The role of Enterococcal Surface Protein in the pathogenesis of *Enterococcus faecium* infections

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Cover picture: Esp of *E. faecium* involved in biofilm formation – designed by Mirjam Heikens

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The role of Enterococcal Surface Protein in the pathogenesis of *Enterococcus faecium* infections

De rol van het enterokokken oppervlakte eiwit in de pathogenese van *Enterococcus faecium* infecties

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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- Voor mijn ouders,
Herman & Agnes -

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General introduction

General introduction into enterococci

Enterococci are Gram-positive cocci that occur in pairs (diplococci) or as short chains. They are facultative anaerobic bacteria, catalase-negative and typically exhibit alpha-hemolysis on sheep's blood agar. The genus *Enterococcus* consists currently of more than 30 species (http://www.bacterio.cict.fr/e/enterococcus.html). Enterococci are widespread in nature and commonly found as commensal bacteria in the gastro-intestinal tract of humans and animals as well as in soil, water, food, and on plants (80). In the human gut they are minority players of the human intestinal flora belonging to the genus *Enterococcus* (80). Especially, *Enterococcus faecium* and *Enterococcus faecalis* colonize the human intestine, but also *Enterococcus avium* and *Enterococcus durans* can be present in the human gut. Enterococci are able to withstand harsh environmental conditions as they are able to grow in 6.5% NaCl, in temperatures from 10 to 45°C, and in a pH range from very acidic (pH 4.8) to very alkaline (pH 9.6) as in the small intestine. In addition, they can withstand chemicals inside the human body like bile salts (25,85) and they can survive for weeks outside the human body on virtually everything in the health-care environment enabling cross-transmission (6,70).

For years, enterococci were considered harmless inhabitants of the gastro-intestinal tract of humans and animals, only causing opportunistic infections in critically ill patients. However, since the first identification of vancomycin-resistant enterococci (VRE) in 1986 in the United Kingdom and France (45) and subsequent rise of invasive infections with VRE a dramatic change in the perspective on enterococcal infections occurred and clinical and public interest substantially increased. From that time on enterococci were no longer considered as colonizers of the gastro-intestinal tract that occasionally cause infections when immunity of the patient is low, but as emerging nosocomial pathogens with high-level resistance to multiple antibiotics (63).

Clinical epidemiology of multi-resistant enterococci

Since the first identification of VRE in 1986, the numbers of patients colonized and infected with VRE have risen steadily in hospitals in the United States. Currently, enterococci are the third most common pathogen isolated from nosocomial

bloodstream infections and the most common pathogen in surgical-site infections reported from intensive care units in the United States (8,31). Besides surgical-site infections and bacteremias, enterococci cause peritonitis, urinary tract infections, endocarditis, and a variety of device-related infections (11,23,46,56,61,63,64,77,89, 90). Nowadays, 33% of device-associated nosocomial enterococcal infections in the United States are caused by VRE (31). In contrast, in Europe VRE were initially identified from healthy people and farm animals most probably due to the use of avoparcin (a glycopeptide) as growth promoter in the veterinary industry. Since the European Union banned the use of avoparcin in 1997, prevalence rates in the community declined from 12% in 1997 to 6% in 1999 (7,43). In the United States, where avoparcin was never used, colonization in the community is absent. It was until the turn of the century that VRE emergence occurred in European hospitals (24,78,88,104) followed by hospitals in South America, Asia, and Australia (4,39,40,55), illustrating the pandemic spread of VRE. In Europe, the proportion of invasive *E. faecium* isolates resistant to vancomycin is highest in Portugal, Ireland, Greece, Italy, Germany, and the United Kingdom (EARSS annual report 2007) although these prevalence rates are not as high as in the United States. In the Netherlands the prevalence of VRE among blood stream isolates is very low (<1%), probably due to the conservative use of antibiotics and stringent infection control measures for patients colonized or infected with VRE. Higher antibiotic use in the United States, specifically higher use of cephalosporines and vancomycin, compared to some European countries, might have contributed to the difference in VRE prevalence in the United States and most of the European countries (7,22,38).

Importantly, the pandemic spread of VRE was preceded by the emergence of ampicillin-resistant *E. faecium* (ARE) in the early 1980s in the United States, followed by the rapid emergence of ARE in European countries ten years later. Also in The Netherlands, where, as mentioned above, VRE prevalence is low in hospitals, a significant increase in invasive ARE infections has occurred (12,99). The proportion of ARE among all enterococcal blood stream isolates increased from 4% in 1994 to 20% in 2005 (99) and 50% in 2008 in the UMC Utrecht. Furthermore, a recent study revealed intestinal colonization rates with ARE as high as 40% in hospital wards (12). *E. faecalis* and *E. faecium* are the main cause of enterococcal infections in hospitalized

patients. In the beginning of the 1990s, 80 to 90% of the clinical enterococcal isolates were *E. faecalis* and only 5 to 10% were *E. faecium* (100). Nowadays, approximately 40% of enterococcal nosocomial infections in European and United States hospitals are caused by *E. faecium* (36,100). This increase in nosocomial *E. faecium* infections not only resulted in an increase in the total burden of hospital-associated enterococcal infections, but also in a partial replacement of *E. faecalis* by *E. faecium* (36,98,100) (www.earss.rivm.nl).

The higher incidence of infections with multi-resistant enterococci has partly been contributed to an increase in immunocompromised and critically ill patients, but mainly to abundant use of antibiotics (2,60). Enterococci are intrinsically resistant to low levels of aminoglycosides and to cephalosporines. In addition, enterococci are able to acquire resistance against a broad range of antibiotics belonging to different antibiotic classes through a combination of mutation and horizontal gene transfer (HGT). Examples of acquired-resistance through HGT include resistance to aminoglycosides, macrolides, streptogramin, chloramphenicol, and vancomycin, the last one being the most important. In particular, *E. faecium* acquired high-level multi-antibiotic resistance to high levels of aminoglycosides, β-lactam-antibiotics, and vancomycin (7,43,95). This resulted in an almost complete penetration of ampicillin and vancomycin resistance, with 80% and 90% of all *E. faecium* isolates considered causative of infections being resistant to vancomycin and ampicillin, respectively (31,62,100). In contrast, in *E. faecalis* vancomycin and ampicillin-resistance is far less prevalent with only 0.5% to 7% of all hospital-associated *E. faecalis* isolates being vancomycin resistant (31,62,100).

Molecular epidemiology of *E. faecium*

Molecular epidemiological analysis of *E. faecium* isolates recovered from human and nonhuman sources and community and hospital reservoirs, using a variety of molecular typing techniques, indicated the existence of a specific polyclonal *E. faecium* subpopulation, designated clonal complex 17 (CC17), responsible for the majority of nosocomial infections and hospital outbreaks, and most probably acquired during hospitalization. Based on comparative genomic hybridization using a mixed whole-genome microarray, these hospital-acquired *E. faecium* isolates appear

to be genetically distinct from indigenous intestinal isolates and share more than 100 genes, including genes encoding resistance to ampicillin and ciprofloxacin, but also putative virulence genes, and insertion sequence elements (44). Potential virulence genes and elements specifically found in this subpopulation are a pathogenicity island containing the enterococcal surface protein gene *esp* (42,108), hyl_{efm} (hyaluronidase) (76), genes encoding surface-exposed LPXTG-like cell-wall-anchored proteins, called *sgrA* (serine-glutamate repeat containing protein A) and *ecbA* (*E. faecium* collagen binding protein A), and genes required for the biogenesis of pili, called *pilA* and *pilB* (28,30). This set of more than 100 genes are not clustered but spread over the genome, indicating that acquisition of these genes probably has occurred in multiple steps and has been responsible for the progressive development of *E. faecium* from a harmless commensal of the gastro-intestinal tract towards a nosocomial pathogen with an increased fitness and enhanced propensity of spread in and between hospitals.

Colonization and infection with multi-resistant enterococci

Colonization is the first (and key) step in the development of clinical infection (9). Enterococci predominantly colonize the gastro-intestinal tract, but colonization has also been documented in groin, axilla, and oropharynx. Colonized patients without clinical symptoms may remain colonized for long periods and may serve as a silent reservoir for transmission to other patients (113). Transmission may occur either via hands of health-care workers or by direct contact with contaminated environment, such as toilet seats, monitoring devices, doors, and medical equipment (6,12,71). Once patient colonization has become endemic within a hospital ward, it is extremely difficult to effectively control further transmission. No antimicrobial regimen has been effective in eradicating gastro-intestinal colonization with multi-resistant hospital-acquired enterococci. Most important risk factor for colonization is antibiotic therapy (12,27,98,102). Especially, β-lactam antibiotics, more specific third-generation cephalosporines, and quinolone use is associated with higher risk of colonization.

Severe infections with multi-resistant enterococci, especially VRE, occur most often in intensive care units, hematology and organ transplant patients (3,9,37,110,113),

which are for multiple reasons severely immunocompromised. In these patients colonization may proceed to clinical infection due to the presence of indwelling (urinary and intravascular) catheters or wounds, or through bacterial translocation from the intestinal lumen to extraintestinal sites (59,103), as seen in neutropenic patients with mucositis (110). Infections with VRE are associated with considerable morbidity and high mortality, because of limited therapeutic options (3). Mortality rates vary with the population at risk. For instance, mortality rates among patients with VRE blood stream infection have been reported as 30% (63), 37% among those with cancer, and 70% among critically ill and liver transplant patients (9,19,110).
Linezolid is the antibiotic of first choice during serious infections caused by VRE, followed by quinupristin/dalfopristin (3,48,49,72). The combination quinupristin/dalfopristin is only active against *E. faecium* and not *E. faecalis*. Both linezolid and quinupristin/dalfopristin can have important toxic side effects such as bone marrow suppression, myalgia, and inflammation at the infusion site. Other antibiotics that can be used are daptomycin and tigecycline (3,112). Failures of daptomycin therapy have been reported and concern has risen about the use of tigecycline in bacteremia, because of its low blood levels. However, resistance to all these newer antibiotics has already been observed (3,79,105,106). Better understanding of virulence of enterococci is, therefore, necessary in order to develop novel treatment strategies and control further spread.

Virulence factors of *E. faecium*

As reported above, infections with *E. faecium* are primarily seen in critically ill and immunocompromised patients. This indicates that *E. faecium* is not as virulent as *Corynebacterium diphtheriae* or *Vibrio Cholerae*, which are capable of causing severe infections in healthy people. Most probably, the pathogenicity of *E. faecium* resides in a combination of factors that increase fitness and enhance survival in the gastro-intestinal tract and on the skin of hospitalized patients, as well capacities to adhere, survive and form biofilms on inanimate objects like stents and catheters (107).
Potential virulence genes of *E. faecium* include *acm* (adhesin of collagen of *E. faecium*), *scm* (second collagen adhesin of *E. faecium*), *sagA* (secreted antigen A), and the earlier mentioned genes hyl_{efm}, *sgrA*, *ecbA*, *esp*, *pilA*, and *pilB*, which are

specifically associated with hospital-acquired *E. faecium*. Acm, Scm, SgrA, and EcbA are surface-exposed cell-wall-anchored proteins. Surface exposed proteins are considered to play important roles during the establishment of many infections as they bind to specific protein ligands located in the host tissue, such as extracellular matrix molecules, that determine both bacterial host range and site of infection (21,97). Acm binds to collagen type I and to a lesser extent to collagen type IV (66,69). Presence of *acm* is widespread among clinical and non-clinical *E. faecium* isolates, however, a functional *acm* gene, promoting adherence to collagen type I and IV, is predominantly present in clinical isolates. A non-functional *acm* gene, containing an insertion element, is mainly present in non-clinical isolates (67). Interestingly, Acm contributes to the pathogenesis of experimental endocarditis. Furthermore, anti-Acm IgG antibodies were detected in sera of patients who suffered from *E. faecium* endocarditis, indicating that Acm is expressed *in vivo* and elicits a human immune response (65). Scm, EcbA, and SgrA also bind to extracellular matrix molecules. Both Scm and EcbA bind to collagen type V (29,84). SgrA binds to nidogen-1 and nidogen-2, which are components of the basal lamina. In addition, both SgrA and EcbA bind to fibrinogen. However, SgrA targets the alpha and beta chain, whereas EcbA binds to the gamma chain of fibrinogen (29). SagA is yet another extracelullar matrix binding protein of *E. faecium*. In contrast to Acm, Scm, SgrA, and EcbA, this protein is secreted by *E. faecium*. It exhibits broad-spectrum binding to extracellular matrix proteins, including fibrinogen, collagen type I, collagen type IV, fibronectin, and laminin (94). Furthermore, SagA is potentially involved in *E. faecium* growth and cell wall metabolism. Both *pilA* and *pilB* encode for major pilin subunits that polymerize into distinct multimeric pilus structures (28). Pili of Gram-positive bacteria have been implicated in adhesion to multiple types of human cells and biofilm formation, two processes critical in the pathogenesis of bacterial disease (1,52,54,68) (and see below). Expression of PilA and PilB was demonstrated in a hospital-acquired *E. faecium* isolate, proving that *E. faecium* can express two different types of pili at the surface of a single cell. Expression of these pili appeared to be temperature and growth condition dependent. It remains to be determined whether these pili are implicated in virulence of *E. faecium*. The gene hyl_{efm}, specifically enriched in hospital-acquired *E. faecium* (76), has homologies to hyaluronidase genes of *Streptococcus*

pyogenes (20). *In S. pyogenes, Streptococcus pneumoniae*, and *Staphylococcus aureus* hyaluronidase has been proposed as a virulence factor capable of breaking down hyaluronate, a glycosaminoglycan widely distributed throughout connective, epithelial, and neural tissues, to initiate infections at the skin or mucosal surfaces (34,35). In addition, hyaluronidase in *S. pneumoniae* may contribute to invasion of the nasopharynx (5,74). So far no function has been identified in *E. faecium*.

As mentioned above, the ability to adhere and to form biofilm on (a)biotic surfaces are considered important virulence properties of *E. faecium* (15,17). A biofilm is an assemblage of microbial cells associated with a surface and enclosed in a matrix of primarily polysaccharide material. Microbial biofilms are found in humans on the skin, in the mouth, in the gut, and on medical devices (51). Enterococci have been associated with biofilms in endocarditis, urinary tract infections, root canal infections, and ocular infections, and in a variety of device-related infections related to artificial hip prostheses, intrauterine devices, (prosthetic) heart valves, catheters, and stents (11,14,17,46,58,77,111). It is hypothesized that the defined architecture of biofilm provides an optimal environment for the exchange of genetic material between bacteria. In addition, biofilms presumably enable bacteria to withstand shear forces resulting from the flow of urine or material in the gut, but also to withstand chemical agents, antibiotics and the human immune response (16,17,51). One of factors implicated in biofilm formation in *E. faecalis* is the Enterococcal Surface Protein, Esp.

Enterococcal surface protein Esp

The enterococcal surface protein gene *esp* was initially identified in a virulent gentamycin-resistant *E. faecalis* isolate, MMH594, that caused a hospital ward outbreak in the mid-1980s (33,83). A few years later, it was found to exist on a large, 153-kb genetic element (81). This genetic element has all the characteristics of a pathogenicity island (PAI) with a significant lower GC content compared to the rest of the *E. faecalis* genome and the presence of genes encoding transposases, transcriptional regulators, and virulence determinants. In 2001, an *esp* homolog was found in *E. faecium* (18,108), also part of a large PAI, which appeared to be genetically distinct from the PAI in *E. faecalis* (42). Esp of *E. faecium* shares up to 90% identity with *esp* of *E. faecalis*. Both enterococcal Esp proteins belong to a family of gram-

positive surface-exposed proteins with repetitive structures such as the alpha C (57) and Rib (87) proteins of *Streptococcus agalactiae*, the R28 protein of *S. pyogenes* (86), and the Bap protein of *S. aureus* (10). All these proteins are involved in virulence and in conferring protective immunity (47). Both Esp proteins are expressed at the surface of the bacterium and have a molecular mass of about 200 kDa. Furthermore, they both contain an N-terminal signal sequence and a variable N-terminal domain of approximately 700 amino acids, followed by three repeat domains designated A, B, and C. The C-terminal end of Esp consists of a (Y/F)PXTG motif instead of a LPXTG motif, which can presumably be recognized by sortase (53), resulting in anchoring of Esp to the peptidoglycan of the cell wall. The number of A, B, and C repeats in Esp varies among strains and some isolates may even lack A repeats (18,42).

In *E. faecalis*, Esp has been identified as a putative virulence factor involved in colonization of the urinary tract. When mice were challenged transurethrally with either an Esp-expressing *E. faecalis* strain or its isogenic Esp-deficient mutant, significantly less Esp-deficient *E. faecalis* were recovered from urine and bladders (82). Conflicting results have been reported about the role of *E. faecalis* Esp in biofilm formation. For instance, in one study biofilm formation in *E. faecalis* was reduced in isogenic Esp-deficient strains (91). Furthermore, the N-terminal domain of Esp appeared to be sufficient to enhance biofilm formation (92). Biofilm formation was significantly reduced in an *E. faecalis* mutant expressing Esp without the N-terminal domain. Furthermore, a mutant that only expressed the N-terminal domain of Esp produced similar amounts of biofilm as the wild-type strain. In other studies, however, no role of *E. faecalis* Esp in biofilm formation could be demonstrated. For example in different studies no correlations were found between the presence or absence of the *esp* gene in clinical *E. faecalis* isolates and biofilm formation (13,26,41,75). And in another study the effect of insertional mutagenesis in *E. faecalis* appeared strain-dependent, ranging from a complete loss of the biofilm formation phenotype to no apparent effect, indicating that besides Esp other factors play a role in this process (96). Besides *esp*, several other genetic determinants have been identified in *E. faecalis* that are involved in the process of biofilm formation, including secreted metalloprotease GelE (26,41,73), the sugar-binding transcriptional regulator BopD (32), the two-component quorum-sensing locus *fsr* (26), the *bee*-locus encoding three

surface proteins and two sortases (93), and the *ebp*-locus encoding endocarditis and biofilm-associated pili (68).

Up till now little is known about the function of *E. faecium* Esp. Interestingly, in contrast to *E. faecalis esp*, which is widely spread among strains, *E. faecium esp* is predominantly present in hospital-acquired isolates and almost absent in community isolates (42,109), suggesting a role of *esp* in virulence. It has been shown that Esp expression is affected by changes in environmental conditions, being highest in conditions that mimic the microenvironment of the human large intestines: 37°C and anaerobioses (101). In addition, bloodstream isolates of *E. faecium* enriched with *esp* appeared to have increased adherence to human colorectal adenocarcinoma cells (Caco-2 cells) (50). These data suggest a role of Esp in intestinal colonization, which is assumed to be a first and key step that precedes clinical infection. Furthermore, expression of Esp was quantitatively correlated with initial adherence to polystyrene and biofilm formation (101). Moreover, when the N-terminal domain of Esp was added to *E. faecium* cells with high Esp expression, primary attachment to polystyrene was blocked, suggesting a possible role of Esp in initial adherence and possibly biofilm formation.

To summarize, our knowledge on the role of Esp in the pathogenesis of *E. faecium* infections is limited. Elucidating the role of Esp may lead to novel insights into how these bacteria initiate infections and interact with their environment. Improved understanding may also contribute to the development of novel strategies to prevent infections and spread of multi-resistant *E. faecium* in hospitals.

Outline of this thesis

This thesis mainly focuses on elucidating the role of Esp in the pathogenesis of different *E. faecium* infections. In **chapter 2** we aimed to investigate the role of Esp in biofilm formation, by using an Esp-deficient mutant. Subsequently in chapters 3 till 6, this Esp-deficient mutant and its Esp-expressing parent strain are tested *in vitro* for adherence to different cell lines and *in vivo* in animal models mimicking different clinical infections. In **chapter 3** the role of Esp in adherence to intestinal epithelial cells and intestinal colonization of *E. faecium* in mice is investigated, while in **chapter 4** the role of Esp in adherence to uroepithelial cells and urinary tract infection and

peritonitis in mice is assessed. In **chapter 5** the role of Esp in *E. faecium* endocarditis in rats is studied. In addition, the presence of antibodies against Esp in *E. faecium* bacteremia and endocarditis patient sera is assessed. In **chapter 6** the contribution of Esp to *E. faecium* bacteremia in mice is studied. In addition, it is investigated whether Esp is a target of opsonic antibodies in infection with *esp*-positive *E. faecium*.
In an attempt to detect recently acquired DNA by hospital-acquired *E. faecium*, additionally to the genes described above, a relatively novel method was used, the δρ-Web model that allows whole-genome composition analysis to visualize anomalous gene clusters in a prokaryotic genome based on differences on both GC content and dinucleotide frequencies, which was the research topic of **chapter 7.**

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2

Enterococcal surface protein Esp is important for biofilm formation of *Enterococcus faecium* E1162

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Abstract

Enterococci have emerged as important nosocomial pathogens with resistance to multiple antibiotics. Adhesion to abiotic materials and biofilm formation on medical devices are considered important virulence properties. A single clonal lineage of *Enterococcus faecium*, complex-17 (CC17), appears to be a successful nosocomial pathogen, and most CC17 isolates harbor the enterococcal surface protein gene, *esp*. In this study, we constructed an *esp* insertion-deletion mutant in a clinical *E. faecium* CC17 isolate. In addition, initial adherence and biofilm assays were performed. Compared to the wild-type strain, the *esp* insertion-deletion mutant no longer produced Esp on the cell surface and had significantly lower initial adherence to polystyrene and significantly less biofilm formation, resulting in levels of biofilm comparable to those of an *esp*-negative isolate. Capacities of initial adherence and biofilm formation were restored in the insertion-deletion mutant by *in trans* complementation with *esp*. These results identify Esp as the first documented determinant in *E. faecium* CC17 with an important role in biofilm formation, which is an essential factor in infection pathogenesis.

Introduction

Enterococci are considered normal inhabitants of the gastrointestinal tract of humans and animals. In the last two decades, though, enterococci have emerged as important nosocomial pathogens, with high-level resistance to antibiotics, such as ampicillin, aminoglycosides, and vancomycin (22). Enterococci are currently the third most frequent nosocomial pathogen isolated from intensive care unit patients in the United States (25). Since the turn of the century, the prevalence of enterococci has been rising in European hospitals too. The majority of enterococcal infections are caused by *Enterococcus faecalis* (http://www.earss.rivm.nl). However, in parallel with the increase in nosocomial enterococcal infections, a partial replacement of *E. faecalis* by *Enterococcus faecium* took place in European and United States hospitals (12,37,38) (http://www.earss.rivm.nl). It is unlikely that these ecological changes result exclusively from increased resistance to antibiotics. A better understanding of the virulence of enterococci, therefore, is necessary to control further spread and to develop new treatment strategies.

The ability to form biofilms on abiotic surfaces is considered to be an important virulence property of enterococci (5,7). A biofilm is an assemblage of microbial cells associated with a surface and enclosed in a matrix of primarily polysaccharide material. The defined architecture of the biofilm provides an optimal environment for the exchange of genetic material between bacteria and increases the innate resistance of the bacterium to antibiotics and activities of the host immune response (6,7). Enterococci have been associated with biofilms in endocarditis, urinary tract infections, root canal infections, and ocular infections (4,7,21,46) and in a variety of device-related infections in which biofilms were found on artificial hip prostheses, intrauterine devices, prosthetic heart valves, catheters, and stents (2,7,18,29).

Compared to *E. faecalis*, relatively little is known about virulence and pathogenesis of *E. faecium*. Previously, we described the evolutionary descent among *E. faecium* isolates obtained from human sources (from community as well as hospital reservoirs) and nonhuman sources in five continents with multi locus sequence typing. Most hospital outbreak and invasive *E. faecium* isolates belong to a single clonal lineage, complex-17 (CC17) (45). Furthermore, by using a mixed whole-genome microarray,

we recently identified a specific *E. faecium* clade largely overlapping with CC17, highly specific for nosocomial outbreaks and infections, and containing more than 100 clade-specific genes (17). The ecological success of CC17 in the hospital environment is not understood. It seems to be partly related to resistance to penicillins and quinolone antibiotics (16,45). Apart from antibiotic resistance and the clade-specific genes, CC17 is correlated with the presence of a putative pathogenicity island, which carries the enterococcal surface protein gene, *esp* (15,45). In *E. faecalis*, Esp is also located on a pathogenicity island, is expressed on the surface of the bacterium (30,31), and is thought to be an adhesin contributing to colonization of urinary tract epithelial cells and biofilm formation (21,31,32,35). Esp of *E. faecium* shares a homology up to 90% with Esp of *E. faecalis*, but its function is unknown. Interestingly, it is predominantly present in isolates associated with infections and hospital outbreaks (13,15,42). Furthermore, expression of Esp varies between strains, is growth condition dependent, and is quantitatively correlated with initial adherence to polystyrene and biofilm formation (41). Based on these findings, Esp may be an important determinant in adhesion and biofilm formation of *E. faecium*. However, the definitive role of Esp in these processes could not be determined, as an isogenic *esp* deletion mutant was lacking. Up till now, an *esp* mutant in an *E. faecium* clinical isolate has not been successfully constructed. In general, it has been extremely difficult to inactivate genes in *E. faecium* by allelic exchange due to poor transformation capacity, plasmid incompatibility, and the lack of selective markers because of multiple antibiotic resistances in clinical relevant strains. Recently, Nallapareddy et al. were the first to construct an insertion-deletion mutation, in the adhesion-encoding *acm* gene, in a clinical *E. faecium* isolate by using an improved temperature-sensitive vector (24). We used the same approach to generate an *esp* insertion-deletion mutant in a clinical isolate of *E. faecium* in order to assess the role of Esp in biofilm formation.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani broth or agar. Enterococci were grown in either brain heart infusion (BHI), Todd-Hewitt, or Trypticase soy (TS) broth or agar, or Sheep red blood agar containing Tryptic soy agar with 5% sheep red blood cells (BD, Alphen aan den Rijn, The Netherlands) at 37°C, unless a different growth temperature is specified. For enterococci, the antibiotics chloramphenicol, gentamicin, and erythromycin were used in concentrations of 10 µg/ml, 125 µg/ml, and 10 µg/ml, respectively. For *E. coli*, the antibiotic chloramphenicol was used in a concentration of 10 µg/ml. All antibiotics were obtained from Sigma-Aldrich (Saint Louis, MO). Growth was determined by measurements of the optical density at 660 nm (OD_{660}).
An improved temperature-sensitive vector (pTEX5500ts), designed by Nallapareddy et al. (24), was used to introduce an insertion-deletion mutation in the *esp* gene of a clinical *E. faecium* isolate, E1162, which was isolated from the blood of a patient. E1162 is a strain from CC17. The pAT18 vector (39) was used for complementation studies.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. faecium		
E135	Community surveillance feces isolate; Amp^s Van^r Chl^r Gen^s Ery^r; *esp* negative	(40)
E1162	Clinical blood isolate; CC17; Amp^r Van^s Chl^s Gen^s Ery^s; Esp^+	(40)
E1162Δ*esp*	*esp* insertion-deletion mutant of E1162; Chl^r Gen^s; Esp^-	This study
E1162EspUp/EspDn:pEF2	*esp* single-crossover mutant of E1162; Chl^r Gen^r	This study
E1162Δ*esp*:pEF3	*esp*-complemented strain of E1162Δ*esp*; Ery^r Chl^r Gen^s; Esp^+	This study
E. coli		
DH5α	*E. coli* host strain for routine cloning	Invitrogen
Plasmids		
pTEX5500ts	Shuttle plasmid; ts in gram-positive hosts; Chl^r Gen^r	(24)
pEF1	pTEX5500ts:EspUp, pTEX5500ts with a cloned *esp* gene fragment flanking the *cat* gene; Chl^r Gen^r	This study
pEF2	pTEX5500ts:EspUp-EspDn, pTEX5500ts with cloned *esp* gene fragments flanking the *cat* gene, plasmid for generating an *esp* insertion-deletion mutation; Chl^r Gen^r	This study
pAT18	Shuttle plasmid; Ery^r	(39)
pEF3	pAT18:*esp*; Ery^r	This study

Amp, ampicillin; Van, vancomycin; Chl, chloramphenicol; Gen, gentamicin; Ery, erytromycin; ts, temperature sensitive; ^{s}, susceptible; ^{r}, resistant; Esp^+, positive for Esp expression; Esp^-, negative for Esp expression.

