Cell Wall-anchored Proteins of *Enterococcus faecium*: Exploring a Novel Surface

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PhD Thesis University Utrecht, The Netherlands.

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A glimpse on the surface of *Enterococcus faecium*

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Cell Wall-anchored Proteins of *Enterococcus faecium*: Exploring a Novel Surface

Celwandverankerde Eiwitten van *Enterococcus faecium*: Verkenning van een Nieuw Oppervlak

(met een samenvatting in het Nederlands)

Proefschrift

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General introduction

Adapted from "LPXTG Surface Proteins of Enterococci".

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General introduction into enterococci.

Enterococci are Gram-positive facultative anaerobic lactic acid ovococci, that occur singly, in pairs or in short chains and are ubiquitous in nature, where they live in the gastrointestinal tracts of humans and animals as well as in soil, water and food[1]. Enterococci are catalase-negative organisms and able to withstand harsh environmental conditions as they are able to grow in 6.5% NaCl, in 40% bile salts, in a wide range of pH values and at temperatures between 10°C and 45°C, with an optimum at 35°C[2]. Among the more than 30 enterococcal species, *Enterococcus faecalis* and *Enterococcus faecium* are most commonly encountered in humans. They account for 1% of the large intestinal microflora, which makes them minor players in the distal colon. Enterococci also have the ability to survive on inanimate objects for weeks, which may facilitate adaptation to any environment and may have contributed to their nosocomial transmission[3,4].

Emergence of multi-antibiotic resistant enterococci.

Traditionally, enterococci were regarded as relatively harmless inhabitants of the gut flora, only sporadically associated with opportunistic infections, including bacteremia, surgical site infections, endocarditis, urinary tract infection and peritonitis among critically ill patients[5]. However, the first isolation of 57 vancomycin-resistant enterococci (VRE) (50 *E. faecium* and 5 *E. faecalis*) in the United Kingdom and France in 1986 boosted public and clinical interest, since therapeutic options for treating infections with VRE were almost exhausted[6,7]. Since then, VRE have emerged globally, though with some remarkable epidemiological differences between continents. In the USA, VRE epidemiology was initially characterized by sporadic nosocomial outbreaks. During the 1990s the prevalence of VRE among enterococcal bloodstream infections in intensive care units, but also among non-intensive care unit patients such as those receiving hemodialysis or in hematology-oncology departments[8], increased rapidly leading to VRE endemicity in US hospitals and long-stay health care facilities[9]. Currently, in the US 30% of nosocomial enterococcal infections are caused by VRE[10]. Higher antibiotic use, specifically higher use of cephalosporines and a five to ten-fold higher use of vancomycin, compared to some European countries, might have contributed to the observed differences in VRE prevalence in the US and most of the European countries[11-13]. In contrast, in Europe VRE were initially identified from a community-reservoir (healthy humans and animals), which presumably resulted from the widespread use of avoparcin as growth promoter in animal husbandry. Avoparcin is a glycopeptide antibiotic that may cause cross-resistance against vancomycin. After the use of avoparcin was banned in Europe in 1997, prevalence of VRE in the community declined. However, VRE prevalence rates in European hospitals emerged since the start of this millennium, even in countries with traditionally low antimicrobial agent resistance levels[14-18]. Nosocomial VRE outbreaks are also reported with increasing frequency from other parts of the world, including Latin-America, Asia and Australia[19-22]. Importantly, the VRE pandemic was

preceded by the emergence of ampicillin-resistant *E. faecium* (ARE) in the early 80s in the USA, which was followed by the rapid emergence of ARE in several European countries, 10 years later[23-27].

Rates of colonization and infection with VRE increased steadily for years, and now, in the US, *Enterococcus* spp. rank third among the leading causes of healthcare-associated infections of humans, which are frequently associated with intravascular devices, such as cathethers and stents[10]. Traditionally, the majority of clinical enterococcal infections were caused by *E. faecalis* (80-90%), while *E. faecium* was found less frequently (10%)[28-31]. Yet, after the turn of the century the proportion of *E. faecium* gradually increased to 40%, thereby partly replacing *E. faecalis*[32-36]. This shift in the number of enterococcal infections in the last 20 years has partly been attributed to changes in medical care, *e.g.* increasing numbers of immunocompromized and critically ill patients and the increased use of indwelling medical devices. In addition, the intrinsic resistance to abundantly used antibiotics like aminoglycosides and cephalosporins, as well as the abilities to acquire resistance genes like vancomycin resistance transposons, probably contributed to the increase of enterococcal infections[37,38]. This resulted in an almost complete penetration of ampicillin and vancomycin resistance, especially in *E. faecium*, with 90% and 80% of all *E. faecium* isolates harvested from infections being resistant towards ampicillin and vancomycin, respectively[10,35,39]. In contrast, ampicillin and vancomycin-resistance is far less prevalent in *E. faecalis*, with only 0.5% to 7% of all health-care associated *E. faecalis* isolates being vancomycin-resistant[10,35,39]. The reasons for this difference between both species are not well understood.

Evolutionary development of hospital-adapted *E. faecium*.

Enterococci are not true pathogens such as *Bacillus anthracis* or *Corynebacterium diphtheriae*, which are capable of causing disease in otherwise healthy persons. As stated above, specifically patients with an impaired immune system are susceptible for enterococcal infections. This implies that the pathogenicity of enterococci most probably resides in a combination of factors that enhance fitness, such as multi-antibiotic resistance determinants and proteins involved in handling environmental stresses, as well as in adhesion[40]. Examples of putative virulence determinants of *E. faecalis* are a bile salt hydrolase, the cytolysin toxin, a gelatinase, capsular polysaccharides, glycolipids, the microbial surface component recognizing adhesive matrix molecules (MSCRAMM) Ace, enterococcal surface protein Esp, surface exposed fibres called Ebp-pili, and aggregation substance[41-45].

For *E. faecium* the list of putative virulence determinants is much shorter. Population biological studies using amplified fragment length polymorphism analysis, multiple-locus variable-number tandem repeat analysis and multilocus sequence typing documented the existence of a specific genetic subpopulation in *E. faecium*, also referred to as clonal complex-17, responsible for the majority of hospital infections and nosocomial outbreaks[46]. Based on comparative genomic

hybridization data, this subpopulation of hospital-adapted *E. faecium* appeared to share an important part of its accessory genome content (> 100 genes), including genes encoding resistance to ampicillin and ciprofloxacin[47]. Other genes specifically found in this subpopulation included genes encoding novel metabolic pathways, and several putative virulence genes, such as the gene encoding the enterococcal surface protein Esp, implicated in biofilm formation and contained on a putative pathogenicity island[48-50], hyl_{Efm}, encoding a putative hyaluronidase[51], genes encoding surface-exposed LPXTG-like cell-wall-anchored proteins and genes required for the biogenesis of pili (see below)[52,53]. The large number of acquired elements and the large differences in GC-content of these genes suggest that acquisition of these adaptive elements has been a multi-step process in which *E. faecium* progressively evolved from a commensal of the normal human gut microbiota to a nosocomial pathogen.

This thesis is focussed on surface proteins of *E. faecium*, as they are thought to play an important role in the pathogenesis of *E. faecium* infections. Elucidating functions and make-up of surface proteins may lead to novel insights into how enterococci initiate disease and interact with their environment. In the following paragraphs existing knowledge on enterococcal surface proteins is summarized.

Surface proteins of enterococci: attachment of LPXTG surface proteins to the cell wall.

A well studied class of surface proteins in Gram-positive bacteria are the cell wall-anchored Leu-Pro-X-Thr-Gly (LPXTG) proteins. The prototype LPXTG-like surface protein contains an N-terminal signal sequence peptide to allow Sec-dependent translocation of the precursor surface protein across the plasma membrane and a C-terminal cell wall sorting signal. The cell wall sorting signal is comprised of a conserved LPXTG sortase substrate motif (where X denotes any amino acid) followed by a hydrophobic domain and positively charged amino acids. After translocation, the LPXTG-motif is cleaved between the threonine (T) and glycine (G) residue by a class A sortase, which covalently immobilizes the cleaved surface protein via the threonine residue to the amino group of the cross bridge within the peptidoglycan by transpeptidase activity[54].

Aggregation substance, a multifunctional surface protein implicated in virulence of *E. faecalis*.

Aggregation substance (AS) was the first described LPXTG surface protein in enterococci and it is a well characterized adhesin with respect to its many functions and contribution to the virulence of *E. faecalis*. The genes *asp1*, *asc10*/*prgB* and *asa1* encode the AS proteins from the three most-studied pheromone-inducible conjugative plasmids pPD1, pCF10, and pAD1, respectively. The expression of the AS genes is induced by an oligopeptide autocrine pheromone, which is secreted by other enterococci[55,56]. Expression of AS at the surface of the cell directs tight physical

contact between donor and recipient cell, thereby allowing the high frequency transfer of virulence plasmids. AS contains a lipoteichoic acid (LTA) aggregation domain, a variable region, a central aggregation domain and two Arg-Gly-Asp (RGD) motifs (Figure 1). The three AS proteins share over 90% amino acid sequence identity throughout most of the protein, although a centrally located variable region displays only 30 to 40% similarity. Biochemical studies on AS have long been hampered by the inability to purify AS as a full-length protein. However, it was demonstrated that purified full-length Asc10 and the LTA aggregation domain located close to the N-terminus of Asc10 bound to *E. faecalis* LTA in a dose-dependent manner, whereas the central aggregation domain did not[57]. Furthermore, in frame deletions in *prgB* revealed that the LTA aggregation domain was not only required for LTA binding, but also for internalization into HT-29 enterocytes[58]. No structure of AS has been revealed, thus the mechanism by which AS binds to its ligand remains to be determined. AS is also involved in adhesion and invasion of cells derived from the colon and duodenum indicating that AS may play a role in the translocation of *E. faecalis* through the intestinal wall, leading to systemic infection[59,60]. Asc10 increased adherence, internalization and intracellular survival in polymorphonuclear leukocytes[61,62]. It has been suggested that the RGD motifs of AS mediate the interactions with eukaryotic cells, since binding of *E. faecalis* was reduced when the eukaryotic cells were pre-incubated with a chemically synthesized RGDS peptide or with monoclonal antibodies to CR3, CD47 and L-selectin, suggesting that these proteins may be recognized by AS[62,63]. Similarly, Asa1 increases the adherence to and survival inside macrophages and mediates adherence to renal tubular epithelial cells[63].

Both Asc10 and Asa1 have been implicated in binding to components of the extracellular matrix (ECM), such as fibrin, fibronectin, thrombospondin, vitronectin and collagen type I. Deletions in the variable region of the *asa1* gene lead to reduced binding to fibronectin, suggesting the variable region is an ECM binding domain[64]. In contrast, purified full length Asc10 protein did not bind to ECMs, but a mutant Asc10 protein lacking the N-terminal aggregation domain did bind to fibrinogen as well as to vitronectin. An *E. faecalis* strain carrying plasmid pCF500, a derivative of pCF10, and expressing AS did not contribute to urinary tract colonization in a mouse model of upper and lower urinary tract infection[65]. Asc10 and Asa1 have been shown to play a role in experimental endocarditis (a severe infection caused by biofilm vegetation on heart valves), where the proteins increase the severity of *E. faecalis* vegetations on rabbit aortic heart valves[66]. Analysis of an N-terminal aggregation domain deletion and a double RGD mutant of *asc10/prgB* in a rabbit model for endocarditis, showed significant reduction in virulence. Active immunization of rabbits with an N-terminal fragment of AS did not elicit protective immunity when rabbits were challenged in an endocarditis model[67]. In conclusion, both *in vitro* and *in vivo* data indicate that AS is a multifunctional and prominent virulence factor of *E. faecalis*.

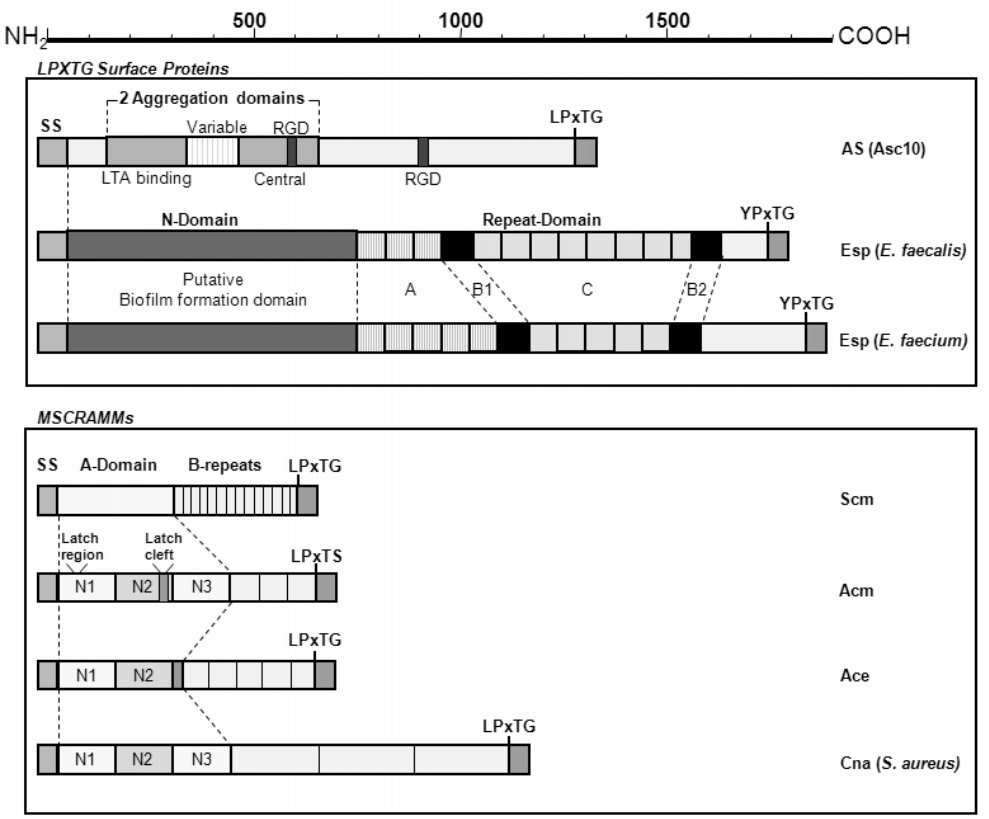

Figure 1. LPxTG surface proteins and MSCRAMMs of enterococci. Structural organization of the LPxTG surface proteins (upper part) and MSCRAMMs (lower part) of enterococci. Signal sequences (SS) are depicted in dark green and start at the N-terminus. The LPxTG-like cell wall sorting signal is marked red. The N-terminal domain of Esp of *E. faecium* and *E. faecalis* is depicted in purple and the aggregation domains of AS are in blue. The RGD motifs, implicated in binding to eukaryotic cells are depicted in dark blue. The collagen binding A-domains of the enterococcal MSCRAMMs are yellow and divided into subdomains, designated N1, N2 and N3 each adopting a variant of the IgG-like fold. Repeat regions of the MSCRAMMs are depicted in light blue. A latching cleft identified in Acm and Ace is depicted in grey. The scale starts at the N-terminal end of the protein and ends at the C-terminus, with the number of amino acids indicated. See appendix for color figure.

The enterococcal surface protein is a virulence factor involved in biofilm formation.

Clinically relevant *E. faecalis* and *E. faecium* both harbor a different pathogenicity island containing the *esp* gene. The Esp proteins are large enterococcal cell wall-anchored proteins, with a molecular weight of approximately 200 kDa. Clinical isolates of *E. faecalis* and *E. faecium* carry the *esp* gene more frequently than non-clinical isolates, which suggests that Esp has a role in nosocomial infections[43,49]. The overall amino acid identity between the Esp proteins of *E. faecium* and *E. faecalis* is high (90%) and strongly suggests that they share similar functions in

both organisms. They both contain an N-terminal signal sequence and a variable N-terminal domain of approximately 700 amino acids, followed by three repeat domains termed A, B and C (Figure 1). The number of A, B, and C repeats in Esp varies among isolates[43,49]. The C-terminal end of Esp is formed by a (Y/F)PXTG motif, which can presumably be recognized by sortase, leading to the cell wall-anchoring of Esp. The Esp proteins exhibits similar global organization to a group of surface proteins (alpha, Rib, Bap) in several Gram-positive bacteria, including pathogenic streptococci and *Staphylococcus aureus*. The biological function of this group of proteins is poorly defined, although there are indications that these proteins play a role in modulating the immune response to the bacterial cell[68]. Whether Esp also protects enterococci against the host immune system, remains to be determined.

The functional characterization of Esp has focused on the role of this surface protein in biofilm formation. Insertional inactivation of the *esp* gene of both *E. faecalis* and *E. faecium* revealed that Esp is involved in initial adherence and 24-hours biofilm formation on abiotic surfaces[48,69]. It should be noted that in *E. faecalis* some controversy exists concerning the role of Esp in biofilm formation[70]. We interpret these data as evidence that Esp is not the sole determining factor for biofilm formation in *E. faecalis* and that several other determinants, such as surface charge[71], glycolipids[41] and the presence of Ebp pili[44] (see below) also play important roles. Analysis of wild-type Esp expressing strains and isogenic mutants in murine intestinal colonization models failed to demonstrate that Esp is involved in gut colonization or adhesion to human colorectal adenocarcinoma cells[72,73], despite the fact that Esp is highly expressed at 37°C and under anaerobic conditions[50]. When mice were challenged transurethally with an Esp positive strain and its isogenic Esp-deficient mutant, significantly less Esp-deficient *E. faecalis* were recovered from urine and murine bladders, demonstrating a role of Esp during urinary tract infection[74]. Possibly, Esp of *E. faecium* may fullfill a similar role during urinary tract infections and because of its important contribution to the ability to form biofilm, it may be involved in other enterococcal infections in which biofilms are implicated, such as infective endocarditis.

The exact functions of the different domains of Esp remain to be determined. There is some evidence that the N-terminal domain of Esp has an important role in the formation of biofilms. A mutant *E. faecalis* strain expressing Esp without its N-terminal domain was significantly impaired in biofilm formation compared to the wild-type strain[75]. In addition, a mutant which only expressed the N-terminal domain of Esp at the surface produced wild-type-like biofilms. In line with these results, addition of the N-terminal domain of Esp of *E. faecium* to cells which highly express Esp, blocked primary attachment of the cells to polystyrene[50]. Taken together, these data indicate that the N-terminal domain may contribute to Esp-mediated biofilm formation and that Esp is important for enterococcal urinary tract infections.

Enterococcal collagen-binding MSCRAMMs.

Colonization of human tissues is believed to occur by interactions of protein ligands located in the ECM and a particular class of LPXTG surface proteins termed microbial surface components recognizing adhesive matrix molecules, or in short, MSCRAMMs. The publicly available genome sequences of *E. faecalis* V583 and *E. faecium* TX0016 revealed the presence of 17 and 15 MSCRAMMs[76,77], respectively, indicating that enterococci have evolved numerous strategies for interacting with host structures. Three enterococcal MSCRAMMs have been characterized in detail and mediated binding with high affinity to different types of collagen and other components of the ECM (Figure 1). Ace (adhesin of collagen from *E. faecalis*) was the first described MSCRAMM among enterococci and mediates binding to collagen type I, collagen type IV, laminin and dentin[42,78,79]. Acm (adhesin of collagen from *E. faecium*) interacts with collagen type I and to a lesser extent with collagen type IV[80,81]. Scm (second collagen adhesin of *E. faecium*) was able to bind to collagen type V and fibrinogen[77]. These three collagen-binding MSCRAMMs were found ubiquitously among enterococcal isolates of clinical and non-clinical origin. Interestingly, while a functional *acm* gene is predominantly present in clinical isolates promoting adherence to collagen type I, it often occurred as an insertion element-interrupted pseudogene in isolates of non-clinical origin; these isolates did not bind collagen type I[82].

The three enterococcal collagen-binding MSCRAMMs share several characteristics, including an N-terminal signal peptide sequence followed by a high affinity ligand-binding A-domain consisting of one or multiple variants of the immunoglobulin (IgG)-like fold, a B-repeat domain, and a C-terminal LPXTG-like cell wall sorting signal. The collagen binding activity of these three MSCRAMMs is localized to the A-domain. Ace and Acm share sequence similarity with a large family of collagen binding MSCRAMMs of Gram-positive pathogens, of which Cna of *Staphylococcus aureus* is the prototype. The A-domain of Cna consists of three subdomains, named N1, N2 and N3. The subdomains form a variant of the IgG-like fold, which is composed of two antiparallel β-sheets and two short α-helices[83]. The ligand binding domain of Ace was predicted to have two subdomains, which consist of N1 and N2, the Acm A-domain is composed of three subdomains, designated N1, N2 and N3, and the Scm A-domain is possibly comprised of a single domain. The minimal sequence of the A-domain of Cna, Ace and Acm which is required for collagen binding appeared to be the N2 domain. The structure of Cna has been characterized in detail and Ace was shown to be structurally similar[84]. In particular, backbone superposition of the crystal structure of the N2 domain of Ace and the N2 domain of Cna showed a nearly identical fold. Although no crystal structures for Acm have been resolved, the N1N2 domain of Acm displays similarity with the N1N2 domain of Cna and Ace, respectively. Far-UV circular dichroism spectroscopy revealed that the N1N2 subdomain of Acm, the A-domain of Scm and the N2 subdomain of Ace had a very similar overall secondary structure composition[77]. Furthermore, Acm is predicted to contain a conserved latch (ASGGVNG) region at the C-terminus of

the N2 domain, which likely interacts with a cleft (VEGWGQF) formed in the N1 domain to complement a β-sheet to close the MSCRAMM N1 and N2 domains[85]. For these reasons it seems likely that Acm also binds to collagen according to the Collagen Hug model, as has been experimentally demonstrated for Ace and Cna[83,86]. In the Collagen Hug binding mechanism, the N1N2 subdomains of Ace and Cna are predicted to adopt an open conformation in which a collagen triple helix molecule is allowed to dock (Figure 2). The MSCRAMM subsequently wraps around the collagen triple helix to lock the ligand between the N1 and N2 subdomains, followed by insertion of a C-terminal latch extension of the N2 subdomain into a trench of the N1 subdomain to stabilize the adhesin-ligand complex. Scm probably binds to collagen by a different mechanism that remains to be elucidated, because it contains only one IgG-like fold in the A-domain.

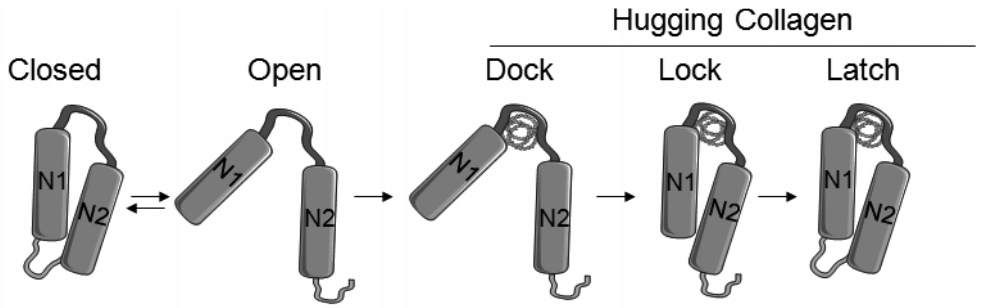

Figure 2. The Collagen hug model. Schematic representation of the Collagen Hug binding mechanism. The N1N2 subdomains of Ace and Cna and likely also of Acm are in a dynamic equilibrium from a closed to open configuration. When the N1N2 subdomains adopt an open configuration, the collagen ligand (dark green) is allowed to dock. The MSCRAMM subsequently hugs around the collagen helix to lock it between the N1 and N2 subdomains. The C-terminal latch extension (light green) of the N2 subdomain inserts into a trench of the N1 subdomain to close the MSCRAMM-collagen complex. Figure was produced using Servier Medical Art. See appendix for color figure.

To further determine the role of the Ace collagen-binding MSCRAMM in human infections, fluorescent microspheres were covalently coated with the A-domain of Ace. These beads bound to human small intestine epithelial cells and human umbilical vein endothelial cells, and these interactions could be specifically inhibited by specific anti-Ace antibodies or free soluble collagen[87]. The described *ace* deletion mutants have not been tested *in vivo* so far, thus the role of Ace of *E. faecalis* during infection is still unresolved. In contrast to Ace and Scm, the *in vivo* function of Acm was thoroughly investigated through animal studies with an *acm* deletion mutant. Acm played an important role in the pathogenesis of experimental *E. faecium* endocarditis[88]. In a rat endocarditis model in which an *acm* deletion mutant and the wild-type were injected intraventricularly in equal measures, the resulting vegetations on the heart valves were mainly consisting of the wild-type (74%) in contrast to the mutant strain (26%). Furthermore, the *acm* mutant was

deficient in initial adherence and colonization one and three hours after infection of damaged heart valves. Acm also elicited a human immune response as anti-Acm A-domain IgG antibodies were detected in sera from patients who suffered from *E. faecium* endocarditis[82]. This strongly indicates that Acm-mediated adherence of *E. faecium* to damaged and collagen-rich heart valves is an important factor in the establishment of endocarditis. There are also some indications that Ace has a role in endocarditis, but this evidence is mostly indirect and animal studies have, so far, not been published[89].

Expression of pili at the surface of enterococci.

Gram-positive surface exposed pili are multimeric fibres of LPXTG surface proteins and are polymerized by a pilus-dedicated class C sortase and subsequently covalently immobilized to the cell wall peptidoglycan by a class A sortase. Surface protein and pilus-mediated adherence to host tissues followed by colonization of mucosal surfaces represents an initial and necessary step in the onset of infection and is a prerequisite for pathogenesis[54].

In the early 1980s, pilus-like structures were detected by transmission electron microscopy at the surface of *E. faecalis* JH-2. At that time, the genetic requirements for pilus assembly in Gram-positives were not revealed. Recent pioneering work in *Corynebacterium*, *Actinomyces*, *Bacillus* and *Streptococcus* revealed that sortase enzymes dedicated to LPXTG surface proteins, which are located in pilin gene clusters (PGCs) are exclusively involved in pilus assembly[54]. Informed by *in silico* analyses, *E. faecalis* and *E. faecium* harbor PGCs which are comprised of multiple open reading frames encoding LPXTG-like cell wall-anchored proteins and sortases (Figure 3). In addition to MSCRAMM-like features, the presumed major pilin subunit proteins also contain conserved HLYPK-like pilin motifs, and ETxAPExY-like E box motifs[44,53]. Conservation of these elements in enterococcal major pilin subunits and in pili from other Gram-positive bacteria, suggest a universal mechanism of pilus assembly[54]. A pilus-dedicated class C sortase, catalyzes the cleavage of the LPXTG motif between the threonine (T) and glycine (G) residues to form an acyl-enzyme between the active site cysteine residue of sortase and the threonine of the enterococcal major pilin subunit. Nucleophilic attack of the amino group of lysine within the HLYPK-like pilin motif of another major pilin subunit putatively resolves the sortase intermediate to generate an amide bond between the C-terminal threonine and the lysine of adjacent major subunits. Following pilus polymerization, a class A sortase anchors the pilus via the threonine residue of the last pilin subunit to the side chain amino group of the lipid II precursor, which leads to the incorporation of pili into the enterococcal cell wall envelope[90].

The identified enterococcal PGCs closely resemble previously characterized pilus loci of other Gram-positive organisms. *E. faecalis* appears to harbor two PGCs, designated as the endocarditis and biofilm-associated pili (*ebp*) operon and the biofilm enhancer in enterococci (*bee*) locus[44,91]. The *bee* locus is located on a conjugative

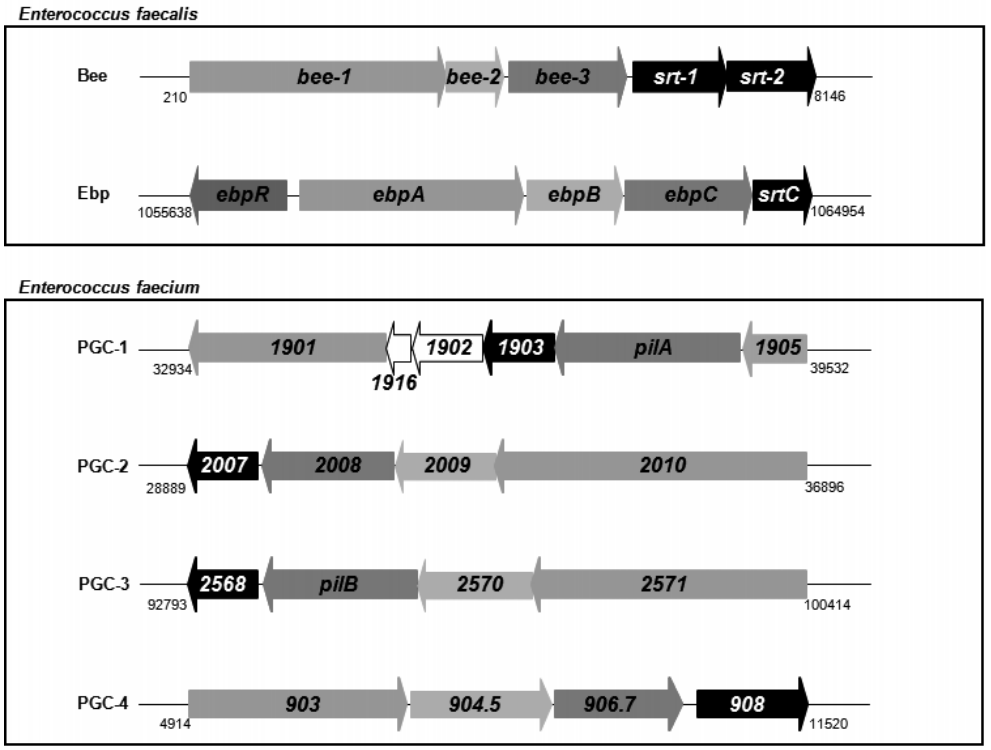

Figure 3. Enterococcal pilin gene clusters. Schematic representation of the organization of the putative pilin gene clusters identified in *E. faecalis* and *E. faecium*. The position and the orientation of the genes is indicated by arrows and the gene name is depicted in the arrows. The black arrows indicate putative pilus dedicated class C sortase genes, while the green arrow indicates a class A housekeeping sortase. The red arrows indicate putative major pilin genes, light blue and dark blue arrows indicate putative minor or tip pilin genes, respectively. The white arrows indicate proteins with unknown function. The *ebpR* transcriptional regulator is depicted in grey. The numbers at the beginning and the end of the pilin gene clusters are positions of the operons on the chromosome. The figure is not to scale. See appendix for color figure.

plasmid and was only sporadically detected (5%) among *E. faecalis* isolates, whereas the *ebp* locus was found ubiquitously[92]. While *E. faecalis* contains only two PGCs, *E. faecium* harbors four PGCs, which were named PGC-1 to -4 and were found to be predominantly present in human hospital-acquired *E. faecium* isolates[53]. The presence of four distinct types of PGCs may provide hospital-acquired *E. faecium* with a selective advantage. Of all the putative pilin proteins encoded from the PGCs of *E. faecalis* and *E. faecium*, only for the EbpA, EbpB, EbpC (in *E. faecalis*), and PilA and PilB proteins (in *E. faecium*) experimental evidence has been obtained, showing that these proteins can assemble into high molecular weight structures. In the case of *E. faecalis*, assembly of Ebp pilus-structures is SrtC-dependent, while expression of this PGC is dependent on EbpR, a transcriptional regulator[93]. Transmission immuno electron microscopy using specific antisera indicated that the putative EbpC, PilA and PilB proteins represent the major pilin subunits (Figure 4). Peculiarly, not

all enterococcal cells analyzed by electron microscopy expressed pili. Ebp and PilB pilus structures were detected in only 10 to 20%, and PilA pili in 40% of the cells. Differential pili expression among enterococcal cell populations suggest that epigenetic processes mediate expression of pili on the cell surface, analogous to the pyelonephritis-associated pili in *Escherichia coli*[94].

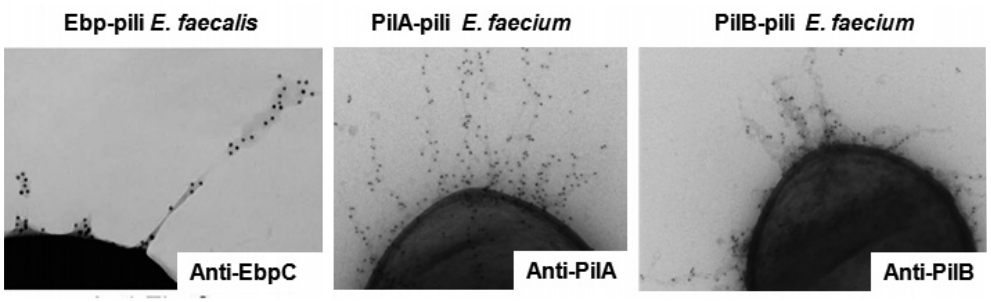

Figure 4. Transmission electron microscopy micrographs of enterococcal pili.
Expression of Ebp pili at the surface of *E. faecalis* (left), PilA pili (middle) and PilB pili (right) of *E. faecium* show phenotypic different types of pili. The three types of enterococcal pili were labelled with specific antisera directed against EbpC, PilA and PilB-type of pili followed by protein A-gold (10 nm) particles and negative staining. Bar = 200 nm.

The first clue concerning a possible function of Ebp pili was that high antibody titers against Ebp pilin proteins were detected in sera collected from patients with an *E. faecalis* endocarditis, suggesting expression of these proteins during human infection[76]. Using a set of *ebpA*, *ebpB*, *ebpC*, and *srtC* disruption and deletion mutants in a laboratory model for biofilm formation to polystyrene, it was demonstrated that Ebp pili are involved in initial adherence and biofilm formation[44]. In a rat endovascular infection model, in which a nonpiliated *ebpA* deletion mutant and the wild-type OG1RF were injected as an equal mixture, the mutant could not compete with the wild-type. Furthermore, in an *in vivo* murine urinary tract infection model in which mice were transurethrally challenged by either a nonpiliated *srtC* mutant or the wild-type OG1RF, the mutant was outnumbered by the wild-type[95,96]. These animal experiments showed a role of Ebp pili during endocarditis and urinary tract infection, which are both biofilm-mediated infections. The role of *E. faecium* PilA- and PilB-type pili in the pathogenesis of *E. faecium* infections remains to be investigated. However, the observation that these pili were expressed at 37 degrees and not at 21 degrees[53], and display high similarities with the genes of the *ebp* locus of *E. faecalis*, suggests a possible role of these pili in interactions with the mammalian host.

Outline of this thesis.

To date, little is known about cell wall-anchored LPXTG-type surface proteins and pili of *E. faecium.* The overall aim of this thesis was to identify and to analyze expression of surface exposed LPXTG-type cell wall-anchored proteins and pilus-like structures at the surface of *E. faecium* and to determine the function of these surface proteins. More specifically, the following research questions were addressed:

- Is Esp expressed at the surface of hospital acquired *E. faecium* and displays Esp of *E. faecium* similar functions as Esp of *E. faecalis*, *e.g.* adherence to abiotic materials and biofilm formation (chapter 2)?

- Do hospital acquired *E. faecium* isolates contain a distinct set of actively expressed LPXTG-type cell wall-anchored surface proteins that distinguish these isolates from non-hospital acquired strains (chapter 3) and what is the function of these surface proteins (chapter 4)?

- Do *E. faecium* isolates harbor the genetic requirements to express distinct pilus-like structures at their surface and what environmental growth conditions influence pilus expression and pilus assembly among strains of different origin (chapter 5 + 6)?

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Chapter 2

Growth Condition-Dependent Esp Expression by *Enterococcus faecium* Affects Initial Adherence and Biofilm Formation

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ABSTRACT.

A genetic subpopulation of *Enterococcus faecium*, called clonal complex 17 (CC-17), is strongly associated with hospital outbreaks and invasive infections. Most CC-17 strains contain a putative pathogenicity island encoding the *E. faecium* variant of enterococcal surface protein (Esp). Western blotting, flow cytometric analyses, and electron microscopy showed that Esp is expressed and exposed on the surface of *E. faecium*, though Esp expression and surface exposure are highly varied among different strains. Furthermore, Esp expression depends on growth conditions like temperature and anaerobioses. When grown at 37°C, five of six *esp*-positive *E. faecium* strains showed significantly increased levels of surface-exposed Esp compared to bacteria grown at 21°C, which was confirmed at the transcriptional level by real-time PCR. In addition, a significant increase in surface-exposed Esp was found in half of these strains when grown at 37°C under anaerobic conditions compared to the level in bacteria grown under aerobic conditions. Finally, amounts of surface-exposed Esp correlated with initial adherence to polystyrene (R^2 = 0.7146) and biofilm formation (R^2 = 0.7535). Polystyrene adherence was competitively inhibited by soluble recombinant N-terminal Esp. This study demonstrates that Esp expression on the surface of *E. faecium* (i) varies consistently between strains, (ii) is growth condition dependent, and (iii) is quantitatively correlated with initial adherence and biofilm formation. These data indicate that *E. faecium* senses and responds to changing environmental conditions, which might play a role in the early stages of infection when bacteria transit from oxygen-rich conditions at room temperature to anaerobic conditions at body temperature. In addition, variation of surface exposure may explain the contrasting findings reported on the role of Esp in biofilm formation.

INTRODUCTION.

Over the last 2 decades, enterococci have emerged as important nosocomial pathogens resistant to virtually all antibiotics, including vancomycin (1, 35). Resistance to clinically relevant antibiotics, such as ampicillin and vancomycin, is increasing and found primarily in *E. faecium*. This may explain its rapid emergence as an etiological agent of nosocomial infections, noticed first in the United States and, more recently, in Europe and Asia (24, 39; see also the European Antimicrobial Resistance Surveillance System at http://www.earss.rivm.nl). Molecular epidemiological studies of *E. faecium* isolates, resistant and susceptible to vancomycin and derived from different ecological niches, identified a specific clonal complex, designated CC-17, strongly associated with nosocomial outbreaks in five continents (45). Almost all CC-17 isolates are resistant to β-lactam antibiotics, and a substantial proportion contains a putative pathogenicity island, which carries the *E. faecium* variant of the enterococcal surface protein (*esp*) gene (17, 45). Esp of *E. faecium* shares a homology of up to 90% with the previously described Esp protein of *E. faecalis*, also located on a pathogenicity island (30) and expressed on the surface of the bacterium (31). Little

is known about the role of Esp in the pathogenesis of enterococcal infections. For *E. faecalis*, *esp*-positive stains were more frequently found among isolates associated with invasive infections than among isolates colonizing the gut (32). Furthermore, insertional inactivation of the *esp* gene attenuated *E. faecalis* virulence in an ascending urinary tract infection mouse model (31), but no role of Esp could be demonstrated in a mouse intestinal colonization model (26). Conflicting results have also been reported about the role of *esp* in biofilm formation and adherence to polystyrene. In several studies, Esp of *E. faecalis* appeared to be important in the initial adherence to polystyrene and biofilm development (20, 36, 41). In a recent study, the N-terminal region of Esp, covering approximately half of the protein in *E. faecalis*, was identified as the region of the protein involved in biofilm formation (38). In other studies, however, biofilm development appeared to be independent of Esp (6, 12, 16, 27). In *E. faecalis*, several other factors have been associated with biofilm development, like the sugar-binding transcriptional regulator BopD (13), the quorum-sensing locus *fsr* (12), heterogeneity in surface charge (43), the *bee* locus (37), and the secreted metalloprotease GelE (12, 16, 25). Yet absence of a correlation between gelatinase and biofilm development has also been found (6, 21, 43).

Currently, little is known about *esp* of *E. faecium*. As mentioned, *esp* of *E. faecium* is predominantly present in clinical isolates. Di Rosa et al. (6) report that, although some *esp*-negative strains developed biofilm, *esp*-positive strains produced thicker biofilms. Furthermore, the presence of both *esp* and biofilm development was found only in strains from clinical settings. To explore the role of Esp in the pathogenesis of *E. faecium* infections, we studied Esp expression using Western blotting and flow cytometry under different growth conditions, like temperature and anaerobioses. We also assessed the association between the amount of cell surface-associated Esp and the ability to adhere to polystyrene and biofilm development.

MATERIALS AND METHODS.

Bacterial strains and culture conditions.

Eight *E. faecium* strains— E135, E155, E300, E470, E745, E1165, E1172, and E1176 (2, 9, 40, 42, 44, 45)—were used in this study. All bacteria were of human origin, and except for E135, all were *esp* positive. E135, E300, E155, E470, and E745 were vancomycin resistant. E135 was a community surveillance isolate. E155, E300, E470, and E745 were isolates from hospital outbreaks in the United States and The Netherlands. Finally, E1165, E1172, and E1176 were all clinical isolates recovered from wounds, urine, and the respiratory tract, respectively (Table 1). For the cloning and expression of Esp, *Escherichia coli* TOP10F′ and BL21(DE3)pLysS were used. *E. faecium* was grown on sheep red blood agar (SRA) containing tryptic soy agar with 5% sheep red blood cells (BD, Alphen aan den Rijn, The Netherlands), brain heart infusion, brain heart infusion agar, tryptic soy broth (TSB), and TSB supplemented with 0.25% glucose. Bacteria grown on SRA were incubated for 72 h at 21°C or 18 h at 37°C under aerobic conditions or in a container with an anaerobic atmosphere (5% H_2, 10% CO_2, and 85% N_2). Before each experiment, bacteria were

initially grown at 21°C on SRA. *E. coli* was grown either on Luria-Bertani agar or on Luria-Bertani agar supplemented, if necessary, with 50 μg/ml ampicillin.

TABLE 1. Bacteria used in this study

Strain	*esp*	*vanA*	Source of isolation	Reference(s)
E135	−	+	Community surveillance	44, 45
E155	+	+	Hospital outbreak	3, 45
E300	+	+	Hospital outbreak	9, 45
E470	+	+	Hospital outbreak	40, 45
E745	+	+	Hospital outbreak	44, 45
E1165	+	−	Clinical isolate, wound	45
E1172	+	−	Clinical isolate, urine	45
E1176	+	−	Clinical isolate, respiratory tract	45

Cloning N-terminal Esp.

A fragment of the N-terminal domain of Esp was cloned using the primer set Esp-1 (5′-ATGGGAACGCCTTGGTATG-3′) and Esp-2 (5′-TACTGCTAAATCGGTCGTG-3′). Both Esp-1 and Esp-2 are based on *esp* of *E. faecalis* (AF034779) and start at positions 999 and 2295, respectively. In Esp-1, an additional 5′ ATG was added for the in-frame expression of Esp. PCRs were performed with 25 μl using HotStarTaq DNA polymerase (QIAGEN Benelux B.V., Venlo, The Netherlands), with 10 pmol of each primer and 10 nmol of E300 chromosomal DNA. DNA was isolated as described previously (30). PCR conditions were as follows: initial denaturation at 95°C for 15 min followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min, followed by an extension at 72°C for 7 min. PCR fragments were directly cloned in the pCRT7/CT-TOPO TA expression vector (Invitrogen, Breda, The Netherlands) and were used to transform One Shot TOP10F′ chemically competent *E. coli* (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. Plasmids were isolated with a plasmid purification kit (QIAGEN Benelux B.V., Venlo, The Netherlands) and checked by sequencing using the BigDye Terminator reaction kit and an ABI PRISM 3700 DNA analyzer (both from Applied Biosystems, Foster City, CA). Plasmid DNA containing the right constructs was used to transform One Shot BL21(DE3)pLysS chemically competent *E. coli* (Invitrogen, Breda, The Netherlands). The recombinant N-terminal Esp domain with a His tag at its C-terminal end (rN-Esp) was induced by isopropyl-β-d-thiogalactopyranoside (IPTG) and isolated using a nickel column (Probond, Invitrogen, Breda, The Netherlands), all according to the manufacturer's instructions.

Raising polyclonal antibodies and isolation of immunoglobulin G.

Polyclonal antibodies to *E. faecium* rN-Esp were raised by immunizing two rabbits. For the initial doses, 150 μg rN-Esp in incomplete Freund's adjuvant was injected subcutaneously. Subsequently, a booster dose of 150 μg was administered at day 28. Rabbits were exsanguinated at day 42, and their sera were collected and stored at −20°C. All experiments were performed according to Dutch regulations on animal experiments.

