive transconjugants from 4 tested for the *E. faecalis* strain, respectively. The *E. faecalis* donor strain UW 3410 has transferred the gene with a lower frequency, with only 3 *esp*-positive transconjugants from 10 tested (Table 3.5). The deduced number of A repeats amplified with this primer pair would be 1 for UW 3114 and its transconjugants, 2 repeats for UW 3410 and 5 A repetitive units for UW 3308 and its transconjugants.

From the 5 transconjugants of the donor strain UW 3410 which have acquired both erythromycin and oxytetracycline resistance determinants, only 3 have also acquired the *esp* gene.

Table 3.4 *Enterococcus faecalis* and *Enterococcus faecium* strains used in conjugation experiments

The donor strains	*The recipient*	*Selection plates*	*Transconjugants phenotype*	*Mating rate/recipient*
UW 3114 *E. fc.*	JH2-2	OTE/RIF	ERY,OTE	$3{,}28 \times 10^{-5}$
UW 2981 *E. fc.*	JH2-2	OTE/FUS	-	-
UW 2982 *E. fc.*	JH2-2	OTE/FUS	-	-
UW 1978 *E. fm.*	64/3	VAN/RIF OTE/RIF	OTE,VAN OTE,VAN	$0{,}063 \times 10^{-5}$ $0{,}031 \times 10^{-5}$
UW 2384 *E. fm.*	64/3	VAN/RIF OTE/RIF	OTE,VAN,ERY OTE	$0{,}168 \times 10^{-5}$ $0{,}179 \times 10^{-5}$
UW 2834 *E. fm.*	64/3	VAN/RIF OTE/RIF	VAN,OTE,ERY VAN,OTE,ERY	$0{,}339 \times 10^{-5}$ $0{,}349 \times 10^{-5}$
UW 3308 *E. fm.*	64/3	VAN/RIF	VAN,ERY	$1{,}063 \times 10^{-5}$
UW 3185 *E. fm.*	64/3	VAN/FUS ERY/FUS	-	-
UW 3410 *E. fc.*	JH2-2	OTE/RIF ERY/RIF	ERY,OTE ERY,OTE	$0{,}0325 \times 10^{-5}$ $0{,}0355 \times 10^{-5}$
UW 2840 *E. fc.*	JH2-2	OTE/RIF ERY/RiIF	- -	$0{,}0122 \times 10^{-5}$ $0{,}0231 \times 10^{-5}$
UW 3181 *E. fm.*	64/3	VAN/FUS OTE/FUS	-	-
UW 3183 *E. fm.*	64/3	VAN/FUS OTE/FUS	VAN,OTE,ERY VAN,OTE,ERY	n.d. n.d.
UW 3311 *E. fm.*	64/3	VAN/FUS OTE/FUS	- -	- -
UW 3440 *E. fm.*	64/3	VAN/FUS	VAN	$0{,}904 \times 10^{-5}$

VAN = vancomycin; OTE = oxytretracycline; ERY = erythromycin; FUS = fusidic acid; RIF = rifampicin

Antibiotic concentrations used for selection are

VAN 5 µg/ml; OTE 15 µg/ml; ERY 20 µg/ml; FUS 20 µg/ml; RIF 30 µg/ml n.d.=not determined

The *esp* gene has been transferred along with resistance determinants for erythromycin, in some transconjugants also with OTE resistance or VAN resistance. On the other hand, transfer of any resistance determinant did not exclusively promote the transfer of the *esp* gene, as demonstrated by the transconjugants that have acquired the resistance determinants but not the *esp* gene. These results demonstrate that the gene is not genetically related to any resistance marker.

Table 3.5 Transconjugants tested by PCR for the transfer of the *esp* gene from donor strains

Strains tested	No.of transconj. tested	*esp* positive	Length of *esp*[a]	Deduced no. of A repeat units	Resistance determinants acquired
E.fc.T-3114	4	3	750 bp	1	ERY, CLI, OTE
E.fm.T-1978	6	-	-		OTE, VAN, TEI
E.fm.T-2384	6	-	-		ERY, CLI, OTE, VAN, TEI
E.fm.T-2834	6	-	-		ERY, CLI, OTE, VAN, TEI
E.fm.T-3308	15	13	1700 bp	5	GEN[b], STR, ERY, CLI,VAN,TEI
E.fc.T-3410	10	3	1000 bp	2	ERY, CLI, OTE, CMP
E.fm.T-3183	8	-	-		STR, ERY, CLI, OTE, VAN, TEI
E.fm.T-3440	6	-	-		VAN, TEI

[a] length of the *esp* fragment is approximated;

[b] GEN high-level resistance detected only in one transconjugant

CLI-clindamycin; TEI-teicoplanin; GEN-gentamicin; STR-streptomycin

3.4.3 RAPD analysis

Four donor strains, two *E. faecalis* (UW 2840, UW 3410) and two *E. faecium* strains (UW 3183, UW 3440) together with the transconjugants and the recipients were further typed by RAPD (random amplified polymorphic DNA) analysis (Figure 3.6 and Figure 3.7). One primer was used, RAPD 10 with the sequence TGCTCTGCCC, for amplification of DNA. The genomic DNA was isolated, and the concentration of DNA was determined. Fifty ng of DNA were used for amplification.

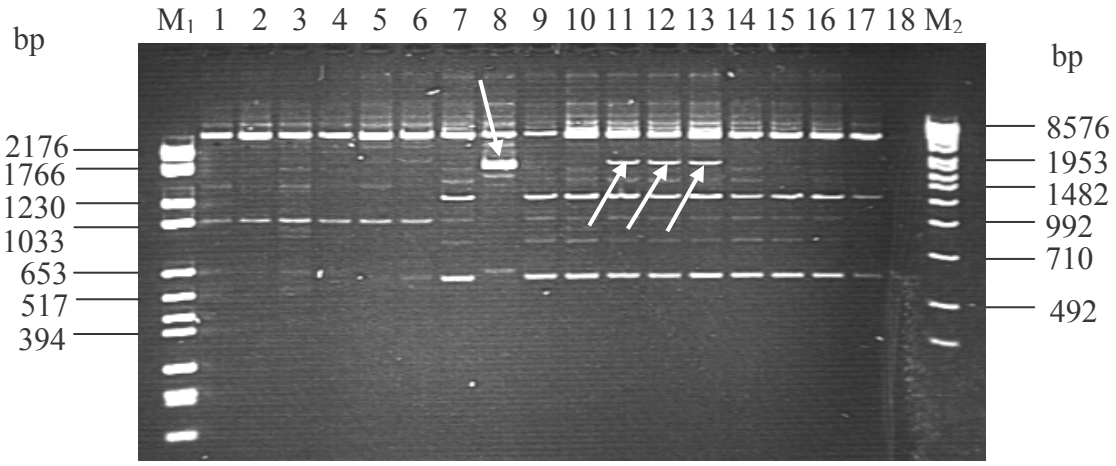


Figure 3.6 The banding pattern of donor strains UW 2840, UW 3410 and their transconjugants as determined by RAPD analysis

M1- Mol. Marker VI (Roche Mannheim, Germany);

lane 1 – UW 2840; lane 2 – T2840(1); lane 3 – T2840(2)

lane 4 – T2840(3); lane 5 - T2840(4); lane 6 - T2840(5); lane 7 – recipient *E.fc*. JH2-2;

lane 8 – UW 3410; lane 9 – T3410(3); lane 10 – T3410(4);

lane 11 - T3410(5); lane 12 - T3410(6); lane 13 - T3410(10); lane 14 - T3410(11);

lane 15 - T3410(12); lane 16 - T3410(14); lane 17 - T3410(21);

lane 18 - T3410(22); M2 – Mol.marker VII (Roche Mannheim, Germany)

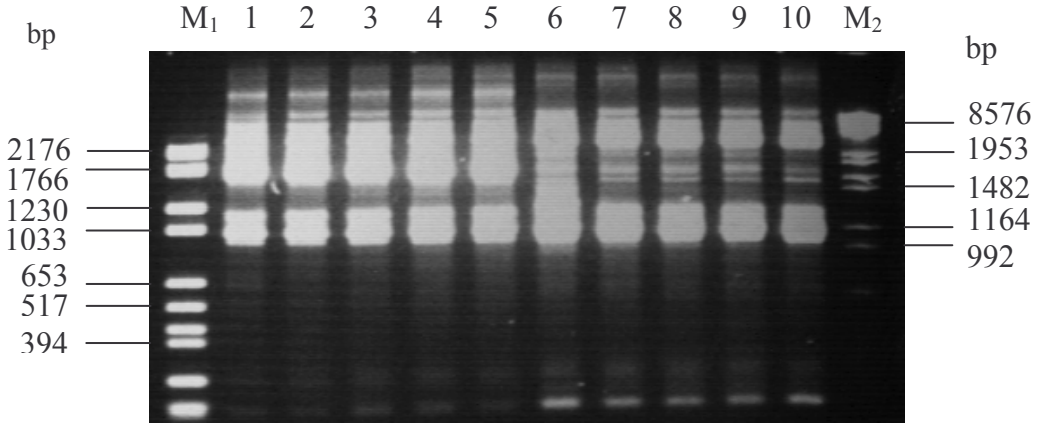


Figure 3.7 The RAPD patterns of donor strains UW 3183, UW 3440, and the transconjugants generated after mating with the recipient *E. faecium* 64/3

M1 – Mol.marker VI (Roche Mannheim, Germany);

lane 1 – UW 3183; lane 2 – T3183(1); lane 3 - T3183(2); lane 4 - T3183(9); lane 5 - T3183(11);

lane 6 – UW 3440; lane 7 – T3440(1); lane 8 - T3440(5); lane 9 - T3440(11);

lane 10– recipient *E.fm.* 64/3; M2 – Mol.marker VII (Roche Mannheim, Germany)

The fragments generated by T2840 are identical to that of the donor strain, suggesting that no mating has taken place between the donor and the recipient. The transconjugants of the donor strain *E. faecalis* UW 3410 have the same banding pattern as the recipient but in addition, three transconjugants, T3410-5, T3410-6 and C3410-10 show a fragment in common with the donor strain (white arrows from Figure 3.6). As the PCR analysis has revealed, the 3 transconjugants have acquired the *esp* gene. The RAPD patterns of T3410-4 and T3410-21 which have acquired the resistance determinants for ERY and OTE, show complete similarity to that of the recipient strain. These transconjugants have not acquired the *esp* gene.

The fragments generated by transconjugants T3183 are identical to that of the donor strain suggesting that no transfer has taken place between the donor and the recipient strain (Figure 3.7).

The transconjugants 3440 show great homology to the recipient strain, these having acquired the VAN resistance determinant but not the *esp* gene.

3.4.4 PFGE analysis

Five donor strains and their transconjugants: UW 3308, UW 3114 with the *esp* (+) and the *esp* (-) transconjugants; UW 2834, UW 2384, UW 1978 and their *esp* (-) transconjugants were further analysed by means of PFGE.

The DNA was isolated and subjected to restriction with *Sma*I enzyme. The macrorestriction patterns generated by the transconjugants T2834, T2384 and T1978 are identical to those generated by the recipient 64/3. The restriction fragments obtained from the transconjugants T3308 and T3114 differ from that of the recipient 64/3 and JH2-2, respectively (Figure 3.8).

Transconjugants T3308-8 and T3308-5 have acquired the *esp* gene, while T3308-7 is *esp*-negative. The transconjugant T3308-8 has acquired along with ERY and VAN resistance determinants, also resistance to gentamicin (high level) and streptomycin. The transconjugants T3308-5 and T3308-7 have acquired only streptomycin resistance together with ERY and VAN. The *esp*-negative transconjugant has a band of about 95 kbp in addition when compared with the recipient's banding patterns. Two bands can be detected in the *esp*-positive transconjugants that are not present in the PFGE-pattern of the recipient. In the transconjugant T3308-8, the two bands have about 90 and 117 kbp, while transconjugant T3308-5 present also a band of about 90 kbp and the other one of 110 kbp, respectively, these two transconjugants differing from each other in gentamicin resistance, as already mentioned.

The PFGE patterns of the T3114 containing the *esp* gene show a missing band (~200 kbp) when compared with those of the recipient and of the *esp* (-) transconjugant (Figure 3.8).

