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The *Escherichia coli* G-Fimbrial Lectin Protein Participates Both in Fimbrial Biogenesis and in Recognition of the Receptor *N*-Acetyl-D-Glucosamine

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The *gafD* gene encoding the *N*-acetyl-D-glucosamine-specific fimbrial lectin (adhesin) protein GafD of uropathogenic *Escherichia coli* was cloned and subjected to genetic analysis. The corresponding gene product was isolated as a MalE fusion protein. The lectin gene was identified with the aid of deletion mutagenesis; mutations in *gafD* impaired either receptor binding or both receptor binding and fimbria production, depending on the mutation created. All mutants converted to wild-type expressors when complemented in *trans* with the cloned intact *gafD* gene. The predicted 354-amino-acid sequence of GafD, deduced from the nucleotide sequence, is closely related to those of the fimbria-associated F17-G and F17b-G proteins coded for by enterotoxigenic and invasive *E. coli* strains. Isolated GafD was shown to recognize *N*-acetyl-D-glucosamine by virtue of specific binding to an immobilized receptor, thus proving directly that GafD is a sugar-binding protein. Our results indicate that GafD as such is sufficient for receptor recognition and that the protein also participates in fimbrial biogenesis.

Colonization of host epithelial cell surfaces is a prerequisite for many bacterial infections (5, 9, 17). Such colonization is promoted by bacterial adherence to receptor structures present on host epithelial surfaces. Among enteric bacteria, binding to host receptors is often mediated by adhesion organelles called fimbriae or pili (5, 9, 17) expressed on the bacterial cell surface.

Fimbriae are filamentous polymers of protein subunits called fimbrillins (5, 9). P fimbriae of uropathogenic *Escherichia coli* strains (10, 31, 33) as well as S (8, 30, 37) and the common type 1 fimbriae (1, 17) possess a receptor-binding molecular organelle attached to the fimbrial filament. This organelle consists of three to four different protein subunits (9, 10, 15–21, 31). One of these proteins, also referred to as the adhesin or lectin, recognizes the receptor (1, 10, 18, 20). The other subunits participate in fimbrillin polymerization or in adapting the lectin protein to the fimbrial filament (9, 10, 15–20, 31, 40). Although the lectin proteins constitute the functional part of the fimbrial filament responsible for receptor recognition, these proteins are, in many instances, dispensable for fimbrial biogenesis itself; lectin-deficient mutants produce fimbriae devoid of their characteristic binding property (9, 29).

A wide variety of host structures may function as receptors for fimbriae (5, 9, 17, 32, 39). Yet, the receptor specificity of individual fimbrial lectin proteins is comparable to that of plant lectins or antibodies. The specificity of the type 1 fimbrial lectin for mannosides corresponds to that of concanavalin A (7), whereas the P-fimbrial lectin specifically recognizes the α-D-Gal*p*-(1,4)-β-D-Gal*p* moiety present in the globoseries of glycolipids (38). The high binding specificity of bacterial lectins makes these proteins interesting tools for protein-ligand interaction studies and, in an analogy with plant lectins, as potential tissue and blood group markers (32).

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Furthermore, bacterial lectin proteins have been suggested as vaccine antigen candidates (27). Such vaccines are expected to block bacterial colonization of the host by evoking antibodies directed against the fimbrial adhesin. Few isolated bacterial lectin proteins have been available for detailed analyses (10, 30). Bacterial lectins are present in only scant amounts on the bacterial surface and even then as integral components of fimbrial filaments (9, 10, 12–20, 30). Therefore, it has been difficult to isolate these lectins in larger quantities free of other fimbrial proteins.

The closely related F17 and F17b fimbriae are present on bovine enteropathogenic and septicemic *E. coli* strains (23–26, 29). Bacteria expressing these fimbriae adhere to intestinal brush borders, and this attachment can be inhibited with *N*-acetyl-D-glucosamine (23, 24). A human pyelonephritogenic *E. coli* isolate possessing a related adhesion phenotype has also been documented (39). The fimbriae of this strain, which is responsible for glucosamine-sensitive hemagglutination of endo-β-galactosidase-treated human erythrocytes, have been termed G fimbriae (32, 39). As reported here, we have identified the lectin protein gene *gafD* of *E. coli* G fimbriae, expressed the gene, isolated the gene product, and established the receptor-binding ability of the isolated lectin protein. The data obtained from these studies and the genetic analysis of *gafD* indicate that GafD is a bifunctional fimbrial lectin protein that is also needed for fimbrial biogenesis.

MATERIALS AND METHODS

Bacterial strains and cultivation. The *E. coli* strains used were HB101 (3) and TB1 (New England Biolabs, Beverly, Mass.). The HB101 strain (HB101/pKTH3020) expressing the KS71A P fimbria (33, 34) was obtained from our own collection. Bacteria were grown on Luria plates or in Luria broth medium supplemented with ampicillin or tetracycline hydrochloride (supplied by Sigma Chemical Corporation, St. Louis, Mo.). For induction of the *tac* promoter, cultures were grown to the early logarithmic growth phase in Luria medium containing 0.2% D-glucose (Merck, Darmstadt, Germany), after which IPTG (isopropyl-β-D-thiogalactopyranoside) (Bio-Rad Laboratories, Heralds, Calif.) was added to a final concentration of 1 mM and the cultivation was continued for 3 h.

