Hyperadhesive Mutant of Type 1-Fimbriated *Escherichia coli* Associated with Formation of FimH Organelles (Fimbriosomes)

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The relationships of the genes and gene-products mediating D-mannose-specific attachment of type 1 fimbriae of *Escherichia coli* to eucaryotic cells were investigated by deletion mutation analysis of recombinant plasmid pSH2, which carries the genetic information for the synthesis and expression of functional type 1 fimbriae. Mutant pUT2004 was derived by a deletion remote from the structural gene encoding the 17-kilodalton (kDa) subunit protein of type 1 fimbriae. Phenotypically, the mutant demonstrated an eightfold-higher mannose-specific hemagglutination titer than the parent strain. On electron microscopy, the mutant strain expressed the same number of fimbriae as the parent strain. However, numerous 10-nm-diameter rounded structures (fimbriosomes) were observed both closely associated with fimbriae and in the culture medium. Fimbriosomes isolated from the medium agglutinated guinea pig erythrocytes in a mannose-sensitive manner. Dissociation of the fimbriosomes yielded a single 29-kDa protein, as demonstrated by sodium dodecyl sulfate gel electrophoresis. Antibodies raised against fimbriosomes reacted with a 29-kDa protein on immunoelectroblots of dissociated type 1 fimbriae and also blocked the adherence of other strains of type 1 fimbriated *E. coli* to eucaryotic cells. These findings suggest that the enhanced adhesive properties of the mutant pUT2004 strain are associated with overproduction of the 29-kDa FimH in the form of fimbriosomes which contain the determinant of the D-mannose-sensitive adhesion of type 1 fimbriae.

Type 1 fimbriae are hairlike appendages that are produced by *Escherichia coli* and by other members of the family *Enterobacteriaceae*. These fimbriae are characterized by their ability to bind to D-mannose-containing receptors on eucaryotic cells (7, 21). The role of type 1 fimbriae in mediating adherence of bacteria to mucosal surfaces of animals has led to the conclusion that these adhesins are important determinants of bacterial virulence (1, 8). The biosynthesis, assembly, and expression of type 1 fimbriae are determined by a cluster of genes which map at 98 min on the *E. coli* chromosome. At least eight genes have been identified in the *fim* cluster, and the functions of some of them are now becoming clear. The gene *fimA* encodes a 17-kilodalton (kDa) subunit which makes up most of the fimbrial structure (9). *fimC* and *fimD* are thought to be involved in fimbrial assembly, because mutants lacking these genes fail to produce fimbriae even though they synthesize and process the *fimA* gene product (12, 20). *fimB* and *fimE* determine fimbrial phase variation; products of these genes control the orientation of a 300-base-pair invertible phase switch that contains the promoter for *fimA* (5, 10). Recent studies have demonstrated that the intact type 1 fimbria is composed of at least two additional but minor 14-kDa (FimG) and 29-kDa (FimH) subunits (3). These proteins are encoded by genes which also map in the *fim* cluster (11, 17).

Recently we undertook a study to identify the genetic determinants responsible for mediating the D-mannose-binding properties of type 1 fimbriae of *E. coli*. These studies involved deletion mutation analysis of a recombinant plasmid, pSH2, which carries the genes necessary for the expression of functional fimbriae, followed by immunochem-

ical analysis of the resulting mutants (18). We found that a mutant of pSH2, pUT2002, containing a deletion remote from the *fimA* gene failed to mediate D-mannose-specific binding even though fimbriae morphologically identical to those of the wild type were expressed (18). Immunochemical analysis of the nonadhesive mutant fimbriae revealed no change in the immunological and chemical properties of FimA, but the two ancillary FimG and FimH proteins were found to be missing (3, 18). This finding indicated that the D-mannose-binding property of type 1 fimbriae did not depend on FimA, the major subunit, but on one or both ancillary subunits. Similar conclusions were reached independently by Maurer and Orndorff (16, 17), who recently provided additional evidence, from analysis of nonhemagglutinating mutants, that the *fimH* gene product (PilE) is responsible for mannose binding (16, 17).

