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# Identification of Two New Hemagglutinins of *Escherichia coli*, *N*-Acetyl-D-Glucosamine-Specific Fimbriae and a Blood Group M-Specific Agglutinin, by Cloning the Corresponding Genes in *Escherichia coli* K-12

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**Genes encoding the *Escherichia coli* IH11165 hemagglutinins with specificity for terminal *N*-acetyl-D-glucosamine and blood group M antigen, respectively, were cloned by a cosmid cloning procedure. A 22-kilobase-pair subclone expressed both hemagglutination specificities in the nonhemagglutinating *E. coli* HB101 recipient strain. Derivatives obtained by insertion and deletion mutagenesis expressed either one of the two hemagglutination specificities. Both agglutinins were purified; the agglutinin recognizing terminal *N*-acetyl-D-glucosamine was associated with a new type of fimbria (G fimbria) with an apparent subunit molecular mass of 19.5 kilodaltons, whereas the blood group M agglutinin (M agglutinin) was nonfimbrial and had an apparent subunit mass of 21 kilodaltons.**

To avoid the mechanical host defense mechanisms of peristalsis, ciliary movement, and secretion, infectious bacteria adhere to the surface of epithelial cells (9, 13, 14). The importance of adhesion has been realized in infectious diseases caused by *Escherichia coli* such as urinary tract infection and diarrhea (24). In these instances filamentous surface antigens called fimbriae have been identified as the adhesive organelles of *E. coli*, but it is apparent that nonfimbrial adhesins, for example, the "O75X" hemagglutinin, also occur on pathogenic *E. coli* strains (31, 54).

Although the importance of fimbriae in pathogenesis is well documented, the receptor structures for the various fimbrial types are known in only a few cases. The common type 1 fimbriae bind mannosides, the optimum receptor corresponding to a trisaccharide (12). P fimbriae of *E. coli*, which mediate adhesion to urinary tract epithelial cells, are a major virulence factor in childhood pyelonephritis (21, 28, 53). The minimal structure recognized by the P fimbriae is the α-D-Gal-(1 → 4)-β-D-Gal carbohydrate moiety of the P-blood-group antigens (51). *E. coli* strains associated with neonatal septicemia and meningitis possess fimbriae (S fimbriae) recognizing sialyl galactosides (29, 30).

A novel blood group-specific agglutinin was recently identified on the pyelonephritogenic *E. coli* strain IH11165 (57) that differentiated between the human M- and N-blood-group alleles by agglutinating MM and MN, but not NN, erythrocytes. This agglutination was inhibited by the isolated blood group M substance glycophorin $A^M$. The presence of a second agglutinin on the same *E. coli* strain was implicated by the observation that NN erythrocytes became agglutinable when treated with endo-β-galactosidase, a treatment that exposes terminal *N*-acetyl-D-glucosamine residues on the erythrocyte surface. This agglutination reaction was inhibited by *N*-acetyl-D-glucosamine (56). In this communication we describe the molecular cloning of an *E. coli* IH11165 DNA fragment that encodes both of these adhesins. In addition, both adhesins were isolated and characterized starting from clones expressing one of them only.

## MATERIALS AND METHODS

**Bacterial strains and cultivation.** *E. coli* IH11165 (O2) was isolated from the urine of a patient with acute pyelonephritis (57) and strain KS300 (56) was donated by G. Källenius (National Bacteriological Laboratory, Stockolm, Sweden). Both strains cause a blood group M-specific hemagglutination; strain IH11165 also agglutinates endo-β-galactosidase-treated NN erythrocytes. *E. coli* strains producing type 1 fimbriae (strain 2131), S fimbriae (IH3084), and O75X hemagglutinin and recombinant strains producing KS71A (P) or KS71C (type 1C) fimbriae were available from previous works (29, 42, 43, 54). *E. coli* HB101 (*recA hsd lac*) was used as recipient strain in all cloning experiments. Wild-type *E. coli* strains were grown on colonization factor antigen agar (10), and *E. coli* HB101 and all of its derivatives were grown in Luria broth or on Luria agar plates (34) supplemented, when necessary, with ampicillin (100 μg $ml^{-1}$), chloramphenicol (20 μg $ml^{-1}$) or tetracycline (15 μg $ml^{-1}$). All cultures were grown for 16 to 18 h at +37°C.