Western blotting.

After growth at 21°C or 37°C on SRA plates (bacteria grown at 37°C were stored under both aerobic and anaerobic conditions), bacteria were collected from the plates and resuspended in phosphate-buffered saline (PBS), generating an optical density at 660 nm (OD_{660}) of 1.0 (1×10^9 bacteria/ml). For each sample, 1 ml (1×10^9 bacteria) was harvested at 6,500 × *g* for 1 min, and the bacteria were lysed by incubating the cell pellet in 200 μl buffer containing 50 μg/ml linezolid, 0.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml lysozyme, and 20% sucrose in PBS at 37°C. After 2 h, samples were centrifuged (10,000 × *g*, 5 min), and total protein content was quantified by OD_{280} measurements. Samples were normalized for protein content, and Western blotting was performed as described previously (28). Esp was detected with anti-Esp rabbit immune serum followed by horseradish peroxidase-conjugated goat anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA).

Real-time quantitative PCR.

After growth at 21°C or 37°C under aerobic conditions on SRA plates, bacteria were collected from the plates and resuspended in PBS to an OD_{660} of 1.0 (1×10^9 bacteria/ml). For each sample, 3 ml (3×10^9 bacteria) was taken and pelleted by centrifugation (6,500 × *g*, 1 min). RNA species were isolated according to procedures outlined by Cheung et al. (4). RNA purification and cDNA generation were done as described by Nallapareddy et al. (22) with some modifications. Total RNA was treated three times with 20 U of RQ1 DNase (Promega Corp., Madison, WI) for 30 min at 37°C, and RNA was isolated using an RNeasy minikit (QIAGEN). From 1.5 μg total RNA, cDNA was synthesized with the SuperScript II first-stand synthesis system (Invitrogen Corp., Carlsbad, CA) using random primers according to the instructions of the manufacturer. Three primer-probe sets were used in this study, one to detect cDNA of 23S rRNA, one to detect *esp* of E470, and one to detect Esp cDNA of the other *esp*-positive strains. The DNA region encoding the Esp N-terminal domain is too diverse to use one primer-probe combination for all *esp*-positive strains. The sequences of the primer-probe combinations are as follows: for 23S rRNA-F, 5′-CCAGGTTGAAGGTGCGGT-3′; for 23S rRNA-R, 5′-CCTCATCCCCGCACTTTTC-3′; for 23S rRNA-probe, (6-carboxyfluorescein [6-FAM]-CACTGGAGGACCGAACCCACGG-6-carboxytetramethylrhodamine [TAMRA]); for EspE470-F, 5′-TTGGTCTTATCTTTGGAGCAACTG-3′; for EspE470-R, 5′-TTCGTAGCTGTTGCCAATATTTTG-3′; for EspE470-probe, 6-FAM-AGCTGTTAATGCACAAGGCAACTTTTCTTCAA-TAMRA; for EspE300-F, 5′-GGTGATGGAAACCCTGACGA-3′; for EspE300-R, 5′-CTTTCCCCTTAACTGTTGTGTCAAC-3′; and for EspE300-probe, 6-FAM-AAGAAGAGAGCGGAGACACGAATCCATATATCG-TAMRA.

Real-time PCR was performed using TaqMan Universal PCR master mix (Applied Biosystems). The concentrations of the three primer-probe combinations were optimized and were 300 μM for 23S rRNA-F, 900 μM for 23S rRNA-R, 100 μM for 23S rRNA-probe, 300 μM for EspE470-F, 300 μM for EspE470-R, 50 μM for EspE470-probe, 300 μM for EspE300-F, 900 μM for EspE300-R, and 50 μM for EspE300-probe. The chosen PCR conditions were 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 94°C for 15 s and 60°C for 1 min on an ABI PRISM 7700 sequence detector (Applied Biosystems). The $\Delta\Delta CT$ method of Livak et al. (18) was used to calculate the difference in Esp mRNA present in bacteria grown at 37°C and at 21°C using 23S rRNA as an internal control. Analyses were performed in triplicate.

Detection of Esp on the surface of *E. faecium*.

After the appropriate incubation, bacteria were collected from the plates and resuspended in RPMI 1640 (Cambrax Bioscience) containing 0.05% human serum albumin (HSA) to an OD_{660} of 1.0 (1×10^9 CFU/ml). For each sample, 100 μl (1×10^7 CFU) was taken and pelleted by centrifugation (6,500 × *g*, 1 min) and 50 μl RPMI 1640-HSA containing 1/100 anti-Esp rabbit immune serum was added. Bacteria were resuspended and incubated on ice. After 30 min, 1 ml cold RPMI 1640-HSA was added, and the bacteria were isolated by centrifugation (6,500 × *g*, 1 min). Bacteria were resuspended in 50 μl RPMI 1640-HSA containing a 1/50 dilution of goat anti-rabbit fluorescein isothiocyanate (Sigma-Aldrich, Saint Louis, MO) and left for 30 min on ice. Bacteria were washed with 1 ml cold RPMI 1640-HSA, and after centrifugation (6,500 × *g*, 1 min), bacteria were resuspended in 50 μl RPMI 1640-HSA. Before analyses in a FACSCalibur (BD, Alphen aan den Rijn, The Netherlands), bacteria were resuspended in 300 μl RPMI 1640-HSA. All measurements were performed with the same machine using the same parameters. The data were normalized for bacterial size, and experiments were performed at least three times. The mean fluorescence (mean fluorescence channel 1) was used as a measure for cell surface-associated Esp. Pooled rabbit preimmune serum and bacteria incubated without anti-Esp rabbit immune serum were used as negative controls. The specificity of the anti-Esp rabbit immune serum was demonstrated by blocking the binding of serum to Esp on the bacterial cell surface after preincubating 1/300-diluted anti-Esp serum in RPMI 1640-HSA with serial dilutions of rN-Esp, starting with 100 μg/ml, for 15 min prior to incubation with the bacteria.

Electron microscopy.

Bacteria were grown on SRA at 37°C and treated as follows. A drop of 1×10^9 CFU/ml in PBS was placed on Parafilm, and a 200-mesh Formvar-carbon-coated copper grid was floated on the surface for 10 min. Grids were washed three times by floatation for 5 min on drops of 0.02 M glycine in PBS and blocked by floatation for 15 min on drops of 1% bovine serum albumin in PBS (PBSb). Esp was labeled by floating the grids for 1 h on drops containing a 1/100 dilution of anti-Esp rabbit immune serum in PBSb. Grids were washed four times by floating them for 2 min on drops of 0.1% bovine serum albumin in PBS. Antibodies were labeled by floating the grids for 20 min on drops of proteinA-Gold (15 nm) (34) in PBSb. Grids were washed by floatation four times for 2 min on drops of PBS, fixed by floatation on drops of 1% glutaraldehyde, and washed again by floatation eight times for 2 min on drops of H_2O. Bacteria were stained by floating the grids for 5 min on drops containing 1.8% methylcellulose (25 centipoises; Sigma-Aldrich, Saint Louis, MO) and 0.4% uranyl acetate (pH 4) and subsequently air dried for 10 min. Bacteria used as negative controls were treated in a similar way except that the incubation with anti-Esp rabbit immune serum was left out. Grids were examined for bacteria by using a Jeol 1010 transmission electron microscope (Jeol-Europe, Amsterdam, The Netherlands) at a magnification of ×30,000.

Initial adherence assay.

The initial adherence assay was performed according to Hufnagel et al. (13). Briefly, plate-grown bacteria were resuspended in TSB at a concentration of 5×10^7 CFU/ml. To the wells of a polystyrene 96-well plate (Corning Inc., Corning, NY), 100-μl bacterial suspensions were added in triplicate, and the plate was incubated at 37°C. In inhibition studies, inhibitors were added directly to the bacterial suspension. After incubation for 1 h, bacteria were removed, and the wells were gently washed three times with 200 μl PBS. The plates were dried by incubating them for 1 h at 60°C. To each well, 50 μl Gram's crystal violet solution (Merck, Darmstadt, Germany) was added. After 2 min, the stain was taken off and the plates were washed

in tap water. Finally, the plates were dried for 10 min at 60°C, and the OD_{595} was measured with an enzyme-linked immunosorbent assay reader. Experiments were performed three times in triplicate.

Blocking of adherence by rN-Esp.

Strains were grown for 72 h on SRA at 21°C and for 18 h at 37°C. Subsequently, strains were suspended at different bacterial concentrations (5×10^7, 2.5×10^7, and 1.25×10^7 CFU) in TSB containing different concentrations of rN-Esp (0.01 to 100 μg/ml) and assayed for primary attachment.

Biofilm assay.

Biofilm development was assayed in a way similar to the way initial adherence was assayed, except that the test was performed with TSB supplemented with 0.25% glucose, and 1×10^5 CFU of bacteria/ml were left for 24 h at 37°C in 96-well polystyrene plates.

Statistics.

Student's *t* test was used to assess statistically significant differences.

RESULTS.

Growth conditions influence Esp expression.

To determine the influence of growth conditions on Esp expression, strains were grown on SRA at 21°C (72 h) or 37°C (18 h) under aerobic conditions and at 37°C (18 h) under anaerobic conditions and analyzed by Western blotting using the anti-Esp polyclonal rabbit serum. Esp expression at 37°C increased relative to that at 21°C in all *esp*-positive strains, though expression differed substantially among strains. In five of six *esp*-positive strains, Esp expression increased when they were grown under anaerobic conditions at 37°C, relative to what occurred under aerobic conditions at 37°C (Fig. 1).

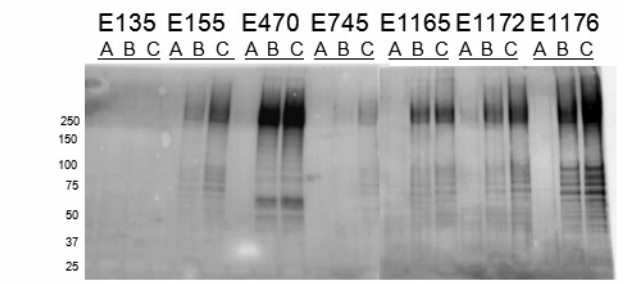

Figure 1. Western blot analyses of a 10% sodium dodecyl sulfate-polyacrylamide gel using the rabbit polyclonal anti-Esp serum. The Western blot shows growth culture-dependent expression of Esp in one *esp*-negative *E. faecium* strain (E135) and six *esp*+ *E. faecium* strains (E155, E470, E745, E1165, E1172, and E1176). Bacteria listed were grown on SRA for 72 h at 21°C under aerobic conditions (lanes A), 18 h at 37°C under aerobic conditions (lanes B), or 18 h at 37°C under anaerobic conditions (lanes C). Numbers to the left of the gel are molecular weights (in thousands).

Growth condition-dependent Esp exposure on the surface of *E. faecium*.

To show that Esp of *E. faecium* is exposed on the surface of the cell, bacteria were grown overnight at 37°C, incubated with anti-Esp polyclonal rabbit serum, and assayed by flow cytometry. Surface exposure of Esp was demonstrated in all six *esp*-positive strains (data not shown). This was confirmed for the *esp*-positive strain E470 and the *esp*-negative strain E135 by transmission electron microscopy on negatively stained immunogold-labeled bacteria (Fig. 2). On strain E470, gold particles are

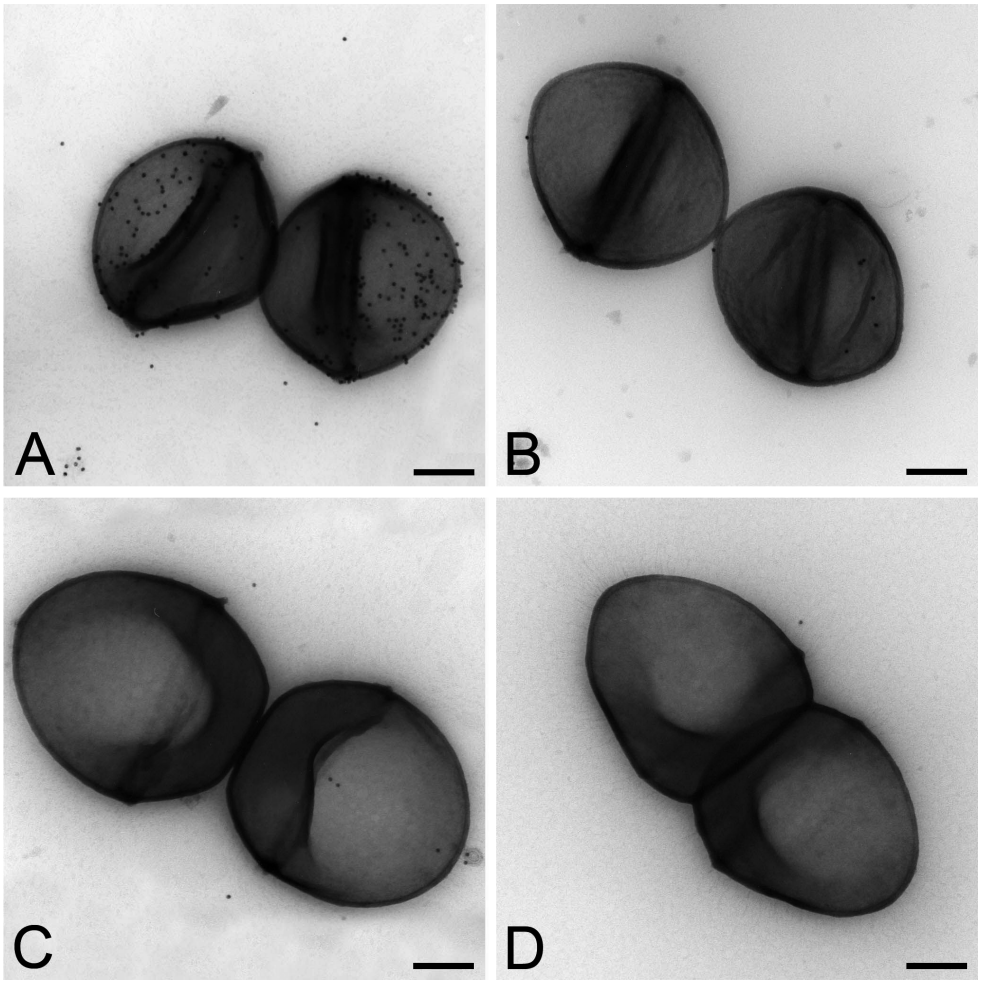

Figure 2. Transmission electron microscope picture at a magnification of x30,000 of E470 and E135 *E. faecium* strains negatively stained by methylcellulose uranyl acetate and labeled with immunogold (15 µm) using anti-Esp antiserum. The inserted bar indicates a length of 200 nm. E470 incubated with anti-Esp polyclonal rabbit serum and proteinA-Gold (A), E470 incubated with proteinA-Gold (B), E135 incubated with anti-Esp polyclonal rabbit serum and proteinA-Gold (C), and E135 incubated with proteinA-Gold (D).

clearly associated with the bacterial cell wall, demonstrating the association of Esp with the bacterial cell wall (Fig. 2A). On the surface of strain E135 (Fig. 2C), gold particles were found in small amounts comparable to those found in the negative controls without the anti-Esp polyclonal rabbit serum (Fig. 2B and D).

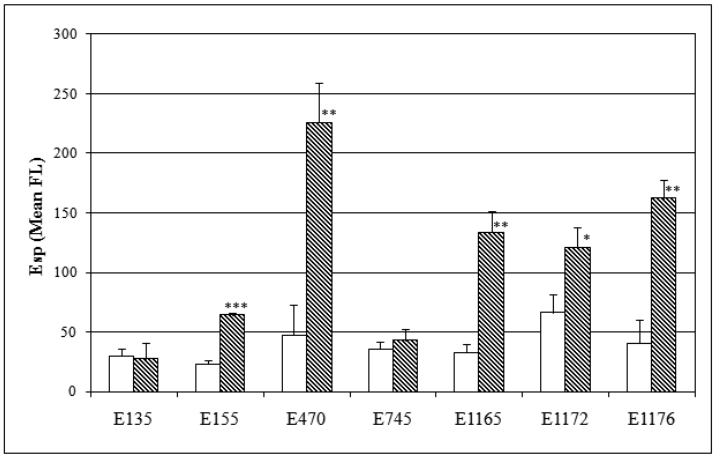

Figure 3. Temperature-dependent expression of cell wall-associated Esp of *E. faecium.* Shown are the means of the mean FL1 from three independent experiments. Bacteria listed were grown on SRA for 72 h at 21°C (open bars) or 18 h at 37°C (dashed bars). Error bars denote standard deviations. *, P < 0.05; **, P < 0.005; and ***, P < 0.0001.

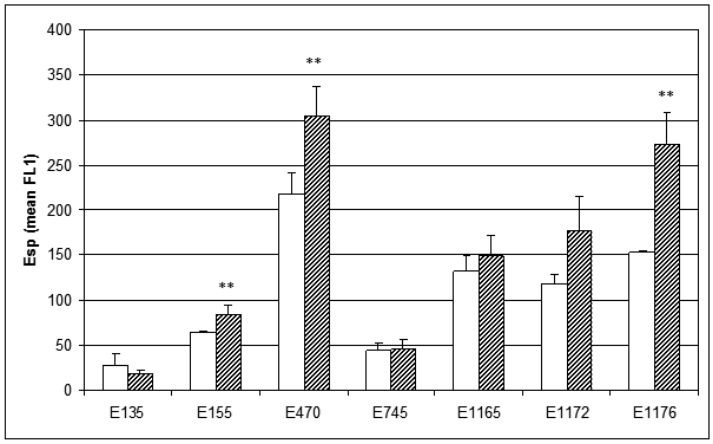

Figure 4. Effect of anaerobiosis on the expression of cell wall-associated Esp of *E. faecium.* Shown are the means of the mean FL1 from three independent experiments. Bacteria listed were grown on SRA for 18 h at 37°C under aerobic (open bars) or anaerobic (dashed bars) conditions. Error bars denote standard deviations. **, P < 0.005.

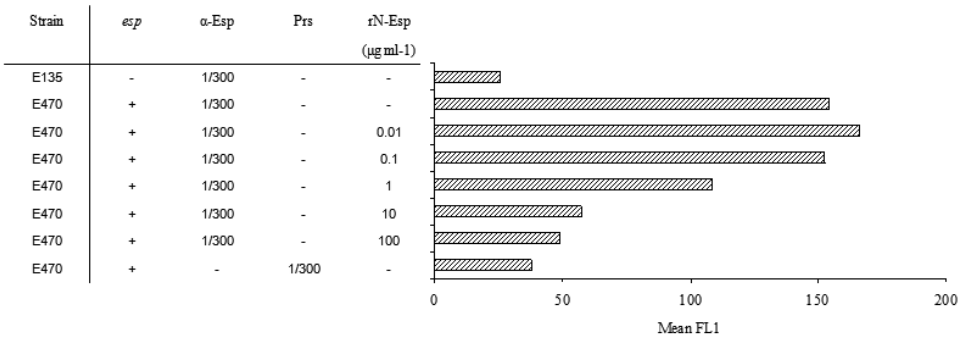

Figure 5. Histograms showing the Esp-specific binding of the rabbit polyclonal anti-Esp serum. Strains were grown for 18 h on SRA at 37°C. E470 was incubated with anti-Esp (α-Esp) serum pretreated with increasing amounts of rN-Esp. As negative controls, E135 (*esp* negative) incubated with the anti-Esp serum and E470 incubated with pooled rabbit preimmune serum (Prs) were used. On the x-axis, the mean fluorescence (mean FL1) is indicated.

To quantify the influence of growth conditions on surface-associated Esp, all seven strains were grown at two different temperatures, 21°C and 37°C, and at 37°C under aerobic and anaerobic conditions, before they were assayed by flow cytometry. Five of six *esp*-positive strains had significantly increased levels of surface-associated Esp when grown at 37°C compared to bacteria grown at 21°C (Fig. 3). Only E745 had low-level surface-associated Esp at 21°C and 37°C. A similar effect of temperature-dependent, elevated, surface-associated Esp expression was obtained when bacteria were grown on brain heart infusion agar medium (data not shown), indicating that the level of surface-associated Esp on *E. faecium* is growth temperature dependent but independent of the growth media tested. Three of the six *esp*-positive strains (E155, E470, E1176) had significantly elevated levels of surface-associated Esp when grown under anaerobic conditions (Fig. 4). Binding of anti-Esp antiserum could be blocked by adding an excess of the N-terminal domain of Esp (Fig. 5), indicating that the anti-Esp antiserum specifically recognized Esp.

Temperature-dependent transcription of *esp*.

Growth temperature-dependent expression of Esp was confirmed at the transcriptional level using real-time PCR. All *esp*-positive strains showed increased Esp mRNA levels when grown at 37°C compared to bacteria grown at 21°C. Based on the $\Delta\Delta CT$ values, 6.63-, 1.03-, 3.14-, 2.51-, 1.40-, and 5.35-fold increases of Esp mRNA were found for strains E470, E155, E745, E1165, E1172, and E1176, respectively. These data also show that strains E470 and E1176, which demonstrated the largest differences in levels of surface-exposed Esp upon the growth temperature shift (Fig. 3), also showed the highest increases of Esp mRNA, namely, 6.63- and 5.35-fold increases, respectively. As expected for the *esp*-negative strain E135, no Esp mRNA could be detected. These data indicate that growth temperature-dependent surface expression of Esp is regulated at the transcriptional level.

Surface-exposed Esp expression and binding to polystyrene.

To investigate whether surface exposure of Esp correlates with initial adherence and biofilm formation, *E. faecium* strains were grown at 21°C (72 h) and 37°C (18 h) under aerobic conditions on SRA and subsequently exposed to polystyrene. Surface expression of Esp, as determined by flow cytometry, and initial adherence to polystyrene were clearly associated ($R^2 = 0.7146$) (Fig. 6). Strain E470 had the highest increase in adherence and Esp expression upon shifting the temperature from 21°C to 37°C. Correlation was close to linear ($R^2 = 0.9887$) if the results for E1176, which had a relatively small increase in initial adherence when grown at 37°C compared to 21°C, were not included. The amount of surface-exposed Esp at the start of biofilm development (0 h), determined by flow cytometry, positively correlated with biofilm development, determined at 24 h ($R^2 = 0.7535$) (Fig. 7). This strongly suggests that, under the conditions tested, the amount of Esp on the surface of *E. faecium* is indicative of the binding of polystyrene and the ability to develop biofilms.

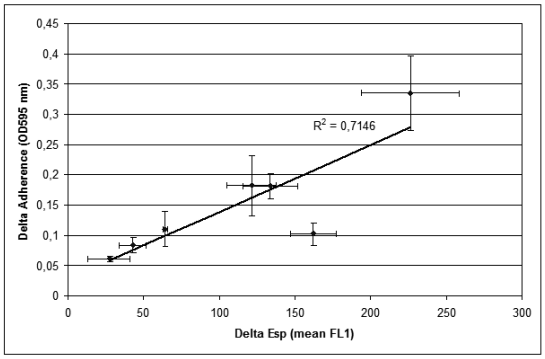

Figure 6. Correlation between Esp expression and initial adherence to polystyrene. Difference in Esp expression and adherence between bacteria grown at 21°C and 37°C are indicated at the x- and y-axes, respectively. Error bars denote standard deviations.

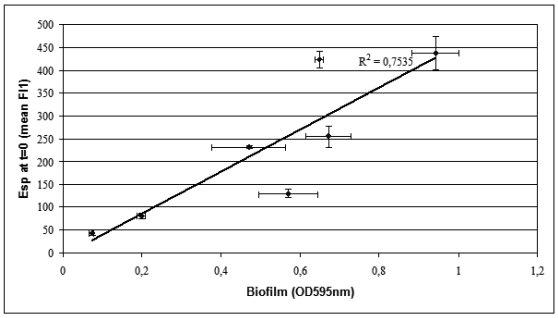

Figure 7. Correlation between Esp expression and biofilm formation. The x-axis indicates the Esp expression at 0 h (t = 0), and the y-axis indicates the biofilm development measured after 24 h. Error bars denote standard deviations.

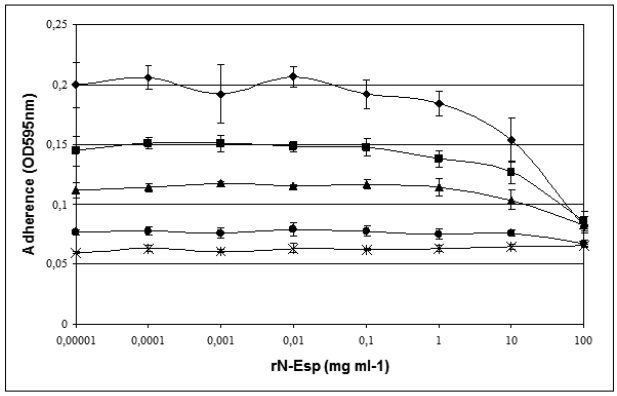

Figure 8. Inhibition of initial adherence of *E. faecium* to polystyrene by the N-terminal Esp domain. The ability to block initial adherence by N-terminal Esp was studied using two different strains. Prior to the assay, strains were grown at different temperatures, and in the assay different bacterial concentrations were used. Diamonds, 5 × 10^7 CFU of E470 grown at 37°C; squares, 2.5 × 10^7 CFU of E470 grown at 37°C; triangles, 1.25 × 10^7 CFU of E470 grown at 37°C; circles, 5 × 10^7 CFU of E470 grown at 21°C; asterisks, 5 × 10^7 CFU of E135 grown at 37°C.

The N-terminal domain of Esp blocks the adherence of *E. faecium* to polystyrene.

The *esp*-negative strain (E135) and the strain with the largest amounts of surface-associated Esp (E470) were used to determine whether adherence could be blocked by rN-Esp. Primary attachment of E470 grown at 37°C was blocked by rN-Esp in a concentration-dependent manner (Fig. 8). Although maximal adherence was bacterial concentration dependent, the concentrations of rN-Esp needed to block attachment were similar for all bacterial concentrations. This suggests that the N-terminal domain of Esp is sufficient for binding to polystyrene.

DISCUSSION.

Using Western blotting and FACscan analysis, we have shown that the amounts of Esp expressed on the cell surfaces of *esp*-positive *E. faecium* strains varied considerably from strain to strain. Furthermore, in almost all strains, the amount of Esp on the surface of *E. faecium* correlated with the ability to adhere to polystyrene, which could be blocked by exogenous N-terminal Esp, suggesting a role for the N-terminal domain of Esp in initial adherence to polystyrene. This is in line with recent observations by Tendolkar and coworkers, who showed that, by constructing in-frame-deletion mutants, the minimal region contributing to Esp-mediated biofilm enhancement in *E. faecalis* was confined to the N-terminal domain (38). The observed dose dependency further supports a role for Esp in initial adherence and the biofilm formation of *E. faecium*.

With both *E. faecalis* and *E. faecium*, there is controversy about the role of Esp in biofilm development. Although Esp appeared to be important for biofilm development (20, 36, 41) in some studies, *E. faecalis* isolates lacking the *esp* gene did produce biofilm in vitro (6, 12, 16, 27). Furthermore, for both *E. faecium* and *E. faecalis*, isolates were found that carried the *esp* gene but that failed to produce biofilms. Esp-independent biofilm formation by *E. faecalis* is probably mediated by multiple additional factors, like GelE (12, 16, 25), BopD (13), the *fsr* locus (12), the *bee* locus (37), and heterogeneity in surface charge (43). The observations that biofilms produced by *E. faecalis* are much thicker than those produced by *E. faecium* and that the biofilm development of *E. faecalis* is much more sensitive to the growth medium used (29) may indicate that in *E. faecalis*, additional factors aside from Esp are involved.

From data presented in the current study, the lack of biofilm formation in *esp*-positive strains can be explained by absent or only low-level expression of functional Esp on the cell surface despite the presence of its gene. Furthermore, detectable levels of Esp on the surface of *E. faecium* does not indicate in all cases availability for adherence to polystyrene, as was illustrated for strain E1176. This could be the result of shielding of Esp by other surface structures, like polysaccharide capsules. In general, however, the amount of surface-exposed Esp correlates well with the ability to adhere to polystyrene and develop biofilm.

Temperature-dependent regulation of expression, as shown for Esp in *E. faecium*, is a characteristic feature of pathogens alternating between a vector or an environmental reservoir and a mammalian host. Pathogens like *Bordetella*, *Borrelia*, *Clostridium*, *Escherichia*, *Salmonella*, *Shigella*, *Vibrio*, and *Yersinia* spp. (14, 15) respond to temperature transitions by inducing the expression of temperature-regulated genes that often encode virulence factors. *E. faecium* shares these ecological features. Besides being a common colonizer of the gastrointestinal tracts of humans and animals, it is also able to survive, for prolonged periods of time, in environmental reservoirs, both outside hospitals (in soil and sewage) and inside hospitals (on thermometers, bed rails, over-the-bed tables, bed linen, urinals, bedpans, hands of health care workers, and patients' skin) (3). We postulate that growth temperature-dependent expression of Esp is a niche-dependent adaptation mechanism of *E. faecium*. As such, Esp may contribute to the early stages of colonization and subsequent infection.

Furthermore, in five of six *esp*-positive strains, Esp expression increased under anaerobic conditions. *E. faecium* is a facultative anaerobic bacterium, which allows the bacterium to alternate between environmental reservoirs and its host. In *E. faecalis*, up-regulation of household genes and the virulence factor cytolysin were found under anaerobic conditions (5, 33). Our observation that Esp expression is up-regulated under anaerobic conditions once more suggests a role for Esp in the early stages of *E. faecium* colonization and infection.

Within hospital settings, enterococci have been described as triple-threat pathogens combining characteristics of *Enterobactericeae* (i.e., gut colonization), *Staphylococcus aureus* (i.e., skin colonization), and *Clostridium difficile* (prolonged survival on inanimate environments) (3). The combination of skin colonization and survival on inanimate environments has probably been instrumental in the rapid nosocomial spread of these bacteria. Furthermore, extensive colonization of the skin is an important risk for the development of intravascular-device-related infections and subsequent bacteremia, which are among the most frequently occurring enterococcal infections (7). Migration of bacteria from the skin along the catheter into the bloodstream, accompanied by a shift in temperature from 21°C to 37°C, might be the signal for *E. faecium* to up-regulate Esp expression, which will result in initial adherence to the catheter and subsequent biofilm development. Once part of a biofilm on the surface of an invasive medical device, bacterial cells are shielded against the detrimental activities of the host immune response and antibiotics (8, 10, 11).

Whether Esp in *E. faecium* serves as an adhesin to abiotic materials or is involved in colonization remains to be determined. *Esp*-positive blood isolates adhered well to Caco-2 human colon cancer cells (19), suggesting a role for Esp in gut colonization. In contrast, in *E. faecalis*, Esp appeared not to be instrumental in a mouse intestinal colonization model (26).

In this study, we have shown that Esp is expressed on the surface of *E. faecium* and that Esp expression may vary considerably between *esp*-positive isolates, which may explain previous conflicting results on the relation between *esp* and adherence or biofilm formation. For the first time, we demonstrated, quantitatively, that Esp expression levels correlate with initial adherence to polystyrene and biofilm formation. Furthermore, it was shown for the first time that *E. faecium* senses and responds to environmental changes. These findings support the idea of a role for Esp in the early stages of *E. faecium* colonization. Naturally, definitive claims of Esp functions await conformation of our findings with *esp* knockout strains. Until now, we and others have not been successful in constructing an *esp* knockout strain of *E. faecium*. Recent findings reported by Nallapareddy et al. (23) on improved temperature-sensitive vectors with which *acm* mutations were constructed in poorly transformable clinical *E. faecium* strains might be instrumental in overcoming this problem.

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Chapter 3

Five Genes Encoding Surface-Exposed LPXTG Proteins Are Enriched in Hospital-Adapted *Enterococcus faecium* Clonal Complex 17 Isolates

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ABSTRACT.

Most *Enterococcus faecium* isolates associated with hospital outbreaks and invasive infections belong to a distinct genetic subpopulation called clonal complex 17 (CC17). It has been postulated that the genetic evolution of CC17 involves the acquisition of various genes involved in antibiotic resistance, metabolic pathways, and virulence. To gain insight into additional genes that may have favored the rapid emergence of this nosocomial pathogen, we aimed to identify surface-exposed LPXTG cell wall-anchored proteins (CWAPs) specifically enriched in CC17 *E. faecium*. Using PCR and Southern and dot blot hybridizations, 131 *E. faecium* isolates (40 CC17 and 91 non-CC17) were screened for the presence of 22 putative CWAP genes identified from the *E. faecium* TX0016 genome. Five genes encoding LPXTG surface proteins were specifically enriched in *E. faecium* CC17 isolates. These five LPXTG surface protein genes were found in 28 to 40 (70 to 100%) of CC17 and in only 7 to 24 (8 to 26%) of non-CC17 isolates ($P < 0.05$). Three of these CWAP genes clustered together on the *E. faecium* TX0016 genome, which may comprise a novel enterococcal pathogenicity island covering *E. faecium* contig 609. Expression at the mRNA level was demonstrated, and immunotransmission electron microscopy revealed an association of the five LPXTG surface proteins with the cell wall. Minimal spanning tree analysis based on the presence and absence of 22 CWAP genes revealed grouping of all 40 CC17 strains together with 18 hospital-derived but evolutionary unrelated non-CC17 isolates in a distinct CWAP-enriched cluster, suggesting horizontal transfer of CWAP genes and a role of these CWAPs in hospital adaptation.

INTRODUCTION.

Enterococcus faecium is a commensal organism of the mammalian gastrointestinal tract, but during the last 2 decades it has been widely recognized as an opportunistic pathogen causing serious infections in immunocompromised patients (11, 13). In these patients, *E. faecium* is responsible for urinary tract infections, surgical site infections, bacteremia, and endocarditis. The emergence of *E. faecium* infections was associated with increasing resistance towards different classes of antibiotics, e.g., penicillins, aminoglycosides, and glycopeptides (9). Recent studies have shown that *E. faecium* isolates responsible for the vast majority of clinical infections and hospital outbreaks belong to a distinct genetic subpopulation designated clonal complex 17 (CC17), which has spread globally (46). Key features of CC17 *E. faecium* are high-level resistance to ampicillin and ciprofloxacin and the presence of a putative pathogenicity island harboring the *esp* virulence gene, suggesting that CC17 not only is multiresistant to antibiotics but also may be more virulent than non-CC17 *E. faecium* isolates (6, 15). The genetic evolution of CC17 has probably been a multistep process involving the sequential acquisition of multiple adaptive mechanisms (14). These adaptive mechanisms include resistance genes as well as genes encoding novel metabolic pathways (16), putative virulence genes

such as hyl_{Efm} (29), and the *esp* gene (45). Surface-exposed Esp expression in *E. faecium* CC17 isolates quantitatively correlates with initial adherence to polystyrene and biofilm formation (40). In addition, the collagen adhesin Acm, which is associated with increased collagen type I binding, is predominantly expressed at the surfaces of clinical *E. faecium* isolates (22, 24). Both Esp and Acm represent cell wall-anchored surface proteins (CWAPs) which may provide *E. faecium* with a selective advantage in the hospital setting, for instance through biofilm formation and better adherence to extracellular matrix molecules.

CWAPs typically contain an N-terminal signal sequence peptide and a C-terminal cell wall sorting signal (CWS). CWSs consist of a conserved Leu-Pro-X-Thr-Gly (LPXTG) sortase substrate motif (where X denotes any amino acid) followed by a hydrophobic domain and positively charged amino acids (31). After the translocation of the precursor CWAP across the plasma membrane, it becomes covalently anchored to the cell wall peptidoglycan by sortase-mediated transpeptidase activity (18, 19). Various CWAPs and MSCRAMM (*m*icrobial *s*urface *c*omponents *r*ecognizing *a*dhesive *m*acro*m*olecules) of *Staphylococcus aureus*, *Enterococcus faecalis*, and *E. faecium* have been recognized as important virulence factors involved in adhesion, biofilm formation, and invasion (12, 24, 33, 37, 40, 43, 44).

To gain insight in the adaptive mechanisms that may have favored the emergence of CC17 *E. faecium*, we aimed to identify novel actively expressed LPXTG surface proteins in addition to Esp and Acm that are specifically enriched in CC17.

(Part of this study was presented at the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, Sep. 27 to 30, 2006 [abstr. G-0147].)

MATERIALS AND METHODS.

Bacterial strains and growth conditions.

A total of 131 *E. faecium* isolates collected from 20 countries worldwide, representing clinical (n = 31) and hospital outbreak (n = 18) isolates from hospitalized patients, surveillance isolates from hospitalized patients (n = 30) and from nonhospitalized persons (n = 30), 12 isolates from various animals (bison [n = 1], calves [n = 2], cat [n = 1], dogs [n = 2], pigs [n = 2], poultry [n = 2], rodent [n = 1], and ostrich [n = 1]), 2 isolates from animal food products, and 8 environmental isolates, were used in this study. The isolates were obtained from various isolation sites, i.e., bile (n = 1), blood (n = 22), carcass (n = 1), catheter (n = 2), environment (n = 6), feces (n = 72), food (n = 2), liquor (n = 1), peritoneal fluid (n = 2), urine (n = 4), and wound (n = 1), and 18 were from undetermined isolation sites. All 131 *E. faecium* isolates were typed previously by our group using multilocus sequence typing (MLST) (46). Based upon MLST, 40 isolates belonged to the hospital-adapted and multiresistant CC17, and 91 strains represented non-CC17 isolates. All bacterial strains were grown aerobically at 37°C on Trypticase soy agar II (TSA) plates supplemented with 5% sheep blood (Becton Dickinson, Alphen aan den Rijn, The Netherlands). *E. faecalis* strain V583 was used as the negative control (30).

Genome search for genes encoding LPXTG-like cell wall-associated proteins.

The draft assembly of the *E. faecium* TX0016 (TEX16, DO) genome deposited at DDBJ/EMBL/GenBank under the project accession number AAAK00000000 was searched for proteins annotated as cell wall surface anchor family, von Willebrand factor type A, Cna B type, or surface proteins from gram-positive cocci (anchor region). Furthermore, CWAPs were identified by searching for the presence of an N-terminal signal peptide sequence within the first 70 amino acids or a CWS. The CWS typically consists of a conserved LPXTG sortase substrate motif (where X denotes any amino acid), a hydrophobic domain, and at least one positively charged amino acid within the last eight residues of the C terminus. The presence of an N-terminal signal sequence was analyzed using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP) (SignalP score of >0.6) (2). The presence of the conserved LPXTG motif, hydrophobic amino acids, and one or more positively charged amino acids within the last 45 residues of the C-terminal end was examined visually. The LPXTG motif was initially compared to the consensus L[FV]PXT[AL]G[N] sortase cleavage motif of the closely related species *E. faecalis* V583 published at the sortase substrate database website (http://bamics3.cmbi.kun.nl/cgi-bin/jos/sortase_substrates/index.py) (3). In another approach, the LPXTG motif was compared to the [FILMPSVY][AP]X [ATS][GAKNS] and NPX[ST][DGNS] patterns representing distinct sortase substrates (10). To identify similarities, BLAST analyses on protein sequences were performed at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

PCR analysis.

The presence or absence of 22 genes encoding putative CWAPs in the 131 *E. faecium* isolates was determined in first instances by PCR. *E. faecium* cell lysates for PCR were prepared as described previously (38). A total of 5 μl of 1:10-diluted lysate was used in the PCR. To confirm the uniform quality of the lysates used in this study, a control PCR on the housekeeping gene *ddl* (encoding D-alanine, D-alanine ligase) was performed with primers ddl_fw and ddl_rv, which are specific for *E. faecium* prior to use of the lysate (26). In all PCRs, *E. faecium* TX0016 was used as a positive control and *E. faecalis* V583 as a negative control. Reactions were performed in 25-μl volumes with HotStar *Taq* polymerase and HotStar MasterMix buffers (QIAGEN Inc., Venlo, The Netherlands). The oligonucleotide PCR primers were designed from the region encoding the N-terminal region of the 22 putative CWAPs. The sequences of the oligonucleotides used in this study are listed in Table 1. The primers used for the detection of the CWAP genes share the same names as the CWAP genes unless otherwise stated. All oligonucleotides were purchased from Invitrogen Corporation (Breda, The Netherlands).

Southern blot and dot blot analysis.

Southern blot and dot blot analysis was performed on chromosomal DNA isolated from 131 *E. faecium* isolates to determine the presence or absence of CWAP genes and to confirm PCR results. Chromosomal DNA isolation was performed as described previously with some minor modifications (1). In brief, a loop of *E. faecium* cells was resuspended in 200 μl 10 mM Tris EDTA plus 10 μl lysozyme (50 mg/ml; Fluka Biochemika, Buchs, Switzerland) and incubated for 15 min at 37°C. After incubation, 30 μl 10% sodium dodecyl sulfate (SDS) and 20 μl proteinase K were added and incubated for 1 h at 65°C. Subsequently, chromosomal DNA was isolated using the DNeasy tissue kit (QIAGEN Inc.) according to the manufacturer's instructions. *E. faecium* TX0016 DNA was used as a positive control and *E. faecalis* V583 DNA as a negative control. Chromosomal DNA was digested with EcoRI (Roche Diagnostics),

and fragments were separated by agarose gel electrophoresis and capillary blotted onto a Hybond N$^+$ nylon membrane (GE Healthcare, Diegem, Belgium). For dot blot analysis, the chromosomal DNA was denatured with 0.5 M NaOH in a 96-well microtiter plate for 15 min, transferred to a 96-well Bio-Rad Bio dot apparatus, and vacuum blotted onto a Hybond N$^+$ nylon membrane. For both methods, DNA was fixed onto the membrane by incubation for 2 min in 0.4 M NaOH followed by a neutralization in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 1 min. Membranes were hybridized overnight at 42°C with a 100-ng probe. For Southern blot analysis, probes were generated by PCR in 50-μl reaction volumes by use of primer pairs 903_fw and 903_rv2, 905_fw and 905_rv2, 907_fw and 907_rv2, 2351_fw and 2351_rv2, and 2430_fw and 2430_rv. For dot blot analysis, gene-specific probes for *orf371*, *orf418*, *orf773*, *orf884*, *orf1901*, *orf1904*, *orf1996*, *orf2008*, *orf2009*, *orf2010*, *orf2109*, *orf2356*, *orf2514*, *orf2515*, *orf2569*, *orf2570*, and *orf2571* were amplified by PCR using primers depicted in Table 1. Amplified DNA probes were purified with a PCR purification kit (QIAGEN Inc.) and labeled according to directions for the ECL nucleic acid labeling kit (GE Healthcare). The membranes were exposed to Hyperfilm ECL (GE Healthcare).