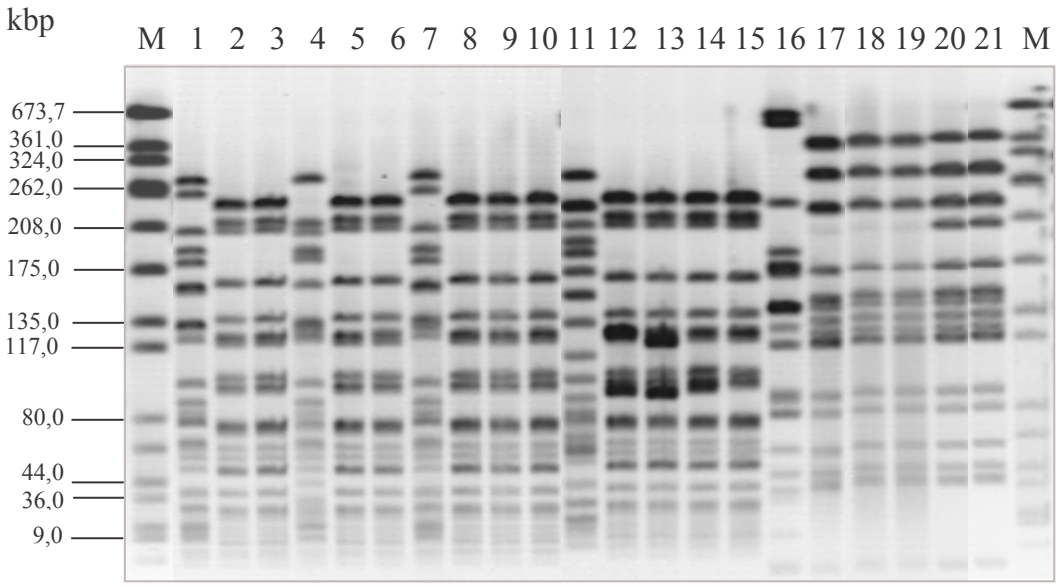


Figure 3.8 *Sma*I restriction fragments generated by donor strains, their transconjugants, and the two recipients, lanes 1-21

Lanes: M - *Staph. aureus* NCTC 8325 (molecular mass standard);

lane1 - UW 2834 ; lane2 - T2834-4 ; lane3 - T2834-13 ;

lane4 - UW 2384 ; lane5 - T2384-1 ; lane6 - T2384-15 ;

lane7 - UW 1978 ; lane8 - T1978-3 ; lane9 - T1978-16 ; lane10 – recipient 64/3;

lane11 - UW 3308 ; lane12 - T3308-8 (Gen^R; *esp*+); lane13 - T3308-5 (Gen^S; *esp*+); lane14 - T3308-7 (Gen^S; *esp*-); lane15 - recipient 64/3; lane16 - UW 3114; lane17 - T3114-1(*esp*+); lane18 - T3114-2 (*esp*+); lane19 - T3114- 3 (*esp*+); lane20 - T3114-4 (*esp*-); lane21 - recipient JH2-2

3.5 Hybridization experiments

The location of the *esp* gene within the genome was first demonstrated for the donor strains used in the mating experiments, after hybridization of the genomic DNA and plasmid DNA (Figure 3.9). The labeled probes were recovered from *E. faecium* strain UW 3308 and *E. faecalis* strain UW 3114. For hybridization, digested genomic DNA and non-digested plasmid DNA were used. Transfer of DNA onto nitrocelulose filters was acomplished by capillary blot.

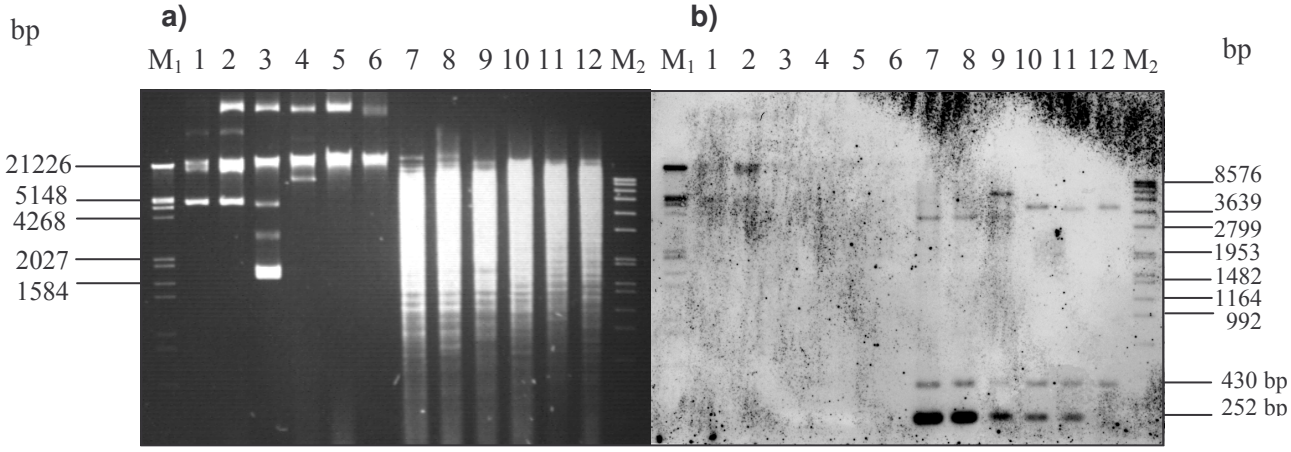


Figure 3.9 Southern hybridization of plasmid DNA and *Hind*III-digested genomic DNA of *E. faecalis* and *E. faecium* donor strains

a) Restriction patterns resolved in 1% agarose gel electrophoresis

b) Corresponding Southern blot hybridized with a labeled *esp* probe

Lanes: M1 - DNA marker III (Roche Mannheim, Germany), M2 - DNA marker VII (Roche Mannheim, Germany)

Lanes 1 to 6 – plasmid DNA: lane1 – *E.fm*.UW 1978 ; lane 2 – *E.fm*. UW 2384 ;

lane 3 – *E.fm*. UW 3308 ; lane 4 – *E.fc*. UW 2981 ; lane 5 – *E.fc*. UW 2982 ;

lane 6 – *E.fc*. UW 3114.

lanes 7 to 12 – genomic DNA of the strains in the same order

No band was detected on the plasmid DNA of the strains, instead three bands were visible on genomic DNA, suggesting that our gene is located on the chromosome.

The restriction map for *Hind*III of the *esp* gene described by Shankar et.al. (1999) in *E. faecalis* demonstrates that the enzyme makes one cut at nucleotide position 1998 and it cuts once within each A repeat, so that different fragments are obtained for the above mentioned gene: one including the first 1997 nucleotides of the N-terminal domain, one fragment of 430 bp upstream of the A repeats, two fragments of 252 bp, and the last one containing the 2860 nucleotides from the carboxy terminus, which includes the C repeat region.

For each strain tested here, three bands were visible on the preparation of genomic DNA. The largest fragment detected includes probably the nucleotides from the 3' end and has different sizes: 3000 bp for UW 1978 and UW 2384, 5500 bp for UW 3308, and about 4200 bp for UW 2981, UW 2982, and UW 3114. The middle band of 430 bp, including the nucleotides upstream of the A repeats was detected in all the strains. The smallest band of 252 bp, representing the size of one A repeat, had different intensities depending on the number of the A repeats within the gene of each strain. For *E. faecium* strains UW 1978 and UW 2384 the band had the highest intensity corresponding to a larger number of A repeat units. The next three strains (*E.fm*. UW 3308, *E.fc*. UW 2981 and UW 2982) showed a 252 bp-fragment with a lower intensity, while the last strain, *E.fc*. UW 3114 showed no signal for the corresponding DNA fragment. The amplified PCR-product from UW 3114, containing the A repeat region, was also very small (750 bp) corresponding to one A repeat, which does not give a detectable signal after hybridization. The intensity of the 252 bp-band correlates with the size of the PCR-product for each strain: 2100 bp product for the first two strains, UW 1978 and UW 2384 (six A repeats), 1700 bp-PCR product for UW 3308 and UW 2981 (five A repeats), 1300 bp product amplified from UW 2982 (three A repeats) and 750 bp for UW 3114 (one A repeat) which gave no signal on the membrane, as above mentioned.

A second hybridization of the plasmid DNA was performed for *E. faecium* UW 3308, *E. faecalis* UW 3114 and the transconjugants T3308-8 and T3114-1, both of them *esp*-positive (Figure 3.10). The plasmid DNA was isolated and digested with *Hind*III. One fragment was detected on the plasmid of T3308-8 which harbour the *esp* gene. The size of this fragment was 5500 bp. No band was detected on the plasmid DNA of the transconjugant T3114-1. To demonstrate the location of the gene on the chromosome of the *esp*-positive transconjugants and to determine the size of the transferred fragment, Southern hybridization was further performed after *Sma*I-digestion of the genomic DNA and PFGE of the donor strain UW 3114, the transconjugants T3114-1 and T3114-4, and the recipient *E. faecalis* JH2-2 (Figure 3.11).

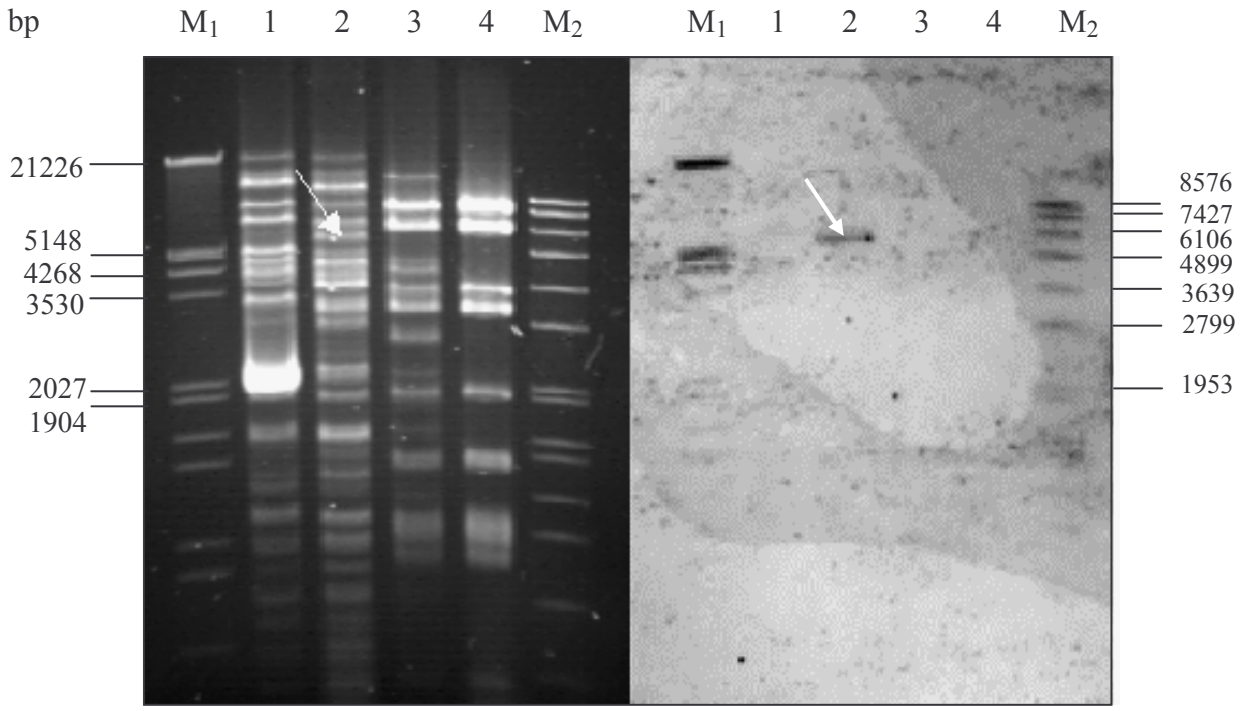


Figure 3.10 ***Hind*III-restricted plasmid DNA of UW 3308, T3308-8 (*esp*+), UW 3114, T3114-1 (*esp*+) (lanes 1 to 4) and the corresponding Southern blot onto nylon membrane**

Lanes M1 - DNA marker III (Roche Mannheim, Germany); M2 – DNA marker VII (Roche Mannheim, Germany)

The white arrow indicate the fragment on the plasmid DNA of the transconjugant which hybridized to the labeled probe on the right membrane.

