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Enterococcal Surface Protein Esp Is Important for Biofilm Formation of *Enterococcus faecium* E1162[▽]

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

Enterococci are considered normal inhabitants of the gastrointestinal tracts of humans and animals. In the last 2 decades, though, enterococci have emerged as important nosocomial pathogens, with high-level resistance to antibiotics, such as ampicillin, aminoglycosides, and vancomycin (22). Enterococci are currently the third most frequent nosocomial pathogen isolated from intensive care unit patients in the United States (25). Since the turn of the century, the prevalence of enterococci has been rising in European hospitals, too. The majority of enterococcal infections are caused by *Enterococcus faecalis* (http://www.earss.rivm.nl). However, in parallel with the increase in nosocomial enterococcal infections, a partial replacement of *E. faecalis* by *Enterococcus faecium* took place in European and U.S. hospitals (12, 37, 38) (http://www.earss.rivm.nl). It is unlikely that these ecological changes result exclusively from increased resistance to antibiotics. A better understanding of the virulence of enterococci, therefore, is necessary to control further spread and to develop new treatment strategies.

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

Compared to *E. faecalis*, relatively little is known about the virulence and pathogenesis of *E. faecium*. Previously, we described the evolutionary descent among *E. faecium* isolates obtained from human sources (from community, as well as hospital, reservoirs) and nonhuman sources in five continents with multilocus sequence typing. Most hospital outbreak and invasive *E. faecium* isolates belong to a single clonal lineage, complex 17 (CC17) (45). Furthermore, by using a mixed whole-genome microarray, we recently identified a specific *E. faecium* clade largely overlapping with CC17, highly specific for nosocomial outbreaks and infections, and containing more than 100 clade-specific genes (17). The ecological success of CC17 in the hospital environment is not understood. It seems to be partly related to resistance to penicillins and quinolone antibiotics (16, 45). Apart from antibiotic resistance and the clade-specific genes, CC17 is correlated with the presence of a putative pathogenicity island, which carries the enterococcal surface protein gene, *esp* (15, 45). In *E. faecalis*, Esp is also located on a pathogenicity island, is expressed on the surface of the bacterium (30, 31), and is thought to be an adhesin contributing to colonization of urinary tract epithelial cells and biofilm formation (21, 31, 32, 35). Esp of *E. faecium* shares up to 90% homology with Esp of *E. faecalis*, but its function is unknown. Interestingly, it is predominantly present in isolates associated with infections and hospital outbreaks (13, 15, 42). Furthermore, expression of Esp varies between strains, is growth condition dependent, and is quantitatively correlated with initial adherence to polystyrene and biofilm formation (41). Based on these findings, Esp may be an important determinant of adhesion and biofilm formation of *E. faecium*. However, the definitive role of Esp in these processes could not be determined, as an isogenic *esp* deletion mutant was lacking and an *esp* mutant in an *E. faecium* clinical isolate has not been

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TABLE 1. Bacterial strains and plasmids used in this study

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

[a] Amp, ampicillin; Van, vancomycin; Chl, chloramphenicol; Gen, gentamicin; Ery, erytromycin; ts, temperature sensitive; Esp$^+$, positive for Esp expression; Esp$^-$, negative for Esp expression.

successfully constructed. In general, it has been extremely difficult to inactivate genes in *E. faecium* by allelic exchange due to poor transformation capacity, plasmid incompatibility, and the lack of selective markers because of multiple antibiotic resistances in clinically relevant strains. Recently, Nallapareddy et al. were the first to construct an insertion-deletion mutation, in the adhesion-encoding *acm* gene, in a clinical *E. faecium* isolate by using an improved temperature-sensitive vector (24). We used the same approach to generate an *esp* insertion-deletion mutant in a clinical isolate of *E. faecium* in order to assess the role of Esp in biofilm formation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani broth or agar. Enterococci were grown in either brain heart infusion (BHI), Todd-Hewitt, or Trypticase soy (TS) broth or agar or sheep red blood agar containing tryptic soy agar with 5% sheep red blood cells (BD, Alphen aan den Rijn, The Netherlands) at 37°C, unless a different growth temperature is specified. For enterococci, the antibiotics chloramphenicol, gentamicin, and erythromycin were used in concentrations of 10 μg/ml, 125 μg/ml, and 10 μg/ml, respectively. For *E. coli*, the antibiotic chloramphenicol was used in a concentration of 10 μg/ml. All antibiotics were obtained from Sigma-Aldrich (Saint Louis, MO). Growth was determined by measurements of the optical density at 660 nm (OD_{660}).