**Cloning procedures and restriction mapping.** Whole-cell DNA was isolated from a logarithmic-phase culture of *E. coli* IH 11165 as described by Hull et al. (18). Plasmid DNA was isolated from cell lysates by ethidium bromide-cesium chloride equilibrium gradient centrifugation (7, 37). For screening purposes, the plasmids were isolated by the alkaline lysis method of Birnboim and Doly (4). Cloning vector pACYC184 has been described by Chang and Cohen (6). The cosmid cloning procedure was carried out as described by Collins and Hohn (8), using the pHC79 cosmid vector (16) and a bacteriophage λ packing kit (Amersham International, Amersham, Buckinghamshire, U.K.). Plasmid DNA was transformed as described by Mandel and Higa (36). Restriction endonucleases and T4 DNA ligase were purchased from Boehringer, Mannheim, Federal Republic of Germany, and used as recommended by Maniatis et al. (37). For restriction mapping, fragments obtained by single or double digests of

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TABLE 1. Properties of recombinant *E. coli* HB101 strains

| Plasmid in *E. coli* HB101 | Presence of major polypeptide in fimbrial extracts | | Agglutination of human erythrocytes[a] | | Auto-agglutination |
|---|---|---|---|---|---|
| | 19.5 kDa | 21 kDa | MM | EbG-NN | |
| pRR-1 to -4, pRR-401 to -404, pRR-411 to -413 | + | + | + (SS)[a] | + (GS) | + |
| pRR-405 to -410, pRR-6 and -7 | − | + | + (SS) | − | − |
| pRR-414, pRR-5 | + | − | − | + (GS) | + |
| pRR-415, pRR-8 | − | − | − | − | − |

[a] SS, Inhibited by 0.1 M L-serine; GS, inhibited by 0.1 M *N*-acetyl-D-glucosamine.

plasmid DNA were analyzed in gels containing 0.5 to 1% agarose depending on the size of the fragments separated.

**Transposon mutagenesis.** Insertion mutagenesis was performed with transposon Tn*1725* by the method of Altenbuchner et al. (1).

**Protein chemical methods.** Protein was estimated by the modified Lowry procedure (38), using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 15% slab gels as described by Laemmli (33). Gels were stained with Coomassie brilliant blue R250 (Serva Feinbiochemica, Heidelberg, Federal Republic of Germany).

**Hemagglutination tests.** Hemagglutination assays with human MM, MN, or NN erythrocytes were carried out as described before (25, 42). Enzyme treatments of erythrocytes were performed as recommended by Finne (11) and Väisänen-Rhen et al. (56). *Escherichia freundii* endo-β-galactosidase was purchased from Seikagaku Kogyo Co. Ltd., Tokyo, Japan, and bovine trypsin was from E. Merck AG, Darmstadt, Federal Republic of Germany. Hemagglutination inhibitors were from Merck and Sigma.

**Purification of fimbriae and O75X hemagglutinin.** Fimbriae and hemagglutinins were purified from *E. coli* cultures as described by Korhonen et al. (26) and Väisänen-Rhen (54). For screening purposes, fimbrial extracts were prepared according to Rhen et al. (44).

**Purification of blood group M agglutinin.** *E. coli* HB101 harboring pRR-6 was grown on Luria agar plates supplemented with ampicillin at +37°C for 18 h. Cells were suspended in 10 mM Tris hydrochloride buffer, pH 7.5 (TB), to a density of $10^{10}$ bacteria $ml^{-1}$, whereafter the agglutinin was detached from the bacterial cells by passing the suspension several times through a hypodermic needle. Bacteria were removed by centrifugation (10,000 × *g*, 15 min), and solid ammonium sulfate was added to the supernatant to 50%