The *esp* gene was found on the 638 kbp *Sma*I fragment of the donor strain. In the transconjugant T3114-1, the transferred fragment containing the *esp* gene has integrated into the 200 kbp *Sma*I fragment of the recipient resulting in the generation of a 380 kbp fragment which runs together with the largest *Sma*I fragment of the recipient genome. It seems that the fragment transferred by conjugation is about 180 kbp and recently it was demonstrated that the *esp* gene in *E. faecalis* is part of a pathogenicity island, the size of which is 153 kbp.

For UW 3410 and the *esp*-positive transconjugant T3410-5 and *esp*-negative T3410-21 hybridization experiments were also performed (data not shown) using plasmid DNA digested with *Hind*III. No hybrid fragment was visible for any of the strains, suggesting that both in donor strain and in the transconjugant 3410-5 *esp* is found on the chromosome.

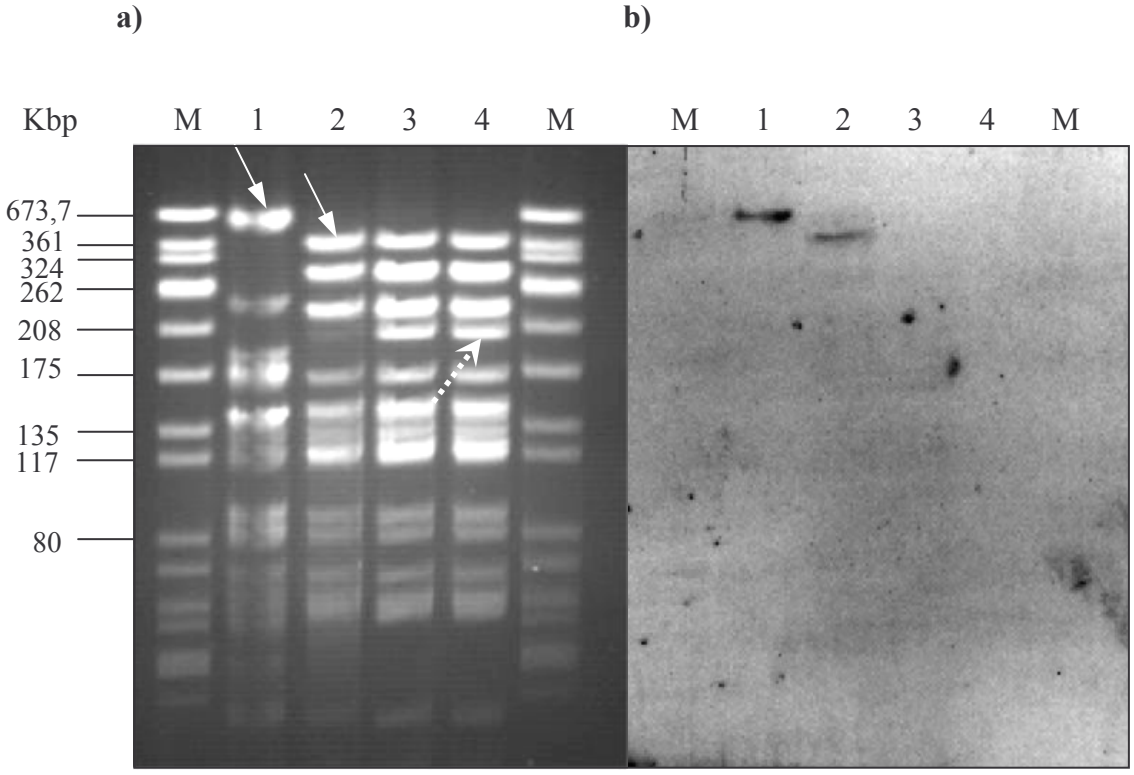


Figure 3.11 Southern hybridization of the *Sma*I-digested genomic DNA of the donor strain UW 3114 and the corresponding *esp*-positive and *esp*-negative transconjugants

a) PFGE of the *Sma*I-digested genomic DNA

b) Corresponding Southern blot probed with a labeled *esp* gene

Lanes: M - *Staph.aureus* NCTC 8325 (molecular mass standard);
lane1 - donor UW 3114; lane2 -transconjugant T3114-1 (*esp*+); lane3 -T3114-4 (*esp*-);
lane4 - recipient JH2-2

The arrows show the fragments from the donor and *esp*-positive transconjugant which hybridized with the labeled probe.

The dotted arrow shows the fragment in which the *esp*-containing DNA was integrated.

In humans, about 80-90% of the enterococcal infections are caused by *Enterococcus faecalis* and most of the remaining by *Enterococcus faecium* and they are now the second most common cause of hospital-acquired infections after *Escherichia coli* (Klare et al., 2001). *E. faecalis* remains the dominant causative agent of enterococcal infections, but recently there have been an increase in the number of *E. faecium* infections, most likely due to emergence of vancomycin-resistant strains (Eaton & Gasson, 2002; Hancock & Gilmore, 2002).

The role of a number of virulence factors in pathogenesis, including Esp, was supported by the fact that these determinants were only found in *E. faecalis* strains (Eaton and Gasson, 2001). This novel surface protein was first identified in *E. faecalis* MMH594 that caused multiple infections within a hospital ward. It was characterized as a marker of infection-derived isolates, since it was found with high frequency among clinical isolates and only rarely among fecal isolates of healthy volunteers (3%; n=1/34) (Shankar et al., 1999). The *esp* gene described by Eaton & Gasson (2002) in *E. faecium* (esp_{fm}) is similar in sequence and global organization to the *E. faecalis esp* gene (esp_{fs}) and it is likely that *esp* has a similar function in *E. faecium.* Moreover, the considerable identity of the *esp*-signal sequence with many Gram-positive adhesin protein signal sequences and with the biofilm-associated Bap-protein of *Staphylococcus aureus* provides support for a functional role of Esp_{fs} and Esp_{fm} as an adhesin-type protein (Eaton & Gasson, 2002).

The purpose of this study was to investigate whether the *esp* gene can be considered as an epidemiological marker, to establish whether the gene can be transferred between strains and whether the transfer is correlated to any antibiotic resistance determinant.

Since nosocomial infections caused by *E. faecium* have been reported worldwide and have frequently been associated with vancomycin resistance, we focused our search on the distribution of *esp* among *E. faecium* isolates. We have screened for this genetic determinant medical, commensal, animal, and environmental isolates of *E. faecium,* and additionally a small set of clinical *E. faecalis* isolates.

4.1 Frequency of *esp* in *Enterococcus faecium* isolates from different sources and correlation with their macrorestriction patterns

A first set of clinical *E. faecium* isolates was recovered from different hospitals in Germany, most of them (91%) exhibiting resistance to vancomycin type A, two isolates with

VanB type resistance, and the rest of 12 isolates being vancomycin-sensitive. We found the gene in 49% of the VAN^R clinical isolates, many of them representing clonally related outbreak strains (30 from 70 isolates *esp*-positive and VAN^R). A large part of the *esp*-positive outbreak strains from Germany (n=19) were found in one PFGE cluster (cluster C), together with one outbreak strain from the U.S. [outbreak strains being generally defined as isolates that are both epidemiologically related (e.g., by time, place, and common source of infection) and genetically related]. Most of the remaining clinical isolates associated with infections were found in cluster B, which also included four outbreak isolates recovered from different German hospitals, three of them were *esp*-positive; six outbreak isolates from outside Germany (The Netherlands, U.S., U.K., and Australia), of which five were positive for *esp*. Two *esp*-positive outbreak isolates were included in cluster A (UW 2368 and UW 1984, representing two different hospitals), and other two outbreak isolates which were *esp*-negative grouped in cluster E (UW 2490 recovered from a German hospital) and cluster D (UW 3321-The Netherlands, clone no. 2-3), respectively (Table 3.3, Figure 3.2, Appendix). All of the isolates are vancomycin-resistant *van*A type, with the exception of 4 isolates in cluster B and two isolates from cluster D, which are susceptible to VAN, of which one is *esp*-positive (UW 3264, cluster B). Similarly, one isolate was identified among the outbreak isolates from cluster C, which was resistant to VAN with *van*B type resistance. These data suggest that particular *E. faecium* clones are adapted to the hospital environment, widely disseminated among different hospitals, and some of them have subsequently acquired the *esp* gene. Two from the 12 remaining VAN^S isolates were identified as *esp*-positive. The frequency of *esp* reported here for VAN^R isolates is lower than that reported by Willems et al. (2001) (70%; n=115/165). This group was the first to describe the presence of a variant *esp* gene in a distinct genetic lineage of vancomycin-resistant *E. faecium* (VREF) associated with nosocomial infections, recovered from different geographical areas. Moreover, they reported that none of the non-epidemic isolates were *esp*-positive and that the epidemic VREF genogroup was distinct from the non-epidemic hospital isolates and from isolates recovered from healthy individuals. After analysis of the macrorestriction patterns generated by the clinical isolates in our study, the outbreak isolates from Germany and from outside Germany were mainly found in three genetically related clusters (clusters A, B and C), which could suggest the existence of a more virulent subset of *E. faecium* which is hospital-adapted and is involved in nosocomial infections. Most of the outbreak isolates from Germany grouped in a single cluster. These clusters comprised also clinical isolates for which we do not know whether they were involved in outbreaks or whether they only caused sporadic infections. However, small sets of two isolates recovered from the same hospital, with indistinguishable genotypes can

be identified within the clusters B and C, and for some of these sets, the two isolates are heterogenous on the presence of *esp*. This demonstrates that the gene was acquired via horizontal transfer [e.g., UW 3180-1 and UW 3178 – C. (Saxony); UW 3002 and UW 3175 – L. (Saxony)]. It is possible that the outbreaks are caused by particular *E. faecium* clones that are widely disseminated in different hospitals in Germany.

A high prevalence of *esp* in VREF was also reported by Woodford et al. (2001) for the U.K. isolates (61%; n=17/28) and it was the first group to report the existence of *esp* also in vancomycin-sensitive *E. faecium* (VSEF) strains. In the present study, *esp* was found not only in epidemic strains, but also in non-epidemic strains, which is consistent with other reports (Woodford et al., 2001; Baldassari et al., 2001).

The hospital isolates studied here included urine isolates, blood isolates, wound swab isolates, fecal isolates, and isolates recovered from other sites. We found a high frequency of *esp* in blood and wound swab isolates (67% for both sites; n=12/18 for blood isolates and n=14/21 for wound swab isolates, respectively), and a lower frequency in fecal isolates from hospitalized patients (38%, n=17/45) and urine isolates (47%, n=9/19). A similar prevalence in blood isolates was reported by Eaton & Gasson (2002), but they reported a higher percentage in urine and wound swab isolates (80% for both sites). This can be due, again, to the small number of isolates tested (n=5 isolates from blood, 5 urine and 5 wound swab isolates). The study of Woodford et al. (2001) showed a similar high occurrence of *esp* in urine isolates (n=27, 85%), indicating a role for Esp from *E. faecium* (Esp_{fm}) in infection at this site, similar to that found for Esp in *E. faecalis* (Esp_{fs}). It was reported that one third of *E. faecalis* urine isolates were positive for *esp*, and it was demonstrated in an animal model of ascending urinary tract infection that *esp* contributes to colonisation of and persistance in the urinary tract (Shankar et al., 2001).

It seems likely that in *E. faecium*, Esp contributes to infection at all these sites. A high incidence in wound swab isolates and in blood and urine isolates related to a catheter may be associated with the ability of *esp*-positive isolates to form a surface biofilm. In *E. faecalis* the association between the ability of this species to produce a biofilm and the presence of *esp* was analysed (Toledo-Arana et al., 2001). A highly significant association between the presence of *esp* and the ability to produce a biofilm in vitro was observed. The same group reported that none of the *esp*-deficient strains tested was able to produce a biofilm, whereas 94% of the strains harbouring *esp* had this property. Furthermore, the study of Toledo-Arana et al. (2001) revealed that five out of six *esp*-positive, biofilm-negative *E. faecalis* isolates did not produce Esp, strongly suggesting that the absence of biofilm formation in these strains was associated with the lack of Esp expression. However, interuption of the *esp* gene in the strong biofilm-producing

strains did not affect this property, thus indicating the presence of other traits involved in attachment and biofilm formation in these strains. This is consistent with more recent studies, that could find no direct correlation between *esp* detection and biofilm-forming ability in *E. faecalis* strains (Archimbaud et al., 2002; Sandoe et al., 2003). The results of Duprè et al. (2003), however, confirmed this association for *E. faecalis*, but not for *E. faecium* isolated in Italy.