An improved temperature-sensitive vector (pTEX5500ts), designed by Nallapareddy et al. (24), was used to introduce an insertion-deletion mutation in the *esp* gene of a clinical *E. faecium* isolate, E1162, which was isolated from the blood of a patient. E1162 is a strain from CC17. The pAT18 vector (39) was used for complementation studies.

Standard molecular techniques. Chromosomal DNA from *E. faecium* was prepared as described elsewhere (43, 44). The primers used in this study were purchased from Isogen Life Science (IJsselstijn, The Netherlands) and are listed in Table 2. PCRs were performed with a 9800 Fast Thermal Cycler (Applied Biosystems, Foster City, CA), and the PCR amplification conditions were as

TABLE 2. Primers used in this study

Primer name	Primer sequence[a,b]	Start position
EspUpdelF	5′-CTA **GCT AGC** GCT CCG TAC AAG TAG GTG ACA	3281[c]
EspUpdelR	5′-CCC **AAG CTT** CCC GCT ACA TAT GGA ACT AAT C	4103[c]
EspDndelF	5′-CCG **GAA TTC** CAT CTT TGA TTC TTG GTT GTC G	4392[c]
EspDndelR	5′-TCC **CCC GGG** TTG TTC CAG TAA TCG GCT CC	5284[c]
EspUpF2	5′-TAC GGG CTA CTT TTT AAC AGA	3825[c]
EspDnR2	5′-TGA ATC TAC ACC CGT AAA TTC	4739[c]
CmF	5′-GAA TGA CTT CAA AGA GTT TTA TG	509[d]
CmR	5′-AAA GCA TTT TCA GGT ATA GGT G	610[d]
pRIE298.6F	5′-GAA GAA GGA ATT TGA AGT CAC	2697[c]
Esp.11R	5′-GGT AGC CTG CAG GAA TG	5395[c]
EspcompF	5′-CCG **GAA TTC** GCT TGC ATC AAA ATA AAC TAC ATG GGT ATA ATA GCA ATG AAA TGC ATT TCA AAA ATA TTT TGA GGA GAA TTT AGT ATG GTT AGC AAG AAT AAT AAG AG	3091[c]
EspcompR	5′-CCG **GAA TTC** CCT CTT TTC AGA GAA GAT T	8827[c]

[a] Restriction sites are in boldface.
[b] Regions −35 and −10, the ribosome binding site from the *bacA* promoter, and the ATG codon of *esp* are underlined (9, 36).
[c] Nucleotide reference positions relative to the *E. faecium* PAI sequence (GenBank accession no. AY322150).
[d] Nucleotide reference positions relative to the shuttle plasmid pTEX5500ts sequence (GenBank accession no. DQ208936).