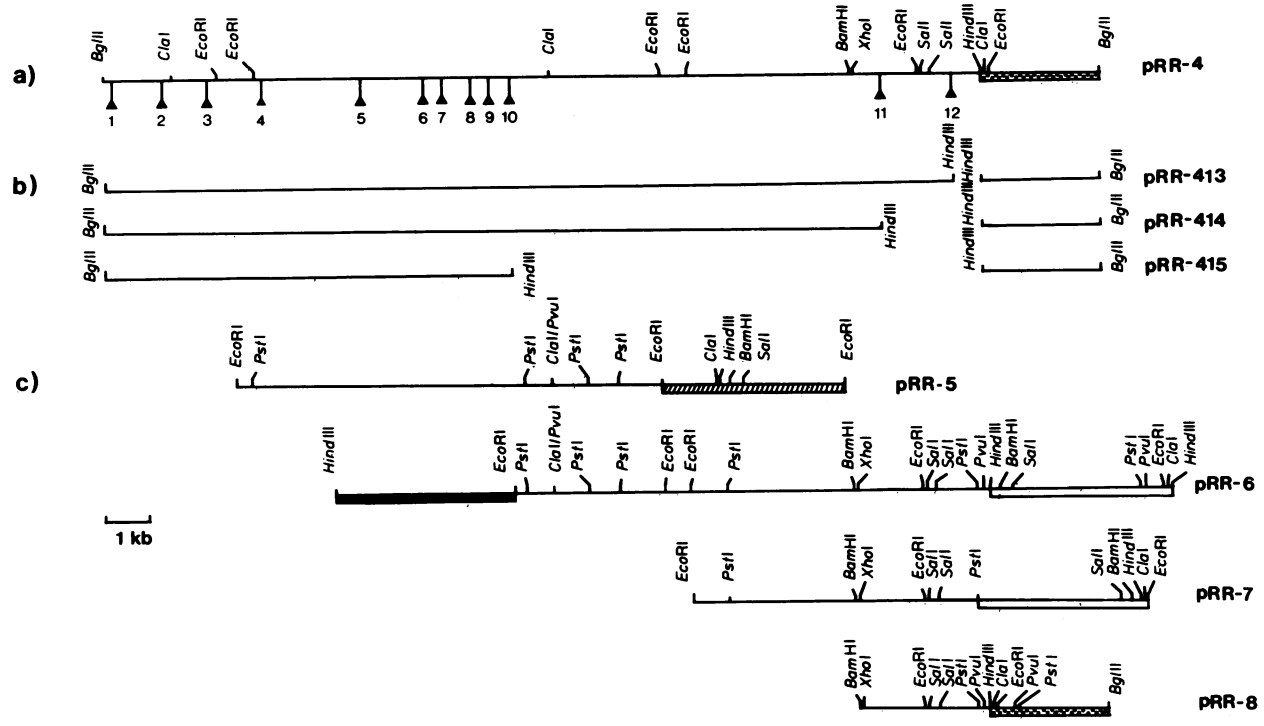


FIG. 1. Restriction endonuclease maps of recombinant plasmid pRR-4 and of its various derivatives. (a) Restriction map of pRR-4. The arrows (numbered 1 to 12) underneath the line indicate the positions of independent Tn*1725* insertions (the corresponding mutant plasmids were termed pRR-401 to -412). (b) Maps of deletion derivatives prepared from the pRR-4 insertion mutant derivatives. The pRR-4 regions present in the mutant plasmids are indicated by the three lines. (c) Restriction maps of the pRR-4 subclones pRR-5 to -8. Bar, 1 kb. Symbols: ——, inserted DNA (in c, nondeleted DNA); ▬, pHC79 DNA; ▨, pACYC184 DNA; ▭, pBR322 DNA. The 4.4-kb *Hin*dIII-*Eco*RI fragment in pRR-6 (▬) originates from Tn*1725*. *Pst*I sites in the apparent 6-kb *Pst*I fragment of pRR-5 were not mapped.

saturation. The pellet was collected by centrifugation (10,000 × *g*, 20 min) and dissolved in TB. After dialysis against TB, the suspension was made 0.5% in deoxycholate and incubated at +4°C for 16 h, whereafter insoluble material was removed by centrifugation (15,000 × *g*, 20 min). The supernatant was concentrated to 1 ml in an Amicon ultrafiltration cell and applied to a sucrose gradient consisting of 4 ml of 60% and 6 ml each of 50, 40, 30, 20, and 10% (vol/wt) sucrose in TB containing 0.5% deoxycholate. After centrifugation (100,000 × *g*, 24 h), the hemagglutinating material was found at a density of 1.08 to 1.09 g $ml^{-1}$. Sucrose and deoxycholate were removed by dialysis against TB, and the protein was concentrated by ammonium sulfate precipitation at 50% saturation. Finally, the material was passed through a 30-ml Sepharose G-75 column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.14 M NaCl–0.01 M phosphate buffer, pH 7.1. The hemagglutinating material was eluted in the void volume. Gel filtration experiments were also carried out on a 30-ml Sepharose 4B column (Parmacia) in 0.14 M NaCl–0.01 M phosphate buffer, pH 7.1.

**Enzyme-linked immunosorbent assay.** Rabbit antisera against type 1, S, KS71A, and KS71C fimbriae and against O75X hemagglutinin were available from previous works (29, 42, 43, 54). Antisera against fimbriae recognizing *N*-acetyl-D-glucosamine ("G fimbriae") and against blood group M-specific hemagglutinin ("M agglutinin") were produced in rabbits by immunization with purified proteins as described previously (26).

Immunological cross-reactions were quantitated by enzyme-linked immunosorbent assay (42), using alkaline phosphatase-conjugated swine anti-rabbit immunoglobulin G (Orion Diagnostica, Helsinki, Finland).

**Amino acid analysis.** Samples of the proteins were hydrolyzed for 24, 48, and 72 h in 6 M HCl containing 0.1% phenol (22). Amino acid analyses were performed on Waters high-pressure liquid chromatography equipment, using postcolumn treatment with *ortho*-phthaldialdehyde. Trypthophan was not identified.

**Amino acid sequence analysis.** Samples of 2 nmol were sequenced on an Applied Biosystems model 470A gas-phase sequenator, using the supplied standard program. The *ortho*-phthaldialdehyde-amino acid products were identified on Waters high-pressure liquid chromatography equipment with 254- and 313-nm detectors. The chromatographic separation was achieved on a Sperisorp S5-ODS2 column, using a methanol-ethanol gradient in acetate buffer.

**Electron microscopy.** Electron microscopy was performed as described previously (26). Electron micrographs were taken at the Department of Electron Microscopy, University of Helsinki, Helsinki, Finland.