Standard molecular techniques

Chromosomal DNA from *E. faecium* was prepared as described elsewhere (43,44). The primers used in this study were purchased from Isogen Life Science (IJsselstijn, The Netherlands) and are listed in Table 2. PCRs were performed with a 9800 Fast Thermal Cycler (Applied Biosystems, Foster City, CA), and the PCR amplification conditions were as follows: initial denaturation at 95°C for 15 min, followed by 10 touchdown cycles starting at 94°C for 30 s, 60°C for 30 s, and 72°C (the time depending on the size of the PCR product) with the annealing temperature decreasing by 1°C per cycle, followed by 25 cycles with an annealing temperature of

52°C. The PCRs were, unless otherwise specified, performed in 25-µl volumes with HotStarTaq Master Mix (QIAGEN Inc., Valencia, CA). PCR products were purified using the QIAquick PCR purification Kit (QIAGEN Inc.) according to the manufacturer's instructions. Restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Ligation was performed by using T4 DNA ligase (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Plasmid DNA from *E. coli* was purified by using the QIAprep Spin Miniprep Kit (QIAGEN Inc.) according to the manufacturer's instructions. Plasmids were transformed into *E. faecium* by electroporation using a Gene Pulser unit (Bio-Rad Laboratories, Richmond, CA) as described elsewhere (23). For Southern hybridization, chromosomal DNA was digested with TaqI, separated by agarose gel electrophoresis (0.7% agarose gels), and transferred onto a Hybond-N^+ nylon membrane (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). A 916-bp PCR fragment obtained with primers EspUpF2 and EspDnR2 was used as a DNA probe. Labeling of the probe and DNA hybridization were performed according to the protocol supplied with the ECL Direct Nucleic Acid labeling and detection System (GE Healthcare).

Forward and reverse DNA sequencing were performed by using the forward primers pRIE298.6F, EspUpDelF, EspUpF2, CMF, and EspDnDelF; the reverse primers Esp.11R, EspDnR2, CMR, and EspUpDelR; and the BigDye Terminator reaction kit and an ABI PRISM 3700 DNA analyzer (both from Applied Biosystems).

Table 2. Primers used in this study

Primer name	Primer sequence[a,b]	Start position
EspUpdelF	5'-CTA**GCTAGC** GCT CCG TAC AAG TAG GTG ACA	3281[c]
EspUpdelR	5'-CCC**AAGCTT** CCC GCT ACA TAT GGA ACT AAT C	4103[c]
EspDndelF	5'-CCG**GAATTC** CAT CTT TGA TTC TTG GTT GTC G	4392[c]
EspDndelR	5'-TCC**CCCGGG** TTG TTC CAG TAA TCG GCT CC	5284[c]
EspUpF2	5'-TAC GGG CTA CTT TTT AAC AGA	3825[c]
EspDnR2	5'-TGA ATC TAC ACC CGT AAA TTC	4739[c]
CmF	5'-GAA TGA CTT CAA AGA GTT TTA TG	509[d]
CmR	5'-AAA GCA TTT TCA GGT ATA GGT G	610[d]
pRIE298.6F	5'-GAA GAA GGA ATT TGA AGT CAC	2697[c]
Esp.11R	5'-GGT AGC CTG CAG GAA TG	5395[c]
EspcompF	5'-CCG**GAATTC**GCTTGCATCAAAATAAACTACATGGGTATAATAG CAATGAAATGCATTTCAAAAATATTTTGAGGAGAATTTAGTATG GTT AGC AAG AAT AAT AAG AG	3091[c]
EspcompR	5'-CCG**GAATTC** CCT CTT TTC AGA GAA GAT T	8827[c]

[a] Restriction sites are boldface.

[b] Regions -35, -10, the ribosome binding site from the *bacA* promoter, and the ATG codon of *esp* are underlined (9,36).

[c] Nucleotide reference positions relative to the *E. faecium* PAI sequence (Genbank accession no. AY322150).

[d] Nucleotide reference positions relative to the shuttle plasmid pTEX5500ts sequence (Genbank accession no. DQ208936).

Construction of an insertion-deletion mutation in the *esp* gene

To introduce an insertion-deletion mutation in the *esp* gene, the same protocol described by Nallapareddy et al. (24) was used with some minor modifications. In brief, an 850-bp-long internal *esp* fragment designated EspUp, coding for a region at the beginning of the N-terminal domain of *esp*, was amplified from genomic DNA of E1162 by using the primers EspUpdelF and EspUpdelR, including the restriction sites NheI and HindIII, respectively (Table 2). The PCR product was digested with NheI and HindIII and ligated to similarly digested pTEX5500ts, resulting in pEF1. In a similar way, an 830-bp-long fragment designated EspDn, coding for a region encompassing the end of the N-terminal domain of *esp*, was amplified by using the primers EspDndelF and EspDndelR, including the restriction sites EcoRI and SmaI, respectively. This PCR product was digested with EcoRI and SmaI and ligated to similarly digested pEF1, resulting in pEF2, pTEX5500ts with cloned *esp* gene fragments flanking the

chloramphenicol acetyltransferase (*cat*) gene. The recombinant plasmids pEF1 and pEF2 were transferred into *E. coli* DH5α cells (Invitrogen) for propagation and plasmid purification. The recombinant plasmid pEF2 was introduced into E1162 by electroporation to generate an insertion-deletion mutation in the *esp* gene. After transformation, the cells were allowed to recover for 4 h at the permissive temperature of 28°C, after which the cells were plated on Todd-Hewitt agar plates with 20% sucrose and gentamicin at 28°C to select for transformants. Gentamicin-resistant colonies were picked and grown overnight in BHI broth supplemented with gentamicin at an elevated temperature (42°C) to cure the plasmid. The cells were plated on BHI agar plates with chloramphenicol at 37°C. Single-crossover integration into EspUp and EspDn regions was tested by PCR with the primers pRIE298.6F and CmR, and CmF and Esp.11R, respectively. Single-crossover mutants were saved and grown for six serial overnight passages in chloramphenicol-BHI culture at 42°C to completely cure free recombinant plasmid. The cultures were serially diluted, plated on chloramphenicol-BHI agar plates and replica plated on gentamicin-BHI agar plates to select for double-crossover recombination. Double-crossovers were colonies that retained the *cat* gene but lost the *aph(2")-Id* gene, which encodes an aminoglycoside phosphotransferase that mediates high-level resistance to gentamicin, by plasmid excision, resulting in an insertion-deletion mutation of the *esp* gene. Correct generation of the insertion-deletion mutation in the *esp* gene was checked by PCR with the primers pRIE298.6F and Esp.11R, by Southern hybridization, and by DNA sequencing (as described above).

Complementation studies

To complement the *esp* mutant strain with wild-type *esp***,** the *esp* gene of E1162 was amplified from genomic DNA by using the Expand Long Template PCR system (Roche Diagnostics, Mannheim, Germany) with the forward primer EspcompF and the reverse primer EspcompR. The forward primer EspcompF included the -35 and -10 promoter regions and the ribosome binding site of the constitutive promoter of the *bacA* gene of *E. faecalis* (9,36), as well as an EcoRI restriction site, facilitating cloning of this fragment. The reverse primer also included an EcoRI restriction site. The resulting PCR product containing the *esp* gene and *bacA* promoter sequences

was digested with EcoRI and ligated to similarly digested pAT18 (39), resulting in pEF3 (pAT18:*esp*). The recombinant plasmid pEF3 was introduced into the *esp* mutant strain by electroporation.

Flow cytometry and electron microscopy

Flow cytometry and electron microscopy were performed as described previously (41). Flow cytometry experiments were repeated twice independently.

Whole-cell ELISA

Plate-grown bacteria were resuspended in phosphate-buffered saline (PBS) to an OD_{660} of 0.1 (1×10^8 CFU/ml). From each bacterial suspension, 100 µl was added to wells of a 96-well polystyrene microtiter plate (Corning Inc., Corning, NY). The bacteria were allowed to bind overnight at 4°C. The wells were washed three times with PBS containing 0.05% Tween 20. After being washed, the wells were blocked with 4% Bovine serum albumin (BSA) in PBS with 0.05% Tween 20 for 1 h at 37°C. Esp was assayed by incubation for 1 h at 37°C with rabbit anti-Esp immunoglobulin Gs (IgGs) (collected using a protein-G column; GE Healthcare) (41) in a dilution range from 10 µg/ml to 0.01 µg/ml. Bound antibodies were detected by incubation with a peroxidase-conjugated goat anti-rabbit IgG (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 37°C. Both antibodies were diluted in PBS with 1% BSA and 0.05% Tween 20. To each well, 50 µl of 0.11 M acetate buffer with 1.6% 3,3',5,5'-tetramethylbenzidine and 0.8% ureumperoxide was added, and the reaction was stopped after 10 min with 50 µl 0.5 M sulphuric acid. The absorbance at 450 nm was measured with an enzyme-linked immunosorbent assay (ELISA) reader. The whole-cell ELISA was performed twice.

Western blotting

Plate grown bacteria were resuspended in PBS to an OD_{660} of 1.0 (1×10^9 CFU/ml). Cells were harvested by centrifugation (1560 × *g*; 5 min) and resuspended in 50 µl PBS. Electrophoresis sample buffer (1×) supplemented with 50 mg/ml ditriothreitol was added. Samples were boiled for 5 min and loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. The bacterial proteins were transferred to

nitrocellulose using a Bio-Rad Trans-Blot Cell tank transfer unit at 12 V overnight in 20 mM Tris, 0.15 M glycine, and 20% methanol at pH 8.3. Nonspecific sites in the blot were blocked by incubation for 1 h at 37°C with 4% skim milk powder in PBS with 0.1% Tween 20. Esp was assayed by incubation for 1 h at 37°C with rabbit anti-Esp immune serum (1:5,000) (41) as the primary antibody, followed by incubation for 1 h at 37°C with horseradish peroxidase-conjugated goat anti-rabbit (1:5,000; Santa Cruz Biotechnology) as the secondary antibody. Both antibodies were diluted in PBS with 1% Tween 20 and 1% BSA. Esp was detected by using light-emitting ECL Western Blotting Detection Reagents (GE Healthcare).

Initial adherence assay to polystyrene

The initial adherence assay was performed as described previously (1). In brief, plate-grown bacteria were resuspended in TS broth to an OD_{660} of 0.5 (5×10^8 CFU/ml). To each well of a 96-well polystyrene microtiter plate (Corning Inc.) 100 µl bacterial suspension (5×10^7 CFU) was added in triplicate and incubated for 2 h at 37°C. After incubation, the bacteria were removed and the wells were washed with 200 µl PBS. The plates were dried for 1 h at room temperature. After 1 h, 100 µl 0.2% Gram's crystal violet solution (Merck, Darmstadt, Germany) was added to each well. After 15 min, the stain was removed and the plates were washed three times with 200 µl PBS. The plates were dried for 15 min at room temperature and the absorbance at 595 nm was measured directly with an ELISA reader. The experiment was repeated two times.

Biofilm formation assay

The biofilm assay was performed similarly to the initial adherence assay, except that the assay was performed in TS broth supplemented with 0.25% glucose and 1×10^5 CFU bacteria were incubated for 24 h at 37°C in a 96-well polystyrene microtiter plate (Corning Inc.).

Confocal laser scanning microscopy (CLSM)

Bacteria were grown in TS broth supplemented with 0.25% glucose to mid-log phase. Nitrocellulose membranes (0.45 µm; diameter 25 mm; Bio-Rad) were put on TS

agar plates with 0.25% glucose, and 200 µl bacterial suspension (5×10^6 CFU) was inoculated onto the nitrocellulose membranes and grown for 24 h at 37°C. After 24 h, the nitrocellulose membranes were washed three times in PBS and the biofilms were chemically fixed in 3.7% formaldehyde (Merck) in PBS for 15 min. Nonspecific sites were blocked by incubation for 1 h at 37°C with 10% skim milk powder in PBS. The biofilms were stained by incubating the nitrocellulose membranes (Bio-Rad) for 15 min at room temperature in 0.1% acridine orange (Merck) in PBS. After incubation, nitrocellulose membranes were washed three times with PBS and transferred to glass microscope slides and covered with glass cover slips (Marienfeld, Lauda-Königshofen, Germany). The biofilms were examined by using an inverted fluorescence microscope (Leica DMRXA2) equipped with an oil plan-neofluor ×100/1.4 objective and confocal images (scans) were developed with an MRCF-1000 laser (488 nm) scanning confocal imaging system (Bio-Rad). The acquired image stacks were viewed by using Leica Confocal Software (version 6.1). The maximum thickness of the biofilms was measured at five randomly chosen positions with the software.

Statistical analysis

For analysis of cell surface expression of Esp, initial adherence, and biofilm formation, a two-tailed Student's *t* test was applied.

Results

Construction of an insertion-deletion mutation in the *esp* gene

esp gene fragments were cloned into pTEX5500ts flanking the *cat* gene, resulting in pEF2, and this recombinant plasmid was used to introduce an insertion-deletion mutation in the *esp* gene of a clinical *E. faecium* isolate (E1162). The *esp* mutant strain (E1162Δ*esp*) was constructed in two steps. First, single-crossover mutants (E1162EspUp/EspDn: pEF2), in which pEF2 was integrated, were selected. Only left single-crossover integration was found. This was followed by selection of double-crossover events, in which the wild-type *esp* gene was replaced by the mutated *esp* gene and plasmid sequences were lost. Double-crossover mutants were expected to

be chloramphenicol resistant and gentamicin susceptible. In total, ~600 colonies were screened by replica plating. Of these colonies, eight colonies were putative double-crossover mutants. In two of the eight colonies, PCR indicated correct insertion-deletion mutation (data not shown). DNA-sequencing results confirmed that a 287-bp-long fragment of *esp* (positions 4105 to 4392 based on the *E. faecium* PAI sequence deposited in Genbank under accession number AY322150) was replaced by a 993-bp-long *cat* gene (data not shown). Southern blot analysis results confirmed correct insertion-deletion mutation in the *esp* gene in these two colonies (Figure 1). E1162Δ*esp* colonies appeared to have the same size as those of the wild-type strain (E1162) when grown overnight on sheep red blood agar plates. To further characterize the behavior of E1162Δ*esp*, growth was monitored by OD_{660}. No difference in growth rates was observed between E1162 and E1162Δ*esp* (data not shown).

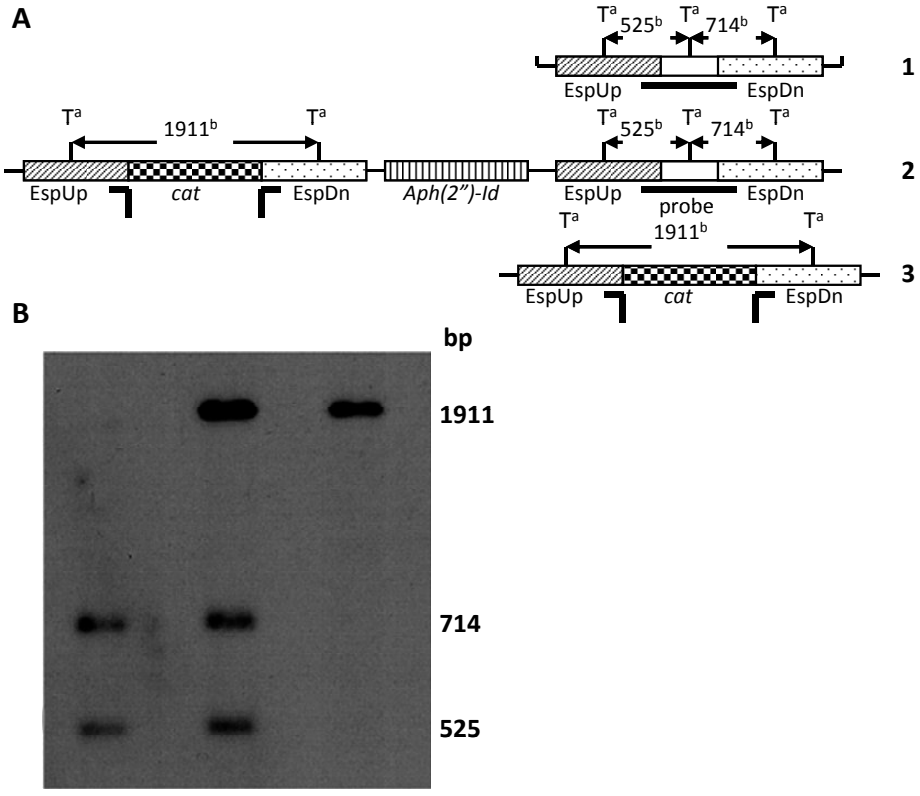

Figure 1. Confirmation of correct insertion-deletion mutation in the *esp* gene by Southern blot analysis. (A) Schematic representation of the 5' end encoding the N-terminal domain of the wild-type *esp* gene (1), the *esp* single-crossover insertion (2), and the *esp* double-crossover insertion-deletion (3). The box with squares represents the *cat* gene coding for chloramphenicol resistance, the striped box represents the *aph(2")-Id* gene coding for gentamicin resistance, the hatched box represents the EspUp fragment used for recombination, the stippled box represents the EspDn fragment used for recombination, and the black line represents the DNA probe. [a] TaqI restriction site; [b] Fragments obtained after digestion with TaqI; [c] Nucleotide reference positions relative to the *E. faecium* PAI sequence (Genbank accession no. AY322150). (B) Hybridization results of Southern blot analysis of TaqI-digested genomic DNA of *esp* wild-type strain (lane 1), *esp* single-crossover mutant strain (lane 2), and *esp* double-crossover mutant strain (lane 3).

Cell surface expression of Esp

Cell surface expression of Esp was analyzed by flow cytometry using rabbit anti-Esp immune serum (Figure 2). Esp expression in E1162Δ*esp* was significantly reduced ($P < 0.001$) compared to E1162 and close to the background levels found in a community surveillance strain (E135) not carrying the *esp* gene. Whole-cell ELISA (Figure 3) and electron microscopy (Figure 4) confirmed the lack of cell surface Esp in E1162Δ*esp*. Western blot analysis indicated that intracellular Esp expression was also abolished in E1162Δ*esp* (data not shown).

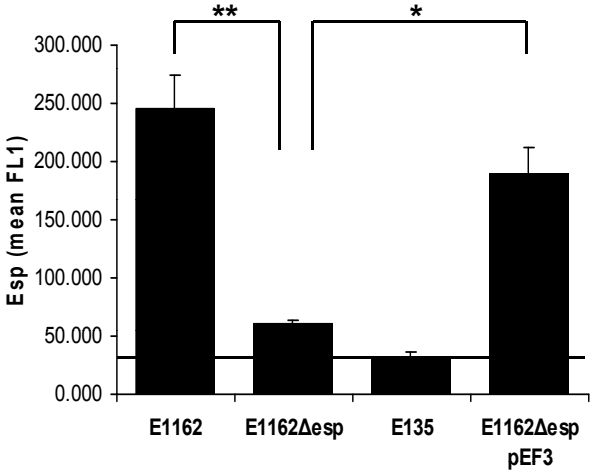

Figure 2. Cell surface expression of Esp by flow cytometry. Shown is analysis of cell surface expression of Esp by flow cytometry using rabbit anti-Esp immune serum for *esp* wild-type strain (E1162), *esp* mutant strain (E1162Δ*esp*), *esp*-negative strain (E135), and *esp*-complemented strain (E1162Δ*esp*:pEF3). Horizontal line represents background level. Mean values and standard deviations are shown. *$P < 0.001$; **$P < 0.0005$.

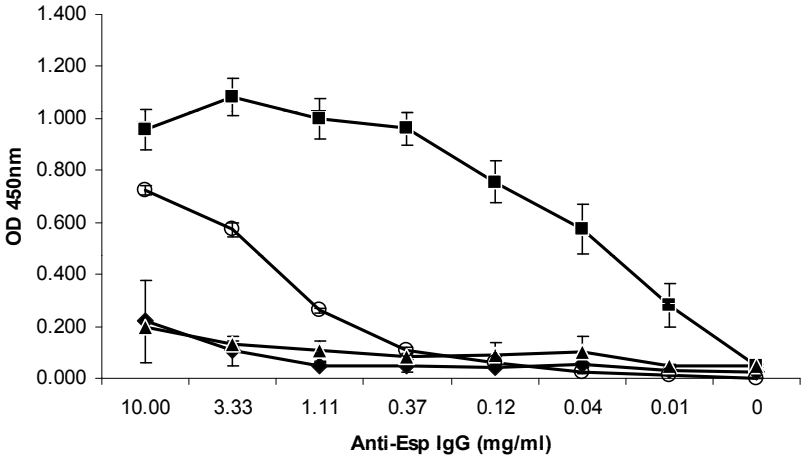

Figure 3. Cell surface expression of Esp by whole-cell ELISA. Shown is analysis of cell surface expression of Esp by whole-cell ELISA using rabbit anti-Esp IgGs in different dilutions for *esp* wild-type strain (E1162) (squares), *esp* mutant strain (E1162Δ*esp*) (triangles), *esp*-negative strain (E135) (diamonds), and *esp*-complemented strain (E1162Δ*esp*:pEF3) (circles). Mean values and standard deviations are shown.

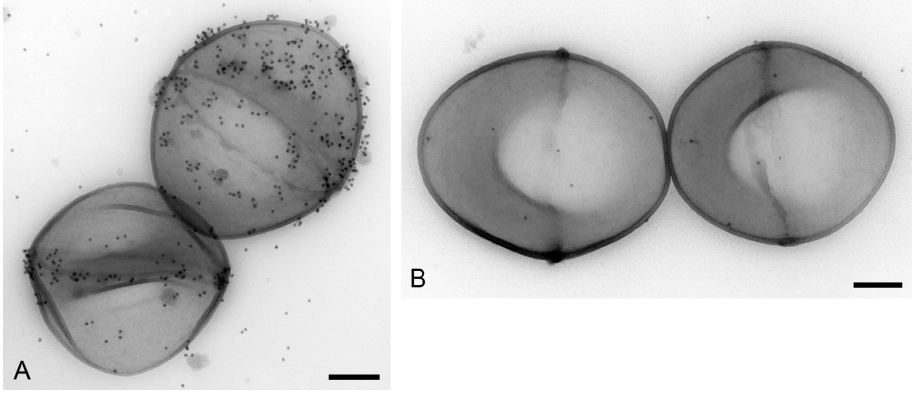

Figure 4. Electron microscopy. Shown are electron micrographs at a magnification of ×60,000. The *esp* wild-type strain (E1162) (A) and the *esp* mutant strain (E1162Δ*esp*) (B) were incubated with rabbit anti-Esp immune serum, followed by protein-A-Gold. Bars, 200nm.

Initial adherence assay to polystyrene and biofilm formation

E1162, E1162Δ*esp*, and E135 were investigated for their ability to adhere to polystyrene and for biofilm formation. Strain E1162 exhibited high adherence to polystyrene and high levels of biofilm formation, while the *esp*-negative strain, E135, showed only low-level binding and biofilm formation (Figure 5). Both initial adherence to polystyrene ($P < 0.0005$) and biofilm formation ($P < 0.001$) were significantly reduced in the E1162Δ*esp* relative to E1162 and dropped to levels seen in E135**.**

CLSM was used to examine biofilms formed by E1162 and E1162Δ*esp* on nitrocellulose membranes. Consistent with the biofilm assay on polystyrene, biofilm formation was highly reduced in E1162Δ*esp* compared to E1162 (Figure 6). The mean maximum thickness of biofilms formed by E1162Δ*esp* was significantly ($P < 0.0001$) lower than that of E1162.

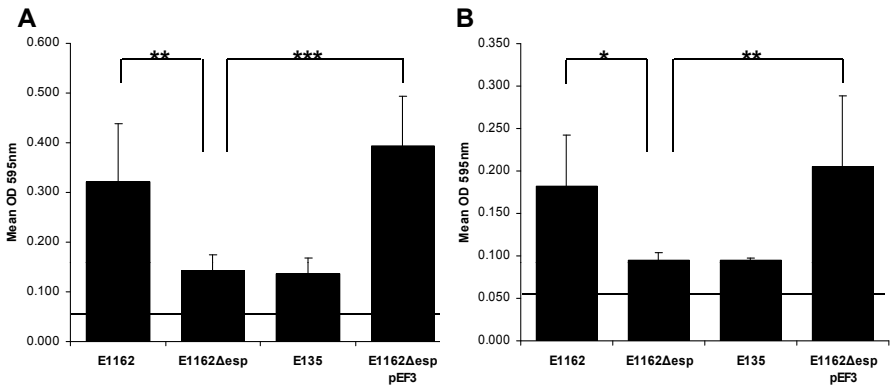

Figure 5. Initial adherence and biofilm formation. Shown are the abilities to adhere to polystyrene (A) and to form biofilm (B) of the *esp* wild-type strain (E1162), *esp* mutant strain (E1162Δ*esp*), *esp*-negative strain (E135), and *esp*-complemented strain (E1162Δ*esp:*pEF3). The horizontal lines represent background OD levels when wells possessing no bacteria were stained with crystal violet. Mean values and standard deviations are shown. $^{**}P < 0.0005$ and $^{***}P < 0.0001$ (A); $^{*}P < 0.001$ and $^{**}P < 0.0005$ (B).

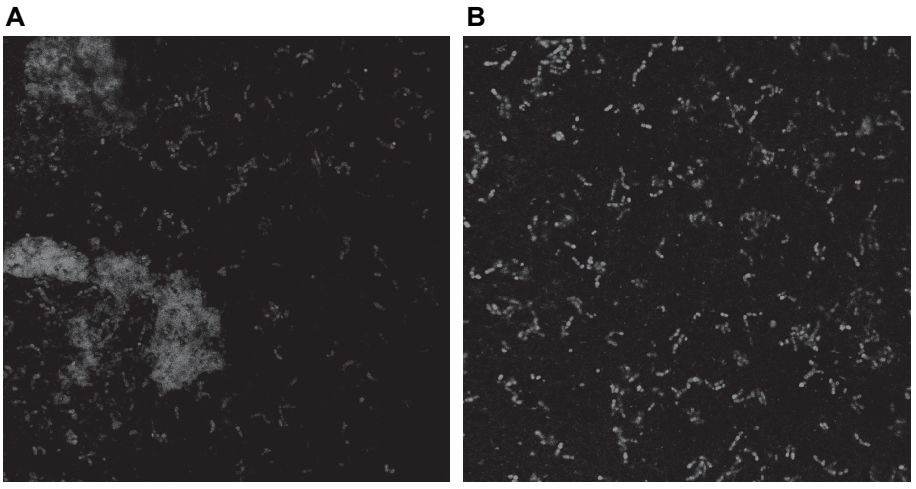

Figure 6. Confocal laser scanning microscopy. CLSM images of the *esp* wild-type strain (E1162) (A) and the *esp* mutant strain (E1162Δ*esp*) (B) grown on nitrocellulose for 24 h. The images represent the layer in a Z-stack that has the maximum bacterial coverage. The maximum thickness of biofilms was measured at five randomly chosen positions, resulting in a mean maximum thickness of 11.01 (± 0.91) µm for E1162 and 6.43 (± 0.81) µm for E1162Δ*esp*. This difference was significant ($P < 0.0001$).

Complementation studies

Complementation experiments were performed to determine whether *in trans* expression of Esp from a plasmid was able to restore initial adherence and biofilm formation in the *esp* mutant strain. Because the promoter of *esp* has not been mapped, *esp* was expressed under the control of a constitutive promoter of the *bacA* gene of *E. faecalis* (9), as described in the Materials and Methods. After transformation of *E. coli* DH5α cells with recombinant plasmid pEF3, the cells were highly unstable, suggesting that Esp expression had a toxic effect on *E. coli* cells. Therefore, the ligation mixture was transferred directly to the *E. faecium esp* mutant by electroporation, resulting in an *esp*-complemented strain (E1162Δ*esp*:pEF3). Cell surface expression of Esp was analyzed by flow cytometry using rabbit anti-Esp immune serum. Esp expression was significantly enhanced ($P < 0.0005$) in E1162Δ*esp*:pEF3 compared to E1162Δ*esp* and was comparable to, though slightly

less than, amounts found in E1162 (Figure 2). Whole-cell ELISA using rabbit anti-Esp IgGs confirmed significantly enhanced Esp expression in E1162Δ*esp*:pEF3 compared to E1162Δ*esp* (Figure 3), as did Western blot analysis (data not shown). Additionally, initial adherence to polystyrene ($P < 0.0001$) and biofilm formation ($P < 0.0005$) were significantly enhanced in E1162Δ*esp*:pEF3 compared to E1162Δ*esp* and were comparable to the levels found in E1162 (Figure 5).

Discussion

Successful insertion-deletion mutation of *esp* in a clinical CC17 *E. faecium* isolate resulted in abolished cell surface Esp expression, significantly lower initial adherence to polystyrene, and reduced biofilm formation. The capacities of initial adherence and biofilm forming were restored in the insertion-deletion mutant by *in trans* complementation with *esp*. Esp is the first documented CC17-specific *E. faecium* determinant implicated in biofilm formation. Our findings suggest that Esp has played an important role in the evolutionary development of CC17 *E. faecium* from avirulent commensal to an important globally spread nosocomial pathogen.