DNA manipulations. DNA isolations and manipulations were performed according to instructions provided by the enzyme supplier (Promega Corporation, Madison, Wis.) and to the methods of Sambrook et al. (35). Plasmids were introduced into *E. coli* by a method described by Mandel and Higa (28). Amplification of DNA by PCR was conducted by the method outlined by Sambrook et al. (35). The oligonucleotides used for the amplification were 5′-GCGGATC CGCAGTTTCATTTATTGG-3′ and 5′-AACAGCTATGACCATG-3′, whereas isolated pHUB113 was used as a template. Oligonucleotides were synthesized and purified at our institute by standard methods and with standard chemicals.

DNA sequencing. DNA sequencing was performed by the chain termination technique (36) with the Sequenase Kit Version II (United States Biochemicals, Cleveland, Ohio). Templates were obtained by cloning selected DNA fragments into M13mp18 and mp19 vectors (41). The radiolabeled ^{35}S-dATP was from Amersham International, Buckinghamshire, United Kingdom.

Detection of fimbriae. Fimbrial extracts were prepared from bacterial cultures by mechanical detachment (32) and analyzed by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE) (22, 34). For immunoblotting, the separated extracts were transferred to Immobilon sheets (Millipore Corporation) and subsequently treated with rabbit anti-G fimbria serum (1:1,000) and peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako A/S, Glostrup, Denmark).

Bacteria were also analyzed with an electron microscope, with whole bacteria negatively stained with potassium platinate (32) being used for the analysis. For immunoelectron microscopy, copper grids coated with Pioloform and carbon were floated in suspensions of bacteria. The grids were then floated in phosphate-buffered saline (PBS) containing 0.1% (vol/wt) bovine serum albumin (BSA). Excess liquid was removed, and the grid was immediately placed face down onto a drop of a suitable dilution of anti-G fimbria serum for 15 min. After thorough washings, the grids were placed on drops of a suitable dilution of a protein A-colloidal gold conjugate (AuroProbe EM Protein A G10; Amersham International) in PBS–0.1% BSA for 15 min. After thorough washings with PBS–0.1% BSA and distilled water, the grids were stained with 1% potassium platinate at pH 7.3. Grids incubated without antiserum were used as controls. The grids were then examined with a JEOL JEM-100CX transmission electron microscope at an operating voltage of 60 kV.

Receptor recognition was assayed by agglutination of human erythrocytes treated with endo-β-galactosidase (32, 33). The enzyme was provided by Seikagadu Kogyo Co., Ltd., Tokyo, Japan.

Isolation of GafD. *E. coli* harboring pHUB114 was grown to early logarithmic growth phase in Luria broth supplemented with 0.2% (wt/vol) glucose and 1 mM IPTG. The bacteria were pelleted by centrifugation, and the pellet was suspended in 20% (wt/vol) glucose in 10 mM Tris-HCl–20 mM EDTA buffer, pH 7.5, at room temperature. After 10 min, the bacteria were repelleted and rapidly suspended into ice-cold Tris-EDTA buffer. After 5 min on ice, the osmotic shock fluid was recovered by pelleting the bacteria.

The osmotic shock fluid was dialyzed against 10 mM Tris-HCl–150 mM NaCl buffer, pH 7.5 (TBS), and passed through an *N*-acetyl-D-glucosamine–Sepharose column (Sigma). After extensive washing with TBS, the bound material was eluted with 5% (wt/vol) *N*-acetyl-D-glucosamine (Sigma) in TBS. Affinity chromatography with amylose-Sepharose (New England Biolabs) was performed in a similar manner, except that the elution was performed with 5% (wt/vol) maltose (Merck).

MalE and GafD were separated by mixing the fusion protein with coagulation factor Xa (New England Biolabs) at 2 U of the factor per 100 μg of protein in TBS supplemented with 10 mM $CaCl_2$. After incubation at +37°C for 16 h, the mixture was passed through the *N*-acetyl-D-glucosamine column. The attached material, eluted with 5% (wt/vol) GlcNAc, was finally passed through the amylose resin in order to remove uncleaved fusion protein.

Binding assay for GafD. Isolated GafD was iodinated by the chloramine T method (11) with ^{125}I from Amersham. GafD (50,000 cpm) was mixed with 100 μl of erythrocytes or GlcNAc-Sepharose for 20 min on ice. After that, the suspensions were washed twice in TBS and the pellets were assayed for radioactivity in an LKB gamma counter. For inhibition studies, the first incubation was performed in TBS containing either 5% (wt/vol) GlcNAc, D-glucose, or D-mannose (Merck).

Nucleotide sequence accession number. The nucleotide sequence obtained from pRR-5 has been submitted to the EMBL Gene Bank under accession number L 33969.

RESULTS

Mutations in *gafD* that affect receptor recognition. The DNA fragment expressing G fimbriae of the *E. coli* isolate IH11165 has previously been cloned as a 7-kb *Eco*RI fragment in cloning vector pACYC184 (32) (pRR-5 in Fig. 1). In order to localize the lectin gene in pRR-5, we constructed a set of deletions in pRR-5 and looked for mutations that would abolish receptor binding but that would, in a manner analogous to that of lectin mutants of P- (31) and type 1-fimbriated bacteria (17), allow a wild-type-level expression of fimbriae. Mutations were produced by deleting restriction fragments from pRR-5 or by deleting single-stranded DNA overhangs, created by restriction enzyme cleavage, with nuclease S1.