follows: initial denaturation at 95°C for 15 min, followed by 10 touchdown cycles starting at 94°C for 30 s, 60°C for 30 s, and 72°C (the time depended on the size of the PCR product) with the annealing temperature decreasing by 1°C per cycle, followed by 25 cycles with an annealing temperature of 52°C. The PCRs were, unless otherwise specified, performed in 25-μl volumes with HotStarTaq Master Mix (QIAGEN Inc., Valencia, CA.). PCR products were purified using the QIAquick PCR purification kit (QIAGEN Inc.) according to the manufacturer's instructions. Restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Ligation was performed by using T4 DNA ligase (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Plasmid DNA from *E. coli* was purified by using the QIAprep Spin Miniprep Kit (QIAGEN Inc.) according to the manufacturer's instructions. Plasmids were transformed into *E. faecium* by electroporation using a Gene Pulser unit (Bio-Rad Laboratories, Richmond, CA) as described elsewhere (23). For Southern hybridization, chromosomal DNA was digested with TaqI, separated by agarose gel electrophoresis (0.7% agarose gels), and transferred onto a Hybond-N^+ nylon membrane (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). A 916-bp PCR fragment obtained with primers EspUpF2 and EspDnR2 was used as a DNA probe. Labeling of the probe and DNA hybridization were performed according to the protocol supplied with the ECL Direct Nucleic Acid labeling and detection System (GE Healthcare).

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

Construction of an insertion-deletion mutation in the *esp* gene. To introduce an insertion-deletion mutation in the *esp* gene, the same protocol described by Nallapareddy et al. (24) was used with some minor modifications. In brief, an 850-bp-long internal *esp* fragment designated EspUp, coding for a region at the beginning of the N-terminal domain of *esp*, was amplified from genomic DNA of E1162 by using the primers EspUpdelF and EspUpdelR, including the restriction sites NheI and HindIII, respectively (Table 2). The PCR product was digested with NheI and HindIII and ligated to similarly digested pTEX5500ts, resulting in pEF1. In a similar way, an 830-bp-long fragment designated EspDn, coding for a region encompassing the end of the N-terminal domain of *esp*, was amplified by using the primers EspDndelF and EspDndelR, including the restriction sites EcoRI and SmaI, respectively. This PCR product was digested with EcoRI and SmaI and ligated to similarly digested pEF1, resulting in pEF2, pTEX5500ts with cloned *esp* gene fragments flanking the chloramphenicol acetyltransferase (*cat*) gene. The recombinant plasmids pEF1 and pEF2 were transferred into *E. coli* DH5α cells (Invitrogen) for propagation and plasmid purification. The recombinant plasmid pEF2 was introduced into E1162 by electroporation to generate an insertion-deletion mutation in the *esp* gene. After transformation, the cells were allowed to recover for 4 h at the permissive temperature of 28°C, after which the cells were plated on Todd-Hewitt agar plates with 20% sucrose and gentamicin at 28°C to select for transformants. Gentamicin-resistant colonies were picked and grown overnight in BHI broth supplemented with gentamicin at an elevated temperature (42°C) to cure the plasmid. The cells were plated on BHI agar plates with chloramphenicol at 37°C. Single-crossover integration into EspUp and EspDn regions was tested by PCR with the primers pRIE298.6F and CmR, and CmF and Esp.11R, respectively. Single-crossover mutants were saved and grown for six serial overnight passages in chloramphenicol-BHI culture at 42°C to completely cure free recombinant plasmid. The cultures were serially diluted, plated on chloramphenicol-BHI agar plates, and replica plated on gentamicin-BHI agar plates to select for double-crossover recombination. Double crossovers were colonies that retained the *cat* gene but lost the (*aph2"-Id* gene, which encodes an aminoglycoside phosphotransferase that mediates high-level resistance to gentamicin, by plasmid excision, resulting in an insertion-deletion mutation of the *esp* gene. Correct generation of the insertion-deletion mutation in the *esp* gene was checked by PCR with the primers pRIE298.6F and Esp.11R, by Southern hybridization, and by DNA sequencing (as described above).

Complementation studies. To complement the *esp* mutant strain with wild-type *esp*, the *esp* gene of E1162 was amplified from genomic DNA by using the Expand Long Template PCR system (Roche Diagnostics, Mannheim, Germany) with the forward primer EspcompF and the reverse primer EspcompR. The forward primer EspcompF included the −35 and −10 promoter regions and the ribosome binding site of the constitutive promoter of the *bacA* gene of *E. faecalis* (9, 36), as well as an EcoRI restriction site, facilitating cloning of this fragment. The reverse primer also included an EcoRI restriction site. The resulting PCR product containing the *esp* gene and *bacA* promoter sequences was digested with EcoRI and ligated to similarly digested pAT18 (39), resulting in pEF3 (pAT18: *esp*). The recombinant plasmid pEF3 was introduced into the *esp* mutant strain by electroporation.