In this communication, we describe the physical, biological, and immunological characteristics of a hyperadhesive mutant of pSH2 and report evidence that its hyperadhesive properties are due to overproduction of the ancillary fimbrial subunit FimH.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* ORN103 [*thr-1 leu-6 thiΔ(argF-lac)U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 fhuA2 minA minB recA13Δ(fimABCDEFGH)*], which is deleted for all of the *fim* genes, was used as the host strain for plasmid pSH2 and its derivative pUT2004. This strain was the kind gift of Paul Orndorff. For best expression of fimbriae, ORN103(pSH2) and ORN103(pUT2004) were cultured in Luria broth under static conditions for 48 h. To maintain the plasmid in the host, chloramphenicol (30 µg/ml) was used in the culture medium. Other *E. coli* strains expressing type 1 fimbriae used in this study were CSH50

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[*araΔ(lac-pro) rps L thi*], a well-characterized K-12 strain (4-6), and C15, isolated from a patient with a urinary tract infection (1).

Recombinant DNA techniques. Plasmid purification, restriction endonuclease digestion, mapping, isolation of restriction fragments, ligation, transformation, and agarose gel electrophoresis of plasmid DNA were performed by standard methods (15).

Isolation and purification of type 1 fimbriae. Type 1 fimbriae were isolated from all strains of *E. coli* by the method of Dodd and Eisenstein (6). The purity of the fimbrial preparations was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by electron microscopy.

Immunization of rabbits. New Zealand White rabbits were injected subcutaneously with 100 μ g of the fimbriosomes (see Results) which had been emulsified in complete Freund adjuvant. Blood was obtained before the initial injection and at 2-week intervals thereafter. At 4 and 10 weeks the animals were given booster injections of 100 μ g of the purified protein in phosphate-buffered saline.

Assays of antibody reactivity. Detection and titration of antibodies were performed with an enzyme-linked immunosorbent assay (ELISA) of the antiserum. The assay was performed as described previously (2) except that purified fimbriosomes (see Results) were absorbed to ELISA trays as the solid-phase antigen. Because these antibodies demonstrated weak cross-reactivity with the structural subunit FimA, the antiserum was preabsorbed with FimA as described previously (3).

SDS-PAGE. SDS-PAGE was performed in a 1.5-mm thick 15% acrylamide slab gel by the system of Laemmli (13). Samples of fimbriae were dissociated before electrophoresis by heating in acid (6).

Immunoelectroblots of fimbriae. After SDS-PAGE, the bands of fimbrial protein were electrophoretically transferred onto nitrocellulose paper strips as previously described (4). The nitrocellulose strips were then incubated with a 1:200 dilution of antiserum, followed by peroxidase-labeled goat anti-rabbit immunoglobulin G antibody and finally by the substrate (2).

Electron microscopy. Samples of purified type 1 fimbriae or type 1 fimbriated bacteria were applied to Formvar-coated copper grids and placed in a drop of phosphotungstic acid. The samples were viewed in a Zeiss electron microscope. To examine the binding of antibodies to the fimbrial structure, grids coated with type 1 fimbriae were incubated in a drop of 0.5% bovine serum albumin for 15 min and then incubated in a drop of antiserum, diluted 1:40, for 20 min at ambient temperature. The grids were then washed several times in deionized water and incubated for another 20 min at ambient temperature in a drop of gold-conjugated protein A, diluted 1:30. The grids were then washed several times and finally stained with phosphotungstic acid and viewed under the electron microscope.

Adherence inhibition assays. The ability of antibodies evoked against the fimbriosomes to block attachment of type 1 fimbriated *E. coli* to human buccal epithelial cells was examined with a previously described ELISA procedure (19).

Hemagglutination assays. Twofold serial dilutions of a bacterial suspension were mixed with an equal volume of 2% guinea pig erythrocytes. The hemagglutination titer was determined after 2 h of incubation at 4°C.

FIG. 1. Restriction map of the *fim* locus in *E. coli*. The *fim* genes are indicated as heavily stippled rectangles, and their location is based on the DNA sequence determined by Klemm and Christiansen (11). Arrowheads P, C, and B indicate the locations of recognition sites for the restriction endonucleases *Pvu*II, *Cla*I, and *Bam*HI, respectively. The heavy line below the bar indicates the region deleted in pUT2004. kb, Kilobase. A through F, Products of the genes indicated by the heavily shaded areas.

