Role of *Enterococcus faecalis* Surface Protein Esp in the Pathogenesis of Ascending Urinary Tract Infection

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Enterococcus faecalis bacteria isolated from patients with bacteremia, endocarditis, and urinary tract infections more frequently express the surface protein Esp than do fecal isolates. To assess the role of Esp in colonization and persistence of *E. faecalis* in an animal model of ascending urinary tract infection, we compared an Esp⁺ strain of *E. faecalis* to its isogenic Esp-deficient mutant. Groups of CBA/J mice were challenged transurethrally with 10⁸ CFU of either the parent or mutant strain, and bacteria in the urine, bladder, and kidneys were enumerated 5 days postinfection. Significantly higher numbers of bacteria were recovered from the bladder and urine of mice challenged with the parent strain than from the bladder and urine of mice challenged with the mutant. Colonization of the kidney, however, was not significantly different between the parent and mutant strains. Histopathological evaluations of kidney and bladder tissue done at 5 days postinfection did not show marked histopathological changes consistent with inflammation, mucosal hyperplasia, or apoptosis, and there was no observable difference between the mice challenged with the parent and those challenged with the mutant. We conclude that, while Esp does not influence histopathological changes associated with acute urinary tract infections, it contributes to colonization and persistence of *E. faecalis* at this site.

The pathogenesis of complicated and uncomplicated urinary tract infection (UTI) is complex and influenced by many host biological and behavioral factors and by properties of the infecting uropathogens. Leading etiological agents of UTIs include *Escherichia coli*, *Candida albicans*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* (27). The incidence of UTIs due to *E. faecalis* has risen steadily over the years, and infections due to multiple-drug-resistant strains present a significant medical problem (11). *Enterococcus* spp. rank third among the most common pathogens isolated from intensive care unit patients with UTIs (23) and are a common cause of chronic or recurrent UTIs, especially those associated with structural abnormalities and instrumentation (5, 17). In spite of the role of *E. faecalis* as a leading cause of nosocomial UTI, little is known about the bacterial factors involved in such infections.

The interaction between enterococci and uroepithelial tissue has been examined previously (16) with the goal of identifying a role for plasmid-encoded aggregation substance in the adhesion of enterococci to renal epithelial cells in vitro. In a study of *E. faecalis* isolates from patients with UTI and endocarditis, Guzman and coworkers (6) showed that UTI isolates adhered efficiently to urinary tract epithelial cells and less effectively to Girardi heart cells. The adherence of UTI isolates to Girardi heart cells was, however, enhanced eightfold by growth of the bacteria in human serum. The nature of the interaction of

enterococci with uroepithelial tissue appears to be quite complex, with a role for bacterial cell surface carbohydrate and protein (6, 26).

About one-third of *E. faecalis* isolates from patients with bacteremia and UTIs express the Esp protein, compared to its rare occurrence in fecal isolates, suggesting that this surface protein may play an important role during these infections (25). The unique architecture of the Esp protein, with multiple repeat motifs, is characteristic of many bacterial surface protein adhesins involved in binding to host ligands (1, 7, 21). It was hypothesized, therefore, that Esp may play a role similar to that of the fimbriae of *E. coli* and *P. mirabilis* in serving as a colonization factor promoting adherence to uroepithelium. To test the role of Esp during UTIs, we constructed an isogenic Esp-deficient mutant by allelic replacement of the *esp* gene with a chloramphenicol resistance cassette. The wild-type and isogenic mutant strains were then compared in a mouse model of ascending UTI, for their ability to colonize and persist at anatomical sites of the urinary tract.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. faecalis* MMH594 is a clinical bacteremia isolate that caused multiple infections in a hospital ward outbreak and is positive for Esp expression (12, 25). The isogenic Esp-deficient mutant (MMH594b) was created by allelic replacement of the *esp* gene with a chloramphenicol resistance cassette. *E. faecalis* strains were routinely cultivated in brain heart infusion (Difco Laboratories, Detroit, Mich.), whereas Luria-Bertani broth (24) was used for cultivation of *E. coli* strains. *E. coli* strain XL1-Blue was obtained from Stratagene (La Jolla, Calif.), and DH5 α was obtained from Life Technologies (Gaithersburg, Md.). Antibiotics (Sigma, St. Louis, Mo.) used for selection of *E. faecalis* strains included gentamicin (500 μ g/ml) for the wild-type strain and gentamicin (500 μ g/ml) plus chloramphenicol (20 μ g/ml) for the mutant. For maintenance of recombinant constructs in *E. coli*, ampicillin at 100 μ g/ml, chloramphenicol at 20 μ g/ml, and tetracycline at 15 μ g/ml were used