Initial adherence and biofilm formation are both considered important pathogenic properties of enterococci (5,7). The relevance of biofilms in enterococcal infections has been demonstrated for a variety of infections (2,4,7,18,21,29,46). In *E. faecalis*, different bacterial surface proteins and genes, such as GelE (10,14,27), BopD (11), the *fsr* locus (10), the *bee* locus (34), and Esp (21,32,33,35), are involved in this process. For instance, biofilm formation in *E. faecalis* was reduced in isogenic Esp-deficient strains (32). Furthermore, the N-terminal domain of Esp appeared to be sufficient to enhance biofilm formation (33). However, in another study, the effect of insertional mutagenesis in *E. faecalis* was strain dependent, ranging from a complete loss of the biofilm formation phenotype to no apparent effect, indicating contribution of additional cell surface proteins (35). Moreover, no correlations were found between the presence or absence of the *esp* gene in clinical *E. faecalis* isolates and biofilm formation in other studies (3,10,14,28).

Both initial adherence and biofilm formation were significantly reduced in the *esp* mutant *E. faecium* strain and restored in the *esp*-complemented strain, indicating

that Esp is important for initial adherence of *E. faecium* to polystyrene and subsequent development of biofilm. The *esp* mutant strain, comparable with the *esp*-negative strain, exhibited a low but measurable degree of initial adherence and biofilm formation, which indicates that other factors besides Esp play minor roles in these processes. The reduced initial adherence in the *esp* mutant strain suggests that Esp is important in the primary attachment to abiotic surfaces in order to initiate biofilm formation. Whether Esp is also involved in adhesion to biotic components, like epithelial cells and extracellular matrix molecules necessary for gut colonization and infection, remains to be determined. In one study, bloodstream isolates of *E. faecium* enriched with *esp* had increased adhesion to Caco-2 human colon cancer cells (20), suggesting a role of Esp in gut colonization. In contrast, adherence of *E. faecium* to Caco-2 cell lines was not associated with the presence of *esp* in another study (8). The restored biofilm formation in the *esp*-complemented strain indicates that the biofilm-reduced phenotype of the *esp* mutant is due to the mutated *esp* gene and not to a polar effect on genes located downstream. The *esp*-complemented strain had slightly less expression of Esp than the *esp* wild-type strain. Nevertheless, both initial adherence to polystyrene and biofilm formation abilities were similar. Perhaps a specific amount of Esp at the surface of the bacterium is already sufficient to induce these processes.

The presence of *esp* in *E. faecium* has been associated with higher conjugation frequencies than in *esp*-negative isolates (19). This suggests that either Esp plays a direct role in cell-cell interaction or that Esp may serve as a marker for strains with enhanced potential to acquire new genetic elements. Furthermore, conjugative transfer of the *esp* gene among *E. faecium* isolates has been described *in vitro* by integration of *esp* into a conjugative plasmid (26), suggesting that the *esp* gene can be transferred horizontally and spread among *E. faecium* isolates. Previously, we have shown that Esp expression was elevated under conditions permissive for lumen gut colonization, such as 37°C and anaerobiosis, while expression was reduced under aerobic conditions and at 20°C, mimicking environmental conditions (41). All these data suggest that Esp plays an important role in the pathogenesis of *E. faecium* infections. Because of the specific linkage of *esp* to CC17, we hypothesize that *esp* is one of the important determinants that explains the ecological success of this clonal complex in the hospital environment.

In conclusion, establishing an isogenic *esp* mutant, as performed in the present study, represents only the second successful insertion-deletion mutation experiment in *E. faecium*. Inactivation of *esp* resulted in completely abolished Esp expression on the cell surface and significantly reduced initial adherence to polystyrene and biofilm formation. Esp, therefore, plays an important role in these processes, which are considered important factors in the infection pathogenesis. Esp could be a promising therapeutic target for preventing CC17 *E. faecium* infections.

Acknowledgements

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3

Enterococcal surface protein Esp is not essential for cell adhesion and intestinal colonization of *Enterococcus faecium* in mice

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Abstract

Background: *Enterococcus faecium* has globally emerged as a cause of hospital-acquired infections with high colonization rates in hospitalized patients. The enterococcal surface protein Esp, identified as a potential virulence factor, is specifically linked to nosocomial clonal lineages that are genetically distinct from indigenous *E. faecium* strains. To investigate whether Esp facilitates bacterial adherence and intestinal colonization of *E. faecium*, we used human colorectal adenocarcinoma cells (Caco-2 cells) and an experimental colonization model in mice. Results: No differences in adherence to Caco-2 cells were found between an Esp-expressing strain of *E. faecium* (E1162) and its isogenic Esp-deficient mutant (E1162Δ*esp*). Mice, kept under ceftriaxone treatment, were inoculated orally with either E1162, E1162Δ*esp* or both strains simultaneously. Both E1162 and E1162Δ*esp* were able to colonize the murine intestines with high and comparable numbers. No differences were found in the contents of cecum and colon. Both E1162 and E1162Δ*esp* were able to translocate to the mesenteric lymph nodes. Conclusion: These results suggest that Esp is not essential for Caco-2 cell adherence and intestinal colonization or translocation of *E. faecium* in mice.

Introduction

Enterococci are normal inhabitants of the human gastrointestinal (GI) tract, but have emerged as important nosocomial pathogens with high-level resistance to antibiotics, such as ampicillin, aminoglycosides, and vancomycin (1). They can cause a wide spectrum of diseases, including bacteremia, peritonitis, surgical wound infections, urinary tract infections, endocarditis, and a variety of device-related infections (1-11). The majority of the enterococcal infections are caused by *Enterococcus faecalis*. However, in parallel with the increase in nosocomial enterococcal infections, a partial replacement of *E. faecalis* by *Enterococcus faecium* has occurred in European and United States hospitals (12-14) (http://www.earss.rivm.nl).

Molecular epidemiological studies indicated that *E. faecium* isolates responsible for the majority of nosocomial infections and hospital outbreaks are genetically distinct from indigenous intestinal isolates (15,16). Recent studies revealed intestinal colonization rates with these hospital-acquired *E. faecium* as high as 40% in hospital wards, while colonization in healthy people appeared to be almost absent (13,15,16). It is assumed that adherence to mucosal surfaces is a key process for bacteria to survive and colonize the GI tract. Intestinal colonization of nosocomial *E. faecium* strains is a first and key step that precedes clinical infection due to fecal contamination of catheters or wounds, and in the minority of infections, through bacterial translocation from the intestinal lumen to extraintestinal sites (17,18). It is not known which factors facilitate intestinal colonization of nosocomial *E. faecium* strains. The enterococcal surface protein Esp, located on a putative pathogenicity island (19,20), is specifically enriched in hospital-acquired *E. faecium* and has been identified as a potential virulence gene. Esp is involved in biofilm formation (21) and its expression is affected by changes in environmental conditions, being highest in conditions that mimic the microenvironment of the human large intestines: 37°C and anaerobioses (22). Furthermore, in one study, bloodstream isolates of *E. faecium* enriched with *esp* had increased adherence to human colorectal adenocarcinoma cells (Caco-2 cells) (23), suggesting a role of Esp in intestinal colonization. In contrast, adherence of *E. faecium* to Caco-2 cell lines was not associated with the presence of *esp* in another study (24). In *E. faecalis*, Esp is also located on a pathogenicity island,

although the genetic content and organization of the *E. faecium* and *E. faecalis* PAI is different. Esp of *E. faecalis* is also expressed on the surface of the bacterium (25,26) and is important in colonization of urinary tract epithelial cells (25). By using a mouse model, Pultz et al. (27) showed that Esp does not facilitate intestinal colonization or translocation of *E. faecalis* in mice, however this does not automatically predict a lack function for *E. faecium* Esp in murine colonization. First data suggest that the function of Esp in both enterococcal species might be different. Esp of *E. faecium* is clearly involved in biofilm formation (see above) while there is controversy about the role of *E. faecalis* Esp in biofilm formation (28-31). Furthermore, studies so far indicate that *E. faecalis* harbors more virulence determinants then *E. faecium*. For instance, besides Esp different determinants (GelE, BopD, *fsr* locus, and *bee* locus) are putatively involved in biofilm formation (32-34). This suggests that virulence factors in *E. faecalis* play somewhat redundant or partially overlapping roles such that the absence of a single virulence factor, like Esp, has only minimal effect. To elucidate the role of Esp of *E. faecium* in bacterial adhesion and intestinal colonization, we studied an Esp-deficient mutant, constructed and described recently (21), and its Esp-expressing parent strain for their ability to adhere to intestinal epithelial cells and intestinal colonization by using Caco-2 cells and a mouse model.

Materials and Methods

Bacterial strains and growth conditions

In this study *E. faecium* strains E135, E1162 and E1162Δ*esp* were used. E135 is an *esp* negative community surveillance feces isolate, while strain E1162 is a hospital-acquired blood isolate, positive for Esp expression. The isogenic Esp-deficient mutant, E1162Δ*esp*, was previously constructed by introduction of a chloramphenicol resistance cassette (*cat*) resulting in an insertion-deletion mutation of the *esp* gene (21). *E. faecium* strains were grown in either Todd-Hewitt (TH) or Brain Heart Infusion (BHI) broth or on Tryptic Soy Agar (TSA) with 5% sheep red blood cells (Difco, Detroit, MI). Slanetz and Bartley (SB) agar plates were used to selectively grow enterococci. *E. faecium* strain E1162 and its isogenic mutant are high-level resistant to ceftriaxone (minimum inhibitory concentration > 32 µg/ml).

Caco-2 cell cultures

Human colorectal adenocarcinoma cells, Caco-2 cells, were obtained from the American Type Culture Collection (HTB-37, ATCC, USA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (Integro B.V, Zaandam, The Netherlands), 1% non-essential amino acids (Gibco), 2 mM glutamine (Gibco), and 50 µg/ml gentamicin (Gibco). Cells were collected every 7th day by washing the monolayer twice with 0.022% disodium-ethylenediamine tetra acetic acid (di-Na-EDTA; Acros Organics, Morris Plains, NJ) in PBS and trypsinizing the cells using 50 µg/ml trypsine (Gibco), in 0.022% di-Na-EDTA in PBS. Cells were seeded at 1 x 10^6 cells in 10 ml DMEM in 75 cm^2 culture bottles (Costar, Corning, NY) and incubated in a humidified, 37˚C incubator with 5% CO_2. The culture medium was refreshed every 4th day after passage of the cells. Differentiated Caco-2 cells were prepared by seeding cells from passage 25 to 45 in 12-wells tissue culture plates (Costar) at 1.6 x 10^5 cells/ml in DMEM. To each well 1 ml of this suspension was added and plates were incubated at 37˚C with 5% CO_2 for 14-16 days before use to allow the Caco-2 cells to differentiate. The medium in the wells was replaced by fresh medium three times a week.

Adherence assay

Overnight-grown cultures of E135, E1162 and E1162Δ*esp* in BHI broth were diluted (1:50) and grown at 37°C to an OD_{660} of 0.4, while shaking. Bacteria were harvested by centrifugation (6,500 × g; 3 min) and resuspended in DMEM to a concentration of 1 × 10^7 CFU/ml. For each strain, 1 ml bacterial suspension was added to the wells (100 bacteria to 1 Caco-2 cell). Plates were centrifuged (175 × g; 1 min) and incubated for 1 h at 37˚C in 5% CO_2 to allow adherence to the Caco-2 cells. After incubation, monolayers were rinsed 3 times with DMEM and cells were lysed with 1% Triton X-100 (Merck, Darmstadt, Germany) in PBS for approximately 5 min at room temperature. Adherent bacteria were quantified by plating serial dilutions onto TSA plates and counting resultant colonies. Also the inoculum was plated to determine viable counts. The assay was performed simultaneously in 3 separate wells in duplicate and repeated on 3 different days.

Mice

Specific pathogen-free 10-week-old female C57BL/6 mice (14 mice in total) were purchased from Harlan Sprague-Dawley (Horst, The Netherlands). The animals were housed in individual cages in rooms with a controlled temperature and a 12-h light-dark cycle. They were acclimatized for 1 week prior to usage, and received standard rodent chow and water ad libitum. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Induction of intestinal colonization

Mice were administered subcutaneous injections of ceftriaxone (Roche, Woerden, The Netherlands; 100 µl per injection, 12 mg/ml) 2 times a day, starting 2 days before inoculation of bacteria and continuing for the duration of the experiment. Two days after the initiation of the antibiotic treatment 2 x 10^9 CFU of E1162 or E1162Δ*esp* in 300 µl TH broth was inoculated by orogastric inoculation using an 18-gauge stainless animal feeding tube. In addition, in one experiment mice were administered a mixture of an equal amount (1.5 x 10^9 CFU) of E1162 and E1162Δ*esp* simultaneously. For all experiments, plate-grown bacteria were inoculated in TH broth and grown at 37˚C to an OD_{620} 1.0, while shaking. The inoculum was plated to determine viable counts. Mice were sacrificed after 10 days of colonization. Seven mice per group were examined.

Collection of samples

Stool samples were collected from naive mice, 2 days after antibiotic treatment and 1, 3, 6 and 10 days after bacterial inoculation. Per mice, 2 stool pellets were collected, pooled, weighed (50-129 mg), and 1 ml of sterile saline was added. After 10 days of colonization mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium; active ingredients fentanyl citrate and fluanisone) and midazolam (Roche, Meidrecht, The Netherlands), blood was drawn by cardiac puncture and transferred to heparin-gel vacutainer tubes. Mesenteric lymph nodes (MLN) were excised, weighed and collected in 4 volumes of sterile saline. Subsequently, the intestines were excised, opened and fecal contents of small bowel, cecum, and colon were weighed and 1 ml of sterile saline was added.

Determination of bacterial outgrowth

The number of *E. faecium* CFU was determined in stool, MLN, blood, and fecal contents of small bowel, cecum, and colon. Stool, MLN, and fecal contents were homogenized at 4°C using a tissue homogenizer (Biospec Products, Bartlesville, UK). CFU were determined from serial dilutions of the homogenates and undiluted blood. Twenty µl of each dilution and 50 µl of undiluted blood, was plated onto SB agar plates and grown at 37˚C for 44 h with 5% CO_2. Colonies were counted, tested by PCR to confirm species identity, and corrected for the dilution factor to calculate CFU per gram of stool/MLN/fecal contents. MLVA was performed to confirm strain identity.

PCR analysis to confirm species

Stool samples from naïve mice and from mice treated for 2 days with ceftriaxone were examined for presence of *E. faecium*. The lowest dilutions of stool homogenates that contained well-separated colonies were chosen and each colony of that dilution (12-24 CFU/20 µl diluted stool homogenate) was tested by PCR for presence of the housekeeping gene *ddl* (encoding D-alanine, D-alanine ligase) using the *E. faecium* specific primers ddlF (5'-GAG ACA TTG AAT ATG CCT) and ddlR (5'-AAA AAG AAA TCG CAC CG) (43). The colonies were directly diluted in 25-µl-volumes with HotStarTaq Master Mix (QIAQEN Inc., Valencia, CA). PCR's were performed with a 9800 Fast Thermal Cycler (Applied Biosystems, Foster City, CA) and the PCR amplification conditions were as follows: initial denaturation at 95°C for 15 min, followed by 10 touchdown cycles starting at 94°C for 30 s, 60°C for 30 s, and 72°C (the time depended on the size of the PCR product) with the annealing temperature decreasing by 1°C per cycle, followed by 25 cycles with an annealing temperature of 52°C. All primers used in this study were purchased from Isogen Life Science (IJselstijn, The Netherlands).

For mono infection, colonies obtained from stool (1, 3, 6, and 10 days after bacterial inoculation), MLN, and fecal contents from small bowel, cecum, and colon were examined to confirm species identity. Colonies were randomly picked and presence of the *ddl* gene, in case E1162 was inoculated, or the *cat* gene, in case E1162Δ*esp* was inoculated, was assessed by PCR using primer pairs ddlF - ddlR and CmF (5'-GAA TGA CTT CAA AGA GTT TTA TG) – CmR (5'-AAA GCA TTT TCA GGT ATA GGT G)

(21), respectively. When both strains were inoculated simultaneously, all colonies from the lowest dilution with well-separated colonies were picked (3-28 CFU/20 µl diluted homogenate). Species identity and the number of E1162 and E1162Δ*esp* were determined by multiplex PCR using primer pairs ddlF – ddlR and CmF - CmR. In PCR's, a colony of E1162 and E1162Δ*esp* was used as positive control and a colony of *E. faecalis* V583 (44) was used as negative control.

MLVA to confirm strain identity

For both mono infection and mixed infection, colonies obtained from stool (1, 3, 6, and 10 days after bacterial inoculation), MLN, and fecal contents from small bowel, cecum, and colon were randomly picked and Multiple-Locus Variable-Number Tandem Repeat Analysis (MLVA) was performed to confirm strain identity. MLVA was performed as described previously (45).

Histological examination

Small bowel, cecum and colon tissue were fixed in 4% buffered formalin and embedded in paraffin. Four-micrometer-thick sections were stained with hematoxylin-eosin and analyzed.

Statistical analysis

Adherence data are expressed as the mean CFU per ml ± the standard deviation (SD). A two-tailed Student's *t* test was applied. Mouse colonization data are expressed as medians of CFU per gram of stool/fecal contents. Two group comparisons were done by Mann-Whitney *U* test. A *P*-value < 0.05 was considered statistically significant.

Results

Adherence assay to Caco-2 cells

To determine whether Esp contributes to adherence of intestinal epithelial cells, the Esp-expressing *E. faecium* strain E1162, its isogenic Esp-deficient mutant (E1162Δ*esp*), and an *E. faecium esp*-negative strain (E135) were investigated for their

ability to adhere to differentiated 14 days old Caco-2 cells. Strain E1162 exhibited high adherence to Caco-2 cells, while the *esp*-negative strain, E135, showed only low-level binding to Caco-2 cells (Figure 1). This difference in adherence was significant ($P < 0.005$). However, no significant difference in adherence to Caco-2 cells was observed between E1162 and E1162Δ*esp*.

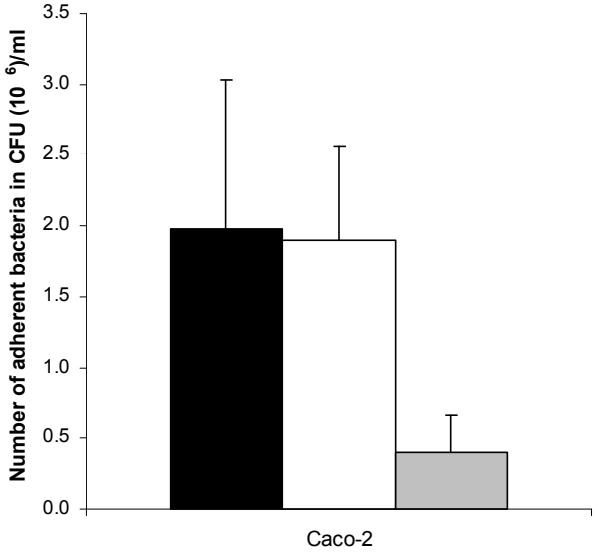

Figure 1. Adherence to Caco-2 cells. Adherence of E135 (grey bars), E1162 (black bars) and E1162Δ*esp* (white bars) to differentiated Caco-2 cells (14 days old). Adherence levels are expressed as the mean number of CFU per ml ± the standard deviation (SD).

Intestinal colonization

To investigate the role of Esp in intestinal colonization and translocation to MLN, the Esp-expressing E1162 and its isogenic Esp-deficient mutant (E1162Δ*esp*) were inoculated orally in mice separately or simultaneously in a mixed inoculum. Mice were kept under ceftriaxone treatment the entire experiment. Prior to any intervention no *E. faecium* was cultured from stools of mice. The mean enterococcal contents of the stool of naïve mice was $5 \times 10^5 \pm 2 \times 10^5$ CFU/gram, these colonies were specified being *E. faecalis*.

Both E1162 and E1162Δ*esp* were able to colonize the intestinal tract with comparable high numbers of cells for the entire 10 days of the experiment. One day after inoculation E1162 reached a median of 5.2 (range 2-15) x 10^8 CFU/gram of stool and E1162Δ*esp* of 5.1 (1.6 - 8.2) x 10^8 CFU/gram. Ten days after inoculation, the amount of both strains slightly reduced to 3.7 (1.3-10) x 10^6 and 2.7 (0.2-25) x 10^6 CFU/gram of stool, respectively (Figure 2A). Similar amounts of E1162 and E1162Δ*esp* were found in the stool of mice colonized when the mixed inoculum was administered (data not shown). After 10 days of colonization, all mice were sacrificed and *E. faecium* colonies obtained from small bowel, cecum, and colon contents were calculated. In both cecum and colon comparable amounts of E1162 (cecum contents 6.9 (0.04-7.3) x 10^6 and colon contents 3.9 (1.3-11) x 10^6 CFU/gram) and E1162Δ*esp* (cecum contents 10 (0.4-200) x 10^6 and colon contents 2.7 (0.2-24) x 10^6 CFU/gram) were isolated, from both separate (Figure 2B) and mixed inocula (data not shown). Significantly more E1162Δ*esp* (8.4 (0.5-300) x 10^6 CFU/gram) compared to E1162 (6.5 (0.5-52) x 10^4 CFU/gram) was isolated from the small bowel contents of mice when inoculated separately with E1162 wild type and the Esp-deficient mutant strain (P = 0.002). This difference was not found in mice inoculated with the mixture of E1162 and E1162Δ*esp* (data not shown).

Both E1162 and E1162Δ*esp* were able to translocate to the MLN. From both of the separately inoculated groups of mice, three out of seven MLN were found positive for either E1162 or E1162Δ*esp*. No bacteria were cultured from blood. No pathological changes in the intestinal wall were observed in any of the colonized mice.

For both mono infection and mixed infection, randomly picked colonies were tested by MLVA to confirm strain identity. All colonies had the same MLVA profile belonging to *E. faecium* E1162(Δ*esp*).

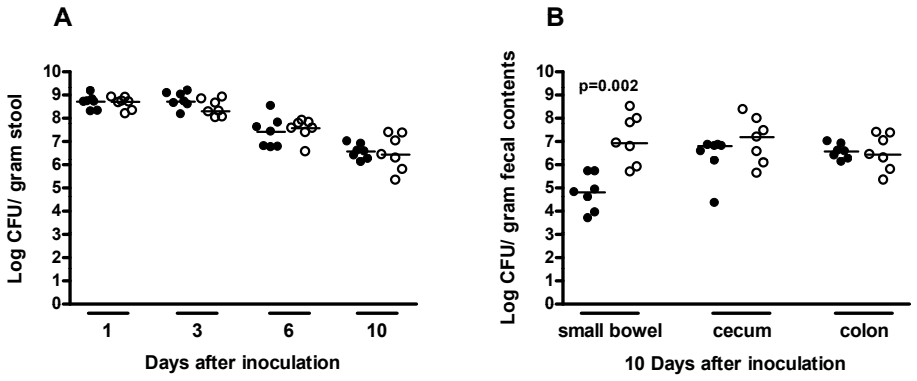

Figure 2. Intestinal colonization. Mice were orally inoculated with E1162 (black circles) or E1162Δ*esp* (open circles). (A) Numbers of E1162 and E1162Δ*esp* were determined in stool of mice at different time points after *E. faecium* inoculation. (B) After 10 days of colonization, numbers of E1162 and E1162Δ*esp* were determined in small bowel, cecum and colon. Data are expressed as CFU per gram of stool/fecal contents and medians are shown for 7 mice per group.

Discussion

Nosocomial *E. faecium* infections are primarily caused by specific hospital-selected clonal lineages, which are genetically distinct from the indigenous enterococcal flora. High rates of colonization of the GI tract of patients by these hospital-selected lineages upon hospitalization have been documented (13,15). Once established in the GI tract these nosocomial strains can cause infections through bacterial translocation from the GI tract to extraintestinal sites (35,36). The mechanism which promotes supplementation of the commensal enterococcal population by these nosocomial strains is not known. Destabilization of the GI tract through antibiotic therapy may provide nosocomial strains enhanced opportunities to gain a foothold in the GI tract. However, the effect of antibiotics is probably not the sole explanation for the emergence of nosocomial *E. faecium* infections since many antibiotics used in hospitals have relatively little enterococcal activity. This implicates that nosocomial *E. faecium* strains may possess traits that facilitate colonization of portions of the GI

tract that the indigenous flora cannot effectively monopolize. Cell surface proteins like Esp, implicated in biofilm formation and specifically enriched in nosocomial strains, could represent one of these traits. Previously, it was shown that *E. faecium* is able to adhere to human and mouse intestinal mucus *in vitro* and becomes associated *in vivo* with the intestinal mucus layer of clindamycin treated mice (37-39). This suggests an interaction between the bacterium and the mucus or with the epithelium itself. To examine the role of Esp in intestinal adherence and colonization, an Esp-expressing strain of *E. faecium* (E1162) and its isogenic Esp-deficient mutant (E1162Δ*esp*) were studied for adherence to differentiated Caco-2 cells and colonization of murine intestines. E1162, a hospital-acquired strain, exhibited significantly higher adherence to Caco-2 cells than E135, a representative of the indigenous flora. These results are consistent with an earlier study performed by Lund et al. (23). However, no difference in adherence to Caco-2 cells between the E1162 and the E1162Δ*esp* was found, indicating that Esp is not the determining factor responsible for the observed difference in Caco-2 cell adherence between nosocomial and indigenous *E. faecium* strains. This also implies that other determinants present in hospital-acquired *E. faecium* strains contribute to adhesion to intestinal epithelial cells. Comparative genomic hybridizations of 97 *E. faecium* nosocomial, commensal and animal isolates identified more than 100 genes that were enriched in nosocomial strains, including genes encoding putative adhesins, antibiotic resistance, IS elements, phage sequences, and novel metabolic pathways (40).

In addition, similar levels of intestinal colonization or translocation were found after inoculation with E1162 wild-type or the isogenic Esp-deficient mutant E1162Δ*esp*. These data are in accordance with a study performed by Pultz et al. (27) in which they showed that Esp did not facilitate intestinal colonization or translocation of *E. faecalis* in clindamycin-treated mice. Only from the small bowel contents of mice when inoculated separately with E1162 wild-type and the Esp-mutant strain significantly more E1162Δ*esp* compared to E1162 was isolated. This was an unexpected observation and we have no explanation for the fact that the levels of E1162Δ*esp* in the small bowel are as high as in the cecum. Relatively lower levels as seen for E1162 are more typical for the small bowel.

Conclusion

Our data clearly demonstrate that Esp is not essential for high density colonization of the GI tract by nosocomial strains. Other possible candidate traits implicated in this process could include novel adhesins, like the novel cell surface proteins recently identified (41), bacteriocins, factors that resist specific or non-specific host defence mechanisms, and/or the ability to utilize new growth substrates. It is interesting in this respect that we recently identified a novel genomic island highly specific for nosocomial strains that tentatively encodes novel sugar uptake system (42).

For nosocomial *E. faecium* clones the GI tract serves as staging area from which they can disperse in and between patients, ultimately causing hospital-wide outbreaks. It is therefore of utmost importance to gain insight into the processes and determinants that promote intestinal colonization of nosocomial *E. faecium* strains. Only then we will be able to impede subsequent spread of these nosocomial clones.

Acknowledgements

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4

Enterococcal surface protein transiently aggravates *Enterococcus faecium* induced urinary tract infection in mice

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Abstract

Background: *Enterococcus faecium* has globally emerged as a multi-resistant nosocomial pathogen causing a wide spectrum of infections including urinary tract infections (UTI) and peritonitis. Knowledge about the role of virulence factors in these infections is limited. The enterococcal surface protein Esp, linked to nosocomial *E. faecium*, has been identified as a putative virulence factor involved in biofilm formation. Methods: An Esp-expressing strain of *E. faecium* (E1162) and its isogenic Esp-deficient mutant (E1162Δ*esp*) were tested for their adherence capacity to uroepithelial cells *in vitro* and *in vivo* in mouse models for UTI and peritonitis. Results: Esp expression by *E. faecium* enhanced *in vitro* binding to bladder and kidney epithelial cells. In mice, higher numbers of E1162 were cultured from kidneys and bladders one and three days after induction of UTI compared to E1162Δ*esp*. This was accompanied by a higher frequency of bacteremia, higher levels of proinflammatory cytokines in kidney tissue, and renal insufficiency. No differences in urine cultures were found. Esp expression had no impact on the course of or inflammatory response during a model of *E. faecium* peritonitis. Conclusions: These results indicate that Esp of *E. faecium* negatively influences the course of an UTI, thereby facilitating subsequent bloodstream infection.