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

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

Western blotting. Plate-grown bacteria were resuspended in PBS to an OD_{660} of 1.0 (1×10^9 CFU/ml). Cells were harvested by centrifugation ($1{,}560 \times g$; 5 min) and resuspended in 50 μl PBS. Electrophoresis sample buffer (1×) supplemented with 50 mg/ml dithiothreitol was added. Samples were boiled for 5 min and loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. The bacterial proteins were transferred to nitrocellulose using a Bio-Rad Trans-Blot Cell tank transfer unit at 12 V overnight in 20 mM Tris, 0.15 M glycine, and 20% methanol at pH 8.3. Nonspecific sites in the blot were blocked by incubation for 1 h at 37°C with 4% skim milk powder in PBS with 0.1% Tween 20. Esp was assayed by incubation for 1 h at 37°C with rabbit anti-Esp immune serum (1:5,000) (41) as the primary antibody, followed by incubation for 1 h at 37°C with horseradish peroxidase-conjugated goat anti-rabbit (1:5,000; Santa Cruz Biotechnology) as the secondary antibody. Both antibodies were diluted in PBS with 1% Tween 20 and 1% BSA. Esp was detected by using light-emitting ECL Western Blotting Detection Reagents (GE Healthcare).

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

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

Confocal laser scanning microscopy (CLSM). Bacteria were grown in TS broth supplemented with 0.25% glucose to mid-log phase. Nitrocellulose membranes (0.45 μm; diameter, 25 mm; Bio-Rad) were put on TS agar plates with 0.25% glucose, and 200 μl bacterial suspension (5×10^6 CFU) was inoculated onto the nitrocellulose membranes and grown for 24 h at 37°C. After 24 h, the nitrocellulose membranes were washed three times in PBS, and the biofilms were chemically fixed in 3.7% formaldehyde (Merck) in PBS for 15 min. Nonspecific sites were blocked by incubation for 1 h at 37°C with 10% skim milk powder in PBS. The biofilms were stained by incubating the nitrocellulose membranes (Bio-Rad) for 15 min at room temperature in 0.1% acridine orange (Merck) in PBS. After incubation, the nitrocellulose membranes were washed three times with PBS and transferred to glass microscope slides and covered with glass coverslips (Marienfeld, Lauda-Königshofen, Germany). The biofilms were examined by using an inverted fluorescence microscope (Leica DMRXA2) equipped with an oil plan-neofluor ×100/1.4 objective, and confocal images (scans) were developed with an MRCF-1000 laser (488 nm) scanning confocal imaging system (Bio-Rad). The acquired image stacks were viewed by using Leica Confocal Software (version 6.1). The maximum thickness of the biofilms was measured at five randomly chosen positions with the software.