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where appropriate. Custom oligonucleotides were obtained from Integrated DNA Technologies (Coralville, Iowa). Restriction and modifying enzymes were purchased from New England Biolabs Inc. (Beverly, Mass.). Plasmids were introduced into electrocompetent *E. coli* or *E. faecalis* cells using a Gene Pulser unit (Bio-Rad Laboratories, Hercules, Calif.).

Construction of the isogenic mutant deficient in Esp expression. A conditionally replicating shuttle-suicide vector (pNS110) was constructed in multiple steps and targeted to the *esp* gene as follows. In order to generate Esp arms to target the *cat* cassette to the *esp* gene, inverse PCR was performed on purified, *Pst*I restricted, and self-ligated MMH594 DNA using the outward-facing primers Esp15B (GAGAgcgccgGATAGGTCGTGGACTAGCATTAGC) and Esp24N (GAGAgcgccgCCACGAGTTAGCGGGACAGGT). Inverse PCR amplification was performed using the Takara LA PCR kit, as suggested by the manufacturer (Panvera Corp., Madison, Wis.). Primers Esp15B and Esp24N corresponded to nucleotide positions 1242 to 1219 and 1551 to 1572 of the *esp* gene, respectively (25). The ~4-kb inverse PCR-amplified DNA product was gel purified and restricted with *Bss*HII and *Nor*I to cleave restriction sites built into the primers Esp15B and Esp24N, respectively. An 851-bp chloramphenicol resistance (CAT) determinant was amplified from plasmid pGB354 (30) using the primer pairs GAGAgcgccgGGCAACGTGAATTAGGTTTGA and GAGAgcgccgGATCACTTACGTGTATAAAATTA, and the amplification product was restricted with *Bss*HII/*Nor*I and subsequently gel purified. The CAT determinant was then ligated to the *Bss*HII/*Nor*I-cut inverse PCR product obtained above from MMH594. Primers Esp58E (GAGAggaattcGGTGTAGGCCTTGTT TTTGGGG; nucleotide positions 187 to 208) and Esp26X (GAGActcgagCGT GCCTACAGAACCATTCTTG; nucleotide positions 2280 to 2260 of the *esp* gene) were then used to amplify from this construct a 2.6-kb DNA segment that consisted of the 851-bp CAT determinant flanked by 1,063-bp and 720-bp regions of the *esp* structural gene. This 2.6-kb amplified product was restricted with *Eco*RI and *Xho*I and cloned into the plasmid vector pBluescript II SK(–) to generate pSK5826, and plasmid DNA was prepared from transformed *E. coli* XL1-Blue cells.