Introduction

Enterococci have globally emerged as important multidrug resistant nosocomial pathogens. They can cause a wide spectrum of infections including device-related-infections, bacteremia, and endocarditis, but also urinary tract infections (UTI) and peritonitis (1-3). Enterococci rank third among pathogens isolated from hospitalized patients with UTI (4) and have been associated with increased mortality in peritonitis (5,6). In the past, most enterococcal infections were caused by *Enterococcus faecalis*, but infections with *Enterococcus faecium* have increased dramatically in the last two decades. This can largely be attributed to the ability of *E. faecium* to adapt to the abundant use of antibiotics in hospitals by acquiring resistance to high dose aminoglycosides, beta-lactam antibiotics and vancomycin (7,8). Molecular epidemiological studies have indicated that *E. faecium* clones responsible for the majority of nosocomial infections and hospital outbreaks are genetically distinct from indigenous intestinal isolates (8,9). Additionally to antibiotic resistance genes, these *E. faecium* clones acquired several genes and elements encoding potential virulence factors and adaptive mechanisms that are thought to enhance survival in hospitalized patients (10-15).

It is not known, which virulence factors play a role in the pathogenesis of *E. faecium* UTI and peritonitis. The enterococcal surface protein Esp, located on a putative pathogenicity island (PAI), is specifically enriched in hospital-acquired *E. faecium* and has been identified as a potential virulence factor involved in biofilm formation (8,10,14-16). A significant relationship was found between *E. faecium* urinary isolates and the presence of *esp*, suggesting a role of Esp in UTI (17). So far, nothing is known about the role of Esp in *E. faecium* peritonitis.

In this study we investigated the virulence potential of *E. faecium* expressing Esp in UTI and peritonitis, by comparing an Esp-expressing strain of *E. faecium* and its isogenic Esp-deficient mutant, we recently generated (16). We tested the adherence capacity to uroepithelial cells *in vitro* and colonization and persistence *in vivo* in a mouse model for UTI. Furthermore, we tested a possible pathogenic role of Esp in a murine peritonitis model we recently developed to investigate the normal immune response during primary *E. faecium* peritonitis (18).

Materials and Methods

Bacterial strains and growth conditions

E. faecium strains E1162 and E1162Δ*esp* were used. E1162 is a clinical blood isolate and positive for Esp expression. The isogenic Esp-deficient mutant, E1162Δ*esp*, was previously constructed by introduction of a chloramphenicol resistance cassette (*cat*) resulting in an insertion-deletion mutation of the *esp* gene (16). *E. faecium* strains were grown in either Todd-Hewitt (TH) or brain heart infusion (BHI) broth or on Tryptic Soy Agar (TSA) with 5% sheep red blood cells (Difco, Detroit, MI). Slanetz-Bartley (SB) agar plates were used to selectively grow enterococci. No difference in growth rate was observed between the E1162 and E1162Δ*esp* (16).

Cell lines, media, and culture conditions

Human bladder carcinoma T24 cells were obtained from American Type Culture Collection (ATCC, HTB-4) and cultured in Eagle's minimal essential medium (EMEM; BioWithaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (Integro B.V., Zaandam, The Netherlands) and 50 µg/ml gentamicin (Gibco, Invitrogen, Paisley, UK). Madin-Darby canine kidney (MDCK) epithelial cells were obtained from ATCC as well (CCL-34) and cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal calf serum (Integro B.V.), 1% non-essential amino acids (Gibco), 2 mM glutamine (Gibco), and 50 µg/ml gentamicin (Gibco). T24 and MDCK cells were prepared by seeding cells in 12-wells tissue culture plates (Costar, Corning, NY) at 1 x 10^5 and 0.5 x 10^4 cells/ml, respectively, in EMEM or DMEM. One ml of these suspensions was added to each well and plates were incubated at 37°C in 5% CO_2 for 3 days to a confluent monolayer.

Adhesion assay

Overnight-grown cultures of E1162 and E1162Δ*esp* in BHI broth were diluted (1:50) and grown at 37°C to an OD_{660} of 0.4, while shaking. Bacteria were harvested by centrifugation (6,500 × g; 3 min) and resuspended in EMEM or DMEM with the goal to get a concentration of 1 × 10^7 CFU/ml. The inoculum was plated to determine viable

counts. For each strain, 1 ml bacterial suspension was added to the wells. Actual CFU of E1162 and E1162Δ*esp* added to the wells were 1.8×10^7 and 1.8×10^7 in EMEM, 2.3×10^7 and 2.2×10^7 in DMEM, 2.0×10^7 and 1.9×10^7 in EMEM, 2.4×10^7 and 2.5×10^7 in DMEM, 5.7×10^6 and 5.2×10^6 in EMEM, 4.7×10^6 and 5.3×10^6 in DMEM, respectively. Plates were centrifuged (175 × g; 1 min) and incubated for 1 h at 37˚C in 5% CO_2. After incubation, non-adherent bacteria were withdrawn from the wells, cells were washed 3 times with EMEM or DMEM, and subsequently lysed with 1% Triton X-100 (Merck, Darmstadt, Germany) in PBS for approximately 5 min at room temperature. Adherent bacteria were quantified by plating serial dilutions onto TSA plates and counting resultant colonies. Also the inoculum was plated to determine initial viable counts. Adherence was calculated for both E1162 and E1162Δ*esp* as follows: $(CFU_{t=1} / CFU_{t=0}) \times 100$, where $CFU_{t=0}$ refers to the initial number of viable bacterial cells per ml and $CFU_{t=1}$ to the number of adherent viable bacterial cells per ml after 1 h. The proportion E1162Δ*esp* relative to E1162 was calculated by using the equation: (percentage $adherence_{E1162\Delta esp} \times 100$) / percentage $adherence_{E1162}$, where the percentage adherence of E1162 was transformed to 100. The assay was performed simultaneously in 3 separate wells in duplicate and repeated on 3 different days. The results of all individual experiments were combined. Controls without eukaryotic cells were used to confirm that 1% Triton X-100 did not kill the *E. faecium* strains.

Mice

Specific pathogen-free 10-wk-old female C57BL/6 mice were purchased from Harlan Sprague-Dawley (Horst, The Netherlands). The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Induction of urinary tract infection

Parent (E1162) and *esp* mutant (E1162Δ*esp) E. faecium* strains were tested in a mouse UTI model. Mice were transurethrally infected, similar to a UTI model described by Kau et al. (19); an inoculum volume of 200 µl is used to facilitate direct delivery of the bacteria to both the bladder and kidneys. Both *E. faecium* strains were grown overnight on TSA plates and then grown in TH broth to midlogarithmic phase at

37˚C, while shaking. Cells were washed and resuspended in saline. The inoculum was plated to determine viable counts. The UTI was induced under general anesthesia (0.07 mL/10g mouse of FFM mixture), containing 1.25 mg/mL midazolam (Roche, Mijdrecht, The Netherlands), 0.08 mg/mL fentanyl citrate and 2.5 mg/mL fluanisone (Janssen Pharmaceutica, Beerse, Belgium). Urinary bladders were emptied by gently pressing the abdomen, before transurethral administration of 1.2 x 10^8, 1.5 x 10^8, or 1.3 x 10^8 CFU of E1162 or 1.2 x 10^8, 1.4 x 10^8, or 1.4 x 10^8 of E1162Δ*esp*, through a 0.55 mm catheter (Abbott, Zwolle, The Netherlands). Twelve, 10 or 11 mice were used for 1-, 3- and 5-days experiments, respectively. During the 5-day experiment, 1 mice of the E1162 group died.

Induction of peritonitis

Bacteria were treated as described above, and mice were injected intraperitoneally (i.p.) with 7 × 10^7 CFU of either E1162 or E1162Δ*esp* in 200 µl saline. This bacterial dose of another clinical isolate of *E. faecium,* E155, is gradually cleared by normal C57BL/6 mice and is not associated with lethality (18). Seven to 8 mice per group were sacrificed 2 and 24 hours after infection.

Collection of samples

Mice were anesthetized by inhalation of isoflurane (Abbot, Laboratories Ltd., Kent, UK)/ 0_2 (2%/ 2 liter). In the UTI experiments mice were bled by cardiac puncture and blood was transferred to heparin-gel vacutainer tubes. The abdomen was opened and urine was collected by puncturing the bladder, then bladder and kidneys were harvested. In the peritonitis experiments a peritoneal lavage was performed first, with 5 ml PBS using an 18-gauge needle, then blood was drawn and liver and lungs were harvested.

Determination of bacterial outgrowth

In the UTI experiments the number of *E. faecium* CFU was determined in urine, blood, bladder, and kidney homogenates. In the peritonitis experiments CFU numbers were determined in peritoneal lavage fluid (PLF), blood, liver, and lung homogenates. To correct for the difference in organ weight, organs were weighed, diluted 4-fold,

and homogenized at 4°C using a tissue homogenizer (Biospec Products, Bartlesville, OK). Serial dilutions were made and 50 µl of each dilution was plated onto SB plates and incubated at 37°C in 5% CO_2. After 44 hrs colonies were counted (detection limit 20 CFU/ml homogenate/PLF/blood). For cytokine measurements in kidneys and bladders, the homogenates were diluted with an equal volume of lysis buffer [300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 1% Triton X-100, and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, EDTA, Pepstatin A, and Leupeptin, pH 7.4] and incubated for 30 min. Homogenates were centrifuged (1500 x *g*) at 4°C for 10 min. All supernatants of organ homogenates, PLF and plasma were stored at -20°C until measurement of cytokines and markers for organ damage were performed.

Cell counts and differentials of PLF

Cells in the PLF samples were counted using a Coulter Counter (Beckman coulter, Fullerton, CA). Differential cell counts for the determination of neutrophils, macrophages and lymphocytes were performed on cytospin preparations, stained with Giemsa (Diff-Quick; Dade Behring, Leusden, The Netherlands).

Histology

Bladders and kidneys were fixed in 4% formalin and embedded in paraffin. For day 1 and 3, 2 kidney-pairs and 4 bladders were used from both E1162 and E1162Δ*esp* groups. For day 5, 3 kidney-pairs and 3 bladders were used from both E1162 and E1162Δ*esp* group. Sections of 4 µm were stained with haematoxylin and eosin. Slides were coded and scored by a pathologist without knowledge of the type of treatment. Renal inflammation and damage were scored by the amount of inflammatory cell influx and examining tubular injury, characterized by necrosis, dilatation, edema and purulent cast deposition. Inflammation of bladder was scored by the amount of neutrophil influx and thickness of the epithelium.

Assays

Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, IL-12p70, interferon (IFN)-γ and monocyte chemoattractant protein (MCP)-1 were measured in kidney and bladder homogenates, PLF and plasma by using a commercially available cytometric

bead array (CBA) multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturer's recommendations. Plasma levels of creatinin and urea were determined with commercially available kits (Sigma-Aldrich, St. Louis, MO), using a Hittachi analyzer (Boehringer Mannheim, Mannheim, Germany).

Statistical analysis

Adhesion data are expressed as the proportion of adherent E1162Δ*esp* relative to adherent E1162 ± the standard deviation (SD). Differences in proportions were tested for significance by the Fisher Exact Test. *In vivo* data are expressed as means ± standard errors of the mean (SEM). Differences between groups were analyzed by Mann-Whitney *U* test using GraphPad Prism version 4.0, GraphPad Software (San Diego, CA). A *P*-value < 0.05 was considered statistically significant.

Results

***In vitro* adherence to T24 and MDCK cells**

To determine whether Esp plays a role in the ability of *E. faecium* to adhere to urinary tract epithelial cells, adherence of the Esp-expressing *E. faecium* strain E1162 and its isogenic Esp-deficient mutant (E1162Δ*esp*) to human bladder epithelial cells (T24) and Madin-Darby canine kidney (MDCK) epithelial cells was investigated. The proportion of E1162Δ*esp* that adhered to T24 cells and to the MDCK cells was significantly lower (25% and 35%, respectively) compared to E1162 ($P < 0.001$) (Figure 1).

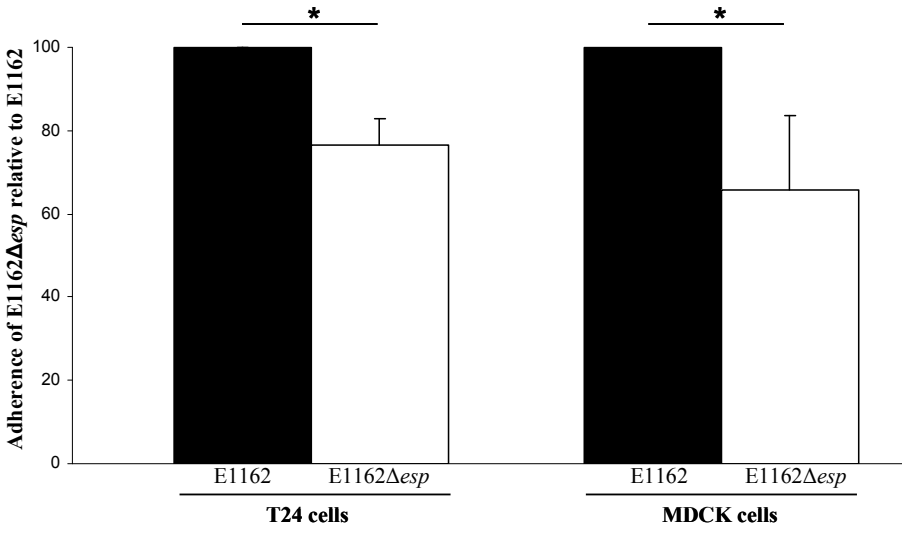

Figure 1. *In vitro* binding to bladder and kidney epithelial cells. Adhesion of E1162 (black bars) and E1162Δ*esp* (white bars) to human bladder epithelial cells (T24) and Madin-Darby canine kidney (MDCK) epithelial cells. Adhesion levels are expressed as the proportion adherent E1162Δ*esp* relative to adherent E1162 ± SD, * $P < 0.001$ versus E1162.

In vivo model of UTI

The role of Esp during UTI was examined by infecting mice with either E1162 or E1162Δ*esp* via transurethral inoculation. One and three days after the infection, significantly more E1162 CFU were cultured from kidneys and bladders than E1162Δ*esp* ($P < 0.05$) (Figure 2). A similar, though not significant, trend ($P = 0.06$ and 0.07 on day 1 and 3, respectively*)* was seen for urine cultures (Figure 2). Furthermore, significantly more mice infected with E1162 had positive blood cultures on day one (7/12) ($P < 0.05$), than mice infected with E1162Δ*esp* (1/12). On day 3 this was three out of ten for mice challenged with E1162 and one out of ten for E1162Δ*esp* challenged mice. Over time, the number of CFU of both strains in kidneys, bladders and urine declined. Five days after start of the infection, kidneys and bladders were still positive for both *E. faecium* strains, but with no differences between wild-type and Esp-deficient mutant in the amount of bacteria cultured, while all urine cultures became sterile.

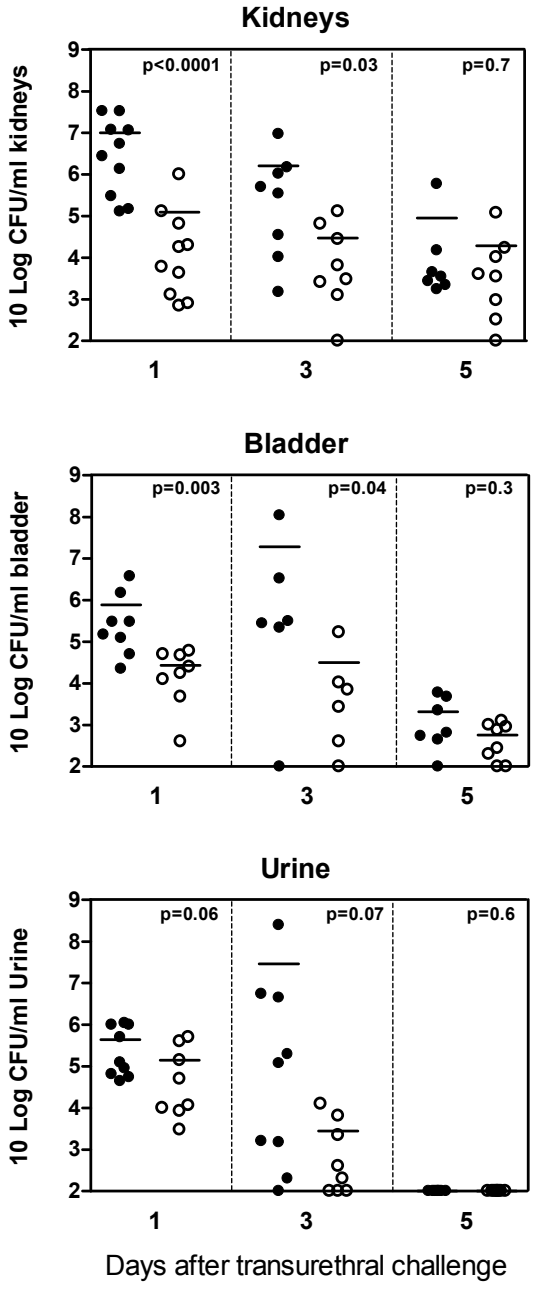

Figure 2. Outgrowth of Esp-expressing E1162 and the isogenic Esp-deficient mutant in kidney and bladder. Mice were transurethrally infected with approximately 10^8 CFU of E1162 (closed circles) or E1162Δ*esp* (open circles). Mice were sacrificed 1, 3 and 5 days after the infection. Bacterial outgrowth is shown for kidney and bladder homogenates, and urine. Horizontal bars represent means.

Cytokine production during UTI

In mice infected with E1162 significantly higher levels of TNF-α, IL-6 and MCP-1 levels were measured in kidney homogenates one day after the start of the infection compared to mice infected with E1162Δ*esp* ($P < 0.05$). This difference was still significant for IL-6 at day 3 ($P < 0.05$) (Figure 3). IL-10 levels in kidneys were comparable in mice challenged with either strain and increased during the experiment, while TNF-α, IL-6, and MCP-1 levels decreased during the course of the infection. Kidney IFN-γ and IL-12p70 and plasma and bladder TNF-α, IL-6, MCP-1, IL-10, IFN-γ, and IL-12p70 were all were all below detection limit (data not shown).

The increased cytokine levels on day 1 were accompanied by higher levels of plasma creatinin and urea in mice infected with E1162 compared to mice infected with E1162Δ*esp* (Figure 4), indicative of a more disturbed kidney function. Histological examination of kidney tissue revealed no differences between kidneys infected with either E1162 or E1162Δ*esp* (data not shown). The pathological changes at all time points in the kidney were variable, ranging from quite extensive in some kidneys, with edema and inflammatory cells lining the entire pelvis, to small, localized spots of inflammation in other kidneys. The cellular infiltrate in the kidneys was primarily neutrophilic, determined by histological features. Tissue of the bladder displayed near to normal histology with minor neutrophil influx at all time points (data not shown), which fits the observation of undetectable cytokine levels in this organ.

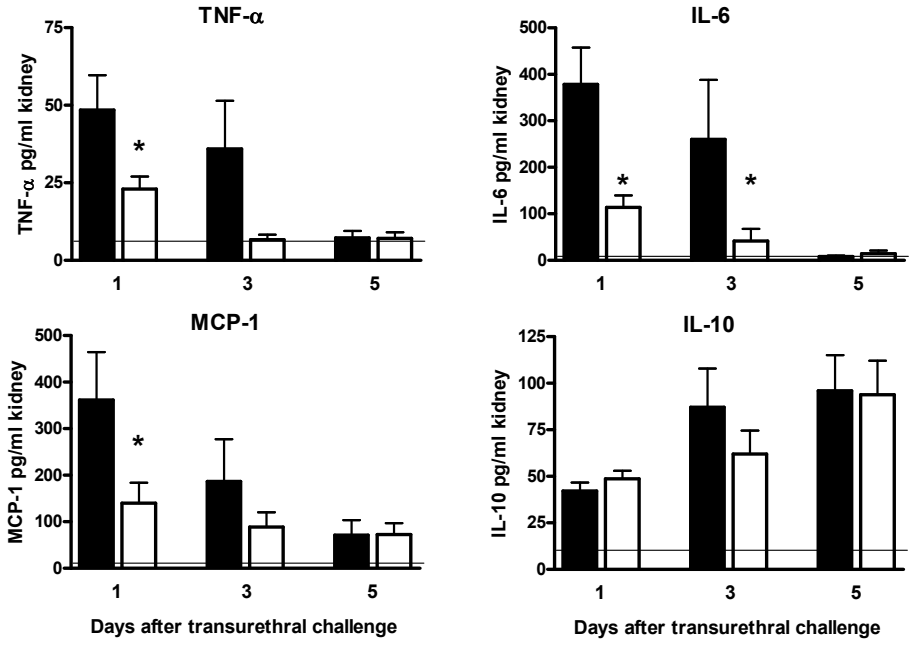

Figure 3. Cytokine levels in kidneys infected with Esp-expressing E1162 and the isogenic Esp-deficient mutant. Mice were transurethrally infected with approximately 10^8 CFU of E1162 (closed bars) or E1162Δ*esp* (open bars). Mice were sacrificed 1, 3 and 5 days after the infection. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1, and IL-10 are shown. Data are means ± SEM, * $P < 0.05$ versus E1162.

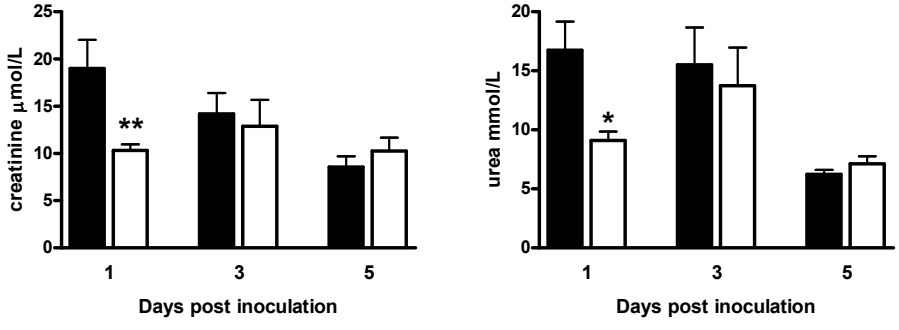

Figure 4. Plasma creatinin and urea levels during UTI. Mice were transurethrally infected with approximately 10^8 CFU of E1162 (closed bars) or E1162Δ*esp* (open bars). Mice were sacrificed 1, 3 and 5 days after the infection. Creatinin and urea levels were measured in plasma. Data are mean ± SEM, * $P < 0.05$ versus E1162.

In vivo model of non-lethal peritonitis

To study additional roles for Esp in the pathogenesis of *E. faecium* infections, mice were intraperitoneally infected with either E1162 or E1162Δ*esp*. No differences were found in *E. faecium* outgrowth in PLF, blood, liver or lung (data not shown) between wild-type E1162 and the Esp-deficient mutant (Figure 5). Furthermore, a rapid reduction was seen in CFU counts of both strains and none of the mice died. On microscopic examination of PLF preparations comparable numbers of peritoneal neutrophils and macrophages were counted in both groups at both time points (Figure 5). The strains were found phagocytosed by peritoneal macrophages and neutrophils to a similar extent (data not shown). Furthermore, no differences were observed in peritoneal and plasma cytokine levels two hours post-infection (Table I). Twenty-four hours after the infection, cytokine levels were below the level of detection.

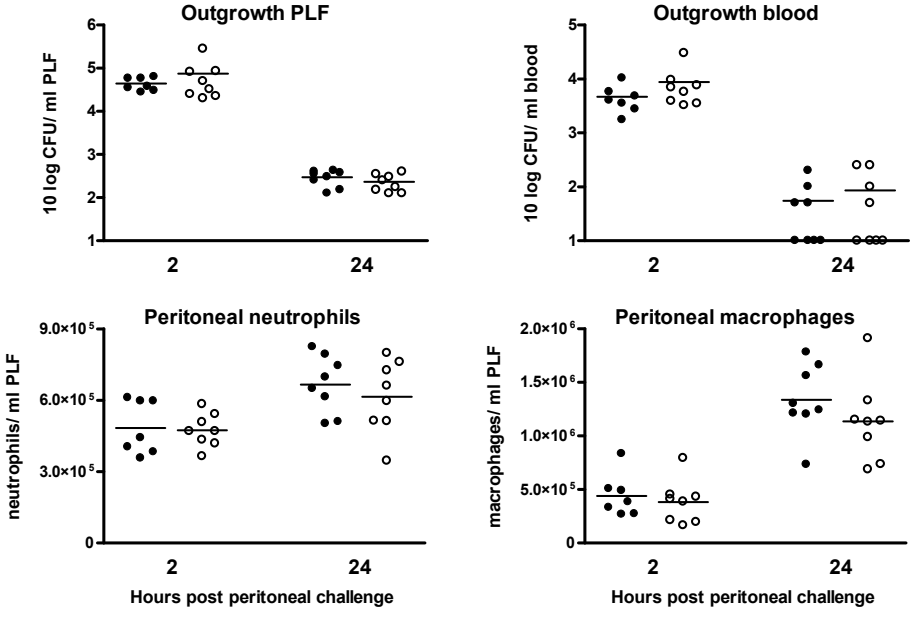

Figure 5. *E. faecium* peritonitis. Mice were intraperitoneally infected with 1×10^7 CFU of E1162 (closed circles) or E1162Δ*esp* (open circles) and sacrificed 2 or 24 hours post-inoculation. Bacterial outgrowth in peritoneal lavage fluid (PLF) and blood, and peritoneal neutrophil and macrophage influx are shown. Horizontal bars represent means.

Table I. Concentrations of cytokines in PLF and plasma in mice with *E. faecium* peritonitis

	PLF concentrations		Plasma concentrations	
Cytokines (pg/ml)	E1162	E1162Δ*esp*	E1162	E1162Δesp
TNF-α	132 ± 28	171 ± 49	79 ± 10	93 ± 20
IL-6	2733 ± 602	2863 ± 678	1347 ± 319	1586 ± 543
IL-10	223 ± 50	305 ± 60	90 ± 27	83 ± 20
MCP-1	1245 ± 375	1159 ± 209	401 ± 43	311 ± 56

Mice were infected intraperitoneally with 7×10^7 CFU of E1162 or E1162Δ*esp* and sacrificed 2 hours post-infection. Data are means ± SEM.

Discussion

While infections with multi-resistant *E. faecium* are emerging, our knowledge on virulence factors expressed by *E. faecium* is very limited. Recently, some potential virulence genes have been described for *E. faecium*: *esp*, *hyl*, *acm,* genes encoding additional surface-exposed LPXTG proteins and genes required for the biogenesis of pili. They were all found more frequently in clinical isolates than in fecal isolates or non-human isolates, yet the exact role of the proteins expressed by these genes in the pathogenesis of *E. faecium* infections is not known (8,12-15,20-26). In this study we showed for the first time an important role for Esp in the pathogenesis of *E. faecium* infection. In an experimental model of UTI in mice the *E. faecium* isogenic mutant deficient in Esp expression was clearly attenuated. Higher bacterial outgrowth of wild-type *E. faecium* relative to the Esp-deficient mutant in bladder and kidney facilitated subsequent bloodstream infection and was accompanied by higher levels of proinflammatory cytokines in kidney tissue and more pronounced renal insufficiency displayed by increased plasma levels of creatinin and urea. These results indicate that Esp expressed by *E. faecium* aggravates the course of an UTI, thereby facilitating subsequent bloodstream infection. Of note, the presence of Esp only influenced the initial course of UTI, indicating that in this model the effect of Esp transient is. The finding that the Esp-deficient strain is still able to colonize kidneys and bladders, albeit to a lesser extent than wild-type *E. faecium*, and could still be

isolated 5 days after challenge, implies important roles for additional factors as well. In addition, we demonstrated that Esp is involved in *in vitro* binding of bladder and kidney epithelial cells. Together, these data strongly suggest that *E. faecium* Esp is an important virulence factor in experimental UTI.