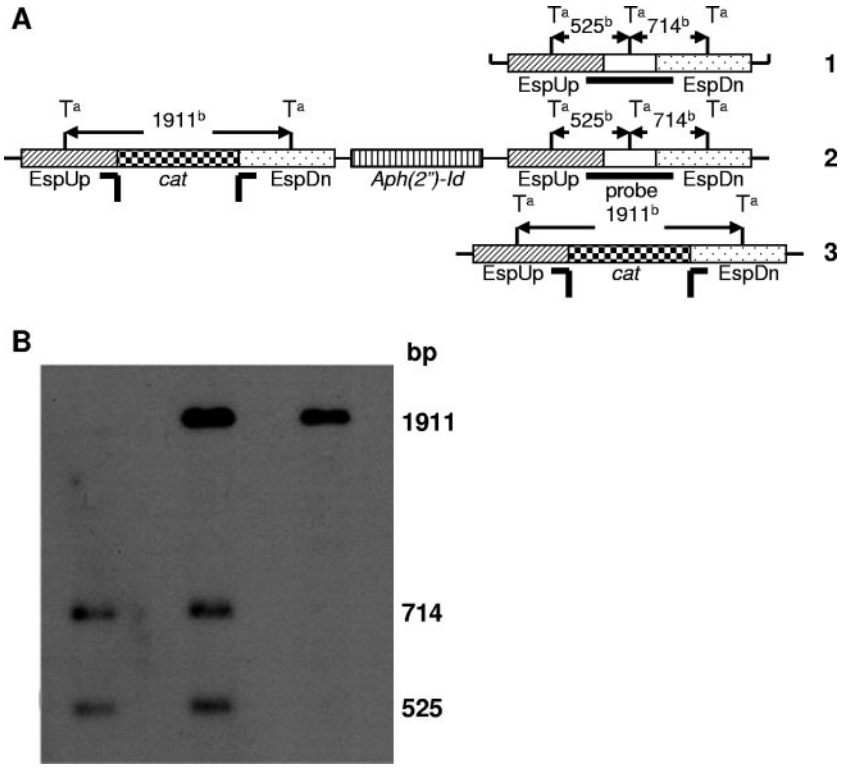

FIG. 1. Confirmation of correct insertion-deletion mutation in the *esp* gene by Southern blot analysis. (A) Schematic representation of the 5′ end encoding the N-terminal domain of the wild-type *esp* gene (1), the *esp* single-crossover insertion (2), and the *esp* double-crossover insertion-deletion (3). The box with squares represents the *cat* gene coding for chloramphenicol resistance, the striped box represents the *aph2″-Id* gene coding for gentamicin resistance, the hatched box represents the EspUp fragment used for recombination, the stippled box represents the EspDn fragment used for recombination, and the black line represents the DNA probe. [a], TaqI restriction site; [b], fragments obtained after digestion with TaqI; [c], nucleotide reference positions relative to the *E. faecium* PAI sequence (GenBank accession no. AY322150). (B) Hybridization results of Southern blot analysis of TaqI-digested genomic DNA of the *esp* wild-type strain (lane 1), *esp* single-crossover mutant strain (lane 2), and *esp* double-crossover mutant strain (lane 3).

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

RESULTS

Construction of an insertion-deletion mutation in the *esp* gene. *esp* gene fragments were cloned into pTEX5500ts flanking the *cat* gene, resulting in pEF2, and this recombinant plasmid was used to introduce an insertion-deletion mutation in the *esp* gene of a clinical *E. faecium* isolate (E1162). The *esp* mutant strain (E1162Δ*esp*) was constructed in two steps. First, single-crossover mutants (E1162EspUp/EspDn: pEF2), in which pEF2 was integrated, were selected. Only left single-crossover integration was found. This was followed by selection of double-crossover events, in which the wild-type *esp* gene was replaced by the mutated *esp* gene and plasmid sequences were lost. Double-crossover mutants were expected to be chloramphenicol resistant and gentamicin susceptible. In total, ~600 colonies were screened by replica plating. Of these colonies, eight were putative double-crossover mutants. In two of the eight colonies, PCR indicated correct insertion-deletion mutation (data not shown). DNA-sequencing results confirmed that a 287-bp-long fragment of *esp* (positions 4105 to 4392 based on the *E. faecium* PAI sequence deposited in GenBank under accession number AY322150) was replaced by a 993-bp-long *cat* gene (data not shown). Southern blot analysis results confirmed correct insertion-deletion mutation in the *esp* gene in these two colonies (Fig. 1).