We screened for this virulence gene also small sets of commensal isolates (n=36), animal isolates (food and manure; n=38) and environmental isolates (sewage; n=24) which were found completely free of this determinant. Our results correlate well with those of Eaton & Gasson (2002) who showed that none of the commensal and food isolates investigated were positive for *esp*. A low incidence of the gene (6%, n=2/33) among commensal isolates was reported by Coque et al. (2002) for the Spanish isolates. In *E. faecalis* commensal isolates, a frequency of *esp* in 3% (n=1/34) of the U.K. strains was reported by Shankar et al. (1999) and 40% (n=35) reported by Waar et al. (2002a) for the isolates from The Netherlands. The latter group has also reported a high frequency of *esp* among isolates from liver transplant patients, which were mostly isolated from feces. These findings, together with the identification of an epidemic esp^+ strain among liver transplant patients demonstrate the role of Esp in colonization and spread of *E. faecalis* in liver transplant patients.

We included in our investigation a low number of isolates from food (poultry meat and pork; n=3) and our results correlate well with those of Eaton & Gasson (2001) who did not find the gene in any of the *E. faecium* food isolates tested (n=11), including also cheese and milk isolates. However, Franz et al. (2001) reported the presence of *esp* in a low number of *E. faecium* isolated mostly from cheeses (2.1%, n=1/48). The same studies have reported the presence of the *esp* gene in *E. faecalis* food isolates, with a higher frequency than that reported for *E. faecium* isolated from food. Thus, the above mentioned groups have found a similar prevalence of *esp* in *E. faecalis* food isolates, 33% (n=3/9; Eaton & Gasson,2001) and 36,2% (n=17/47; Franz et al., 2001), respectively.

The absence of the *esp* gene in animal isolates was first demonstrated by Willems et al. (2001) and by the results of Coque et al. (2002). In *E. faecalis* isolated from animals, the absence of the *esp* gene was also reported. However, a group from Denmark (Hammerum et al., 2002) have recently shown that 8% (n=6/75) of the *E. faecalis* isolated from pigs were *esp*-positive, but none of the isolates from poultry.

None of the environmental isolates investigated in this study carried the *esp* gene. The same result for environmental strains (n=0/20, 0%) was reported by Coque et al. (2002) but in contrast to this, Eaton & Gasson (2002) showed a high incidence of the gene among

environmental isolates (40%; n=2/5), which, however, can be partly explained by the low number of strains tested (n=5).

The presence of *esp* in clinical isolates, especially the high prevalence of the gene in blood and wound swab isolates, and absence of the gene in all non-clinical isolates in this study reflect a role for *esp* in infection. Also the absence of the gene in commensal isolates but its presence in fecal isolates from hospitalized patients obviously indicate that the strains were acquired in the hospital environment and that *esp* might be involved in the spread of the strains. We found in our collection of clinical strains that *esp* was present in most of the outbreak isolates, but its detection also in non-epidemic strains (which, however, can be the begin of an outbreak) suggests that other traits are also important in the nosocomial spread. One of these factors could be (combined) enterocin production, since it was detected mostly in the *esp*-positive isolates (29 ent+/esp+ vs. 9 ent+/esp-), and the isolates positive for both traits were mainly outbreak isolates.

4.2 Occurrence of *esp* in clinical *Enterococcus faecalis* and *Enterococcus faecium* recovered from five intensive care wards at Charité University Hospital in Berlin

The distribution of *esp* among clinical *E. faecium* and *E. faecalis* strains isolated over a period of several years was investigated prospectively in 5 ICUs, as part of the SIR study. A high number of *esp*-positive isolates was found among *E. faecalis* strains (65%, n=60/93), but a high frequency was also found in *E. faecium* strains (55%, n=22/40). All *E. faecalis* isolates tested were vancomycin-susceptible and only 4 *E. faecium* isolates were resistant to VAN (VanA-type).

The *E. faecalis* isolates were grouped after macrorestriction analysis in eight clusters (according to the guidelines of Tenover et al., 1995; Figure 3.3, Appendix). The *esp* gene was found among strains from six clusters, the other two being free of the determinant. Four clusters (I, II, IV and V) are comprised mostly from *esp*-positive isolates recovered from different units. Most of the strains were isolated from two or more patients over a period of > 5 days, and only a few isolates from single patients. We have identified two outbreak strains that belong to cluster VII and cluster VIII, respectively, which are all *esp*-positive. Identification of isolates of the same genotype from different units (e.g., UW 3378/UW 3286, cluster VIII) and of strains that are closely related to the outbreak strain but isolated from other units (e.g., UW 3218/UW 3290; UW 3378/UW 3286, cluster VIII) suggest the transmission of the strains from one unit to another. In addition, identification within one cluster of strains with closely related genotypes

which are positive and negative for *esp* demonstrates the acquisition of the gene via horizontal transfer (e.g., UW 3211/UW 3209, cluster V).

Existence of differently sized *esp* fragments, as determined by PCR, even within one cluster (e.g., UW3020/UW3021, 1000 bp/750 bp, clusterV), supports the idea of recombination between the tandem units, as described by Shankar et al. (1999). Such genetic events result in deletions or duplications of the repeats, which might serve to retract the protein from the surface, hiding the protein from the immune system (Toledo-Arana et al., 2001). A possible role for *esp* in evasion of the immune system is also supported by the overall sequence similarity of Esp with Rib and C alpha proteins, known to possess this function in *S. agalactiae* (Shankar et al., 1999; Eaton & Gasson, 2002).

Overall, we found a higher prevalence of *esp* in *E. faecalis* clinical isolates than that reported by Shankar et al. (1999) (32%, n=43/133) and Eaton & Gasson (2001) (44%, n=4/9). From the blood isolates tested in this study, 75% (n=18/24) were *esp*-positive, compared with 29% (n=29/100) reported by Shankar et al. (1999) and with 45% (n=44) reported by Waar et al. (2002a) for the isolates from U.S. and The Netherlands, respectively. Most of the *E. faecalis* blood isolates in this study were related to catheter usage (n=14 esp^+/18). Similarly, 19 from 30 urine isolates were related to catheter usage, from which 11 were found to be *esp*-positive, thus confirming a role for Esp in adherence to abiotic surfaces. A high frequency of *esp* was also found in wound swab isolates.

In *E. faecium* clinical isolates recovered from the same hospital, 55% were *esp*-positive, of which most were vancomycin-sensitive and only one strain vancomycin-resistant. One outbreak was identified with six isolates with identical genotypes recovered from six patients, represented by cluster 4 (all six *esp*-positive; Figure 3.4, Appendix). We have also identified three isolates from different units possibly related to the outbreak strain (UW3054, UW2903, UW3050), one of them positive for *esp*. This, together with the identification of genetically related strains but heterogenous with respect to the presence of *esp* (e.g., UW 2900/UW 2884, cluster 7; UW 3279/UW 3094, cluster 8) could suggest the transmission of strains between units and aquisition of *esp* via horizontal transfer.

A significantly higher prevalence of *esp* was found among epidemic strains (75%, n=6/8 epidemic strains) compared with non-epidemic strains (30%, n=6/20), suggesting again a role for *esp* in spread of the strains, but other virulence traits might also be involved in nosocomial spread. In this set of *E. faecium* strains, we found a low incidence of enterocin production and almost all the strains were VAN susceptible, indicating that still other factors may contribute to the nosocomial spread of *E. faecium* strains.

Willems et al. (2001) noted a variant *esp* gene as a marker of a distinct genetic lineage of VREF associated with hospital outbreaks. The same group reported that none of the non-epidemic clones tested was positive for *esp*. Subsequently, the *esp* gene was reported in vancomycin-sensitive *E. faecium* and in non-epidemic strains, as well. Thus, Woodford et al. (2001) have shown that 64% (n=36/56) of the clinical VSEF isolates from U.K. were *esp*-positive, which is similar to the results reported here.

In Italy, where nosocomial infections involving VRE and the prevalence of VRE is not so common, the incidence of the *esp* gene is lower at 33% (n=6/18) in clinical non-epidemic VSEF, which is similar to the prevalence of the gene reported here for non-epidemic strains. However, a more recent study (Duprè et al., 2003) has shown that 71.9% (n=23/32) clinical isolates, mostly VAN^S, recovered from a hospital in Sardinia (Italy) were positive for *esp*.

Coque et al. (2002) have found the *esp* gene in 26% (n=25/96) of the clinical VSEF from Spain, with a higher frequency among ampicillin (AMP) resistant than AMP-susceptible strains, remarking that the esp^+ AMP^R strains were more prevalent than the esp^- AMP^R strains. We found the *esp* gene in AMP^S as well as in AMP^R strains, but most of the investigated strains were AMP^R. Recently, the study of Hammerum et al. (2002) on the distribution of *esp* in *E. faecalis* and *E. faecium* isolates from Denmark has suggested a possible genetic relation between *esp* and resistance determinants for GEN (high level), STR and ERY. We found *esp* in 75% of GEN^R (high level) *E. faecium* strains from the SIR study and a similar frequency of *esp* was found among GEN^R *E. faecium* clinical strains from other hospitals, which could suggest a possible connection between *esp* and high level resistance to GEN. On the other hand, 62.5% of the *esp*-positive clinical isolates from different hospitals exhibited high level resistance to GEN, while 50% of the *esp*-positive *E. faecium* isolates from the SIR study were resistant to high levels of GEN. Resistance to AMP, ERY and STR was found in both *esp*-negative and *esp*-positive isolates, susceptible or resistant to VAN.

The *E. faecium* clinical strains tested here were mostly isolates from wound swab (n=30) and we found that 63% of them were positive for *esp,* suggesting one more time the possible role of Esp in biofilm formation.

When VAN^S *E. faecium* strains from the SIR study (n=12) were compared with VAN^R clinical strains from different other hospitals (n=31), most of the *esp*-positive isolates were grouped in one genomic group, which included also the German outbreak isolates recovered from different hospitals, and three outbreak isolates from U.S., U.K., and The Netherlands, respectively (Figure 3.5, Appendix). In this genogroup were found most of the VAN^S isolates from the SIR study, including the outbreak isolate (UW 3060). Vancomycin resistance and

susceptibility was detected in isolates with closely related genotypes (e.g., UW 3097 from SIR study and the outbreak isolate UW 901) suggesting, as for *esp*, the acquisition of the *vanA* gene cluster via horizontal transfer. These data also indicate that obviously hospital-adapted virulent *E. faecium* strains were already spread in hospitals before acquisition of the *vanA* gene cluster. It seems likely that these strains, which are mostly *esp*-positive, belong to a subset of the species *E. faecium* with special adaptation to the hospital environment. The isolates negative for *esp* were found in a separate genogroup, which, however, included also a few *esp*-positive isolates (5 from 15 isolates).

4.3 Comparison of the detection methods for *esp* used by different research groups

For detecting the *esp* gene in isolates of this study, we used a primer pair (esp5/6) kindly provided by Rob Willems from The Netherlands. The primer pair was based on a consensus sequence of different *esp* alleles from various *E. faecium* strains (R. Willems, unpublished data). In our case, the PCR results were further confirmed by dot blot hybridization, which have shown that a larger number of isolates were *esp*-positive compared with the PCR results (55% after hybridization versus 20% by PCR), which can suggest the sequence divergence between different variants of the gene among *E. faecium* clinical strains. Willems et al. (2001) and Baldassari et al. (2001) have sequenced a small segment from the N-terminal domain of the *esp* gene isolated from *E. faecium* and they found a large number of mutations compared with the *esp* gene in *E. faecalis*. Later, Eaton & Gasson (2002) have sequenced a variant *esp* gene from *E. faecium* and found 89% similarity with the gene from *E. faecalis* MMH594.

Different groups screened *E. faecium* isolates for the presence of *esp* using mostly the primer pair esp11/12 described by Shankar et al. (1999) for the *esp* gene from *E. faecalis*. These primers do not possess 100% identity with most of the *esp* alleles described/sequenced in *E. faecium* strains, thus raising the question whether studies using this primer pair were able to detect all *esp*-positive *E. faecium* strains. Our results suggest that for detection of *esp* in *E. faecium*, the PCR analysis should allways be accompanied by a less stringent method, such as dot blot hybridization.

4.4 Transfer of the *esp* gene and detection within the genome of the donors and the transconjugants

The mating experiments have shown that the *esp* gene can be transferred from one strain to another, at least within the same species. We have used in our conjugation experiments 5 *E. faecalis* donor strains and 9 *E. faecium* donor strains which were mated with *E. faecalis* and *E. faecium* recipients, respectively. Only three mating experiments were succesfull with respect to the transfer of *esp*. Two matings from 5 with *E. faecalis* as donors and only 1 from 9 matings between *E. faecium* strains have led to transconjugants positive for *esp*. However, the transfer of resistance determinants was accomplished in 5 from 9 matings with *E. faecium*, and in two matings with *E. faecalis*, the later ones resulting in *esp*+ transconjugants as well.