## RESULTS

**Construction of a genomic library from *E. coli* IH 11165 DNA.** The whole-cell DNA isolated from *E. coli* IH 11165 was partially digested with the *Sau*3A restriction endonuclease and subsequently fractionated by agarose gel electrophoresis. DNA fragments in the 35- to 50-kilobase (kb) size range were ligated with *Bam*HI-treated pHC79 DNA and packaged in vitro into bacteriophage λ particles used to infect the nonhemagglutinating *E. coli* HB101. A total of 2,000 $Amp^r$ transfectant colonies were obtained.

**Isolation and characterization of hemagglutinating recombinant strains.** A total of 1,000 transfectant colonies were recultured and tested for their ability to agglutinate human MM, MN, and NN erythrocytes as well as endo-β-galactosidase-treated NN cells (EbG-NN). Three strains, carrying recombinant cosmids pRR-1, pRR-2, and pRR-3, were found to cause a strong hemagglutination of MM and MN erythrocytes; none of them agglutinated NN erythrocytes (Table 1). Only these three strains agglutinated EbG-NN erythrocytes. The three hemagglutinating strains were also strongly autoagglutinating.

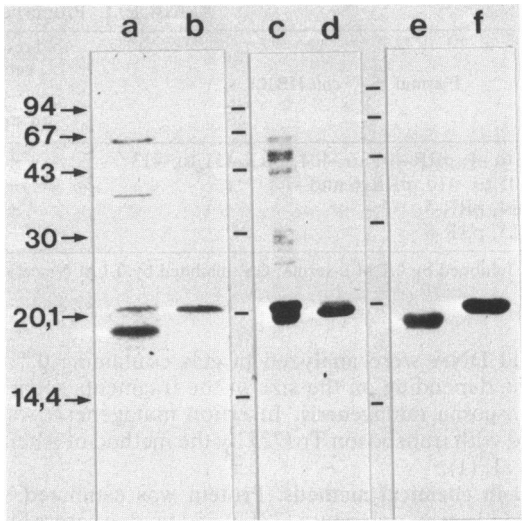


FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of "fimbrial extracts" and hemagglutinin preparations. Lanes a and b show the fimbrial extracts from wild-type strains IH11165 and KS300, respectively. Lane c contains a fimbrial extract from *E. coli* harboring pRR-1; lane d contains a purified fimbria preparation from the same strain. Lane e contains hemagglutinin purified from *E. coli* HB101 harboring pRR-5; lane f contains hemagglutinin purified from *E. coli* harboring pRR-6. The positions of standard proteins in the gels are given at the left in kilodaltons. The G-fimbrial peptide (19.5 kDa) is too weakly expressed in *E. coli* IH11165 to be seen in fimbrial extracts (lane a). The 18.5-kDa peptide originates from a second fimbrial type (unpublished observation).

The sizes of the inserted DNA fragments in pRR-1, pRR-2, and pRR-3 were 46, 35, and 37 kb, respectively, which is in the size range expected for recombinant cosmids (16). Subclone pRR-4, prepared from pRR-1, contained the 19.5-kb *Bgl*II-*Hin*dIII fragment of pRR-1 in the *Bgl*II-*Hin*dIII site of pHC79 (Fig. 1a). HB101 cells harboring pRR-4 still agglutinated MM, MN, and EbG-NN erythrocytes. Cells harboring pRR-4 also formed visible autoaggregates (Table 1).

For a more detailed analysis of the DNA segment that encoded the hemagglutinins, Tn*1725* insertions were produced in pRR-4 (Fig. 1a; plasmids containing insertions 1 to 12 were correspondingly termed pRR-401 to -412). Insertions 1 to 4 and 11 and 12 did not affect its agglutination properties. Insertions 5 to 10 blocked both autoagglutination and the ability of the bacteria to agglutinate EbG-NN erythrocytes, while the agglutination of MM erythrocytes remained intact (Table 1). We next deleted the DNA fragment situated between the *Hin*dIII site of pHC79 and the transposon (1, 47) from insertion derivatives pRR-410 to -412 by ligating the corresponding *Hin*dIII digests (Fig. 1a and b). Of these deletion derivatives (termed pRR-413 to -415, Fig. 1b), pRR-413 behaved like pRR-4. Bacteria harboring pRR-414 did not express the blood group M agglutinin but still agglutinated EbG-NN erythrocytes (Table 1). Bacteria har-