Esp is a cell wall-associated protein that is characterized by multiple repeat motifs, structurally similar to many bacterial surface protein adhesins involved in binding to host ligands (20,27-31). *Esp* is found in both *E. faecium* and *E. faecalis*, and located on a PAI (8-10,14,20). Shankar et al. (32) demonstrated in a mouse model that Esp contributes to colonization and persistence of *E. faecalis* in the urinary tract. In this study, Esp expression in an *E. faecalis* ascending UTI resulted in more outgrowth in bladder and urine, compared to the isogenic Esp-deficient mutant strain. In contrast to our study, they did not find significantly higher outgrowth in the kidneys, although a trend was observed. This difference in outcome may be explained by the fact that the UTI model reported by Shankar et al. differed from ours with respect to the mouse strain used and volume of bacterial inoculation. A volume of 50 µl, as used by Shankar, will only reach the bladder and from there, bacteria might cause an ascending UTI. In our own unpublished experiments, inoculation of 50 µl of *E. faecium* strain E1162 into the bladder resulted in inconsistent and only low graded kidney infection. Similar observations were described previously for *E. faecalis* by Kau et al. (19) and Singh et al. (33). We therefore used the model described by Kau et al. (19) and Singh et al. (33) in which 200 µl was introduced transurethrally in order to reach both the bladder and the kidneys. In hospitalized patients, one can imagine a continuous bacterial challenge from a biofilm formed on a catheter placed in situ for several days. However, such a model is difficult to simulate in mice.

Interestingly, Shankar et al. (32) did not find differences between the binding of Esp-expressing *E. faecalis* strains and the isogenic Esp-deficient mutant strains to the porcine renal tubular cell line LLC-PK1. Our observation that, in contrast to the findings in *E. faecalis*, the *E. faecium* Esp-deficient mutant was significantly impaired in cell line binding might be explained by the fact that we used different cell lines or by a different role of Esp in the two *Enterococcus* species. Although Esp of *E. faecalis* and *E. faecium* share 89% sequence similarity (20,34), there are some striking differences between *E. faecalis* and *E. faecium* Esp with respect to structure and

function. First, a number of sequence differences exist within the N- and C- termini and in the arrangement of the repeat region (20). Second, in *E. faecium*, Esp is clearly involved in biofilm formation while the role of Esp in *E. faecalis* biofilm formation is still a matter of debate (16,35-39). Third, there are important differences in the distribution of Esp positive strains: while Esp-positive *E. faecium* strains are confined to hospitalized patients, Esp-positive *E. faecalis* strains are more widely distributed and are also found in the animal reservoir (14,15,20,40).

As opportunistic pathogens, *Enterococcus* species are frequently isolated from urine and may cause chronic UTI as they form biofilms on inanimate surfaces, like catheters (4,41,42). Currently, they rank third among the most common pathogens isolated from ICU patients (4). The observation that *E. faecium* fails to elicit a strong induction of inflammatory cytokines in the bladder and causes only minor pathology is consistent with findings by Kau et al. (19) for *E. faecalis*-induced UTI. Furthermore, clinical data published by Wong et al. (42) indicate that the majority of patients with *E. faecium* UTI have an asymptomatic bacteriuria.

Knowledge on how the innate immune system deals with an *E. faecium* infection is limited. In previous studies we described the normal immune response during primary *E. faecium* peritonitis (18). In a non-lethal murine model of *E. faecium* peritonitis we found a fast and brisk peritoneal neutrophil influx and a consecutive rapid decline in peritoneal and systemic enterococcal load (18). This was accompanied by a modest peritoneal and systemic cytokine response. To further investigate the pathogenesis of *E. faecium* infections we tested the role of Esp in this peritonitis model. No differences were found in the course of the infection between wild-type E1162 and the Esp-deficient mutant, as shown by comparable clearance of the two isogenic species and similarly induced inflammatory responses. In *E. faecalis*, Dupont et al. (43) found that 46% (30/65) of *E. faecalis* strains isolated from peritoneal fluid of patients with peritonitis contained the *esp* gene. Furthermore, the presence of *esp* in combination with cytolysin, gelatinase, and aggregation substance was independently associated with mortality in patients with severe peritonitis (43).

Differences in levels of attenuation of the Esp-deficient mutant *E. faecium* in a model of UTI and peritonitis suggests a niche specific role of Esp in the pathogenesis of *E. faecium* infections. With the increase in multiresistance of *E. faecium*, development

of antibodies directed against Esp might be a valuable addition in the treatment of UTI caused by *E. faecium*.

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5

Contribution of the enterococcal surface protein Esp to pathogenesis of *Enterococcus faecium* endocarditis

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Abstract

Enterococcus faecium has globally emerged as a multi-antibiotic resistant nosocomial pathogen causing difficult-to-treat diseases, including endocarditis. The enterococcal surface protein Esp, specifically linked to nosocomial *E. faecium*, is involved in biofilm formation and plays a role in the pathogenesis of urinary tract infection in mice. To assess the role of *E. faecium* Esp in endocarditis, a assumed to be biofilm-associated infection, we compared an Esp-expressing *E. faecium* strain (E1162) and its Esp-deficient mutant (E1162Δ*esp*) in an experimental infective endocarditis model in rats. In addition, we investigated the presence of antibodies against Esp in *E. faecium* bacteremia and endocarditis patient sera. Heart valve vegetations were produced in rats by inoculating E1162, E1162Δ*esp*, or a mixture of E1162 and E1162Δ*esp* through a catheter into the left ventricle. Numbers of bacteria recovered from vegetations at autopsy 24 hours after inoculation were lower for E1162Δ*esp* than for E1162 (P = 0.0117 and P = 0.0503). In an other experiment, three hours after infection, however, only small numbers of CFU could be recovered from heart valves in half of the rats, with no significant difference between wild-type and Esp-deficient mutant. After inoculating a mixture of E1162 and E1162Δ*esp* similar amounts of both bacteria were recovered from vegetations 72 hours after infection, suggesting that Esp-deficient mutant cells are incorporated in heart valve vegetations of wild-type cells. In patients, antibodies against Esp were detected in four of five after bacteremia and in two of four after endocarditis caused by Esp-positive *E. faecium*. In conclusion, the results from the mono infection experiments indicate that Esp contributes to colonization and persistence of *E. faecium* at the heart valves in experimental endocarditis. Furthermore, systemic infection elicits an Esp-specific antibody response in humans. Esp of *E. faecium* is expressed *in vivo*, is immunogenic, and contributes to the pathogenesis of *E. faecium* endocarditis.

Introduction

Enterococci have globally emerged as important multi-antibiotic resistant nosocomial pathogens (26). They can cause a wide spectrum of diseases, including bacteremia, endocarditis, peritonitis and surgical wound, urinary tract, and device-related infections (4,10,11,21,23,25,26,28,36,41,42). Although *Enterococcus faecalis* infections are more common, infections with *Enterococcus faecium*, which are more difficult to treat due to the accumulation of multiple antibiotic resistance traits, have increased in the last two decades. Molecular epidemiological studies have indicated that *E. faecium* clones that are responsible for the majority of nosocomial infections and hospital outbreaks are genetically distinct from indigenous intestinal isolates (19,49). In addition to several antibiotic resistance genes, these hospital-acquired *E. faecium* clones have acquired potential virulence factors that may enhance its pathogenicity in hospitalized patients. Potential virulence genes specifically found in this subpopulation are *esp* (enterococcal surface protein) (12,18,47,48), *sgrA* (serine-glutamate repeat containing protein A), and *ecbA* (*E. faecium* collagen binding protein A) encoding surface-exposed proteins (14,15), hyl_{efm} encoding a potential hyaluronidase (35), and genes required for the biogenesis of pili (13,15). Other genetic determinants, not specifically linked to this subpopulation, but also implicated in virulence of *E. faecium* are *acm* (adhesin of collagen of *E. faecium*) (29-31,33) and *scm* (second collagen adhesin of *E. faecium*) (39), both encoding surface-exposed proteins, and the secreted antigen A (*sagA*) (45). The exact role of these putative virulence factors is unknown, but some appear to be involved in adherence to extracellular matrix molecules, human cells, and biofilm formation. Adherence to a(biotic) surfaces and subsequent biofilm formation are key processes for colonization and initiation of infection (6-8). Enterococci have been associated with biofilms in a variety of infections, including urinary tract infections, root canal infections, ocular infections, device-related infections, and endocarditis (4,5,8,21,24,36,50).

Endocarditis, an infection of the heart valves or inner lining, is a assumed to be biofilm-associated infection that is difficult to treat, especially when caused by multi-resistant *E. faecium*, and as a result associated with high morbidity and mortality (2,26,28). Bacteria may adhere to the heart valves at sites of injury and subsequently

form biofilms leading to heart valve vegetations and valvular destruction (8). Little is known about the pathogenesis of *E. faecium* endocarditis. Recent data indicate that the collagen adhesin Acm (30,31,33) is involved in the primary adherence of *E. faecium* to the collagen-rich heart valves (29). In *E. faecalis*, the *ebp* operon, encoding endocarditis and biofilm associated pili, is involved in biofilm formation and contributes to the pathogenesis of endocarditis (32), as is the enterococcal surface protein (Esp) (43,44,46) (37,38). Endocarditis isolates of *E. faecalis* more frequently expressed Esp than fecal isolates (38), and the presence of the *esp* gene in endocarditis isolates was associated with higher amounts of biofilm (24). In *E. faecium*, Esp is specifically enriched in hospital-acquired *E. faecium* isolates (9,18,48). By constructing an Esp-deficient mutant, we demonstrated that, in *E. faecium,* Esp is involved in initial adherence and biofilm formation (12) and contributes to the pathogenesis of urinary tract infection (20) and bacteremia in mice (chapter 6 of this thesis). To assess the role of *E. faecium* Esp in infective endocarditis, we compared the Esp-deficient mutant and its Esp-expressing parent strain for their ability to colonize the heart valves in an experimental endocarditis model in rats. In addition, we investigated whether Esp is expressed *in vivo* and immunogenic by determining the presence of antibodies against Esp in sera of patients with *E. faecium* bacteremia and endocarditis.

Materials and Methods

Bacterial strains and growth conditions

E. faecium strain E1162 is a clinical blood isolate and positive for Esp expression (12). The isogenic Esp-deficient mutant, E1162Δ*esp*, was previously constructed by introduction of a chloramphenicol resistance cassette (*cat*) resulting in allelic replacement in the *esp* gene (12). *E. faecium* strains were grown in brain heart infusion (BHI) broth or on BHI Agar (Difco Laboratories). No difference in growth rates was observed between E1162 and E1162Δ*esp* (12).

Rats

Male Sprague-Dawley rats (~250 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). The Animal Welfare Committee of the University of Texas Health Science Center at Houston approved all experiments.

Induction of endocarditis

Plate-grown bacteria were inoculated into BHI broth and grown at 37˚C for 24 hrs, while shaking. Cells were washed and resuspended in saline and OD_{660} adjusted (see below) prior to inoculation. Surgical procedures were carried out under general anesthesia. Vegetations were produced in rats by inserting a polyethylene catheter (Intramedic PE 10; Braintree Scientific Inc.) via the right carotid artery, across the aortic valve into the left ventricle. After twenty minutes, 2 different inocula (OD_{660} adjusted with the aim to infect with 5×10^6 and 1×10^7 CFU) of both E1162 and E1162Δ*esp*, or a mixture of E1162 and E1162Δ*esp* (OD_{660} adjusted with the aim to infect with 2×10^7 CFU in total in which the wild-type would be ≤ 50% of the inoculum) were injected intraventricularly through the catheter. Subsequently, the catheter was heat sealed, imbedded in subcutaneous tissue, and the skin was closed. After the injection the inoculum was plated to determine the actual number of CFU used for infection. Rats were sacrificed 3 or 24 hrs after inoculation for mono infection and 72 hrs after inoculation for mixed infection. Aortic vegetations were excised with aseptic technique, weighed, and homogenized in 1 ml saline. Serial dilutions were made and 50 µl of each dilution was plated onto BHI plates and incubated at 37°C for 24 hrs. For mono infection, colonies were counted and corrected for the dilution factor to calculate CFU per gram of vegetation. For mixed infection, randomly picked colonies (188 per rat) from each vegetation were replica plated onto BHI agar with or without 10 µg/ml chloramphenicol, along with E1162 and E1162Δ*esp* controls. In addition, these colonies were also tested by colony hybridization using *acm*, *esp* and *cat* probes to confirm the strain identity. Proportions of E1162 and E1162Δ*esp* were calculated and data were expressed as percentages CFU per vegetation.

PFGE to confirm strain identity

For both mono infection and mixed infection, a total of 15 colonies obtained from vegetations were randomly picked and pulsed-field gel electrophoresis (PFGE) was performed to confirm strain identity. PFGE analysis was performed as described previously (27) with agarose plugs containing genomic DNA, which was digested with SmaI and separated by electrophoresis using a clamped homogeneous electric field (CHEF-DRII, Bio-Rad Laboratories), with ramped pulse times beginning with 2 s and ending with 50 s, at 200 V for 23 hrs.

Bacteremia and endocarditis patient sera

Serum was obtained from four patients with documented infective endocarditis and from five patients with bacteremia caused by *esp*-positive *E. faecium* strains. In addition, serum was obtained from one patient with endocarditis caused by an *esp*-negative *E. faecium* isolate and from healthy adult volunteers. Sera from healthy volunteers were pooled before testing. Whole blood was allowed to clot at room temperature and centrifuged at 4°C, and sera were stored in aliquots at - 70°C. The sera were investigated for the presence of IgM and IgG antibodies against Esp.

Cloning N-terminal Esp

A 2545-bp-long DNA fragment of the N-terminal domain of *esp* was amplified from genomic DNA of TX2465 by using a Platinum® Pfx DNA polymerase (Invitrogen) with the primers EspExpF2 (5'- CGCAGATCTCAGGTCGATCCAAAGAAAGGAATTG) and EspExpR1 (5'-CGGGGTACCTTAAGTTACTGCTAAATCGGTCGTGC) (introduced restriction sites are underlined). The PCR product, digested with BglII and kpnI, was cloned into the pQE30 expression vector (Invitrogen), digested with BamHI and KpnI, followed by electroporation into *E. coli* DH5α cells (Invitrogen). Electroporation was carried out using a Bio-Rad gene pulser as described previously (22). Plasmid DNA was purified by using the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions and electroporated into *E. coli* host M15(pREP4) cells (QIAGEN), and one of the resulting colonies was verified by sequencing. Recombinant N-terminal Esp (rN-Esp) was overexpressed and purified as described elsewhere (33).

Western blotting

For western blotting 100 ng rN-Esp in electrophoresis sample buffer (100 mM Tris-HCl, 5% dithiothreitol, 2% SDS, 0.004% bromophenol blue, and 20% glycerol) was boiled for 5 min and electrophoresed through a 10% SDS-polyacrylamide gel and transferred to nitrocellulose using a Bio-Rad Trans-Blot Cell tank transfer unit at 12 V overnight in 20 mM Tris, 0.15 M glycine, and 20% methanol at pH 8.3. Non-specific sites in the blot were blocked by incubation for 1 h at room temperature with 5% skim milk powder in PBS with 0.05% Tween 20. Binding of antibodies to rN-Esp was assayed by overnight incubation at 4°C with bacteremia or endocarditis patient sera (1:500 dilution) as primary antibody, followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-human IgG or goat anti-human IgM (both 1:5000 dilution; Santa Cruz Biotechnology) as secondary antibody. Both primary and secondary antibodies were diluted in PBS with 1% skim milk powder and 0.05% Tween 20. rN-Esp was detected by using light-emitting ECL™ Western Blotting Detection Reagents (GE Healthcare).

Statistical analysis

For mono infection, the geometric means of CFU of E1162 and E1162Δ*esp* per ml inoculum or per gram vegetation were calculated. For mixed infection, the median of the percentages of CFU of E1162 and E1162Δ*esp* per inoculum or vegetation was calculated. Differences between groups were analyzed by Mann-Whitney *U* test using GraphPad Prism version 4.0, GraphPad Software (San Diego, CA). A *P*-value < 0.05 was considered statistically significant.

Results

Experimental endocarditis

To investigate the role of Esp in experimental infective endocarditis, the Esp-expressing strain (E1162) or its isogenic Esp-deficient mutant (E1162Δ*esp*) were injected intraventricularly through a catheter in 16 and 11 rats, respectively. Vegetations recovered at autopsy 24 hours after inoculation (0.99×10^7 to $1.87 \times$

10^7 CFU of E1162 and 1.08 × 10^7 to 1.59 × 10^7 CFU of E1162Δ*esp*) contained less E1162Δ*esp* (8 × 10^4 CFU/g) than E1162 (9 × 10^6 CFU/g) ($P < 0.05$) (Figure 1A). This was confirmed in a second experiment with inoculations of 2.35 x 10^6 to 5.6 x 10^6 CFU of E1162 in 20 rats and 3 x 10^6 to 9.5 x 10^6 CFU of E1162Δ*esp* in 20 rats, in which vegetations again contained less E1162Δ*esp* (7 × 10^3 CFU/g) than E1162 (2 × 10^5 CFU/g) ($P = 0.0503$) (Figure 1B), demonstrating that Esp contributes to infective endocarditis in rats. To test whether Esp also adds to initial adherence of *E. faecium* to heart valves, an additional 12 rats were inoculated with 2.4 × 10^7 CFU of E1162 (6 rats) or 4.2 × 10^7 CFU of E1162Δ*esp* and CFU counts of viable bacteria were determined three hours after infection. Vegetations from six rats, three inoculated with E1162 and three inoculated with E1162Δ*esp*, contained comparable amounts of bacteria (5 × 10^2, 1.56 × 10^3, or 2.67 × 10^4 CFU/g of E1162 and 8.88 × 10^2, 1.5 × 10^3, or 1.77 × 10^3 CFU/g of E1162Δ*esp*). From six rats, three inoculated with E1162 and three with E1162Δ*esp*, no bacteria were recovered from vegetations. PFGE analysis confirmed correct strain identity of the E1162 and E1162Δ*esp*.

When rats (n = 10) were injected with approximately equal mixture of E1162 (2.7 × 10^7 CFU; 51.90% of the inoculum) and E1162Δ*esp* (2.5 × 10^7 CFU; 48.10% of the inoculum), no significant difference in the proportional distribution of wild-type and Esp-deficient mutants was observed (Figure 1C). In CFU counts recovered from vegetations 72 hours after mixed infection, the wild-type/mutant ratio was 54%/46%, which was similar to the ratio of the inoculum. This at least indicates no difference in *in vivo* growth rate between wild-type and Esp-deficient mutant.

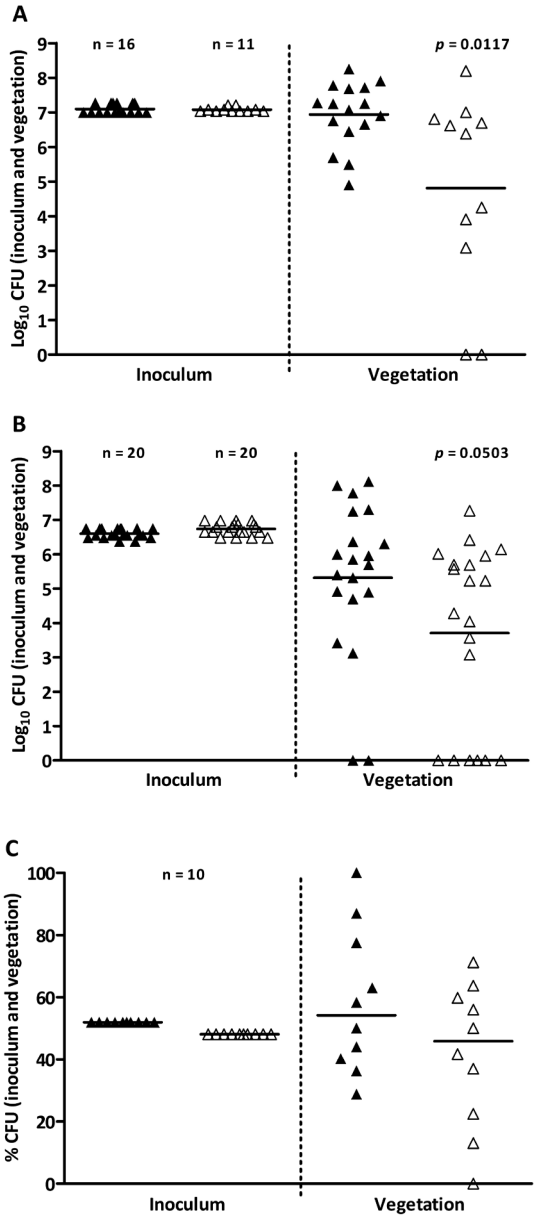

Figure 1. Recovering of E1162 wild-type and its Esp-deficient mutant from heart valve vegetations in experimental endocarditis in rats. Panel A and B depict the number of wild-type E1162 (closed triangles) and Esp-deficient mutant E1162Δ*esp* (open triangles) recovered from inocula (CFU/ml) and from vegetations (CFU/gram) 24 hours after infection with approximately 1 × 10^7 and 5 × 10^6 CFU, subsequently. Horizontal lines represent geometric means. Panel C depicts percentages CFU of E1162 (closed triangles) and E1162Δ*esp* (open triangles) in inocula and vegetations 72 hours after infection with a mixture of E1162 and E1162Δ*esp*. Horizontal lines represent medians.

Determination of antibodies against Esp in human patient sera

To investigate whether Esp is expressed *in vivo* and is immunogenic, the presence of antibodies against Esp was assessed in endocarditis and bacteremia sera from patients infected with *esp*-positive *E. faecium* isolates by Western blotting. Of five endocarditis sera, strong reactivity (lane 4) was observed in one and weak reactivity in another one (lane 3) (Figure 2A). There was no reactivity in the endocarditis serum from a patient infected with *esp*-negative *E. faecium* isolate (lane 1). Of five bacteremia sera, strong reactivity (lane 7 and 9) and moderate reactivity (lane 6 and 8) were observed in two sera, each (Figure 2B). No reactivity could be demonstrated in pooled sera from healthy human volunteers (lane 11).

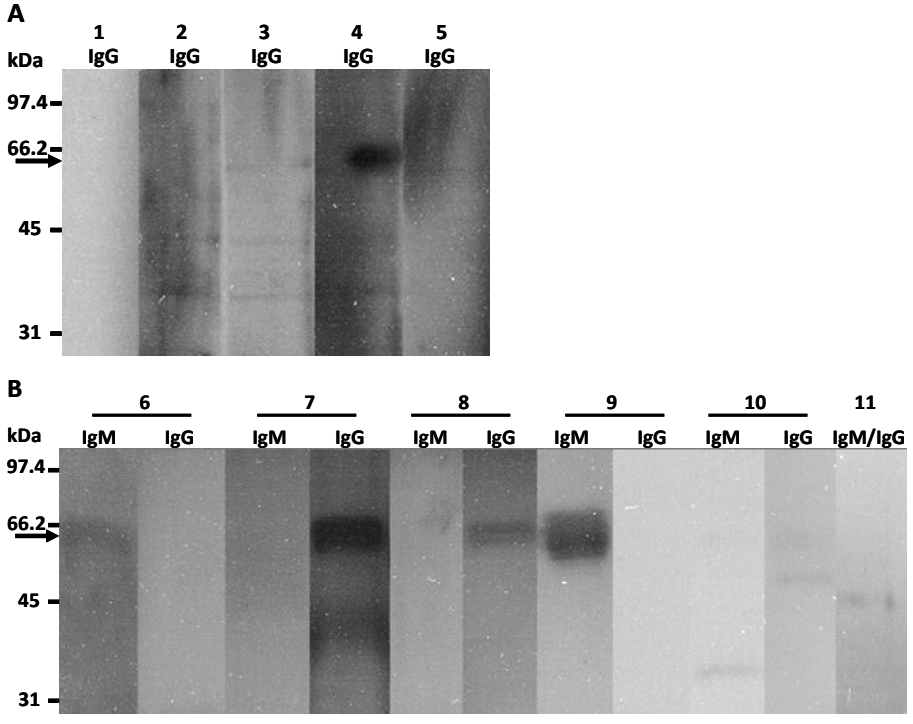

Figure 2. Western blots of recombinant Esp protein (molecular mass of 65 kDa) probed with five endocarditis (lane 1-5) (A) and five bacteremia patient sera (lane 6-10) (B). Lanes 1 and 11 represent the negative controls, endocarditis sera from a patient infected with an *esp*-negative *E. faecium* isolate and pooled sera of healthy human volunteers, respectively. Numbers depicted on the left indicate molecular masses in kDa; arrow indicates the predicted position of the recombinant Esp protein; 100 ng of Esp was used for each lane.

Discussion

Infections caused by multi-resistant *E. faecium* have increased since the late 1980s. In most *E. faecium* infections, like urinary tract infections, root canal infections, ocular infections, device-related infections, and endocarditis, adherence to a(biotic) surfaces and subsequently biofilm formation is the first step in the pathogenesis. Previous studies have demonstrated that Esp, specifically associated with hospital-acquired multi-resistant *E. faecium* (18,48), is involved in initial adherence and biofilm formation to abiotic material (12). To investigate the contribution of Esp in the pathogenesis of endocarditis, a thought to be biofilm-associated infection, we studied an Esp-deficient mutant and its Esp-expressing parent strain in an experimental endocarditis model in rats. When rats were challenged with either the Esp-deficient mutant or the wild-type strain, vegetations recovered after 24 hours contained significantly fewer Esp-deficient mutants than wild-types indicating that Esp is important in this endovascular infection. Although the Esp-deficient mutant was significantly attenuated, in 23 of 31 rats, Esp-deficients mutants were nonetheless recovered from vegetations demonstrating that the Esp-deficient mutant strain was able to colonize the heart valves of these rats. This indicates that, besides Esp, other determinants are involved in adherence to and biofilm development on heart valves. For instance, Acm also contributes to the pathogenesis of experimental endocarditis caused by *E. faecium*. The Acm-deficient mutant (TX0082Δ*acm*) (30) was highly attenuated compared to the wild-type strain (TX0082, an endocarditis isolate) (30) in both established vegetations (72 hours) and colonization of the heart valves, one and three hours after infection (29). Other possible factors that could be implicated in this process are a set of potential adhesins described recently including novel cell surface proteins or pili (13,15,39,40). It is interesting in this respect that PilA of *E. faecium* (13) exhibits high similarity with the EbpC pilin protein of *E. faecalis*, which is involved in biofilm formation and endocarditis (32).

Subsequent analysis of vegetations obtained at three hours post-inoculation with Esp-deficient mutants or wild-types demonstrated low level colonization or no (detectable) colonization of the heart valves, indicating that strain E1162, independent of the presence of Esp, is only low potential capable to colonize the heart

valves within three hours after infection. This is in contrast to the above mentioned *E. faecium* strain TX0082, which was used in the same experimental endocarditis model. Presumably, genotypic and phenotypic differences between the *E. faecium* E1162 and TX0082, like differences in growth rate or differences in expression of determinants involved in initial adherence (e.g. Acm or Esp), account for the observed difference in colonization during early stages of infection. When rats were challenged with a mixture of E1162 wild-type and its *esp* mutant, the proportion of wild-types recovered from vegetations 72 hours after inoculation was approximately the same as for the Esp-deficient mutant. One could speculate that, during the mixed infection, the Esp-deficient mutant coaggregates with the Esp-expressing wild-type strain in the growing biofilm on the heart-valve, resulting in similar numbers of wild-type and Esp-deficient mutant. Growing and developing biofilms are microbial communities that rely on bacterial cell-cell interactions and can involve coggregation of cells even from different species (16,17). This has especially been studied in biofilms in the oral cavity, where biofilms are composed of numerous different bacteria (1). For example, in a multispecies oral biofilm, a *S. oralis* strain bound via a lectin-like adhesin to a *S. gordonii* strain and coaggregated also with a *Veillonella* sp strain (3). In addition, it has been demonstrated that actinomyces bacteria bind via their pili to streptococci resulting in co-infection of the oral mucosa (34). Considering these data, coaggregation of the Esp-deficient mutant with the Esp-expressing wild-type strain might mask a contribution of Esp during mixed infection.

In order to assess whether Esp is expressed in humans during a natural infection, we screened four endocarditis and five bacteremia patient sera for a serological response against Esp by Western blotting. In six of nine patient sera, we were able to detect anti-Esp antibodies indicating that Esp is expressed and recognized by the immune system yielding an anti-Esp response, at least in some patients. Anti-Esp antibodies could not be detected in sera from a patient with endocarditis caused by an *esp*-negative *E. faecium* strain nor in pooled sera from healthy human volunteers. These results indicate that reactivity is specific and probably not caused by cross-reacting antibodies.

In conclusion, the identification of Esp as an immunogenic surface protein and its association with biofilm formation and endocarditis confirms the importance of Esp in the pathogenesis of infections caused by hospital-acquired *E. faecium* strains. Further elucidating the role of Esp has improved our understanding how this emerging nosocomial pathogen initiates infections and interacts with its environment. Improved understanding may contribute to the development of novel intervention strategies, possibly targeting Esp, to prevent infections with and the spread of multi-resistant *E. faecium* in hospitals.