TABLE 1. Oligonucleotides used in this study

ORF	Primer name[a]	Oligonucleotide sequence (5′→3′)	Start in sequence (5′→3′)[b]
orf371	371_fw	TGACTTCCAATGTACCGACA	161
	371_rv	GCTGCTGCGACTAACACAC	830
orf418	418_fw	CTAACTGGTAACTATGGCTTGT	121
	418_rv	GTCCGTGCTGTCACTTGT	1230
orf773	773_fw	GCATCAGTCATTAACCAGAGTA	139
	773_rv	CCCTGTCAAAGGAATAACG	1050
orf884	884_fw	AATCAGACAGTCCACACAGAG	61
	884_rv	ATGATTCCGCTCCACAGTA	1229
orf903	903_fw	TCAACGGACATACCATACCA	311
	903_rv	TCAGTTGGATTCCATGTGAT	1400
	903_rv2	CTTACCATCAACGATCTGCC	720
orf904	904s_fw	AGACGAGGAAGAGGGCAT	360
orf905	905_fw	GTGACAGATTCATCTAATCAT	1
	905_rv	TCATTTTATTTCCCTCCTATTG	1005
	905_rv2	GTGTGAAATGTATGAATCTTGTC	281
	905s_rv	CTTTGGATACAAGTGGATGG	51
orf906	906s_fw	GACACAGCAGTTCCAGCA	271
orf907	907_fw	GTGACCGGTTTTGATGAAAAC	1
	907_rv	TTAAGCTTCTGTTTCTTGATGGC	816
	907_rv2	GTTGTATTTTCCCGATTGAC	290
orf1901	1901_fw	CCGTCAAATCCAATCCAG	121
	1901_rv	CAGTACCTTGACCAAACTTCG	940
orf1904	1904_fw	AGGCAGATTATGGTGATGTT	251
	1904_rv	GGCTGTTGGTTCTTTATCTG	870
orf1996	1996_fw	TGAAGTTGCTGCTAACGAAC	81
	1996_rv	GCAACTTCGATTCCTTCTAC	1040
orf2008	2008_fw	CTACGGATTTTGGTGAAGAA	191
	2008_rv	TCGCACTTCTATTTCCTGAT	900
orf2009	2009_fw	GCTTGGCTATCTCTGAGGA	182
	2009_rv	TCATAGGCAGAGACTGGAAC	1100
orf2010	2010_fw	GTAGCGAAGAAAATGAGATGG	80
	2010_rv	TAACTTGACTGAATCGGTGC	1101
orf2109	2109_fw	GCAGGTGCAACTTATACATTAG	61
	2109_rv	CTTGATCCGTCGTAATATTGA	811
orf2351	2351_fw	AATGAACGGGCAAATGAG	82
	2351_rv	CTTTTGTTCCTTAGTTGGTATGA	753
orf2356	2356_fw	GTTGGTCATCTTATTTGCTGTA	30
	2356_rv	TGCCCTGCTCCTTCTACTA	1370
orf2430	2430_fw	GCAGTTTACAATGGTGTGAAGCAA	217
	2430_rv	CGGCTAATGAGTATTTGTCGTTCC	1180
	2430_rv2	AGAACCCTCAGTTTCCACAT	840
orf2514	2514_fw	AGTTCCAGTTGCGAGTCAGA	131
	2514_rv	ATGTAGTCGGATTCGGTGC	1120
orf2515	2515_fw	GTGGTAGAATTGACGAAAGA	1
	2515_rv	AACAAGTAGCACACCCAATA	990
orf2569	2569_fw	GTGTTTGCAGAGGAGACAGC	61
	2569_rv	GACAGAATAATTTACTGGGTCG	1182
orf2570	2570_fw	GGTAGCAACAGAAACAGAAAC	66
	2570_rv	GAATACTGCGCCAGCTAATC	1026
orf2571	2571_fw	ATGACGACCACAGGGAAGAA	1
	2571_rv	CCGCTATCTGCTAAAGTATC	980
ddl	ddl_fw	GAGACATTGAATATGCCTTATG	334
	ddl_rv	AAAAAGAAATCGCACCG	893

[a] Annotation: fw, forward; rv, reverse.
[b] Relative to the start codon of the open reading frame.

DNA sequencing.
PCR products were sequenced by using the BigDye Terminator 3.1 reaction kit and an ABI PRISM 3100 capillary DNA sequencer (both from Applied Biosystems, Foster City, CA).

Western blot analysis.
To detect 904.5 and 906.7 LPXTG protein expression, plate-grown bacteria were resuspended in phosphate-buffered saline (PBS) and harvested by centrifugation (1,560 × *g*, 5 min). To detect 904.5 protein expression, cell pellets were suspended in 70% formic acid and incubated at 65°C for 30 min as described previously (7). For the 906.7 protein, cell pellets were resuspended in 50 μl PBS plus 50 μl sample buffer (100 mM Tris-HCl, 5% dithiothreitol, 2% SDS, 0.004% bromophenol blue, and 20% glycerol) and boiled for 5 min. Equal amounts of protein samples of E135 (negative control) and E380 *E. faecium* were electrophoresed through a 10% SDS-polyacrylamide gel. Prior to electroblotting, the membranes were incubated for 10 min in blot buffer (20 mM Tris, 150 mM glycine, and 20% methanol, pH 8.3). Samples were electroblotted overnight using a Trans-Blot cell tank transfer unit at 15 V onto Trans-Blot nitrocellulose membranes (0.45 μm; Bio-Rad Laboratories Inc., Veenendaal, The Netherlands). The membranes were blocked with 4% skim milk (Campina Holland, Alkmaar, The Netherlands) in PBS-0.1% Tween 20 for 1 h at 37°C. Incubation with mouse anti-904 and mouse anti-906 immune sera (both at a 1:3,000 dilution) was carried out for 1 hour in 1% bovine serum albumin (BSA) in PBS-1% Tween 20 at 37°C, followed by two washes, each of 10 min, in PBS-0.1% Tween 20 at 37°C. As a control, blots were incubated with mouse preimmune sera with similar serum dilutions. Subsequently, membranes were incubated for 1 h with goat anti-mouse immunoglobulin G (IgG) (heavy plus light chains)-horseradish peroxidase (Bio-Rad) in 1% BSA in PBS (PBSb)-1% Tween 20 at 37°C. Membranes were washed twice with PBS-0.1%, Tween 20, and the 904.5 and 906.7 proteins were visualized using the ECL plus Western blotting detection system (GE Healthcare) and exposed to film (Hyperfilm ECL; GE Healthcare).

Peptide mouse antisera.
Polyclonal mouse antisera were prepared by Eurogentec (Seraing, Belgium) according to their protocol by immunizing mice with 15-amino-acid-residue keyhole limpet hemocyanin-conjugated peptide directed against the N-terminal regions of proteins 903 (H_2N-QTTEETNSPPYSEIQ-$CONH_2$), 904 (H_2N-CRMFESDKMPSISQND-$CONH_2$), 906 (H_2N-CGTELTFPNSEPLNG-$CONH_2$), 2351 (H_2N-CERANENLSFTVKTDR-$CONH_2$), and 2430 (H_2N-EFTPGYEKNPLPDMSC-$CONH_2$) of the *E. faecium* TX0016 genome sequence.

Generation of cDNA from total mRNA.
Eight *E. faecium* isolates, E135, E155, E380, E470, E745, E1165, E1172, and E1176 (4, 36, 39, 45, 46), were assayed for mRNA expression of the five CC17-enriched putative CWAP genes (*orf903*, *orf905*, *orf907*, *orf2351*, and *orf2430*). E135 is a community surveillance isolate and was used as a negative control. E155, E470, and E745 were isolates from hospital outbreaks in the United States and The Netherlands, while E380, E1165, E1172, and E1176 were all clinical isolates recovered from blood, wound, urine, and respiratory tract, respectively. Bacterial strains were grown overnight on TSA at 37°C, scraped off of the plates, resuspended into 3 ml PBS to an optical density at 660 nm of 1.0 (~1 × 10^9 CFU/ml), and pelleted by centrifugation (6,500 × *g* for 1 min). Total RNA was isolated according to the method of Cheung et al. (5). RNA purification and cDNA generation was done according to the method of Nallapareddy et al.

(21). In brief, total RNA was treated three times with 20 U of RQ1 DNase (Promega Corp., Leiden, The Netherlands) for 30 min at 37°C, and RNA was isolated using an RNeasy minikit (QIAGEN Inc.). From 5 μg total RNA, cDNA was synthesized with the SuperScript II first-strand synthesis system (Invitrogen Corp.) using random primers according to the instruction of the manufacturer. cDNA was used as the template for PCR using primer pairs 903_fw and 903_rv2, 905_fw and 905_rv2, 907_fw and 907_rv2, 2351_fw and 2351_rv, and 2430_fw and 2430_rv2 as shown in Table 1. As an internal control, the housekeeping gene *ddl* (encoding D-alanine, D-alanine ligase) was amplified using primers shown in Table 1. RNA samples not treated with reverse transcriptase were used as a control to detect DNA contamination in the total RNA preparations.

Electron microscopy and immunogold labeling.

Transmission immunoelectron microscopy was performed as described previously with some modifications (40). In brief, copper grids (mesh Formvar-carbon coated) were incubated for 30 min with carbon side on a drop of 1×10^9 CFU/ml of *E. faecium* E380 cells (CC17 isolate containing *orf904.5* allele 2 and *orf906.7* insertion A) to detect surface-exposed 903, 904.5, and 906.7 protein expression and E470 and E745 cells to detect surface-exposed 2351 and 2430 protein expression, respectively. The E135 strain was used as a negative control. Grids were washed three times for 5 min on drops of 0.02 M glycine in PBS and subsequently blocked for 30 min on drops of 1% PBSb. The five LPXTG surface proteins were labeled for 1 h on drops with 1:100 (for the 903, 904, and 906 sera)-, 1:250 (for the 2430 serum)-, or 1:300 (for the 2351 serum)-diluted specific peptide mouse immune sera in PBSb. The preimmune sera were diluted similarly. Grids were washed four times for 2 min on drops of 0.1% PBSb. Then, grids were incubated for 20 min on drops with 1:250-diluted rabbit anti-mouse IgG antibody (Dako, Glostrup, Denmark) in PBSb and washed four times for 2 min on drops of 0.1% PBSb. Antibody complexes were labeled by incubation for 20 min on drops with 1:55-diluted protein A-gold label (15 nm) in PBSb. Grids were washed four times for 2 min on drops of PBS, fixed by incubation for 5 min on drops of 1% glutaraldehyde in PBS, and washed again eight times for 2 min on drops of H_2O. Bacteria were stained by incubation of the grids for 5 min on drops containing 1.8% methylcellulose (25 centipoises; Sigma-Aldrich, St. Louis, MO) and 0.4% uranyl acetate (pH 4) and subsequently air dried for 10 min. Grids were examined using a Jeol 1010 transmission electron microscope (Jeol Europe, Amsterdam, The Netherlands) at a magnification of ×65,000.

MST analysis.

The BioNumerics software, version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium), was used to generate a minimum spanning tree (MST) as described previously (32). The MST was based on the presence and absence of the 22 putative CWAP genes. The categorical coefficient was used to calculate the MST. CWAP gene profile types were linked by the eBURST priority rule by first linking profiles that differ in the presence or absence of only one CWAP gene. When two types have an equal distance to a linkage position in the tree, the type with the highest number of single CWAP gene variants is linked first.

Nucleotide sequence accession numbers.

The DNA sequences of the junction regions of the *904.5* and *906.7* (types A, B, and C) open reading frames (ORFs) reported in this article have been deposited in the GenBank nucleotide sequence database under accession numbers EU122157, EU122158, EU122159, EU122160, EU122161, and EU122162.

RESULTS.

Genome search for ORFs encoding putative CWAPs.

The partially sequenced and annotated genome of *E. faecium* TX0016 was searched for ORFs encoding putative CWAPs. Putative CWAPs were identified by the presence of a C-terminal CWS domain. The genome search identified 22 putative CWAP ORFs, of which 18 contained an N-terminal signal sequence and a CWS domain, 4 ORFs only a CWS and no N-terminal signal sequence, and 1 ORF an N-terminal signal sequence but no positively charged amino acids in the CWS (Table 2). Eleven of the 22 proteins contained the most abundant canonical LPXTG motif, while the remaining proteins had deviant cell wall anchor motifs.

TABLE 2. Characteristics of the 22 putative LPXTG cell wall-associated proteins

ORF	Signal peptide (probability)	Cleavage site probability	C-terminal cell wall sorting sequence[a]	Accession no.
orf371	Yes (1.000)	0.995	**LPKTN**EKTASVSYSLAGAGVLVAAAYGII<u>RKKK</u>	ZP_00604782
orf418	Yes (1.000)	0.976	**LPKTG**SQSNNWITLAGVILLVIGLRITFMSY<u>KKSKR</u>	ZP_00604835
orf773	Yes (0.923)	0.853	**IPLTG**ITFPFLSQGGSSLLMLSICVGFVLNISADE<u>KRKS</u>LGL	ZP_00604269
orf884	Yes (0.804)	0.798	**LPKSG**ESQNKVILWSGII LLSIATMLSA<u>KR</u>F<u>K</u>QNRSL	ZP_00603974
orf903	Yes (0.840)	0.787	**LPSTG**GIGLLPFVFLGLIFIFSGFFYFIH<u>RKKK</u>AGEQ<u>R</u>	ZP_00604460
orf905	No	0.000	**FPKTS**EEMLGGFSILGLLLVLSTGTAWFYKKQQ<u>K</u>GNN<u>RR</u>EI<u>K</u>	ZP_00604462
orf907	No	0.000	**LPSTG**GMGIIVFILVGTALVGGAVIYF<u>KKR</u>HQETEA	ZP_00604464
orf1901	Yes (0.999)	0.999	**VPKTG**STHLVTISAVSLLLLVLATFSYAVL<u>R</u>YI	ZP_00603526
orf1904	Yes (0.999)	0.999	**VPMTG**SNGFQTYVLISCLLLGAGALSAVVYF<u>KKK</u>A	ZP_00603529
orf1996	Yes (1.000)	0.914	**LPKTG**EFNNPLLALAGGILLIGVVVYVMK<u>Q</u><u>RKK</u>	ZP_00603029
orf2008	Yes (1.000)	0.999	**LPATG**GNGLLAFLLIGISLMIGAYSWY<u>RKSKMKS</u>EV	ZP_00603041
orf2009	Yes (0.999)	0.797	**FPQTN**EARTLISLLGILLLLGAVLLI<u>KKER</u>GE<u>K</u>NE	ZP_00603042
orf2010	No	0.000	**LPATG**GSGRLFYILLAAFFMALAGAAYGLFV<u>R</u>QS<u>KK</u>EGA<u>R</u>	ZP_00603043
orf2109	Yes (0.993)	0.512	**QPITN**TLLEQGQKKLKITAKDTSINITTDQEDNETD	ZP_00602865
orf2351	Yes (1.000)	0.782	**LPETG**SRTLNKLITWMGVLLILIVGASYF<u>R</u>SLFH<u>R</u>V<u>K</u>	ZP_00602747
orf2356	Yes (0.989)	0.938	**LPKTG**EKKHSLLIYSGGFLSVSTIITWIW<u>RRKR</u>G	ZP_00602752
orf2430	Yes (1.000)	1.000	**FPQTG**EKNSNVLLFIGFTLIFATAGYYFWN<u>RR</u>N	ZP_00603098
orf2514	Yes (1.000)	0.978	**LTPGG**VVLTKIDDQSGEILQELFLSYRTE<u>K</u>F<u>KR</u>Y<u>K</u>PV	ZP_00602630
orf2515	No	0.000	**LPKTG**ETIISQFILSILGVLLVFISIKF<u>RKKR</u>NI	ZP_00602631
orf2569	Yes (1.000)	0.997	**LPSTG**GKGIYVYIGAGVVLLLIAGLYFA<u>RRK</u>HSQI	ZP_00602687
orf2570	Yes (0.678)	0.516	**LPKTN**ETKNTLLGVVGMVFASFAIWLFI<u>KKR</u>TGV<u>KK</u>	ZP_00602688
orf2571	Yes (1.000)	0.997	**LPETG**GIGRLGIYLVGMIGCAFSIWYLFL<u>KKER</u>GGS	ZP_00602689

[a] The LPXTG-like motifs are depicted in bold, and the positively charged C-terminal amino acids are underlined.

Distribution of 22 putative CWAP-encoding genes among *E. faecium* isolates.

The presence of the 22 identified putative CWAP-encoding genes among 131 *E. faecium* isolates was determined by PCR and Southern blot and dot blot hybridizations. Based on this, 17 of the 22 putative CWAP genes were found to be widespread and highly prevalent in the *E. faecium* population (Table 3, lower part). Three ORFs, *orf371*, *orf773*, and *orf2109*, were detected in all 131 isolates, thus presumably belonging to the *E. faecium* core genome. Four ORFs, *orf418*, *orf884*, *orf1996*, and *orf2356*, were detected in 100% of the CC17 isolates and in 91.2 to 98.9% of the non-CC17 isolates. The ORFs *orf371*, *orf773*, *orf1901*, *orf1904*, *orf2008*, *orf2009*, *orf2010*, *orf2109*, *orf2514*, *orf2515*, *orf2569*, *orf2570*, and *orf2571* were detected in 90 to 100% of the CC17 *E. faecium* isolates and in 40.7 to 85.7% of the non-CC17 isolates (Table 3). Five of the 22 putative CWAP genes were specifically enriched in CC17 *E. faecium* isolates recovered from clinical sites or associated with hospital outbreaks (Table 3, upper part). Three putative CWAP genes, *orf903*, *orf905*, and *orf907*,

TABLE 3. Incidence of 22 putative CWAP genes in hospital-adapted *E. faecium* CC17 and non-CC17 isolates

Description	ORF	Distribution [no. (%)] of the 22 putative CWAP genes among:	
		CC17 isolates (n = 40)	Non-CC17 isolates (n = 91)
CC17 enriched	*orf903*	40 (100)	22 (24.2)
	orf905	40 (100)	20 (22.0)
	orf907	40 (100)	20 (22.0)
	orf2351	40 (100)	24 (26.4)
	orf2430	28 (70)	7 (7.7)
Widespread	*orf371*	40 (100)	91 (100)
	orf418	40 (100)	84 (92.3)
	orf773	40 (100)	91 (100)
	orf884	40 (100)	91 (98.9)
	orf1901	36 (90)	38 (41.8)
	orf1904	39 (97.5)	78 (85.7)
	orf1996	40 (100)	91 (98.9)
	orf2008	38 (95)	77 (84.6)
	orf2009	37 (92.5)	72 (79.1)
	orf2010	37 (92.5)	37 (40.7)
	orf2109	40 (100)	91 (100)
	orf2356	40 (100)	83 (91.2)
	orf2514	40 (100)	68 (74.7)
	orf2515	40 (100)	70 (76.9)
	orf2569	40 (100)	70 (76.9)
	orf2570	40 (100)	67 (73.6)
	orf2571	40 (100)	70 (76.9)

clustered together on contig 609 (gi 68194716) on the *E. faecium* TX0016 genome and were detected in all (40/40) of the CC17 *E. faecium* isolates (Fig. 1A). This cluster was present only in a minor subset of non-CC17 isolates, *orf903* in 24.2% (22/91) and *orf905* and *orf907* in 22.0% (20/91). The 20 non-CC17 isolates that contain this cluster of putative CWAP genes represented hospital outbreak isolates (3), clinical isolates (6), hospital surveillance isolates (5), and community surveillance isolates (6). Thus, the majority (14/20) of these non-CC17 isolates were also hospital derived. In addition, the *orf903* gene was also found in one extra hospital surveillance isolate and one community surveillance isolate. The predicted 903 protein is 773 amino acids in size, and the C-terminal part has the highest sequence similarity with a CWAP of *Listeria monocytogenes* strain 1/2a F6854 designated LMOf6854_0833, with an unknown function (Table 4). The predicted 905 protein is 338 amino acids in size and contains one Cna B-type domain in the C-terminal part of the protein. This Cna B-type domain was found in the *S. aureus* collagen binding protein but is not involved in collagen binding (34). It has the highest similarity with the endocarditis- and biofilm-associated pilin protein EbpB (previously known as EF1092), a CWAP of *E. faecalis* OG1RF (Table 4) (23). The predicted 907 protein is 371 amino acids in size and contains two Cna B-type domains, one located in the N-terminal part and one in the C-terminal part of the protein, and has the highest similarity with the biofilm enhancer in enterococcus 3 protein (Bee3) of *E. faecalis* (Table 4) (35). The presence of putative transcriptional regulators upstream of *orf903* (LuxR; *orf902*) and downstream of *orf907* (MgA-like; *orf910*, MerR; *orf912*, LysR; *orf913*, and *orf914*), direct repeats upstream of *orf901* and downstream of *orf911*, and an integrase (*orf901*) may imply that this region encompasses a distinct genomic island that is acquired through horizontal gene transfer.

Putative CWAP gene *orf2351* was detected in all (40/40) of the CC17 *E. faecium* isolates and in 26.4% (24/91) of the non-CC17 isolates. Of the 91 non-CC17 isolates,

the 24 which harbor *orf2351* included clinically relevant (2 hospital outbreak and 7 clinical isolates) as well as less clinically relevant (9 surveillance, 3 animal, and 3 environmental *E. faecium* isolates) isolates. The predicted 2351 protein is 324 amino acids in size and contains a serine-rich repeat region close to the C terminus. It has the highest similarity to a CWAP of *E. faecalis* V583 designated EF0093 (Table 4). The serine-rich repeat region of the 2351 protein is highly similar to the serine-rich repeat domain of the clumping factor B protein of *Staphylococcus aureus* subsp. *aureus* NCTC 8325.

orf2430 was detected in 70% (28/40) of the CC17 *E. faecium* isolates and in only 7.7% (7/91) of the non-CC17 isolates. The majority of the non-CC17 *E. faecium* isolates (six of seven) harboring *orf2430* are hospital-derived isolates (two hospital outbreak isolates, three clinical isolates, and one hospital surveillance isolate). The predicted 2430 protein is 1075 amino acids in size and contains four conserved Cna B domains. The protein has the highest similarity with a CWAP of *E. faecalis* V583 designated EF1896 (Table 4) and a collagen adhesion protein of *Bacillus cereus* ATCC 14579.

TABLE 4. Homologies of the five LPXTG surface proteins enriched in CC17 *E. faecium* isolates as determined by BLAST

Protein	BLAST hit	Function	Organism	% Amino acid identity
903	LMOf6854_0833	Unknown	*L. monocytogenes* 1/2a F6854	40
905	EbpB	Biofilm formation	*E. faecalis* OG1RF	36
904.5	EbpB	Biofilm formation	*E. faecalis* OG1RF	40
907	Bee3	Biofilm formation	*E. faecalis*	29
906.7	Bee3	Biofilm formation	*E. faecalis*	28
2351	EF0093	Unknown	*E. faecalis* V583	57
2430	EF1896	Unknown	*E. faecalis* V583	90

Sequence heterogeneity in the *orf903-907* gene cluster.

The *orf904* gene in the *orf903-907* cluster of the sequenced *E. faecium* TX0016 genome is predicted to encode a protein with an N-terminal signal peptide sequence (signal peptide probability, 1.000; cleavage site probability, 1.000) but no CWS. In contrast, *orf905* is predicted to encode a protein with no detectable signal peptide sequence but with an intact CWS (Fig. 1A). Detailed examination of the *orf904-905* junction region suggests that *orf904* in the sequenced *E. faecium* TX0016 genome contains a premature TAA stop codon at position 7688 of contig 609 (Fig. 1B, allele 1). The region encompassing this stop codon was amplified by PCR in all isolates positive for *orf905* (60 of 131) by use of primers 904s_fw and 905s_rv to yield a 191-bp DNA fragment that was subsequently sequenced. Sequencing revealed four different alleles in the *orf904-905* junction region. In 13 of the 60 isolates, a single thymine-to-cytosine point mutation changed the TAA stop codon into CAA, resulting in a merging of the *orf904-905* genes, leading to a potential functional CWAP gene designated *orf904.5*, encoding a protein with an N-terminal signal sequence and a CWS (Fig. 1A and B). The T-to-C point mutation was found in two hospital outbreak isolates, four clinical isolates, two hospital surveillance isolates, and five community surveillance isolates.

Similarly to *orf904-905*, *orf906* encodes a putative protein with an N-terminal

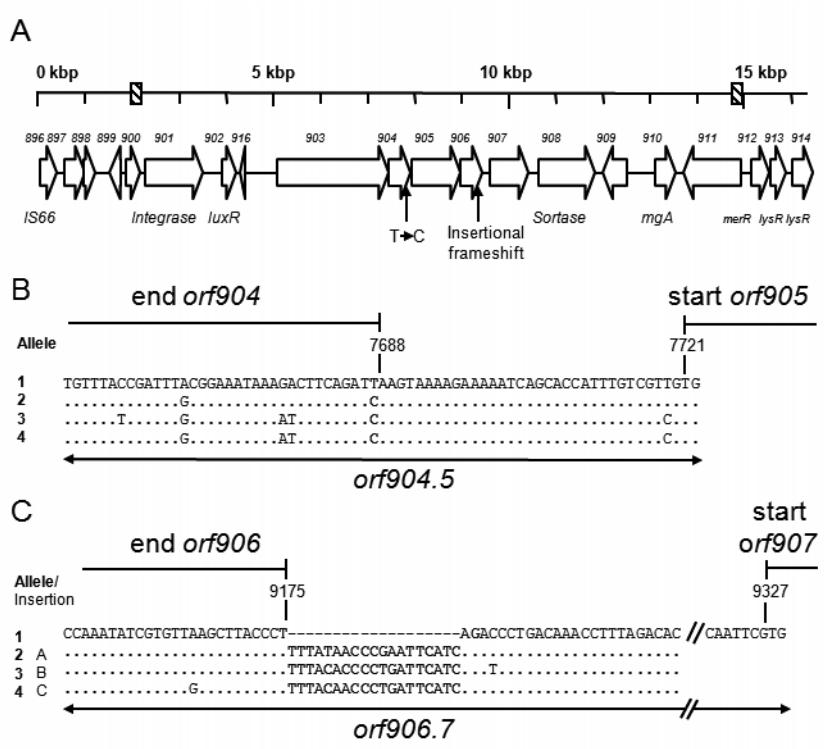

Figure 1. The genomic cluster (*E. faecium* contig 609) enriched in hospital adapted *E. faecium* CC17 isolates. (A) The genomic organization of contig 609 as published at DDBJ/EMBL/Genbank. Direct repeats (DR) are indicated as dashed boxes in the scale at position 2030 and 14751 of contig 609. The thymine to cytosine point mutation and the 19 bp oligonucleotide insertion are indicated with black arrows. The position and presumed direction of transcription of ORFs has been indicated by open arrows. (B) Alignment of the *orf904-905* junction region. The TAA stop codon is depicted in bold in strain TX0016 at position 7688 (Allele 1). In alleles 2, 3 and 4 a thymine to cytosine point mutation lead to a merged *orf904.5*. (C) Different alleles in the *orf906-907* junction region. Allele 1 is found in 29 *E. faecium* isolates including TX0016. Alleles 2, 3 and 4 resulted from three different 19 bp insertions (A, B and C) and were found in 26, 3 and 2 isolates respectively. The TAG stop codon is depicted in bold in strain TX0016 at position 9175 (Allele 1) and is disrupted by the 19 bp insertion to yield *orf906.7*.

signal peptide sequence (signal peptide probability, 1.000; cleavage site probability, 0.975) but no CWS, while *orf907* encodes a putative protein with no detectable signal peptide sequence but with an intact CWS. Examination of the *orf906-907* junction region suggests that *orf906* contains a premature TAG stop codon (Fig. 1C, allele 1). The region encompassing this stop codon was amplified by PCR in all isolates positive for *orf907* (60 of 131) by use of primers 906s_fw and 907_rv2 and yielded a 616-bp product. Sequencing of this fragment revealed that in 31 of the 60 isolates, a 19-bp oligonucleotide insertion at position 9175 of contig 609 caused a frameshift which merged the *orf906-907* genes, leading to a putative functional CWAP gene

designated *orf906.7* encoding a protein with an N-terminal signal sequence and a CWS (Fig. 1C). In total, four different alleles were found. Allele 1 is present in TX0016, while alleles 2 to 4 resulted from three different types of 19-bp oligonucleotide insertions. Twenty-six isolates contained the TTTATAACCCGAATTCATC insertion (type A), three isolates contained the TTTACACCCCTGATTCATC insertion (type B), and two contained the TTTACAACCCTGATTCATC insertion (type C). All three (A, B, and C) insertions putatively encode different amino acid sequences, i.e., FYNPNSS, FYTPDSS, and FYNPDSS, respectively. The predominant insertion A was found in nine clinical, seven outbreak-associated, six hospital surveillance, and four community surveillance isolates. The majority of the strains (22/26) with this insertion are hospital-derived isolates. Insertion B was found in one outbreak-associated isolate, one hospital surveillance isolate, and one community surveillance isolate. Insertion C was found in one clinical and in one community surveillance isolate. Only 12 of the 131 isolates (9.2%) carry both the T-to-C point mutation in the *orf904-905* junction and the insertion in the *orf906-907* junction. Seven of the 12 of these isolates are hospital-associated isolates.

Newly identified *orf904.5* and *orf906.7* encode 904.5 and 906.7 LPXTG proteins.

To confirm that *orf904.5* and *orf906.7* encode 904.5 and 906.7 LPXTG proteins, Western blotting was performed on *E. faecium* strain E380 (containing *orf904.5* allele 2 and *orf906.7* insertion A) and strain E135 (negative control). Mouse antipeptide immune sera raised against the 904 and 906 proteins reacted with 48-kDa and ~70-kDa protein bands, respectively, in protein extracts of E380 but not in negative control E135 (Fig. 2), while mouse preimmune sera did not react with these proteins (data not shown). The observed molecular mass of the 904.5 protein correlated well with the predicted molecular mass of 48.05 kDa (after posttranslational modification). In contrast, the observed molecular mass of the 906.7 protein is higher than the predicted molecular mass (44.62 kDa with a pI of 4.4 after posttranslational modification). Interestingly, a similar effect was also observed for the Acm LPXTG surface protein of *E. faecium* (24). This aberrant migration in SDS-polyacrylamide gel electrophoresis is possibly due to an association with peptidoglycan or the acidic nature of the 906.7 LPXTG protein.

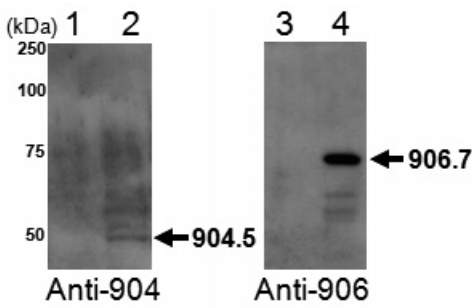

Figure 2. Western blots of protein extracts of *E. faecium* E135 and E380 isolates probed with mouse anti-904 (left part) and mouse anti-906 (right part) immune sera. The western blots show expression of the 904.5 and 906.7 LPXTG proteins in *E. faecium* E380 (lane 2 and 4) as depicted by arrows and not in the negative control E135 (lane 1 and 3). Numbers depicted on the left indicate molecular weights in kilo Daltons.

mRNA expression of the five CC17-enriched genes after growth *in vitro* on TSA.

Total RNA was isolated of one community surveillance isolate (E135; negative control), three outbreak-associated isolates (E155, E470, and E745), and four clinical *E. faecium* isolates (E380, E1165, E1172, and E1176) after growth on TSA plates overnight at 37°C. The mRNA expression of the five CC17-enriched putative CWAP genes (*orf903*, *orf905*, *orf907*, *orf2351*, and *orf2430*) and of *ddl* (internal housekeeping control) was analyzed by PCR on cDNA generated from total mRNA. mRNA transcripts of *orf903*, *orf905*, *orf907*, and *orf2351* were detected in seven CC17 isolates which harbor these ORFs, indicating that these ORFs are expressed and do not represent silent genes (Fig. 3A to E). mRNA transcripts of *orf2430* were detected in E155, E470, E745, E1165, and E1172, although at levels lower than those of *orf903*, *orf905*, *orf907*, and *orf2351*, suggesting that *orf2430* transcription is low during growth on TSA at 37°C (Fig. 3B). As expected, no *orf2430* mRNA transcripts were detected for E380 and E1176 (lanes 3 and 8), two strains deficient for *orf2430*. No DNA contamination was detected in control *ddl* reactions of the DNase-treated total mRNA preparations in which the reverse transcriptase reaction was omitted (Fig. 3F). The control *ddl* housekeeping gene was expressed in all isolates analyzed (Fig. 3G).

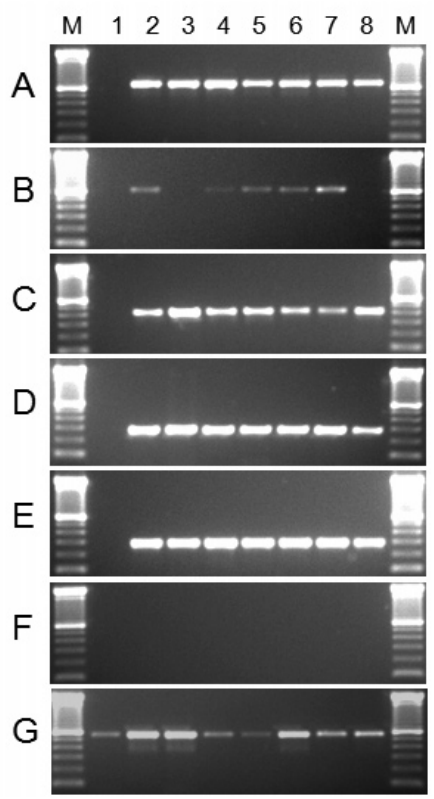

Figure 3. mRNA expression of *orf903*, *orf905*, *orf907*, *orf2351*, and *orf2430*. The mRNA is depicted of three outbreak associated isolates E155, E470 and E745 (lanes 2, 4 and 5) and four clinical isolates E380, E1165, E1172 and E1176 (lanes 3, 6-8). Lane 1 contains mRNA of the E135 negative control, a community *E. faecium* isolate, lacking all five CC17 enriched putative CWAP genes. Strains E380 and E1176 (lane 3 and 8) are deficient for *orf2430*. Panel A-E shows mRNA expression of the putative CWAP genes *orf2351*, *orf2430*, *orf903*, *orf905* and *orf907* respectively. Control *ddl* PCR reactions (panel F) on total mRNA preparations in which the RT reaction was omitted were all negative, demonstrating absence of DNA contamination. Control *ddl* RT-PCR reactions (internal housekeeping control, panel G) with *E. faecium* specific *ddl* primers were all positive. The results are presented as amplified PCR products electrophoresed on the same ethidium bromide-stained 1.5% agarose gel.

Surface-exposed expression of the 903, 904.5, 906.7, 2351, and 2430 LPXTG proteins.

Surface expression of all five CC17-enriched putative surface proteins was demonstrated by transmission electron microscopy of negatively stained immunogold-labeled bacteria. By use of peptide antisera directed against the five surface proteins, gold particles were clearly associated with the cell walls of strains positive for the respective genes (Fig. 4A to E). In contrast, no gold particles were associated with the cell wall when cells were incubated with the individual mouse preimmune sera (Fig. 4F). No gold particles were detected at the cell wall when immune sera were omitted (conjugate control; rabbit anti-mouse IgG plus protein A-gold); this finding was comparable to that for strain E135 (negative control; deficient for *orf903-906.7*, *orf2351*, and *orf2430*), when E135 cells were incubated with each antiserum separately (Fig. 4G and H).

Enrichment of 22 putative CWAP genes among clinical and outbreak-associated isolates.

Although the 22 putative CWAP genes studied are found in *E. faecium* isolates from different ecological niches or genetic backgrounds, they are not equally distributed (Fig. 5A). On average, clinical and outbreak-associated isolates contain 19 and 21 of the 22 identified putative CWAP genes, respectively. Human hospital surveillance isolates harbor on average 17 of the 22 CWAP genes and human community surveillance isolates harbor 14, while animal and environmental isolates contain only 13 and 15 of the 22 putative CWAP genes, respectively.

These results suggest that variant numbers or various combinations of putative CWAP genes contribute to survival and growth in different ecological niches. It is interesting in this respect that isolates not belonging to the hospital-adapted genetic complex CC17 contain significantly fewer (P = 0.02) of the 22 putative CWAP genes than isolates that do belong to CC17 (Fig. 5B). On average, CC17 isolates contain 21 of the 22 studied CWAP genes, whereas non-CC17 isolates contain on average only 15 of these CWAP genes.

Indication of horizontal gene transfer of putative CWAP genes.

An MST based upon clustering on the presence and absence of the 22 putative CWAP genes revealed a grouping of 58 isolates in a distinct CWAP-enriched cluster (Fig. 6A). Isolates belonging to this cluster belong predominantly to CC17 (n = 40/58; 70%) (Fig. 6A) and contain the highest proportion of CC17-enriched CWAP genes (Fig. 6B). However, the CWAP-enriched cluster also contains 18 non-CC17 isolates which are not evolutionary linked to CC17. Fourteen of these 18 are hospital derived. This strongly indicates horizontal transfer of putative CWAP genes among *E. faecium* isolates of distinct genetic backgrounds. The facts that isolates within the CWAP-enriched cluster are predominantly hospital related (55/58; 95%) and that of all hospital isolates 87% (55/63) group in this cluster suggest that the acquisition

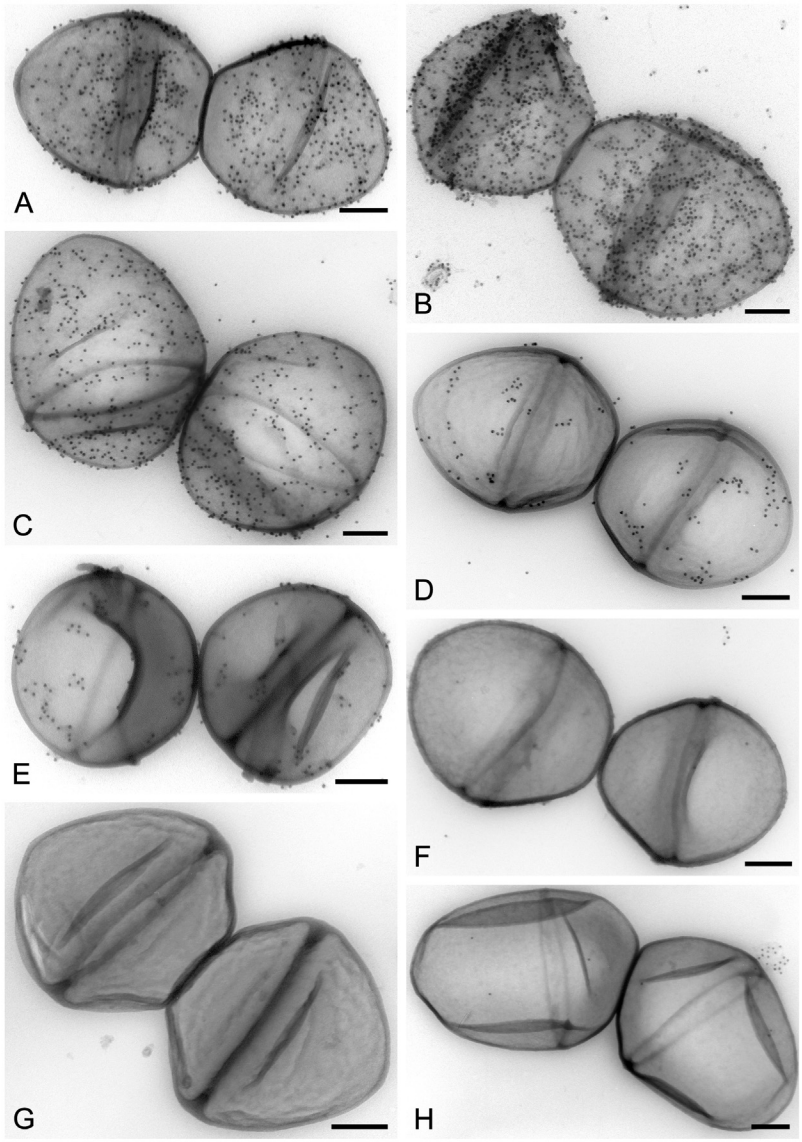

Figure 4. Transmission electron microscope micrographs. *E. faecium* isolates E135, E380, E470 and E745 were negatively stained and labeled individually with five peptide immune sera directed against the five CC17 enriched LPXTG surface proteins followed by rabbit anti-mouse IgG and protein A-gold (15nm). Panel A indicates E380 *E. faecium* cells (containing *orf904.5* allele 2 and *orf906.7* insertion A) incubated with anti-903 mouse immune serum, (B) E380 cells incubated with anti-904 mouse immune serum, (C) E380 cells incubated with anti-906 mouse immune serum, (D) E470 cells incubated with anti-2351 mouse immune serum and (E) E745 cells incubated with anti-2430 mouse immune serum. (F) E745 cells incubated with mouse pre-immune serum. (G) E470 cells incubated with only rabbit anti-mouse IgG and protein A-gold (conjugate control). (H) E135 cells (negative control; deficient for *orf903-906.7*, *orf2351* and *orf2430*) incubated with anti-903 immune serum (incubation with anti-904, 906, 2351 and 2430 immune sera, which also did not display gold labeling, are not shown). Bar = 200 nm.

of CWAP genes increases the survival and spread of *E. faecium* in the hospital environment.

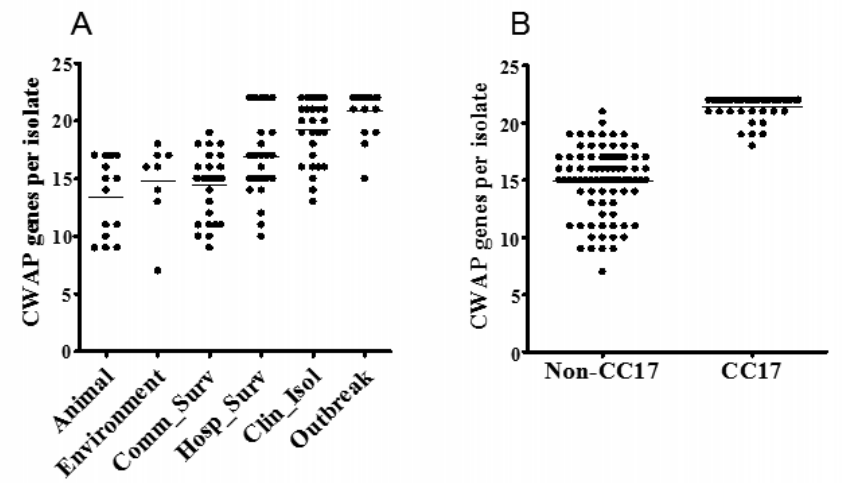

Figure 5. Distribution of putative CWAP genes among *E. faecium* isolates. Each black circle represents an *E. faecium* isolate with a specific number of putative CWAP genes. Horizontal lines indicate the average number of putative CWAP genes in each epidemiological class of isolates. (A) Distribution among distinct epidemiological classes. The epidemiological classes are ordered with increasing clinical relevance. Annotation: Comm_surv, community surveillance isolates from human volunteers not connected to hospitals. Hosp_surv, hospital surveillance isolates from hospitalized patients not associated with enterococcal infection or outbreak. Clin_Isol, isolates from clinical sites from hospitalized patients. Outbreak, isolates from hospital outbreaks. (B) Distribution of putative CWAP genes among CC17 and non-CC17 *E. faecium* isolates. On average CC17 isolates contain 21 of the 22 CWAP genes, whereas non-CC17 isolates contain 15 of the 22 CWAP genes in their genome.

DISCUSSION.