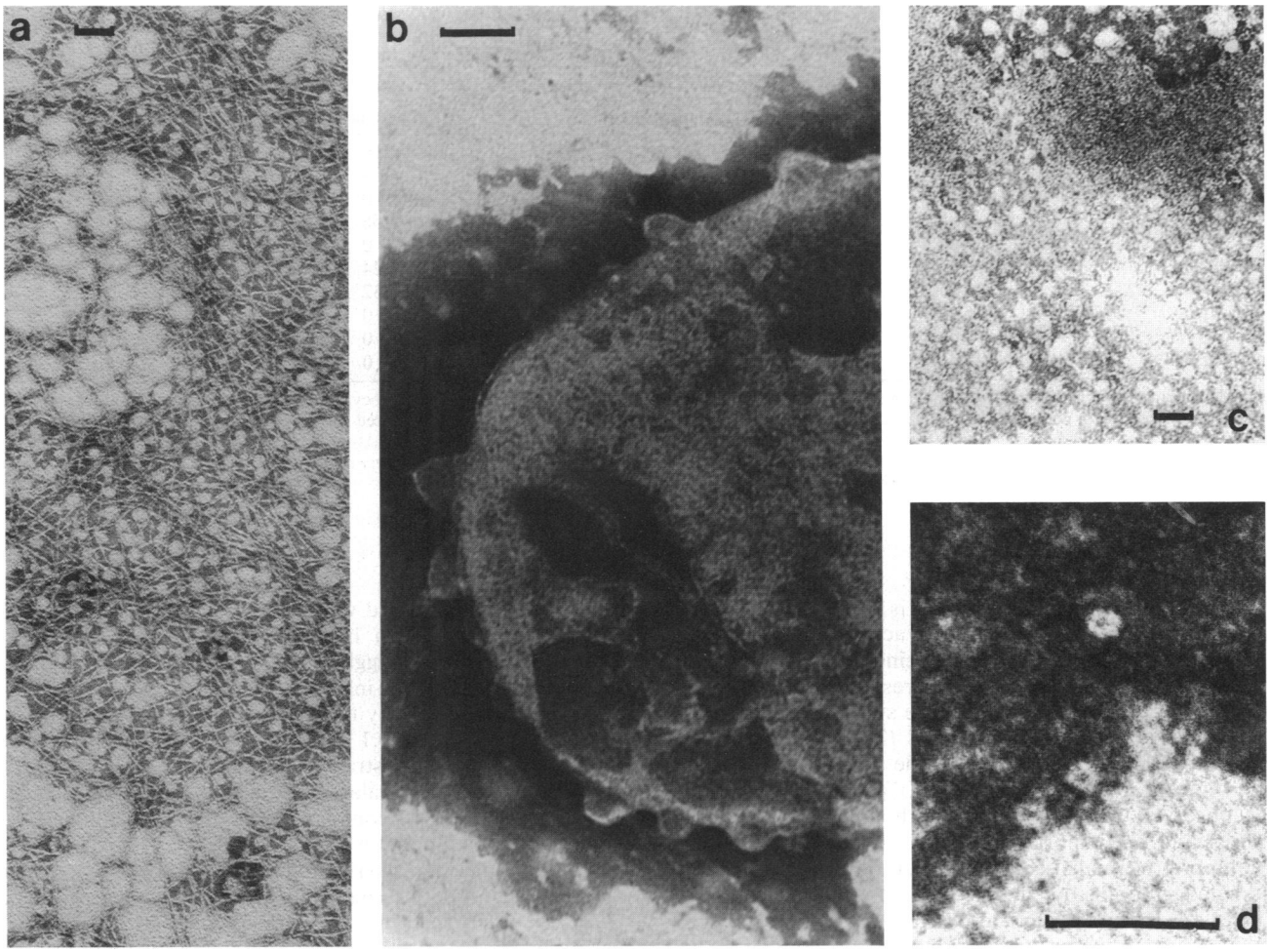


FIG. 3. Electron micrographs of purified fimbria (a) encoded by pRR-5 and (b) showing part of the surface of *E. coli* HB101 harboring pRR-6. (c) and (d) show purified blood group M agglutinin (note ringlike structures in panel d). Bar, 100 nm.

boring pRR-415 were completely nonhemagglutinating (Table 1).

We also prepared subclones that expressed only one of the two agglutinins; pRR-5 contains the 10-kb *Eco*RI fragment of pRR-4 and pRR-6 contains the 14.5-kb *Hin*dIII fragment situated between insertion 10 and the *Hin*dIII site of pRR-4 in the corresponding cloning sites of pACYC184 and pBR322, respectively (Fig. 1c). HB101 harboring pRR-5 was autoagglutinating and agglutinated EbG-NN erythrocytes, whereas HB101 harboring pRR-6 agglutinated MM and MN but not EbG-NN erythrocytes (Table 1). pRR-7 (Fig. 1c) was obtained by ligating a partial *Pst*I-*Eco*RI digest of pRR-6 with *Pst*I-*Eco*RI-treated pBR322. The 3.4-kb *Bam*HI-*Hin*dIII fragment of pRR-6 was further subcloned in the *Bgl*II-*Hin*dIII site of pHC79 to get pRR-8. Of these two plasmids, pRR-7, but not pRR-8 (Fig. 1d), expressed the blood group M agglutinin (Table 1). From these experiments we concluded that the DNA segment needed for expression of the blood group M agglutinin was situated within the 6.5-kb *Pst*I-*Eco*RI fragment of pRR-6 and that the genes for the EbG-NN-specific hemagglutinin were situated within the 10-kb *Eco*RI fragment of pRR-4 and pRR-5 (Fig. 1a to c).