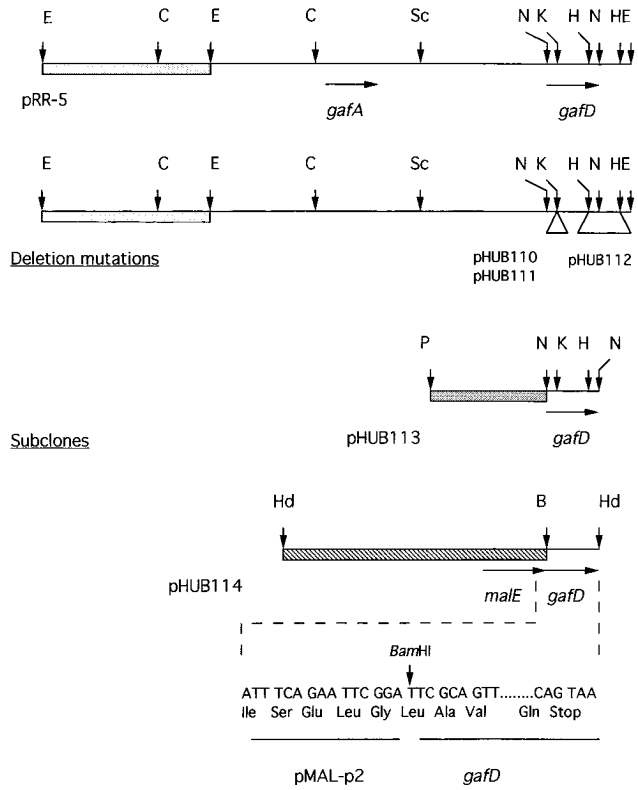

FIG. 1. Restriction map of pRR-5 and of deletion derivatives and subclones derived from it. pRR-5 contains a 7-kb *Eco*RI fragment of the uropathogenic *E. coli* strain IH11165 in the corresponding cloning site of pACYC184 (4). Deletion derivatives pHUB110 and pHUB111 were obtained by opening pRR-5 at the unique *Kpn*I site (K) and by subsequently treating the plasmid with S1 nuclease and DNA ligase. pHUB112 is an *Hpa*I cutback derivative of pRR-5. pHUB113 contains the *Nsi*I fragment of pRR-5 in the *Pst*I site of cloning vector pUC19 (41). pHUB114 is a derivative of pMAL-p2 containing the *malE* gene under the *tac* promoter. Thick boxes represent cloning vectors, and thin lines represent inserted DNA. Horizontal arrows underneath the map lines represent the locations of the *gafA*, *gafD*, and *malE* open reading frames. The location of the *gafA* fimbrial subunit gene was verified by nucleotide sequence analysis. Triangle symbols represent deletions created in pRR-5. Restriction endonuclease abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; Hd, *Hin*dIII; H, *Hpa*I; K, *Kpn*I; N, *Nsi*I; P, *Pst*I; Sc, *Sac*I.

Potential lectin gene mutants were screened for receptor binding and production of fimbriae. G-fimbriated bacteria agglutinate erythrocytes rich in terminal *N*-acetyl-D-glucosamine residues (32). GlcNAc is readily exposed on erythrocytes treated with endo-β-galactosidase (39). Receptor binding was therefore assayed by agglutination of endo-β-galactosidase-treated human erythrocytes in bacterial suspensions. Production of fimbriae was demonstrated by immunoblotting of fimbrial extracts and electron-microscopic investigation of bacterial surfaces.

One mutation in pRR-5 resulted in the expression of nonhemagglutinating fimbriae. This construct, termed pHUB110, was obtained by first linearizing pRR-5 with *Kpn*I, removing the single-stranded overhangs by mild S1 nuclease treatment, and subsequently self-ligating the plasmid. In an *E. coli* HB101 background, pHUB110 produced normal amounts of fimbriae (Fig. 2a and b and 3 [lanes a and b]), which were shown by