Although knowledge of the adaptive mechanisms of CC17 *E. faecium* has gradually increased, the complete nature of the process of hospital adaptation remains poorly understood. In this study, we show that clinical and outbreak *E. faecium* isolates belonging to CC17 as well as 14 hospital-derived isolates not belonging to CC17 contain on average 19 and 21 of 22 analyzed putative CWAP-encoding genes identified in the incomplete genome sequence of an endocarditis *E. faecium* isolate (TX0016) belonging to CC17. In contrast, non-CC17 isolates e.g., animal, human community, and environmental *E. faecium* strains, contain on average only 13, 14, and 15 of the 22 putative CWAP genes, respectively. The enrichment of putative CWAP genes in clinical and outbreak-associated *E. faecium* isolates was striking. The presence of variant numbers and combinations of CWAP genes by distinct epidemiological class may contribute to successful adaptation to the different ecological niches and thus may provide a selective advantage for these isolates in the hospital setting. Five LPXTG surface protein genes were specifically

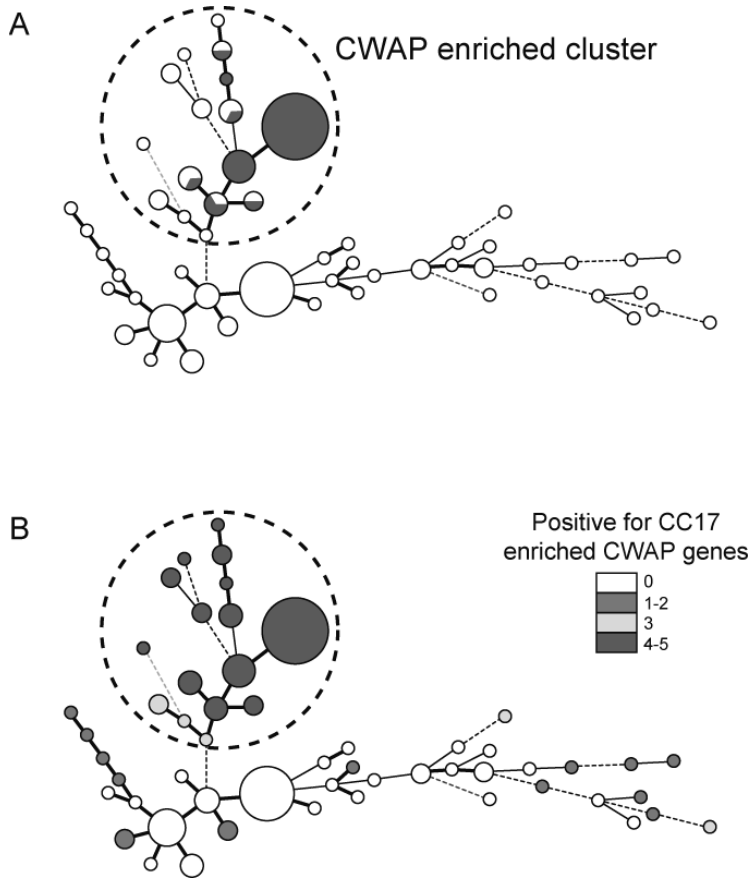

Figure 6. MST based on clustering on the presence and absence of the 22 putative CWAP genes. A categorical coefficient and the eBURST priority rule of the highest number of single-locus changes were used for the clustering. Circles represent putative CWAP gene profile and the size of the circles indicate the number of isolates. Thick short lines connecting two CWAP gene types denote types differing by a single CWAP gene, thin lines connect double CWAP gene variants, and dotted lines indicate the most likely connection between two types differing by more than two CWAP genes. (A) All 40 CC17 isolates are marked red and cluster together with 18 non-CC17 clinical isolates (marked white) in a CWAP enriched cluster. (B) The presence of 0, 1-2, 3, 4-5 CC17 enriched CWAP genes is indicated with colours depicted in the legend. See appendix for color figure.

enriched in CC17 *E. faecium* isolates, and expression in terms of mRNA level and at the surface of the cell was confirmed. These CC17-enriched genes encoding surface-exposed LPXTG proteins do not belong to the *E. faecium* core genome, as they were absent from the majority of the non-CC17 isolates. The ORFs *orf903*, *orf905*, and *orf907* clustered together on the *E. faecium* genome on a novel genomic island covering contig 609. Two lines of evidence support this assumption. First, this region does not harbor housekeeping genes but putatively encodes accessory functions. Second, it is most likely acquired by horizontal gene transfer, as it is found in isolates that are

(based upon MLST) not closely evolutionary linked. Furthermore, we hypothesize that this genomic island constitutes a putative pathogenicity island, since it is largely missing from nonclinical non-CC17 isolates. The deduced protein sequences of this region revealed an N-terminal signal sequence in the 903, 904, and 906 proteins and an LPXTG-like motif followed by a hydrophobic domain at the C termini in the 903, 905, and 907 proteins. The presence of a thymine-to-cytosine point mutation in the *orf904-905* junction and a 19-bp insertion in the *orf906-907* junction merging *orf904-905* into *orf904.5* and *orf906-907* into *906.7* was found only in a subset (9.8%) of the 131 isolates. Insertion A, the most frequently found 19-bp insertion, which removed the stop codon in *orf906*, possibly occurred through a site-specific recombinatorial event. The other two insertions, B and C, may have arisen by multiple-nucleotide mutations of insertion A. For these isolates, *orf904.5* and *orf906.7* are predicted to encode a CWAP with an N-terminal signal sequence and a CWS. For *E. faecium* strain E380, this was demonstrated using Western blotting and immunoelectron microscopy. The presence of transcriptional regulators and a sortase downstream of *orf906.7* suggests that the three putative CWAP genes are part of a functional transcriptional unit and that the 904.5 and 906.7 proteins are anchored to the cell wall by a sortase-dependent mechanism. The 903 and 904.5 proteins have similarities to uncharacterized putative CWAPs of *L. monocytogenes* and *E. faecalis*. Furthermore, the 906.7 protein is homologous to the recently discovered Bee3 protein, which plays a role in biofilm formation in *E. faecalis* (35), and therefore we hypothesize that the 903, 904.5, and 906.7 proteins may also play a role in biofilm formation of *E. faecium*. The ability to form biofilm is clinically highly relevant, as the pathogeneses of many bacterial infections have been attributed to this process (27).

The serine-rich repeat region of the 2351 LPXTG surface protein displays high similarity with the serine-rich repeat domain of the cell wall-anchored clumping factor B protein (ClfB) of *S. aureus*. The ClfB protein is an MSCRAMM involved in fibrinogen binding (25). Fibrin and fibrinogen are major components of blood clots and are the major plasma proteins deposited on implanted foreign devices. The ability of *S. aureus* to adhere to fibrinogen/fibrin is of importance in the initiation of intravascular catheter-related infections (8, 20, 41, 42). Thus, the presence of the putative 2351 LPXTG fibrinogen binding protein could be a beneficial factor for CC17 *E. faecium* in establishing foreign body-associated infections.

The 2430 LPXTG surface protein has 94% homology to the EF1896 protein of *E. faecalis* V583, which may suggest that the gene encoding this protein has been acquired via horizontal gene transfer and that this may have occurred recently. This may explain its low prevalence (7%) in non-CC17 isolates and in only a subset (70%) of the CC17 isolates. In both *E. faecium* and *E. faecalis*, the 2430 protein has not been characterized. However, its ortholog in *E. faecalis*, EF1896, was enriched in clinical isolates (17). The exact role of the five CC17-enriched LPXTG surface proteins remains to be elucidated but will be determined by making insertional knockouts in the genes by use of the improved temperature-sensitive vectors described by

Nallapareddy et al. (22).

The sequence homology and surface-exposed expression of the five CC17-enriched gene products suggest that these genes encode factors involved in bacterial adhesion and biofilm formation. Attachment of a bacterium to host tissues or indwelling abiotic medical devices like stents or catheters, whether mediated through CWAPs or not, is the initial event in the pathogenesis of microbial infections (28). Therefore, the five identified CC17-enriched LPXTG surface proteins may play a role in the pathogenesis of CC17 *E. faecium* in hospital-related infections and as such may have contributed to the rapid emergence of CC17 *E. faecium* as a nosocomial pathogen over recent decades. The clear distinction of the CWAP gene profile of *E. faecium* CC17 make the 903, 904.5, 906.7, 2351, and 2430 LPXTG surface proteins possible targets for novel active or passive immunization therapies.

ACKNOWLEDGEMENTS.

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Chapter 4

SgrA, a Nidogen-binding LPXTG Surface Adhesin Implicated in Biofilm Formation, and EcbA, a Collagen Binding MSCRAMM of Hospital Acquired *Enterococcus faecium*

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ABSTRACT.

Hospital acquired *Enterococcus faecium* isolates responsible for nosocomial outbreaks and invasive infections are enriched in the *orf2351* and *orf2430* genes, encoding the SgrA and EcbA LPXTG-like cell wall-anchored proteins, respectively. These two surface proteins were characterized to gain insight into their function, as they may have favored the rapid emergence of this nosocomial pathogen. We are the first to identify a surface adhesin among bacteria (SgrA) that binds to the extracellular matrix molecules nidogen-1 and nidogen-2, which are constituents of the basal lamina. EcbA is a novel *E. faecium* MSCRAMM (microbial surface component recognizing adhesive matrix molecules) that binds to collagen type V. In addition, both SgrA and EcbA bound to fibrinogen, however, SgrA targeted the alpha and beta chains, whereas EcbA bound to the gamma chain of fibrinogen. An *E. faecium sgrA* insertion mutant displayed reduced binding to both nidogens and fibrinogen. SgrA did not mediate binding of *E. faecium* cells to biotic materials such as human intestinal epithelial cells, human bladder cells and kidney cells, while this LPXTG surface adhesin is implicated in *E. faecium* biofilm formation. The *acm* and *scm* genes, encoding two other *E. faecium* MSCRAMMs were expressed at the mRNA level together with *sgrA* during all phases of growth, whereas *ecbA* was only expressed in exponential and late exponential phase, suggesting orchestered expression of these adhesins. Expression of these surface proteins that bind to extracellular matrix proteins and involved in biofilm formation (SgrA) may contribute to the pathogenesis of hospital acquired *E. faecium* infections.

INTRODUCTION.

Enterococcus faecium has emerged as an important opportunistic Gram-positive pathogen responsible for nosocomial infections and hospital outbreaks world-wide (1,30,48). *E. faecium* can cause a variety of infections in immunocompromised patients, such as urinary tract infections, surgical site infections, bacteremia, and endocarditis. Hospital aquired *E. faecium* isolates recovered from clinical sites and isolates associated with nosocomial outbreaks clearly differ from clinically non-relevant *E. faecium* (25). These hospital acquired *E. faecium* strains are high-level resistant to ampicillin and ciprofloxacin (24), are enriched in putative virulence genes such as *esp* (23), which encodes the enterococcal surface protein and is implicated in biofilm formation (16,46), genes putatively encoding PilA and PilB pilus-like structures (17) and three cell wall-anchored LPXTG surface proteins designated as Orf903, Orf2351 and Orf2430 (18). These three surface proteins have in common that they contain a N-terminal signal peptide, a non-repetitive A-domain, and a C-terminal cell wall sorting signal (CWS), which is comprised of an highly conserved LPXTG-like (Leu-Pro-X-Thr-Gly, where X denotes any amino acid) sortase substrate motif and a hydrophobic domain followed by positively charged amino acids (27,40). After translocation of the precursor protein, the LPXTG motif is cleaved by sortase, which subsequently anchors the surface protein to the cell wall peptidoglycan (27,28).

A class of LPXTG surface proteins are the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (37,38), which contain in addition to characteristics of Gram-positive cell wall-anchored surface proteins at least one DE variant of the immunoglobulin (IgG)-like fold in the N-terminal A-domain (9). MSCRAMMs are often bifunctional proteins that adhere to one or more components of the host extra cellular matrix (ECM). The ECM is a complex three-dimensional structure surrounding cells in mammalian tissue and critical site for initial bacterial attachment and colonization. To date, two MSCRAMMs have been described for *E. faecium* that bind to components of the ECM, named Acm (adhesin of collagen from *E. faecium*) and Scm (second collagen adhesin of *E. faecium*). Acm interacts with collagen type I and to a lesser extent with collagen type IV (31,35), and Scm binds to collagen type V and fibrinogen (41). These two collagen-binding MSCRAMMs were found ubiquitously among enterococcal isolates of clinical and non-clinical origin. However, while a functional *acm* gene is predominantly present in clinical isolates promoting adherence to collagen type I, it often occurred as an insertion element-interrupted pseudogene in isolates of non-clinical origin (33). These isolates did not bind collagen type I. In a rat endocarditis model in which equal amounts of an *acm* deletion mutant and the wild-type were injected intraventricularly, the mutant strain was outnumbered by the wild-type on the heart valve vegetations (32).

In this study we functionally characterize two LPXTG surface proteins, Orf2351 and Orf2430 we recently described (18) and which are now renamed to SgrA and EcbA, respectively. We assessed whether these surface proteins can bind to protein ligands of the ECM. Furthermore, we investigated the function of a *sgrA* mutant in adhesion to biotic and abiotic surfaces.

(Part of this study was presented as a poster at the 108th General Meeting of the American Society for Microbiology, Boston, MT, June 1 to 5, 2008 [abstr. 08-GM-A-2548-ASM])

MATERIALS AND METHODS.

Bacterial strains, growth conditions and reagents.

The bacterial strains and plasmids used in this study are listed in Table 1. Rosetta-gami (DE3) pLysS *Escherichia coli* cells, containing the pRSETB vector (Invitrogen Corporation, Breda, The Netherlands), were used for expression of recombinant protein and were grown at 37°C in Luria Bertani broth or agar, supplemented with carbenicillin (50 μg/ml) and chloramphenicol (34 μg/ml). *E. faecium* strains were grown aerobically at 37°C on Trypticase Soy Agar II (TSA) plates supplemented with 5% sheep blood (Becton Dickinson, Alphen aan den Rijn, The Netherlands) or in Brain Heart Infusion broth, and when appropriate, the antibiotics chloramphenicol and gentamicin were used in concentrations of 10 μg/ml, and 125 μg/ml, respectively. Antibiotics were obtained from Sigma-Aldrich (Saint Louis, MO, USA). A

temperature-sensitive vector (pTEX5500ts) was used to introduce an insertion-deletion mutation in the *sgrA* gene of *E. faecium* E1162, a clinical bloodstream isolate. Extracellular matrix molecules, collagen type I to type V, laminin (ultra-pure), fibronectin, fibrinogen (plasminogen free) and vitronectin were all from human plasma (Sigma-Aldrich B.V., Zwijndrecht, The Netherlands). Bovine serum albumin (BSA) was purchased from Serva (Heidelberg, Germany). Human recombinant nidogen-1 and nidogen-2 containing an N-terminal His-tag were purchased from R&D Systems (Abingdon, United Kingdom).

TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[a]	Reference
Strains		
E. coli		
TOP10F	*E. coli* host strain for routine cloning	Invitrogen
DH5α	*E. coli* host strain for routine cloning	(14)
Rosetta-Gami	(DE3) pLysS for high-level recombinant protein expression	Novagen
E. faecium		
E135	Community surveillance faeces isolate; *esp*, *sgrA* and *ecbA* negative	(49,50)
E155	Hospital outbreak-associated isolate; *esp*, *sgrA* and *ecbA* positive	(4,50)
E300	Hospital outbreak-associated isolate, from urine; *esp*, *sgrA* and *ecbA* positive	(12,50)
E470	Hospital outbreak-associated isolate, *esp*, *sgrA* and *ecbA* positive	(43,50)
U0317	Clinical isolate, from urine, *esp*, *sgrA* and *ecbA* positive	This study
E1162	Clinical blood isolate; Chl^s Gen^s; *esp*, *sgrA* and *ecbA* positive	(16)
E1162Δ*sgrA*	E1162 with a disrupted *sgrA* gene; Chl^r Gen^s; *esp* and *ecbA* positive	This study
TX0016	Clinical endocarditis isolate; *sgrA* and *ecbA* positive	(2)
Plasmids		
pRSETB	Expression vector	Novagen
pAH2351	pRSETB derivative, to express rSgrA protein (residues 76W to 297N)	This study
pAH2430	pRSETB derivative, to express rEcbA protein (residues 35E to 347T)	This study
pTEX5500ts	Shuttle plasmid; temperature-sensitive in gram-positive hosts; Chl^r Gen^r	(31)
pEF4	pTEX5500ts:*sgrA*-Up, pTEX5500ts with a cloned *sgrA-Up* gene fragment; Chl^r Gen^r	This study
pEF9	pTEX5500ts:*sgrA*-Up-*sgrA*-Dn, pTEX5500ts with cloned *sgrA* gene fragments flanking the *cat* gene, for generating an *sgrA* insertion-deletion mutation; Chl^r Gen^r	This study

[a]Chl, chloramphenicol; Gen, gentamicin; ts, temperature sensitive; ^{s}, sensitive; ^{r}, resistant.

PCR amplification and cloning of *sgrA* and *ecbA*.

Fragments of *sgrA* (encoding residues 76W to 297N) and *ecbA* (encoding residues 35E to 347T) were amplified by PCR using primer sets SgrA_fw and SgrA_rv and EcbA_fw and EcbA_rv (Table 2). PCR reactions were performed in 25-µl volumes using HotStarTaq HiFidelity DNA polymerase (Qiagen Benelux B.V., Venlo, The Netherlands), with 10 pmol of each primer and 10 nmol of *E. faecium* TX0016 chromosomal DNA. DNA was isolated as described previously (3,18). Restriction sites for *Nde*I and *Bam*HI enzymes (New England Biolabs, Ipswich, MA, USA) were incorporated at the 5′ ends of the primers to facilitate directional cloning. In SgrA_fw and EcbA_fw a 5′ ATG and 6x CAT was added for the in frame expression of recombinant SgrA and EcbA. In SgrA_rv and EcbA_rv a TGA stopcodon was added. The two PCR fragments were purified by use of the PCR purification kit (Qiagen), cloned into the pRSETB expression vector (Invitrogen) resulting in pAH2351 and pAH2430, respectively, and were subsequently used to transform One Shot TOP10F′ chemically competent *E. coli* (Invitrogen) according to the manufacturer's instructions. Recombinant plasmid was isolated with a plasmid purification kit (Qiagen) and analysed by sequencing.

Expression and purification of recombinant SgrA and EcbA.

The plasmids pAH2351 and pAH2430 were used to transform Rosetta-Gami (DE3) pLysS chemically competent *E. coli* (Novagen, Gibbstown, NJ, USA). These *E. coli* cells were grown to an OD_{660} of ~0.7 in 200 ml Luria Bertani broth supplemented with 0.02 M glucose. Expression of recombinant SgrA and EcbA containing a 6x his-tag at its N-terminal end (rSgrA and rEcbA) was induced by 10 mM isopropyl-β-D-thiogalactopyranoside. The recombinant fusion proteins were purified by use of immobilized Ni^{2+} affinity

chromatography (Probond, Invitrogen), all according to the manufacturer's instructions. Purified rSgrA and rEcbA were dialyzed overnight against 0.1x PBS at 4°C, concentrated by lyophilization and purity was analyzed by SDS-PAGE.

TABLE 2. Oligonucleotides used in this study.

Primer name[a]	Oligonucleotide sequence (5'-3')
ddl_fw	GAGACATTGAATATGCCTTATG
ddl_rv	AAAAAGAAATCGCACCG
Scm_fw	CTAACTGGTAACTATGGCTTGT
Scm_rv	GTCCGTGCTGTCACTTGT
Acm_fw	TCAGCAGTAATGTCACTTCGTTG
Acm_rv	GAATAGGCTGTTCATCTGCTCG
SgrA_fw	GGAATTCCATATGCGGGGTTCTCATCATCATCATCATCATGG-TTGGGATGGTAATGGAAGTTCA
SgrA_rv	CGCGGATCCGCGTCAGTTCAAGGTTCTACTACCAGT
EcbA_fw	GGAATTCCATATGCGGGGTTCTCATCATCATCATCATCATGA-AATTACTCATCCACAAACGGTA
EcbA_rv	CGCGGATCCGCGTCATGTAGTGTCAATCGTATAAGG
SgrA_fw2	AATGAACGGGCAAATGAG
SgrA_rv2	CTTTTGTTCCTTAGTTGGTATGA
EcbA_fw2	GCAGTTTACAATGGTGTGAAGCAA
EcbA_rv2	CGGCTAATGAGTATTTGTCGTTCC
SgrA-Up_fw	TTCCGCGGCCGCTATGGCCGACGTCGTCGACGCGTGGCTGA-GTATAATTGCAG
SgrA-Up_rv	CGCGGATCCGCGCAAATTCTCCGTGATCGTCAT
SgrA-Dn_fw	AACTGCAGAACCAATGCATTGGGAGAAATGCAGGGAGCAAC
SgrA-Dn_rv	GCCTTAAGGCTTCACTCGATGGAAGAGAGAAC
SgrA_fw3	ATGAAG AAAACAGCGACCGT
Orf2352_rv	TTATTCAATTTTAGATCTGT
SgrA_ser_F	AGTTGGACAGTTGTTGGACC
SgrA_ser_R	CTCGTGCTTCCTGTGCTACT
Cam_F	TTTAATAAAATTGATTTAGA
Cam_R	CCTGAATAGAGTTCATAAAC

[a]Annotation: fw, forward and rv, reverse.

SDS-PAGE, western blot and ligand-affinity blot analysis.

Protein samples were equally mixed with sample buffer (100 mM Tris-HCl, 5% dithiotreitol, 2% SDS, 0,004% Broomphenol blue and 20% glycerol) and boiled for 5 mins. Equal amounts of protein was electrophoresed through a 10% SDS polyacrylamide gel. Western blotting was carried out as described previously (18). Membranes were blocked with 4% skim milk (Campina Holland, Alkmaar, The Netherlands) in PBS-0.1% Tween 20 for 1 hour at 37°C. Incubation with primary antibody was carried out for one hour in 1% BSA in PBS-1% Tween 20 at 37°C followed by two washes of each 10 mins in PBS-0.1% Tween 20 at 37°C. Subsequently, membranes were incubated for 1 hour with Goat anti-mouse IgG (H+L)-horseradish peroxidase (HRP) (Bio-Rad Laboratories, Veenendaal, The Netherlands) in 1% BSA in PBS-1% Tween 20 at 37°C. Membranes were washed twice with PBS-0,1% Tween 20 and proteins were visualized using the ECL plus Western blotting detection system (GE Healthcare, Diegem, Belgium) and exposed to film (Hyperfilm ECL, GE Healthcare). For the ligand affinity blots, 20 μg of ECMs were separated by SDS-PAGE, blotted onto nitrocellulose, and after blocking the membranes were incubated for 1 hour at room temperature with 5.0 μg/ml of rSgrA or rEcbA, followed by one hour incubation with anti-His IgG-HRP antibodies (Qiagen) at 37°C. For the non-reducing ligand affinity blots, SDS was omitted from buffers.

Biotinylation of recombinant proteins.

Recombinant nidogen-1, nidogen-2 and rSgrA were biotinylated using sulfo-*N*-Hydroxysuccinimide-biotin according to the manufacturers instructions (Pierce Biotechnology, Rockford, IL, USA).

mRNA expression analysis by reverse transcription (RT-) PCR.

For mRNA expression of the *sgrA*, *ecbA*, *acm* and *scm* genes, E135 and E1162 cells were resuspended into PBS to an OD_{660} nm of 1.0 (~1x10^9 CFU/ml) and pelletted by centrifugation (6,500 x *g* for 1 minute). Total RNA was isolated, purified and cDNA synthesis was done as described previously (6,18). cDNA was used as template for PCR using primer pairs SgrA_fw2 and SgrA_rv2, EcbA_fw2 and EcbA_rv2, Acm_fw and Acm_rv, and Scm_fw and Scm_rv as depicted in Table 2. As an internal control, the housekeeping gene *ddl* (encoding D-alanine, D-alanine ligase) was amplified using primers ddl_fw and ddl_rv (Table 2). RNA samples not treated with reverse transcriptase were used as control to detect DNA contamination in the total RNA preparations.

ELISA and whole-cell ELISA.

Polystyrene microtitre plates (Greiner Microlon from Greiner Bio-one, Alphen aan den Rijn, The Netherlands) were coated overnight at 4°C with 10 μg/ml of ECMs in 50-μl volumes and BSA was used as a negative control. After washing three times with washbuffer (PBS-0.05% Tween 20), non-specific sites were blocked with 100-μl blocking buffer (4% BSA in PBS-0.05% Tween 20) for 1 hour at 37°C. Various concentrations of recombinant protein in 1% BSA in PBS-0.05% Tween 20 were added to the wells and incubated at 37°C. After 2 hours, unbound protein was removed by washing three times. Bound proteins were detected by anti-His IgG-HRP antibodies (Qiagen) in 1% BSA in PBS-0,05% Tween 20 (1:5,000 dilution) for 1 hour at 37°C. Binding of E1162 and the *sgrA* mutant strain, E1162Δ*sgrA*, to immobilized nidogen-1, fibrinogen, laminin and fibronectin (all 25 μg/ml) was assayed three times in duplicate by whole-cell ELISA. Plate-grown bacteria were resuspended in PBS to an OD_{660} of 0.2 (2 x10^8 CFU/ml) and 100 μl was added to wells of a microtiter plate and allowed to bind overnight at 4°C. The wells were washed three times with wash buffer, and subsequently blocked with blocking buffer for 1 h at 37°C. Binding of E1162 and E1162Δ*sgrA E. faecium* was assayed by incubation for 1 h at 37°C with rabbit anti-enterococcal serum (kindly provided by J. Huebner). Bound antibodies were detected by incubation with a peroxidase-conjugated goat anti-rabbit IgG (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 37°C. Both antibodies were diluted in PBS with 1% BSA and 0.05% Tween 20. For both types of ELISAs, 50 μl of 0.11 M acetate buffer with 1.6% 3,3,5,5-tetramethylbenzidine and 0.8% ureumperoxide was added to each well, and the reaction was stopped after 10 min with 50-μl 0.5 M H_2SO_4. The absorbance was measured at 450 nm with an ELISA reader.

Mutagenesis of *sgrA* by insertional inactivation.

The *sgrA* gene of *E. faecium* E1162 was disrupted by construction of an insertion-deletion mutation as described previously (16,31). In brief, a 373-bp *sgrA* fragment designated SgrA-Up was amplified from genomic *E. faecium* E1162 DNA by using primers SgrA-Up_fw and SgrA-Up_rv, which contain *Sal*I and *Bam*HI restriction sites respectively (Table 2). The PCR product was digested with *Sal*I and *Bam*HI and ligated to similarly digested pTEX5500ts, resulting in pEF4. Similarly, a 218-bp fragment designated SgrA-Dn was amplified by using primers SgrA-Dn_fw and SgrA-Dn_rv, including restriction sites *Nsi*I and *Eco*RI incorporated at the 5' ends of the primers to facilitate directional cloning into pEF4, resulting in pEF9 (Table 1). The pEF9 plasmid was introduced into competent *E. faecium* E1162 by electroporation. After transformation, gentamicin-resistant colonies were picked and grown overnight at 42°C in BHI broth supplemented with gentamicin and subsequently plated on BHI agar plates with chloramphenicol and grown at 42°C. Single-crossover integration into SgrA-Up and SgrA-Dn complementary sequences of *E.*

faecium E1162 was tested by PCR. Single-crossover mutants were grown overnight for eight serial passages in BHI culture supplemented with chloramphenicol at 42°C to cure pEF9. Selection for double-crossover recombination was performed by replica plating on BHI agar plates containing either chloramphenicol or gentamicin. The insertional inactivation of the *sgrA* gene in double crossover mutants was analysed by PCR with the primers SgrA_fw3 and Orf2352_rv, sequencing and Southern hybridization.

Southern blot analysis.

Southern blot analyis was performed on chromosomal DNA isolated from *E. faecium* E1162 and E1162Δ*sgrA* to confirm disruption of the chromosomal *sgrA* gene. Chromosomal DNA was digested with *Eco*RI (Roche diagnostics, Almere, The Netherlands), fragments were separated by agarose gel electrophoresis and blotted onto an Hybond N^+ nylon membrane (GE Healthcare, Diegem, Belgium). DNA was fixed onto the membrane by incubation for 2 minutes in 0.4 M NaOH followed by a neutralisation in 10x SSC for 1 minute. The membrane was hybridized overnight at 42°C with 100 ng probe. Probes were generated by PCR in 50-µl volumes using primer pairs Cam_F and Cam_R and SgrA_ser_F and SgrA_ser_R from pTEX5500ts and chromosomal DNA of E1162, respectively, as depicted in Table 2. Amplified DNA probes were purified with a PCR purification kit (Qiagen) and labelled according to the ECL nucleic acid labelling kit (GE Healthcare). The membranes were exposed to Hyperfilm ECL (GE Healthcare).

Cell surface hydrophobicity assay.

Cell surface hydrophobicity was measured using a hexadecane extraction of E1162 and E1162Δ*sgrA* cultures as previously described (39). Hydrophobicity is expressed as the percentage of cells that are extracted by the hexadecane as measured by OD.

Adherence to biotic and abiotic materials.

Human colorectal adenocarcinoma cells, (Caco-2) and Madin-Darby canine kidney cells (MDCK) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated foetal calf serum (Integro B.V, Zaandam, The Netherlands), 1% non-essential amino acids (Gibco), 2 mM glutamine (Gibco), and incubated in a humidified, 37°C incubator with 5% CO_2. Human bladder carcinoma cells (T24) were cultured in Eagle's Minimal Essential Medium (EMEM, BioWhittaker/Lonza, Breda, The Netherlands) supplemented with 50 µg/ml gentamicin (Gibco) and 10% heat-inactivated foetal calf serum (Integro B.V, Zaandam, The Netherlands). 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. 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 with all supplements and replacing the culture medium every second day. Differentiated Caco-2 cells were used 14 – 16 days after seeding. 12-well plates with MDCK or T-24 cells were prepared three days before use by seeding 1.6 x 10^5 cells/ml in DMEM or EMEM with the necessary supplements. The medium was replaced after two days.

Overnight-grown cultures of E135, E1162 and E1162Δ*sgrA* in BHI broth were diluted (1:50) and grown at 37°C to an OD_{660} of 0.4. Bacteria were harvested by centrifugation (6,500 × g; 3 min) and resuspended in DMEM or EMEM to 1 × 10^7 CFU/ml. For each strain, 1 ml bacterial suspension was added to the wells (100 bacteria to 1 cell). Plates were centrifuged (175 × g; 1 min) and incubated for 1 h at 37°C

in 5% CO_2 to allow bacterial adherence to the Caco-2, MDCK and T24 cells. After incubation, monolayers were rinsed 3 times with DMEM/EMEM 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 CFUs. The inoculum was also plated to determine viable counts. The assay was performed in duplicate and repeated 3 times. Adherence of E135, E1162 and E1162Δ*sgrA* to abiotic material (polystyrene) was assayed as described previously (20,46).

Statistics.

The Students' *t* test was used to assess statistical significant differences.

DNA Sequencing.

PCR products were sequenced by use of the BigDye Terminator 3.1 reaction kit and an ABI PRISM 3100 capillary DNA sequencer (both from Applied Biosystems, Foster City, CA).

RESULTS.

Structural organization of the SgrA and EcbA LPXTG surface proteins.

Recently, we identified a 975-bp gene (*orf2351*; ZP_00602747) and a 3228-bp gene (*orf2430*; ZP_00603098) encoding a 325 amino acid (aa) and 1076-aa surface exposed LPXTG protein, which were specifically enriched in hospital acquired *E. faecium* isolates (Fig. 1) (18). The *orf2351* gene is renamed to *sgrA*, for serine-glutamate repeat containing protein A, and *orf2430*, to *ecbA* for *E. faecium* collagen binding protein A (see below). *In silico E. faecium* TX0016 analysis revealed the absence of a sortase gene in the vicinity of the *ecbA* and *sgrA* genes, suggesting that these surface proteins may be anchored to the cell wall by the putative Orf2128 housekeeping class A sortase.

The SgrA LPXTG surface protein contains a 28-aa N-terminal signal sequence (region S), followed by a 126-aa non-repetitive region A, which may be

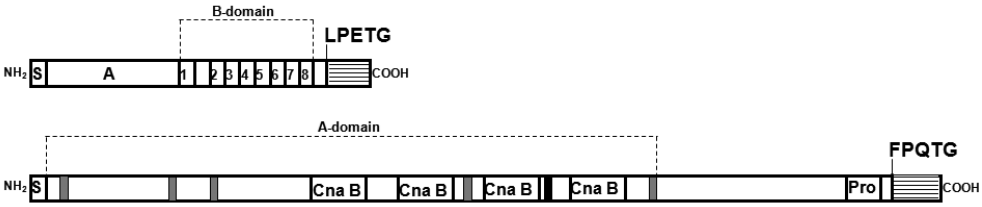


Figure 1. Schematic representation of the SgrA LPXTG surface protein and the EcbA MSCRAMM. Organization of the SgrA and EcbA proteins identified from the *E. faecium* TX0016 genome sequence as published at DDBJ/EMBL/Genbank. Signal sequences are depicted by an "S", a putative ligand binding domain is indicated by an "A", the SSESST repeats are numbered. The cell wall sorting signal is depicted as a striped box, starting at the LPXTG or FPQTG sortase substrate motif. The putative latching regions are depicted in grey, the Cna B domains are indicated by white boxes, the phenylalanine box is indicated in black and the proline rich region is depicted by "Pro".

a putative ligand-binding domain. Putative IgG-like folds were not present in this domain, suggesting that the SgrA protein can not be classified as an MSCRAMM. Close to the C-terminal end a 77-aa B-repeat-domain was identified which contains eight Ser-Ser-Glu-Ser-Ser-Thr (SSESST) repeats and at the C-terminus a typical CWS, containing an LPETG motif. Secondary structure prediction of SgrA using the 3D-PSSM/Phyre servers (21) predicted a 95-aa long C-terminal coiled structure from amino acids 119-Pro to 213-Asp (relative to the signal peptide cleavage site) including the B-domain, which is possibly involved to span the cell wall to expose the putative ligand-binding A-domain from the surface to the extracellular environment (8,15). A BLASTP homology search revealed significant similarity of the B-domain of SgrA with a putative LPXTG containing cell wall anchor family protein of *E. faecalis* V583 (EF0093; NP_813896), which contains nine SESST repeats (six SETSNT and three SESSST repeats) close to the C-terminal end, suggesting structural similarity with SgrA.

The EcbA LPXTG surface protein contains a 34-aa N-terminal signal sequence (S), followed by a large 696-aa non-repetitive region A and a C-terminal CWS (Fig. 1) with a FPQTG sortase substrate motif. EcbA is predicted to be an MSCRAMM, as the A-domain contains multiple MSCRAMM-like features, which are also found in the fibrinogen-binding MSCRAMM family of *S. aureus* (13). It contains five low consensus 9 amino acid TYTFTDYVD-like sequences (TVTVELDLA; TVTDTNGLN; TYTIKIDVE; TVTLTLDEK and TVTVPYEKL) which are implicated to be a latching cleft. In addition, EcbA harbors four putative Cna B-domains which are predicted to adopt a variant of an IgG-like fold (8,9). The C-terminus contains a PxxxPxxPxxPxxPxxPxxPxxxxxxxP (where x denotes any amino acid) proline-rich region directly upstream the putative FPQTG sortase substrate motif. Furthermore, a stretch of phenylalanine amino acids (FxFxFFxF) was identified in the central part of the protein and the function, if any, remains to be elucidated. The EcbA protein displays similarity with putative cell wall-anchored proteins of *E. faecalis* (90% with EF1896; AAO81648.1 and 84% with EF2347; AAO82072.1), and *Listeria welshimeri* serovar 6b str. SLCC5334 (30% with lwe0767; YP_848968.1). *E. faecium* E1162 genome analysis (Van Schaik *et al*., manuscript in preparation) revealed that in addition to the *ecbA* gene also an *ecbA*-like gene was present on the E1162 chromosome. The EcbA and EcbA-like proteins share 90% amino acid identity, and the highest sequence heterogeneity occurs within the signal peptide sequence.

Heterogeneity in the *sgrA* gene.

To determine the level of sequence heterogeneity in the *sgrA* gene among *E. faecium* strains, the *sgrA* genes of five strains (E155, E300, E470, E1162 and U0317) were sequenced. The *sgrA* gene did not show any sequence variation among clinically relevant isolates at both DNA and protein level, demonstrating that the *sgrA* gene is highly conserved. PCR amplification of *sgrA* in 64 isolates (25 clinical, 17 outbreak-associated, 10 hospital surveillance, 6 community surveillance, 3 environmental and

3 animal *E. faecium* isolates), identified in 56 isolates the expected 671-bp fragment. However, in eight isolates from various sources aberrant DNA fragments of ~450 bp, ~750 bp, and ~1500 bp were detected and were subsequently sequenced to determine the level of sequence heterogeneity. The occurrence of variant fragment sizes in these isolates was due to variation in the number of B-repeats of *sgrA*, and 4 of the 8 isolates also contained premature stop codons, suggesting that these *sgrA* genes are pseudogenes.

mRNA expression of *ecbA*, *sgrA*, *acm* and *scm* in different stages of growth.

To analyse whether *ecbA* and *sgrA* and the collagen-binding MSCRAMMs *acm* and *scm* are expressed simultaneously in different stages of growth, *E. faecium* E1162 (positive for *sgrA*, *ecbA*, *acm* and *scm*) and E135 (negative for *sgrA* and *ecbA*, positive for *acm* and *scm*) cells were grown in BHI broth. At four different time points in the growth phase, early-exponential (OD_{660} nm = 0.30 after 4.5 hrs), exponential (OD_{660} nm = 0.750 after 5.5 hrs), late exponential (OD_{660} nm = 1.00 after 6.5 hrs) and stationary phase of growth (OD_{660} nm = >1.00 after 8 hrs), cells were harvested and transcription of the five genes, *ecbA*, *sgrA*, *acm*, *scm* and the *ddl* internal control was

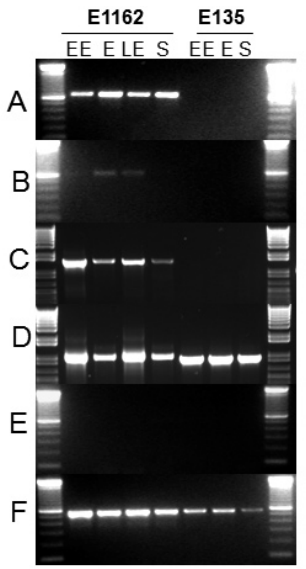


Figure 2. mRNA expression of *sgrA*, *ecbA*, *acm* and *scm* in different stages of growth. Panel A-D shows mRNA expression of *sgrA*, *ecbA*, *acm* and *scm* in E1162 (left part) and E135 (right part) cells isolated in early exponential (EE), exponential (E), late-exponential (LE) and stationary phase of growth (S) in BHI broth at 37°C. The E135 lacks both the *sgrA* and the *ecbA* genes. The *acm* and *scm* genes are expressed in all stages of growth in E1162 (Panel C + D), while *acm* is not expressed in the E135 strain. Control *ddl* PCR reactions (panel E) on total mRNA preparations in which the RT reaction was omitted were all negative, demonstrating absence of DNA contamination. Control *ddl* RT-PCR reactions (internal housekeeping control, panel F) with *E. faecium* specific *ddl* primers were all positive. The results are presented as amplified PCR products electrophoresed on an ethidium bromide-stained 1.5% agarose gel.

analyzed by PCR on cDNA generated from total mRNA. In strain E1162, transcripts of *sgrA*, *acm* and *scm* were detected in all phases of growth, whereas transcripts of *ecbA* were only detected in exponential and late exponential phase (Fig. 2). The E135 strain did not display expression of *ecbA*, *sgrA* and *acm*, while *scm* was expressed. Notably, PCR revealed a larger amplification product for the *acm* gene in E135 (data not shown). Control PCR reactions on DNAse treated total mRNA samples in which the reverse transcriptase reaction was omitted were negative and *ddl* was constitutively expressed in all samples.

The rSgrA protein binds to human nidogen-1, its homolog nidogen-2, and the α- and β-chains of fibrinogen.

To asses whether rSgrA has the ability to bind to proteins of the ECM, fibronectin, fibrinogen, vitronectin and BSA (negative control) were immobilized onto a microtiter plate and the ability of rSgrA to bind to these components was assayed by ELISA (Fig. S1 and 3A). The rSgrA protein bound to immobilized fibrinogen in a dose-dependent and saturable manner, and not or to a lesser extent to fibronectin, vitronectin or BSA. We performed ligand affinity blotting under reducing conditions to confirm binding to fibrinogen and not to fibronectin. Fibrinogen polypeptide chains separated through SDS-PAGE and stained with Coomassie blue appeared as three bands designated Aα (63.5 kDa), Bβ (56 kDa) and γ (47 kDa) while fibronectin is displayed as one single 250 kDa band (Fig. 4). Using this technique, binding of rSgrA could be localized to the α and β chains of fibrinogen. The negative control, fibronectin, did not display binding of rSgrA.

Interestingly, in an unpure laminin preparate we detected binding of rSgrA to an unknown ligand as determined by ligand affinity blotting (data not shown). Glycoproteins designated nidogen-1 (also known as entactin) and nidogen-2 are tightly associated to the γ-chain of laminin and are often co-isolated from basal membrane extracts (5,22,44). We therefore hypothesized that rSgrA, in addition to the binding activity to fibrinogen, may also display binding activity to either human nidogens. Nidogen-1, its homolog nidogen-2 and BSA as negative control were biotinylated, and the ability of these two ECM proteins to bind to rSgrA was assessed by ELISA. Biotinylated nidogen-1 and nidogen-2 bound to the rSgrA protein in a concentration-dependent and saturable manner, while no binding was observed to BSA (Fig. 3B). To confirm the interaction of rSgrA to both nidogens a ligand affinity Western blot was performed. The nidogen-1, nidogen-2 and fibronectin proteins were separated through PAGE under non-reducing conditions, blotted onto nitrocellulose and subsequently incubated with biotinylated rSgrA. The rSgrA protein bound to both nidogens and not to the negative control, fibronectin (Fig. 4A).

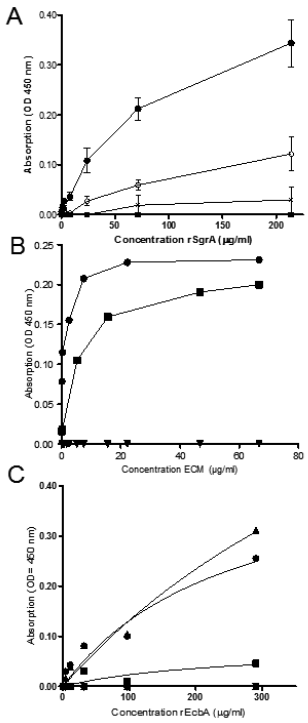


Figure 3. Binding of rSgrA and rEcbA to ligands of the extracellular matrix as assessed by ELISA. Panel A shows concentration-dependent binding of rSgrA to immobilized fibrinogen (black circles) and not to fibronectin (black cross), vitronectin (open circles) or BSA (black squares). For all ELISA's, OD_{450} values were corrected for the response of 6xHis IgG-HRP antibodies with the respective ECM proteins. Data points represent the means of OD_{450} values ± standard deviation of three independent experiments with two different purified rSgrA protein batches. Panel B demonstrates concentration-dependent binding of nidogen-1 (black squares) and nidogen-2 (black circles) to immobilized rSgrA in a saturable manner and not to BSA (black triangles). The data points are representative values of three independent experiments with two different purified rSgrA protein batches. Panel C indicates concentration-dependent binding of rEcbA to immobilized collagen type V (black triangles) and fibrinogen (black circles), and not to collagen types I to IV, vitronectin, laminin or BSA. The data points are representative values of four independent experiments with three different purified rEcbA protein batches.

The rEcbA protein binds to collagen type V and the γ-chain of fibrinogen.

To analyze whether rEcbA has the ability to bind to proteins of the ECM, collagen types I to V, fibrinogen and ultra pure laminin, vitronectin and BSA as negative control were immobilized onto a microtiter plate and the ability of rEcbA to bind to these components was assayed by ELISA (Fig. S1 and 3C). The rEcbA protein bound to immobilized collagen type V and fibrinogen in a dose-dependent manner, and not to the other ECM proteins or BSA. Ligand affinity blotting confirmed binding of rEcbA to these ligands of the ECM (Fig. 4B). The binding site for rEcbA could be localized to the α-chain of collagen type V and the γ chain of fibrinogen. The negative control, fibronectin, did not display binding of rEcbA.

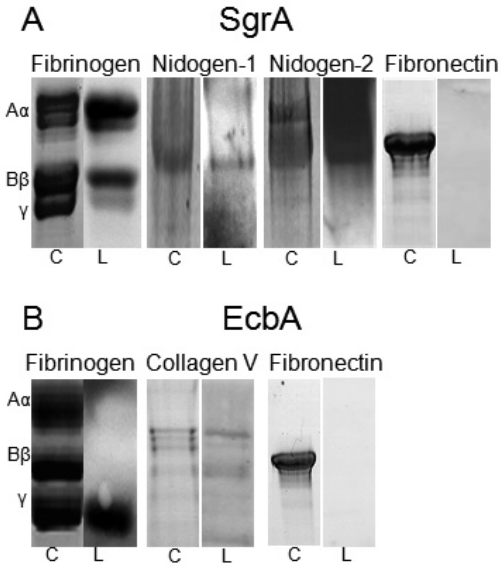


Figure 4. Ligand-affinity blotting demonstrated binding of rSgrA to nidogen and fibrinogen (A) and **rEcbA to collagen type V and fibrinogen** (B). Human fibrinogen, collagen type V, and fibronectin were separated through SDS-PAGE, while nidogen-1 and nidogen-2 were separated through native-PAGE followed by Coomassie blue staining (indicated by an "C"). The ligand-affinity blots were probed with rSgrA (fibrinogen; left part), biotinylated rSgrA (nidogen-1 and nidogen-2) and rEcbA (collagen type V and fibrinogen; right part) and are indicated by a "L".