Fimbrial extracts prepared from the wild-type strains IH11165 and KS300 (both produce blood group M agglutinin [56]) and from all recombinant strains were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The extracts obtained from HB101 harboring pRR-1, pRR-2, or pRR-3 contained two major peptides of 19.5 and 21 kilodaltons (kDa) in size (Fig. 2, lane c; Table 1). Extracts prepared from strains that agglutinated MM and NM erythrocytes (including those of the two wild-type strains) all contained the 21-kDa peptide (Fig. 2, lanes a, b, and c), and those from strains agglutinating EbG-NN erythrocytes (and being autoagglutinating) contained the 19.5-kDa peptide (Table 1, Fig. 2, lane c). Neither of these two peptides was seen in extracts from completely nonhemagglutinating strains (Table 1).

**Isolation and characterization of fimbriae (G fimbriae) recognizing *N*-acetyl-D-glucosamine.** HB101 cells harboring pRR-5 appeared strongly fimbriated when viewed in the electron microscope, whereas those harboring pACYC184 appeared nonfimbriated (not shown). Fimbrial preparations obtained from cells harboring pRR-5 produced a single peptide band with an apparent molecular mass of 19.5 kDa (Fig. 2, lane e). When viewed in the electron microscope, these preparations contained structures with typical fimbrial morphology; i.e., they contained filaments 0.5 to 1 μm in length and 5 to 7 nm in diameter (Fig. 3a). The amino acid

TABLE 2. Effect of type of erythrocyte on hemagglutination caused by recombinant *E. coli* HB101 and purified hemagglutinins

| Type of erythrocyte used[a] | Agglutination[b] by: | | | |
|---|---|---|---|---|
| | HB101 harboring pRR-5 | | HB101 harboring pRR-6 | |
| | Bacteria | Hemagglutinin (μg ml$^{-1}$) | Bacteria | Hemagglutinin (μg ml$^{-1}$) |
| MM | 0 | >250[c] | 256 | 0.01 |
| MN | 0 | >250 | 128 | 0.05 |
| NN | 0 | >250 | 0 | >100[d] |
| EbG-MM | 16 | 60 | 256 | 0.01 |
| EbG-NN | 16 | 60 | 0 | >100 |
| NEU-MM | 0 | >250 | 1,024 | 0.005 |
| TRY-MM | 0 | >250 | 32 | 50 |
| NEU-NN | 0 | >250 | 0 | >100 |
| TRY-NN | 0 | >250 | 0 | >100 |
| EbG-TRY-NN | 64 | 15 | 0 | >100 |

[a] MM, human MM erythrocytes; MN, human MN erythrocytes; NN, human NN erythrocytes; EbG, erythrocytes treated with endo-β-galactosidase; NEU, erythrocytes treated with neuraminidase; TRY, erythrocytes treated with trypsin; EbG-TRY, erythrocytes treated first with endo-β-galactosidase and then with trypsin.

[b] Agglutination is recorded as the reciprocal of the highest dilution of a bacterial suspension ($3 \times 10^9$ bacteria ml$^{-1}$) or the lowest concentration of isolated hemagglutinin still causing hemagglutination.

[c] No hemagglutination at 250 μg ml$^{-1}$.

[d] No hemagglutination at 100 μg ml$^{-1}$.

composition of the fimbrial subunit is given in Table 3. The proportion of hydrophobic amino acids (proline, alanine, valine, methionine, isoleucine, leucine, and phenylalanine) was 40.1%, and the two cysteine residues might originate from an intrapeptide disulfide bridge suggested to occur in a number of other *E. coli* fimbrillins (2, 23, 47). The $NH_2$-terminal amino acid sequence of the fimbrillin is shown in Fig. 4 (where it has been termed "G"). A partial homology was observed when compared with KS71A (a type of P fimbria) and K99 fimbriae.

G fimbria were also compared with other *E. coli* fimbriae and hemagglutinins by enzyme-linked immunosorbent assay (Table 4). Immunological cross-reactions were generally under 1% with heterologous antigens as compared with homologous reactions.

The purified fimbriae did not agglutinate human erythrocytes unless these were treated with endo-β-galactosidase (Table 2). Still, the hemagglutination titer was low. A stronger agglutination reaction was obtained if EbG-NN erythrocytes were also treated with trypsin (Table 2). Such erythrocytes were used in hemagglutination inhibition assays (Table 5). The hemagglutination caused by the purified fimbriae was strongly inhibited by *N*-acetyl-D-glucosamine and weakly inhibited by D-glucose. *N*-Acetyl-D-glucosamine at a concentration of 0.1 M partially inhibited the autoagglutination of the HB101 strain that harbored pRR-1. *N*-Acetyl-D-galactosamine, D-galactose, α-methyl-D-mannopyranoside, L-serine, L-threonine, L-glycine, and L-leucine all appeared to be noninhibitory.