E1162Δ*esp* colonies appeared to have the same size as those of the wild-type strain (E1162) when grown overnight on sheep red blood agar plates. To further characterize the behavior of

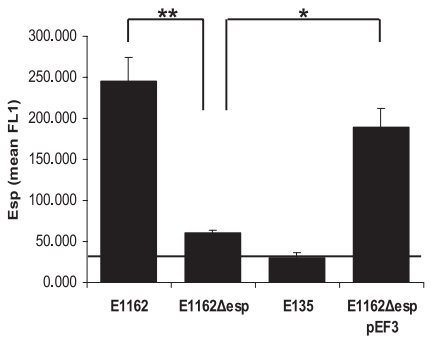

FIG. 2. Cell surface expression of Esp by flow cytometry. Shown is analysis of cell surface expression of Esp by flow cytometry using rabbit anti-Esp immune serum for the *esp* wild-type strain (E1162), *esp* mutant strain (E1162Δ*esp*), *esp*-negative strain (E135), and *esp*-complemented strain. Mean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

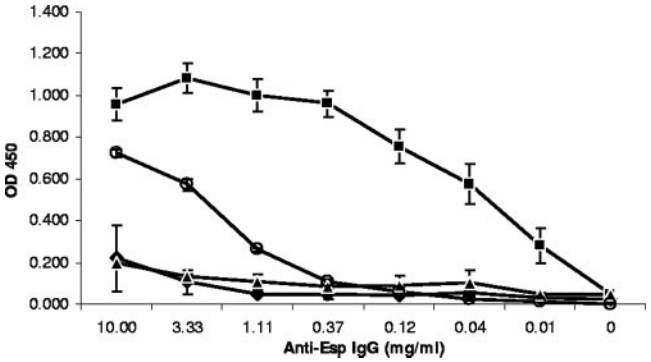

FIG. 3. Cell surface expression of Esp by whole-cell ELISA. Shown is analysis of cell surface expression of Esp by whole-cell ELISA using rabbit anti-Esp IgGs in different dilutions for the *esp* wild-type strain (E1162) (squares), *esp* mutant strain (E1162Δ*esp*) (triangles), *esp*-negative strain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

E1162Δ*esp*, growth was monitored by the OD_{660}. No difference in growth rates was observed between E1162 and E1162Δ*esp* (data not shown).

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

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

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

Complementation studies. Complementation experiments were performed to determine whether in *trans* expression of Esp from a plasmid was able to restore initial adherence and biofilm formation in the *esp* mutant strain. Because the promoter of *esp* has not been mapped, *esp* was expressed under the control of a constitutive promoter of the *bacA* gene of *E. faecalis* (9), as described in Materials and Methods. After transformation of *E. coli* DH5α cells with recombinant plasmid pEF3, the cells were highly unstable, suggesting that Esp expression had a toxic effect on the *E. coli* cells. Therefore, the ligation mixture was transferred directly to the *E. faecium esp* mutant by electroporation, resulting in an *esp*-complemented strain (E1162Δ*esp*:pEF3). Cell surface expression of Esp was analyzed by flow cytometry using rabbit anti-Esp immune serum. Esp expression was significantly enhanced ($P < 0.0005$) in E1162Δesp:pEF3 compared to E1162Δ*esp* and was comparable to, though slightly less than, amounts found in E1162 (Fig. 2). Whole-cell ELISA using rabbit anti-Esp IgGs confirmed significantly enhanced Esp expression in E1162Δesp:pEF3 compared to E1162Δ*esp* (Fig. 3), as did Western blot analysis (data not shown). Additionally, initial adherence to polystyrene ($P < 0.0001$) and biofilm formation ($P < 0.0005$) were significantly enhanced in