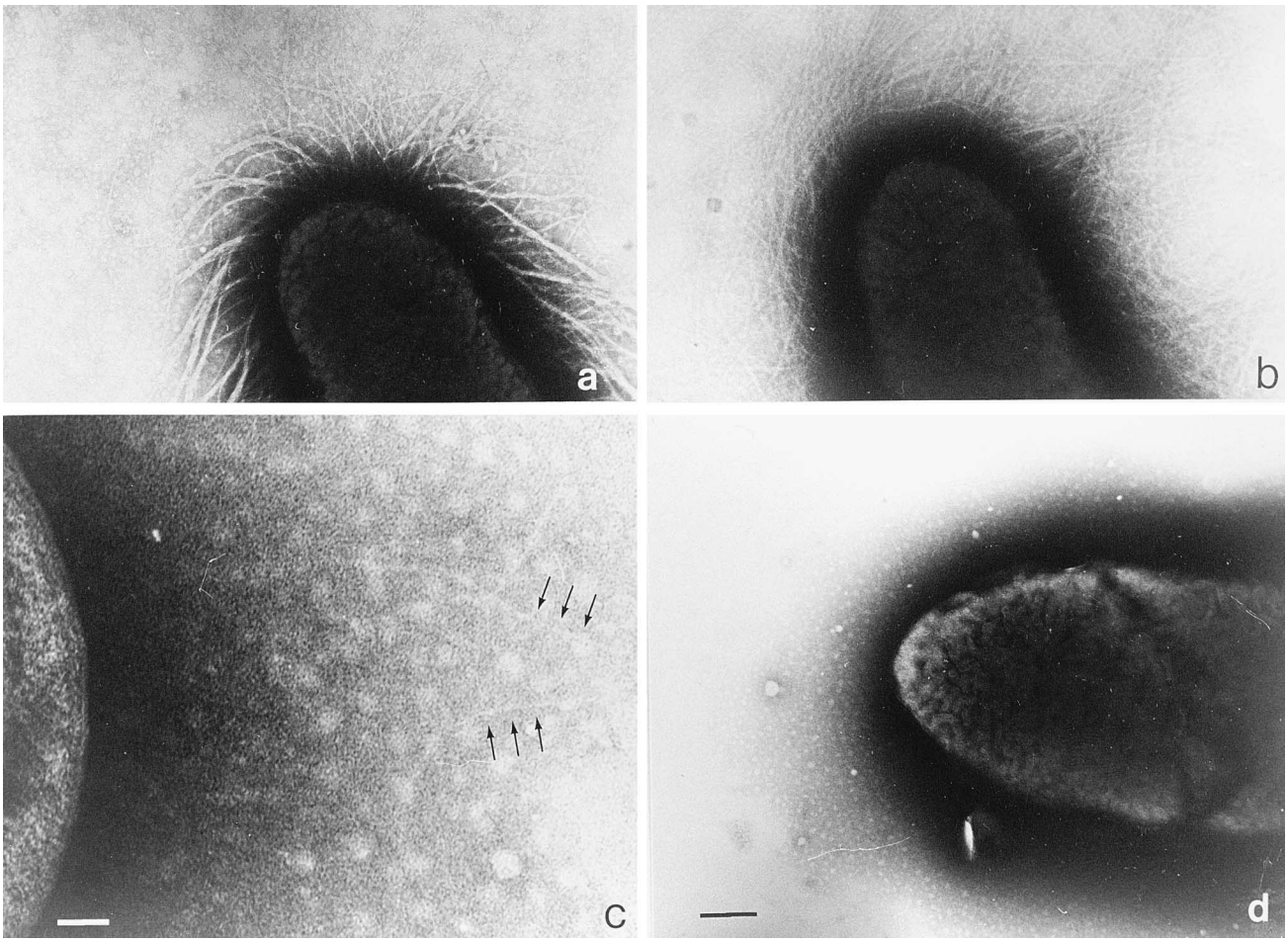

FIG. 2. Electron micrographs of *E. coli* HB101 bacteria harboring pRR-5 (a), pHUB110 (b), pHUB112 (c), and pHUB111 (d). HB101/pRR-5 and HB101/pHUB110 clearly show fimbriae, whereas HB101/pHUB112 possessed scant amounts of fimbria-like structures (arrows). The bars are 100 nm.

immunoelectron microscopy to react with antibodies directed against G fimbriae (Fig. 4). In contrast to HB101/pRR-5, the HB101/pHUB110 strain did not agglutinate endo-β-galactosidase-treated human erythrocytes (Table 1), suggesting a loss of receptor-binding ability. Furthermore, HB101/pHUB110 had also lost the autoagglutinative property (Table 1) associated with G-fimbrial receptor binding (32).

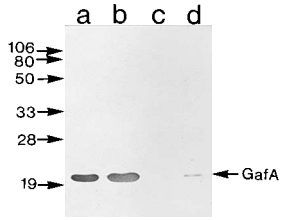

FIG. 3. Immunoblotting of *E. coli* HB101 fimbrial extracts with rabbit anti-G fimbria serum (anti-GafA) and peroxidase-conjugated swine anti-rabbit immunoglobulins. The extracts were prepared by mechanical agitation of bacterial suspensions with equal optical densities. The bacteria were removed by centrifugation, and the supernatant was applied to gels for SDS-PAGE. Lane a, extract from HB101/pRR-5; lane b, extract from HB101/pHUB110; lane c, extract from HB101/pHUB111; lane d, extract from HB101/pHUB112. The numbers at the left indicate the positions of molecular mass markers in kilodaltons, whereas GafA at the right indicates the position of the G-fimbrial subunit protein (25 kDa).

Sequence analysis of wild-type and mutated *gafD*. In order to characterize the mutation in pHUB110, the 1.2-kb *Nsi*I fragments of pRR-5 and pHUB110 were both subjected to sequence analysis. The sequence obtained from pRR-5 revealed a 1,062-bp open reading frame (ORF), *gafD*, capable of encoding a 354-amino-acid protein, GafD, including a putative 22-residue signal sequence (Fig. 5). The predicted amino acid sequence of GafD appeared to be closely related to those of the F17-G and F17b-G fimbrial proteins of enterotoxigenic and invasive *E. coli* (Fig. 5) (24, 29) but distinct from those of the F17A and GafA G-fimbrial subunit proteins (25, 26, 32). Interestingly, all amino acid substitutions observed for GafD and F17-G and F17b-G were located in the N-terminal halves of the respective proteins (Fig. 5).

Upon sequence analysis of the corresponding *Nsi*I fragments from HUB110, the S1 nuclease-treated derivative of pRR-5, the ORF was found to be missing the six nucleotides encoding residues 116 and 117 of GafD (Fig. 5). The observed lack of receptor recognition in HB101/pHUB110 is in accordance with observations of F17-G mutants; deletions of the F17-G gene allow a normal level of fimbria expression but abolish the binding of bacteria to the receptor present on calf intestinal villi (23, 24).