The transfer of *esp* could not be associated with glycopeptide resistance or with any of the resistance determinants since the gene did not transfer preferentially with any of them. Moreover, transconjugants resulting from the same mating pair with the same resistance phenotype were identified as *esp*-positive as well as *esp*-negative. It is known that in *E. faecalis* the *esp* gene is found on a pathogenicity island which does not include any resistance determinant, so that a direct genetic linkage between *esp* and any resistance determinant in this species is improbable. In our mating experiments with *E. faecium*, the esp^+ transconjugants aquired the resistance determinants for STR, ERY, CLI, VAN and TEI, and only one transconjugant aquired, in addition to the above mentioned determinants, the GEN high-level resistance determinant.

The analysis of the *Sma*I-digested patterns of the donors and the transconjugants have shown for the esp^- transconjugants no difference compared with the recipient strain.

However, the *esp*-negative transconjugants from the mating pair UW 3308 x 64/3 made an exception (Figure 3.8). The *E. faecium* esp^+ transconjugants displayed two bands in addition to the recipient's pattern and one band in addition to the *esp*-negative transconjugant. The suggestion that the transferred resistance determinants and the *esp* gene are localized on a plasmid is supported by blot hybridization (Figure 3.10). It seems likely that the transferred plasmid had two (or more) restriction sites for *Sma*I enzyme and the linearized fragments could be resolved and visualized after PFGE. The differences between the *esp*-positive and *esp*-negative transconjugants, as well as between the two *esp*+ transconjugants suggested that the transferred *esp* gene is found on the 117 (110) kbp fragments in the transconjugants and that the GEN resistance determinant is localized on the same fragment with the *esp* gene in the GEN^R transconjugant.

In *E. faecalis*, the PFGE-derived patterns of the *esp*$^{+}$ transconjugants show a missing band when compared to those of the *esp*$^{-}$ transconjugant and the recipient. One explanation would be a recombination event by which a large fragment containing the *esp* gene has integrated into a *Sma*I fragment giving rise to a larger DNA fragment with a similar size to one of the upper bands.

The hybridization experiments showed that the gene is localized on the chromosome of the *E. faecalis* and *E. faecium* donor strains. Thus, the transfer of *esp* can reflect the presence of the gene on a mobile genetic element. In the *E. faecium* transconjugants Southern blot experiments have demonstrated the presence of *esp* on the plasmid, together with the resistance determinants. It seems that the gene moved from the chromosome into the plasmid of the donor strain which was then transferred into the recipient strain, where it remained stable on the plasmid.

In *E. faecalis* the gene has transferred from the chromosome of the donor and stabilized on the chromosome of the recipient. Recently, the *esp* gene has been detected on the chromosome of the *E. faecalis* ward outbreak strain MMH594 within a pathogenicity island, a genetic element previously unknown in this genus, which also codes for other virulence determinants (aggregation substance, cytolysin) and a number of additional, previously unstudied genes that are rare in non-infection derived isolates (Shankar et al., 2002). In our case, the DNA fragment transferred from UW 3114 to the recipient was about 180 kbp in size and the size of the pathogenicity island is 153,571 bp, which could suggest that the entire pathogenicity island was transferred and then integrated into the chromosome of the recipient.

5. Conclusions

Among the virulence factors identified to date in *E. faecalis*, the *esp* gene is the only factor often detected in *E. faecium* strains recovered in the hospital environment. Moreover, the *esp* gene has been associated with a specific subset of *E. faecium* strains causing hospital outbreaks in different countries.

Our data confirm that *esp* is frequently found in *E. faecium* strains from the hospital setting and occur only rarely in the community and food isolates, as demonstrated by other studies, which provides indirect evidence that Esp_{fm} may contribute to virulence.

We found the gene with high frequency in blood and urine isolates associated many times with a catheter, and in wound swab infections, which could provide evidence that *esp* is involved in the primary attachment and biofilm formation. It seems that in *E. faecalis*, *esp* is involved in adherence to urinary tract epithelial cells *in vitro* and the same role can be attributted to *esp* in *E. faecium*, since the gene was found with high frequency also in urine isolates. A possible function for *esp* as an adhesin-type protein is supported by the fact that the Esp protein exhibits sequence similarity at the N-terminal domain with other gram-positive adhesin proteins signal sequences.

The incidence of *esp* among isolates resistant and susceptible to different antibiotics suggests that *E. faecium* did not acquire this gene recently, this trait probably emerged prior to acquisition of resistance not only to vancomycin but also to other antibiotics commonly used in the hospital setting. Moreover, widely disseminated ampicillin resistant *E. faecium* clones have been reported as the source for vancomycin resistance, which has led to the emergence of extended VREF outbreaks. In this study, all vancomycin resistant esp^+ *E. faecium* strains are also resistant to at least three more antibiotics, including ampicillin, streptomycin, erythromycin. It has been suggested that antibiotic resistant variants may arrise frequently under antibiotic selection pressure among *esp*-positive clones reaching ecological abundance in the nosocomial habitat, which is also shown by our results. Identification of epidemic and non-epidemic strains positive for *esp* suggests that in the spread of nosocomial strains, other factors are also important. Enterocin production and vancomycin resistance were found along with *esp* in the *E. faecium* outbreaks represented by the 20 strains from different hospitals, suggesting that these traits highly contribute to nosocomial spread. However, the esp^+ *E. faecium* outbreak strain from the SIR study involving only six patients, was vancomycin-susceptible and did not produce enterocin. This, together with the identification of *esp*-positive non-epidemic strains among the vancomycin-susceptible and vancomycin-resistant isolates, with a much lower frequency than among epidemic strains, and the grouping of the *esp*-positive isolates in one genomic group, suggest that strains harbouring *esp* have a higher potential for spreading in the hospital

environment and possibly belong to a more virulent subpopulation of the species. However, other traits are also involved in the nosocomial spread of infection. Detection of *esp* in *E. faecalis* commensal and pig isolates in a recent study increases the need for more investigation of the function of this protein.

The conjugation experiments have shown for the first time that *esp* can be transferred between strains, which may explain the high number of isolates harbouring this gene in different genomic backgrounds, both among *E. faecalis* and *E. faecium* isolates. Southern blot experiments have demonstrated the presence of the gene on the chromosome also in *E. faecium*, and the possible presence of the gene on a mobile element, since it could transfer by conjugation.

Zusammenfassung

Enterokokken sind Darmkommensale vieler Tiere und des Menschen. Desweiteren können Isolate vor allem der beiden Arten, *Enterococcus faecium* und *Enterococcus faecalis*, als nosokomiale Pathogene auftreten. Was einen harmlosen Darmbesiedler von einem epidemischen, pathogenen Isolat unterscheidet ist in weiten Zügen noch unbekannt. Das *esp* Gen erscheint als einer der wichtigsten, wenn nicht gar der wichtigste Virulenzfaktor zumindest für *E. faecium*. Darüber hinaus besteht ein Zusammenhang zwischen dem Besitz des *esp* Gens und einer spezifischer Klasse von *E. faecium* Stämmen, die für Ausbrüche innerhalb Krankenhäuser in verschiedenen Ländern verantwortlich sind.

Unsere Daten bestätigen, dass *esp* oft in *E. faecium* Krankenhausstämmen gefunden wird, hingegen nur selten in Isolaten aus nicht-hospitalisierte Personen oder Lebensmittel-Isolaten oder aus der Umwelt. Dies weist darauf hin, dass der Besitz des *esp* Gens zur Virulenz beitragen kann.

Wir haben das Gen mit hoher Frequenz in Isolaten aus Blut und Urin (z.T. Katheter-assoziiert) sowie aus Wundinfektionen gefunden. Dies ist ein Hinweis, dass *esp* in der primären Bindung und Biofilmbildung involviert ist. Es scheint, dass in *E. faecalis* das Esp Protein an der Adhäsion an Harnwegepithelzellen beteiligt ist. Die gleiche Rolle kann *esp* in *E. faecium* erfüllen, da das Gen mit hoher Frequenz in Urin-Isolaten gefunden wurde. Unterstützt wird die mögliche Funktion von Esp als Adhesin-ähnliches Protein durch die hohe Sequenzähnlichkeit seiner N-terminalen Domäne mit anderen gram-positiven Adhesin-Protein Signalsequenzen.

Das Vorkommen von *esp* in Antibiotika-resistenten und -empfindlichen Isolaten legt nahe, dass *E. faecium* dieses Gen nicht erst kürzlich erworben hat. Diese Eigenschaft entwickelte sich wahrscheinlich vor dem Erwerb der Resistenz gegenüber Vancomycin und anderen gebräuchlichen Antibiotika. Es wurde von weit verbreiteten Ampicillin-resistenten *E. faecium* Klonen berichtet, welche später Vancomycin Resistenz erwarben und die zum Auftreten von ausgedehnten VREF (vancomycin-resistant *E. faecium*) Ausbrüchen geführt haben. In der vorliegenden Arbeit sind alle Vancomycin-resistenten, *esp*+ *E. faecium* Stämme ebenfalls resistent gegenüber mindestens drei Antibiotika, einschließlich Ampicillin, Streptomycin, Erythromycin. Durch unsere Ergebnisse wird die Vermutung unterstützt, dass in nosokomialen Habitaten Antibiotika-resistente Varianten unter *esp*-positiven Klonen wegen des Selektionsdruckes weitaus häufiger auftreten. Die Identifikation von epidemischen und nicht-epidemischen *esp*-positiven Stämmen lässt den Schluss zu, dass bei der Ausbreitung von nosokomialen Stämmen auch andere Faktoren wichtig sind. Die Enterocinproduktion und Vancomycin-Resistenz wurden auch mit *esp* in den 20 *E. faecium* Ausbruchstämmen von

verschiedenen Krankenhäusern gefunden, was darauf hindeutet, dass diese Eigenschaften anscheinend eine nosokomiale Ausbreitung begünstigt. Jedoch war der *esp*$^{+}$ *E. faecium* Ausbruchstamm in der SIR Studie Vancomycin-empfindlich und produzierte kein Enterocin. Dies, zusammen mit der Identifikation von *esp*-positiven, nicht-epidemischen Stämmen unter den Vancomycin-empfindlichen und Vancomycin-resistenten Isolaten, die eine sehr viel geringere Frequenz als epidemische Stämme aufwiesen, lassen vermuten, dass Stämme, die *esp* tragen, ein höheres Potential zur Ausbreitung in Krankenhäusern haben. Jedoch sind auch andere Eigenschaften in der nosokomialen Ausbreitung involviert. Wie der Nachweis von *esp* in kommensalen und Schweine-Isolaten von *E. faecalis* in jüngeren Studien zeigt, sind weitergehende Untersuchungen zur Verbreitung und Funktion dieses Gens bzw. Proteins notwendig.

Die Konjugationsexperimente haben erstmalig gezeigt, dass *esp* zwischen Stämmen übertragen werden kann. Dies könnte die hohe Anzahl von Isolaten erklären, die dieses Gen in verschiedenen genomischen Hintergründen tragen. Durch Southern Blot Experimente konnte neben der Anwesenheit des Gens auf dem Chromosom von *E. faecium* die mögliche Anwesenheit des Gens auf einem mobilen Element nachgewiesen werden, da es durch Konjugation übertragen wurde.