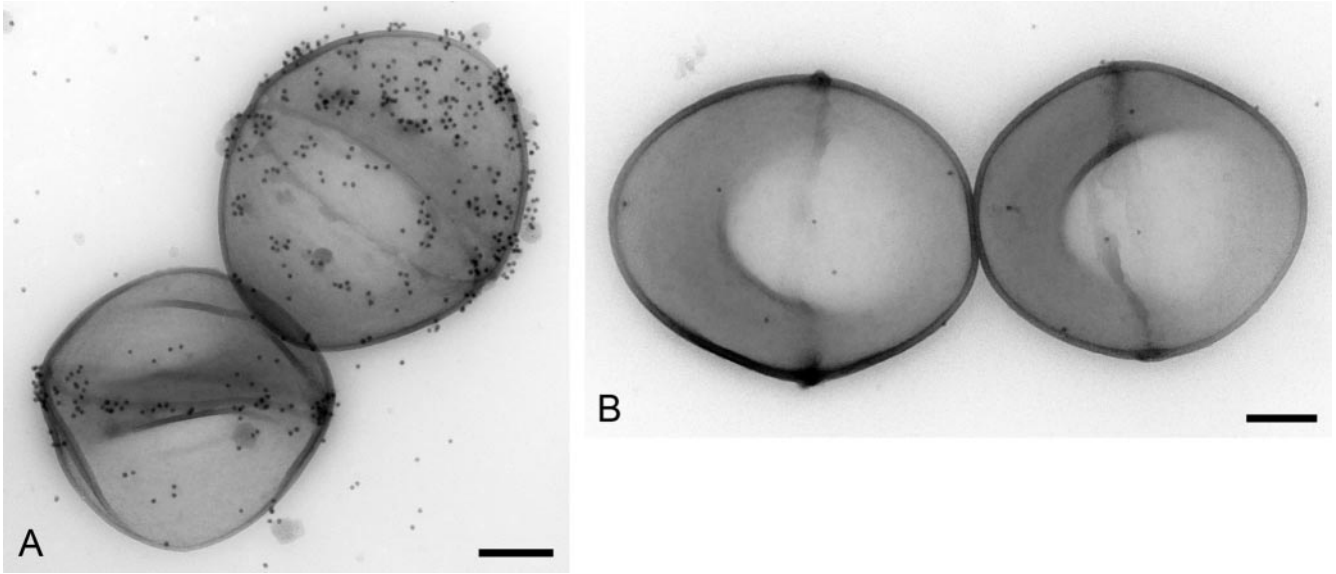

FIG. 4. Electron microscopy. Shown are electron micrographs at a magnification of ×60,000. The *esp* wild-type strain (E1162) (A) and the *esp* mutant strain (E1162Δ*esp*) (B) were incubated with rabbit anti-Esp immune serum, followed by protein-A-Gold. Bars, 200 nm.