RESULTS

Origin of pUT2004. pUT2004 (18) was constructed by subjecting pSH2 to partial digestion with *Pvu*II, followed by religation and transformation. One of the resulting transformants was found to carry a plasmid with a restriction map identical to that of pSH2 except for the loss of a single *Pvu*II fragment (Fig. 1). This plasmid was designated pUT2004 and used in the following studies.

Hyperadhesive properties of *E. coli* ORN103(pUT2004). In static culture, ORN103(pUT2004) grew in large aggregates and formed a massive pellicle on the surface of the culture medium. Although formation of a pellicle on the surface of a liquid culture is characteristic of all type 1-fimbriated bacteria, the size of the pellicle formed by the mutant was much greater than that produced by the parent strain, ORN103 (pSH2). The formation of both bacterial clumps and surface pellicle in culture by the mutant could be neutralized by the incorporation of 1% α -methyl-D-mannoside but not by α -methyl-D-glucoside, indicating that these traits were manifestations of the D-mannose-binding moiety of type 1 fimbriae. Consistent with this observation, the mutant had an eightfold-higher hemagglutination titer than its parent.

When plasmid pUT2004 was introduced into other *E. coli* K-12 strains, such as LE392 and HB101, bacterial clumping and massive pellicle formation were again observed, indicating that the hyperadhesive property was determined by the plasmid and not by factors associated with the bacterial host. It would appear that the mutant ORN103(pUT2004) was exhibiting the same D-mannose-binding property as type 1-fimbriated ORN103(pSH2) *E. coli* but at a much higher level than the parent strain.

Electron microscopy of ORN103(pUT2004). Electron microscopic examination of ORN103(pUT2004) revealed a normal number, length, and diameter of type 1 fimbriae, although many of them were slightly kinked or broken (Fig. 2A and B) compared with the wild-type pSH2-encoded fimbriae (Fig. 2C). However, numerous spherical structures of various diameters appeared closely associated with the fimbriae and in the surrounding medium (Fig. 2). For convenience, we designated these spherical structures fimbriosomes. These observations suggested that the hyperadhesive phenotype of the mutant was related to the presence of fimbriosomes.

Isolation and purification of fimbriosomes. After removal of the bacteria by sedimentation, the culture supernatant was collected and the free fimbriosomes were sedimented by centrifugation at $110,000 \times g$ for 20 h. We found that the fimbriosomes could be further purified by absorption to

FIG. 2. (A and B) Electron micrographs of negatively stained hyperadhesive mutant ORN103(pUT2004) expressing rounded fimbriosomes. On higher magnification (B), note the close association of fimbriosomes to the kinked and broken fimbrial filaments. (C) Electron micrograph of ORN103 containing the parent plasmid pSH2, showing normal fimbria and absence of fimbriosomes. Magnifications: A, $\times 30,000$; B and C, $\times 100,000$.

FIG. 3. Electron micrograph of purified fimbriosomes. Magnification, $\times 120,000$.

yeast cells that contained surface mannan rich in D-mannose. The crude suspension of fimbriosomes was absorbed with three batches of yeast cells, followed by elution of the fimbriosomes from the yeast cells with α -methyl-D-mannoside. After each elution step and before absorption with the next batch of yeast cells, the preparation of fimbriosomes was dialyzed against phosphate-buffered saline to remove all traces of α -methyl-D-mannoside. The material obtained after three cycles of absorption consisted of a homogeneous preparation of fimbriosomes, as determined by electron microscopy (Fig. 3) and by SDS-PAGE. No residual fimbriae were detected in the preparation.

Like type 1 fimbriae, fimbriosomes did not easily penetrate SDS gels and had to be boiled at pH 1.0 for 10 min to dissociate into subunits. SDS-PAGE of the fimbriosome preparation revealed a single 29-kDa band (Fig. 4), suggesting that the fimbriosomes were composed entirely of FimH.