To facilitate the identification of single-crossover integrants compared with double-crossover integrants, a second antibiotic resistance marker was introduced as follows. The 1.7-kb tetracycline resistance (Tet^r) determinant from pT181 (3) was PCR amplified using primers T181-L (GAGAggattcCGCC AGTCGATTTAACGGAC) and T181-R (GAGAggattcATACGTGTGCTCT GCGAGGC), restricted with *Bam*HI, and cloned into *Bam*HI-restricted pSK5826. The recombinant plasmid pSKT5826 was purified from transformed *E. coli* DH5 α . For conditional replication in both gram-positive bacteria and *E. coli*, the temperature-sensitive origin of replication, *rep4*(Ts), of plasmid pTV10K (4) was PCR amplified using the primers Ts-L (CCACTAATAACTACAATA-GAGAGATGTCACCG) and Ts-R (GAGActcgagGCCTTGAAACATTGGTT-TAGTGGG), gel purified, and restricted with *Xba*I and *Xho*I. The *Xba*I/*Xho*I fragment was cloned into pBluescript SK(–) to generate pSKTs, and recombinant plasmid DNA was purified from XL1-Blue transformants. The Ts replicon region from pSKTs and the 4.5-kb insert from pSKT5826 were gel purified after restriction of the respective plasmids with *Xba*I/*Xho*I and ligated together to generate pNS110. The entire construct pNS110 was sequenced using custom Cy5-labeled primers by a standard chain termination method employing the T7 DNA polymerase-based Autoread sequencing kit (Amersham Pharmacia Biotech Inc., Piscataway, N.J.) and determined to be 6,747 bp in size.

An Esp-deficient mutant was generated from parent strain MMH594 by homologous recombination and allele replacement, adopting a protocol reported earlier (18). The single-crossover and double-crossover integrants were analyzed by both PCR and Southern blot hybridization to verify proper integration. The double-crossover integrant (MMH594b) along with the parent strain was used for the studies described in this report.

Antiserum to Esp. Polyclonal rabbit antiserum to purified Esp was raised by immunization of New Zealand White rabbits, and the reactivity and specificity of the antiserum were determined as previously described (25).

Enzyme-linked immunosorbent assay. The expression and localization of Esp at the cell surface of parent and mutant strains were verified using whole-cell enzyme-linked immunosorbent assay as follows. Briefly, 10⁵ CFU of the parent and mutant strains suspended in 50 μ l of 100 mM carbonate buffer, pH 9.6, was coated in triplicate wells of a 96-well microtiter plate and allowed to bind at 4°C overnight. Loosely adherent cells were washed off by gentle rinsing with phosphate-buffered saline (PBS), and exposed areas of the polystyrene wells were blocked with 2% bovine serum albumin in PBS. One hundred microliters of polyclonal Esp-specific rabbit antiserum, diluted 1:500 in PBS containing 0.05% Tween 20 (PBST), was applied to each well and incubated at 37°C for 4 h. The microtiter wells were rinsed three times with PBST using an automated microtiter plate washer (Bio-Rad Laboratories), followed by the addition of 100 μ l of

a 1:10,000 dilution of goat anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase. Incubation was carried out at 37°C for 2 h, the wells were rinsed three times with PBST, and Esp expression was quantified by measuring conversion of the chromogenic substrate *p*-nitrophenyl phosphate in 10 mM diethanolamine buffer, pH 9.5. The absorbance of each well was read at 405 nm after a 30-min incubation at 37°C.

Immunogold labeling of Esp and high-resolution scanning electron microscopy. Esp was visualized on the bacterial cell surface using a combination of colloidal gold immunolabeling and low-voltage scanning electron microscopy (LVSEM) by adopting a protocol previously described for enterococcal aggregation substance (22). Overnight or exponential-phase bacterial cultures were washed twice and resuspended to a concentration of 10⁸ cells per ml in 10 mM PBS (pH 7.4). Glass chips (4 by 8 mm) were cleaned with 95% ethanol and coated with 0.1% poly-L-lysine for 10 min. Excess poly-L-lysine was rinsed off, and 30 μ l of each bacterial suspension was placed on individual chips for 10 min. Excess bacteria were washed off gently using Hanks' balanced salt solution (HBSS) containing 0.5% bovine serum albumin, and 20 μ l of a 1:50 dilution in HBSS of purified IgG (10 mg/ml) from rabbit polyclonal antiserum to Esp was applied for 1 h at 37°C. Bacteria were then gently washed with HBSS containing 0.5% bovine serum albumin, and 20 μ l of a 1:5 dilution of goat anti-rabbit IgG conjugated to 12-nm colloidal gold particles (Jackson ImmunoResearch Laboratories, West Grove, Pa.) was applied for 10 min at room temperature. Finally all samples were washed gently with HBSS and placed in fixative (2.5% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer containing 7.5% sucrose).