Appendix

Table 3.2 Distribution of *esp* among clinical *E. faecium* isolates from different hospitals (A), and among *E. faecalis* (B) and *E. faecium* (C) isolates from SIR study

		esp positive[1]			Enterocin positive[1]	
	Total (%)	VanR	VanS	GHLR[2]	Total	esp+
A. *E. faecium*						
Blood isolates[3]	12/18 (67)	12/17	-/-	7/7	8/12	8/12
Urine isolates	9/19 (47)	8/12	1/7	7/7	6/9	6/7
Wound swab isolates	14/21 (67)	14/18	-/2	9/10	7/10	7/9
Stool isolates	17/45 (38)	17/45	-/-	14/25	9/36	-/10
Others	20/53 (38)	19/50	1/3	8/13	8/30	8/8
Total	72/156 (46)	70/142 (49)	2/12	45/62 (73)	38/97(39)	29/46(63)
B. *E. faecalis*						
Blood isolates[4]	18/24 (75)	-	18/24	n.d.	14/18	12/13
Urine isolates[5]	19/30 (63)	-	19/30	n.d.	12/20	9/15
Wound swab isolates	15/26 (58)	-	15/26	n.d.	12/20	9/12
Others	8/13 (62)	-	8/13	n.d.	6/8	5/5
Total	60/93 (65)		60/93(65)		44/66(67)	35/45(78)
C. *E. faecium*						
Blood isolates[6]	1/5	-/1	1/4	1/2	1/5	-/1
Urine isolates[7]	1/3	-	1/3	-/1	-/3	-/1
Wound swab isolates	19/30 (63)	1/3	18/27	11/13	5/30	4/19
Others	1/2	-	1/2	-	2/2	1/1
Total	22/40 (55)	1/4	21/36 (58)	12/16 (75)	8/40(20)	5/22(23)

[1]number positive / total isolates (%)

[2]Gentamicin high level resistance (> 1000 µg/ml)

[3] 4 isolates from bacteremia (2 *esp* +), 3 isolates from catheter (1 *esp* +), 11 blood isolates (9 *esp* +)

[4] 18 isolates from catheter (14 *esp* +, 78%) and 6 from blood (4 *esp* +)

[5] 19 catheter isolates (11 *esp* +, 58%) and 11 urine isolates (8 *esp* +)

[6] 4 isolates from catheter (0 esp +), 1 blood isolate (*esp* +)

[7] only isolates from catheter

n.d., not determined

Table 3.3 *Enterococcus faecium* clinical isolates from different German hospitals*

	UW-Nr.	Town / County	sample origin	Van genotype	bac	*esp*
	UW 2990	L. (Saxony)	blood	*van* A	n.d.	-
	UW 1500	A. (North Rhine-Westfalia)	/	*van* A	n.d.	-
A	UW 2368	H. (Lower Saxony)	/	*van* A	+	+
A	UW 2987	E. (North Rhine-Westfalia)	blood	*van* A	+	+
A	UW 1984	B. (Baden-Württemberg)	Trachea	*van* A	n.d.	+
A	UW 2988	M. (North Rhine-Westfalia)	blood	*van* A	n.d.	-
B	UW 3409	T. (Baden-Württemberg)	stool	*van* A	-	-
B	UW 3405	T. (Baden-Württemberg)	stool	*van* A	-	-
B	UW 3317	U.K.	/	*van* A	n.d.	+
B	UW 3408	T. (Baden-Württemberg)	stool	*van* A	-	-
B	UW 3406	T. (Baden-Württemberg)	stool	*van* A	-	-
B	UW 2926	D. (North Rhine-Westfalia)	urine	susceptible	n.d.	-
B	UW 3268	E. (Bavaria)	wound swab	*van* A	n.d.	-
B	UW 3308	A. (Bavaria)	wound swab	*van* A	-	+
B	UW 3185	M. (Bavaria)	stool	*van* A	-	+
B	UW 3310	A. (Bavaria)	wound swab	*van* A	n.d.	+
B	UW 3264	B.W. (Hesse)	urine	susceptible	n.d.	+
B	UW 3180-1	C. (Saxony)	stool	*van* A	-	+
B	UW 3178	C. (Saxony)	urine	*van* A	n.d.	-
B	6011	Berlin	clinical site	*van* A	-	-
B	UW 3177	C. (Saxony)	blood	*van* A	-	-
B	UW 3071	W. (Bavaria)	wound swab	*van* A	n.d.	-
B	UW 3080	F. (Hesse)	Trachea	*van* A	n.d.	-
B	UW 3437	H. (Saxony-Anhalt)	stool	*van* A	-	+
B	UW 2957	H. (Saxony-Anhalt)	/	*van* A	+	+
B	UW 1679	Berlin2	stool	*van* A	-	-
B	UW 1342	Berlin2	stool	*van* A	-	-
B	UW 786	N. (Bavaria)	urine	*van* A	-	+
B	UW 3274	G. (Lower Saxony)	blood	*van* A	-	+
B	UW 3076	G. (Lower Saxony)	wound swab	*van* A	-	+
B	UW 3262	W. (Bavaria)	wound swab	susceptible	n.d.	-
B	UW 2486	M. (Bavaria)	urine	*van* A	n.d.	-
B	L 283	L. (Saxony)	clinical site	*van* A	-	-
B	UW 3440	L. (Saxony)	Sputum	*van* A	n.d.	+
B	UW 3352	L. (Saxony)	Sputum	*van* A	-	+
B	UW 805	N. (Bavaria)	blood	*van* A	n.d.	+
B	UW 3078	I.O. (Rhine Land Pfalz)	wound swab	*van* A	n.d.	+
B	UW 2389	I.O. (Rhine Land Pfalz)	urine	*van* A	n.d.	+
B	UW 1803	P. (Brandenburg)	urine	*van* A	-	-
B	70/90	Berlin	/	*van* A	-	-
B	UW 1873	H. (Lower Saxony)	rectal swab	*van* A	-	-
B	2513	S. (Mecklenburg-Vorpommern)	clinical site	*van* A	-	-
B	U 200	M. (Saxony-Anhalt)	clinical site	*van* A	-	-
B	9542	no data	/	*van* A	n.d.	-
B	UW 3319	USA-1	/	*van* A	n.d.	+
B	UW 3318	Australia-1	/	*van* A	n.d.	-
B	UW 1497	A. (North Rhine-Westfalia)	/	*van* A	-	-
B	UW 3175	L. (Saxony)	urine	*van* A	-	-
B	UW 3002	L. (Saxony)	Trachea	*van* A	n.d.	+
B	UW 3311	L. (Saxony)	blood	*van* A	-	+
B	UW 3313	L. (Saxony)	blood	*van* A	-	+
B	UW 3316	The Netherlands3-1	/	*van* A	n.d.	+
B	UW 1987	M. (North Rhine-Westfalia)	wound swab	*van* A	+	+
B	UW 3265	B.W. (Hesse)	urine	susceptible	n.d.	-
B	UW 3315	The Netherlands2-2	/	*van* A	n.d.	+
B	UW 3314	The Netherlands1-1	/	*van* A	n.d.	+
B	UW 3183	W. (North Rhine-Westfalia)	blood	*van* A	+	+
B	UW 2894	Berlin7	Sputum	*van* A	n.d.	-

n.d., not determined

* the similarity analysis of the *Sma* I-macrorestriction patterns of these strains is shown in Figure 3.2
Outbreak isolates from other countries are shown in grey.
Subclusters B1, B2 and B3 are delimitated by dotted lines.

Table 3.3 *Enterococcus faecium* clinical isolates from different German hospitals

	UW-Nr.	Town / County	sample origin	Van genotype	bac	*esp*
C	**UW 3273**	G. (Lower Saxony)	urine	*van* A	+	+
	UW 2683	S. (Mecklenburg-Vorpommern)	urine	*van* A	+	+
	UW 2442	Berlin9	wound swab	*van* A	+	+
	UW 2413	Berlin8	wound swab	*van* A	+	+
	UW 2390	Berlin7	urine-catheter	*van* A	+	+
	UW 2383	Berlin6	blood-catheter	*van* A	+	+
	UW 3182	C. (Brandenburg)	blood	*van* A	+	+
	UW 3181	C. (Brandenburg)	blood	*van* A	+	+
	UW 3069	B. (North Rhine-Westfalia)	wound swab	*vanB*	n.d.	+
	UW 1806	Berlin1	bronchiallavage	*van* A	+	+
	UW 931	G. (Lower Saxony)	/	*van* A	+	+
	UW 1505	A. (North Rhine-Westfalia)	/	*van* A	+	+
	UW 901	D. (Hesse)	/	*van* A	+	+
	UW 3353	G. (Lower Saxony)	urine	*van* A	+	+
	UW 2441	L. (Schleswig-Holstein)	/	*van* A	+	+
	UW 2467	Hamburg	blood	*van* A	+	+
	UW 1983	K. (Hesse)	blood	*van* A	+	+
	UW 1978	K. (Hesse)	wound swab	*van* A	-	+
	UW 2834	M. (Baden-Württemberg)	wound swab	*van* A	+	+
	UW 2444	C. (Saxony)	wound swab	*van* A	+	-
	UW 2384	E. (Brandenburg)	urine	*van* A	+	+
	UW 3320	USA2-2	/	*van* A	-	+
	UW 2320	Berlin3	wound swab	*van* A	+	+
	UW 2321	Berlin4	/	*van* A	+	+
	UW 2322	Berlin5	urine	*van* A	+	+
	UW 2306	Berlin2	bronchiallavage	*van* A	+	+
	UW 2319	F. (Hesse)	wound swab	*van* A	+	+

n.d., not determined

Table 3.3 *Enterococcus faecium* clinical isolates from different German hospitals

	UW-Nr.	Town / County	sample origin	Van genotype	bac	*esp*
D	**UW 3433**	H. (Saxony-Anhalt)	stool	*van* A	-	+
	UW 3432	H. (Saxony-Anhalt)	stool	*van* A	-	+
	UW 3431	H. (Saxony-Anhalt)	stool	*van* A	-	+
	18912	no data	/	*van* A	-	-
	UW 3000	M. (Bavaria)	/	*van* A	-	+
	UW 2376	H. (Lower Saxony)	/	*van* A	n.d.	-
	UW 3423	H. (Saxony-Anhalt)	stool	*van* A	-	+
	UW 3471	H. (Saxony-Anhalt)	wound swab	*van* A	n.d.	+
	UW 3422	H. (Saxony-Anhalt)	stool	*van* A	-	+
	UW 3424	H. (Saxony-Anhalt)	stool	*van* A	-	+
	UW 2780	H. (Saxony-Anhalt)	stool	*van* A	n.d.	-
	UW 2787	H. (Saxony-Anhalt)	stool	*van* A	n.d.	-
	UW 3427	H. (Saxony-Anhalt)	stool	*van* A	+	-
	UW 3430	H. (Saxony-Anhalt)	stool	*van* A	+	-
	UW 3435	H. (Saxony-Anhalt)	stool	*van* A	+	-
	UW 3436	H. (Saxony-Anhalt)	stool	*van* A	+	-
	UW 3267	B.W. (Hesse)	wound swab	susceptible	n.d.	-
	UW 3421	H. (Saxony-Anhalt)	stool	*van* A	-	+
	UW 3438	H. (Saxony-Anhalt)	stool	*van* A	-	+
	UW 3263	B.W. (Hesse)	urine	susceptible	n.d.	-
	UW 2782	H. (Saxony-Anhalt)	stool	*van* A	n.d.	+
	UW 3321	The Netherlands 2-3	/	*van* A	n.d.	-
	UW 1883	H. (Lower Saxony)	rectal swab	*van* A	-	-
	UW 2737	H. (Saxony-Anhalt)	stool	*van* A	n.d.	+
	UW 2818	H. (Saxony-Anhalt)	stool	*van* A	n.d.	+
	UW 2699	H. (Saxony-Anhalt)	stool	*van* A	n.d.	+
	UW 2700	H. (Saxony-Anhalt)	stool	*van* A	n.d.	+
	UW 3428	H. (Saxony-Anhalt)	stool	*van* A	+	-
	UW 3429	H. (Saxony-Anhalt)	stool	*van* A	+	-
	UW 3434	H. (Saxony-Anhalt)	stool	*van* A	+	-
	UW 3439	H. (Saxony-Anhalt)	stool	*van* A	+	-
E	**UW 1889**	H. (Lower Saxony)	rectal swab	*van* A	-	-
	UW 1535	Berlin2	/	*van* A	-	-
	UW 1800	Berlin2	stool	*van* A	-	-
	UW 1530-2	Berlin2	/	*van* A	-	-
	UW 2490	H. (Baden-Württemerg)	blood	*van* A	n.d.	-
	UW 1669	Berlin2	stool	*van* A	-	-
	UW 1536	Berlin2	/	*van* A	-	-
	UW 1711	Berlin2	stool	*van* A	-	-
	UW 1463	K. (Schleswig-Holstein)	/	*van* A	-	-
	UW 1498	A. (North Rhine-Westfalia)	/	*van* A	n.d.	-
	UW 2332	A. (North Rhine-Westfalia)	stool	*van* A	-	-
	UW 2059	J. (Thuringia)	/	*van* A	-	-
	UW 2062	J. (Thuringia)	/	*van* A	-	-
	UW 1699	Berlin2	stool	*van* A	n.d.	-
	UW 1674	Berlin2	stool	*van* A	-	-
	UW 1206	M. (Bavaria)	/	*van* A	n.d.	-
	UW 1944	Hamburg	stool	*van* A	-	-
	UW 2104	I. (Austria)	/	*van* A	-	-
	UW 1534	Berlin2	/	*van* A	-	-
	UW 1445	no data	/	*van* A	-	-
	UW 1924	H. (Lower Saxony)	rectal swab	*van* A	+ +	-
	UW 1448	no data	/	*van* A	-	-
	UW 1208	Berlin4	/	*van* A	-	-