SgrA promotes interaction of *E. faecium* cells with nidogen-1 and -2 and fibrinogen.

To study the role of SgrA in the interaction of *E. faecium* cells with nidogen-1, nidogen-2 and fibrinogen, the *sgrA* gene was inactivated by an insertion-deletion mutation in the clinical *E. faecium* E1162 isolate. Correct insertion-deletion mutation in the *sgrA* gene was confirmed by PCR, sequencing and Southern hybdridization. In addition, Southern blotting using a probe directed against the serine-glutamic acid repeat region and the *cat* cassette, demonstrated that in the double-crossover E1162Δ*sgrA* mutant, the serine-glutamic acid repeat region was replaced by a chloramphenicol (*cat*) cassette (Fig. S2). *In vitro* growth analyses in BHI broth and on solid media did not reveal any growth defects between the mutant and the wild-type E1162 strain (data not shown). Subsequent whole-cell ELISA analysis in which nidogen-1, nidogen-2, fibrinogen and laminin (negative control) were immobilized, showed that the E1162Δ*sgrA* cells displayed significantly reduced binding to nidogen-1 (3.1 fold), nidogen-2 (3.6 fold), fibrinogen (4.5 fold), and a minor, though unexpected reduced binding to ultrapure laminin (1.9 fold) (Fig. 5A). We therefore determined the cell surface hydrophobicity of E1162 and E1162Δ*sgrA* cells using a hexadecane extraction, which revealed a significant lower (2.9 fold) surface hydrophobicity of the E1162Δ*sgrA* strain compared to the wild-type E1162 (Fig. 5B).

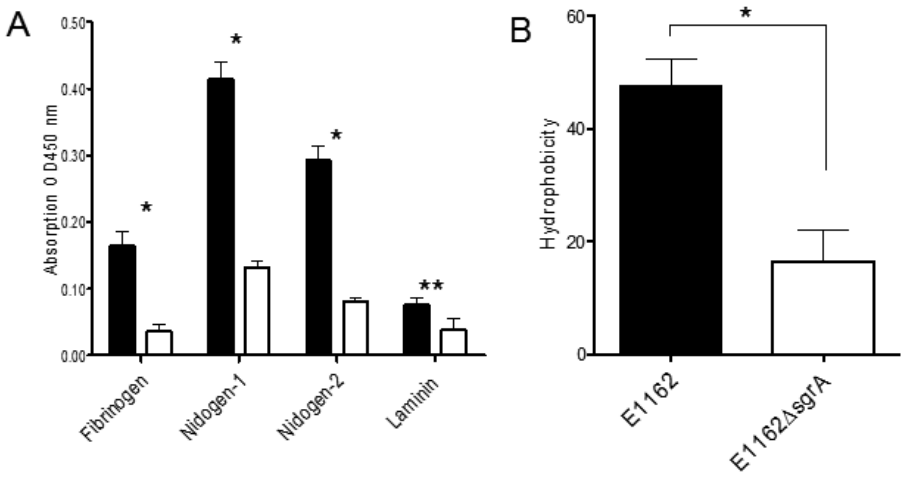


Figure 5. Adherence of the E1162 and the E1162Δ*sgrA* isogenic mutant to extracellular matrix molecules by whole-cell ELISA. Panel A, Fibrinogen, nidogen-1, nidogen-2 and laminin (negative control) were immobilized onto a microtitre plate and E1162 and E1162Δ*sgrA E. faecium* cells were added to the wells and allowed to bind to these components. Adherent bacteria were detected using an anti-enterococcal serum followed by goat anti-rabbit IgG-HRP antibodies. Black bars represent the wild-type *E. faecium* E1162 and white bars indicate the E1162Δ*sgrA* mutant. *, P <0.005; **, P <0.05. Panel B shows cell surface hydrophobicity of the wild-type E1162 and a *sgrA* isogenic mutant (E1162Δ*sgrA*). The experiments were performed three times with similar results and values represent means ± standard deviation of triplicate measurements. *, P= 0.0169

Adhesion to biotic and abiotic surfaces.

To further characterize the function of SgrA it was assessed whether SgrA mediates adherence to biotic surfaces. The wild-type *E. faecium* strain E1162, its isogenic E1162Δ*sgrA* mutant, and an *sgrA*-negative *E. faecium* strain (E135) were assayed for their ability to adhere to intestinal epithelial cells (Caco-2), human bladder carcinoma cells (T24) and Madin-Darby canine kidney cells (MDCK). Strain E1162 exhibited adherence to Caco-2, T24 and MDCK cells, while the E135 strain showed only low-level binding to these cell lines (data not shown). The E1162 and E1162Δ*sgrA* strains displayed comparable binding to Caco-2, MDCK and T24 cells. To analyze whether SgrA is involved in adherence to abiotic material, the E1162, E1162Δ*sgrA*, and E135 were analyzed for the ability to form a 24-hrs biofilm on a polystyrene surface. The E1162 wild-type strain displayed high level, whereas the E135 strain showed low-level of biofilm formation on polystyrene. The E1162Δ*sgrA* strain was significantly impaired in biofilm formation (Fig. 6) compared to the wild-type E1162 (1.6 fold).

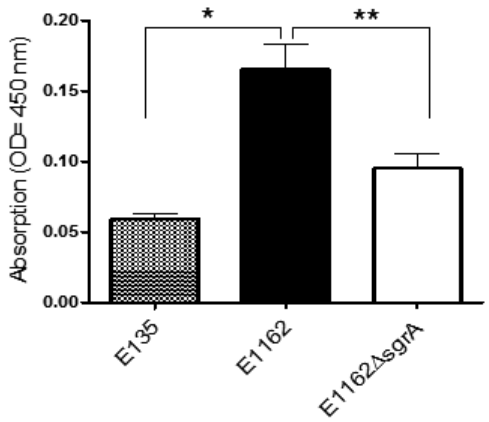


Figure 6. Biofilm formation on polystyrene. The ability of a *sgrA* negative strain E135 (grey bars), wild-type E1162 (black bars) and a *sgrA* isogenic mutant, E1162Δ*sgrA* (white bar), to form a 24-hours biofilm on a polystyrene surface is shown. The experiments were performed twice with similar results and values represent means ± standard deviation of 10 measurements. *, P <0.002; **, P = 0.03.

DISCUSSION.

Here we describe the function of the SgrA LPXTG protein and the EcbA MSCRAMM, two novel surface-exposed adhesins that are specific markers for hospital acquired *E. faecium* isolates that may play a role in the pathogenesis of *E. faecium* infections. We demonstrated that SgrA and EcbA are implicated in adhesion to components of the ECM, that SgrA mediates binding of *E. faecium* cells to its ligands of the ECM and that SgrA is implicated in biofilm formation.

SgrA is the first characterized cell wall-anchored LPXTG surface adhesin of *E. faecium* that binds to components of the ECM. It is an highly conserved protein among multi-resistant hospital acquired *E. faecium*, and binds to fibrinogen and two homologues proteins designated as nidogen-1 and nidogen-2. To our knowledge, we are the first to identify a bacterial cell surface adhesin that has nidogen as its cognate ligand. Nidogen-1 and nidogen-2 are sulfated monomeric glycoproteins of 150 and 200 kDa, respectively (5,22). Nidogens are a major component of the basal lamina, a specialized membrane of extracellular matrix. The basal membrane is a well-organized network and is also comprised of laminin, perlecan (an heperan sulfate proteoglycan) and collagen type IV underlying epithelia, peripheral nerve axons, muscle and fat cells. Fibrinogen is a large (340 kDa) plasma protein, composed of six polypeptide chains (two Aα, two Bβ and two γ) and plays an important role in haemostasis and coagulation (19). Wild-type *E. faecium* E1162 cells bound to fibrinogen, nidogen-1 and nidogen-2, whereas the E1162Δ*sgrA* strain showed reduced binding to these components of the ECM. Peculiarly, the E1162Δ*sgrA* strain also showed reduced binding to ultrapure laminin. This finding may be explained by the fact that E1162Δ*sgrA* cells had a significantly reduced cell surface hydrophobicity, thereby potentially influencing binding efficiencies or binding mechanisms to other

ligands.

The SgrA LPXTG surface adhesin is not involved in binding to biotic surfaces, such as human intestinal epithelial cells, human bladder cells and kidney cells. Instead, SgrA mediates adherence of *E. faecium* cells to a polystyrene abiotic surface as the E1162Δ*sgrA* strain produced significantly less biofilm. Biofilm formation is considered to be an important pathogenic property of enterococci and has been demonstrated for a variety of infections (7,11,26,29). Biofilm formation by the E1162Δ*sgrA* strain was not completely abolished, which is likely due to expression of Esp at the surface (16,46). Alternatively, a different cell surface charge (45), glycolipids (42), or possibly other surface components such as pili (17,34), which are known to be implicated in biofilm formation of *E. faecalis* may contribute to *E. faecium* biofilm formation.

In silico analysis revealed that the EcbA protein, a marker for hospital acquired *E. faecium*, has all structural and typical features of an MSCRAMM. *E. faecium* E1162 genome analysis (van Schaik *et al*, manuscript in preparation) revealed in addition to the *ecbA* gene, the presence of an *ecbA*-like gene, which is highly similar to *ecbA* (90%). Due to the presence of two highly homologues genes and the current limited genetic tools for *E. faecium*, we were unable to construct a double *ecbA* and *ecbA*-like isogenic mutant in *E. faecium* E1162. Therefore, we assessed whether rEcbA bound to components of the ECM. Indeed, rEcbA bound to collagen type V and fibrinogen in a concentration-dependent manner, making EcbA the third MSCRAMM of *E. faecium*. Recently, the Scm MSCRAMM was identified and characterized and showed also to bind to collagen type V and fibrinogen (41). In addition, SgrA bound to fibrinogen as well. The presence of multiple LPXTG surface proteins with similar function has also been described for *S. aureus*, where MSCRAMMs such as clumping factor A and B, the FnbpA and FnbpB proteins bind to fibrinogen, and thereby targeting different fibrinogen chains (36,47). The ligand-affinity blot data suggest that both SgrA and EcbA target also different parts of the fibrinogen molecule. The rSgrA protein bound to the α and β subunits, whereas rEcbA bound only to the γ subunit of fibrinogen. Future experiments including ligand affinity blotting using recombinant fibrinogen subunits isolated and purified from *Lactococcus lactis*, pepscan analysis, and the construction of amino acid substitution mutants will reveal the binding sites of rEcbA and rSgrA to fibrinogen and their other ligands.

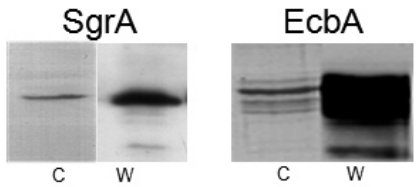
Apparently, *E. faecium* expresses three (SgrA, EcbA and Scm) fibrinogen-binding adhesins at its surface, and for SgrA and EcbA it has been demonstrated that these proteins recognize different parts of the ligand. Possibly, these two different interactions may act synergistically to allow tight attachment to this ligand. Alternatively, the presence of multiple structurally distinct fibrinogen-binding surface proteins may allow *E. faecium* to attach to the ligand in the presence of anti-SgrA, -EcbA or -Scm antibodies. Constitutive mRNA expression of *sgrA*, *acm*, *scm* in different stages of growth, and controlled *ecbA* expression from exponential to late exponential phase suggest that expression of these adhesins in *E. faecium* is fine-

tuned to allow adherence to or trophism for a particular tissue. Given of the aberrant size of the *acm* amplicon, absence of *acm* expression in the E135 strain is likely due to insertion of a transposon in the *acm* gene (33).

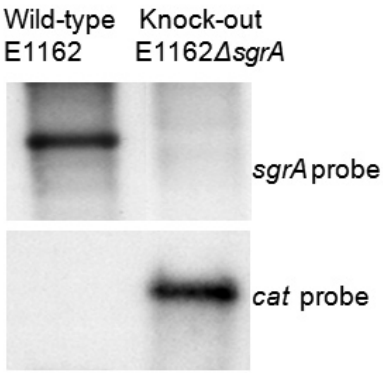
Fibrinogen and fibrin are major components of blood clots during wound healing and are the major plasma proteins deposited on implanted foreign devices. This means that for patients with indwelling medical devices SgrA may have a dual role in the infective process. In these patients, SgrA may facilitate attachment to polymer surfaces with subsequent growth on the device. Subsequently, SgrA and EcbA may mediate binding to ECM, deposited on these abiotic devices (10). As such, these surface adhesins may play a role in the pathogenesis of intravascular catheter-related infections.

Hospital acquired *E. faecium* has multiple intrinsic and acquired antibiotic resistance traits, which seriously hamper treatment of infected patients. Novel alternative treatment and prevention strategies are, therefore, urgently required. In that respect, the development of combinatory vaccines that target LPXTG surface proteins, such as SgrA and EcbA, could be a promising approach.

SUPPLEMENTAL FIGURES.



Supplemental Fig. 1. Recombinant SgrA and EcbA. rSgrA and rEcbA was expressed and purified from E. coli and analysed by SDS-PAGE and Western blotting using anti-His antibodies. The Coommassie stained recombinant proteins are indicated by a “C” and the Westernblots are indicated by an “W”.



Supplemental Fig. 2. Confirmation of correct insertion-deletion mutation in the *sgrA* gene by Southern blot analysis. Hybridization results of Southern blot analysis of *Eco*RI-digested genomic DNA of the E1162 wild-type strain (left lane) and the E1162Δ*sgrA* double-crossover mutant strain (right lane) probed with an *sgrA* probe (upper part) or an *cat* probe (lower part).

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Chapter 5

Expression of Two Distinct Types of Pili by a Hospital-acquired *Enterococcus faecium* Isolate

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ABSTRACT.

Surface filamentous structures designated pili, and implicated in virulence, have been found on the surfaces of several Gram-positive pathogens. This work describes the conditional expression of two phenotypically distinct pilus-like structures, designated PilA and PilB, on the surface of a hospital-adapted *Enterococcus faecium* bloodstream isolate. *E. faecium* is an emerging Gram-positive opportunistic pathogen that can cause severe disease, particularly in immunocompromised patients. Expression of PilA- and PilB-type pili was analysed during different phases of growth in broth culture. During growth, PilA and PilB pilin subunits were expressed around the cross-wall in early-exponential-phase cells. Polymerization and migration of short PilB-type pili towards the poles occurred in cells from the exponential phase and long polymerized pili were expressed at the poles of cells grown to stationary phase. In contrast, PilA-type pili were not expressed in broth culture, but only when cells were grown on solid media. Furthermore, surface expression of the PilA- and PilB-type pili was regulated in a temperature-dependent manner, as polymerization of two distinct types of pili at the surface only occurred when cells were grown at 37°C; no pili were observed on cells grown at 21°C. Hospital-aquired *E. faecium* isolates were specifically enriched in pilin gene clusters, suggesting that conditional expression of pili may contribute to *E. faecium* pathogenesis.

INTRODUCTION.

Enterococci have rapidly emerged as one of the most important nosocomial pathogens causing infections in debilitated patients (Murray, 2000). Although most hospital-acquired infections are still caused by *Enterococcus faecalis*, the ratio of *E. faecalis* to *Enterococcus faecium* infections is rapidly changing in favour of *E. faecium* in hospitals all across the world (Iwen *et al.*, 1997; Murdoch *et al.*, 2002; Top *et al.*, 2007; Treitman *et al.*, 2005). Hospital-acquired *E. faecium* isolates recovered from clinical sites of infection and responsible for nosocomial outbreaks (Leavis *et al.*, 2007; Willems *et al.*, 2005) show high-level resistance to a variety of antibiotics, including ampicillin and ciprofloxacin (Leavis *et al.*, 2006b), and are enriched in a putative pathogenicity island (Leavis *et al.*, 2004), genes encoding LPXTG-like cell-wall-anchored proteins (Hendrickx *et al.*, 2007), insertion sequence elements and genes encoding novel metabolic pathways (Leavis *et al.*, 2007). The cumulative acquisition of resistance determinants and putative virulence genes may have contributed to adaptation and selective advantage of specific *E. faecium* clones in the hospital environment (Leavis *et al.*, 2006a).

Recently, pili (also called fimbriae) have been identified in Gram-positive bacteria (Lauer *et al.*, 2005; Yeung & Ragsdale, 1997; Yeung *et al.*,1998). Pili are proteinaceous, non-flagellar, covalently joined multimers protruding from the bacterial cell wall, and function as a surface organelle (Marraffini & Schneewind, 2006; Ton-That & Schneewind, 2003). Pili of Gram-positive bacteria have been implicated in biofilm formation and endocarditis (*E. faecalis*) (Nallapareddy *et al.*,

2006), macrophage resistance (Maisey *et al*., 2008), invasion of and adherence to brain microvascular endothelial cells (*Streptococcus agalactiae*) (Maisey *et al*., 2007), and adhesion to human tonsil epithelium and skin (*Streptococcus pyogenes*) (Abbot *et al*., 2007) and to human pharyngeal epithelia (*Corynebacterium diphtheriae*) (Mandlik *et al*., 2007). Typical heterotrimeric pili are composed of a major subunit which forms the pilus backbone, a minor subunit and a tip, each protein having characteristics of Gram-positive cell-wall-anchored proteins. The major pilin subunit contains an N-terminal signal sequence for initiation of Sec-mediated translocation across the plasma membrane and a C-terminal cell wall sorting signal (CWS), which comprises an LPXTG-like sortase substrate motif, a hydrophobic domain and positively charged amino acids (Schneewind *et al*., 1992, 1993; Ton-That & Schneewind, 2003). A pilus-dedicated sortase catalyses the cleavage of the LPXTG-like motif and mediates pilus polymerization, which involves specific motifs such as the YPK pilin motif with a lysine (K) residue required for pilin polymerization and a conserved E box sequence (YxLxETxAPxGY) with a glutamate (E) residue to incorporate minor pilin subunits (Gaspar & Ton-That, 2006; Swaminathan *et al*., 2007; Swierczynski & Ton-That, 2006; Ton-That *et al*., 2004). After polymerization, a housekeeping sortase anchors the pilus via the threonine residue of the last pilin to the amino group of the lipid II precursor within the peptidoglycan (Dramsi *et al*., 2006; Ton-That & Schneewind, 2003).

The presence of pili has not been documented previously in *E. faecium*. Here, we report the identification of two genetic clusters encoding two distinct types of pili (designated PilA and PilB, previously referred to as Orf1904 and Orf2569, respectively) at the surface of a clinical *E. faecium* isolate, which are both expressed in a growth condition- and temperature-dependent manner.

MATERIALS AND METHODS.

Bacterial strains and growth conditions.

E. faecium strain E1165 is a hospital-acquired wound isolate and strain E1002 is a faecal isolate from a human healthy volunteer. *E. faecium* strains were grown aerobically at 21°C or 37°C on Trypticase Soy Agar II supplemented with 5 % sheep blood (TSA; Becton Dickinson) or on Brain Heart Infusion (BHI) agar or broth. Growth curves were prepared from cultures in BHI broth inoculated with plate-grown *E. faecium* cells, standardized to OD_{660} 0.1 and allowed to grow at 21°C or 37°C in an environmental air shaker at 225 r.p.m. A total of 131 *E. faecium* isolates collected from 20 countries worldwide were used in this study (Hendrickx *et al*., 2007). These isolates include 49 hospital-acquired *E. faecium* strains, representing clinical (n=31) and hospital outbreak (n=18) isolates from hospitalized patients, and 82 non-hospital-acquired isolates including human surveillance isolates (n=60, from individuals without known history of *E. faecium* infections) and isolates from various animals (n=12), from food products (2) and from the environment (8).

***In silico* search for genes encoding putative major pilin subunits.**

The incomplete *E. faecium* TX0016 (TEX16, DO) genome deposited at DDBJ/EMBL/GenBank under the project accession number AAAK00000000 was searched for proteins annotated as cell wall surface anchor family protein, von Willebrand factor type A, CnaB-type or surface protein from Gram-positive cocci (anchor region). Furthermore, putative major pilin subunits were identified by searching for the presence of an N-terminal signal peptide sequence, a consensus WxxxVxVYPKN pilin motif, a conserved ETxAPxGY E box motif and an LPXTG-like sortase substrate motif (where X denotes any amino acid), a hydrophobic domain and at least one positively charged amino acid within the last eight residues of the C terminus. The LPXTG-like motif was compared to the consensus [FILMPSVY][AP]X[ATS][GAKNS] and NPX[ST][DGNS] patterns representing distinct sortase substrate motifs (Boekhorst *et al.*, 2005). The presence of an N-terminal signal sequence was analysed using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP) (SignalP score >0.6) (Bendtsen *et al.*, 2004). The presence of the conserved pilin, E box and LPXTG-like motifs, hydrophobic amino acids and one or more positively charged amino acids within the last 45 residues of the C terminus was examined visually. To identify similarities, BLAST analyses on protein sequences were performed at the NCBI site (http://www.ncbi.nlm.nih.gov/BLAST/). Alignments were made using the Weblogo 2.8.2 site (http://weblogo.berkeley.edu/).

Identification of pilin gene clusters (PGCs) in *E. faecium* E1165.

PGC-1 and PGC-3 were amplified from strain E1165 using primers 1900_fw and Int1_rv for PGC-1 and primers 2567_fw and Int2_rv (Table 1) for PGC-3 to yield 6980 bp and 8202 bp fragments, respectively. PCR products were sequenced by using the BigDye Terminator 3.1 reaction kit and an ABI PRISM 3100 capillary DNA sequencer (both from Applied Biosystems) to determine sequence similarity to the corresponding clusters of *E. faecium* TX0016.

Table 1. Oligonucleotides used in this study

Target (gene cluster/ORF)	Primer name*	Oligonucleotide sequence (5′–3′)	Start in sequence† (5′–3′)
PGC-1	1900_fw	ATGCCATACACTCTCAGAGT	32701
	Int1_rv	CAGTTGAGACGAACTTATGT	39681
PGC-3	2567_fw	GCAGGAGGAATATGATTTTG	92461
	Int2_rv	TAGTTTCATTCCAACAACCTGAT	100663
pilA	PilA_fw	TTTTTGTGTGACTAATCCAG	38272
	PilA_rv	ATGGCAATAAATCGGTATGTA	38807
pilB	PilB_fw	GTGTTTGCAGAGGAGACAGC	95541
	PilB_rv	GACAGAATAATTTACTGGGTCG	94418
ddl	ddl_fw	GAGACATTGAATATGCCTTATG	334‡
	ddl_rv	AAAAAGAAATCGCACCG	893‡

*fw, forward; rv, reverse.
†Relative to the start of the contig.
‡Relative to the start codon.

Distribution of PGCs among *E. faecium* isolates.

The presence or absence of the putative pilin subunit genes from the PGCs in the 131 *E. faecium* isolates was determined by PCR and Southern hybridizations as described previously (Hendrickx *et al.*, 2007). A chi-squared test was used to assess statistically significant differences.

Anti-peptide polyclonal rabbit antisera.

Highly specific polyclonal rabbit antisera were prepared by Eurogentec (Seraing, Belgium) according to their classic 87 day anti-peptide polyclonal antibody protocol, by immunizing two specific-pathogen-free rabbits with unique 15 amino acid residue keyhole limpet haemocyanin-conjugated peptides directed against the N-terminal region of PilA (H_2N-EDVTQKTPPEKVNITC-$CONH_2$) and PilB (H_2N-CKMTDLPDPLIQNSGK-$CONH_2$) of the *E. faecium* TX0016 genome sequence. Rabbits were exsanguinated at day 87; the serum was collected and stored at –20°C.

Western immunoblot analysis.

To detect PilA and PilB pilin protein expression, E1165 bacteria were resuspended in PBS and harvested by centrifugation (1560 ***g***, 5 min). To extract cell-surface proteins, cell pellets were suspended in 70 % formic acid and incubated at 65°C for 30 min as described previously (Ton-That & Schneewind, 2003; Gaspar & Ton-That, 2006), followed by trichloroacetic acid precipitation and acetone washing. Protein pellets were resuspended in 50 µl PBS+50 µl sample buffer (100 mM Tris/HCl, 5 % dithiothreitol, 2 % SDS, 0.004 % bromophenol blue and 20 % glycerol) and boiled for 5 min. Equal amounts of protein sample of *E. faecium* E1165 were analysed by Western blotting as described previously (Hendrickx *et al.*, 2007).

Dot-blot analysis.

Dot-blot analysis was performed on chromosomal DNA isolated from *E. faecium* E1002 to determine the presence or absence of LPXTG-like surface-protein genes from PGC-1 and PGC-3. Chromosomal DNA was isolated as described previously (Hendrickx *et al.*, 2007). The chromosomal DNA was denatured with 0.5 M NaOH in a 96-well microtitre plate for 15 min, transferred to a Bio-Rad Bio dot apparatus, vacuum blotted onto a Hybond-N^+ nylon membrane and fixed onto the membrane by incubation for 2 min in 0.4 M NaOH followed by neutralization in 10x SSC for 1 min. Membranes were hybridized overnight at 42°C with 100 ng probe. Gene-specific probes were amplified by PCR using primer pairs specific for *orf1901*, *pilA*, *pilB*, *orf2570* and *orf2571* as described previously (Hendrickx *et al.*, 2007). Amplified DNA probes were purified with a PCR purification kit (Qiagen) and labelled according to the ECL nucleic acid labelling kit (GE Healthcare). The membranes were exposed to Hyperfilm ECL (GE Healthcare).

mRNA expression analysis by reverse transcription (RT-)PCR.

For mRNA expression of two putative major pilin subunit genes (*pilA* and *pilB*) cells were resuspended into PBS to an OD_{660} of 1.0 ($1x10^9$ c.f.u. ml^{-1}) and pelleted by centrifugation (6500 ***g*** for 1 min). Total RNA was isolated according to Cheung *et al.* (1994). RNA purification and cDNA generation was done as described previously (Hendrickx *et al.*, 2007; Nallapareddy & Murray, 2006). cDNA was used as template for PCR using primer pairs PilA_fw and PilA_rv and PilB_fw and PilB_rv (Table 1). As an internal control, the housekeeping gene *ddl* (encoding D-alanine–D-alanine ligase) was amplified using primers ddl_fw and ddl_rv (Table 1). RNA samples not treated with reverse transcriptase were used as control to detect DNA contamination in the total RNA preparations.

Electron microscopy and immunogold labelling.

Transmission immunoelectron microscopy was performed as described previously with some modifications (Hendrickx *et al.*, 2007; Van Wamel *et al.*, 2007). In brief, copper grids (mesh Formvar-carbon coated) were incubated for 30 min with the carbon side on a drop containing *E. faecium* E1165 cells

($1x10^9$ c.f.u. ml^{-1}) to detect surface-exposed PilA and PilB expression. Grids were washed three times for 5 min on drops of 0.02 M glycine in PBS and subsequently blocked for 30 min on drops of 1 % BSA in PBS (PBSb). The PilA- and PilB-type pili were labelled for 1 h on drops of undiluted specific peptide rabbit immune serum or pre-immune serum in PBSb. Grids were washed four times for 2 min on drops of 0.1 % BSA in PBS. Pilus–antibody complexes were labelled by incubation for 20 min on drops with 1 : 55 diluted protein A-gold label (10 nm) in PBSb. Grids were washed four times for 2 min on drops of PBS, fixed by incubation on drops of 1 % glutaraldehyde in PBS and washed again eight times for 2 min on drops of H_2O. For double labelling experiments, the labelling was repeated similarly with a second immune serum, and a 1 : 70 diluted protein A-gold label (5 nm). Bacteria were stained by incubation of the grids for 5 min on drops containing 1.8 % methylcellulose (25 centipoise; Sigma-Aldrich) and 0.4 % uranyl acetate (pH 4) and subsequently air-dried for 10 min. Grids were examined using a JEOL 1010 transmission electron microscope (JEOL Europe).

RESULTS.

Expression of putative pilus-like structures at the surface of *E. faecium*.

To examine *E. faecium* for the presence of putative pilus-like appendages at the surface of the cell, transmission electron microscopy (TEM) was performed on *E. faecium* strain E1165, a clinical hospital-acquired isolate. This strain exhibited good binding to the carbon-coated copper grids, in contrast to many other strains. TEM identified putative pilus-like structures, typically 50 nm to 1 μm long, expressed at the poles of the cell (Fig. 1A).

In silico characterization of putative pilin loci of *E. faecium*.

The unsequenced *E. faecium* strain E1165 expressed putative pilus-like structures at its surface. Therefore, we assessed whether the partially sequenced and annotated genome of *E. faecium* TX0016 contains ORFs encoding putative major pilin subunit genes, located in the vicinity of putative sortase genes. Putative major pilin subunits were identified by the presence of an N-terminal signal sequence, a conserved pilin motif, an E box motif and a CWS. The genome search identified four putative major pilin subunit genes, located in four distinct gene clusters containing LPXTG-like surface protein genes and putative pilus-dedicated sortases. Putative *E. faecium* PGC-1 contains six ORFs: *orf1901*, *orf1916*, *orf1902*, *orf1903*, *orf1904* and *orf1905* (Fig. 1B). *orf1901* is predicted to encode a putative cell-wall-associated protein with a VPKTG sortase substrate motif. The predicted *orf1904* gene product, designated PilA, is a putative major pilin subunit with an N-terminal signal sequence, a pilin motif, a motif resembling the conserved E box motif, and a VPMTG sortase substrate motif (Fig. 1C). The putative PilA major pilin protein displays similarities to uncharacterized cell-wall-anchored surface proteins. The *pilA* gene is located between two putative sortase-encoding genes, of which *orf1903* resembles a pilus-dedicated sortase enzyme, and *orf1905* a housekeeping sortase. *orf1903* has low (34 %) similarity to the EF1094 (also designated srtC or Bps) pilus-associated sortase protein of *E.*

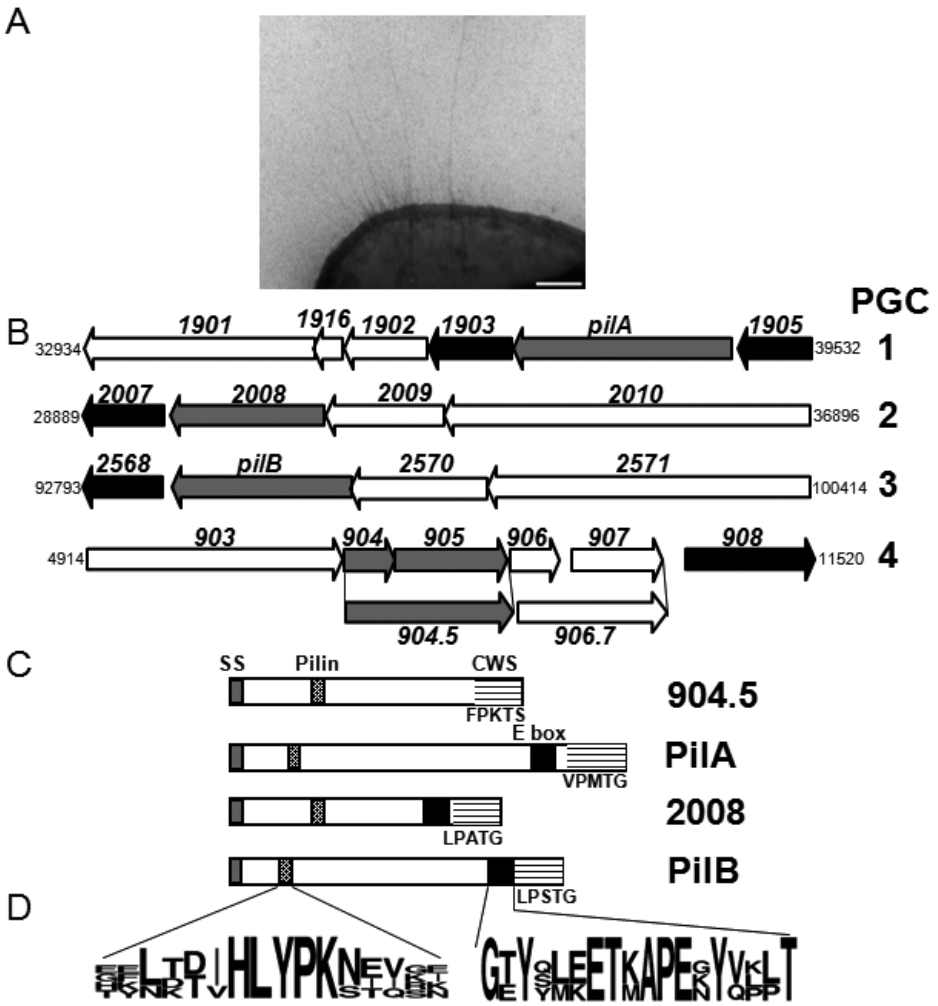

Figure 1. Putative pili, pilin gene clusters and major pilin subunit proteins in *E. faecium*. Panel A displays a transmission electron microscope micrograph of negatively stained *E. faecium* E1165 cells. Bar = 100 nm. (B) Schematic representation of the genomic organization of four putative pilin gene clusters identified from the *E. faecium* TX0016 genome sequence as published at DDBJ/EMBL/Genbank. The position and presumed direction of transcription of ORFs is indicated by arrows and the ORF number is depicted in bold above the arrows. The black arrows indicate putative sortase genes, grey arrows indicate putative major pilin genes, white arrows indicate putative minor or tip pilin genes or genes with unknown function. Thin lines connect genes encoding putative major pilin subunits. The numbers at the beginning and the end of the pilin gene clusters are positions relative to the start of the contig. (C) Structural organization of the putative major pilin proteins. Signal sequences (SS) are depicted in grey, conserved pilin motifs in dotted grey, E box motifs in black and the CWSs are striped. (D) Alignment of regions of the conserved pilin motifs and E box motifs found in the four putative major pilin proteins. Large amino acid sequences are highly conserved.

faecalis V583 (Nallapareddy *et al.*, 2006) and other putative pilus-dedicated sortases of *Corynebacterium diphtheriae* and *Clostridium perfringens* (data not shown). In contrast, the *orf1905* sortase did not display similarities to pilus-dedicated sortase enzymes, but it showed 31 % similarity to the SrtA (EF3056) housekeeping sortase of *E. faecalis* V583.

PGC-2 contains four ORFs, *orf2007*, *orf2008*, *orf2009* and *orf2010* (Fig. 1B). The *orf2007* gene product resembles a putative pilus-dedicated sortase, showing similarity (44 %) to the EF1094 pilus-associated sortase of *E. faecalis* V583. The *orf2008* gene is predicted to encode a major pilin subunit protein with an N-terminal signal sequence, a putative pilin motif, an E box motif, and a canonical LPATG sortase substrate recognition sequence (Fig. 1C). The *orf2008* gene product has 32 % similarity to the Bee protein of *E. faecalis*, which has been implicated in biofilm formation (Tendolkar *et al.*, 2006). Interestingly, *orf2009* also contains, in addition to all features of a Gram-positive LPXTG-like surface protein, a putative pilin motif, and may represent a minor pilin subunit. *orf2010* is predicted to encode the largest LPXTG-like protein in this PGC.

PGC-3 contains four ORFs, *orf2568* to *orf2571* (Fig. 1B). This cluster closely resembles the organization of and has high similarity to the *ebpABC–srtC* (previously designated EF1091–EF1094) PGC of *E. faecalis* V583 (Nallapareddy *et al.*, 2006). A putative sortase (*orf2568*) is located upstream of a putative major pilin subunit, *pilB*, previously designated *orf2569*. PilB contains a pilin and E box motif and exhibits 74 % similarity to the putative major EbpC (EF1093) pilin protein of *E. faecalis* V583 (Fig. 1C). *orf2570* and *orf2571* (the latter contains an E box motif) share similarities of 48 % with *ebpB* and 57 % with *ebpA* of *E. faecalis* and putatively encode a minor subunit and a pilus tip, respectively.

We recently identified a cluster of three LPXTG-like surface protein genes and a sortase gene (*orf903* to *orf908*) located on a putative pathogenicity island, which was specifically enriched in hospital-acquired *E. faecium* (Hendrickx *et al.*, 2007). This cluster possibly constitutes a fourth *E. faecium* PGC (Fig. 1B). In the majority of the hospital-acquired *E. faecium* isolates, the presence of multiple premature stop codons suggests that this is a non-functional locus. However, in 9.2 % of the isolates we analysed, this cluster comprises ORFs encoding three surface-exposed proteins, *orf903*, *orf904.5* and *orf906.7*, of which the *orf904.5* product contains a pilin motif and is a putative major pilin subunit (Fig. 1C). *orf908* tentatively encodes a pilus-dedicated sortase, since it shares 52 % similarity to the EF1094 pilus-associated sortase protein of *E. faecalis* V583.

Alignment of the identified putative major pilin subunits of *E. faecium* did not reveal a significant degree of amino acid identity, except for the pilin and E box motifs, which were highly conserved among the *E. faecium* pilin proteins analysed (Fig. 1D). The consensus pilin motif of putative major pilin proteins of *E. faecium* is LxxIHLYPKNxx, with a conserved HLYPK motif, which is different from the consensus Gram-positive pilin motif, WxxxVxVYPKN (Mandlik *et al.*, 2008). Similarly, the consensus E box motif of *E. faecium*, YxxxETxAPExY, was also different from the consensus E box motif (YxxxETxAPxGY) of other Gram-positive major pilin subunits.

PGCs-1 and -3 of *E. faecium* TX0016 and E1165 are conserved.

In this paper, the focus is on the *pilA* and *pilB* genes located in PGC-1 and PGC-3, respectively. To identify homologues of *pilA* and *pilB* of *E. faecium* TX0016 in strain E1165, PGC-1 and PGC-3 were amplified by PCR from genomic *E. faecium* E1165 DNA and sequenced. PGC-1 and PGC-3 of *E. faecium* E1165 exhibit very high overall similarity (99 and 100 %, respectively) to PGC-1 and PGC-3 of *E. faecium* TX0016. The organization and transcriptional direction of the ORFs in PGC-1 and PGC-3 are similar, demonstrating that both PGCs are conserved in *E. faecium* E1165.

Putative pilin subunit genes are enriched among hospital-acquired *E. faecium* isolates.

The presence of the putative pilin subunit genes from the four PGCs was analysed among 131 *E. faecium* isolates, of which 49 were hospital-acquired and 82 non-hospital-acquired. Based on PCR and Southern hybridization, both *orf1901* and *pilA* (PGC-1) were present in the genomes of 71 % (35/49) of the hospital-acquired isolates and in 48 % (39/82) of the non-hospital *E. faecium* isolates (P=0.004). The three putative pilin subunit genes from PGC-2 (*orf2008–orf2010*) were detected in 80 % (39/49) of the hospital-acquired isolates and in only 43 % (35/82) of the non-hospital strains (P<0.001). The ORFs *pilB* to *orf2571* were present in 94 % (46/49) of the hospital-acquired strains and in 74 % (61/82) of the non-hospital strains (P=0.002). These data, in combination with the previously reported distribution of PGC-4 (Hendrickx *et al.*, 2007), show that hospital-acquired *E. faecium* isolates are significantly enriched for putative pilin genes.

Expression of PilA and PilB multimers at 37°C.

Expression of the PilA and PilB pilin proteins (of PGC-1 and -3) in *E. faecium* strain E1165 grown at 37°C on TSA plates was studied at both the mRNA and protein level. mRNA transcripts of *pilA* and *pilB* were detected in strain E1165 (data not shown). Using specific rabbit anti-peptide immune sera directed against the two putative major pilin subunits, monomeric forms of PilA and PilB pilin proteins were detected at the predicted molecular masses of 64 and 61 kDa respectively (Fig. 2A). In addition, as observed for other Gram-positive pili, bands of high molecular mass were detected corresponding to PilA and PilB multimers, and PilA and PilB monomers coupled to sortase enzymes or associated with peptidoglycan. Rabbit pre-immune sera did not react with these protein monomers or multimeric pilin species (data not shown). The patterns of PilA and PilB multimeric pilin species were different, suggesting that *pilA* and *pilB* encode two different types of pili. To exclude potential cross-reactivity of the rabbit anti-PilA peptide immune serum, the E1002 PilB pili overexpression strain was included in this study. *E. faecium* strain E1002 is deficient for *orf1901* and *pilA* from PGC-1, and harbours the *pilB-orf2570-orf2571* genes from PGC-3 (Fig. 2B) as determined by PCR and DNA–DNA hybridizations using gene specific probes. The anti-PilA peptide immune serum did not react with

PilB monomers or high-molecular-mass pilin species (Fig. 2A, lane 3), whereas the anti-PilB peptide immune serum did react with the PilB momoner and pilin species (Fig. 2A, lane 4).

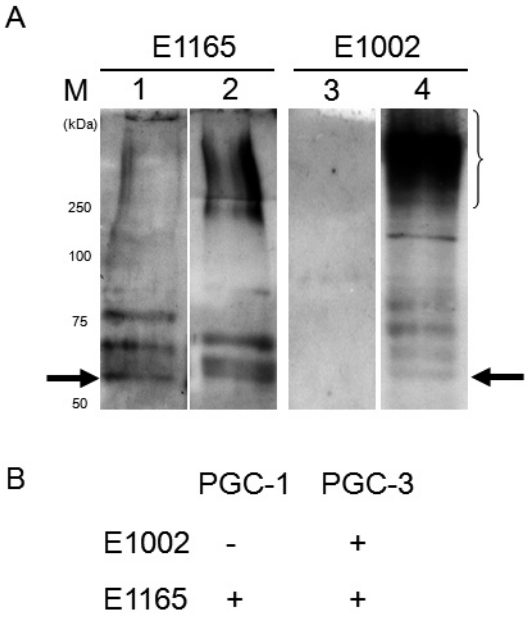

Figure 2. Western blots of surface protein extracts of *E. faecium* E1165 and E1002 grown on TSA plates at 37ºC. (A) Blots were probed with rabbit anti-PilA (lanes 1 and 3) and rabbit anti-PilB (lanes 2 and 4) immune sera followed by Goat anti-rabbit IgG-HRP conjugate. Numbers depicted on the left indicate molecular weights in kilo Daltons (kDa). Predicted monomeric PilA (64kDa) and PilB (61 kDa) pilin proteins expression are indicated by an arrow, high molecular weight pilin species are indicated by an accolade. (B) Presence and absence of PGC-1 and PGC-3 in strain E1165 and E1002 as determined by PCR and dot blot hybridizations. + indicates presence and – indicates absence of the LPXTG-like surface protein genes in the PGC.

Expression of PilA and PilB pili at the surface of *E. faecium* E1165.