For high-resolution LVSEM, the fixative was washed from the samples twice for 10 min in 0.1 M sodium cacodylate with 7.5% sucrose buffer and postfixed for 30 min in 0.1 M sodium cacodylate containing 1% osmium tetroxide and 7.5% sucrose. The samples were then washed twice with 0.1 M sodium cacodylate, dehydrated with ethanol, critical point dried by the CO₂ method, and coated with a 1- to 2-nm discontinuous layer of platinum by using a sputter coating system (VCR Group, South San Francisco, Calif.). *E. faecalis* cells were viewed with a Hitachi S-900 field emission scanning electron microscope operated at low accelerating voltages (1.3 to 5 keV), using a scatter electron detector for conventional topographical imaging and a high-resolution yttrium-argon-garnet back scatter electron detector (29) for the visualization of colloidal gold by atomic number contrast.

Phenotypic characterization of the Esp-deficient mutant. In vitro growth rates in broth cultures were compared for the parent and mutant, in either the presence or the absence of chloramphenicol selection, using standard techniques. The stability of the Cm^r determinant in the absence of antibiotic selection was also assessed. A single colony of the allelic replacement mutant was allowed to undergo approximately 96 doublings in broth culture without antibiotic selection. One hundred colonies from this culture were replica plated on agar plates with and without chloramphenicol, to check for loss of chloramphenicol resistance.

CBA mouse model of ascending UTI. A modified ascending UTI model, as described earlier (13), was used to assess the virulence of parent and mutant *E. faecalis* strains. Six- to eight-week-old CBA/J mice (Jackson Laboratories, Bar Harbor, Maine) were used. All animal experiments were conducted in accordance with relevant federal guidelines and institutional policies. Prior to bacterial challenge, spontaneously voided urine was collected in a sterile petri dish; bacteriuric mice were not used. Mice ($n = 20$) were challenged while anesthetized with methoxyflurane (Metofane; Pitman-Moore, Inc., Washington Crossing, N.J.) by inserting a polyethylene catheter (2.5 cm long; outer diameter, 0.61 mm; Clay Adams, Parsippany, N.J.) into the bladder through the urethra and infusing 0.05 ml of a suspension containing 2×10^8 CFU into the bladder over a 30-s period. Mice were challenged with a suspension of either *E. faecalis* MMH594 or the isogenic Esp-deficient mutant. The urethral catheter was removed immediately after challenge, and mice were cared for by the normal routine. Mice were inspected daily to monitor morbidity and mortality. At 5 days after transurethral challenge, quantitative cultures of the urine, bladder, and kidneys were performed as previously described (13). Segments of bladder and kidneys were preserved in 10% neutral formalin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy. The pathologist examining the tissue sections was blinded to the experimental procedure.

A standard histology scoring system for bladder mucosa and submucosa was followed, and the degree of inflammation was graded as follows: acute, 0, no inflammation; 1+, few neutrophils; 2+, scattered neutrophils not forming microabscesses; 3+, numerous neutrophils in clusters; chronic (based on the degree of lymphocytes and plasma cells in the submucosa), 0, none; 1+, rare; 2+, small aggregates measuring <100 μ m; 3+, larger aggregates. The thickness of the epithelium was evaluated, and the degree of hyperplasia was graded as follows: 0, epithelial morphology identical to that of normal controls (two to three layers);

FIG. 1. Schematic of the strategy used to create an *Esp*-deficient mutant. Gray arrows within pNS110 denote the directions of transcription for these determinants. Black arrows adjacent to the *esp* gene on the MMH594 chromosome indicate the positions of primers used to verify proper integration in the single-crossover and double-crossover mutants.

1+, epithelium with three to four cell layers and normal cytoplasmic surface maturation; 2+, epithelium with three to four cell layers and reduced cytoplasmic volume in surface cells; 3+, irregular epithelial crowding with patchy areas showing more than four cell layers and nuclear crowding; 4+, epithelium with diffuse thickening, more than four cell layers, and nuclear crowding-palisading. Increased epithelial cell turnover was graded, based on the presence of apoptotic bodies, as follows: 0, none; 1+, extremely rare; 2+, occasional; 3+, numerous with mitosis.