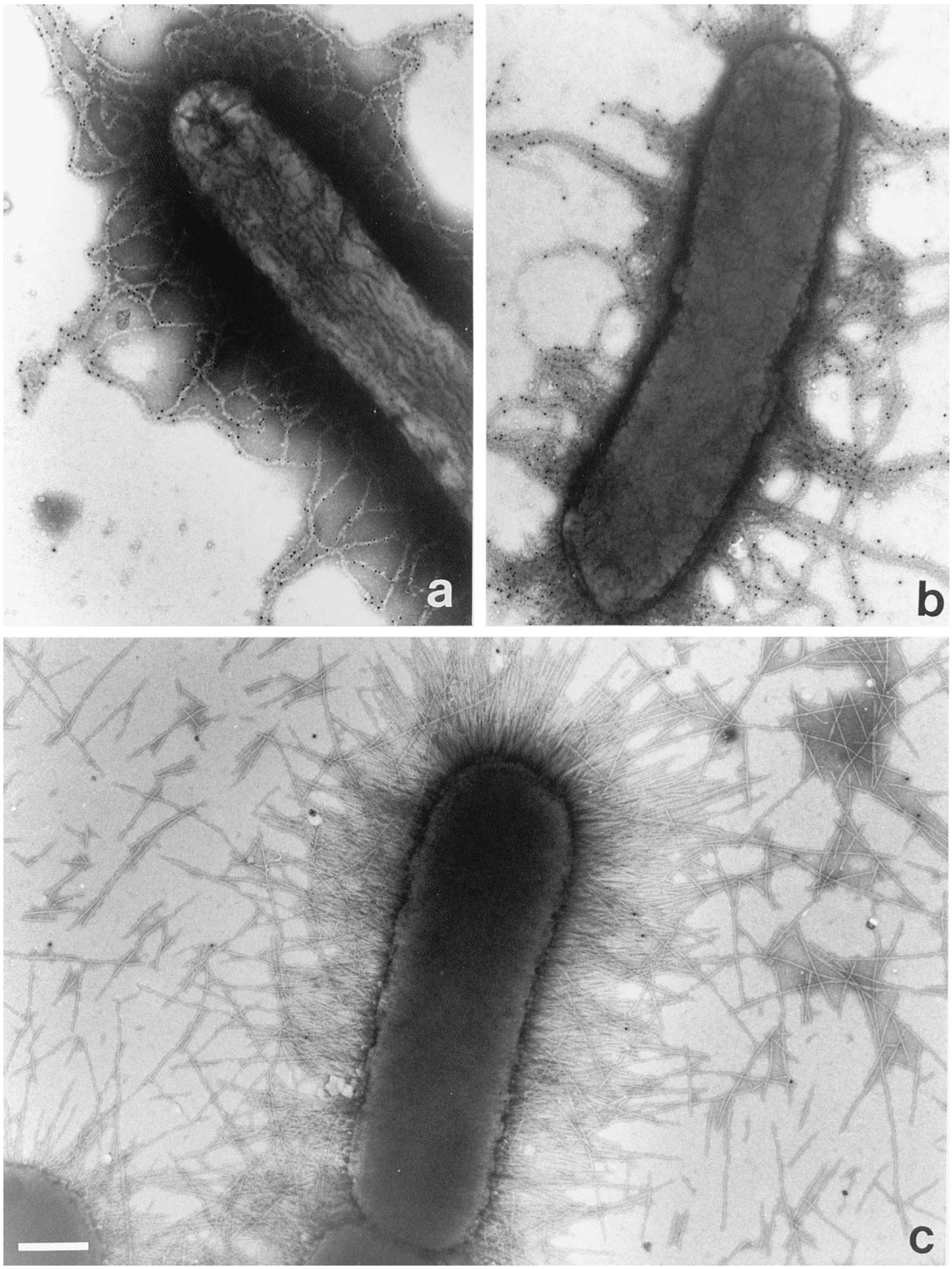

FIG. 4. Immunoelectron microscopy of HB101 carrying pRR-5 (a) or pHUB110 (b). The bacteria were exposed to anti-G fimbria serum and colloidal gold-labeled protein A (10-nm-diameter particles). Note the fimbrial bundling and gold particle accumulation mediated by the antibodies in panels a and b. The control HB101 strain expressing the serologically unrelated KS71A P fimbriae (c) does not show an immunoreaction. The bar is 100 nm.

Mutations in *gafD* that affect formation of fimbriae. In our experiments, aimed at localizing the gene responsible for receptor binding by deletion mutagenesis, we also obtained mutations in *gafD* that resulted in a loss of both receptor recognition and fimbria expression. Specifically, deletions resulting in premature translational terminations of *gafD* were found to interfere with the production of G fimbriae. In pHUB111, the *gafD* ORF is interrupted at the unique *Kpn*I site as a result of S1 nuclease deletion mutagenesis (Fig. 1 and 5). In pHUB112, the last 11 residues of GafD were removed by deleting the 3′

TABLE 1. Agglutination properties of *E. coli* HB101 harboring recombinant plasmids

Plasmid(s) carried	Autoagglutination[a]	Agglutination in anti-GafA serum[b]	Hemagglutination[c]
pRR-5	+	+	+
pHUB110	−	+	−
pHUB111	−	−	−
pHUB112	−	+/−	−
pHUB113	−	−	−
pHUB110/pHUB113	+	+	+
pHUB111/pHUB113	+	+	+
pHUB112/pHUB113	+	+	+

[a] +, autoagglutination; −, no autoagglutination.
[b] Bacteria were mixed with suitable dilutions of anti-GafA antiserum, and the agglutination reaction was recorded after 1 min of agitation. −, no agglutination; +/−, weak agglutination; +, strong agglutination.
[c] Agglutination of human endo-β-galactosidase-treated erythrocytes. −, no hemagglutination; +, hemagglutination.

end of *gafD* from the *Hpa*I site (Fig. 1 and 5). In HB101/pHUB111, fimbrial expression was abolished completely (Fig. 2d and 3 [lane c]), whereas HB101/pHUB112 expressed reduced numbers of fimbriae, as indicated by electron microscopy (Fig. 2c) and immunoblotting (Fig. 3, lane d). Both HB101/pHUB111 and HB101/pHUB112 were unable to hemagglutinate endo-β-galactosidase-treated erythrocytes (Table 1).