To detect expression of PilA and PilB pili at the surface of *E. faecium* E1165, single-labelling TEM experiments were performed on immunogold-labelled (10 nm) and negatively stained cells grown on TSA plates at 37°C. Using the rabbit PilA and PilB anti-peptide immune sera, 10 nm gold particles were clearly associated with distinct pilus-like structures protruding from the *E. faecium* cell wall (Fig. 3A, E). Interestingly, expression of PilA pili was observed at the surfaces in approximately ~40 % of the cells and expression of PilB pili in ~15 % of the cells examined by TEM. Cells without pili expressed cell-wall-associated major pilin subunits or no pilin subunits at all (data not shown) (Dramsi *et al.*, 2006; Nelson *et al.*, 2007). The PilA pili were highly expressed at the poles of dividing enterococci and appeared as rigid, thin (~2 nm and not electron-dense) appendages, which were relatively short (to

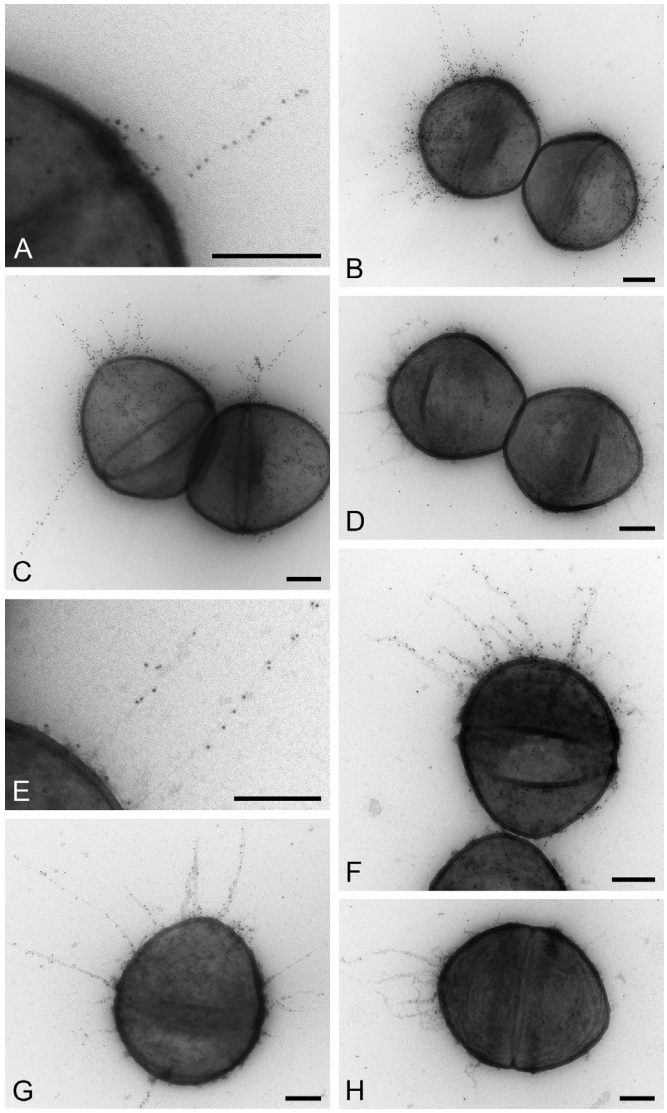

Figure 3. TEM micrographs of PilA-type and PilB-type pili of *E. faecium* E1165. E1165 was grown on TSA plates at 37ºC and subsequently labelled with either anti-PilA or -PilB peptide immune sera followed by protein A-gold (10 nm particles) and negative staining. Panel A indicates *E. faecium* cells incubated with anti-PilA rabbit immune serum, showing details of an PilA pilus, decorated with 10 nm gold particles. (B) Highly expressed PilA-type pili at the poles appearing as rigid and thin (2 nm) appendages, relatively short (to ~300nm) of length. (C) Similarly as for B, but occasionally pili extended up to 1 µm long. (D) *E. faecium* cells incubated with PilA rabbit pre-immune serum not displaying gold labelling. (E) *E. faecium* cells incubated with anti-PilB rabbit immune serum, showing details of a PilB pilus, decorated with 10 nm gold particles. (F) PilB-type pili appear flexible, thick (4 nm) and less abundantly expressed at the poles of the cell compared to PilA-type pili. (G) Peritrichous PilB-type pili expression occurs in single cells. (H) *E. faecium* cells incubated with PilB rabbit pre-immune serum not displaying gold labelling. Bar = 200 nm.

~300 nm) (Fig. 3B), but could extend up to 1 μm long (Fig. 3C). No peritrichous PilA pili expression was observed in the case of single cocci. PilB pili were detected at the poles of the cell (Fig. 3F), and in the case of single cocci, peritrichous pili expression was observed (Fig. 3G). The PilB pili appeared more flexible, extended over 1 μm and were thicker (~4 nm, electron-dense) compared to PilA pili. No gold particles were detected along pilus shafts when cells were incubated with rabbit pre-immune sera (Fig. 3D, H) or with protein A-gold (conjugate control; data not shown).

Pili encoded by *pilA* and *pilB* are two distinct types.

To corroborate that *pilA* and *pilB* encode two distinct types of pili, a double-labelling TEM experiment was performed on cells grown on TSA plates at 37°C. Pili of *E. faecium* E1165 cells were initially labelled with rabbit anti-PilB immune serum followed by protein A-gold (5 nm), fixed with glutaraldehyde, and subsequently incubated with anti-PilA serum and protein A-gold (10 nm). TEM confirmed that the two immune sera bound to distinct pili (Fig. 4) and that cells displayed expression of the two distinct types of pili simultaneously.

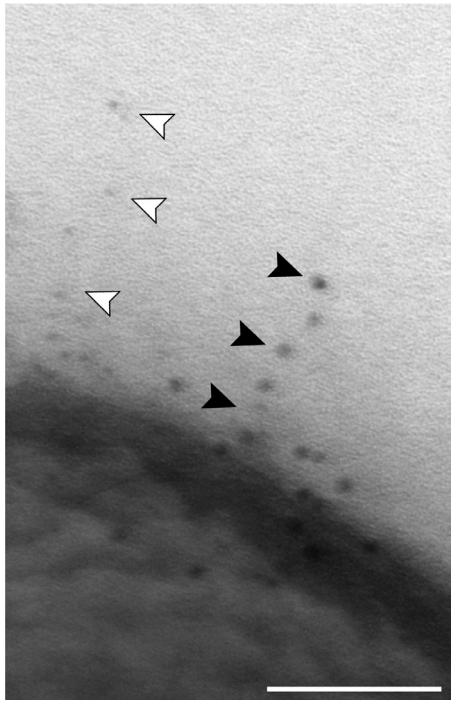

Figure 4. TEM micrograph of PilA- and PilB-type pili expressed on a single cell. *E. faecium* E1165 was labelled with rabbit anti-PilB immune serum, followed by protein A-gold (5 nm particles), fixed and consecutively labelled using anti-PilA immune serum, followed by protein A-gold (10 nm particles). The PilA-type of pili are indicated by black arrows, PilB-type pili by white arrows and are expressed simultaneously on a single cell. Bar = 100 nm.

Temperature-dependent downregulation of expression of PilA- and PilB-type pili at 21°C.

To determine the influence of temperature on expression levels of PilA- and PilB-type pili, *E. faecium* E1165 cells were grown at 21°C on TSA plates, and protein and pili expression levels were analysed by Western blotting and single-labelling TEM experiments using anti-PilA and anti-PilB rabbit antisera. Interestingly, only monomeric forms of PilA and PilB pilin protein were detected at 21°C, at a lower level compared to cells grown at 37°C, and no polymerization into PilA and PilB multimers was observed (compare Figs 2A and 5A). In addition, cells analysed by TEM did not display expression of either PilA- or PilB-type pili. Instead, major pilin subunits were expressed at the surface or cells did not show expression of pilin subunits at all (Fig. 5B). The observation that expression of both types of pili was decreased at 21°C suggests common regulation. The fact that putative major pilin proteins were still expressed at the surface of the cell indicates that polymerization of monomers into pili may readily occur upon a temperature switch.

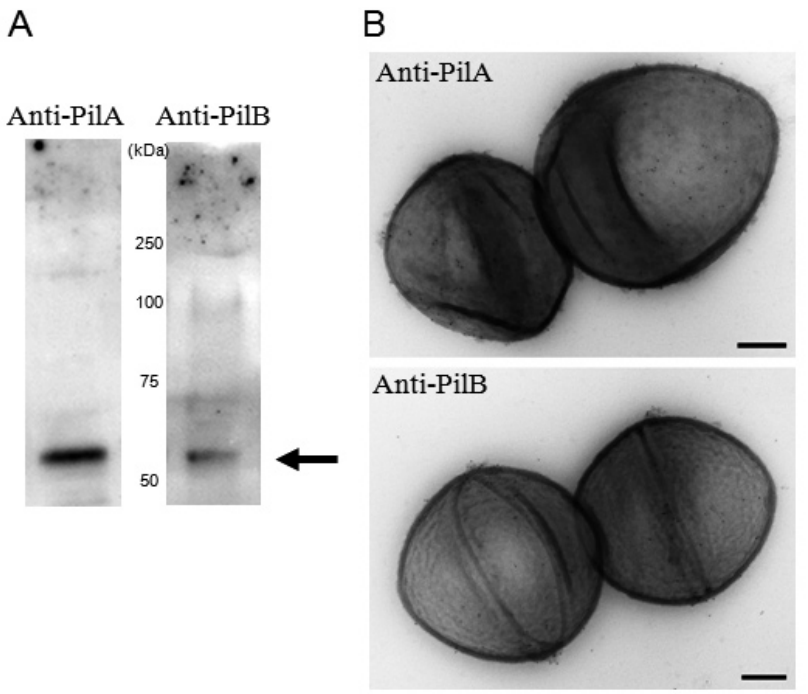

Figure 5. Temperature-dependent down-regulation of PilA- and PilB-type pili expression at 21ºC. *E. faecium* E1165 was grown on TSA plates at 21ºC. Panel A shows surface protein extracts analyzed by Western blots probed with rabbit anti-PilA (left part) and rabbit anti-PilB (right part) immune sera. Only PilA and PilB monomeric pilin subunits are expressed as depicted by an arrow and no multimers were observed compared to Fig. 2. Numbers depicted in the middle indicate molecular weights in kilo Daltons (kDa). (B) TEM micrographs of cells grown at 21ºC and labelled with anti-PilA (upper part) or anti-PilB (lower part) immune serum followed by protein A-gold (10 nm) and negative staining. Major pilin subunits are associated with the cell wall and no surface-exposed pili are expressed. Bar = 200 nm.

PilA- and PilB-type pili expression in different stages of growth.

To analyse *pilA* and *pilB* expression in different stages of growth, *E. faecium* E1165 cells were grown in BHI broth and a growth curve was made (Fig. 6A). At three consecutive time points, early-exponential, exponential and stationary phase of growth, cells were harvested and transcription of the two major pilin genes, *pilA* and *pilB*, and the *ddl* internal control was analysed by PCR on cDNA generated from total mRNA. Transcripts of *pilB* were detected in all phases of growth, but decreased from early-exponential to stationary phase in strain E1165, indicating downregulation of PilB-type pili expression in later stages of growth (Fig. 6B). Control PCRs on DNase-treated total mRNA samples in which the reverse transcriptase reaction was omitted were negative and *ddl* was constitutively expressed in all samples (Fig. 6B). Although

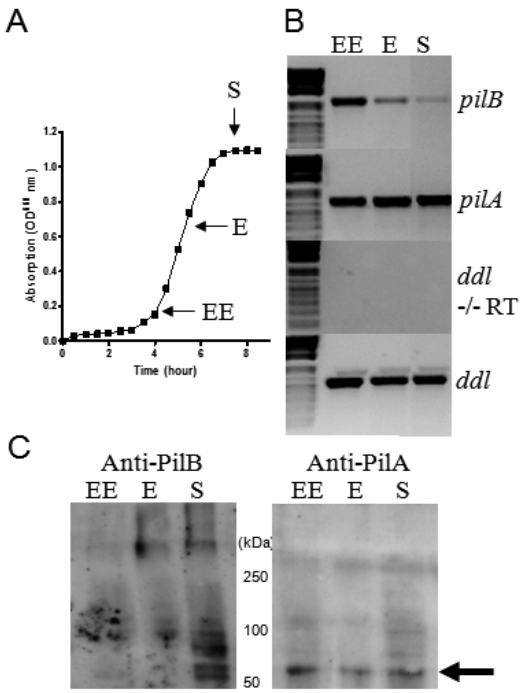

Figure 6. Expression of PilA- and PilB-type pili in different phases of growth. Panel A shows a growth curve of *E. faecium* E1165 in BHI at 37ºC. In early-exponential (EE), exponential (E) and stationary phase (S) cells were harvested as indicated by arrows. (B) mRNA expression of *pilA*, *pilB*, and *ddl* in cells isolated in EE, E and S-phase of growth. In the upper part, mRNA of *pilB* is depicted, which decreased from EE to S-phase grown cells. The *pilA* gene is constitutively expressed in all growth phases. Control *ddl* PCR reactions on total mRNA preparations in which the RT reaction was omitted were all negative, demonstrating absence of DNA contamination. Control *ddl* RT-PCR reactions (internal housekeeping control) with *E. faecium* specific *ddl* primers were all positive. The results are presented as amplified PCR products electrophoresed on the same ethidium bromide-stained 1.5% agarose gel. (C) Surface protein extracts of EE, E- and S-phase grown cells analyzed by western blots incubated with rabbit anti-PilB (left part) and rabbit anti-PilA (right part) immune sera. The PilB pilin protein levels increase from EE to S-phase grown cells. The monomeric PilA pilin protein is constitutively expressed (arrow) while pilin multimers are absent.

high-level mRNA transcripts were detected, low levels of pilin proteins were extracted from cells grown to early-exponential and exponential phase (Fig. 6C) and no long PilB-type pili were expressed at the surface. Instead, major pilin subunits were observed in early-exponential-phase cells close to the septum or cross-wall in the centre of a dividing cell (Fig. 7A), in which already a low level of polymerization of PilB-type pili occurred (Fig. 7B). In some early-exponential-phase cells, expression of PilB pilin subunits was directed more towards the poles, possibly the result of newly synthesized peptidoglycan pushing old peptidoglycan from the cross-wall to the poles. In exponential-phase cells, *pilB* mRNA expression levels decreased (Fig. 6B), whereas expression of PilB multimeric pilin species and polymerized pili, mainly at the 'old poles' increased (Figs 6C and 7C). In these cells, pili were ~400 nm

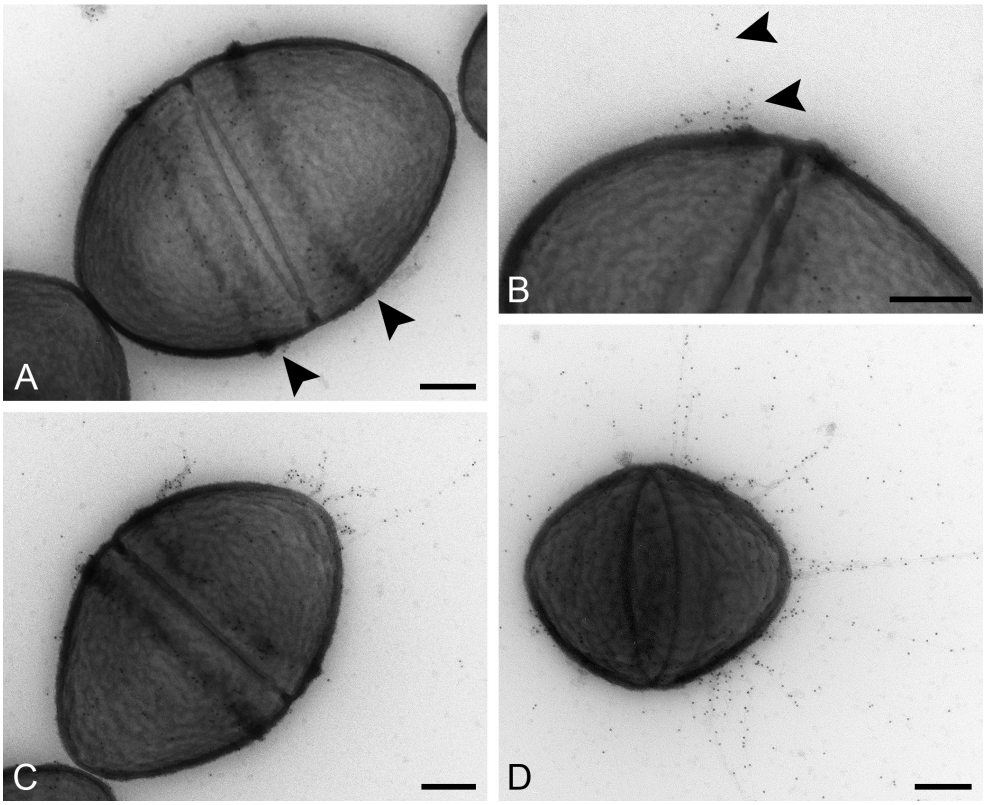

Figure 7. TEM micrographs of PilB-type pili expression in different phases of growth. In early-exponential (EE), exponential (E) and stationary (S) phase, *E. faecium* cells were harvested and labelled with anti-PilB immune serum followed by protein A-gold (10 nm) and negative staining. In panel A, cells harvested from an EE-culture display PilB pilin protein expression around the cross wall (black arrows). (B) Similar to A, detailed micrograph of low-level of PilB-type pili polymerization at the cross wall (black arrows). (C) Cells in E-culture display PilB pilin protein expression around the cross wall, to a lesser extend compared to EE-cells, and pili were observed at the poles. (D) In S-phase cells, cell did not show pilin protein expression around the cross wall, instead PilB pilin subunits and long (1 µm) PilB-type pili were observed. Bar = 200 nm.

in length, and less pilin protein was observed around the cross-wall. In stationary phase, no pilin subunits were observed around the cross-wall. Very long (1 μm) PilB-type pili were expressed and PilB subunits were detected at the poles of the cells (Fig. 7D), suggesting pilin subunit translocation and polymerization, which is in line with detection of mRNA transcripts and high levels of PilB multimers (Fig. 6B, C).

Transcripts of *pilA* were detected in all phases of growth, suggesting constitutive expression of PilA-type pili (Fig. 6B). Monomeric forms of PilA pilin protein were detected by Western blotting, demonstrating constitutive PilA expression during the different phases of growth (Fig. 6C). Interestingly, no polymerization of PilA into PilA multimers was detected at any stage of growth in broth culture; this was subsequently confirmed by TEM (data not shown). Pilin subunits were observed close to the cross-wall in the centre of the cell when cells were grown to early-exponential phase (data not shown). This was also the case for cells grown to exponential or stationary phase. However, polymerized PilA pili were observed when cells were grown on solid TSA and BHI media, instead of liquid BHI medium, suggesting that PilA pilus polymerization is induced in a contact-dependent manner.

DISCUSSION.

We describe conditional expression of pili on the surface of hospital-acquired *E. faecium*. Pili are 'hair-like' surface-exposed structures involved in adhesion to multiple types of human cells and biofilm formation, two processes critical in the pathogenesis of bacterial disease. In contrast to pili of Gram-negative bacteria, little is known concerning conditional pilus expression in Gram-positives. We demonstrate that *E. faecium* can express two distinct types of pili at the surface of a single cell, and that these pili are encoded by two different and physically separated PGCs. Both *pilA* and *pilB* encode two different major pilin subunits that polymerize into distinct multimeric pilus structures. Five lines of evidence support this interpretation. First, *in silico* analysis revealed that the PilA and PilB proteins contain an N-terminal signal sequence to facilitate Sec-mediated translocation, a pilin motif required for pilus polymerization, an E box motif possibly involved in incorporation of minor pilin subunits and a CWS containing an LPXTG-like sortase recognition sequence. Second, expression analysis by RT-PCR detected two specific mRNA transcripts and Western blotting revealed two distinct PilA and PilB multimeric pilin species. Third, the rabbit anti-PilA immune serum did not cross-react with the PilB monomeric subunit or the PilB high-molecular-mass pilin species. Fourth, in TEM experiments, specific anti-PilA and anti-PilB peptide sera reacted with two phenotypically distinct pilus structures at the surface of *E. faecium* cells grown at 37°C. The PilA-type pili were thin, rigid, relatively short and occasionally expressed as long polymers at the poles of dividing cells. The PilB-type pili were thicker than PilA-type pili, more flexible and expressed as long polymers at the poles of dividing cells. The observation that PilB-

type pili are thick suggests incorporation of additional pilin proteins along the pilus shaft or a bundled multi-fibre structure (Hilleringmann *et al.*, 2008). Finally, double-labelling TEM demonstrated expression of two distinct types of pili simultaneously on a single *E. faecium* cell. The fact that PilA- and PilB-type pili were differentially expressed to form a heterogeneous population of *E. faecium* cells expressing PilA- or PilB-type, both types or no pili at all indicates that cells grown in colonies on solid media are in different phases of growth and may favour phase variation, which plays a role in immune evasion by limiting exposure of pilin antigens to the immune system, or adaptation and survival in changing environments (Holden & Gally, 2004; Kearns *et al.*, 2004; van der Woude, 2006).

Expression of PilA- and PilB-type pili at the surface of *E. faecium* was regulated in a temperature-dependent manner. In contrast to high-level expression of both types of pili on surfaces of *E. faecium* cells grown at 37°C, no pili were observed when the bacteria were grown at 21°C. Instead, cell-wall-associated major pilin subunits were detected at the surface. This suggests that during prolonged survival of *E. faecium* in the hospital environment, both types of pili are not expressed. Contamination of skin and indwelling medical devices by *E. faecium*, accompanied by a temperature shift to 37°C, results in upregulation of pili biogenesis and may initiate intimate attachment, followed by colonization and finally infection.

The PilA and PilB major pilin subunits are expressed around the cross-wall in cells grown to early-exponential phase. The cross-wall is a thick layer of newly synthesized peptidoglycan at which the cell separates into two newly formed cells (Giesbrecht *et al.*, 1998). A similar finding was recently reported for the LPXTG-like cell-wall-anchored protein A of *Staphlococcus aureus* (DeDent *et al.*, 2007). This suggests that PilB pilin proteins are possibly translocated around the cross-wall and deposited into newly synthesized murein sacculi during separation of the cross-wall at its midline at cell division. Murein sacculi are rigid exoskeletal organelles in which surface proteins are immobilized (Salton, 1952; Cole & Hahn, 1962; Ghuysen, 1968; Navarre & Schneewind, 1999). Possibly, the pilus-dedicated sortase is also located at these cell division sites, as a low level of polymerization of PilB-type pili was observed, but this remains to be investigated. During further growth, novel murein sacculi arise and migration of old murein sacculi towards the poles occurs. Indeed, labelled PilB pilin proteins migrated towards the poles and the pili were longer than those of early-exponential-phase cells. These pili were typically ~300 nm long and not abundantly expressed at the surface. In stationary phase, cells display high-level pili expression and pilin subunits were detected predominantly at the old poles. The finding of differential PilB expression and biogenesis during growth suggests that pili of this type are expressed in a growth-phase-dependent manner. However, environmentally relevant cues, such as fluctuations in pH, oxygen and carbon dioxide concentrations, increasing cell density, and cell-to-cell signalling in different phases of growth in liquid media may also influence pili expression. Which of these factors affect PilB expression remains to be elucidated.

The PilA-type pili were expressed when the bacteria were grown on solid media, but not in liquid media, and expression was not growth phase-dependent. These pili are possibly expressed upon cell–cell or cell–substratum contact. Alternatively, shearing, enzymic cleavage or degradation of PilA pili may occur in broth cultures, and could account for the lack of polymerized PilA structures at the surface of cells grown in liquid cultures. The localization of *pilA* between a putative housekeeping and a putative pilus-dedicated sortase gene in PGC-1 suggests that polymerization by the pilus-dedicated sortase may be followed instantly by cell wall anchoring of the PilA pilus by the housekeeping sortase. Possibly, the PilA-type pili may be involved in initial adherence of *E. faecium*.

The finding that PGCs were enriched in hospital-acquired *E. faecium* isolates implies that conditional pili expression may confer a selective advantage in the hospital environment and may promote *E. faecium* pathogenesis. Recently, active and passive immunization with recombinant pilus subunits of *Streptococcus pneumoniae* protected mice against a lethal challenge, suggesting that Gram-positive pili are promising novel targets for immune therapy to treat infections (Gianfaldoni *et al.*, 2007). Hospital-acquired *E. faecium* are multi-resistant, which limits the choice of antibiotics and complicates effective therapy to treat infected patients. A novel strategy to combat these multi-antibiotic-resistant bacteria could be the development of vaccines that target pili.

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Chapter 6

Differential Expression of PilA Pili by a Hospital Acquired and a Non-hospital acquired *Enterococcus faecium* Isolate

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ABSTRACT.

Pili are hair-like structures protruding from the cell wall of bacterial cells. PilA- and PilB- pilus-like structures have been detected at the surface of an infection-derived *Enterococcus faecium* isolate. Here we describe the conditional and differential expression of PilA-type pili and the PilE, and PilF proteins putatively encoded from pilin gene cluster-1 at the surface of a hospital acquired (E1165) and a non-hospital acquired (E1039) *E. faecium* isolate at two different temperatures. Sequence analysis of pilin gene cluster-1 of the E1039 and E1165 isolates demonstrated that this gene cluster was highly conserved. Western blotting and transmission immunoelectron microscopy revealed that the PilA, PilE and PilF pilin proteins were expressed as cell wall-anchored proteins in the non-clinical E1039 *E. faecium* isolate at 37°C and were up-regulated at 21°C. In E1039 cells grown to early exponential phase, these proteins were expressed around the septum. In the clinical E1165 strain, PilA and PilF assembled into high molecular weight pilus-like structures at 37°C, whereas PilE was not expressed at both temperatures. At 21°C, PilA and PilF were expressed as cell wall-anchored proteins. Double labelling electron microscopy revealed that PilA forms the pilus backbone, and PilF the minor subunit which was distributed along the PilA pilus shaft and positioned at the tip. PilF was occasionally exclusively positioned at the PilA pilus tip, and at elevated concentration at the pilus base, suggesting that PilF may initiate and terminate PilA pilus assembly. The differential expression of surface proteins from the pilus gene cluster-1 and PilA pilus assembly indicates complex regulatory mechanisms involved in pilus biogenesis in *E. faecium*.

INTRODUCTION.

Enterococci are antimicrobial-resistant opportunistic pathogens causing healthcare-associated infections (31,45). Both *Enterococcus faecium* and *Enterococcus faecalis* have rapidly emerged in hospitals around the world and are responsible for infections in the immunocomprimized host (15,30). Nowadays, enterococci rank third among pathogenic organisms as reported by the National Healthcare Safety Network (15). *E. faecium* isolates associated with hospital outbreaks and associated with clinical sites of infections are frequently high-level resistant to ampicillin and ciprofloxacin and can be distinguished from non-hospital acquired isolates based on their gene content (22,23,46). Hospital acquired *E. faecium* isolates are enriched in genes encoding LPXTG-like cell wall-anchored surface proteins (14), including the gene encoding the enterococcal surface protein Esp (21), a collagen and fibrinogen binding MSCRAMM (microbial surface component recognizing adhesive matrix molecules) called EcbA, a nidogen-adhesin designated SgrA (this thesis), and also LPXTG surface protein genes located near one or more predicted sortase genes in pilin gene clusters (PGC) (13). Recent *in silico* analysis revealed the presence of four PGCs in *E. faecium*, and specific sera directed against two putative major pilin subunits, designated PilA and PilB encoded from PGC-1 and -3, respectively,

reacted with two distinct pilus-like structures on the surface of an infection-derived *E. faecium* isolate (13).

Pili, also referred to as fimbriae, are hair-like proteinaceous filaments protruding from the bacterial cell wall and are implicated in virulence in several Gram-positive pathogens (1,7,8,32,33). In contrast to Gram-negative organisms, pili of Gram-positives are covalently-joined polymers which are tightly immobilized to the peptidoglycan wall (26,43). The prototype heterotrimeric pilus is composed of a major subunit which forms the pilus backbone, a minor pilin subunit distributed along the pilus shaft, and an adhesive tip protein. The putative *E. faecium* major pilin subunits contain an N-terminal signal sequence for initiation of Sec-mediated translocation across the plasma membrane, an HLYPKN pilin motif, an ETxAPExY E box motif and a C-terminal cell wall sorting signal (CWS). The CWS is comprised of an LPXTG-like sortase substrate sequence, a hydrophobic domain followed by positively charged amino acids (36,37,43). Conservation of these sequence elements in putative major pilin subunits of *E. faecium* and in pilin subunits from other Gram-positive bacteria, suggest an universal mechanism of pilus assembly which may also be applied to enterococci. Putatively, a pilus-dedicated class C sortase catalyzes the cleavage of the LPXTG-like motif and mediates fibre assembly, which involves the HLYPKN-like pilin motif with a lysine (K) residue required for pilus polymerization and a conserved E box sequence with a glutamate (E) residue to incorporate minor pilin subunits. After polymerization, an housekeeping class A sortase anchors the pilus via the threonine residue of the LPXTG motif of the last minor pilin subunit to the amino group of the lipid II precursor within the cell wall peptidoglycan (7,8,24,39-42).

To gain an insight in pilus assembly of *E. faecium*, we studied the PilA pilus biogenesis and differential expression of the surface proteins encoded on PGC-1, which are implicated in PilA-type fibre polymerization, at the surface of a clinical and a non-clinical *E. faecium* isolate grown at 21°C and 37°C. We further analyzed the distribution of these pilin proteins at the surface of the cell.

(Part of this study was presented as a poster at the 109th General Meeting of the American Society for Microbiology, Philadelphia, PA, May 17 to 21, 2009 [abstr. 09-GM-A-2001-ASM]).

MATERIALS AND METHODS.

Bacterial strains and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. *E. faecium* strain E1162 and E1165 are clinical isolates, harvested from bloodstream and wound infections, respectively, whereas E1360 and E1391 are clinical isolates with an undetermined isolation site. Strains E128, E1039 and E1133 are faecal isolates from healthy human volunteers. *E. faecium* strains were grown aerobically at 21°C or 37°C on Trypticase Soy Agar II supplemented with 5% sheep blood (Becton Dickinson, Alphen aan den Rijn, The Netherlands) plates (TSA) or in Brain Heart Infusion (BHI) broth at 37°C, unless stated otherwise. When appropriate, the antibiotics chloramphenicol and gentamicin were used in concentrations of 15 to 34 μg/ml, and 125 μg/ml, respectively. Antibiotics were obtained from Sigma-Aldrich (Saint Louis, MO, USA). The oligonucleotides used in this study are listed in table 2.

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics[a]	Reference
E. coli		
DH5α	*E. coli* host strain for routine cloning	(10)
E. faecium		
E128	Community surveillance isolate from faeces, *pilA* and *pilF* negative;	This study
E1039	Community surveillance isolate from faeces, *pilA* and *pilF* positive;	This study
E1133	Community surveillance isolate from faeces, *pilA* and *pilF* positive;	This study
E1162	Clinical blood isolate, *pilA* and *pilF* positive, Chl^s Gen^s;	(12)
E1165	Clinical wound isolate, *pilA* and *pilF* positive, intermediate Chl^r Gen^s	(13)
E1360	Clinical isolate, undetermined isolation site, *pilA* and *pilF* positive;	This study
E1391	Clinical isolate, undetermined isolation site, *pilA* and *pilF* positive;	This study
E1674	Surveillance isolate from faeces, *pilA* and *pilF* positive.	This study

[a]Chl, chloramphenicol; Gen, gentamicin; ts, temperature sensitive; ^{s}, sensitive; ^{r}, resistant

TABLE 2. Oligonucleotides used in this study.

Primer name[a]	Oligonucleotide sequence (5'-3')
PilA_fw1	AACGAAATCCACGCTATTTG
PilA_fw2	TTTACGTATGAAGCTTTTAA
PilA_rv1	ATCAAGGAACGCAATTGAAT
PilA_rv2	GCACAGCTCAAAGCAAACAA
ddl_fw	GAGACATTGAATATGCCTTATG
ddl_rv	AAAAAGAAATCGCACCG

[a]Annotation: fw, forward and rv, reverse.

Identification of pilin gene cluster-1 (PGC-1) in *E. faecium* isolates.

Dot-blot analysis was performed on chromosomal DNA isolated from *E. faecium* E128, E1039, E1133, E1162, E1360, E1391 to determine the presence of the *pilA* and *pilF* genes from PGC-1. Chromosomal DNA isolation was performed as described previously (14). The chromosomal DNA was denaturated with 0.5 M NaOH in a 96 wells microtiter plate for 15 mins, transferred to a Biorad Bio dot apparatus, vacuum blotted onto an Hybond N^+ nylon membrane and fixed onto the membrane by incubation for 2 mins in 0.4 M NaOH followed by a neutralisation in 10x SSC for 1 min. Membranes were hybridized overnight at 42°C with 100 ng probe. Gene specific probes were amplified by PCR using primer pairs specific for *pilA* and *pilF* (Table 2). Amplified DNA probes were purified with a PCR purification kit (Qiagen Inc.) and

labelled according to the ECL nucleic acid labelling kit (GE Healthcare). The membranes were exposed to Hyperfilm ECL (GE Healthcare).

Anti-peptide polyclonal rabbit antisera.

Specific polyclonal rabbit antisera were prepared by Eurogentec (Seraing, Belgium) according to their 87-day anti-peptide polyclonal antibody protocol, by immunizing two specific pathogen free rabbits with a unique 15 amino acid residue keyhole limpet hemocyanin-conjugated peptides directed against the N-terminal region of PilA (Orf1904; H_2N-EDVTQKTPPEKVNITC-$CONH_2$), PilF (Orf1901; H_2N-CEKVIVDPSNPIQNVK-$CONH_2$) and PilE (Orf1902; H_2N-SSDETVDSSTVDSSEE-$CONH_2$) of the *E. faecium* TX0016 genome sequence. Rabbits were exsanguinated at day 87, the serum was collected and stored at -20°C.

Western immunoblot analysis of surface protein extracts.

To detect PilA, PilE and PilF pilin protein expression, E1165 and E1039 bacteria were grown at 21°C and 37°C on TSA agar and resuspended in PBS and harvested by centrifugation (1,560 × *g*, 5 min). To extract the surface proteome including pili, cell pellets were suspended in 70% formic acid and incubated at 65ºC for 30 mins, followed by trichloroacetic acid precipitation and acetone washing (13). Protein pellets were resuspended in 50 µl PBS + 50 µl sample buffer (100 mM Tris-HCl, 5% dithiotreitol, 2% SDS, 0,004% Broomphenol blue and 20% glycerol) and boiled for 5 mins. Equal amounts of protein sample of *E. faecium* E1039 and E1165 was analyzed by Western blotting as described previously (13,14).

FACS analysis.

Bacteria were collected from TSA agar plates and resuspended in RPMI 1640 (Cambrax Bioscience) containing 0.05% human serum albumin (HSA), to an OD_{660} of 1.0 ($1x10^9$ bacteria/ml). For each sample 100 µl ($1x10^7$ bacteria) was taken and pelleted by centrifugation (6,500 × *g*, 1 min.) and 50 µl RPMI/HSA was added containing 1:100 rabbit anti-PilA immune serum. Bacteria were resuspended and incubated on ice. After 30 min. 1ml cold RPMI/HSA was added and the bacteria were isolated by centrifugation (6,500 × *g*, 1 min.). Bacteria were resuspended in 50-µl RPMI/HSA containing 1:50 Goat anti-rabbit fluorescein isothiocyanate (Sigma-Aldrich, Saint Louis, MI) and incubated for 30 min. on ice. Bacteria were washed with 1ml cold RPMI/HSA, and after centrifugation (6,500 × *g*, 1 min.) bacteria were resuspended in 50 µl RPMI/HSA. Before analyses in the FACSCalibur (BD, Alphen aan den Rijn, The Netherlands), bacteria were resuspended in 300 µl RPMI/HSA. All measurements were performed in the same machine using the same parameters. The data were normalized for bacterial size, and experiments were performed two times. The mean fluorescence (mean FL1) was used as a measure for cell surface-associated PilA. Rabbit pre-immune serum and bacteria incubated without anti-PilA rabbit immune serum were used as negative controls.

Electron microscopy and immunogold labelling.

Transmission immunoelectron microscopy was performed as described previously with some modifications (13,14,44). In brief, copper grids (mesh Formvar-carbon coated) were incubated for 30 mins with carbon side on a drop of 1.10^9 CFU ml^{-1} of *E. faecium* E1039, E1162 or E1165 cells to detect surface exposed PilA, PilE, or PilF expression. Grids were washed 3 times for 5 mins on drops of 0.02 M glycine in PBS and subsequently blocked for 30 mins on drops of 1% BSA in PBS (PBSb). The PilA, PilE, and PilF

proteins were labelled for 1 hr on drops of undiluted or 1:100 diluted specific peptide rabbit immune sera or pre-immune sera in PBSb. Grids were washed 4 times for 2 mins on drops of 0.1% BSA in PBS. Surface protein-antibody complexes were labelled by incubation for 20 mins on drops with 1:70 diluted protein A-gold label (10 nm) in PBSb. Grids were washed four times for 2 mins on drops of PBS, fixed by incubation on drops of 1% glutaraldehyde in PBS and washed again 8 times for 2 mins on drops of H_2O. For double labelling experiments, the labelling was repeated similarly with 1:100 dilution of a second immune serum, and a 1:70 diluted protein A-gold label (5 nm). After fixation with 1% glutaraldehyde in PBS, bacteria were stained by incubation of the grids for 5 mins on drops containing 1.8% methylcellulose (25 centipoises; Sigma-Aldrich, Saint Louis, MO) and 0.4% uranyl acetate (pH=4) and subsequently air dried for 10 min. Grids were examined using a Jeol 1010 transmission electron microscope (Jeol Europe, Amsterdam, The Netherlands).

DNA Sequencing.

PCR products were sequenced by use of the BigDye Terminator 3.1 reaction kit and an ABI PRISM 3100 capillary DNA sequencer (both from Applied Biosystems, Foster City, CA).

RESULTS.

Characteristics of the proteins putatively encoded from pilin gene cluster-1 (PGC-1) of *E. faecium* E1165.

E. faecium PGC-1 of E1165 (Genbank accession number EU909697) contains six putative open reading frames (ORFs); *orf1901* (*pilF*), *orf1916*, *orf1902* (*pilE*), *orf1903* (*srt1903*), *orf1904* (*pilA*) and *orf1905* (*srt1905*) (Fig. 1). The *orf1901*, *orf1902* and *orf1904* gene products, designated PilF, PilE and PilA respectively, are predicted to encode cell wall-associated proteins with LPXTG-like sortase substrate motifs. The PilA, PilE and PilF are predicted to contain five, two and four N-glycosylation sites, respectively, suggesting that these putative pilin subunits may be glycosylated. PilA is the putative major pilin subunit containing an HLYPK pilin motif, and an ETxAPExY E box motif. The latter forms intra-molecular isopeptide bonds to stabilize the major subunit and to incorporate minor pilin subunits (19). PilA is possibly polymerized by the Srt1903 class C pilus-dedicated sortase enzyme, and anchored to the peptidoglycan by the Srt1905 class A housekeeping sortase. The small PilE pilin protein, was recently classified as a secreted protein (38). However, this protein contains an N-terminal signal sequence, six SS- and four SD-repeats distributed along the protein, and a putative deviant IVSTG cell wall-anchor motif followed by a hydrophobic transmembrane region and a positively charged tail, suggesting that PilE is a putative serine-aspartic acid rich LPXTG-like surface protein. The IVSTG motif of PilE was compared to the consensus [FILMPSVY][AP]X[ATLS][GAKNS] and NPX[ST][DGNS] patterns representing distinct sortase substrates (3,17). To date, these putative sortase substrate sequences do not allow the valine (V) on the second position of the IVSTG motif. Furthermore, PilE contains a putative YPKSE pilin motif.

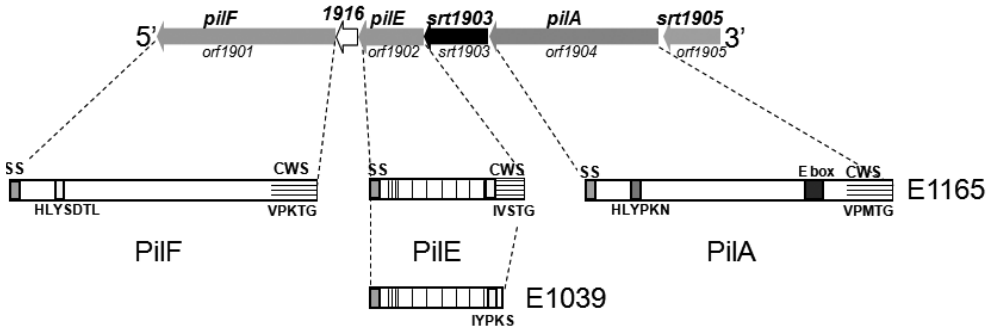

Fig. 1. Schematic representation of the genomic organization of PGC-1 of *E. faecium* E1165 (DDBJ/EMBL/Genbank accession number EU909697). The position and presumed direction of transcription of ORFs is indicated by arrows. The ORF number is depicted below the arrows, while the gene name is depicted in bold above the arrows. The blue arrows indicate minor pilin proteins and the white arrow indicate a gene with unknown function. The red arrow indicates the putative major pilin subunit. The black and green arrow indicates class C and class A sortase genes, respectively. The putative LPXTG-like surface proteins are depicted below the PGC-1. (SS) are depicted in green, the pilin and pilin-like motifs in red and yellow, respectively. The E box motif is in brown and the CWSs are depicted as a (horizontally) striped box. The repeats in PilE are depicted by vertical black lines. See appendix for color figure.

The PilF protein contains in addition to all characteristics of a Gram-positive surface protein also a variant of the pilin motif, designated HLYSDTL. This motif lacks a lysine (K), which appeared essential for pilus polymerization in *Corynebacterium diphtheriae* (42), suggesting that PilF cannot be polymerized into high molecular weight structures by a class C sortase. The *orf1916* open reading frame putatively encodes a small 6.8 kDa protein, containing an N-terminal signal peptide, but not an cell wall anchor, and is possibly a secreted hydrophobic polypeptide.

PilA is expressed as a cell wall-anchored protein in various *E. faecium* isolates.

Recently, we showed that PilA-type pili were expressed on the surface of a clinical *E. faecium* isolate E1165 (13). To analyse PilA expression on the surfaces of cells of other *E. faecium* isolates, three clinical (E1162, E1360, and E1391) and four non-clinical (E128, E1133, E1039, and E1674) isolates were selected and PCR and DNA-DNA hybridizations confirmed the presence of *pilA* in these strains, except in E128, which was used as a negative control. Subsequently, these seven isolates were grown on TSA plates at 37°C, incubated with anti-PilA polyclonal rabbit serum or pre-immune rabbit serum and assayed by flow cytometry. Surface exposure of PilA was demonstrated in five *pilA* positive strains, with PilA expression levels varying among the isolates analysed, and with the highest level at the surface of E1039 (Fig. 2). Strain E1360 displayed low fluoresence, comparable to the *pilA* deficient strain (E128), suggesting that PilA is not expressed in this isolate. The flow cytometry analyses were confirmed by transmission electron microscopy (TEM) on

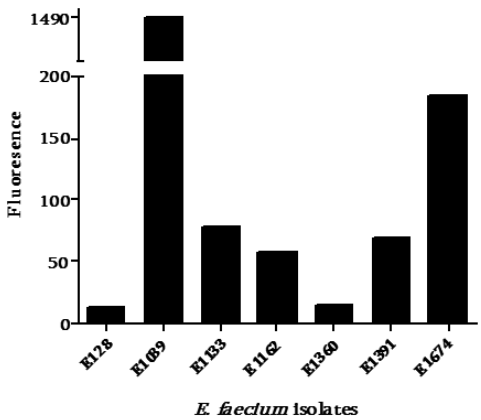

Figure 2. Flow cytometry analysis of expression of PilA at the surface of *E. faecium* strains. Three clinical (E1162, E1360, and E1391) and three non-clinical (E128 [negative control], E1133 and E1039) isolates were grown on TSA at 37ºC at aerobic conditions to analyse PilA expression by flow cytometry. Shown are the means of the mean fluoresence of two independent experiments.

negatively stained and anti-PilA immunogold labelled bacteria. Unexpectedly, gold particles were clearly associated with the bacterial cell wall at different levels, but not associated with pilus-like structures, suggesting differential PilA pilus assembly among *E. faecium* isolates (data not shown). Expression of PilA at the surface of E1039 was the highest compared to the other isolates, and was therefore subjected to further analyses in this study.

Comparison of the PGC-1 sequence of E1039 and E1165 reveals heterogeneity in *orf1916* and *pilE*.

The sequence of the PGC-1 of *E. faecium* strain E1039 (van Schaik *et al.*, unpublished data), which did not assemble PilA pili, was compared to the recently sequenced PGC-1 of *E. faecium* E1165, a strain which expresses PilA pili. In general, the proteins putatively encoded from the ORFs from PGC-1 of both E1039 and E1165 exhibit very high overall similarity of >98% (Table 3). The organization and transcriptional direction of the ORFs in this PGC were similar, demonstrating that PGC-1 is conserved among *E. faecium* E1039 and E1165. The sortase genes *srt1903* and *srt1905* of E1039 and E1165 did not contain mutations leading to amino acid changes in the active site amino acid residues or the TLxTC catalytic pocket (16,27).