The histologic criteria used for evaluation of renal lesions included degree and types of inflammatory infiltrates in renal parenchyma and pelvis (epithelium and subepithelial connective tissue), necrosis of transitional and tubular epithelium, purulent casts in collecting ducts, intraparenchymal abscess formation, parenchymal interstitial fibrosis-tubular atrophy, and pelvic fibrosis (13).

Statistics. Means of quantitative counts of urine, bladder, and kidneys from mice challenged with the parent strain were compared with means from mice challenged with the mutant strain by Student's *t* test. Differences in the number of mice with urine, bladder, or kidneys colonized with the challenge organisms were compared by chi-square analysis.

RESULTS

Characterization of the *Esp*-deficient mutant. An isogenic mutant of MMH594 that was deficient in *Esp* expression was constructed by replacement of an intragenic region of the *esp* gene with a chloramphenicol resistance cassette. As shown in Fig. 1, regions including the 5' and 3' ends of the *esp* gene were cloned into the suicide-shuttle plasmid pNS110, to target the insertion vector to the *esp* gene on the chromosome of

FIG. 2. Ethidium bromide-stained agarose gel electrophoresis of PCR-amplified products from parent (MMH594), single-crossover integrant (SCO), and double-crossover integrant (DCO; MMH594b). Molecular size markers are shown in lane M.

MMH594. Confirmation of the single- and double-crossover mutations was done by PCR, and the amplification products were analyzed by gel electrophoresis (Fig. 2). In separate experiments, genomic DNA from the parent and mutant strains was also analyzed by restriction mapping and Southern blot hybridization, using nucleotide probes to both the *esp* gene and the chloramphenicol resistance determinant. These experiments confirmed the nature of the mutations (data not shown). To prevent any reversion or possible polar effects stemming from plasmid sequences in the single-crossover integrant, only the double-crossover integrant (MMH594b) was used in further studies.

Phenotypic characteristics of the mutant. To confirm that the mutant (MMH594b) was indeed deficient in *Esp* expression as expected, two immunological approaches were employed. In the first instance, specific antiserum to *Esp* failed to bind to MMH594b cells bound to 96-well polystyrene microtiter plates, as evaluated by enzyme-linked immunosorbent assay (data not shown). Secondly, affinity-purified antibodies to *Esp* failed to detect any *Esp* at the cell surface of the mutant strain under conditions where it was readily detected on the surface of the wild-type organism (Fig. 3). These results unambiguously demonstrated that the mutant strain lacked *Esp* on the cell surface.

In vitro growth rates in broth cultures were determined to verify that the inactivation of the *esp* gene did not affect growth and survival of the mutant. As shown in Fig. 4, no difference was observed between parent and mutant during growth in the presence or absence of chloramphenicol selection. Moreover, when a single colony representing the mutant strain was al-

FIG. 3. Back scatter electron imaging of colloidal gold-immunolabeled Esp protein. (A) Representative view of the isogenic mutant strain (MMH594b) which exhibits no binding of colloidal gold to the cell surface. (B) Typical view of the parent strain MMH594.

lowed to undergo 96 doublings in the absence of antibiotic selection, 100 of 100 colonies replica plated on agar plates retained the Cm^r phenotype, confirming its stability.

Model UTI studies. Colonization of both urine and bladder at day 5 after transurethral challenge with 10^8 CFU of *E. faecalis* MMH594 parent strain or the Esp-deficient isogenic

FIG. 4. In vitro growth characteristics of the parent and isogenic mutant strains in brain heart infusion broth. Data points along the growth curve represent the means of three independent measurements from bacteria grown without antibiotic selection. Growth characteristics of parent and mutant strains grown in the presence of antibiotics were identical and superimposable.