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6

Enterococcal surface protein is a virulence factor in bacteremia but is not a target of opsonic antibodies in *Enterococcus faecium* infection

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Abstract

Background: Enterococci have emerged as important nosocomial pathogens with multiple intrinsic and acquired resistances against many antibiotics. In the past, the majority of infections were caused by *Enterococcus faecalis*, however an increase in *Enterococcus faecium* clinical isolates has been observed in recent years. The enterococcal surface protein (Esp) is specifically linked to *E. faecium* clinical isolates, expressed at the surface of the bacterium, and involved in biofilm formation. In this study, an Esp-expressing *E. faecium* strain (E1162) and its previously created Esp-deficient mutant (E1162Δ*esp*) were compared in a mouse bacteremia model. Anti-Esp serum was tested for its capacity to mediate opsonophagocytic killing of E1162 *in vitro* and to protect against *E. faecium* bacteremia. Results: *esp* inactivation attenuated *E. faecium* virulence with reduced numbers of bacteria recovered from the kidneys in animals infected with the mutant compared to the wild-type strain ($P = 0.03$). Passive transfer of rabbit antibodies against the recombinant Esp protein did not protect mice against *E. faecium* bacteremia ($P > 0.05$). In contrast, mice passively immunized with antibodies specific to lipoteichoic acid (LTA) had lower numbers of *E. faecium* E1162 in the blood compared to mice immunized with normal rabbit serum. Conclusions: These results suggest that Esp contributes to the virulence of *E. faecium* in sepsis. However, in contrast to LTA, Esp does not seem to be a target for protective antibodies in *E. faecium* strain E1162 in mouse bacteremia.

Introduction

Enterococcus faecalis and *Enterococcus faecium* are currently the third to fourth most commonly isolated nosocomial pathogens world-wide (34). The gradual replacement of clinical isolates of *E. faecalis* by *E. faecium,* first observed in the United States and recently also found in Europe is of major concern (1,31) because most *E. faecium* strains are resistant to both vancomycin (>90%) and ampicillin (almost 100%) (1). The rapid emergence of resistance to novel antimicrobial compounds by these bacteria significantly reduces options for antibiotic therapies. Epidemic *E. faecium* strains are a genetically distinct population compared to isolates from healthy persons (36). Enterococcal surface protein (Esp) is an important marker in epidemic strains since its presence seems to be limited to hospital acquired *E. faecium* clones (35). In the present study, we investigated the role of Esp in the pathogenicity of bacteremia and its potential role as a target for immunotherapy.

Materials and Methods

Bacterial strains and growth conditions

E. faecium E1162 strain (E1162) is a well characterized clinical blood isolate harbouring the enterococcal surface protein gene (*esp*) (32). An insertion-deletion mutant in the *esp* gene was constructed previously (6). Enterococci were grown at 37°C in tryptic soy broth (TSB or CASO-broth; Merck) without agitation or on tryptic soy agar (TSA or CASO-agar; Merck). No difference in growth rates was observed between E1162 and E1162Δ*esp* (6).

Mouse bacteremia model

Virulence of the *E. faecium* E1162 and *E. faecium* E1162Δ*esp* (E1162Δ*esp*) strains was evaluated in a mouse bacteremia model as described previously (10), with minor modifications. Aliquots of bacterial overnight cultures were washed, resuspended in saline, shock-frozen and stored at -80°C. For each experiment, the concentration of bacterial stocks was confirmed by viable counts. Female BALB/c mice (6-8 weeks

old) were infected intravenously via the tail vein with 1.02 x 10^9 CFU/animal for E1162 wild-type strain and 1.76 x 10^9 CFU/animal for E1162Δ*esp*. After 24 hrs, the animals were sacrificed, blood was collected by cardiac puncture and the kidneys were harvested. The number of bacteria in kidney homogenates was determined by plating serial dilutions onto TSA plates and CFU were counted the next day. The number of bacteria was expressed as CFU/ml homogenized kidney.

Opsonophagocytic activity of the anti-Esp serum

An opsonophagocytic assay was used as previously described to test rabbit anti-Esp serum for the presence of opsonic antibodies directed against strain E1162 (28). Fresh polymorphonuclear neutrophils (PMNs) were isolated from healthy volunteers, immune rabbit serum raised against lipoteichoic acid (LTA) (28) or against the N-terminal part of Esp (33) was used in various concentrations ranging from 1:1000 to 1:25, and newborn rabbit serum (Cedarlane Laboratories, Hornby, Ontario, Canada) diluted 1:15 and absorbed with the target bacterial strain for *E. faecium* E1162Δ*esp* 1 h at 4°C served as a source of complement. Bacteria were prepared by growing them to mid-log phase, with subsequent washing and diluting to obtain a final ratio of bacterium:PMN of 1:1. Tubes lacking complement, PMNs or serum were used as negative controls, while rabbit serum raised against LTA (28) was used as a positive control. The percent killing was calculated by comparing the mean of the negative controls with the colony counts obtained after incubation for 90 min at 37°C (T_{90}) on a rotor rack by use of the following formula: [(mean CFU controls – mean CFU at T_{90})/(mean CFU controls)] x 100.

Protection studies

Rabbit anti-Esp serum raised against the N-terminal portion of the protein (33) was tested for its ability to confer protection against *E. faecium* bacteremia in mice. Two groups of eight BALB/c mice were injected in the tail vein with 200 µl of either rabbit anti-Esp serum or normal rabbit serum, respectively. After 24 hrs, all the mice were challenged i.v. with 10^{10} CFU E1162 and after an additional 24 hrs all the mice were sacrificed; the kidneys were harvested and cultivated quantitatively as described above.

The animal experiments have been conducted according to internationally recognized guidelines (approval Regierungspräsidium Freiburg, 35/9185.81/G-07/15).

Statistical analysis

Opsonophagocytic data are expressed as mean ± the standard error of the mean (SEM). *In vivo* data are expressed as median of CFU per ml. Differences between groups were analyzed by Mann-Whitney *U* test. A *P*-value < 0.05 was considered statistically significant.

Results

Mouse bacteremia model

To study the persistence of strain E1162 and its isogenic Esp-deficient mutant in the kidneys, mice were injected via the tail vein with a high bacterial inoculum of both wild-type and Esp-deficient mutant. In these experiments, the animals were sacrificed after 24 hours because all the mice had completely cleared the bacteria from blood and kidneys after 72 hours - a kinetic also observed for *E. faecalis* strains in previous studies [8] (data not shown). At 24 hours, the bacterial load of Esp-deficient mutant bacteria in homogenized kidneys was significantly lower compared to infection with wild-type bacteria (*P* = 0.03; Figure 1). Quantitative counts of bacteria in the blood, on the other hand, did not differ between E1162 wild-type and E1162Δ*esp* infection (data not shown).

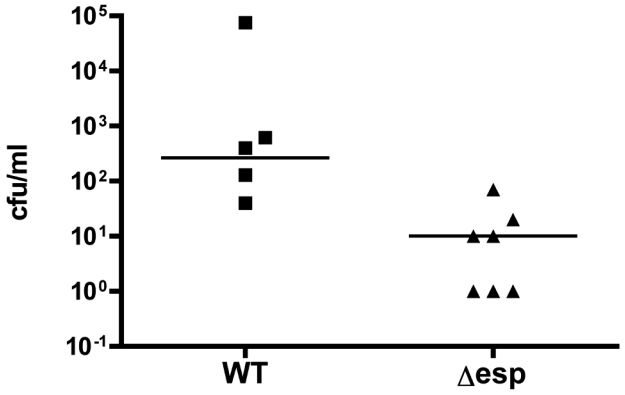

Figure 1. Mouse bacteremia model. Mice were challenged i.v. with the E1162 strain (wt; 1.02 x 10^9 CFU/animal) or with the E1162Δ*esp* strain (Δesp; 1.76 x 10^9 CFU/animal). After 24 hours, the animals were sacrificed and the kidneys were harvested. The number of bacteria in kidney homogenates was expressed as CFU/ml homogenized kidney (P = 0.03). The line represents the median.

Opsonic activity of anti-Esp serum

Since Esp seemed to be associated with virulence in a mouse bacteremia model, we evaluated the efficacy of immune serum raised against (the N-terminal part of) Esp using this serum as a source of antibodies in an opsonic killing assay. However, even at high concentrations of the serum (1:25), no significant bacterial killing (i.e. more than 50%) was observed (Figure 2). In comparison, using serum raised against *E. faecalis* lipoteichoic acid (anti-LTA serum) >75% killing of E1162 even at relatively low serum concentrations (i.e. 1:500) was observed.

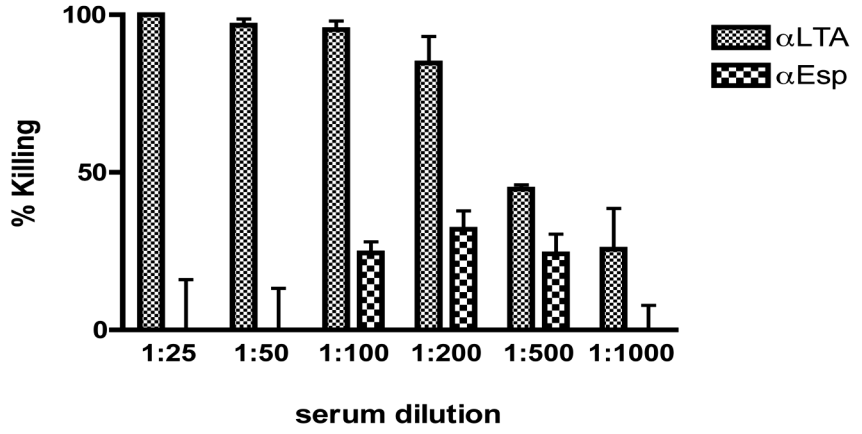

Figure 2. Opsonic activity of the anti-Esp serum (αEsp) compared with anti-LTA serum (αLTA). The target of the opsonic antibodies in E1162 strain was investigated using rabbit anti-Esp and anti-LTA sera in an opsonophagocytic assay. Bars represent the mean of four determinations and error bars represent SEM.

Protection studies

Mice were immunized with anti-Esp serum or with normal rabbit serum and subsequently infected with strain E1162. All mice showed similar bacterial counts in blood and in kidneys 24 hours after infection (Figure 3). This indicates that anti-Esp antibodies do not protect against *E. faecium* infection. In additional experiments, normal rabbit serum and anti-LTA serum were compared for their ability to protect mice against bacterial infection with strain E1162. Passive immunization with anti-LTA serum significantly reduced bacterial counts in blood (6 $\log_{10}$ reduction) and kidneys (4 $\log_{10}$ reduction) (data not shown). These observations confirm the results obtained in the opsonophagocytic assay that lipoteichoic acid represents a target of protective antibodies in E1162 strain while antibodies against Esp are not protective in this model.

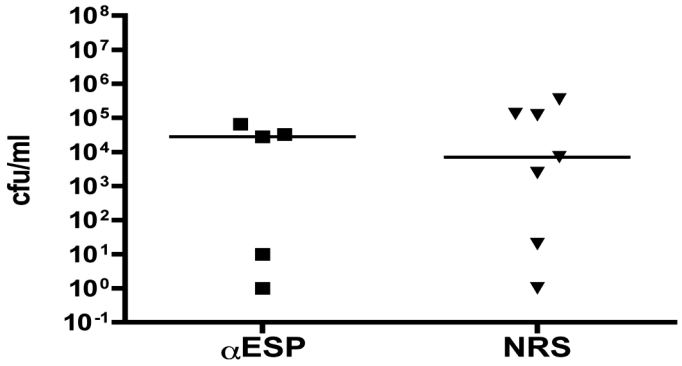

Figure 3. Protection against enterococcal bacteremia using anti-Esp serum (αEsp) and normal rabbit serum (NRS). Mice were challenged with 10^{10} CFU 24 hours after vaccination and sacrificed 24 hours later. The numbers of bacteria in kidney homogenates was expressed as CFU/ml homogenized kidney ($P > 0.05$). The line represents the median.

Discussion

Several factors have been associated with virulence in enterococci (11,13). Shankar and colleagues described an enterococcal surface protein (Esp) in *E. faecalis* with homology to alpha-C and Rib proteins in group B streptococci (23). This protein has been shown to be associated with biofilm formation on polystyrene (27). However, since Kristich and colleagues (14) and Toledo-Arana and coworkers (30) were able to demonstrate that biofilm formation is also present in the absence of Esp, it is likely that Esp represents only one of a number of different mechanisms by which *E. faecalis* produces biofilm.

Willems et al. (35) and Baldassarri and colleagues (2) discovered that epidemic *E. faecium* isolates causing hospital infections carry the *esp* gene more frequently than fecal isolates. In addition, *E. faecium esp* has been shown to be part of a putative pathogenicity island (PAI) that seems to be associated with nosocomial outbreaks (16). Insertion-deletion mutation of *esp* in a hospital-acquired *E. faecium* isolate confirmed the role of Esp in initial adherence and biofilm formation on polystyrene surfaces (6). However, *esp* disruption did not affect bacterial adhesion to colonic

carcinoma cells *in vitro* or intestinal colonization and translocation to lymph nodes in mice (7). These results suggest that *E. faecium* Esp is not essential for intestinal cell adhesion and translocation, reaffirming earlier results obtained with *E. faecalis* (21). Esp was shown to be involved in adhesion of *E. faecium* to uroepithelial cell lines and the presence of this protein prolonged infection in kidneys and the bladders of mice (17). The role of Esp in a mouse urinary tract infection (UTI) model was also demonstrated for *E. faecalis* (24), and together these two studies suggest that Esp is an important virulence determinant in UTIs caused by either *E. faecium* or *E. faecalis*. In addition, Esp of *E. faecium* was also shown to contribute to the pathogenesis of endocarditis in a rat endocarditis model, with wild-type bacteria showing greater ability to colonize the heart valves than the mutant strain (chapter 5 of this thesis). The importance of *E. faecium* Esp in experimental rat endocarditis and mouse urinary tract infections could be explained by the fact that these models represent classical biofilm infections.

In the present study, we investigated invasive infection caused by *E. faecium* using mouse bacteremia model (8). In this model, significantly fewer bacteria could be isolated after 24 hours from the kidneys of mice infected with the mutant strain. In the blood compartment, on the other hand, Esp-expression did not contribute to virulence. Our data suggest a specific role of Esp in invasion in the renal parenchyma and/or persistence in the kidney. In *E. faecalis,* several mutants impaired in biofilm formation were also less virulent in a mouse sepsis model (10,29), suggesting a correlation between the ability to form biofilm and prolonged bacteremia.

Since there is convincing evidence for a role of Esp in virulence, anti-Esp serum was tested for passive immunization of mice, to assess whether antibodies against Esp are able to protect against *E. faecium* bacteremia. Our results suggest that Esp is neither a target of protective antibodies in a mouse bacteremia model nor a target of opsonic antibodies *in vitro*. With the exception of an ABC transporter in *E. faecalis* (3), none of the other enterococcal surface proteins were shown to elicit a protective immune response in an animal model. However, in other gram-positive bacteria, antibodies to homologues of Esp, such as Rib and α-like protein of *Streptococcus agalactiae* have been shown to confer immune protection (26,15).

A variety of other enterococcal surface proteins have been examined on their role on pathogenesis and protective immune response. A plasmid-encoded surface exposed protein, aggregation substance (AS), also contributes to virulence in *E. faecalis,* and there is evidence that it is a virulence factor in endocarditis and in interaction with several eukaryotic cell lines (22,4). Despite all these findings, antibodies against the N-terminal domain of the AS are opsonic *in vitro*, but failed to protect rabbits against endocarditis (19). Pili have been demonstrated to be an attractive vaccine target in several gram-positive pathogens (18,5). In enterococci, pili have been shown to be involved in similar virulence processes as Esp: biofilm formation (20), rat endocarditis (12) or UTI (25). However, so far, no protection studies involving these proteins have been reported.

The susceptibility of E1162 to opsonic killing mediated by anti-LTA antibodies confirms previous studies by our group (8,9) in which LTA was shown to be the predominant target for protective antibodies in about 25% of *E. faecalis* and *E. faecium* strains. In the current study, we were able to confirm that passive immunization of mice with anti-LTA serum offered protection and correlated well with the ability to mediate opsonophagocytic killing *in vitro*. Abundance, accessibility and little variability of the LTA structure among different bacterial species make this glycoconjugate a promising target for therapies by vaccination either conjugated or alone, since it has been shown to induce an adaptive immune response.

In summary, the data presented here confirm the role of Esp in the pathogenicity of *E*. *faecium,* while indicating that this antigen is probably not a suitable vaccine candidate.

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7

Identification of a novel genomic island specific to hospital-acquired clonal complex 17 *Enterococcus faecium* isolates

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Abstract

Hospital-acquired clonal complex 17 (CC17) *Enterococcus faecium* strains are genetically distinct from indigenous strains and are enriched with resistance genes and virulence genes. We identified a genomic island in CC17 *E. faecium* tentatively encoding a metabolic pathway involved in carbohydrate transport and metabolism, which may provide a competitive advantage over the indigenous *E. faecium* microbiota.

Introduction

Enterococcus faecium strains acquired during hospitalization and responsible for the majority of the hospital burden are genetically distinct from indigenous *E. faecium* strains that belong to the normal intestinal flora (11,19). The underlying mechanisms explaining the ecological dominance of these hospital-acquired *E. faecium* strains, currently labeled clonal complex 17 (CC17) based on multilocus sequence typing, are not well understood. Previous studies suggest that the acquisition of antibiotic resistance traits, such as penicillin and quinolone resistance, and cell surface proteins may have contributed to the ecological success of CC17 *E. faecium* (6,7,10,12,17-19). In addition, comparative genomic hybridizations using a mixed whole-genome microarray identified a specific *E. faecium* clade encompassing CC17 *E. faecium* containing more than 100 clade-specific genes, including resistance genes, putative virulence genes, and insertion sequence elements (13).

An additional method to identify CC17 *E. faecium*-acquired genes or gene clusters (GeCs) in a genome is base composition analysis. At the time of transfer, horizontally acquired genes often differ in their codon usages, GC percentages, and dinucleotide frequencies, since horizontally acquired genes share these characteristics with the DNA of the bacterium from which they originated (8,9). A recently described Web-based tool for the detection of horizontally transferred genes and GeCs is the δρ-Web model (16). The δρ-Web model allows whole-genome composition analysis to visualize anomalous DNA in a prokaryotic genome based on differences in both GC percentages and dinucleotide frequencies. Horizontally acquired genes or GeCs, such as genomic islands (GIs), often encode accessory functions, such as additional metabolic activities and antibiotic resistance, or functions involved in microbial fitness, symbiosis, or pathogenesis (1,4). In this study, we used the δρ-Web model as an initial, quick screen to identify anomalous GeCs in the genome of *E. faecium* DO, a CC17 *E. faecium* that may have contributed to increased fitness and enhanced survival of CC17 *E. faecium*. In addition, PCR and dot blotting were performed on a large set of *E. faecium* isolates to confirm whether these anomalous GeCs were CC17 specific.

Materials and Methods, Results and Discussion

Identification of a CC17 *E. faecium*-specific GeC.

In order to submit the publicly available *E. faecium* DO draft genome sequence (Genbank accession no. AAAK00000000) to the δρ-Web model, an in silico concatenated genome sequence was created by linking all contigs larger than 2,000 bp (n = 41), encompassing 64% of the whole genome, in order from large to small. After submission, the concatenated genome sequence was divided in nonoverlapping fragments of 10,000 bp, as recommended by the user guidelines supplied at http://deltarho.amc.uva.nl. The difference in dinucleotide frequency (δ^*-value) between each fragment and the complete sequence and the GC percentage of each fragment were calculated. The model identified five fragments with both a high δ^*-value and an aberrant GC percentage compared to the average genome values for the concatenated *E. faecium* DO contigs. These fragments represented sequences located in contig 608, 624, 638, 654, and 656. Contig 656 was previously identified as a contig harboring many CC17-specific genes (13). Therefore, we chose to focus on the fragments located in the other four contigs. Of each fragment, one gene (*orf877*, *orf1155*, *orf1482*, and *orf2303* of contig 608, 624, 638, and 654, respectively) was chosen and the presence of this gene was assessed by PCR and dot blotting on chromosomal DNA from 134 *E. faecium* isolates, 41 CC17 *E. faecium* isolates and 93 non-CC17 *E. faecium* isolates (Table 1).

Table 1. Strains used in this study

Category	Epidemiology	Country	Year	Source	ST	CC	Strain
Hospital outbreak (n=17)	HO	GBR	1992	urine	18	17	E0013
	HO	USA	1995	unknown	17	17	E0155
	HO	USA	1994	urine	20	non 17	**E0300**
	HO	NLD	1999	unknown	16	17	E0470
	HO	NLD	2000	environment	16	17	E0734
	HO	NLD	2000	faeces	50	non 17	E0772
	HO	USA	2001	faeces	16	17	E1132
	HO	NOR	1999	wound	17	17	E1340
	HO	GRC	1999	blood	65	17	E1435
	HO	GRC	2000	peritoneal fluid	16	17	E1441
	HO	DEU	2002	catheter urine	78	17	E1644
	HO	NLD	2002	faeces	18	17	E1652
	HO	BRA	1998	catheter	114	non 17	**E1679**
	HO	DNK	unknown	unknown	17	17	E1716
	HO	DNK	unknown	unknown	18	17	E1717
	HO	AUS	2000	unknown	173	17	E1760
	HO	AUS	1998	blood	17	17	E0510
Clinical (n=33)	Ci	NLD	1995	faeces	22	non 17	E0073
	Ci	NLD	1995	bile	21	non 17	E0125
	Ci	ISR	1997	blood	80	17	E0333
	Ci	GBR	1997	blood	17	17	E0380
	Ci	FRA	unknown	blood	17	17	E1162
	Ci	POL	1998	urine	99	non 17	**E1172**
	Ci	AUT	1998	blood	78	17	**E1263**
	Ci	POL	1998	blood	17	17	E1302
	Ci	PRT	1998	blood	17	17	E1308
	Ci	USA	2001	unknown	16	17	E1360
	Ci	GBR	2000	unknown	16	17	E1391
	Ci	GBR	2000	unknown	84	non 17	E1403
	Ci	GBR	2000	unknown	94	non 17	E1423
	Ci	ESP	1998	blood	17	17	E1463
	Ci	ESP	1997	blood	18	17	E1467
	Ci	ESP	1999	urine	74	non 17	E1499
	Ci	ESP	1997	blood	18	17	E1500
	Ci	NLD	1957	blood	67	non 17	E1620
	Ci	NLD	1959	blood	86	non 17	E1621
	Ci	NLD	1961	liquor	22	non 17	E1625
	Ci	NLD	1961	blood	106	non 17	E1636
	Ci	TZA	unknown	blood	169	non 17	**E1721**
	Ci	TZA	unknown	blood	132	17	E1728

	Ci	TZA	unknown	blood	18	17	E1731
	Ci	ESP	1995	blood	18	17	E1734
	Ci	ESP	2001	blood	16	17	E1735
	Ci	ESP	1997	blood	18	17	E1737
E. faecium DO	Ci	USA	1991	blood	18	17	E1794
	Ci	GBR	unknown	unknown	157	non 17	E0699
	Ci	DEU	unknown	blood	17	17	E1282
	Ci	DEU	unknown	blood	17	17	E1284
	Ci	ITA	1997	blood	17	17	E1292
	Ci	NLD	1960	pus	22	non 17	E1623
Hospital surveillance (n=31)	HS	FRA	1997	faeces	17	17	E0321
	HS	FRA	1997	faeces	79	non 17	E0322
	HS	NLD	2000	faeces	5	non 17	E0802
	HS	NLD	2000	faeces	21	non 17	E0849
	HS	USA	2001	faeces	117	17	E1133
	HS	NLD	1998	faeces	16	17	E1147
	HS	NLD	1995	faeces	6	non 17	E1149
	HS	NLD	2000	blood	5	non 17	E1316
	HS	NLD	2002	faeces	50	non 17	E1554
	HS	DEU	2002	faeces	78	17	E1643
	HS	BRA	2000	faeces	110	non 17	E1674
	HS	BRA	2001	faeces	111	non 17	E1675
	HS	ESP	2001	faeces	18	17	E1850
	HS	NLD	1995	faeces	87	non 17	E0075
	HS	FRA	1997	faeces	79	non 17	E0323
	HS	NLD	1999	unknown	89	non 17	E0492
	HS	NLD	2000	faeces	5	non 17	E0729
	HS	NLD	2000	faeces	6	non 17	E0767
	HS	NLD	2000	faeces	6	non 17	E0808
	HS	NLD	1995	faeces	123	non 17	E1141
	HS	ESP	2001	faeces	103	17	E1493
	HS	NLD	2002	faeces	5	non 17	E1552
	HS	NLD	1979	faeces	66	non 17	E1627
	HS	NLD	unknown	faeces	5	non 17	E1638
	HS	NLD	unknown	faeces	5	non 17	E1640
	HS	BRA	unknown	faeces	94	non 17	E1669
	HS	BRA	unknown	faeces	94	non 17	E1670
	HS	BRA	unknown	faeces	113	non 17	E1677
	HS	BRA	1998	faeces	97	non 17	E1695
	HS	AUS	2000	unknown	174	17	E1762
	HS	GBR	1992	faeces	146	non 17	E0027
Community surveillance (n=30)	CS	NLD	1996	faeces	147	non 17	E0060
	CS	NLD	1997	faeces	6	non 17	E0092
	CS	NLD	1996	faeces	82	non 17	E0128

	CS	NLD	1996	faeces	6	non 17	E0135
	CS	NLD	1999	faeces	94	non 17	E0980
	CS	NLD	1998	faeces	54	non 17	E1002
	CS	NLD	1998	faeces	42	non 17	E1039
	CS	NLD	2000	faeces	32	non 17	E1071
	CS	ESP	2000	faeces	101	non 17	E1485
	CS	IRL	2001	faeces	163	non 17	E1590
	CS	BEL	1996	faeces	6	non 17	E1764
	CS	BEL	1996	faeces	136	non 17	E1766
	CS	NLD	1996	faeces	6	non 17	E0129
	CS	NLD	1996	faeces	6	non 17	E0130
	CS	NLD	1996	faeces	5	non 17	E0131
	CS	NLD	1996	faeces	6	non 17	E0138
	CS	NLD	1996	faeces	6	non 17	E0139
	CS	NLD	1996	faeces	9	non 17	E0278
	CS	NLD	1997	faeces	15	non 17	E0400
	CS	NLD	1997	faeces	7	non 17	E0403
	CS	NLD	1998	faeces	56	non 17	E1028
	CS	NLD	1998	faeces	97	non 17	E1037
	CS	NLD	1998	faeces	98	non 17	E1046
	CS	NLD	1998	faeces	92	non 17	E1050
	CS	NLD	1998	faeces	118	non 17	E1327
	CS	ESP	2000	faeces	32	non 17	E1488
	CS	ESP	2000	faeces	102	non 17	E1489
	CS	BEL	2001	faeces	59	non 17	E1582
	CS	BEL	2001	faeces	161	non 17	E1583
	CS	BEL	1996	faeces	137	non 17	E1768
Environment (n=8)	Env	NLD	1981	environment	68	non 17	E1628
	Env	NLD	1981	environment	69	non 17	E1630
	Env	FRA	1985	environment	172	non 17	E1759
	Env	GBR	unknown	faeces	176	non 17	E0695
	Env	GBR	unknown	faeces	156	non 17	E0696
	Env	NLD	1981	environment	93	non 17	E1629
	Env	NLD	1981	environment	69	non 17	E1631
	Env	NLD	1982	environment	66	non 17	E1634
Animal (n=15)	A Poultry	GBR	1992	faeces	9	non 17	E0045
	A Pig	NLD	1996	faeces	6	non 17	E0144
	A Calf	NLD	1996	faeces	1	non 17	E0172
	A Calf	NLD	1996	faeces	4	non 17	E0211
	A Poultry	NLD	1997	faeces	8	non 17	E0429
	A Dog	NLD	1996	faeces	27	non 17	E0463
	A Cat	NLD	1996	faeces	21	non 17	E0466
	A Bison	BEL	1994	faeces	21	non 17	E1573
	A Dog	BEL	1995	faeces	27	non 17	E1574
	A Ostrich	ZAF	2001	faeces	159	non 17	E1576

	A Food	NOR	1956	food	76	non 17	E1607
	A Food	NOR	1964	food	70	non 17	E1619
	A Rodent	NLD	1959	faeces	104	non 17	E1622
	A Pig	BEL	2001	carcasses	141	non 17	E1781
	A Pig	ESP	unknown	faeces	137	non 17	E0685

E. faecium isolates, used before by us in the study published by Leavis *et al*. (13), are underlined. The single CC17 *E. faecium* isolate that does not contain the GI and the four non-CC17 *E. faecium* isolates that contain the GI are in bold. A, animal; AUS, Australia; AUT, Austria; BEL, Belgium; BRA, Brazil; CS, community surveillance; CHE, Switzerland; Ci, clinical; DEU, Germany; DNK, Denmark; Env, environmental; ESP, Spain; FRA, France; GBR, Great Britain; GRC, Greece; HO, hospital outbreak; HS, hospital surveillance; IRL, Ireland; ISR, Israel; ITA, Italy; NLD, Netherlands; NOR, Norway; POL, Poland; PRT, Portugal; ST, sequence type determined by MLST; TZA, Tanzania; USA, United States of America; ZAF, Republic of South Africa.