n.d., not determined

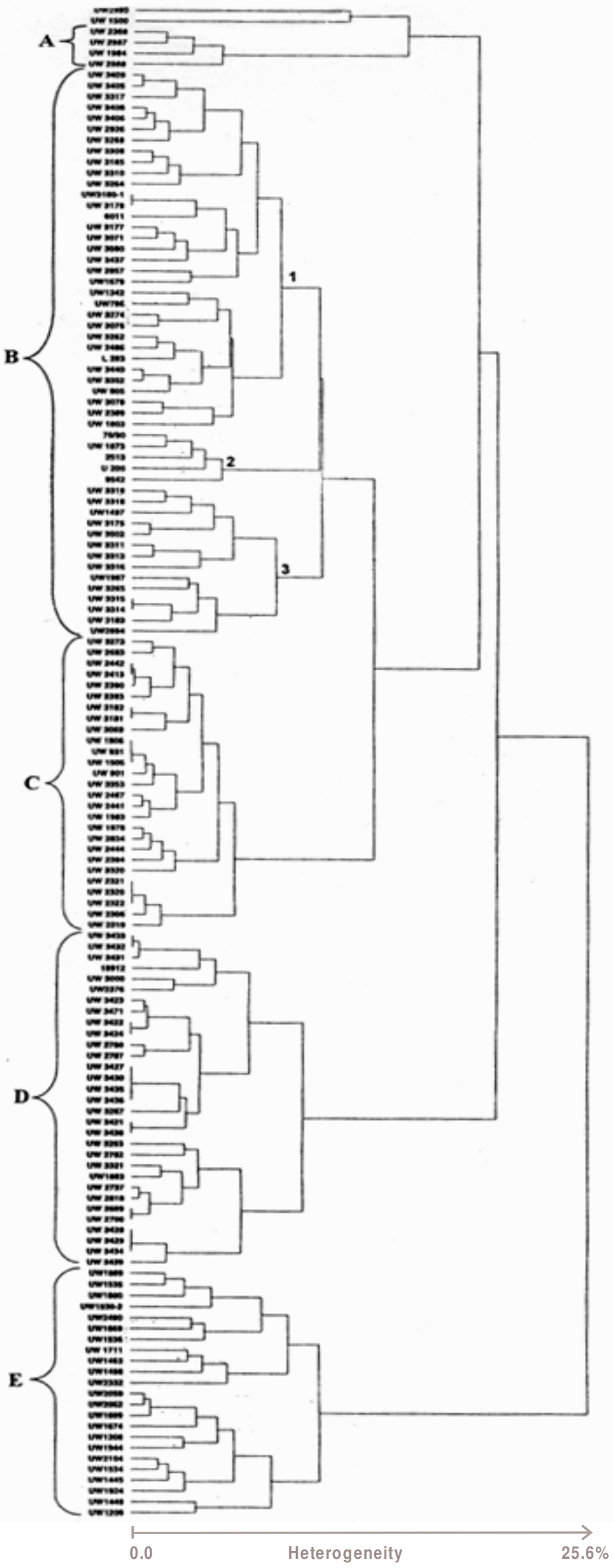


Figure 3.2 Dendogram representing the similarity analysis of *Sma*I-macrorestriction patterns for *Enterococcus faecium* isolates from different German hospitals

Figure 3.3 Similarity analysis of *Sma*I macrorestriction fragments of *Enterococcus faecalis* clinical isolates from SIR Study

	UW-Nr.	Ward	sample origin	n patients	bac	*esp*
I	**UW 3212**	CVK 43	urine	2	-	+
	UW 3379	CVK 9	wound swab	2	-	+
	UW 3204	CVK 1	urine	2	-	-
	UW 3192	CCM 1	blood-catheter	1	-	+
	UW 3382	CVK 43	urine	2	+	+
	UW 2984	CVK 9	wound swab	2	-	-
	UW 3117	CVK 8	wound swab	1	+	+
	UW 2983	CVK 8	wound swab	1	+	-
	UW 3113	CVK 43	urine-catheter	2	-	+
	UW 2879	CVK 8	wound swab	2	-	+
II	**UW 3116**	CVK 43	blood-catheter	2	+	-
	UW 3112	CVK 43	urine	2	+	+
	UW 2979	CVK 43	urine-catheter	2	-	+
	UW 3100	CCM 1	urine	1	-	+
	UW 3221	CVK 43	urine	2	+	-
III	**UW 3026**	CVK 43	wound swab	11	+	-
	UW 3241	CVK 9	blood	4	+	-
	UW 3287	CCM 1	wound swab	2	-	-
	UW 2881	CVK 8	wound swab	18	-	-
	UW 3015	CCM 1	trachaeal-sekret	3	+	-
IV	**UW 3294**	CVK 43	blood	4	-	-
	UW 3029	CVK 43	urine-catheter	2	-	+
	UW 2840	CVK 8	wound swab	6	+	+
	UW 3300		wound swab		-	+
	UW 3234	CVK 8	blood-catheter	1	+	+
V	**UW 3205**	CVK 1	urine	4	+	+
	UW 3020		urine-catheter		-	+
	UW 3021		urine-catheter		+	+
	UW 3022		urine-catheter		+	+
	UW 3385	CVK 43	urine	6	+	+
	UW 3103	CCM 1	urine-catheter	2	+	-
	UW 3211	CVK 1	urine	2	+	+
	UW 3209		urine		+	-
	UW 3397	CCM 1	sputum	3	+	+
	UW 3383		sputum		+	+
	UW 3394		sputum		+	+
VI	**UW 3441**	CVK 43	wound swab	2	-	-
	UW 3122	CVK 9	wound swab	2	-	-
	UW 3106	CVK 43	urine-catheter	1	-	-
	UW 3393	CCM 1	sputum	1	-	-
	UW 2878	CVK43	blood-catheter	1	-	-
	UW 3375	CVK 8	blood	3		
	UW 3377	CCM 1	wound swab	2	+	-
	UW 3380	CVK 43	sputum	1	-	-
VII	**UW 3191**	CCM 1	blood-catheter	5	+	+
	UW 3190		wound swab		+	+
	UW 3018		blood-catheter		+	+
	UW 3017		wound swab		+	+
	UW 3016		blood-catheter		+	+
	UW 3296	CVK 43	Punktat	5	+	+
	UW 2904	CCM 1	wound swab	1	+	+
	UW 3305	CVK 8	wound swab	3	+	+
VIII	**UW 3218**	CVK 43	urine-catheter	7	+	+
	UW 3378	CVK 8	wound swab	2	+	+
	UW 3290	CCM1	blood-catheter	11	+	+
	UW 3286		urine		+	+
	UW 3189		blood-catheter		+	+
	UW 3188		blood-catheter		+	+
	UW 3187		blood		+	+
	UW 3196		blood-catheter		+	+
	UW 3195		trachaeal-sekret		+	+
	UW 3193		blood		+	+
	UW 3194		wound swab		+	+
	UW 3198		wound swab		+	+
	UW 3200		blood-catheter		+	+
	UW 3197	CCM1	blood-catheter	3	+	+

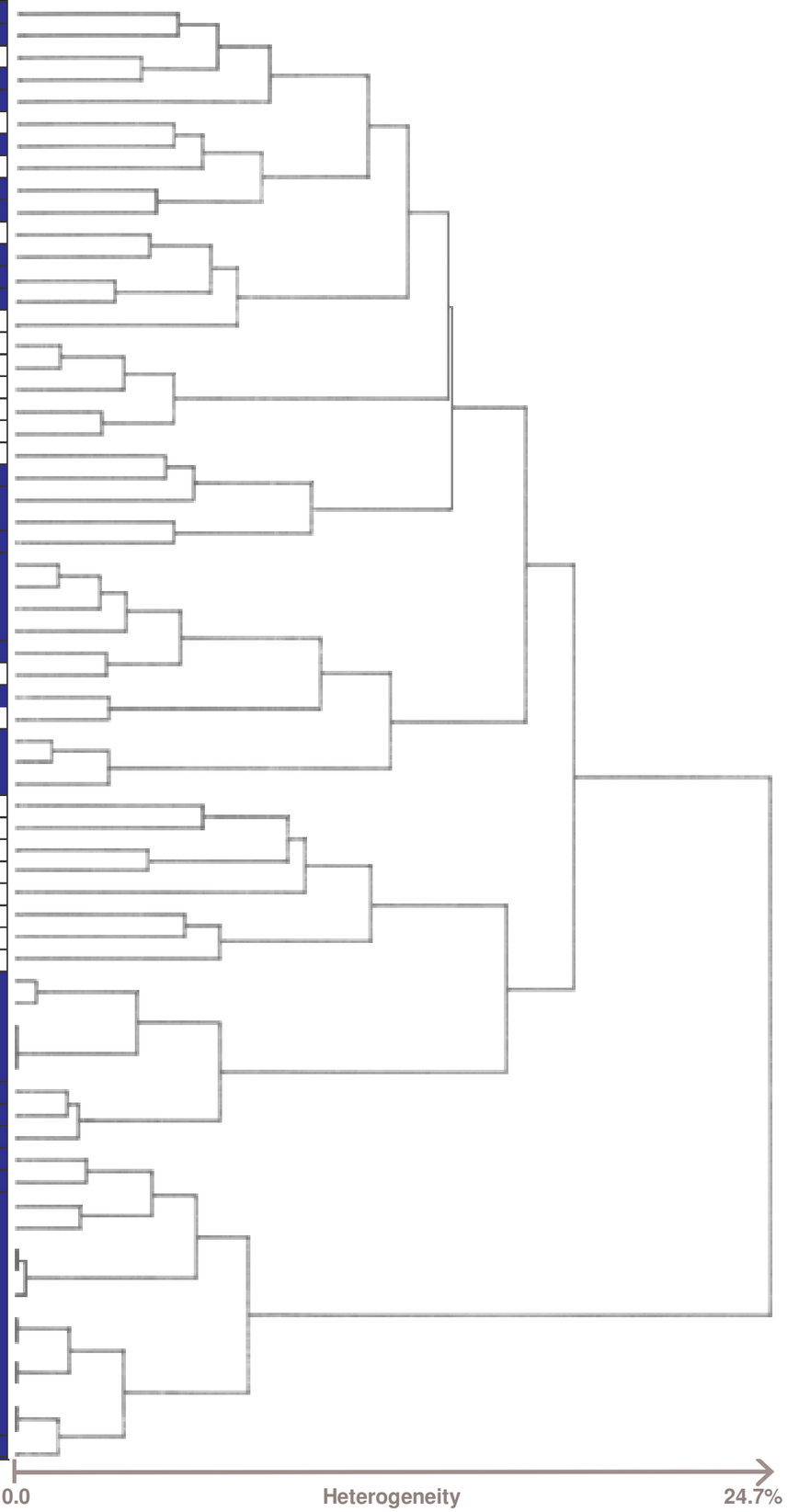
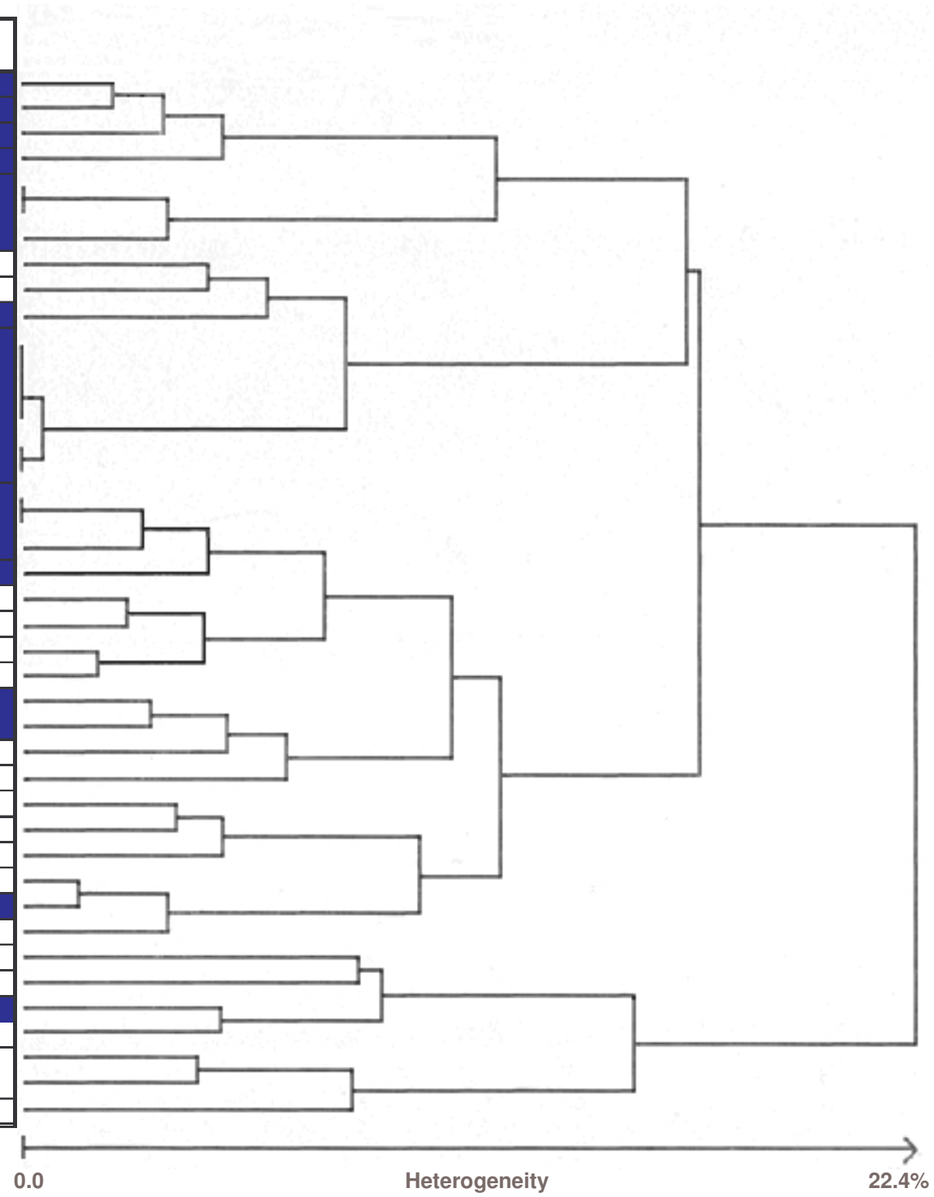