HB101 carrying either pHUB110, pHUB111, or pHUB112 both expressed fimbriae and caused hemagglutination of endo-β-galactosidase-treated erythrocytes when complemented in *trans* with pHUB113, a construct containing the wild-type

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          1                                                  50
F17b-G
 F17-G          F                   Q                 L     R
  GafD  MTNFYKVCLA VFILVCCNIS HAAVSFIGST ENDVGPSQGS YSSTHAMDNL

         51                                                  100
F17b-G                                G
 F17-G      D RNK            H   K
  GafD  PPVVYNTGYNI GYQNANVWRI SGGFCVGLDG KVDLPVVGSL DGQSIYGLTE

        101                                                  150
F17b-G                                    S  .  L
 F17-G          N  K                        A TA        R T
  GafD  EVGLLIWMGD TNYSRGTAMS GNSWENVFSG WCVG.NYVST QGLSVHVRPV
                a   --
                b    ----- ---------- ---------- ----------

        151                                                  200
F17b-G
 F17-G         R
  GafD  ILKRNSSAQY SVQKTSIGSI RMRPYNGSSA GSVQTTVNFS LNPFTLNDTV
        ---------- ---------- ---------- ---------- ----------

        201                                                  250
F17b-G
 F17-G
  GafD  TSCRLLTPSA VNVSLAAISA GQLPSSGDEV VAGTTSLKLQ CDAGVTVWAT
        ---------- ---------- ---------- ---------- ----------

        251                                                  300
F17b-G
 F17-G
  GafD  LTDATTPSNR SDILTLTGAS TATGVGLRIY KNTDSTPLKF GPDSPVKGNE
        ---------- ---------- ---------- ---------- ----------

        301
F17b-G
 F17-G
  GafD  NQWQLSTGTE TSPSVRLYVK YVNTGEGINP GTVNGISTFT FSYQ*
        ---------- ---------- ---------- ---------- ----
                                        c  ------- ----
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FIG. 5. The predicted amino acid sequence encoded by the *gafD* ORF. The sequence for the *gafD* ORF has been submitted to the EMBL Gene Bank under accession number L 33969. Amino acids above the sequence indicate changes by comparison with the predicted F17-G (24) and F17b-G (29) protein sequences. Residues deleted in GafD coded for by pHUB110 (a), pHUB111 (b), and pHUB112 (c) are marked by dashes underneath the sequence.

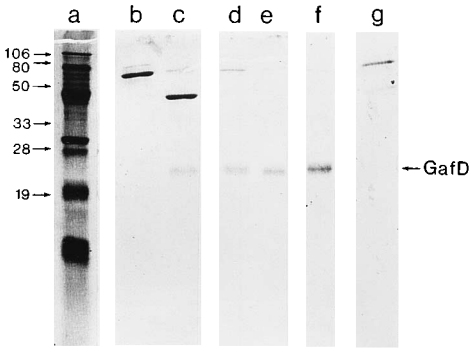

FIG. 6. Osmotic shock fluid recovered from *E. coli* TB1 harboring pHUB114 was fractionated on *N*-acetyl-D-glucosamine and amylose-Sepharose resins and analyzed in SDS–15% PAGE gels. Lane a shows the osmotic shock supernatant. The MalE-GafD fusion protein eluted from the *N*-acetyl-D-glucosamine–Sepharose column is shown in lane b, and the fusion protein after treatment with factor Xa is shown in lane c. Protease-treated material attached to *N*-acetyl-D-glucosamine–Sepharose and eluted with 5% *N*-acetyl-D-glucosamine is shown in lane d. The material from lane d was next passed through an amylose-Sepharose column. The material not attached in this experiment is shown in lane e, the material after concentration is shown in lane f, and the material that was eluted with 5% maltose and that was obtained from the amylose resin is shown in lane g.

gafD gene as a 1.2-kb *Nsi*I fragment in pUC19 (Fig. 1 and Table 1).

Expression, isolation, and characterization of GafD. In order to test whether GafD actually possessed receptor-binding ability, we decided to isolate the GafD protein. Attempts to isolate the protein by separating it from fimbrial preparations from pRR-5/HB101 or by expressing *gafD* from the cloned 1.2-kb *Nsi*I fragment of pHUB113 failed. We therefore expressed GafD as a MalE fusion protein by the use of expression plasmid pMAL-p2 (New England Biolabs). For this purpose, a fragment of *gafD* encompassing the coding region for the mature GafD protein was fused as a translational extension of *malE*. The 3′ extension fragment was generated by PCR using pHUB113 DNA as a template. The resulting fragment was ligated as a *Bam*HI-*Hin*dIII fragment into pMAL-p2. The linkage region between *malE* and *gafD* encoded the recognition sequence of the proteolytic coagulation factor Xa. In this way, GafD was fused to MalE, starting at residue 23 (Fig. 5). The resulting gene fusion construct was termed pHUB114 (Fig. 1).