The preparation of chromosomal DNA and dot blotting were performed as described previously (7). The primers used for PCR and for the generation of DNA probes are listed in Table 2. *E. faecium* DO was used as a positive control and *E. faecalis* V583 as a negative control (15). PCR (data not shown) and dot blotting revealed that one of the four genes, *orf1482* on contig 638, was specific for CC17 *E. faecium* (Table 3). This gene was detected in 97.56% (40/41) of the CC17 *E. faecium* isolates and in only 4.30% (4/93) of the non-CC17 *E. faecium* isolates ($P < 0.0001$; Fisher's exact test). *orf1482* encodes a putative transcriptional regulator belonging to the AraC-family. Transcriptional regulators of the AraC family are widely spread among bacteria and regulate genes with diverse functions, ranging from carbon metabolism and stress response to pathogenesis (3,14). Since AraC-type transcriptional regulators are often found close to or on GIs (5), the presence or absence of genes located upstream and downstream of *orf1482* was determined. This revealed that all isolates that contained *orf1482* also contained a set of seven genes just upstream of *orf1482*, while isolates lacking *orf1482* also lacked this set of genes, indicating that the *araC*-like gene is located in an 8.5-kb GeC, which is specifically enriched in CC17 *E. faecium* (Table 3). This means that the genes of the GeC may also serve as a marker to distinguish CC17 *E. faecium* strains from other *E. faecium* strains. *orf1474* and *orf1483*, flanking this 8.5-kb GeC, belong to the *E. faecium* core genome. The four non-CC17 *E. faecium*

isolates that harbor this GeC represent two hospital outbreak isolates (E300 and E1679) and two clinical isolates (E1172 and E1721). The single CC17 *E. faecium* isolate that does not harbor this GeC represents a clinical isolate (E1263).

Table 2. Primers used in this study

ORF	Primer name	Primer sequence	Start position[a]	Used in
orf1474	1474F	5'-CGA GAC ACT TTC CTG GCT T	591	PCR/DB
	1474F2	5'-GAA AAC CTG GAA GAT CAC G	1807	GI insertion site
	1474R	5'-CTG GAG TGG TAG GAT GCT CA	1264	PCR/DB/RT-PCR[c]
orf1475	1475F	5'-CAA TAT TGG TTG GAC AGA AGG	605	PCR/DB/RT-PCR[b,c]
	1475R	5'-TTG AAT ATG AGC CTG AGC AG	872	PCR/DB/RT-PCR[b,c]
orf1476	1476F	5'-TTG GTC AGC TAT GTT GTG GG	786	PCR/DB/RT-PCR[b,c]
	1476R	5'-GCC AGC TTC ACT CAC CCT A	1587	PCR/DB/RT-PCR[b]
	1476R2	5'-GAC TAA CCC CTT GAT TTC ACC	849	RT-PCR[c]
orf1477	1477F	5'-CAT TAC TGT ATT GGG CTT CGA	479	PCR/DB/RT-PCR[b,c]
	1477R	5'-CTC TAT GGT ATG CTT CTG CTC C	1027	PCR/DB/RT-PCR[b,c]
orf1478	1478F	5'-ATG CTA TCT AGG ATG GTG CC	334	PCR/DB/RT-PCR[b,c]
	1478R	5'-TAG ACT GCG CCC ACA CAT A	796	PCR/DB/RT-PCR[b,c]
orf1479	1479F	5'-GGC TGT AGC TTC CGT ATT CA	393	PCR/DB/RT-PCR[b,c]
	1479R	5'-ATC GTC CAG GCA ATA GCA G	854	PCR/DB/RT-PCR[b,c]
orf1480	1480F	5'-CAA ATA CTG TTT TAC CAG CCA C	26	PCR/DB/RT-PCR[b,c]
	1480R	5'-CAC CAG TTA ATT TTA CGC CG	175	PCR/DB/RT-PCR[b,c]
orf1481	1481F	5'-GTT TAT CAA CAT GCT AGC CCA	708	PCR/DB/RT-PCR[b,c]
	1481R	5'-GCC AAT GAG TTA GAT GTA GCC	1085	PCR/DB/RT-PCR[b,c]/5'RACE
	1481R2	5'-TTT GTA CTG TTG CTG TTC CC	245	5'RACE
orf1482	1482F	5'-CCA TCT TCA TTT ATC TGC TGC T	63	PCR/DB/RT-PCR[b,c]
	1482R	5'-TGA TGG ATC GAA TGA AAT CC	833	PCR/DB/RT-PCR[b]/5'RACE
	1482R2	5'-TTA GTA GTC GAA AAT AGG GAG C	81	5'RACE
orf1483	1483F	5'-TAT GCG GGA ATG ACA GAA CA	1015	PCR/DB
	1483R	5'-GAG ATA CTG CCA GAA GGT GC	1454	PCR/DB/GI insertion site

[a] Nucleotide reference positions relative to the start codon of the open reading frame.

[b] RT-PCR to assess expression of the GI genes.

[c] RT-PCR to confirm cotranscription of *orf1475* to *orf1481*.

DB, dot blotting. Primers were purchased from Isogen Life Science, IJselstijn, The Netherlands.

Table 3. Prevalence of genes in CC17 and non-CC17 *E. faecium* isolates as determined by dot blotting

ORF	CC17 isolates (n=41)	Non-CC17 isolates (n=93)
orf1474	41 (100%)	93 (100%)
orf1475	40 (97.56%)	4 (4.30%)
orf1476	40 (97.56%)	4 (4.30%)
orf1477	40 (97.56%)	4 (4.30%)
orf1478	40 (97.56%)	4 (4.30%)
orf1479	40 (97.56%)	4 (4.30%)
orf1480	40 (97.56%)	4 (4.30%)
orf1481	40 (97.56%)	4 (4.30%)
orf1482	40 (97.56%)	4 (4.30%)
orf1483	41 (100%)	93 (100%)

Organization and genetic features of the CC17 *E. faecium*-specific GeC.

Direct and inverted repeats were found upstream and downstream of the CC17 *E. faecium*-specific GeC (Figure 1). Furthermore, a putative integrase is located downstream of *orf1482*. The presence of a member of the AraC family of transcriptional regulators, of direct and inverted repeats upstream and downstream of this GeC, and of an integrase downstream and the finding that this cluster of genes was found in clinically relevant isolates and not in surveillance isolates suggest that this region encompasses a distinct GI that is acquired by horizontal transfer.

Blast searches in GenBank of the predicted proteins encoded by this GI revealed that the GI genes putatively encode two glycosyl hydrolases, two binding-protein-dependent transporter proteins, a sugar binding protein, two proteins with unknown function, and an AraC transcriptional regulator (Table 4). Considering the putative functions of the predicted GI proteins, this GI may represent a novel metabolic island involved in carbohydrate transport and metabolism, possibly providing CC17 *E. faecium* a competitive advantage over indigenous commensal *E. faecium*, in particular ecological niches. The CC17 *E. faecium*-specific GI proteins share only low-level identity with orthologs in other prokaryotes, and these orthologous proteins originate from a wide range of taxonomically distinct groups. This indicates that this GI originates from an as-yet-unidentified biological source.

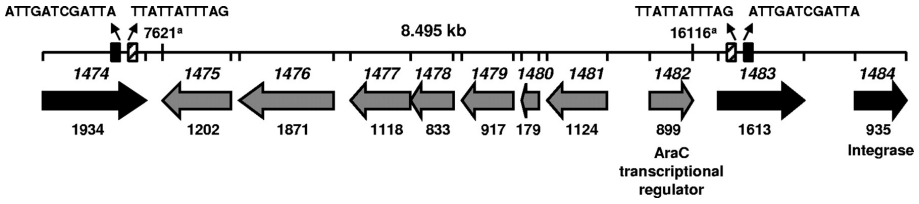

Figure 1. Genomic organization of the 8.5-kb GI (*E. faecium* DO contig 638) specifically enriched in CC17 *E. faecium*. The direction of transcription is indicated by arrows. The gray arrows represent the genes which belong to the GI, and the black arrows represent the flanking genes. The numbers below the arrows indicate gene sizes. Direct (dashed boxes) and inverted (black boxes) repeats were found at positions 7184 and 16391 and positions 7061 and 16803, respectively. Open reading frame numbers are indicated in italics. a, nucleotide reference position relative to that of the *E. faecium* DO contig 638 sequence (GenBank accession no. AAAK03000019).

Table 4. Identities of the predicted proteins encoded by the GI specifically enriched in CC17 *E. faecium* as determined by BLAST

Predicted protein	Annotation	Organism	Amino acid identity (%)[a]
1475	Glycosyl hydrolase	*Citrobacter koseri*	52
1476	Protein with unknown function	*Paenibacillus species*	37
1477	Glycosyl hydrolase	*Paenibacillus species*	50
1478	Binding-protein-dependent transporter protein	*Halothermothrix orenii*	36
1479	Binding-protein-dependent transporter protein	*Petrotoga mobilis*	43
1480	No homologues found		
1481	Sugar binding protein	*Bacillus clausii*	22
1482	AraC transcriptional regulator	*Clostridium bolteae*	36

[a] Amino acid identities represent top BLAST hits. Blast searches were performed in GenBank.

Transcriptional analysis of the GI.

To assess whether this GI is actively expressed, total RNA was isolated from mid-exponential cultures of five CC17 *E. faecium* isolates (E470, E734, E1133, E1162, and *E. faecium* DO) by using Tri Reagent (Ambion, Austin, TX). Residual DNA was removed with Turbo DNase according to the protocol supplied with the Turbo DNA-free kit (Ambion). cDNA was synthesized with a SuperScript III first-strand synthesis system (Invitrogen Corp., Carlsbad, CA), using random hexamers according to the

manufacturer's instructions. The expression levels of the eight GI genes were then assessed by PCR with gene-specific primers (Table 2). Expression was detected at the mRNA level for all the GI genes (data not shown), indicating that these genes are expressed and do not represent silent genes. The secondary structures of the mRNA from the region between *orf1474* and *orf1475* and that from the region downstream of *orf1482* were predicted using Mfold (20), which revealed very stable stem-loop structures with highly negative ΔG values of –8.11 kcal/mol and –14.01 kcal/mol, respectively. This suggests the presence of transcriptional terminators at these sites and that *orf1475* to *orf1481* are part of a single operon. To confirm this, reverse transcription-PCR was performed with cDNA by using gene-specific primer pairs (Table 2) designed to span the entire region, resulting in overlapping amplification products. Products of the expected size were observed with primer pairs covering *orf1475* to *orf1481*, showing that these genes are cotranscribed in a single operon and that *orf1474* and *orf1482* are not part of the operon (Figure 2A). In addition, promoter mapping of *orf1481* and *orf1482* was performed using 5' rapid amplification of cDNA ends (Invitrogen Corp.) according to the manufacturer's instructions. Total RNA from *E. faecium* DO was isolated and reverse transcribed using the primers 1481R and 1482R (Table 2). The subsequent PCR was performed using primers 1481R2 and 1482R2 and the abridged anchor primer provided with the system. Sequencing of the PCR products revealed that two transcriptional start sites were located in the *orf1481-orf1482* intergenic region (Figure 2B). Direct repeats were found between the two promoters (P1 and P2), which may represent a putative binding site of a transcriptional regulator protein (2).

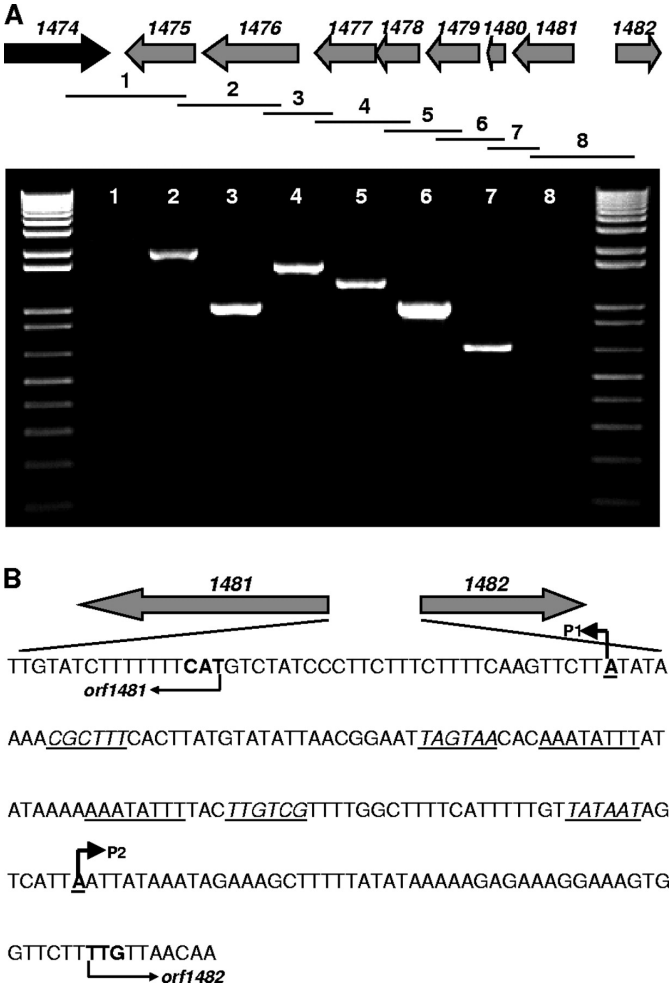

Figure 2. Transcriptional analysis of the GI specifically enriched in CC17 *E. faecium*. (A) Cotranscription of *orf1475* to *orf1481*, demonstrated by using primer pairs designed to span the entire region, resulting in overlapping amplification products. The molecular size marker is the 1-kb ladder (Invitrogen Corp.). (B) Intergenic region of *orf1481-orf1482*, with the start codons and orientations of *orf1481* and *orf1482* in bold and indicated by arrows below the sequence. Transcriptional start sites and directions are in bold, underlined and indicated by arrows above the sequence. Putative –35 and –10 boxes are underlined and in italics. Direct repeats, representing a putative binding site of a transcriptional regulator protein, are between the two promoters (P1 and P2) and are underlined.

GI insertion site.

To analyze the insertion sites of GIs in other CC17 *E. faecium* and non-CC17 *E. faecium* isolates, PCR and DNA sequencing were performed by using primer pair 1474F2-1483R (Table 2), designed from the flanking genes in *E. faecium* DO, *orf1474* and *orf1483*. The insertion site was analyzed for one CC17 *E. faecium* isolate (E1162), one non-CC17 *E. faecium* isolate (E980), the single CC17 *E. faecium* isolate that does not harbor the gene cluster (E1263), and the four non-CC17 *E. faecium* isolates that harbor the gene cluster (E300, E1172, E1679, and E1721). In E1162, E300, E1172, E1679, and E1721, the GI was found to be inserted at exactly the same position as in *E. faecium* DO. In E980 and E1263, *orf1474* and *orf1483*, the flanking genes of the GI, are located directly adjacent to each other. Insertion of the GI resulted in the deletion of a 108-bp fragment located in the intergenic region from *orf1474* to *orf1483* (Figure 3A and B). The observation of an identical insertion site in isolates that carry this GI suggests site-specific recombination.

Conclusions.

By using the δρ-Web model, PCR, and dot blotting, we identified a GI tentatively encoding a novel metabolic pathway involved in carbohydrate transport and metabolism. Our finding that all CC17 *E. faecium* isolates but one harbor this island and none of the non-CC17 *E. faecium* human surveillance, environmental, and animal isolates harbors it indicates that this GI is acquired by CC17 *E. faecium* via horizontal transfer. We hypothesize that this GI may provide CC17 *E. faecium* a competitive advantage over the indigenous commensal *E. faecium* flora by enabling CC17 *E. faecium* to effectively colonize the gastrointestinal tracts of hospitalized patients.

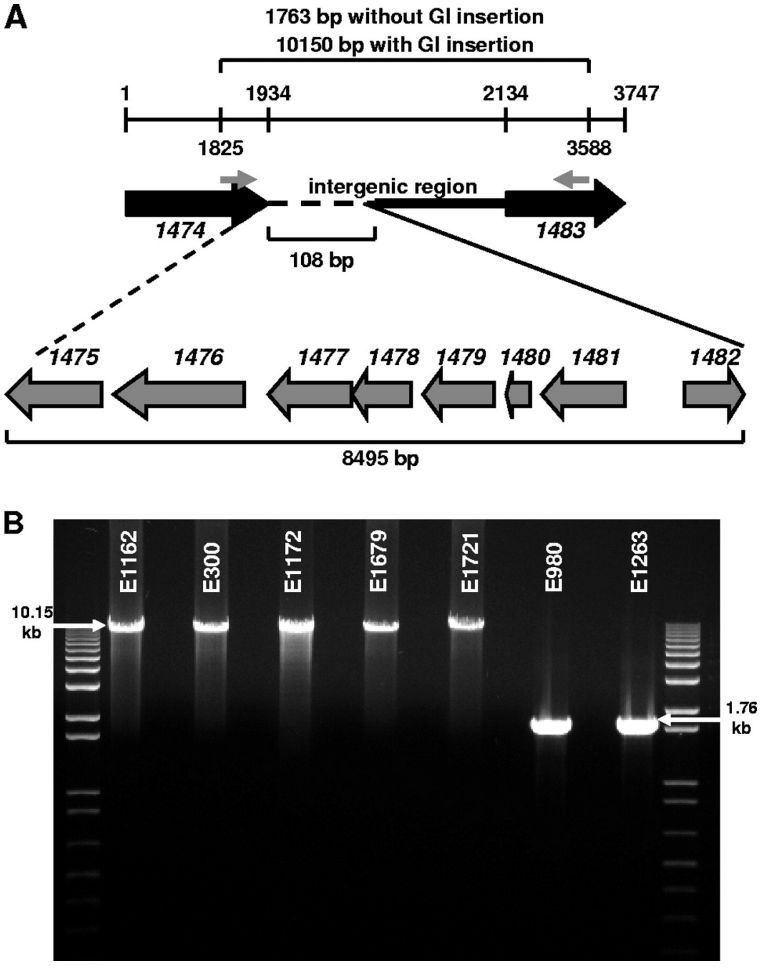

Figure 3. Analysis of the insertion site of the GI specifically enriched in CC17 *E. faecium*. (A) Schematic representation of insertion of the GI in the intergenic region from *orf1474* to *orf1483*, resulting in deletion of a 108-bp fragment (dashed line). The primers used to analyze the insertion site are indicated by small grey arrows. Numbers indicate start and stop positions of *orf1474* and *orf1483* and start position of the primers. The sizes of the two amplicons, with and without GI insertion, the GI, and the deleted fragment are indicated. Open reading frame numbers are indicated in italics. (B) PCR was performed with one CC17 *E. faecium* isolate (E1162), four non-CC17 *E. faecium* isolates that harbor the gene cluster (E300, E1172, E1679, and E1721), one non-CC17 *E. faecium* isolate (E980), and the single CC17 *E. faecium* isolate that does not harbor the GI (E1263). The molecular size marker is the 1-kb ladder (Invitrogen Corp.).

Acknowledgements

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8

General discussion

General discussion

In the last two decades *Enterococcus faecium* has emerged as a nosocomial pathogen in immunocompromised and critically-ill patients (37). Successful treatment is increasingly hampered because of antibiotic resistance, and consequently, *E. faecium* infections in this patient population are associated with increased mortality (1). To prevent infections with and the spread of these multi-resistant emerging nosocomial pathogens, further knowledge of the pathogenesis of *E. faecium* is needed. *E. faecium* isolates responsible for the majority of nosocomial infections and hospital outbreaks are genetically distinct from indigenous intestinal isolates (29,58). These hospital-acquired *E. faecium* strains acquired antibiotic resistance and virulence genes conferring an infectious phenotype to *E. faecium* resulting in increased fitness and enhanced survival of *E. faecium* in hospitalized patients. So far, different putative virulence genes are identified (23,28,30,41,57) that possibly contribute to the pathogenesis of *E. faecium*. However, our knowledge about the functions of these genes and proteins, their possible role in initiating infection and interaction with the environment is still limited. The enterococcal surface protein gene *esp* is one of the identified putative virulence genes (28,57) and has been implicated in initial adherence to polystyrene and biofilm formation (51). We hypothesized that Esp, because of its epidemiological linkage to hospital-acquired *E. faecium* isolates and potential involvement in adherence and biofilm formation, is an important virulence factor for *E. faecium* in initiating infection and crucial in the pathogenesis of *E. faecium* infections (16,17). Therefore, the studies of this thesis mainly focused on elucidating the role of Esp in the pathogenesis of different experimental *E. faecium* infections.

In **chapter 2** we demonstrated, for the first time, that Esp is involved in biofilm formation of a hospital-acquired *E. faecium* strain by constructing an *esp* mutant. The constructions of mutants in *E. faecium* by allelic replacement is complicated and can, certainly, not be considered a routine molecular technique as it is in other Gram positives like, group A and group B streptococci or pneumococci. Only three years ago, Nallapareddy et al. were the first to construct an insertion-deletion mutation, in

the adhesion-encoding *acm* gene, in a clinical *E. faecium* isolate by using an improved temperature sensitive vector (39). Until then, construction of an *E. faecium* knock-out strain was hampered by poor transformation capacity, plasmid incompatibility, and the lack of selective markers because of multiple antibiotic resistances in clinically relevant strains. By using the same approach as Nallapareddy et al. we succeeded in constructing an *esp* mutant, which was the second insertion-deletion mutation in a clinical *E. faecium* isolate. Inactivation of *esp* resulted in abolished cell surface Esp expression, significant lower initial adherence to polystyrene, and reduced biofilm formation compared to the Esp-expressing parent strain. Capacities of initial adherence and biofilm formation were restored in the Esp-deficient mutant by *in trans* complementation with *esp*, which demonstrates that adherence and biofilm-reduced phenotype of the Esp-deficient mutant is due to the mutated *esp* gene and not a polar effect on genes located downstream. Capacities of adherence and biofilm formation were not completely diminished in the Esp-deficient mutant strain indicating that besides Esp other factors play a role in these processes.
Initial bacterial adherence and subsequent biofilm formation are considered key processes for colonization and infection development. Furthermore, the relevance of biofilm in enterococcal infections has been demonstrated for a variety of infections including endocarditis, urinary tract infections, root canal infections, and ocular infections, and in a variety of device-related infections (12,14,17,32,35,42,59). To elucidate the role of Esp in the pathogenesis of *E. faecium* infections, the Esp-deficient mutant and its Esp-expressing parent strain were tested in different experimental infection models mimicking intestinal colonization, urinary tract infection, peritonitis, bacteremia, and endocarditis.

In **chapter 3** the role of Esp in intestinal colonization was investigated. Recent studies revealed intestinal colonization rates with hospital-acquired *E. faecium* to be as high as 40% in hospital wards, while colonization in healthy people appeared to be almost absent (13,49,58). For nosocomial *E. faecium* strains the gastro-intestinal tract serves as staging area from which they can disperse in and between patients. Moreover, colonization is the first and key step that precedes clinical infection. Patients become infected by contaminated catheters or wounds as a result of skin colonization due to

fecal contamination, and, in the minority of infections, through bacterial translocation from the intestinal lumen to extraintestinal sites (36,54). Intestinal colonization not only increases the risk on subsequent infection, the intestines are also an optimal environment for the exchange of genetic material by horizontal gene transfer (HGT). For instance, it was demonstrated that *in vivo* transfer of *vanA* and *vanB* genes occurs in the intestine of mice (5,11) and human volunteers (31). In addition, in hospitalized patients transfer of vancomycin resistance to methicillin-resistant *Staphylococcus aureus* (MRSA) creating a vancomycin-methicillin-resistant *S. aureus* (VRSA) was reported (52,53). So far, the VRSA strains that were reported seem to lack capacity of efficient human to human spread, but continuous high vancomycin-resistant enterococci (VRE) and MRSA prevalence rates among hospitalized patients will increase the likelihood of emergence of an epidemic VRSA, which would have serious clinical consequences. Next to resistance genes also dissemination of virulence genes via HGT may occur within the intestines. *In vivo* transfer of an internal fragment of the pathogenicity island of *Enterococcus faecalis* containing the *esp* gene and the *cytolysin* gene was demonstrated during transient colonization of the gastro-intestinal tract of mice (9). Considering these data, high colonization rates of hospital-acquired *E. faecium* in the gastro-intestinal tract increases the risk of transfer of resistance and virulence genes to other, perhaps more pathogenic bacteria. Or vice versa, *E. faecium* might acquire genes by HGT from more pathogenic bacteria, such as immune evasion genes of *S. aureus*, resulting in more pathogenic behavior of hospital-acquired *E. faecium*. As such, hospital-acquired *E. faecium*, harboring a large reservoir of transferable resistance genes and elements, may be considered an important hub for the spread of antibiotic resistance. It is, therefore, of utmost importance to understand the processes and elucidate determinants that promote intestinal colonization of nosocomial *E. faecium* strains. Only then we will be able to impede subsequent spread of these nosocomial clones. It has been shown that Esp expression is affected by changes in environmental conditions, with expression being highest in conditions that mimic the microenvironment of the human large intestines: 37°C and anaerobioses (51). In addition, bloodstream isolates of *E. faecium* enriched with *esp* appeared to have increased adherence to human colorectal adenocarcinoma cells (Caco-2 cells) (33). These data suggest a role of Esp in intestinal colonization. To

investigate the role of Esp in intestinal colonization, the Esp-deficient mutant and the Esp-expressing wild-type strain were tested for their ability to adhere to intestinal epithelial cells and intestinal colonization by using Caco-2 cells and a mouse model. Results demonstrated that Esp is not essential for intestinal colonization. When mice, kept under ceftriaxone treatment, were inoculated orally with either the wild-type or the Esp-deficient mutant, or with both strains simultaneously, both strains were able to colonize the murine intestines with high and comparable numbers and were able to translocate to the mesenteric lymph nodes. These *in vivo* results were supported by the *in vitro* data, which showed no differences in adherence to Caco-2 cells between the Esp-expressing *E. faecium* strain and its isogenic Esp-deficient mutant, although the wild-type strain exhibited a significantly higher level of adherence to Caco-2 cells than an *esp*-negative community surveillance feces isolate, a representative of the indigenous flora. This implies that other determinants present in hospital-acquired *E. faecium* strains contribute to adhesion to intestinal epithelial cells and intestinal colonization in hospitalized patients. Other possible factors implicated in intestinal colonization may include novel adhesins, like the novel cell surface proteins and pili recently identified (21-23,40,46,47), bacteriocins, factors that resist specific or non-specific host defence mechanisms, and/or the ability to use novel metabolic pathways, which could facilitate exploration of novel niches. It is interesting in this respect that in chapter 7 a novel genomic island highly specific for nosocomial *E. faecium* strains is described that tentatively encodes a novel sugar uptake system.

In **chapter 4** we demonstrated - for the first time - an important role for Esp in the pathogenesis of *E. faecium* infection, which unequivocally proves that *E. faecium* Esp is a virulence factor. In an experimental urinary tract infection (UTI) model in mice the Esp-deficient mutant was attenuated compared to the wild-type strain. When mice were challenged transurethrally with the wild-type or the Esp-deficient mutant, significantly higher CFU numbers of wild-type were cultured from kidneys and bladders, one and three days after induction of UTI compared to the Esp-deficient mutant strain. This was accompanied by a higher frequency of bacteremia, higher levels of proinflammatory cytokines in kidney tissue, and enhanced renal insufficiency displayed by increased plasma levels of creatinin and urea. The *in vivo* data were

supported by *in vitro* data demonstrating enhanced binding of the wild-type strain to bladder and kidney epithelial cells compared to the Esp-deficient mutant strain. The finding that the Esp-deficient mutant was still able to colonize kidneys and bladders, although to a significantly lesser extent than the wild-type strain, and could still be isolated five days after challenge, indicates that other additional determinants are also involved in colonization of the urinary tract. Additional factors that could be implicated in this process are a set of potential adhesins described recently, including novel cell surface proteins or pili (21,23,40,46,47).
Yet, in chapter 4 we failed to demonstrate a role of Esp in a non-lethal peritonitis model in mice, as no differences were found in the course of the infection between the wild-type and the Esp-deficient mutant strain and in the induction of inflammatory responses.