TABLE 3. Amino acid composition of the purified hemagglutinins

| Amino acid | Residues per subunit molecule[a] | |
|---|---|---|
| | G fimbria | M agglutinin |
| Asx | 21 (20.9) | 19 (19.3) |
| Thr | 20 (20.0) | 24 (24.2) |
| Ser | 13 (13.0) | 18 (18.0) |
| Glx | 14 (13.9) | 17 (17.2) |
| Pro | 7 (6.7) | 12 (11.8) |
| Gly | 16 (16.1) | 27 (27.0) |
| Ala | 21 (21.1) | 17 (16.8) |
| Cys | 2 (1.6) | 0 (Tr) |
| Val | 15 (15.1) | 13 (13.0) |
| Met | 2 (1.6) | 2 (1.7) |
| Ile | 8 (7.9) | 5 (5.0) |
| Leu | 13 (13.0) | 11 (11.2) |
| Tyr | 7 (7.0) | 5 (5.0) |
| Phe | 5 (4.6) | 8 (8.2) |
| His | ND[b] | ND |
| Lys | 8 (7.9) | 7 (6.8) |
| Arg | 5 (5.0) | 7 (7.0) |
| Trp | ND | ND |

[a] Experimentally obtained values are given in parentheses.

[b] ND, Not determined.

**Isolation and characterization of a nonfimbrial hemagglutinin (M agglutinin) recognizing the human blood group M antigen.** Fimbriae purified from HB101 harboring pRR-1 agglutinated MM erythrocytes weakly but the amount of the expected 21-kDa peptide in such preparations was low as compared with the amount of this peptide in fimbrial extracts (Fig. 2, lanes c and d). Therefore, another method was used to isolate the presumed blood group M agglutinin (see Materials and Methods). When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the agglutinin preparation gave a single peptide band with an apparent molecular mass of 21 kDa (Fig. 2, lane f). No fimbriae were observed when these preparations were analyzed in the electron microscope (Fig. 3c and d). In accordance with this, HB101 harboring pRR-6 or pRR-7 (Fig. 3b) and the wild-type strain KS300 were nonfimbriated (not shown). The M-agglutinin preparations appeared as granular carpets sometimes containing ringlike structures (Fig. 3c and d). The amino acid composition of the subunit of the agglutinin is given in Table 3. The proportion of hydrophobic amino acids was 32.3%, and the subunit appeared to be devoid of cysteine. The $NH_2$-terminal amino acid sequence of the M agglutinin is shown in Fig. 4 (where it has been termed "M"). There was no significant homology with the other $NH_2$-terminal sequences shown.

The hemagglutinin appeared to be immunologically unrelated to the other *E. coli* fimbriae and hemagglutinins when compared in enzyme-linked immunosorbent assays (Table 4).

Human MM and MN erythrocytes were strongly agglutinated by the purified agglutinin preparation (Table 2). No hemagglutination was observed with NN erythrocytes. The hemagglutination reaction was not affected by treatment of

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                    5                   10                  15                  20                  25
Ala-Ala-Ser-Ile-Pro-Gln-Gly-Gln-Gly-Glu-Val-Ser-Phe-Lys-Glu-Thr-Val-Val-Asp-Ala-Pro-Cys-Gly-Ile-Glu-  KS71A

                          Asn-Thr-Gly-Thr-Ile-Asn-Phe-Asn-Gly-Lys-Ile-Thr-Ser-Ala-Thr-Cys-Ser-Ile-Glu-  K99

                          Thr-Asp-Gly-Thr-Ile-Asn-Phe-Asn-Gly-Lys-Val-Val-Asp-Gln-Thr- X -Ser-Val-Thr-  G

                                Ser-Thr-Val-Thr-Ala-Thr-His-Thr-Val-Glu-Ser-Asp-Ala-Glu-Phe-Thr-Ile-  M

Asn-Phe-Thr-Ser-Ser-Gly-Thr-Asn-Gly-Lys-Val-Asp-Leu-Thr-Ile-Thr-Glu-Glu-Cys-Arg-Val-Thr-Val-Glu-Ser-  AFA-1
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FIG. 4. Comparison of the $NH_2$-terminal amino acid sequences of KS71A, K99, G fimbrillins, M agglutinin, and AFA-1 adhesin. The sequences for the KS71A, K99, and AFA-1 proteins were taken from references 47, 50, and 32, respectively. X indicates unidentified residue. Underlined residues in the G and M proteins are uncertain; i.e., they indicate probable amino acids.

erythrocytes with neuraminidase or endo-β-galactosidase, but trypsinized erythrocytes were agglutinated only weakly (Table 2). L-Serine and L-threonine inhibited the hemagglutination reaction caused by the agglutinin, whereas L-glycine, L-leucine, and all five sugars tested appeared to be noninhibitory (Table 5).

In gel filtration experiments, the hemagglutinating material was eluted in the void volume of a Sephadex G-75 column but as a broad peak starting in the void volume in a Sepharose 4B column. These findings indicated that the 21-kDa peptide occurred as aggregates with an apparent molecular mass of $>10^5$ Da.