mutant per mouse was significantly ($P < 0.01$ for each site) lower in mice challenged with the isogenic mutant ($n = 20$) than in mice challenged with the parent strain ($n = 20$). Numbers of CFU ($\text{log}_{10} \pm$ standard error of the mean) at each site were as follows: urine, mutant, 1.54 ± 0.62 , versus parent, 4.39 ± 0.078 ; bladder, mutant, 1.34 ± 0.47 , versus parent, 3.32 ± 0.50 . While levels of colonization of the kidney by the mutant tended to be lower than those of colonization by the parent (3.16 ± 0.33 versus 3.99 ± 0.29), significance at a level of ≤ 0.05 was not achieved ($P = 0.062$). Figure 5 shows the distribution data for each animal at each site tested. For urine, 15 of 20 mice challenged with the mutant had counts below 10^2 CFU/ml versus 7 of 20 mice challenged with the parent strain ($P = 0.011$, chi-square test). For bladder, 14 of 20 mice challenged with the mutant had counts below 10^2 CFU/ml versus 6 of 20 mice challenged with the parent strain ($P = 0.011$), and 11 of 40 kidneys from mice challenged with the mutant had counts below 10^2 CFU/ml versus 6 of 40 kidneys from mice challenged with the parent strain ($P = 0.17$).

Although statistically significant differences in quantitative colony counts from urine and bladder were observed, no significant differences in histology scores between mutant and wild-type infections were found. Histology scores for acute inflammation in kidneys were as follows: 14 of 40 kidneys from mice challenged with the mutant versus 13 of 40 kidneys from mice challenged with the parent strain had a histology score of 0, 14 of 40 kidneys from mice challenged with the mutant versus 15 of 40 kidneys from mice challenged with the parent strain had a histology score of 1, and 12 of 40 kidneys from mice challenged with the mutant versus 12 of 40 kidneys

ered from the kidneys of mice challenged with the parent strain and those from kidneys of mice challenged with mutant strains. It is possible that other surface adhesins, such as aggregation substance, contribute to binding to renal epithelial cells, making the pathogenesis of *E. faecalis* UTI a multistep, multifactorial process. In the present study, both the parent and mutant strains express aggregation substance. We are currently examining strains possessing various combinations of Esp and aggregation substance for differences in localization, as this hypothesis would suggest.

A novel feature of the Esp protein is the presence of identical, large (82- and 84-amino-acid) repeat motifs encoded by nearly identical tandem repeating units within the structural *esp* gene. Homologous recombination within these repeat units at the genetic level leads to addition or deletion of repeat units, resulting in an alteration in the size of the encoded protein. We have shown previously that *E. faecalis* isolates do indeed express altered forms of the Esp protein that vary in size depending on the number of repeating units (25). It was postulated that this variation in size of Esp at the cell surface could define an environment-specific function for Esp. Consequently, an extended form of the Esp protein might be involved in adhesion functions during the initial stages of infection, facilitating interaction with host receptors. Subsequent to establishment in the host, an extended form of the surface protein may be detrimental to survival and persistence, favoring expression of a less-extended form of Esp to evade the immune response, analogous to the phase variation observed for uropathogenic *E. coli* (14).

The ability of *E. faecalis* to cause pyelonephritis in an experimental mouse model of infection has been reported elsewhere (8). In these experiments, *E. faecalis* alone when used to infect the bladders of mice at a concentration of 10^8 CFU caused pyelonephritis in 50% of infected animals after 7 days. These and other studies (28) have also suggested that *E. faecalis* may enhance the onset and clinical severity of UTIs caused by other uropathogens such as *E. coli* and *P. aeruginosa* during mixed infections. The molecular basis for this synergism during mixed infections remains unexplained. Our histopathological data showed no significant differences between bladder and kidney tissue from mice infected with 10^8 CFU of the parent or mutant *E. faecalis* strain. One explanation may be that the 5-day postinfection point was suboptimal for pathological changes. Alternatively, *E. faecalis* may bind and activate bladder epithelial cells, setting the stage for secondary, more symptomatic infection. Identification of the role of Esp in the pathogenesis of enterococcal UTI is an important first step in dissecting this complex process.

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