Using another infection model we were able to demonstrate that Esp also contributes to the pathogenesis of experimental endocarditis, as described in **chapter 5**. Rats were challenged intraventricularly via a catheter with the wild-type or Esp-deficient mutant. Heart valve vegetation recovered at autopsy 24 hours after inoculation contained significantly less Esp-deficient mutants than wild-types. Comparable with the UTI study, the Esp-deficient mutant was still able to colonize the heart valves 24 hours after inoculation, although to a significantly lesser extent than the wild-type strain, indicating that other determinants are also involved in experimental endocarditis. Again, these could include recently identified adhesins and pili (21,23,40,46,47). For one surface protein, Acm, its role in the pathogenesis of experimental endocarditis has already been demonstrated, both in starting and established vegetations (38).
Differences in levels of attenuation of the Esp-deficient mutant in models of intestinal colonization, UTI, peritonitis, and endocarditis suggest a niche specific role of Esp in the pathogenesis of *E. faecium* infections. The beneficial role of Esp in UTI and endocarditis indicates that Esp is especially involved in biofilm-associated diseases. Biofilms can develop to three-dimensional structures by a complex process that can be divided into relatively distinct phases of attachment, accumulation, maturation, and dispersal (8). The *in vitro* UTI data showed that Esp is already involved during

initial adherence to uroepithelial cells. The endocarditis *in vivo* data demonstrated attenuation of the Esp-deficient mutant 24 hours post-inoculation and low level colonization or no (detectable) colonization of the heart valves three hours post-inoculation for both the wild-type strain and the Esp-deficient mutant strain. Therefore, the contribution of Esp in different stages of biofilm formation remains to be determined. Different types of experiments, like monitoring gene expression of *E. faecium* during biofilm formation may provide further knowledge about the contribution of Esp to different stages of bacterial biofilm development.
In addition, in chapter 5, the serological response of patients to Esp was investigated by determining presence of antibodies against Esp in sera from patients with bacteremia and endocarditis caused by *esp*-positive *E. faecium* isolates. Anti-Esp antibodies were detected in bacteremia and endocarditis sera, indicating that at least in some patients Esp is expressed, recognized by the immune system, and that an anti-Esp response is elicited. This also indicates that Esp may serve as a target for immunotherapy. Alternative therapeutic options are desperately needed since effective antibiotic therapy of *E. faecium* infections is increasingly hampered by the multi-resistance nature of this nosocomial pathogen. Even resistance to recently developed antibiotics such as daptomycin, quinupristin/dalfopristin, linezolid, and tigecycline has already been reported (1,44,55,56) making *E. faecium* infections a clinical challenge in years to come. To investigate whether antibodies against Esp can confer protective immunity, vaccination studies were carried out in mice as described in the following chapter.

In **chapter 6**, the role of Esp in bacteremia was investigated by intravenous inoculation of the wild-type or the Esp-deficient mutant via the tail vein of mice. Kidneys obtained at autopsy 24 hours after inoculation contained significantly more wild-type isolates than Esp-deficient mutants, indicating that dissemination from the blood to the kidney occurs and that Esp, in accordance with the results of the UTI study (chapter 4), contributes to colonization and persistence in kidneys. In *E. faecalis* several mutants impaired in biofilm formation were also less virulent in a mouse bacteremia model (25,48), suggesting a correlation between the ability to form biofilm and prolonged bacteremia.

In addition, opsonophagocytic activity of the anti-Esp serum *in vitro* and protection of anti-Esp serum against *E. faecium* bacteremia *in vivo* were investigated in chapter 6. Anti-Esp serum neither exhibited opsonophagocytic activity by opsonic antibodies nor protected mice from *E. faecium* bacteremia. These findings indicate that Esp is involved in the pathogenesis of different *E. faecium* infections including UTI, endocarditis, and bacteremia but does not confer protective immunity, limiting its suitability as vaccine candidate. An alternative explanation for the absence of protective immunity could be that the rabbit anti-Esp serum used in these studies is raised against a part of Esp that lacks protective epitopes, as only a fragment of the *esp* gene, encoding a part of the N-terminal-end of Esp, was cloned and expressed in order to raise antibodies against Esp in rabbits (51). To further investigate this hypothesis, the whole *esp* gene should be cloned and expressed in order to get full length Esp with correct folding of the protein. Alternatively, a novel display system, previously described, could be used that allows efficient binding of heterologous proteins at the surface of killed non-recombinant *Lactococcus lactis*, designated gram-positive enhancer matrix (GEM) particles, by means of a peptidoglycan binding domain (4). Intranasal immunisation with GEM particles displaying two pneumococcal antigens, SlrA (streptococcal lipoprotein rotamase A) and IgA1 (IgA1 protease), offered significant protection against fatal pneumococcal pneumonia in mice (2). Other possible vaccine candidates might include the recently identified surface exposed proteins (23,38,46,47) or pili (21,47). Especially pili have been shown to be an attractive vaccine candidate in gram-positive pathogens (19,34). For example, a vaccine exclusively constituted by a combination of pilus components provided protection against group B streptococci in a mouse model (34).

In general, the UTI data as well as the peritonitis and bacteremia data demonstrate that *E. faecium*, both wild-type and Esp-deficient mutant, was effectively cleared in mice and rats. In the UTI model, kidneys and bladders were still positive for both *E. faecium* strains five days after infection, although the bacterial load was low for both strains. Furthermore, all urine samples were sterile after five days. In the peritonitis and bacteremia models colony counts of both strains were markedly reduced (compared to the inoculated numbers) in peritoneal lavage fluid and kidneys, indicating effective clearance of both strains by the immune system of mice and rats.

In humans, *E. faecium* infections are especially seen in immunocompromised and critically ill patients. The immune system of healthy people is apparently able to clear *E. faecium* strains or even never get challenged by these strains. Although mice and rats are widely used for *in vivo* immunological studies, it is difficult to extrapolate results obtained in animal studies to the human situation. Important differences in immune system development, activation, and response to infection exist between the animals used in our studies and human beings. Furthermore, we used single bolus inocula to cause infection, as has been done by most other researchers. However, in reality, humans are most often exposed to bacteria during longer periods with infection developing more gradually in time. These conditions are difficult to mimic in animal models. Our intestinal colonization model with subsequently induced peritonitis mimics the human situation better. The endogenous source of infection (i.e., the colonized intestinal tract) remains *in situ* and high numbers of wild-type bacteria and Esp-deficient mutants were still recovered ten days after inoculation.

So far, experiments were aimed to elucidate the role of Esp in the pathogenesis of *E. faecium* infections. Esp is one of the relatively few virulence determinants yet identified in *E. faecium*. In **chapter 7** the δρ-Web model (50), a fast and simple applicable computer program, was used to identify novel putative virulence genes acquired by nosocomial *E. faecium* through HGT, which are, therefore, recognizable by their anomalous GC-content and dinucleotide frequency (26,27). Based on this algorithm, a genomic island (GI) was identified, which appeared highly specific for hospital-acquired *E. faecium* isolates. GIs often carry genes with specific functions, such as metabolic activities, antibiotic resistance, or properties involved in microbial fitness, symbiosis, or pathogenesis (15,20). Therefore, acquisition of a GI may provide bacteria a selective advantage under specific environmental conditions. It may do so, by enhancing microbial transmission and providing bacteria new tools to explore and survive novel ecological niches. Considering the putative functions of the predicted GI proteins, this GI may represent a metabolic island involved in carbohydrate transport and metabolism providing hospital-acquired *E. faecium* alternative metabolic pathways of energy production. Bacteria that colonize the large intestines have access to non-digestible dietary polysaccharides (18) and heavily

glycosylated mucins produced by intestinal globet cells (43). Relatively few enteric bacteria are able to degrade mucins, because these compounds are inherently resistant to bacterial enzymatic degradation. The presence of this GI may provide hospital-acquired *E. faecium* additional sugars for growth by increasing its ability to compete for different kinds of non-digestible polysaccharides and mucins. Besides the importance of sugars as a source of energy, sugars are also involved in capsular polysaccharides, virulence gene induction, and biofilm formation (6,7,10,24,25,45). Right now, we cannot prove that this GI contributes to increased fitness and enhanced survival of hospital-acquired *E. faecium*. Therefore, a GI knock-out strain should be constructed and tested in *in vitro* growth models using different sugars and mucins and *in vivo* in an intestinal colonization model as described in chapter 3.

In **conclusion**, by using different animal models mimicking different *E. faecium* infections our knowledge about the role of Esp in the pathogenesis of *E. faecium* infections has improved considerably. The importance of *E. faecium* Esp in UTI and endocarditis probably results from the fact that these models represent typical biofilm-associated infection models indicating a niche specific role of Esp in the pathogenesis of *E. faecium* infections.

The last two decades incidences of *E. faecium* colonization and infections dramatically increased in hospitalized patients all over the world. Most probably, this rise in incidence of nosocomial *E. faecium* resides in an accumulation of a large number of genes and genetic elements that increase fitness and enhance survival of *E. faecium* in the hospital. Until now, only few genes (potentially) involved in pathogenicity of *E. faecium* have been identified. However, ongoing developments in whole-genome sequencing techniques and molecular tools of mutagenesis will allow genome-wide studies in which more genes and genetic elements that are important for *in vivo* fitness of *E. faecium* can be identified. Ongoing selection for this organism, importantly driven by antibiotic use, may eventually lead to the acquisition of additional virulence determinants, which could further increase *E. faecium* colonization and infection rates, a concept referred to as genetic capitalism (3). The work described in this thesis helped to increase our understanding in the function of Esp and identified a novel genomic island possibly implicated in *E. faecium* fitness. With the limited therapeutic

options available and the expanding population of immunocompromised patients, *E. faecium* will increasingly become a clinical challenge. Improved understanding of the biology and virulence properties of this species will contribute to the development of novel intervention strategies, which may help us to prevent spread and infections of *E. faecium* in hospitals.

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Nederlandse samenvatting

Eenvoudig verteld

Enterococcus faecium werd vroeger gezien als onschuldige darmbacterie. In de afgelopen twee decennia heeft een specifieke *E. faecium* variant zich echter ontwikkeld tot een gevaarlijke ziekenhuisbacterie en is het aantal infecties met deze variant wereldwijd in ziekenhuizen drastisch toegenomen. Deze ziekenhuisvariant onderscheidt zich van de ongevaarlijke *E. faecium* die normaal in de darm voorkomen door de aanwezigheid van resistentie tegen veel verschillende antibiotica, waardoor behandeling van infecties moeilijk is, en specifieke genen die potentieel bijdragen aan ziekmakende eigenschappen. Één van deze genen codeert voor het oppervlakte eiwit Esp. Het onderzoek beschreven in dit proefschrift heeft zich gericht op het ontrafelen van de rol van Esp in verschillende experimentele infecties. Gebleken is dat Esp een belangrijke rol speelt bij aanhechting en de vorming van biofilms, een ophoping van bacteriën vaak afgeschermd met een slijmlaag waardoor bacteriën in een biofilm het menselijk afweersysteem en antibiotica beter kunnen weerstaan. Zowel aanhechting als biofilm vorming zijn cruciale processen die de bacterie in staat stelt infecties te veroorzaken. Tevens is uit dit onderzoek gebleken dat Esp een belangrijke bijdrage levert aan de pathogenese van urineweg en bloedbaan infecties en endocarditis, een ontsteking aan de hartkleppen. De resultaten beschreven in dit proefschrift kunnen bijdragen aan de ontwikkeling van nieuwe medicijnen en vaccins om infecties met deze *E. faecium* ziekenhuisbacterie te behandelen en verspreiding in ziekenhuizen te voorkomen.

Nederlandse samenvatting

Enterococcus faecium werd jarenlang gezien als onschuldige darmbacterie van mens en dier, maar heeft zich de afgelopen 25 jaar wereldwijd ontwikkeld tot nosocomiaal pathogeen die resistent is tegen vele antibiotica. De eerste ontdekking van vancomycine-resistente *E. faecium* (VRE) in 1986 en vooral de daarop volgende stijging van het aantal patiënten gekoloniseerd of geïnfecteerd met VRE, heeft de interesse voor *E. faecium* drastisch doen toenemen. VRE werd in eerste instantie alleen gezien in Amerikaanse ziekenhuizen, echter sinds 2000 is er een stijging van het aantal VRE infecties ook in Europa, Azië en Australië. Uit retrospectief onderzoek blijkt dat de toename van VRE in de jaren ’90 van de vorige eeuw in Amerikaanse ziekenhuizen vooraf werd gegaan door een stijging in het aantal infecties met ampiciline-resistente *E. faecium* (ARE), vanaf 1980. Deze toename van ARE infecties vond in Europa 10 jaar later plaats. Ook in Nederland ziet men in de ziekenhuizen een sterke toename in het aantal invasieve infecties en het aantal patiënten gekoloniseerd met ARE. Deze multi-resistente *E. faecium* kunnen verschillende soorten infecties veroorzaken zoals urineweg-, lijn- en wondinfecties en endocarditis. Uit moleculair epidemiologisch onderzoek is gebleken dat het overgrote deel van deze multi-resistente *E. faecium* behoren tot een specifieke polyclonale subpopulatie, ook wel complex 17 (CC17) genoemd, die zich genetisch onderscheidt van *E. faecium* stammen die normaal in de darm voorkomen. Naast de aanwezigheid van multi-resistentie tegen vele antibiotica, heeft deze *E. faecium* ziekenhuisvariant meer dan 100 genen die potentieel bijdragen aan virulentie en die ontbreken bij normaal in de darm voorkomende *E. faecium* stammen. Potentiële virulentie genen die zijn gevonden in deze *E. faecium* ziekenhuisvariant zijn onder andere genen die coderen voor oppervlakte eiwitten en pilus-structuren. Van beiden wordt gedacht dat ze een belangrijke rol spelen bij het veroorzaken van infecties doordat ze aanhechting van de bacterie aan humane gastheercellen/componenten bewerkstelligen. Één van de oppervlakte eiwitten is het enterokokken oppervlakte eiwit Esp.

Esp is gelokaliseerd op een genetisch element dat alle eigenschappen heeft van een pathogeniciteitseiland en genen bevat die coderen voor transposases, transcriptionele regulatoren en virulentie determinanten. *Enterococcus faecalis*

bevat ook een *esp* gen, welke 90% identiek is aan het *E. faecium esp* gen en ook gelegen is op een pathogeniciteitseiland wat verder echter heel verschillend is van het *E. faecium* eiland. Uit onderzoek is gebleken dat Esp van *E. faecalis* betrokken is bij kolonisatie van de urineweg en biofilm vorming. Naast aanhechting aan gastheercellen en componenten, is biofilm vorming een belangrijke eigenschap die de bacterie in staat stelt infecties te veroorzaken. Microbiële biofilms zijn populaties van micro-organismen omgeven door een extracellulaire polymere matrix, voornamelijk bestaand uit polysaccharides. De biofilm schermt de bacterie af van antibiotica en de humane afweer.
Tot nu toe is weinig bekend over de rol van *E. faecium* Esp. Onderzoek heeft laten zien dat het *esp* gen specifiek voorkomt in de *E. faecium* ziekenhuisvariant en nauwelijks daarbuiten. Tevens heeft men aangetoond dat expressie van Esp is geassocieerd met initiële aanhechting aan en biofilm vorming op plastic materiaal. Al deze bevindingen wijzen erop dat Esp mogelijk een belangrijke virulentie factor is voor *E. faecium* en bijdraagt aan de pathogenese van infecties. Het onderzoek beschreven in dit proefschrift heeft zich gericht op het ontrafelen van de rol van Esp in verschillende infecties. Onderzoek naar de rol van Esp kan leiden tot nieuwe inzichten in hoe *E. faecium* infecties initieert en zijn omgeving beïnvloedt. Verbeterde kennis kan mede leiden tot de ontwikkeling van nieuwe therapie strategieën om infecties met deze multi-resistente bacteriën te behandelen en verdere verspreiding in de ziekenhuizen te voorkomen.

In **hoofdstuk 2** van dit proefschrift tonen we via de constructie van een Esp mutant voor de eerste keer aan dat Esp van *E. faecium* daadwerkelijk betrokken is bij biofilm vorming. Het maken van mutanten in *E. faecium* is niet een routinematige handeling zoals in andere Gram-positieven, maar is gecompliceerd door de afwezigheid van goede genetische 'tools'. Nallapareddy en zijn collega's waren de eersten die succesvol een mutant in een klinisch *E. faecium* isolaat maakten. Door het gebruiken van dezelfde techniek, is het ons gelukt om de tweede *E. faecium* mutant te construeren, een Esp mutant. Inactivatie van het *esp* gen resulteerde in afwezigheid van Esp aan het oppervlak van de bacterie, significant lagere initiële binding aan polystyreen en gereduceerde biofilm vorming vergeleken met de wild-type stam die Esp aan het

oppervlak heeft. Complementatie van de Esp mutant met het *esp* gen resulteerde in herstel van initiële binding en biofilm vorming wat aangeeft dat inactivatie van het *esp* gen verantwoordelijk is voor het veranderde fenotype van de Esp mutant en dat niet het gevolg was van polaire effecten. Initiële binding en biofilm vorming waren niet compleet gereduceerd in de Esp mutant, wat aangeeft dat behalve Esp ook andere determinanten een rol spelen in deze processen.

Zoals boven vermeld, zijn binding en biofilm vorming cruciale eigenschappen voor een bacterie om infecties te veroorzaken. De aanwezigheid van biofilm is aangetoond in verschillende *E. faecium* gerelateerde infecties zoals endocarditis, urineweg-, oog-, en wortelkanaalinfecties en infecties waarbij biofilms werden gevonden op katheters, lijnen en prothesen. Om de rol van Esp te onderzoeken in de pathogenese van *E. faecium* infecties, werden de Esp mutant en de wild-type stam getest in verschillende experimentele diermodellen, waarin darmkolonisatie, endocarditis, urineweginfectie, bacteriëmie en peritonitis werden nagebootst.

In **hoofdstuk 3** werd de rol van Esp in darmkolonisatie onderzocht. Recente studies hebben aangetoond dat darm kolonisatie met de *E. faecium* ziekenhuisvariant op sommige afdelingen 40% bedraagt, terwijl in gezonde mensen kolonisatie van deze variant bijna afwezig is. Vanuit het maag-darmstelsel kan *E. faecium* de huid en vervolgens wonden en katheters koloniseren, wat kan resulteren in infectie en verspreiding van deze bacterie tussen patiënten. Daarnaast biedt het maag-darmstelsel een optimaal milieu voor bacteriën om genetisch materiaal, als resistentie genen, uit te wisselen. De *E. faecium* ziekenhuisvariant draagt een groot aantal virulentie en resistentie genen en vormt daarmee een potentieel gevaar als verspreider van deze genen naar andere, misschien meer pathogene bacteriën. Vanwege bovengenoemde redenen is het daarom van groot belang om meer kennis te verkrijgen over darmkolonisatie van *E. faecium* en de determinanten te identificeren die verantwoordelijk zijn voor dit proces.
Esp expressie is het hoogst bij 37°C onder anaerobe condities, beiden omgevingsfactoren die worden gevonden in de humane dikke darm. Daarnaast blijkt uit literatuur dat *esp*-positieve *E. faecium* stammen geïsoleerd uit het bloed een

verhoogde binding aan humane colorectale adenocarcinoma (Caco-2) cellen hebben in vergelijking met *esp*-negatieve bacteriestammen. Deze data suggereren een rol van Esp in darmkolonisatie. Om dit te onderzoeken werden muizen, behandeld met ceftriaxon, oraal geïnoculeerd met de Esp mutant of de wild-type stam. Beiden stammen waren even goed in staat de darm te koloniseren. Ook was er geen verschil tussen de Esp mutant en de wild-type stam in binding aan Caco-2 cellen. Deze gegevens laten zien dat Esp niet betrokken is bij darmkolonisatie en dat andere determinanten van *E. faecium* betrokken zijn bij dit proces.

In **hoofdstuk 4** lieten we voor de eerste keer zien dat Esp een belangrijke rol speelt in de pathogenese van een *E. faecium* infectie. Wanneer muizen via de urethra werden geïnfecteerd met de Esp mutant of de wild-type stam werden significant meer wild-type bacteriën terug gevonden in het nier- en blaasweefsel, één en drie dagen na infectie, dan Esp mutanten. Daarbij kon de wild-type bij meer muizen uit het bloed worden teruggekweekt en konden in muizen geïnfecteerd met de wild-type stam, hogere cytokine niveaus worden aangetoond en was er in grotere mate sprake van nierinsufficiëntie. Ook *in vitro* werd een significant hogere binding van wild-type bacteriën aan blaas- en niercellen gezien vergeleken met de Esp mutanten. Dat ook de Esp mutant in staat is de urineweg te koloniseren, echter significant minder dan de wild-type stam, geeft aan dat naast Esp ook andere determinanten een rol spelen in de pathogenese van urineweginfecties. Deze andere determinanten zouden pili of andere oppervlakte eiwitten kunnen zijn.
In hoofdstuk 4 lieten we tevens zien dat Esp geen rol speelt in experimentele peritonitis. Er werden geen verschillen gevonden in het beloop van, of de ontstekingsreactie tijdens, een peritonitis veroorzaakt door de Esp mutant of de wild-type bacterie in muizen.

In **hoofdstuk 5** werd de rol van Esp in endocarditis, een ontsteking aan de hartkleppen, bestudeerd. Ratten werden via een katheter intraventriculair geïnfecteerd met de wild-type of de Esp mutant. Na 24 uur werden er significant meer wild-type bacteriën terug gevonden in de vegetaties, afgeschraapt van de hartkleppen, dan Esp mutanten. Net als in het urineweginfectie model was de Esp mutant ook hier in staat

tot kolonisatie, echter in veel mindere mate dan de wild-type. Dit geeft aan dat ook in de pathogenese van endocarditis naast Esp andere determinanten een rol spelen. Een determinant waarvan reeds is bewezen dat deze een rol speelt in endocarditis, is het oppervlakte eiwit Acm.

De verschillende uitkomsten die worden gezien in de hierboven beschreven modellen suggereren een 'niche' specifieke rol van Esp in de pathogenese van *E. faecium* infecties. Zowel urineweginfectie als endocarditis zijn biofilm geassocieerde infecties. Het is daarom aannemelijk dat Esp met name een rol speelt in biofilm gerelateerde infecties.

In hoofdstuk 5 lieten we tevens zien dat patiënten die een bacteriëmie of een endocarditis hebben doorgemaakt, veroorzaakt door een *esp*-positieve *E. faecium* stam, antilichamen tegen Esp hebben. Dit bewijst dat Esp *in vivo* in de mens tot expressie komt en dat een immuun reactie tegen dit oppervlakte eiwit wordt opgewekt. Sinds effectieve therapie met antibiotica steeds moeilijker wordt door de toenemende resistentie bij *E. faecium*, wordt gezocht naar alternatieve therapieën zoals immunotherapie. Om te onderzoeken of Esp mogelijk een kandidaat zou kunnen zijn voor immunotherapie, werd een vaccinatie studie uitgevoerd.

Allereerst werd in **hoofdstuk 6** gekeken of Esp een rol speelt bij bloedbaan infecties in een sepsis model in muizen. Daartoe werden muizen via de staart vene geïnfecteerd met wild-type of Esp mutant bacteriën. Na 24 uur werden meer wild-type bacteriën terug gevonden in de nieren dan Esp mutanten. Deze resultaten laten zien dat verspreiding vanuit de bloedbaan naar de nieren plaats vindt en dat Esp een bijdrage levert aan de kolonisatie van de nieren, zoals reeds gezien werd in het urineweginfectie model.

Daarna werd de opsoniserende werking van anti-Esp serum en de beschermende rol van dit serum door middel van passieve immunisatie in het sepsis model getest. Anti-Esp serum bleek geen opsoniserende werking te hebben en tevens geen bescherming te bieden in het sepsis model. Een verklaring zou kunnen zijn dat het anti-Esp serum wat gebruikt werd, is opgewekt tegen een deel van het Esp eiwit wat mogelijk geen beschermende epitopen draagt. Om deze hypothese verder te kunnen onderzoeken moeten antilichamen opgewekt worden tegen het gehele Esp eiwit.

De hoofdstukken dusver beschrijven de rol van Esp in verschillende infectiemodellen. Tot nu toe zijn er naast Esp relatief weinig virulentie determinanten geïdentificeerd in *E. faecium*. In **hoofdstuk 7** hebben we een makkelijk toepasbaar computer programma gebruikt, genaamd 'δρ-web model', om nieuwe potentiële virulentie factoren te identificeren. Dit model kan aan de hand van abnormale GC percentages en dinucelotide frequenties genen herkennen welke zijn verkregen door horizontale gen overdracht. Met behulp van dit model werd een genen cluster gevonden dat alleen voorkomt in de *E. faecium* ziekenhuisvariant en in slechts zeer geringe mate bij *E. faecium* stammen die normaal in de darm voorkomen. Op grond van DNA homologie is dit genen cluster mogelijk betrokken bij suiker transport en metabolisme. Hypothetisch zou dit genen cluster van voordeel kunnen zijn voor de *E. faecium* ziekenhuisvariant doordat dit genen cluster mogelijk een extra/alternatieve route voor energie productie biedt.

Concluderend hebben de studies beschreven in dit proefschrift ons meer inzicht gegeven over de rol van Esp in de pathogenese van verschillende *E. faecium* infecties. Daarnaast hebben we een genen cluster ontdekt wat specifiek voorkomt in de *E. faecium* ziekenhuisvariant en potentieel een voordeel biedt in overleving. Toenemende kennis over de biologie en virulentie eigenschappen van *E. faecium* kunnen bijdragen aan de ontwikkeling van nieuwe therapie strategieën om infecties met deze ziekenhuisbacterie te behandelen en verspreiding in de ziekenhuizen te voorkomen.

A

Appendix

Dankwoord / Acknowledgements

List of publications

Curriculum vitae

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Esther

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Curriculum Vitae

Esther Heikens was born November 18th 1976, Zwolle, The Netherlands. From 1989 to 1995 she followed her secondary education at the Stedelijk Gymnasium in Apeldoorn. Upon graduation from secondary school in 1995 she started Veterinary Sciences at the University of Gent, Belgium. In 1996 she made a move to Medical Biology at the University of Amsterdam. During Medical Biology two research internships were taken. In 1999 she performed an internship at the Department of Surgery at the Academic Medical Center (AMC) in Amsterdam on 'Complement activation in the liver during ischemia-reperfusion' under supervision of prof. dr. T.M. van Gulik, dr. I.H. Straatsburg, and dr. B.H. Heijnen. In 2000 a second internship was done at the Department of Pathology at the AMC on 'The role of Cd44 splice variants in wound healing of the skin' under supervision of prof. dr. S.T. Pals and dr. R. van der Neut. Upon completion of this internship, she did her MSc thesis titled 'The role of adhesion molecules and chemokines in homing of lymphocytes' at the same department under supervision of prof. dr. S.T. Pals. In 2000 she received her master's degree in Medical Biology and started Medical School at the University of Utrecht. In 2003 she performed a research internship at the Department of Microbiology at the University Medical Center (UMC) in Utrecht on 'Comparison of genotypic with phenotypic methods for identification of coagulase-negative staphylococci' under supervision of dr. A. Fleer, dr. A.C. Fluit, and dr. A. Paauw. In her last year of Medical School she did another research internship at the Department of Microbiology at the UMC on 'Cloning and expression of three genes encoding putative cell surface proteins specifically enriched in Enterococcus faecium CC17 isolates' under supervision of prof. dr. M.J.M. Bonten, dr. R.J.L. Willems, and dr. A.P.A. Hendrickx. After receiving her medical degree in 2006, she started her PhD project at the Department of Medical Microbiology at the UMC under supervision of prof. dr. M.J.M. Bonten and dr. R.J.L. Willems. In 2008 she received an ESCMID grant for training in foreign laboratories and fulfilled a part of her PhD project at the Department of Internal Medicine at the University of Texas Medical School, Houston, Texas, United States, under supervision of prof. B.E. Murray and dr. K.V. Singh. In December 2007 she commenced her residency in Medical Microbiology at the Department of Medical Microbiology at the UMC.