TABLE 3. Similarities between the proteins of PGC-1 of *E. faecium* E1039 and E1165.

Protein	Putative function	% Amino acid identity	% Similarity
Srt1905	Class A housekeeping sortase	98.65	99.09
PilA	Major pilin subunit	98.94	99.35
Srt1903	Class C pilus-dedicated sortase	98.80	99.18
PilE	PilF linker protein	97.84	98.42
Orf1916	Secreted peptide	97.67	98.37
PilF	Minor pilin subunit	99.28	99.55

Furthermore, conserved elements required for pilus assembly, such as the CWS of the putative minor pilin protein PilF, and the HLYPKN pilin motif, the E box sequence and the CWS of the major PilA pilin subunit among the E1039 and E1165 strains were identical. The largest sequence heterogeneity in PGC-1 of both isolates occurs in the putative PilE and Orf1916 proteins. PilE of E1039 did not contain a CWS, but a C-terminal IYPKS sequence, which resembles a pilin motif (Fig. 1). Orf1916 contained two amino acid substitutions in the hydrophobic polypeptide.

Differential PilA, PilE and PilF expression in *E. faecium* E1039 and E1165.

To analyze the expression profile of PilA, PilE and PilF LPXTG-like proteins in *E. faecium* strains E1039 and E1165, grown at different temperatures, western blotting was performed on surface protein extracts of cells grown on TSA plates at 21ºC and 37ºC. This confirmed our previous results that PilA assembles into high molecular weight pilus-like structures in E1165 at 37°C, whereas the monomeric subunit was expressed at predicted molecular weight of 64.8 kDa at the surface when cells were grown at 21°C. In contrast, in *E. faecium* E1039, PilA did not assemble into high molecular weight pili but formed a putative dimer, and expression levels were higher at 21°C than at 37°C (Fig. 3A). In addition, anti-PilA serum also reacted with slightly larger protein bands of ~75 and ~70 kDa in E1039 and E1165 grown at 21°C, respectively, suggesting covalent association of another molecule to PilA.

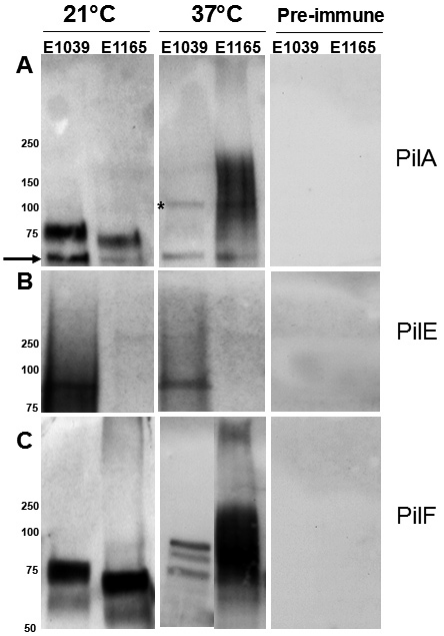

Figure 3. Western blots of surface protein extracts of *E. faecium* E1039 and E1165 grown on TSA plates at 21ºC and 37ºC. (A) Blots were probed with rabbit anti-PilA, (B) rabbit anti-PilE and (C) rabbit anti-PilF immune sera (left part) or with the respective pre-immune sera (right part) followed by Goat anti-rabbit IgG-HRP conjugate. Numbers depicted on the left indicate molecular weights in kilo Daltons (kDa). Predicted monomeric PilA (64.8 kDa) is indicated by an arrow, and the PilA dimer with an asterix.

Anti-PilE serum reacted with a ~90 kDa protein band in surface protein extracts of E1039 at 21°C, and at lower levels at 37°C, while the PilE protein was not detected in cell wall extracts of E1165 at both temperatures (Fig. 3B). The observed molecular weight of PilE did not correlate with the predicted molecular weight of 22.8 kDa (after post-translational modification), suggesting a covalent association of the PilE protein to another protein. In strain E1039, the anti-PilF sera reacted with three bands of ~75, ~90 and ~100 kDa at 37°C, respectively, and with one predominant protein band of ~75 kDa at 21°C. Anti-PilF serum reacted with a smear of protein reactive material in E1165 which ranged into the stacking gel, suggesting that the PilF pilin protein is associated with pili (Fig. 3C). The monomeric PilF subunit was expressed at predicted molecular weight of 70.4 kDa (after post-translational modification) at 21°C. The fact that PilF protein of E1039 migrated slower through reducing SDS-PAGE than the predicted molecular weight, suggest posttranslational modification of PilF. Pre-immune sera were used as negative controls and did not react with any of the protein products.

Expression of PilA, PilE and PilF LPXTG-like pilin proteins at the surface of *E. faecium* E1039 at different environmental conditions.

To confirm expression of the PilA, PilE and PilF proteins at the surface of *E. faecium* E1039, TEM was performed on negatively stained and immunogold labeled E1039 bacteria grown on TSA plates at 21°C and 37°C. Using specific peptide antisera directed against PilA, PilE and PilF, gold particles were associated with the cell wall of strain E1039 at 21°C (Fig. 4A, C, E) and at lower levels at 37°C (Fig. 4B, D, F). The PilA, PilE and PilF proteins were equally distributed in high densities at the surface of the cells. Although the PilE protein in E1039 lacks the deviant IVSTG putative sortase substrate motif, the protein is associated with the cell wall of E1039. No gold particles were associated with the cell wall when cells were incubated with the individual rabbit pre-immune sera (Fig. 4G) or when immune sera were omitted (conjugate control; protein A-gold) (Fig. 4H).

Since the DNA sequences of PGC-1 of E1039 and E1165 were highly similar, we hypothesized that particular environmental conditions relevant for the *in vivo* lifestyle of *E. faecium* may induce PilA pilus assembly in the non-hospital acquired E1039 isolate. Therefore, E1039 and E1165 strains were grown under anaerobic conditions on TSA and BHI agar at 37°C, at aerobic conditions on BHI at 37°C, on TSA and BHI agar at the elevated temperature of 42°C, and in filter sterilized human male urine at 37°C. The E1039 and E1165 cells grown under these different conditions were harvested and subjected to TEM analysis using anti-PilA immune serum. Strain E1165 expressed PilA-type of pili at the surface during all the different growth conditions, whereas in strain E1039, anti-PilA immune serum labelled PilA at the surface, but PilA pilus assembly was not induced (data not shown).

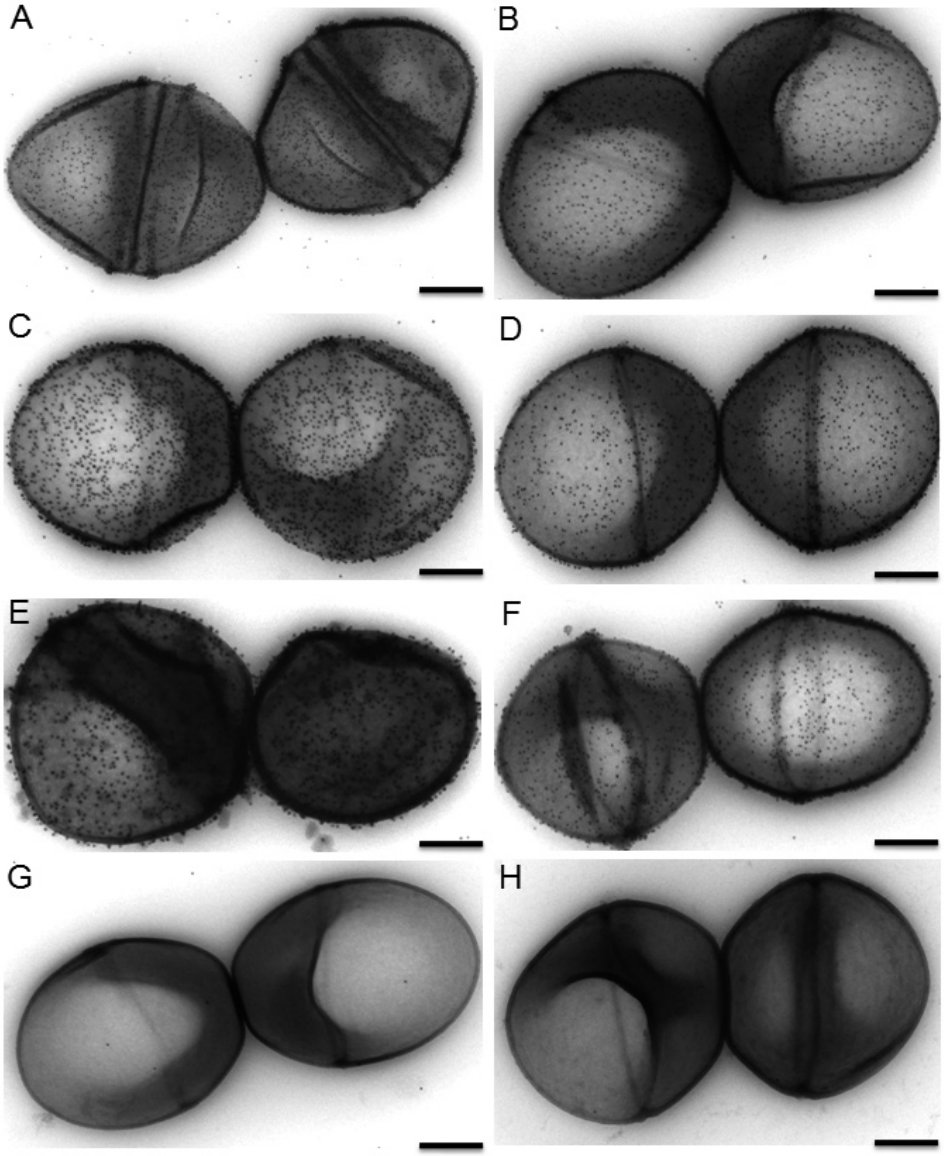

Figure 4. Transmission electron microscope micrographs. *E. faecium* E1039 cells were grown at 21°C and 37°C and labelled individually with rabbit anti-PilA, -PilE or -PilF immune serum, followed by protein A-gold (10nm) and negatively stained. Panel A indicates *E. faecium* E1039 cells grown at 21°C incubated with anti-PilA immune serum, (B) cells grown at 37°C incubated with anti-PilA immune serum (C) E1039 cells grown at 21°C incubated with anti-PilE immune serum, (D) E1039 cells grown at 37°C incubated with anti-PilE immune serum (E) E1039 cells grown at 21°C incubated with anti-PilF immune serum, (F) E1039 cells grown at 37°C incubated with anti-PilE immune serum. (G) Representative micrograph of E1039 cells incubated with rabbit pre-immune serum followed by protein A-gold (10nm). (H) E1039 cells incubated with only protein A-gold (conjugate control). Bars = 200 nm.

PilF, but not PilE, is associated with pili in *E. faecium* E1165.

To corroborate whether the PilE and PilF pilin proteins are associated with the cell wall or with pili, respectively, single labelling TEM experiments were performed on *E. faecium* E1165 cells grown on TSA plates at 21°C and 37°C. In the TEM experiments, cells were labelled with either rabbit anti-PilE or anti-PilF immune serum, followed by protein A-gold (10 nm). In contrast to E1039, no gold particles bound to the cell wall or to pili of E1165 when cells were grown at both temperatures and incubated with anti-PilE serum (data not shown). Using anti-PilF serum, gold particles were either equally distributed along pilus shafts at 37°C (Fig. 5A), associated with pilus tips (Fig. 5B) and with the base of the pilus (Fig. 5C).

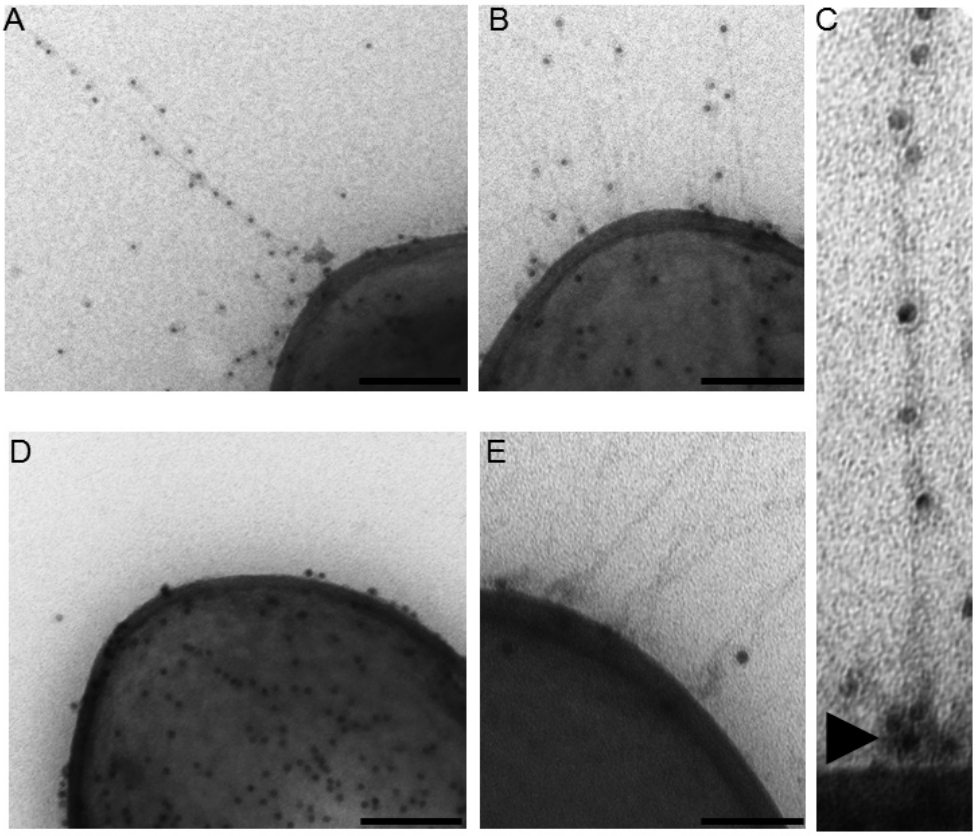

Figure 5. Transmission electron microscope micrographs of PilF associated with pili of *E. faecium* E1165. *E. faecium* E1165 cells were grown at 21°C and 37°C and labelled individually with rabbit anti-PilF immune serum, followed by protein A-gold (10nm) and negatively stained. (A) Depicted is a detail of *E. faecium* E1165 cells grown at 37°C, showing that PilF is associated along a thin pilus of 1µm or (B) that PilF is exclusively positioned at the tip of pilus-like structures. (C) Detail of a pilus, showing that PilF is positioned at the tip, along the pilus shaft, and present in elevated concentration at the base of the pilus (arrow). (D) *E. faecium* E1165 cells grown at 21°C, showing that PilF is associated at the cell wall. (E) *E. faecium* E1165 cells incubated with rabbit pre-immune serum followed by protein A-gold (10nm). Bars = 200 nm.

Association of PilF with pili was observed in ~40% of the cells, and cells without pili expressed cell wall-associated PilF or did not express PilF at all. In line with the western blotting results, PilF was associated with the cell wall at 21°C (Fig. 5D) and did not assemble into pili. Rabbit pre-immune serum did not react with pilus-like structures or with the cell wall (Fig. 5E).

PilF is assembled into PilA-type pili of *E. faecium*.

To investigate whether the PilF pilin subunit was associated with PilA-type pili, a double labelling TEM experiment was performed. *E. faecium* E1165 cells were grown at 37°C and initially labelled with rabbit anti-PilA immune serum, followed by protein A-gold (5 nm), fixed, and subsequently incubated with anti-PilF serum followed by protein A-gold (10 nm). TEM analysis confirmed that both PilA and PilF immune sera bound to the same type of pili. We demonstrated that in PilA-type pili, PilA forms the pilus backbone and PilF the pilus tip. PilF was also found equally distributed along the PilA pilus shaft (Fig. 6A), suggesting that PilF has a dual function. Peculiarly, multiple (typically 3 to 5 gold particles) PilF pilin subunits were also positioned at the base of some PilA pili (Fig. 6B).

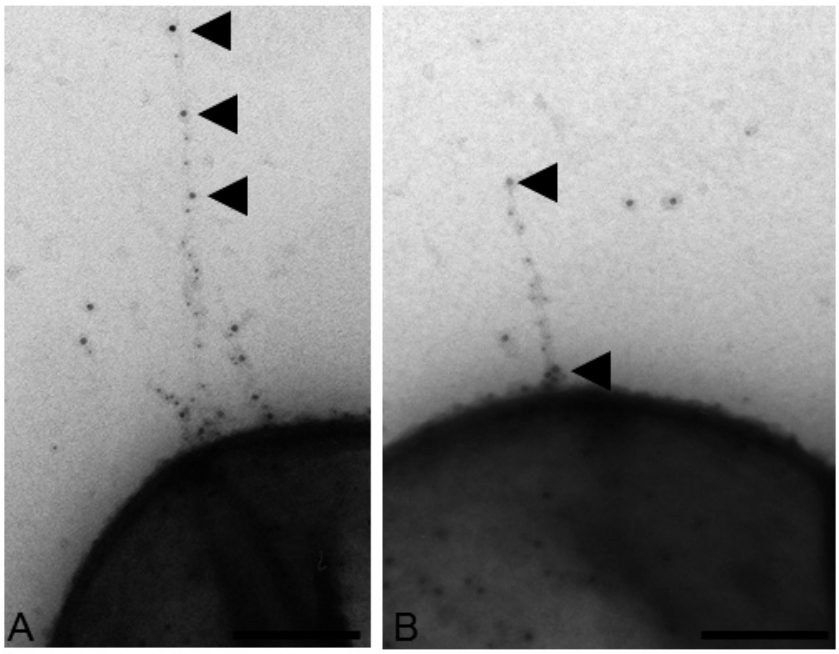

Figure 6. Transmission electron microscope micrograph of PilF associated with PilA-type pili. *E. faecium* E1165 was grown at 37°C labelled with rabbit anti-PilA immune serum, followed by protein A-gold (5 nm particles), fixed and consecutively labelled using anti-PilF immune serum, followed by protein A-gold (10 nm particles). Panel A shows distribution of PilF along the PilA pilus shaft. (B) PilF is positioned at the PilA pilus tip and at the pilus base. PilF is indicated by black arrows. Bar = 100 nm.

Distribution of the PilA, PilE, and PilF proteins at the surface in early stage of growth.

We previously described expression of PilA and PilB pilus subunits close to the septum in the centre of dividing E1165 cells, when cells were grown to early exponential phase (13). To investigate whether this was also true for PilA, PilE and PilF, we analysed the distribution of these pilin proteins of PGC-1 at the surface of strain E1039. The E1039 strain was chosen for this analysis, as this strain exhibits high level of pilin protein expression at the surface. The *E. faecium* E1039 cells were harvested from early-exponential phase of growth and labeled using anti-PilA, –PilE, and –PilF sera, and subsequently subjected to TEM analysis. Using these four sera, gold particles were associated with the cell wall of strain E1039. The PilA, PilE and PilF pilin subunits were expressed with the highest densities on the surface

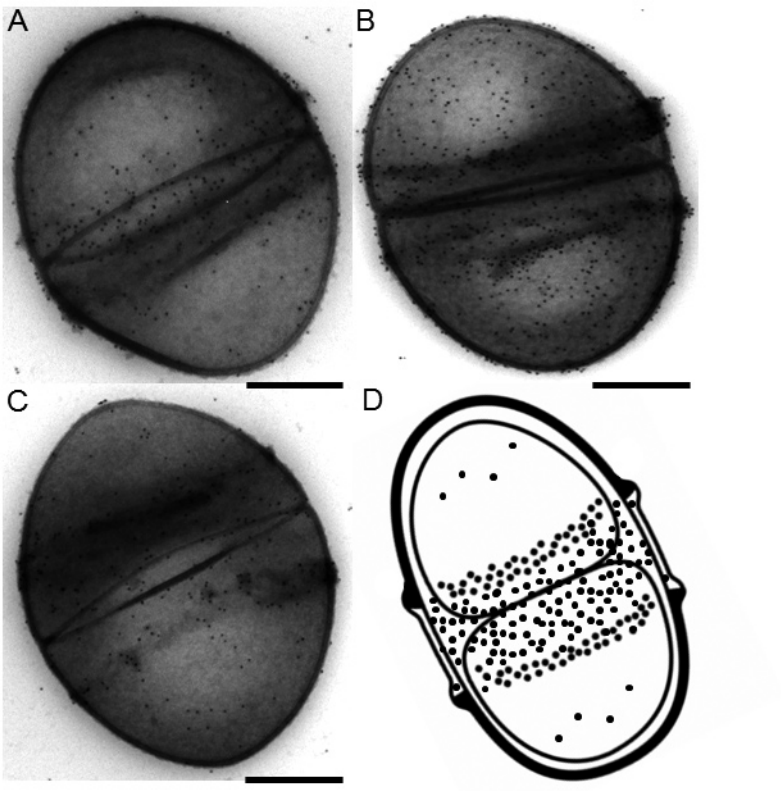

Figure 7. Transmission electron microscope micrographs of PilA, PilE and PilF expression in early-exponential phase of growth. In early-exponential phase of growth in BHI broth at 37°C, *E. faecium* E1039 cells were harvested and labelled with anti-PilA, -PilE, or -PilF immune serum followed by protein A-gold (10 nm) and negative staining. Cells harvested from an early exponential phase culture display PilA major pilin protein expression around the septum (A), PilE is expressed in highest densities around the septum (B), and similarly for the PilF minor pilin protein (C). Panel D displays a schematic representation of an *E. faecium* cell, expressing pilin proteins around the septum, the site at which *de novo* peptidoglycan synthesis occurs (depicted in white). Bars, 200 nm.

close to the septum in the centre of a dividing cell (Fig. 7A, B, C), the site at which newly synthesized peptidoglycan pushes previously produced peptidoglycan from the septum to the poles, which is schematically depicted in Fig. 7D.

DISCUSSION.

We have demonstrated the differential expression of PilA-type pili at the surface of a clinical and a non-clinical *E. faecium* isolate grown at 21°C and 37°C, which provides the first insights in the complexity of PilA pilus assembly and expression in *E. faecium*. Pili are proteinaceous surface filaments of covalently linked subunits and implicated in adhesion to biotic and abiotic material and showed to contribute to the pathogenesis of several Gram-positive bacteria (1,25,33), including *E. faecalis* (32).

Double labelling TEM analysis of the wild-type E1165 showed that the PilA protein is the major component of PilA-type pili and thus forms the pilus backbone. TEM analyses also showed that the PilF protein was found in increased concentrations at the pilus base, is equally distributed along the pilus shaft and is associated with the PilA pilus tip in the clinical *E. faecium* E1165 strain at 37°C. PilF is therefore designated as the minor pilin subunit, which may represent an adhesin that binds to biotic or abiotic surfaces. The fact that PilF was occasionally found to be exclusively positioned at the pilus tip, and in some instances in increased concentrations at the pilus base close to the cell wall, suggests that PilF may initiate and terminate PilA-type pilus polymerization. Budzik *et al.* demonstrated that the sorting signal IPNTG of the BcpB minor pilin subunit of *Bacillus cereus* is specifically cleaved by the pilus-dedicated sortase and not by the housekeeping sortase, thereby initiating polymerization (4). The sortase-specific cleavage of the minor pilin protein determines that BcpB is positioned at the BcpA pilus tip. Initiation of PilA pilus polymerization may be more complex in *E. faecium.* The fact that both the PilA and PilF proteins harbor VPXTG sorting motifs, suggests that another signal, in addition to the sorting substrate motif which determines sortase specificity, is required to initiate pilus polymerization.

Recently, Ton-That and colleagues revealed that the minor pilin subunit (SpaB) is required to stop SpaA-type pilus polymerization of *C. diphtheriae* and to anchor pili polymers to the cell wall through incorporation of the minor pilin subunit in the base of the growing pilus shaft (24). Our observation of accumulation of PilF at the base of PilA pili might indicate that PilF is required to terminate pilus polymerization. The fact that not all PilA fibres contained PilF at the base of the filament, may suggest that for several PilA pili, pilus assembly was not terminated upon labelling of the pili. These data imply a dual role for PilF in PilA pili biogenesis: initiation and termination of pilus assembly.

The PilE protein was not associated with PilA-type pili or expressed at the cell wall of *E. faecium* E1165 at 21°C or 37°C. Furthermore, the PilA and PilF proteins

were associated with the E1165 cell wall at 21°C and assembled into high molecular weight pilin species at 37°C. In contrast, the PilA, PilE and PilF pilin proteins are cell wall-anchored proteins at the surface of the non-clinical *E. faecium* isolate E1039 at 37°C, and all three proteins were up-regulated at 21°C. Thus, in the non-clinical isolate pilus assembly was not only abrogated, but the level of surface protein expression was also differentially regulated when compared to strain E1165. To date, the E1165 strain is the only isolate that we analyzed that assembles PilA-type pili at the surface and we have not identified the environmental cues leading to PilA pilus assembly in the E1039 strain or in the other tested *E. faecium* isolates. Which environmental conditions relevant for the lifestyle of *E. faecium* induce PilA pilus polymerization, such as growth in microbial networks in the gastrointestinal tract or in bile or blood, or growth under fluctuating pH or carbon dioxide concentrations, remains to be elucidated.

PilE, a small protein lacking the deviant IVSTG motif in E1039 migrated slowly through reducing SDS-PAGE, which suggest a covalent association of PilE to another protein. Possibly, PilE is associated with PilF in E1039 as multiple protein bands were observed when this strain was analyzed for PilF expression. PilE is a serine-glutamic acid rich protein and the SD-repeats or serine-rich repeat domains of multiple MSCRAMMs are predicted to adopt a long coiled structure to expose the ligand-binding domain to the extracellular environment (5,11). The presence of SD-repeats and serine-rich repeats in PilE suggest that this protein may be a linker protein to expose PilF at the surface in *E. faecium* E1039. Since cell surface exposure of PilE correlates with absence of assembled pili in E1039, we speculate that surface expression of PilE, possibly via an interaction with PilF to form a hetero PilE-PilF dimer, abrogates pilus biogenesis. To date, attempts to construct an isogenic *pilF* mutant using the pTEX5500ts vector failed (data not shown), thus whether PilE is associated to PilF and whether the IYPKS pilin-like motif of PilE mediates such an interaction with PilF remains to be elucidated.

The PilA, PilE and PilF pilin proteins in E1039 and 1165 at 21°C did not migrate at the predicted mass calculated from their sequences. Possibly, one or multiple copies of the small (6.8 kDa) Orf1916 protein, a putative secreted hydrophobic polypeptide, may be associated to these pilin proteins. The finding that the PilA, PilE and PilF proteins contain multiple predicted N-glycosylation sites, suggest that these pilins are posttranslationally modified by glycans. Modification by glycosylation of bacterial and archaeal flagellins and bacterial pilins has been described previously and aberrant migration of these glycosylated proteins through SDS-PAGE appeared to be a common finding (34,35).

The PilA, PilE and PilF pilin proteins are expressed at the septum of cells grown to early-exponential phase. At this site, *de novo* peptidoglycan synthesis occurs to separate the cell in parallel planes, perpendicular to their long axis, into two daughter cells (47). We recently reported a similar finding for the putative PilB major pilin subunit of *E. faecium*. This suggests that the *E. faecium* PilA, PilE, and PilF pilin

subunits, and in addition the PilB protein, are likely translocated and incorporated at the septum into newly synthesized peptidoglycan. We propose that the class A housekeeping sortases of *E. faecium* (designated *srt1905* and *srt2128*) may be located at this cell division site around the septum as well, to covalently immobilize these surface proteins to the peptidoglycan. Whether putative conserved motifs in *E. faecium* signal peptides or a C-terminally located positively charged domain identified in *Staphylococcus aureus* and *E. faecalis* surface proteins, respectively, target these pilin proteins to the septum and other potential LPXTG-type surface proteins to different locations in the cell envelope is currently under investigation (6,20).

E. faecium has multiple intrinsic and acquired resistances to the three commonly used classes of antimicrobials, i.e. the aminoglycosides, β-lactams, and glycopeptides (45). Treatment of these infections will become even more challenging, when resistance against antibiotics from other, recently introduced classes of antimicrobials, such as daptomycin and linezolid emerges (2,18,28). Therefore, novel alternative treatment and prevention strategies such as passive or active immunization are urgently required. Gram-positive pilin proteins are promising novel targets for immune therapy (9,29). Although PilA, PilE, and PilF are differentially expressed and assembled among clinical relevant and non-relevant strains, these pilin proteins may be novel vaccine candidates.

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Chapter 7

General Discussion and Future Perspectives for *E. faecium* Surface Protein Research

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Summarizing Discussion.

Enterococcus faecium was considered to be a harmless member of the mammalian gastrointestinal flora, but has rapidly emerged as a multi-resistant opportunistic pathogen in hospitals around the world where it is responsible for urinary tract infections, surgical site infections, and bacteremia[1,2]. Nowadays, *E. faecium* ranks third among Gram-positive pathogens causing life-threatening hospital-associated infections, which are associated with increased morbidity, higher mortality and rising healthcare costs[3]. Previous studies on the molecular epidemiology and population structure of *E. faecium*, suggested the existence of a genetic lineage, often referred to as clonal complex-17 (CC17), hospital clade or hospital acquired (HA-) *E. faecium* that included clones capable of endemic and epidemic spread within and between hospitals around the world that are distinct from non-HA isolates based on gene content[4,5]. The HA-*E. faecium* isolates have multiple intrinsic and acquired resistances to aminoglycosides, β-lactams, and glycopeptides[2,3,6]. Therefore, treatment of infections caused by these bacteria has become increasingly challenging, especially since resistance for recent newly developed and introduced antibiotics has already been reported[3,7-9]. It is, therefore, warranted to design novel treatment strategies, such as surface protein-based passive or active immunization, in order to counter the global threat of multi-resistant HA-*E. faecium*.

Despite our increase in knowledge on differences in the genetic repertoire between HA- and non-HA-*E. faecium,* functional characterization of genes and proteins enriched in hospital clones is still greatly lacking. In this thesis, we focussed on cell wall-anchored surface proteins and pilus-like structures since surface protein-mediated adherence to host tissues and subsequent colonization of mucosal surfaces represents an initial step in the onset of infection and is a prerequisite for pathogenesis. Deciphering the role that surface proteins of *E. faecium* play during the infectious process and subsequent interference in this interaction, will lead to novel insights into how these antibiotic-resistant bacteria initiate disease and may reveal novel therapeutic options to treat HA-*E. faecium* infections.

This thesis describes the first exploration, *i.e.* the identification, the initial characterization and expression analysis of surface-exposed LPXTG cell wall-anchored proteins and pilus-like structures of HA-*E. faecium* that potentially have contributed to the enhanced pathogenicity and relative fitness of *E. faecium* strains of clinical origin. These studies also provide novel insights in the LPXTG surface protein heterogeneity, and pilus assembly mechanisms in Gram-positive bacteria.

In **chapter 2**, we describe that Esp is expressed at the surface of HA-*E. faecium* and we were the first to show that *E. faecium* senses and responds to environmental changes[10]. Levels of surface-exposed Esp were up-regulated at anaerobic conditions, compared to aerobic conditions, or when the temperature was shifted from 21°C to 37°C. Growth temperature- and oxygen-dependent expression of Esp is possibly a niche dependent adaptation mechanism of HA-*E. faecium* suggesting a role of Esp in the early stages of HA-*E. faecium* colonization and infection. *E. faecium* is a facultative

anaerobic bacterium, and adapting its surface protein composition or amount of surface-exposed proteins likely allows alternation between environmental reservoirs and its host. Furthermore, we provided the first evidence that levels of cell wall-anchored Esp correlated in a dose-dependent manner with initial adherence to polystyrene and biofilm formation which could be competitively inhibited by soluble exogenous recombinant N-terminal Esp, indicating a role for this domain in initial adherence and biofilm formation. Recently, due to the availability of a temperature sensitive suicide vector, insertional inactivation of the *esp* gene in a clinical HA-*E. faecium* isolate confirmed our initial findings that Esp plays an important role in initial adherence and biofilm formation[11,12].

Microbial biofilms are concentrated populations of microorganisms typically surrounded by an extracellular polymeric substance matrix. Biofilms develop to three-dimensional structures according to several subsequent steps: (1) primary attachment to a particular surface, (2) accumulation, (3) maturation and (4) dispersal. Biofilm formation is considered to be an important pathogenic property of enterococci, as it facilitates colonization on indwelling medical devices such as ureteral stents, intravascular catheters, biliary stents and silicone gastrostomy devices[13-16]. Furthermore, these microbial communities are relatively less permeable for antimicrobial agents, thereby hampering antibiotic treatment[17].

To gain further insights in characteristics that may have favored the emergence of HA-*E. faecium* we aimed to identify novel actively expressed LPXTG surface proteins that were specifically enriched in clinical and outbreak associated *E. faecium* isolates[18]. In **chapter 3** we describe the investigation of LPXTG-type surface protein genes in HA- and non-HA-*E. faecium* isolates by screening 131 *E. faecium* isolates for the presence of 22 putative surface protein genes identified from the *E. faecium* TX0016 genome. Clinical and outbreak associated *E. faecium* isolates contained on average 19 and 21 of 22 putative cell wall-anchored protein encoding genes, whereas non-HA-*E. faecium* strains, such as animal, human community and environmental isolates contained on average only 13, 14 and 15 of the 22 analyzed putative cell wall-anchored protein genes, respectively. PCR and Southern hybridization revealed that five LPXTG surface protein genes, designated as *sgrA*, *ecbA, orf903*, *orf904.5*, and *orf906.7*, were specifically enriched in HA-*E. faecium* isolates, were actively expressed at the mRNA level, and expressed at the surface of the cell. The enrichment of the analyzed LPXTG surface protein genes in HA-*E. faecium* isolates was striking. Sequence homology of the five surface proteins suggests that these genes encode factors involved in bacterial adhesion and biofilm formation and, therefore, may play a role in the pathogenesis of HA-*E. faecium* infections. The presence of variant numbers and combinations of surface protein genes per distinct epidemiological class may have contributed to successful adaptation to different ecological niches and may have enhanced fitness of these isolates in the hospital setting. Possibly, the set of LPXTG surface proteins displayed at the surface of specific *E. faecium* clones determines the fate and success of initial colonization of the niche the bacterium

encounters.

SgrA and EcbA are two of the five LPXTG surface proteins that were specifically enriched in HA-*E. faecium* isolates, and these were functionally characterized to elucidate their functions (**chapter 4**). SgrA is the first characterized cell wall-anchored LPXTG surface adhesin of HA-*E. faecium*, which lacks IgG-like folds in the putative ligand-binding domain, and binds to the α- and β-chains of fibrinogen and two homologues proteins designated as nidogen-1 and nidogen-2[19-21]. We were the first to identify a bacterial cell surface adhesin that binds to these ligands from the basal membrane. In line with these results, wild-type *E. faecium* E1162 cells bound to fibrinogen, nidogen-1 and nidogen-2, whereas an isogenic mutant E1162Δ*sgrA* strain showed reduced binding to these components of the extracellular matrix. It was further demonstrated that SgrA, like Esp, was implicated in biofilm formation on a polystyrene surface, while it did not mediate adherence to human intestinal epithelial cells, human bladder cells and kidney cells. SgrA is predicted to have a dual role in the infective process: attachment to abiotic surfaces, such as cathethers and stents, followed by binding to fibrinogen and the nidogens deposited on these devices and subsequently to form a biofilm.

EcbA is, like SgrA, also a novel marker for HA-*E. faecium*, and is an MSCRAMM that contains four IgG-like folds in the putative ligand-binding domain and binds to the γ-chain of fibrinogen and collagen type V. To prove that EcbA mediates binding of HA-*E. faecium* cells to fibrinogen and collagen type V, an isogenic mutant should be constructed followed by *in trans* complementation. Due to the presence of an *ecbA*-like gene on the chromosome of E1162 (Van Schaik, *et al.* manuscript in preparation), which is highly similar (90%) to *ecbA,* and with the current limited genetic tool box for *E. faecium*, we were unable to construct a double *ecbA* and *ecbA*-like isogenic mutant in *E. faecium* E1162, yet.

Scm, recently identified as another MSCRAMM of *E. faecium*, also binds to collagen type V and fibrinogen[22]. Both SgrA and EcbA bound to fibrinogen as well, and our ligand-affinity blot data show that these surface proteins target different chains of the fibrinogen molecule. Multiple MSCRAMMs of *S. aureus* such as clumping factor A and B, the FnbpA and FnbpB proteins, exhibit similar functions and bind to fibrinogen, thereby targeting different chains[23,24]. Possibly, the two different interactions of the structurally distinct SgrA and EcbA proteins with fibrinogen act synergistically to promote tight attachment to this ligand or allow adherence to fibrinogen in the presence of anti-SgrA, -EcbA or -Scm antibodies.

The presence of pili has been documented for *E. faecalis* and multiple other pathogenic Gram-positive organisms[25-30]. Pili are 'hair-like' surface-exposed structures involved in (initial) adherence to multiple types of human cells and biofilm formation[29,31-35], two processes which are critical in the pathogenesis of enterococcal disease. In **chapter 5** we report the identification of four *E. faecium* pilin gene clusters (PGC) containing all the genetic requirements for sortase-mediated pilus assembly from cognate LPXTG surface proteins[36]. The presence of the putative pilin subunit

genes from the PGCs was analyzed among 131 isolates of which 49 were HA- and 82 non-HA-*E. faecium*. PCR and Southern hybridization revealed that the surface protein genes from PGC-1 to -4 were significantly enriched in HA-*E. faecium* strains, favoring the hypothesis that conditional pili expression among these isolates potentially promote adherence to biotic and abiotic surfaces and pathogenesis.

We characterized two PGCs by use of transmission electron microscopy (TEM), and showed that specific immune sera reacted with phenotypically distinct PilA- and PilB-type pilus-like structures at the surface of an HA-*E. faecium* E1165 isolate grown at 37ºC. The PilA-type of pili were thin and rigid, whereas PilB-type of pili were thicker and more flexible, suggesting the formation of a bundled multi-PilB-fibre structure. Expression of these two types of pili was regulated in a temperature-dependent manner, as monomeric pilin subunits were cell wall-associated when grown at 21°C. Furthermore, using TEM we demonstrated simultaneous expression of PilA- and PilB-type pili on a single E1165 cell at 37 °C, which supported by western blotting, confirmed absence of cross reaction of the two sera. Interestingly, differential expression of PilA- or PilB-type pili resulting in a heterogeneous population with cells expressing both types or no pili at all, suggest that epigenetic processes mediate expression of pili, analogous to the pyelonephritis-associated pili in *Escherichia coli*[37] to limit exposure of pilin antigens to the immune system.

In **chapter 6** we further characterized PilA-type pili. To gain insights in pilus assembly of *E. faecium*, we studied the differential expression of the PilA, PilE and PilF LPXTG cell wall-anchored proteins putatively encoded from PGC-1, at the surface of an HA- and the E1039 non-HA-*E. faecium* isolate grown at 21°C and 37°C. At both these temperatures, the small PilE protein was not expressed in E1165. TEM analysis of the HA-*E. faecium* E1165 strain demonstrated that the PilA protein forms the pilus backbone of PilA-type pili, whereas the PilF minor pilin protein was found in increased concentrations at the pilus base, is equally distributed along the pilus shaft, and is associated with the PilA pilus tip at 37°C. Both proteins were expressed at the cell wall at 21°C. The fact that the minor PilF pilin protein was exclusively positioned at the pilus tip, and in increased concentrations at the pilus base close to the cell wall, implies a dual role for PilF in PilA pilus biogenesis: initiation and termination of pilus assembly. Such a dual role for pilin minor subunits in Gram-positive bacteria has previously been demonstrated by others[38,39]. In contrast to our findings in strain E1165, the PilA, PilE and PilF pilin proteins are cell wall-anchored proteins at the surface of the non-HA-*E. faecium* isolate E1039 at 37°C, and all three proteins were up-regulated at 21°C. Thus, in E1039 pilus assembly was not only abrogated, but the level of surface protein expression was also differentially regulated compared to the HA-*E. faecium* strain E1165. Since cell surface exposure of PilE correlates with absence of assembled pili in E1039, we speculate that surface expression of PilE, possibly via an interaction with PilF to form an hetero PilE-PilF dimer, abrogates pilus biogenesis. Which environmental conditions relevant for the lifestyle of *E. faecium* induce PilA pilus polymerization is currently under

investigation.

The PilA, PilB, PilE, and PilF pilin proteins were expressed at the cross wall of cells grown to early-exponential phase. At this site, *de novo* peptidoglycan synthesis occurs to separate the cell into two daughter cells[40]. This suggests that these LPXTG pilin subunits are translocated and incorporated at the cross wall into newly synthesized murein sacculi. Likely, a class A housekeeping sortase of *E. faecium* is co-localized at these sites to covalently immobilize these surface proteins to the peptidoglycan. Whether putative conserved motifs in *E. faecium* signal peptides or a C-terminally located positively charged domain identified in *Staphylococcus aureus* and *E. faecalis* surface proteins, respectively, target these pilin proteins to the septum and other potential LPXTG-type surface proteins to different locations in the cell envelope is currently under investigation[41,42]

Future perspectives for *E. faecium* surface protein research.

Enterococci have increasingly become resistant to antibiotics and emerged as nosocomial pathogens in hospitals throughout the world. However, as compared to other Gram-positive pathogens, research of fundamental aspects of enterococcal biology is seriously lagging behind. This is particularly true for *E. faecium*, which -due to its multi-resistant nature- is difficult to study using standard molecular techniques and for which no complete genome sequence is currently available. The advances described in this thesis have expanded the *E. faecium* surface protein research and provided insights into the knowledge regarding cell wall-anchored LPXTG-type surface proteins and pili of this bacterium.

Alternatives to antibiotic treatment for *E. faecium* infections are needed. The Esp, SgrA, EcbA and Orf903, Orf904.5 and Orf906.7 LPXTG-type surface proteins and the components of PilA and PilB pilus-like structures identified and characterized in this thesis are potential targets for the development of active and passive immunization strategies to treat HA-*E. faecium* infections. Because of their presumed role in pathogenesis, these LPXTG surface proteins will remain an important research focus in the future. However, it is currently unclear whether active immunization with these proteins or passive immunotherapy elicits protective immunity and can prevent infection with *E. faecium*. Logical next steps following this thesis are the following:

1. What is the role and function of the Esp, SgrA, EcbA, Orf903, Orf904.5 and Orf906.7 LPXTG-type surface proteins and the components of PilA and PilB pilus-like structures in the pathogenesis of enterococcal disease?

To assess whether the Esp, SgrA, EcbA, Orf903, Orf904.5 and Orf906.7 LPXTG-type surface proteins and the PilA, PilB, PilE, and PilF pilin proteins contribute to enhanced virulence, isogenic *E. faecium* variants with knockout mutations in these genes have to be assessed in murine models, such as intestinal colonization,

peritonitis, bacteremia and endocarditis. *E. faecium* wild-type strains and the mutants have to be tested for their abilities to adhere to human epithelial cells or biomaterials and for their capability to form biofilms. Furthermore, the effectiveness of antibodies directed against these surface proteins to block adhesion of *E. faecium* to epithelial cell lines, biomaterials or to prevent biofilm formation should be tested.

Novel therapeutic options directed against biofilm could include the N-terminal part of Esp, or specific monoclonal anti-Esp antibodies, which may disrupt the development of the multicellular structure of the biofilm. It has been demonstrated that monoclonal antibodies directed to particular epitopes of CNA, a collagen-binding MSCRAMM, could detach *S. aureus* from a collagen substrate[43]. Potentially, monoclonal anti-N-terminal Esp antibodies may detach Esp-expressing HA-*E. faecium* cells from its abiotic substrate.

2. Is it possible to prevent *E. faecium* infections in animals by passive or active immunization based on the Esp, SgrA, EcbA, Orf903, Orf904.5, Orf906.7, PilA, PilB, PilE, and PilF LPXTG surface proteins? Furthermore, is it possible to develop full human monoclonal antibodies directed against these proteins that may be used as immune prophylaxis in humans?