The osmotic shock fluid of *E. coli* harboring pHUB114 (Fig. 6, lane a) was passed through an *N*-acetyl-D-glucosamine–Sepharose column in TBS. After extensive washing with TBS, a protein with an apparent size of 70 kDa was eluted with 5% *N*-acetyl-D-glucosamine in TBS (Fig. 6, lane b). Treatment of this protein with factor Xa resulted in 43- and 25-kDa fragments (Fig. 6, lane c). On the basis of the apparent molecular masses, the 43-kDa protein would represent the maltose-binding protein MalE. As can be predicted from the amino acid sequence, the GafD component would migrate at around 32 to 33 kDa (Fig. 5). The appearance of a 25-kDa fragment could be explained by abnormal migration of the GafD component in SDS-PAGE or by proteolytic cleavage of the protein before its isolation.

Next, the factor Xa-treated material was refractionated with the *N*-acetyl-D-glucosamine column. Now, only the 25-kDa GafD protein bound to the resin, whereas the 43-kDa protein passed through the column. The 25-kDa protein was detached from the column upon elution with 5% *N*-acetyl-D-glucosamine in TBS (Fig. 6, lane d). There was no binding of the 25-kDa protein fragment to amylose-Sepharose (Fig. 6, lanes e

and g). Thus, since amylose is a homopolymer of α-1-4-linked D-glucose, isolated GafD did not bind a plain glucose polymer. In contrast, the 43-kDa protein component did attach to the amylose resin, confirming its identification as MalE.

DISCUSSION

The expression of P, S, K99, and type 1 fimbriae is coded for by complex gene clusters of about 10 kb in size and including 8 to 11 genes each (2, 6, 8, 15–21, 31, 40). On the basis of functional properties, these genes can be divided into three groups. One group, occupying no more than two genes, transcriptionally regulates the expression of the fimbrial gene clusters. The second group of genes encodes structural proteins of the fimbrial filament, that is, the fimbrillin itself, accessory fimbrial proteins needed as polymerization initiators and as adaptors, and finally the lectin protein (1, 9, 14, 19, 30). The third group includes genes encoding components of a translocation machinery, such as molecular chaperones and ushers, that specifically directs fimbrial components from the periplasm into or through the outer membrane (10).

F17, F17b, and G fimbriae are expressed by gene segments with sizes of around 5 kb (23–26, 29, 32), suggesting a more simple gene organization. Indeed, the F17 gene cluster is known to include only four genes (23, 24). Preliminary genetic analysis of pRR-5 suggests that the G-fimbrial gene cluster also would consist of no more than four genes (34a). This raises the question of how, in view of the complex biosynthetic pathway proposed for other *E. coli* fimbriae (9, 13), the biosynthesis and biogenesis of F17 and G fimbriae are carried out.

G fimbriae mediate two agglutination properties in laboratory *E. coli*. Autoagglutination is seen when G-fimbriated bacteria are mixed in a buffer on a glass slide; the bacteria strongly agglutinate without the addition of any antibodies. G-fimbriated bacteria also agglutinate endo-β-galactosidase-treated human erythrocytes (39). Autoagglutination and hemagglutination are both inhibited in the presence of *N*-acetyl-D-glucosamine (32), suggesting that G fimbriae bind to a common receptor structure present on both cell types. We are in the process of testing whether *N*-acetyl-D-glucosamine residues in the lipopolysaccharide core structure actually serve as the autoagglutination receptor structure. In order to identify the gene encoding the putative G-fimbrial lectin protein, we produced directed mutations in the G fimbria gene cluster and searched for mutants which lacked the ability to autoagglutinate and to agglutinate endo-β-galactosidase-treated human erythrocytes. In our attempt to produce such mutants, we obtained two classes of mutations in a single gene, *gafD*, that caused a simultaneous loss of the autoagglutinating and hemagglutinating properties of the bacteria. An internal deletion in *gafD*, removing two amino acid residues from GafD, still allowed expression of fimbriae (Table 1 and Fig. 2). In contrast, 3′ end deletions of *gafD* additionally abolished fimbrial fiber formation (Table 1 and Fig. 2). The impaired expression of both fimbriae and hemagglutinin in HB101/pHUB111 and HB101/pHUB112, the two mutants with 3′ deletions in *gafD*, was unexpected on the basis of previous data for P (31) and F17 fimbriae (23, 24). The mechanism of this interference with fimbrial production might be explained by one of the following possibilities. First, C-terminal truncations of GafD might block the translocation of the fimbrial transport apparatus by, for example, irreversible binding to the translocation components, such as a putative chaperone. However, strains carrying either pHUB111 or pHUB112 could be converted to a wild-type phenotype when complemented in *trans* with the cloned wild-type *gafD* gene (Table 1), suggesting that no irreversible blocking of the putative translocation apparatus had taken place in the *gafD* mutants. Second, GafD might directly participate in the translocation or in an indispensable modification of the fimbrillin or a putative lectin protein. Finally, GafD could possess a dual function by participating in fimbrial filament biogenesis and by acting as a fimbrial lectin.

In order to test whether the *gafD* gene product actually could perform receptor recognition, we set out to isolate and characterize GafD. The *gafD* gene was expressed as a 3′ end translational fusion to the *malE* gene in the expression vector pMAL-p2 (Fig. 1), which results in periplasmic expression of a MalE-GafD fusion protein due to the presence of the MalE signal sequence. The fusion protein was isolated from an osmotic shock fluid preparation, consisting of periplasmic contents and some amount of cell debris, by the use of an *N*-acetyl-D-glucosamine resin (Fig. 6). Other attempts to express and isolate *gafD* failed or yielded only minute amounts of the protein. The MalE and GafD components of the fusion protein were separated by protease treatment, and the MalE component was removed from the suspension with an amylose resin (Fig. 6).