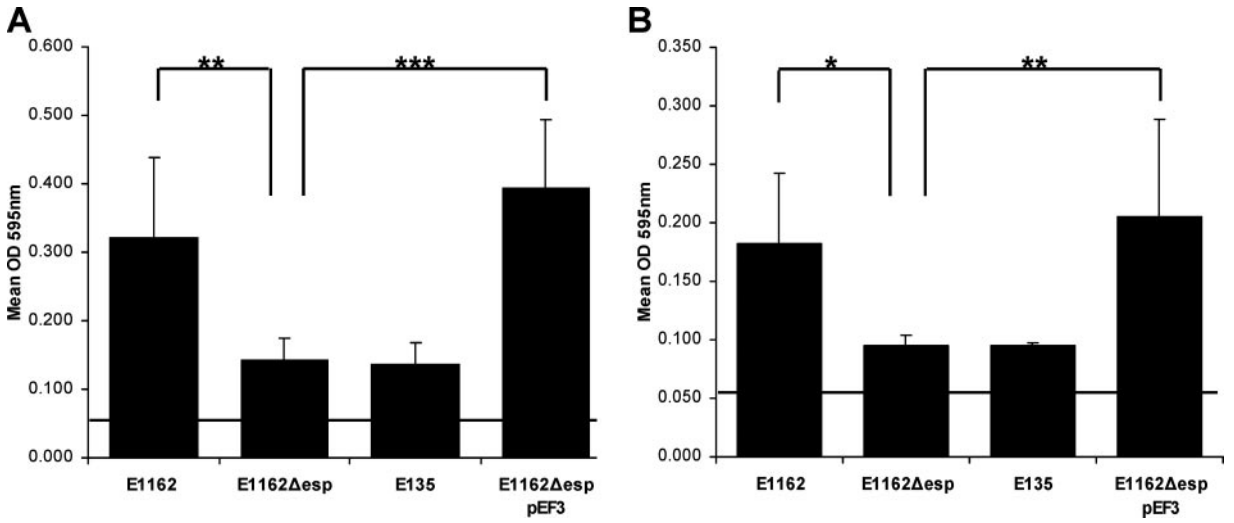

FIG. 5. Initial adherence and biofilm formation. Shown are the abilities to adhere to polystyrene (A) and to form biofilm (B) of the *esp* wild-type strain (E1162), *esp* mutant strain (E1162Δ*esp*), *esp*-negative strain (E135), and *esp*-complemented strain (E1162Δ*esp:*pEF3). The horizontal lines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

E1162Δesp:pEF3 compared to E1162Δ*esp* and were comparable to the levels found in E1162 (Fig. 5).

DISCUSSION

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

Initial adherence and biofilm formation are both considered important pathogenic properties of enterococci (5, 7). The

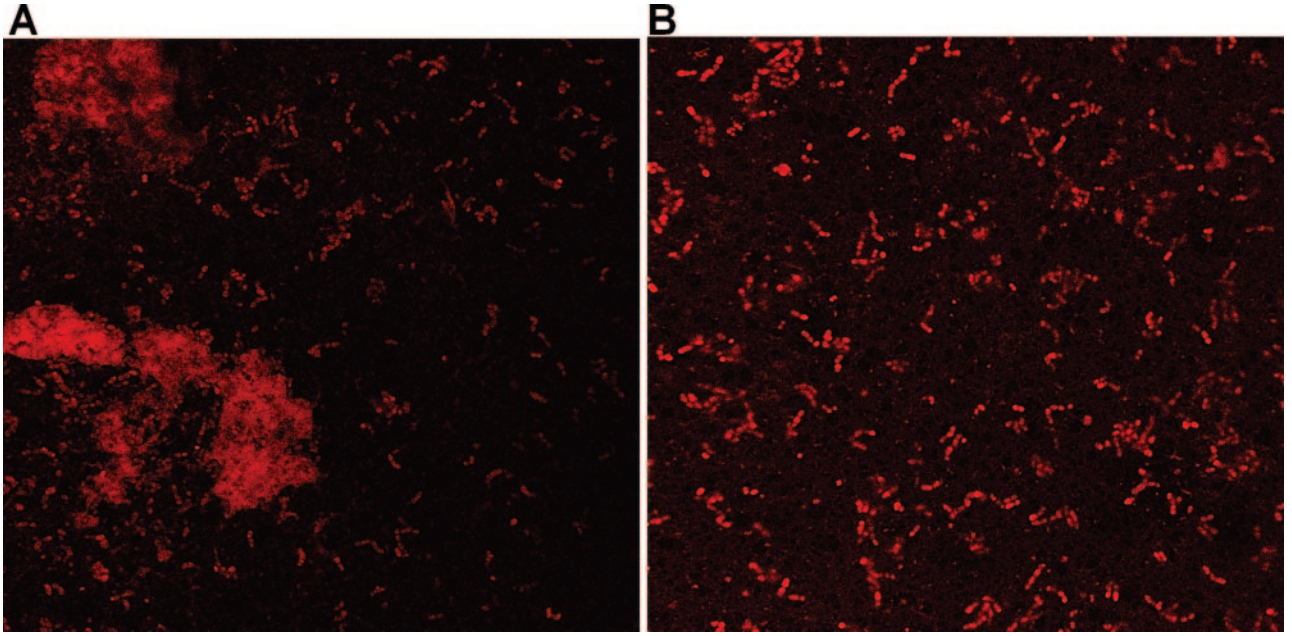

FIG. 6. CLSM images of the *esp* wild-type strain (E1162) (A) and the *esp* mutant strain (E1162Δ*esp*) (B) grown on nitrocellulose for 24 h. The images represent the layer in a Z-stack that has the maximum bacterial coverage. The maximum thickness of biofilms was measured at five randomly chosen positions,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

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

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

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

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

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