Immunological cross-reactivity between fimbriosomes and the FimH subunit of type 1 fimbriae. To see whether the 29-kDa fimbriosome protein was similar to FimH, we reacted antibodies raised against fimbriosomes with dissociated fimbriae from *E. coli* ORN103(pSH2). We found that the antibody reacted with a doublet band of protein in the region of 29 kDa in a Western blot (immunoblot) (Fig. 5, lane A); whether the lower-molecular-weight band represents a truncated form of the 29-kDa protein was not determined. Similar reactions were seen when type 1 fimbrial preparations from a K-12 (CSH50) and a clinical strain (CI5) of *E. coli* were exposed to antibodies against fimbriosomes (Fig. 5, lanes B and C). Moreover, antibodies raised against synthetic peptides corresponding to the amino-terminal region

FIG. 4. SDS-PAGE of dissociated fimbriosomes, showing a single Coomassie blue-staining band at 29 kDa.

of FimH (3) reacted with a 29-kDa protein band of dissociated fimbriosomes on a Western blot (data not shown). These findings suggest that these fimbriosomes have molecular masses and antigenic epitopes similar to those of the FimH protein from different type 1-fimbriated strains of *E. coli*.

Antiadhesive properties of antibodies evoked against fimbriosomes. We had previously demonstrated that antibodies directed at a synthetic peptide of the FimH protein inhibited binding of type 1-fimbriated *E. coli* to human epithelial cells (3). Antibodies raised against the fimbriosomes were similarly antiadhesive (Table 1). The antifimbriosome antiserum inhibited *E. coli* ORN103(pSH2) binding by 87%. Furthermore, the antibodies also inhibited the binding of two additional strains, CSH50 and CI5, of type 1-fimbriated *E. coli* by 89 and 85%, respectively. The antiserum had no

FIG. 5. Western blots of dissociated type 1 fimbriae. Lane A, *E. coli* ORN103(pSH2); lane B, CSH50; lane C, CI5. Each lane was probed with antiserum raised against fimbriosomes. The antiserum reacted strongly with a 29-kDa band and weakly with a 28-kDa band in each of the fimbrial preparations.

TABLE 1. Inhibition by antibodies to fimbriosomes^a of adhesion of type 1-fimbriated *E. coli* to human buccal epithelial cells

<i>E. coli</i> strain	% Inhibition of adhesion
ORN103(pSH2).....	87
CSH50.....	89
CI5.....	85
CI6 (Pap-fimbriated control).....	9

^a Subagglutinating concentrations of antibodies were added to the test *E. coli* strains (all were type 1 fimbriated except the control strain) and incubated in the microtiter well coated with human buccal epithelial cells. Adherence inhibition was measured as described in Materials and Methods.

effect on the binding of a Pap-fimbriated strain of *E. coli* C16 (Table 1). Thus, antibodies to fimbriosomes were potent inhibitors of the adherence of type 1-fimbriated *E. coli*, and our findings are consistent with the notion that fimbriosomes are composed, at least in part, of FimH and that FimH mediates the D-mannose-binding property of type 1 fimbriae and fimbriosomes.

Determination of the stoichiometric ratio of FimH to FimA in ORN103(pUT2004) fimbriae. Fimbriae from the mutant ORN103(pUT2004) appeared to be crooked and often broken, indicating an abnormality in the structure of these fimbriae. To determine whether this abnormality was caused by a difference in the relative abundance of fimbrial proteins and in particular by an increase in the amount of FimH, we estimated the relative amounts of fimbrial proteins in SDS

FIG. 6. Densitometric scans of fimbriae isolated from ORN103(pUT2004) (A) and ORN103(pSH2) (B) after SDS-PAGE.

FIG. 7. Immunoelectron micrographs of fimbrial filaments of ORN103(pUT2004) (A) and ORN103(pSH2) (B) reacted with antibodies directed against fimbriosomes, followed by gold-conjugated protein A. Notice the crooked ORN103(pUT2004) fimbria and the large number of gold particles associated with it. Magnification, $\times 90,000$.

gels by densitometry of approximately similar amounts of dissociated fimbriae. The ratio of FimH subunits to FimA subunits in the wild-type pSH2 fimbriae was 1:60 (Fig. 6). In marked contrast, the ratio was as high as 1:15 in the pUT2004 mutant fimbriae (Fig. 6). Thus, the mutant fimbriae contained at least a fourfold increase in FimH.