First, human IgM and IgG immune responses directed against the identified proteins described in this thesis should be determined in patients who have suffered an *E. faecium* infection to assess whether these proteins are expressed and immunogenic during natural infection. This can be done by western blotting and Enzyme Linked Immuno Absorbent Assays (ELISA) analyses. For these experiments, serum samples should be collected prospectively from patients. Serum samples have to be obtained preferably at the day of diagnosis, and one and two weeks after onset of disease to examine the development of specific antibody titers.

Mouse IgG antibodies directed against the Esp, SgrA, EcbA, Orf903, Orf904.5, Orf906.7, PilA, PilB, PilE, and PilF LPXTG surface proteins will be used in passive protection experiments to assess whether these antibodies can prevent *E. faecium* infections in mice. Promising vaccine candidates among the surface proteins discussed in this thesis are the pilin proteins, as they have already been shown to elicit protective immunity in other Gram-positives[44,45]. Furthermore, purified recombinant proteins have to be tested in active immunization experiments to elicit a protective immune response in mice. By performing Western blot and ELISA analysis we have to investigate whether the Esp, SgrA, EcbA, Orf903, Orf904.5, Orf906.7, PilA, PilB, PilE, and PilF LPXTG surface proteins elicit mouse antibodies. As a first step in the development of a novel therapeutic option based on active and passive immune prophylaxis protection after challenge with *E. faecium* in murine infection models could be evaluated.

Using the technology to generate and develop fully human monoclonal antibodies from the blood of infected patients, it should be aimed to develop several human monoclonal antibodies against HA-*E. faecium* strains. After whole blood donation, the *in vitro* procedure includes isolating human B lymphocytes, expanding them and (electro)fusing them with a suitable fusion partner. The resulting fusion products will be screened for relevant specificities and will, when positive for relevant antigens, be cloned to monoclonal hybridomas and stabilized. In addition, RNA will be isolated from the hybridomas for the expression of the antibody-encoding genes. A selection of the most promising antibodies can then, after *in vitro* efficacy studies, be subjected to pre-clinical and clinical research for the purpose of developing an antibody based drug for the treatment of HA-*E. faecium* infections.

3. What is the three-dimensional structure of the Esp, SgrA, EcbA, Orf903, Orf904.5, Orf906.7, PilA, PilB, PilE, and PilF proteins?

Elucidating the three-dimensional structure of these proteins, their binding mechanisms to protein ligands, and in addition the domains responsible for binding to their cognate ligands, host cell receptors, or abiotic substances may provide novel targets for treatments that interfere with this interaction.

4. Can PilA and PilB pilus-like structures (and other yet uncharacterized *E. faecium* pilus structures) be assembled *in vitro* using their purified cognate pilus-dedicated sortases (catalysts), and the major and minor pilin proteins? Unravelling the *E. faecium* pilus assembly mechanism may provide targets for treatments that interfere with pilus polymerization.

For *in vitro* assembly of monomeric components into pilus-like filaments, and determination of the three-dimensional structure, the recombinant pilin proteins and sortase proteins have to be purified. The establishment of the correct molecular organization and the appropriate intra- and intermolecular bonds within major subunits and between major subunits can be analyzed by Electrospray Ionization Fourier Transform Mass Spectrometry and Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry. As proposed here, the development of an *in vitro* system for pilus assembly would provide new experimental opportunities and insights into the molecular mechanisms and the order of assembly reactions that form the pilus, which may lead to therapeutics that can specifically disrupt *E. faecium* pilus assembly.

In summary, this thesis has provided new insights in the unique repertoire and capabilities of cell wall-anchored LPXTG surface proteins of *E. faecium* clones responsible for the majority of nosocomial *E. faecium* infections and outbreaks. The most important challenge in the near future now resides in translating these improved insights into novel therapeutics to combat emergence of hospital-

associated infections caused by multiresistant *E. faecium*. In addition, despite our increased knowledge, further research is needed to fully understand the mechanisms that have lead to the rapid evolutionary development and dominance of these clones in hospitalized patients.

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Dutch Summary/Nederlandse Samenvatting

Samenvatting voor leken.

De afgelopen 25 jaar zijn infecties met de multiresistente ziekenhuisbacterie *Enterococcus faecium* bij ernstig zieke patiënten in ziekenhuizen sterk toegenomen. Deze toename werd voor het eerst waargenomen in de Verenigde Staten in de jaren '80 van de vorige eeuw, gevolgd in dit millennium door Europa, Azië en Australië. *E. faecium* werd aanvankelijk gezien als een ongevaarlijke darmbacterie, maar door de toename van het aantal infecties bij ziekenhuispatienten wordt *E. faecium* nu beschouwd als één van de belangrijkste ziekenhuispathogenen. Deze toename van infecties is zeer waarschijnlijk het gevolg van het ontstaan van een bepaalde *E. faecium* variant die zich aan het ziekenhuis heeft aangepast. Deze ziekenhuisvariant onderscheidt zich van de *E. faecium* soorten die normaal in de darm voorkomen door resistentie tegen bepaalde antibiotica en door de aanwezigheid van genen coderend voor de oppervlakte eiwitten Esp, SgrA en EcbA en genen betrokken bij de assemblage van haarachtige structuren op de celwand, de zogenaamde pili. Het onderzoek beschreven in dit proefschrift heeft zich gericht op de identificatie, expressie en functie van oppervlakte eiwitten en pili aan het oppervlak van deze *E. faecium* ziekenhuisvariant. Hierbij bleek dat de oppervlakte eiwitten SgrA en EcbA binden aan specifieke moleculen in het menselijk lichaam. Esp en SgrA zijn daarbij tevens betrokken bij de vorming van biofilm, een complexe driedimensionale structuur van bacteriën die vrijwel inert is voor behandeling met antibiotica. Verder zijn twee typen pili (PilA en PilB) geïdentificeerd aan het oppervlak van deze ziekenhuisvariant wanneer deze groeit bij de menselijke lichaamstemperatuur van 37°C. De aanwezigheid van de specifieke oppervlakte eiwitten en pili aan het oppervlak van de ziekenhuisvariant verklaart mogelijk waarom deze variant infecties kan veroorzaken. Daarnaast kunnen deze oppervlakte eiwitten misschien gebruikt worden voor nieuwe laboratoriumtests, nieuwe medicijnen en vaccins om infecties met *E. faecium* te behandelen en te voorkomen.

Dutch summary/Nederlandse samenvatting.

Enterococcus faecium werd aanvankelijk gezien als een onschuldige darmbacterie, maar heeft zich de laatste twee decennia snel verspreid en ontwikkeld tot een multiresistente opportunistiche pathogeen in ziekenhuizen overal ter wereld en is verantwoordelijk voor met name urineweginfecties, lijninfecties, en bacteriemiën. Tegenwoordig is *E. faecium* één van de meest voorkomende Gram-positieve pathogenen die ziekenhuisinfecties veroorzaakt en is geassocieerd met een verhoogd onderliggend lijden, hogere sterfte en toenemende kosten in de gezondheidszorg. Uit moleculaire epidemiologische studies blijkt dat de meerderheid van *E. faecium* infecties en ziekenhuisuitbraken wordt veroorzaakt door een specifieke genetische variant, die "klonaal complex-17 (CC17)-" of "ziekenhuisvariant-" wordt genoemd. Deze ziekenhuisvariant is in staat om zich endemisch en epidemisch te verspreiden binnen en tussen ziekenhuizen over de gehele wereld en is genetisch verschillend van de *E. faecium* stammen die normaal in de darm van mens en dier voorkomen. De ziekenhuis *E. faecium* variant heeft verscheidene intrinsieke en verkregen antibioticum resistenties tegen aminoglycosiden, β-lactams en glycopeptiden. Hierdoor zijn de mogelijkheden om infecties met deze bacteriën te behandelen beperkt, vooral omdat ook tegen de nieuwste antibiotica, zoals linezolid, daptomycine en tigecycline reeds resistentie is waargenomen. Het is daarom essentieel om nieuwe behandelingsmethoden te ontwikkelen, zoals op oppervlakte eiwit gebaseerde passieve of actieve immunisatie.

Ondanks de toenemende kennis over het verschil in genetisch repertoire tussen de multiresistente ziekenhuisvariant en de stammen die van nature in onze darm voorkomen (de niet-ziekenhuis varianten), is er nog maar weinig bekend over de functie van specifieke genen en eiwitten die verrijkt zijn in ziekenhuis *E. faecium* klonen. Het onderzoek in dit proefschrift richt zich op celwandverankerde oppervlakte eiwitten en pilus-achtige structuren, aangezien oppervlakte eiwit gemedieerde adhesie aan gastheerweefsels en kolonisatie de eerste stappen zijn in de pathogenese. Onderzoek naar de rol die oppervlakte eiwitten van *E. faecium* spelen in de pathogenese kan leiden tot nieuwe inzichten hoe deze antibioticaresistente bacteriën infectie veroorzaken. Dit kan vervolgens leiden tot nieuwe behandelingsmethoden om infecties met deze ziekenhuisvariant bij mensen te bestrijden.

In dit proefschrift beschrijven we de eerste verkenning —de identificatie, karakterisatie en expressie analyse— van celwandverankerde LPXTG oppervlakte eiwitten en pilus-achtige structuren aan het oppervlak van de *E. faecium* ziekenhuisvariant. Deze studies leverden tevens nieuwe inzichten in de heterogeniteit in LPXTG oppervlakte eiwitten en pilus assemblage mechanismen van Gram-positieve bacteriën.

In **hoofdstuk 2** beschrijven we dat het Esp eiwit tot expressie komt aan het oppervlak van de ziekenhuisvariant en toonden we als eersten aan dat *E. faecium* reageert op veranderingen in zijn omgeving. Bij groei onder anaerobe condities en

bij groei bij 37˚C vonden we meer Esp aan het oppervlak van *E. faecium* dan bij aerobe condities en bij 21˚C. Groeitemperatuur- en zuurstofafhankelijke Esp expressie is mogelijk een niche afhankelijk aanpassingsmechanisme van de ziekenhuisvariant en dit suggereert een rol voor Esp tijdens de eerste fase van adhesie, infectie en kolonisatie. *E. faecium* is een facultatief anaerobe bacterie en aanpassing aan de hoeveelheid en samenstelling van oppervlakte eiwitten draagt mogelijk bij aan het overleven van deze bacterie in de omgeving of humane gastheer. Wij hebben als eerste kunnen aantonen dat de hoeveelheid Esp aan het oppervlak van de cel correleert met de mate van initiële aanhechting aan polystyreen en biofilm vorming. Deze aanhechting kon competitief geremd worden met oplosbaar exogeen recombinant N-terminaal Esp, wat suggereert dat dit domein verantwoordelijk is voor initiële aanhechting en biofilm vorming. Recentelijk konden deze resultaten worden bevestigd door de constructie van een Esp mutant.

Microbiële biofilms zijn geconcentreerde populaties van micro-organismen die omgeven zijn door een extracellulaire polymere matrix. Biofilms ontwikkelen tot driedimensionale structuren door de volgende achtereenvolgende stappen: (1) primaire aanhechting, (2) ophoping, (3) rijping en (4) dispersie. Biofilm vorming door enterococcen is een belangrijke eigenschap, omdat dit proces optreedt gedurende kolonisatie op bijvoorbeeld intravasculaire en urethrale katheters. Tevens zijn deze microbiële biofilms relatief slecht permeabel voor antimicrobiële middelen waardoor antibioticabehandeling bemoeilijkt wordt.

Om inzicht te krijgen in eigenschappen die mogelijk hebben bijgedragen aan de verspreiding en opkomst van de ziekenhuisvariant, werden LPXTG oppervlakte eiwit genen geïdentificeerd die verrijkt voorkomen in klinisch relevante en ziekenhuisuitbraak geassocieerde *E. faecium* isolaten. In **hoofdstuk 3** beschrijven we het onderzoek naar de aan- en afwezigheid van 22 LPXTG oppervlakte eiwit genen die, in eerste instantie geïdentificeerd werden in het *E. faecium* TX0016 genoom, in een collectie van 131 *E. faecium* isolaten, bestaande uit ziekenhuis verworven en commensale stammen. Klinische en ziekenhuisuitbraak geassocieerde *E. faecium* isolaten bevatten gemiddeld 19 en 21 van de 22 oppervlakte eiwit genen, terwijl commensale stammen, zoals isolaten uit dieren, humane vrijwilligers en het milieu respectievelijk 13, 14 en 15 van de 22 LPXTG oppervlakte eiwit genen bevatten. PCR en Southernblot hybridisaties bevestigden dat vijf LPXTG oppervlakte eiwit genen, genaamd *sgrA*, *ecbA*, *orf903*, *orf904.5* en *orf906.7*, specifiek verrijkt waren in de klinische en ziekenhuisuitbraak geassocieerde *E. faecium* isolaten. Deze genen kwamen actief tot expressie op mRNA niveau en aan het oppervlak van de cel. Het specifiek voorkomen van deze vijf oppervlakte eiwit genen in ziekenhuis verworven stammen is bijzonder interessant, omdat de sequentie similariteit van deze vijf oppervlakte eiwitten suggereert dat deze genen coderen voor factoren die betrokken zijn bij adhesie en biofilm vorming en daarom mogelijk een rol spelen in de pathogenese van *E. faecium* infecties. De aanwezigheid van variable hoeveelheden en combinaties van oppervlakte eiwit genen per verschillende epidemiologische klasse heeft mogelijk

bijgedragen aan de succesvolle aanpassing aan verschillende ecologische niches en heeft mogelijk de fitness van deze ziekenhuisstammen verhoogd. De set van LPXTG oppervlakte eiwitten die geëxposeerd zijn op de celwand van specifieke *E. faecium* klonen bepaald mogelijk het lot en succes van initiële kolonisatie van de niche waar deze bacteriën in kunnen voorkomen.

SgrA en EcbA zijn twee van de vijf LPXTG oppervlakte eiwitten die specifiek verijkt waren in klinische en ziekenhuisuitbraak geassocieerde *E. faecium* isolaten. Deze twee eiwitten werden functioneel gekarakteriseerd om hun functie(s) te ontrafelen (**hoofdstuk 4**). SgrA is het eerste LPXTG oppervlakte adhesine dat gekarakteriseerd is van *E. faecium*; het bevat geen IgG-folds in het mogelijke ligand bindingsdomein en bindt aan de α- en β-subunits van fibrinogeen en twee homologe eiwitten die nidogeen-1 en nidogeen-2 worden genoemd. We karakteriseerden als eersten een bacterieel oppervlakte adhesine dat kan binden aan nidogenen van de *lamina basalis*. In overeenstemming met deze resultaten bleek dat wildtype *E. faecium* E1162 cellen aan fibrinogeen, nidogeen-1 en nidogeen-2 kon binden, terwijl een isogene SgrA mutant, in mindere mate bond aan deze componenten van de extracellulaire matrix. Verder kon worden aangetoond dat SgrA, net als Esp, betrokken is bij biofilm vorming op een polystyreen oppervlak, terwijl dit oppervlakte eiwit niet betrokken is bij adhesie aan humane darmepitheel cellen, humane urineblaascellen en niercellen. SgrA speelt dus mogelijk een dubbelrol gedurende infectie, namelijk aanhechting aan abiotische oppervlakten, zoals katheters, en binding aan fibrinogeen en nidogenen die afgezet worden op deze oppervlakten.

EcbA is net zoals SgrA een nieuwe marker voor klinische en ziekenhuis-uitbraak geassocieerde *E. faecium* isolaten. EcbA is een MSCRAMM (microbial surface component recognizing adhesive matrix molecules) en bevat vier IgG-folds in het mogelijke ligand bindingsdomein en bindt aan de γ-subunit van fibrinogeen en collageen type 5. Om definitief aan te tonen dat EcbA binding van *E. faecium* cellen aan fibrinogeen en collageen type 5 mediëert, is het nodig om een isogene *ecbA* mutant te construeren. Omdat er een *ecbA*-like gen aanwezig is in het genoom van E1162 (Van Schaik *et al.*, manuscript in preparation), welke voor 90% gelijk is aan *ecbA* én door de huidige beperkte genetische toolbox voor *E. faecium*, zijn wij nog niet in staat gebleken om een dergelijke mutant te genereren.

Scm is een recent geïdentificeerde MSCRAMM van *E. faecium* en bindt aan collageen type 5 en fibrinogeen. Zowel SgrA als EcbA bonden ook aan fibrinogeen en onze ligand-affinity blot data laten zien dat deze oppervlakte eiwitten aan verschillende subunits van het fibrinogeen molecuul kunnen binden. Verschillende MSCRAMMs van *Staphylococcus aureus* zoals clumping factor A en B, de FnbpA en FnbpB eiwitten hebben overeenkomstige functies en binden ook aan verschillende subunits van fibrinogeen. De twee verschillende interacties van de twee in structuur verschillende SgrA en EcbA eiwitten met fibrinogeen, werken mogelijk synergistisch om zo sterker aan dit ligand te hechten of laten binding aan fibrinogeen toe in de aanwezigheid van anti-SgrA, -EcbA of –Scm antilichamen.

Pili zijn "haarachtige" structuren die uitsteken van het oppervlak van de cel en zijn onder andere betrokken bij initiële aanhechting aan verschillende typen humane cellen en de vorming van biofilm. Dit zijn twee processen die waarschijnlijk cruciaal zijn in de pathogenese van infecties met enterococcen. Pili zijn beschreven in *Enterococcus faecalis* en verscheidene andere pathogene Gram-positieve organismen, maar nog niet in *E. faecium*. In **hoofdstuk 5** beschrijven we de identificatie van vier pilus genen clusters (PGC) in *E. faecium* die alle genetische benodigdheden bevatten voor sortase-gemediëerde assemblage van LPXTG oppervlakte eiwitten tot pili. PCR en Southern hybridisaties toonden aan dat de oppervlakte eiwit genen van PGC-1 tot PGC-4 significant verrijkt waren in de klinische en ziekenhuisuitbraak geassocieerde *E. faecium* isolaten. Dit suggereert dat conditionele pili expressie van deze ziekenhuis verworven isolaten mogelijk bijdragen aan adhesie aan biotische of abiotische oppervlakten en pathogenese van *E. faecium* infecties.

We hebben twee van de vier PGCs gekarakteriseerd met behulp van transmissie elektronen microscopie (TEM) en hiermee aangetoond dat specifieke immuunsera reageerden met verschillende pilus-achtige structuren, PilA- en PilB-type, aan het oppervlak van een klinisch *E. faecium* E1165 isolaat gegroeid bij 37°C. De PilA-type pili zijn dun en rigide, terwijl PilB-type pili dikker en flexibeler zijn. Dit suggereert dat PilB pili zijn opgebouwd uit multi-PilB-structuren. Het bleek dat expressie van deze twee typen pili temperatuurafhankelijk werd gereguleerd. Tijdens groei bij 21°C konden alleen celwand geassocieerde monomere subunits worden aangetoond en geen volledig gesynthetiseerde pili. Verder hebben we aangetoond dat PilA- en PilB-typen pili tegelijk tot expressie kunnen komen op één enkele E1165 cel bij 37°C. Differentiële expressie van PilA- en PilB-type pili kan resulteren in een heterogene populatie met cellen die beiden typen of geen pili aan het oppervlak bevatten, en suggereert dat epigenetische processen pili expressie reguleren, zoals bij pyelonephritis-geassocieerde pili van *Escherichia coli* om zo de blootstelling van pilus antigenen aan het immuun systeem te limiteren.

In **hoofdstuk 6** hebben wij de PilA-type pili verder gekarakteriseerd. Om inzicht te krijgen in pilus assemblage van *E. faecium* hebben we de differentiële expressie van de PilA, PilE en PilF LPXTG celwand geassocieerde eiwitten bestudeerd aan het oppervlak van een klinisch *E. faecium* isolaat (E1165) en een commensale *E. faecium* stam (E1039) gegroeid bij 21°C en 37°C. Bij deze twee temperaturen kwam het kleine PilE eiwit niet tot expressie aan het oppervlak van E1165. TEM analyses van E1165 gegroeid bij 37°C toonden aan dat het PilA eiwit de pilus schacht vormt van PilA-pili, terwijl PilF werd gedetecteerd zowel aan de basis van de pilus, als gelijkmatig verdeeld langs de pilus schacht en geassocieerd met het uiteinde van de PilA pilus. Zowel PilA als PilF waren geassocieerd met de celwand bij 21°C. Het feit dat PilF geassocieerd was met het pilus uiteinde en in verhoogde concentratie aan de basis van de pilus suggereert een dubbele rol voor PilF gedurende PilA pilus biogenese: initiatie en terminatie van pilus assemblage. Een dergelijke dubbele functie is eerder gesuggereerd voor minor pilus componenten bij andere bacteriële species, maar

was niet eerder met behulp van TEM zichtbaar gemaakt. In tegenstelling tot onze bevindingen in de E1165, zijn de PilA, PilE en PilF oppervlakte eiwitten geassocieerd met de celwand en niet met pili in stam E1039 wanneer deze was gegroeid bij 37˚C. Expressie van deze drie eiwitten in deze stam bleek te zijn opgereguleerd wanneer E1039 werd gegroeid bij 21˚C. In de E1039 stam was pilus assemblage dus niet alleen afwezig, de expressie van deze eiwitten was ook differentieel gereguleerd in vergelijking tot het klinische E1165 isolaat. Omdat expressie van PilE aan het oppervlak correleert met de afwezigheid van geassembleerde pili in de E1039, veronderstellen we dat PilE mogelijk een interactie aangaat met PilF om zo een hetero-PilE-PilF dimeer te vormen, wat pilus assemblage blokkeert. Of en onder welke condities *E. faecium* stam E1039 PilA pilus assemblage wordt geïnduceerd wordt momenteel onderzocht.

De PilA, PilB, PilE en PilF pilus eiwitten komen tot expressie rond het septum van *E. faecium* cellen die tot de vroeg exponentiële fase waren gegroeid. Rond het septum van de cel vindt *de novo* peptidoglycaansynthese plaats om zo de cel te delen in twee dochtercellen. Dit suggereert dat deze LPXTG oppervlakte eiwitten getransloceerd en ingebouwd worden in het peptidoglycaan rond het septum. Mogelijk is op deze plek van de cel tevens een klasse A sortase gelokaliseerd om deze oppervlakte eiwitten covalent te immobiliseren in het peptidoglycaan. Momenteel wordt onderzocht of geconserveerde motieven in signaalpeptiden of een C-terminaal positief geladen domein, zoals geïdentificeerd in oppervlakte eiwitten van *S. aureus* en *E. faecalis*, bijdragen aan de specifieke lokalisatie van deze oppervlakte eiwitten naar het septum van de cel.

Dit proefschrift heeft geleid tot nieuwe inzichten in het unieke repertoire van oppervlakte eiwitten en hun functies van *E. faecium* stammen die verantwoordelijk zijn voor de meerderheid van ziekenhuisinfecties en -uitbraken. Een grote uitdaging is nu om deze nieuwe inzichten te vertalen naar nieuwe behandelmethoden om zo de wereldwijde verspreiding van ziekenhuisinfecties veroorzaakt door multiresistente *E. faecium* te stoppen. Ondanks deze nieuwe inzichten is vervolgonderzoek essentieel om de mechanismen die hebben geleid tot de snelle toename van ziekenhuisinfecties met *E. faecium* beter te doorgronden.

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Acknowledgements/Dankwoord.

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wegging. Je hebt nu bijna je master biomedische wetenschappen op zak en succes met je verdere carrière! **Claudia Schapendonk** en **Angela Kragten** (2007-2008), jullie kwamen tegelijk je afstudeerstage doen voor het HLO uit Breda en Utrecht, en dat was toch drukker voor me dan ik had verwacht! Angela, we hebben soms pittige aanvaringen gehad, maar je hebt een belangrijke bijdrage geleverd aan het opstarten van het enterococcen pili onderzoek. Claudia, of te wel "Clau", je ontwikkelde je erg snel en je kon al snel netjes, goed en geheel zelfstandig je experimenten uitvoeren. Het was daarom ook hartstikke leuk dat je na je stage onze groep kwam versterken als analist! Hartelijk bedankt voor het maken van al die Westerns, maar voornamelijk de EM experimenten, die was je op een gegeven moment wel zat volgens mij! Ook zonder jouw hulp –vooral tijdens de laatste loodjes van mijn onderzoek– was ik niet zo ver gekomen! Bedankt voor je hulp! **Britta Bouwman** (2008), hoewel je tijdens je HLO-afstudeerstage eigenlijk begeleid en beoordeeld werd door Miranda, heb je erg veel experimenten gedaan voor mijn onderzoek in het UMCU, maar ook in het RIVM, die je erg goed en netjes deed. Succes met je master opleiding en buitenlandstage!

De enterococcen Janetta Top, Helen Leavis, Willem van Schaik, **Marieke de Regt en Esther Heikens.** Beste Janetta, je bent de "Moeder van het enterococcenlab", jouw jarenlange onderzoekservaring en ervaring met het laboratorium en moleculaire technieken zijn zeer indrukwekkend, hierdoor heb ik erg veel van je geleerd! Je stond daarbij ook altijd voor mij klaar. Het was bijzonder gezellig tijdens onze Duck Tour, in een roze Duck door Boston! Helen, ik ben altijd onder de indruk geweest van je onuitputtelijke enterococcen literatuurkennis! We waren samen met Janetta de eerste AIO's in de groep en hebben een bijzonder gezellige tijd gehad tijdens congressen, borrels, uitgaan en labuitjes. Vooral het enterococcen congres in Helsingør was leuk hè?! Beste Willem, "Muziek en film, vraag het Willem" klopt inderdaad, daardoor was je hulp tijdens het maken van promotiefilmpjes zeer nuttig en gewenst! Je hebt de afgelopen 2 jaar een belangrijke invloed gehad op mijn presentaties, artikelen en werk in het lab. Je kritische visie, commentaar en feedback heb ik altijd erg gewaardeerd. Wanneer begin je je eigen onderzoeksgroep? Marieke, we hebben samen al flink wat plekken van de wereld gezien, van San Francisco tot Barcelona; en in moeilijke tijden waren we meestal een begripvol en luisterend oor voor elkaar. Pas je wel op met het oplopen van handblessures?! Esther, altijd druk-druk-druk, maar kom je een keer langs in Chicago? Ik haal je dan wel op van het vliegveld, want autorijden in de USA is niet je sterkste kant ;-). We keep in touch!

De superanalisten Erik Heezius, Piet Aerts, Maartje te Brake-Ruyken, Marc Jansen, **Annemarie Kuipers, Ingrid Boer, Stephanie Zuurveen** en **Bertie Dekker**. Hartelijk bedankt voor jullie hulp in het lab aan mij, maar ook aan al mijn studenten die ik geregeld aan hun lot over liet en dus vaak bij jullie terecht kwamen!

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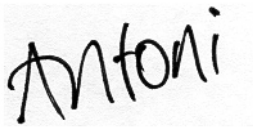

Antoni (Toon)

Curriculum Vitae

Curriculum Vitae.

The author of this thesis was born on March 24, 1980 in Amsterdam, The Netherlands. He grew up in Almere where he obtained his HAVO diploma at high school "Het Baken" in 1998. In the same year, he started his study Biology and Medical Laboratory Research (HLO, molecular biology) and obtained his B.Sc. degree in 2002 at the Institute of Life Sciences and Chemistry at the Hogeschool van Utrecht after completion of his internship under supervision of Prof. dr. David A. Low at the University of California Santa Barbara, U.S.A.. At the University of California, he investigated the role of two-component signal transduction systems in gut colonization by uropathogenic *Escherichia coli*. His work was rewarded with the predicate "Best Undergraduate of 2002" at the Hogeschool van Utrecht.

He continued his education with the master Biomedical Sciences in 2002 at the Vrije Universiteit in Amsterdam, The Netherlands. He performed his first internship under supervision of Prof. Dr. Rogier Versteeg and Dr. Arjen Koppen at the Academical Medical Center in Amsterdam, where he studied the regulation of genes of the N-*myc* pathway in human neuroblastoma. His second internship was in the laboratory of Prof. Dr. Hans V. Westerhoff and Dr. Rob J. van Spanning at the Vrije Universiteit where he was to work on the function of genes of the *nos* operon of *Paracoccus denitrificans*, after which he graduated in 2004. After obtaining his M.Sc. degree, he was a teaching assistant at the Vrije Universiteit for the courses biological chemistry and microbiology until he started in 2005 with his Ph.D. training at the Department of Medical Microbiology under supervision of Prof. Dr. Marc J.M. Bonten and Dr. Rob J. L. Willems. The author of this thesis is author on the publications described in this thesis, presented his work on national and international congresses, won multiple prizes including the "ASM Richard and Mary Finkelstein Award", and is author of a patent on the use of enterococcal pilin proteins as an vaccine for enterococcal infections.

In the summer of 2009 he will pursue his postdoctoral career at the Department of Microbiology at the University of Chicago, U.S.A., where he will examine the role of pili of *Bacillus anthracis* in the host-pathogen interaction.

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List of Publications

List of Publications.

Kankainen M, Paulin L, Tynkkynen S, von Ossowski I, Reunanen J, Partanen P, Satokari R, Vesterlund S, **Hendrickx AP**, Hämäläinen T, Laukkanen S, Salovuori N, Ritari J, Alatalo E, Korpela R, Mattila-Sandholm T, Lassig A, Hatakka K, Kinnunen KT, Karjalainen H, Saxelin M, Laakso K, Surakka A, Palva A, Salusjärvi T, Auvinen P, and de Vos WM.
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van Schaik W, Top J, Riley D, Boekhorst J, Vrijenhoek JE, **Hendrickx AP**, Nijman IJ, Bonten MJ, Tettelin H, and Willems RJ.
Genome Analysis of the Nosocomial Pathogen *Enterococcus faecium* and Identification of a Large Transferable Pathogenicity Island.
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Hendrickx AP, Schapendonk CM, van Luit-Asbroek M, Bonten MJ, and Willems RJ.
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Hendrickx AP, van Luit-Asbroek M, Schapendonk CM, Van Wamel WJ, Braat JC, Wijnands LM, Bonten MJ, and Willems RJ.
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Appendix - Color Figures

Appendix/Color figures.

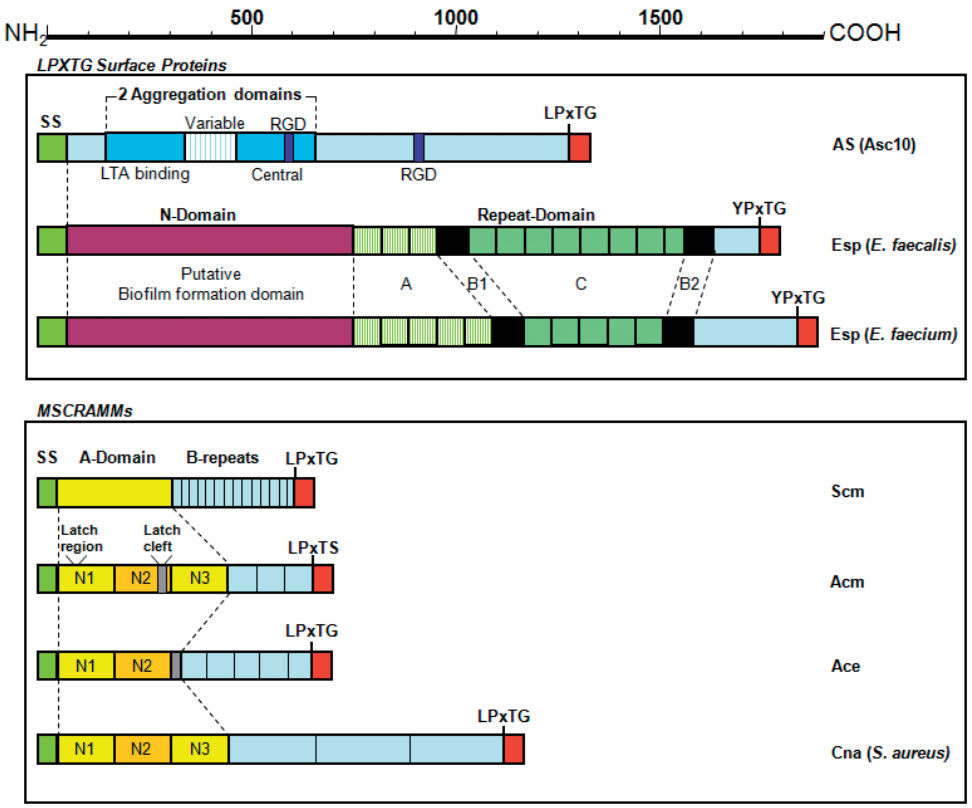

Figure 1. LPxTG surface proteins and MSCRAMMs of enterococci. Structural organization of the LPxTG surface proteins (upper part) and MSCRAMMs (lower part) of enterococci. Signal sequences (SS) are depicted in dark green and start at the N-terminus. The LPxTG-like cell wall sorting signal is marked red. The N-terminal domain of Esp of *E. faecium* and *E. faecalis* is depicted in purple and the aggregation domains of AS are in blue. The RGD motifs, implicated in binding to eukaryotic cells are depicted in dark blue. The collagen binding A-domains of the enterococcal MSCRAMMs are yellow and divided into subdomains, designated N1, N2 and N3 each adopting a variant of the IgG-like fold. Repeat regions of the MSCRAMMs are depicted in light blue. A latching cleft identified in Acm and Ace is depicted in grey. The scale starts at the N-terminal end of the protein and ends at the C-terminus, with the number of amino acids indicated.

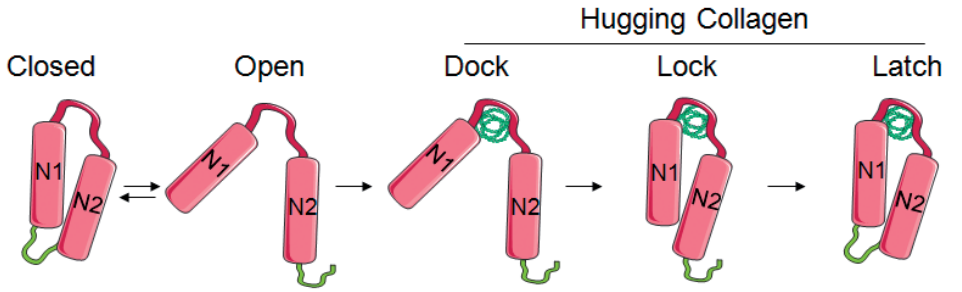

Figure 2. The Collagen hug model. Schematic representation of the Collagen Hug binding mechanism. The N1N2 subdomains of Ace and Cna and likely also of Acm are in a dynamic equilibrium from a closed to open configuration. When the N1N2 subdomains adopt an open configuration, the collagen ligand (dark green) is allowed to dock. The MSCRAMM subsequently hugs around the collagen helix to lock it between the N1 and N2 subdomains. The C-terminal latch extension (light green) of the N2 subdomain inserts into a trench of the N1 subdomain to close the MSCRAMM-collagen complex. Figure was produced using Servier Medical Art.

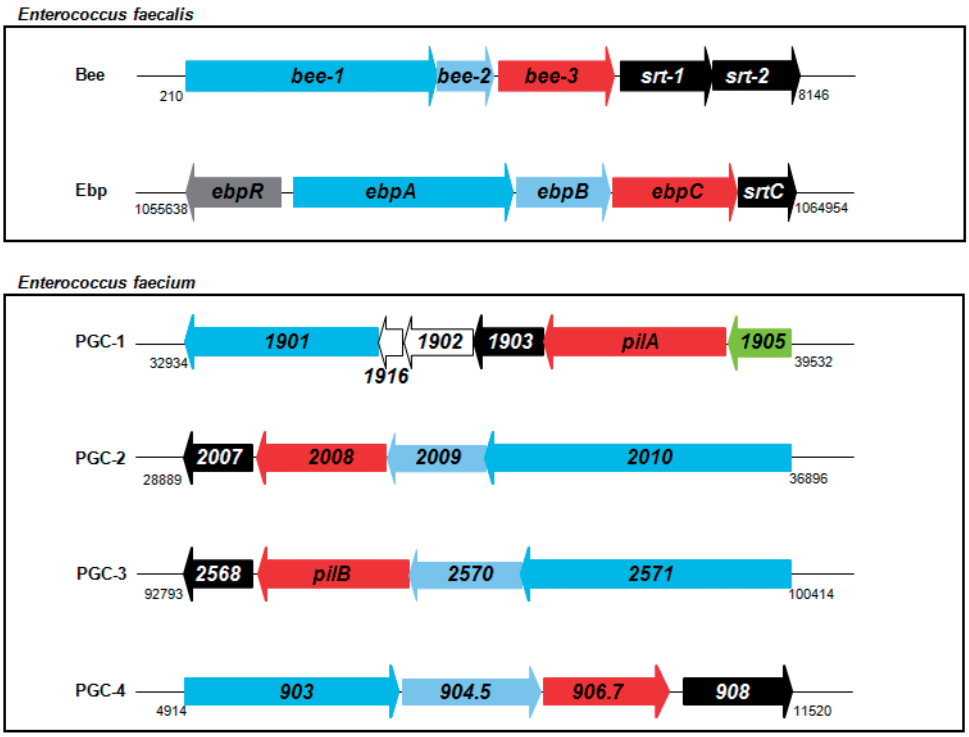

Figure 3. Enterococcal pilin gene clusters. Schematic representation of the organization of the putative pilin gene clusters identified in *E. faecalis* and *E. faecium*. The position and the orientation of the genes is indicated by arrows and the gene name is depicted in the arrows. The black arrows indicate putative pilus dedicated class C sortase genes, while the green arrow indicates a class A housekeeping sortase. The red arrows indicate putative major pilin genes, light blue and dark blue arrows indicate putative minor or tip pilin genes, respectively. The white arrows indicate proteins with unknown function. The *ebpR* transcriptional regulator is depicted in grey. The numbers at the beginning and the end of the pilin gene clusters are positions of the operons on the chromosome. The figure is not to scale.

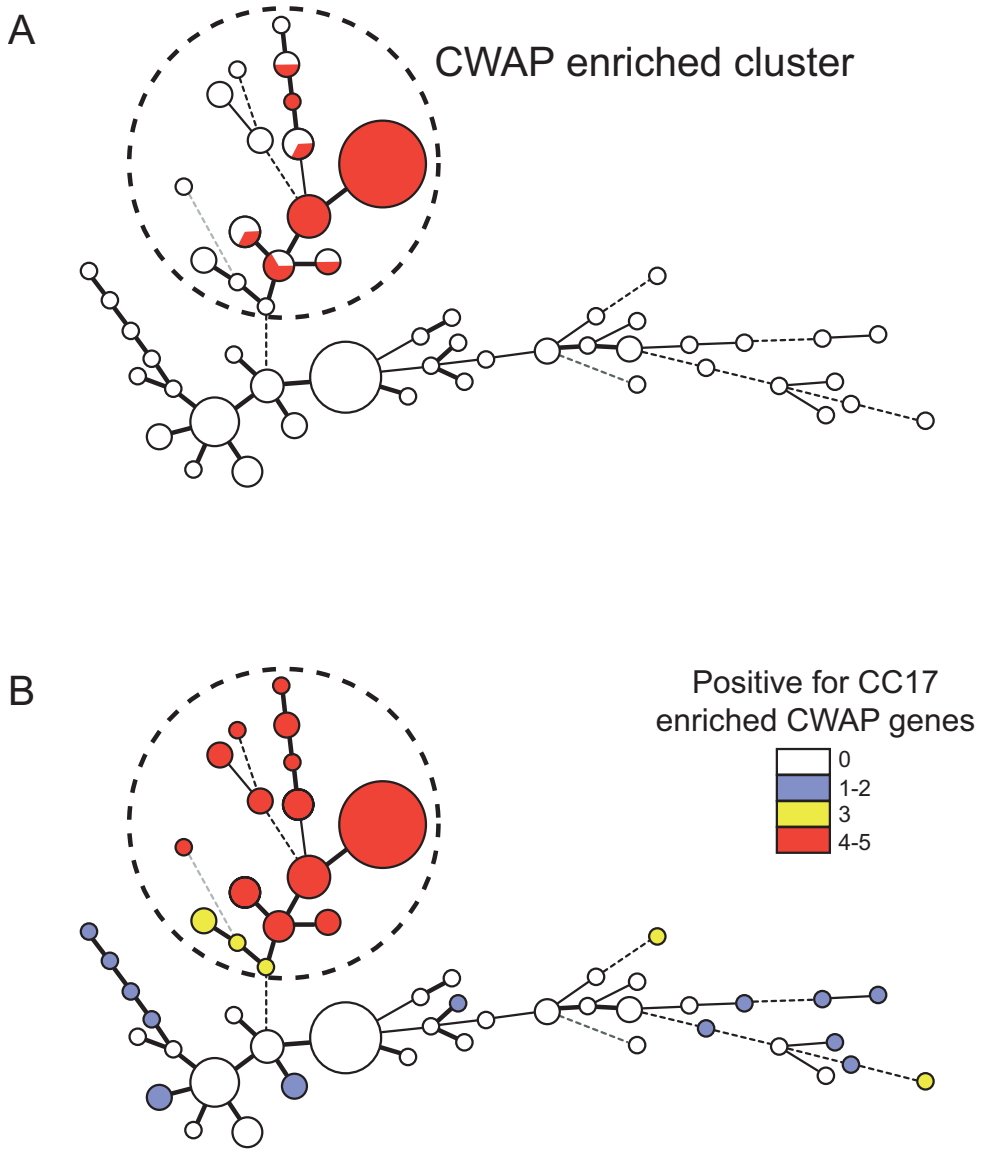

Figure 6. MST based on clustering on the presence and absence of the 22 putative CWAP genes. A categorical coefficient and the eBURST priority rule of the highest number of single-locus changes were used for the clustering. Circles represent putative CWAP gene profile and the size of the circles indicate the number of isolates. Thick short lines connecting two CWAP gene types denote types differing by a single CWAP gene, thin lines connect double CWAP gene variants, and dotted lines indicate the most likely connection between two types differing by more than two CWAP genes. (A) All 40 CC17 isolates are marked red and cluster together with 18 non-CC17 clinical isolates (marked white) in a CWAP enriched cluster. (B) The presence of 0, 1-2, 3, 4-5 CC17 enriched CWAP genes is indicated with colours depicted in the legend.

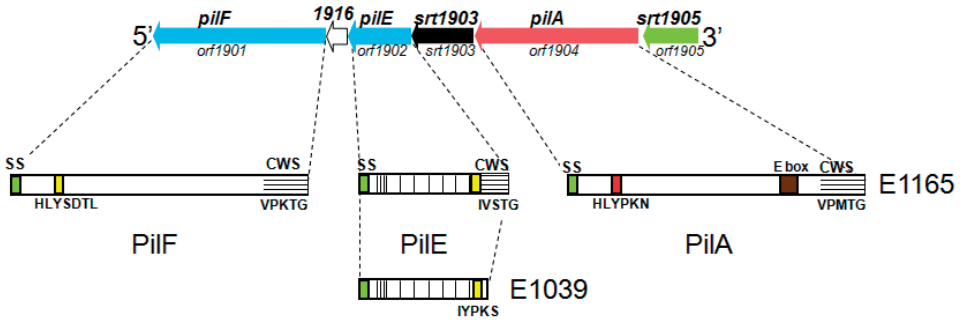

Fig. 1. Schematic representation of the genomic organization of PGC-1 of *E. faecium* E1165 (DDBJ/EMBL/Genbank accession number EU909697). The position and presumed direction of transcription of ORFs is indicated by arrows. The ORF number is depicted below the arrows, while the gene name is depicted in bold above the arrows. The blue arrows indicate minor pilin proteins and the white arrow indicate a gene with unknown function. The red arrow indicates the putative major pilin subunit. The black and green arrow indicates class C and class A sortase genes, respectively. The putative LPXTG-like surface proteins are depicted below the PGC-1. (SS) are depicted in green, the pilin and pilin-like motifs in red and yellow, respectively. The E box motif is in brown and the CWSs are depicted as a (horizontally) striped box. The repeats in PilE are depicted by vertical black lines.