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 10⁶ 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 XLI-Blue was obtained from Stratagene (La Jolla, Calif.), and DH5α was obtained from Life Technologies (Gaithersburg, Md.). Antibiotics (Sigma, St. Louis, Mo.) used for selection of E. faecalis strains included gentamicin (500 µg/ml) for the wild-type strain and gentamicin (500 µg/ml) plus chloramphenicol (20 µg/ml) for the mutant. For maintenance of recombinant constructs in E. coli, ampicillin at 100 µg/ml, chloramphenicol at 20 µg/ml, and tetracycline at 15 µg/ml were used.

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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, at 405 nm. 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 agglutination substance (22). Overnight or exponential-phase bacterial cultures were washed twice and resuspended in 100 mM PBS, pH 7.4. Infection of 0.5 to 10⁷ CFU/ml of bacteria per cell results in approximately 10³ to 10⁴ PFBs (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 μl of each bacterial suspension was placed on individual chips for 10 min. Excess bacteria were washed off gently using Hank’s balanced salt solution (HBSS) containing 0.5% bovine serum albumin, and 20 μl of a 1:50 dilution in HBSS of purified IgG (10 mg/ml) from rabbit polyclonal antisera to Esp was applied for 1 h at 37°C. Bacteria were then gently washed with HBBS containing 0.5% bovine serum albumin, and 20 μl 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 HBBS and placed in fixative (2.5% glutar- aldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer containing 7.5% sucrose).

For high-resolution LVSEM, the fixative was washed from the samples twice with 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 continuous layer of platinum by using a sputter field ion beam gun (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⁺ 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 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; bacterial urine was 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 suspending solution containing 2 × 10⁶ 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 procured, verified using routine stain techniques, stained with 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; and 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 μm; 3⁺, large 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);
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 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-
allowed to undergo 96 doublings in the absence of antibiotic selection, 100 of 100 colonies replica plated on agar plates retained the Cm' 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 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 ($\log_{10} \pm$ standard error of the mean) at each site were as follows: urine, mutant, $1.54 \pm 0.62$, versus parent, $4.39 \pm 0.078$; bladder, mutant, $1.34 \pm 0.47$, versus parent, $3.32 \pm 0.50$. While levels of colonization of the kidney by the mutant tended to be lower than those of colonization by the parent ($3.16 \pm 0.33$ versus $3.99 \pm 0.29$), significance at a level of $P < 0.05$ 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 strain 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 strain versus 12 of 40 kidneys...
from mice challenged with the parent strain had a histology score of 2. No other histologic changes were observed.

**DISCUSSION**

Despite the recognition that *E. faecalis* has emerged as an important uropathogen, much remains to be learned about the pathogenicity of this infection. Numerous studies of the two leading causes of community-acquired UTI, *E. coli* and *P. mirabilis*, have identified unique traits that are expressed by specific UTI isolates (2, 9, 10, 14, 19, 20, 27). Uropathogenic strains are highly adapted and possess specific factors that promote bladder colonization, survival in the urinary tract, and often the ability to induce tissue damage, including P fimbriae, hemolysin, serum resistance, and encapsulation. It is well established that adhesion to the bladder epithelium is a key initial step in UTI pathogenesis (20, 32). In *E. coli*, type 1 fimbriae, P and related fimbriae, and F1C fimbriae mediate the initial adherence preventing washout by urinary flow. Type 1 fimbriae bind to mannose-containing receptors, and the P group fimbriae bind to the Galα(1-4)Gal moiety of the P blood group and related receptors, which are widely distributed on the uroepithelium (31). The high-affinity binding of F1C fimbriae to the GalNAcβ1-4Galβ sequence of glycolipids, asialo-GM₁ (GgO₂Cer) and asialo-GM₂ (GgO₃Cer), and low-affinity binding to carbohydrate structures GlcNAcβ1-3Galβ, Galβ1-4Glc, Gal, and Glc of glycolipids have been demonstrated recently (15).

The Esp protein of *E. faecalis* is displayed on the cell surface. We previously showed a significant association of the Esp protein with *E. faecalis* isolated from patients with UTI compared to fecal isolates (25; N. Shankar, unpublished data). This localization and enrichment among UTI-derived isolates suggested a possible role for Esp in adherence and colonization. The results of this study support such a role and show that the effect is primarily localized in the bladder. Higher numbers of bacteria recovered from the urine of mice challenged with the parent strain reflect a bacteriuria resulting from colonization of the bladder.

Plasmid-encoded aggregation substance was found to contribute to *E. faecalis* adhesion to renal epithelial cells in vitro (16). However, to our knowledge, no reports have demonstrated a role for aggregation substance in colonization or persistence in the urinary tract during infection. Guzman et al. (5, 6) have shown that *E. faecalis* from patients with UTI adhered to urinary tract epithelial cells in vitro and suggested that carbohydrate antigens on the bacterial cell surface were responsible for this adherence. In a recent study (26), it was shown that 5 of 30 *E. faecalis* isolates from the urine of patients with UTI adhered efficiently to freshly isolated human bladder mucosa and to T-24 bladder carcinoma cells in culture. The adhesiveness of these isolates was inhibited by treatment with fibronectin or trypsin, implying that a specific protein on the bacterial cell surface was responsible for the adhesion. It is apparent from the studies described above that the nature of the interaction between enterococci and uroepithelial tissue can be quite complex, involving surface adhesins of a protein and/or carbohydrate nature.

Preliminary studies in our laboratory have shown no observable differences between the binding of Esp⁺ parent strains and that of isogenic Esp-deficient mutant strains to the porcine renal tubular cell line LLC-PK₁. This observation is not surprising given that the in vivo studies reported here found no significant differences between the number of bacteria recov-
tered 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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