Localization of FimH on *E. coli* ORN103(pUT2004) fimbriae. The binding of antibodies evoked by fimbriosomes to ORN103(pUT2004) and ORN103(pSH2) fimbriae was examined by immunoelectron microscopy. We found that a higher number of antibody-binding sites were associated with pUT2004-encoded fimbriae, as judged by the relative amount of protein A-conjugated gold particles that were associated with each fimbrial filament (Fig. 7). The gold particles appeared to bind along the fimbrial filament at intervals, suggesting that FimH is interspersed periodically among the 17-kDa major subunits. Interestingly, the ratio between the average interval of the mutant pUT2004 fimbriae and that of the wild-type pSH2 fimbriae was approximately 6:1, in approximate agreement with the ratio found by gel scan analysis (see above).

DISCUSSION

Recent studies have provided evidence that the fimbrial appendages of *E. coli* possess minor components within their quaternary structures that mediate adhesion to sugar-containing receptors on eucaryotic cells. Whereas evidence has been presented that these minor proteins reside at the tips of Pap fimbriae (14), we have recently shown that the minor protein FimH resides not only at the tips but also at long intervals along the length of type 1 fimbriae (3). Antibodies against both these minor proteins have proved to be antiadhesive (3, 14).

Although considerable knowledge has been gained on the regulation of the major fimbrial subunits, the regulation of the minor fimbrial proteins remains unclear. Our data suggest that a negative regulatory element resides beyond the known fimbrial gene cluster within a 1.5-kilobase *PvuII* fragment adjacent to FimH. Deletion of this fragment markedly increased the production of FimH out of proportion to the production of FimA, the major structural subunit of type 1 fimbriae. However, we cannot discount the possibility that, in addition to the deletion, pUT2004 has a mutation at another site, for example, in the ribosomal binding site for *fimH*, which could account for this phenomenon.

The overproduction of FimH is associated with the production of peculiar structures that we have named fimbriosomes because of their association with fimbriae and their rounded shape. From our data thus far, fimbriosomes appear to be composed entirely of the 29-kDa FimH protein. The structures appear to be associated rather loosely with the fimbriae because of the ease by which they can be separated from the fimbriae. According to Klemm and Christensen, *fimH* appears to require either of two ancillary genes, *fimF* or *fimG*, to mediate adhesion (11). Although we did not detect the *fimF* or *fimG* gene products in our fimbriosome preparation, it is possible that they were also secreted in the culture medium by the mutant but failed to adsorb to yeast cells in the isolation process. Interestingly, no increase in either of these gene products was detected in the comparative gel scans of dissociated mutant and wild-type fimbriae.

Immunization of rabbits with purified fimbriosomes evoked anti-FimH antibodies in high titer. It should be noted that the immune sera also contained antibodies that reacted with FimA, but such cross-reactions can be explained on the basis that FimA and FimH contain certain sequence homologies (11). Cross-absorption of the antifimbriosome antibodies with mutant nonadhesive fimbriae lacking FimH rendered the immune sera FimH specific. That the fimbriosomes were contaminated with minute amounts of FimA or other fimbrial components such as FimG cannot be completely excluded. However, only the 29-kDa FimH protein could be detected in dissociated fimbriosomes separated on SDS gels and stained with silver nitrate or in Western blots reacted with polyclonal antifimbriosome antibodies.

The finding that antibodies raised against the fimbriosomes were antiadhesive for other type 1-fimbriated strains of *E. coli* is in agreement with our previous findings with anti-FimH antibodies (3) and has important implications for the development of antiadhesive vaccines. In contrast to FimA, FimH appears to be antigenically conserved, and therefore vaccines composed of FimH may be broadly protective against the colonization of mucosal surfaces by a variety of type 1-fimbriated strains of *E. coli*. Fimbriosomes composed entirely of FimH may prove useful in the formulation of such a vaccine.

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