Figure 3.4 Similarity analysis of *Sma*I macrorestriction patterns of *Enterococcus faecium* clinical isolates from SIR Study*

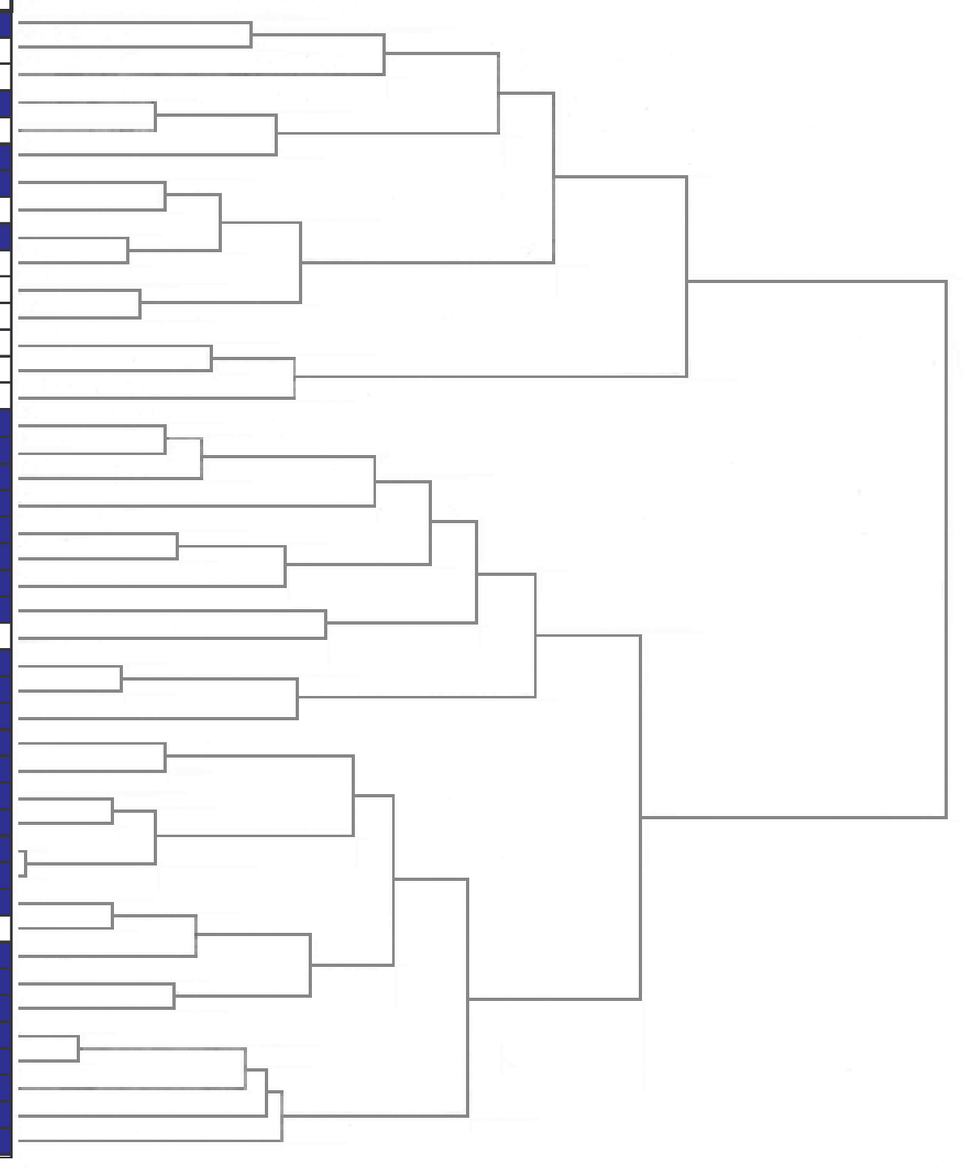
	UW-Nr.	Ward	sample origin	n patients	bac	*esp*
1	**UW 3047**	CVK 43	urine-catheter	4	-	+
	UW 3096	CVK 9	wound swab	3	-	+
	UW 2901	CVK 8	wound swab	4	-	+
	UW 3098	CVK 9	wound swab	1	-	+
2	**UW 3367**	CVK 8	wound swab	3	-	+
	UW 3056		blood		-	+
	UW 3053		galle		+	+
3	**UW 3054**	CVK 8	wound swab	1	-	-
	UW 2903	CCM 1	wound swab	1	-	-
	UW 3050	CVK 43	wound swab	1	+	+
4	**UW 3061**	CVK 9	wound swab	5	-	+
	UW 3058		wound swab		-	+
	UW 3059		wound swab		-	+
	UW 3060		wound swab		-	+
	UW 3062		wound swab		-	+
	UW 3057		wound swab		-	+
5	**UW 2969**	CVK 9	wound swab	3	-	+
	UW 2888		wound swab		-	+
	UW 3097		wound swab		-	+
	UW 3048	CVK 43	wound swab	2	+	+
	UW 3052	CVK 8	wound swab	2	-	-
	UW 3092	CCM1	blood-catheter	2	-	-
	UW 2966	CVK 8	wound swab	3	-	-
	UW 3370	CCM 1	Punktat	4	+	-
6	**UW 3256**	CVK 8	wound swab	2	+	+
	UW 3093		wound swab		+	+
	UW 3281	CVK 43	urine-catheter	1	-	-
	UW 1806	Reference strain				
7	**UW 3252**	CVK 43	urine-catheter	2	-	-
	UW 3259	CVK 9	wound swab	1	+	-
	UW 3283	CCM 1	blood-catheter	1	+	-
	UW 2900	CVK 43	blood-catheter	2	-	-
	UW 2884	CCM 1	wound swab	1	-	+
	UW 3365	CVK 9	wound swab	1	-	-
8	**UW 3045**	CCM 1	wound swab	1	-	-
	UW 3044	CCM 1	wound swab	1	-	-
	UW 3279	CVK 8	wound swab	2	-	+
	UW 3094		wound swab		-	-
9	**UW 3278**	CVK 8	wound swab	2	-	-
	UW 3119		blood-catheter		-	-
	UW 3255	CVK 8	wound swab	1	-	-



*All isolates are susceptible to vancomycin, with the exception of UW 3048, UW 2900, UW 3365, UW 3045 and UW 1806 (reference-outbreak strain)

Figure 3.5 Similarity analysis of *Sma*I macrorestriction patterns of *Enterococcus faecium* clinical isolates (VanS and VanR)

	UW Nr.	*origin / material*	resistance to vancomycin	bac	esp
1	UW 3279	**SIR study** / wound swab	sensitive	-	+
	UW 3255	**SIR study** / wound swab	sensitive	+	-
	UW 1669	**Berlin2** / -	VanA	-	-
2	UW 3319	**USA-1*** / -	VanA	n.d	+
	UW 3318	**Australia-1*** / -	VanA	n.d	-
	UW 3316	**The Netherlands 3-1*** / -	VanA	n.d.	+
3	UW 2737	**H. (Saxony-Anhalt)** / stool	VanA	n.d.	+
	UW 3321	**The Netherlands 2-3*** / -	VanA	n.d.	-
	UW 3432	**H. (Saxony-Anhalt)** / stool	VanA	-	+
	UW 3430	**H. (Saxony-Anhalt)** / stool	VanA	+	-
	UW 3370	**SIR study** / -	sensitive	+	-
	UW 3429	**H. (Saxony-Anhalt)** / stool	VanA	+	-
4	UW 2059	**J. (Thuringia)** / -	VanA	-	-
	UW 1535	**Berlin2** / -	VanA	-	-
	UW 1924	**H. (Lower Saxony)** / -	VanA	+	-
5	UW 3096	**SIR study** / wound swab	sensitive	-	+
	UW 3308	**A. (Bavaria)** / wound swab	VanA	-	+
	UW 3093	**SIR study** / wound swab	sensitive	+	+
	UW 2957	**H. (Saxony-Anhalt)** / -	VanA	+	+
	UW 3183	**W. (North Rhine-Westfalia)** / blood	VanA	+	+
	UW 3422	**H. (Saxony-Anhalt)** / stool	VanA	-	+
	UW 2884	**SIR study** / wound swab	sensitive	-	+
6	UW 3180-1	**C. (Saxony)** / stool	VanA	-	+
	UW 1342	**Berlin2** / -	VanA	-	-
7	UW 3053	**SIR study** / -	sensitive	+	+
	UW 3056	**SIR study** / blood	sensitive	-	+
	UW 3000	**M. (Bavaria)** / -	VanA	-	+
8	UW 3097	**SIR study** / wound swab	sensitive	-	+
	UW 931	**G. (Lower Saxony)** / -	VanA	+	+
	UW 3181	**C. (Brandenburg)** / blood	VanA	+	+
	UW 2322	**Berlin5** / urine	VanA	+	+
	UW 2368	**H. (Lower Saxony)** / -	VanA	+	+
	UW 2442	**Berlin9** / wound swab	VanA	+	+
	UW 2834	**M. (Baden-Württemberg)** / wound swab	VanA	+	+
	UW 2444	**C. (Saxony)** / wound swab	VanA	+	-
	UW 3320	**USA 2-2*** / -	VanA	-	+
	UW 3317	**UK** / -	VanA	n.d.	+
	UW 3314	**The Netherlands 1-1*** / -	VanA	n.d.	+
9	UW 3060	**SIR study** / wound swab	sensitive	-	+
	UW 3440	**L. (Saxony)** / sputum	VanA	n.d.	+
	UW 3078	**I.O. (Rhine Land Pfalz)** / wound swab	VanA	n.d.	+
	UW 3048	**SIR study** / wound swab	VanA	+	+
	UW 3050	**SIR study** / wound swab	sensitive	+	+



n.d., not determined
* number denotes number of the epidemic clone according to Willems et al. (2001)
The outbreak strains from other countries are marked in grey

6. Literature

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Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe.

Claudia Oancea

Frankfurt, den 16.09.2004