From the nucleotide sequence, we may predict the nascent GafD to include 354 amino acid residues, 22 of which are contained in a putative signal sequence. Yet, isolated GafD, which was predicted to include 331 residues of the mature protein, moved as a 25-kDa band in Coomassie blue-stained gels subjected to SDS-PAGE (Fig. 6). Possibly, GafD possesses an abnormal movement in gels subjected to SDS-PAGE. Such a behavior would not be exceptional for a fimbrial protein; the KS71A fimbrial subunit protein, for example, moves at 22 kDa in gels subjected to SDS-PAGE, though it includes only 166 residues (33, 34). These abnormal movements could originate from regions of fimbrial proteins constituting strong conformational domains. Fimbrial proteins are also known to be proteolytically degraded in the periplasm in the absence of their specific chaperone proteins (13, 21). Here, the GafD fusion protein was expressed into the periplasm in the absence of such a chaperone. Thus, as the MalE-GafD complex possessed an apparent mass of 70 kDa instead of a calculated mass of some 80 kDa, the apparently reduced size of GafD could also be explained by a carboxy-terminal proteolytic truncation.

The MalE-GafD fusion protein possessed two binding specificities, as evidenced by its binding to both amylose and *N*-acetyl-D-glucosamine resins (Fig. 6). The fact that the individual MalE and GafD components, after separation by protease treatment, bound differently to these resins suggested that each counterpart of the fusion protein possessed a different receptor specificity, with GafD binding to *N*-acetyl-D-glucosamine and MalE binding to amylose (Fig. 6). Isolated GafD, labeled with ^{125}I, was additionally shown to bind *N*-acetyl-D-glucosamine–Sepharose in a manner that was inhibited by 5% *N*-acetyl-D-glucosamine but not by 5% D-mannose or 5% D-glucose (data not shown). This indicates that GafD as such, without any accessory fimbrial components, is capable of recognizing *N*-acetyl-D-glucosamine linked to Sepharose via a C_5 linker. G fimbriae isolated from HB101/pRR-5 agglutinate endo-β-galactosidase-treated erythrocytes in an *N*-acetyl-D-glucosamine-sensitive manner (32). This observation, together with results showing that expression of G fimbriae as such is not sufficient for receptor recognition (compare data for HB101/pRR-5 and HB101/pHUB110 in Table 1), whereas isolated GafD does bind *N*-acetyl-D-glucosamine (Fig. 6), suggests that GafD, in a manner analogous to those of the P- (10) and S-fimbrial lectin proteins (30), would function as a minor fimbrial lectin protein. The apparently weak intensity of the GafD protein in SDS-PAGE (Fig. 6) could indicate that the

protein is poorly stained with Coomassie blue. This would explain why the GafD component has so far not been detected as a minor component in SDS-PAGE analyses of a purified G-fimbrial preparation (32). Yet, isolated 25-kDa GafD protein failed to agglutinate endo-β-galactosidase-treated erythrocytes, and we could not convincingly demonstrate the binding of ^{125}I-labeled GafD to erythrocytes. It is possible that the isolated lectin possessed a slightly altered affinity or specificity for *N*-acetyl-D-glucosamine-containing receptors in comparison with fimbria-bound lectin and therefore did not bind to erythrocytes.

The P-fimbrial lectin proteins are proposed to consist of two functional domains: an N-terminal domain mainly involved in receptor recognition (9, 10, 14) and a C-terminal domain linking the lectin, via the adapter proteins, to the fimbrial filament (10). Mutations in the very 3′ end of the lectin gene are also known to affect receptor recognition (14), suggesting that both domains may interact to form a functional lectin protein. Two lines of evidence suggest a two-domain structure for GafD also. First, all amino acid changes observed for the F17-G, F17b-G, and GafD proteins were located in their N-terminal halves, creating a group of proteins with an 87% identity within the first 160 residues and with a 100% identity within the remaining 194 residues. The absolute conservation of the C termini suggests a strict structural or functional role for this part of the protein. Second, nonpolar mutations in the N- and C-terminal halves of GafD affected fimbrial expression differently. A deletion in the N-terminal half of GafD abolished receptor recognition but allowed expression of G fimbriae, whereas deletions affecting the C-terminal region abolished both expression of G fimbriae and receptor recognition (Table 1). Although they are not conclusive, the results from the mutational analysis of *gafD* make it tempting to suggest that the N terminus of GafD would participate in receptor recognition and that the C terminus would participate mainly in the biogenesis of the fimbrial filament or in the integration of GafD into the fimbrial filament. The observed sequence variability in the N termini of the F17-G lectin proteins could result in variations in the receptor-binding specificities of the individual lectins.

During the biogenesis of P fimbriae, two proteins, PapK and PapF, are needed as polymerization initiators of fimbrillin monomers (9, 12). In a manner analogous to that of this assembly pathway, the interaction between the fimbrial subunit and GafD could be crucial, for example, for the polymerization initiation of G fimbrillin monomers. If so, then GafD would perform a dual role in the formation of G fimbriae by acting as both a lectin and an assembly protein. A multifunctional nature of fimbrial proteins could explain how fimbrial filament biogenesis can be carried out with a small amount of genes.

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