Cell-free supernatants from broth cultures of recombinant strains expressing the M agglutinin showed hemagglutination titers of up to 1:128 on microtiter plates. However, no attempts were made to purify the M agglutinin from such supernatants because of its apparently low concentration (0.5 to 1 μg $ml^{-1}$).

## DISCUSSION

Recent studies have shown that the most common hemagglutinin among pyelonephritogenic *E. coli* strains is the P-blood-group antigen-binding P fimbria (28, 55). Some of these strains also agglutinate erythrocytes lacking the P-blood-group antigens; such binding specificities were termed X adhesins (53). Fimbrial X adhesins recognizing sialyl galactosides have been described before (29). In P- and S-fimbriated strains, fimbriation and hemagglutination have been genetically separated (15, 40, 45, 59), but at least in P-fimbriated strains the hemagglutinating principle appears to be physically associated with the fimbrial filament (45, 60). To date, no such hemagglutinin has been isolated and its properties have been characterized by studying hemagglutination properties of bacteria and isolated fimbriae.

The isolation and characterization of other X adhesins have been seriously hampered by the simultaneous expression of many different hemagglutinins with similar biochemical properties by most uropathogenic *E. coli* strains (27, 42, 43, 48). We therefore cloned the genetic determinants for the X adhesins of *E. coli* IH11165 and obtained a DNA fragment that converted the nonfimbriate recipient strain into a strain agglutinating human erythrocytes carrying the blood group M antigen or terminal *N*-acetyl-D-glucosamine residues or both. These two agglutination specificities were further separated by subcloning, and strains expressing one or the other of the two hemagglutinins were obtained (Fig. 1; Table 1). This enabled us to purify the agglutinins and confirm the presence of two separate hemagglutinins in *E. coli* IH11165. Genetic analyses of several multifimbriate *E. coli* strains have so far shown that genes for the adhesins are encoded by unlinked gene clusters (18, 43). In contrast, the genes encoding the two *E. coli* IH11165 hemagglutinins were linked.

The fimbriae isolated from a recombinant strain recognizing *N*-acetyl-D-glucosamine also agglutinated endo-β-galactosidase-treated erythrocytes (Table 2). The hemagglutination pattern of the isolated fimbriae closely resembled that of the parental *E. coli* IH 11165 strain (56). We propose the term G fimbriae for this fimbrial type. Whether the hemagglutinative activity resides in the subunit itself or in an acessory component remains to be eludicated. Another protein recognizing *N*-acetyl-D-glucosamine is the *Tritium vulgare* lectin known as wheat germ agglutinin (5, 39). It also agglutinates untreated erythrocytes, but their agglutinability is enhanced by protease treatment (3). In contrast to wheat germ agglutinin, G fimbria did not agglutinate erythrocytes unless they were treated with endo-β-galactosidase (Table 2), which may indicate a difference in the affinity or in the precise receptor structure of these lectins. The autoagglutination observed among G-fimbriate recombinant strains (Table 1) may be due to binding to terminal *N*-acetyl-D-glucosamine residues known to be present, for example, in the lipopolysaccharide of *E. coli* K-12 (41).

The isolated blood group M hemagglutinin, which we propose to call the M agglutinin, appeared to be nonfimbrial.

TABLE 4. Immunological cross-reactions between fimbrial and nonfimbrial adhesins[a]

| Solid-phase antigen | Antibody titer of antiserum against: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Type 1 fimbria | Type 1C fimbria | KS71A fimbria | S fimbria | G fimbria | O75X agglutinin | M agglutinin | Preimmune serum |
| Type 1 | 6.5 | 2.0 | <2 | 3.2 | 3.5 | 3.2 | 3.0 | <2 |
| Type 1C | 3.3 | 5.2 | <2 | 3.0 | <2 | 3.0 | <2 | <2 |
| KS71A | 3.1 | 3.8 | 5.6 | <2 | 2.1 | 3.2 | 2.2 | <2 |
| S | 2.8 | 3.3 | <2 | 4.4 | 2.0 | 3.4 | 3.1 | <2 |
| G | 3.0 | <2 | <2 | <2 | 5.4 | 3.3 | 3.2 | <2 |
| O75X | 2.2 | <2 | 2.2 | <2 | <2 | 5.5 | 2.8 | <2 |
| M | 3.0 | <2 | <2 | 3.0 | 3.2 | 3.3 | 6.2 | <2 |

[a] Enzyme-linked immunosorbent assay; the antibody titre is given as the logarithm of the reciprocal of the highest dilution of the antiserum giving an $A_{400}$ 0.5.

TABLE 5. Inhibition of hemagglutination caused by purified hemagglutinins

| Inhibitor[a] | Hemagglutination titer (μg of protein $ml^{-1}$) of isolated hemagglutinins from *E. coli* HB101 harboring: | |
|---|---|---|
| | pRR-5 | pRR-6 |
| None | 15 | 0.005 |
| L-Serine | 15 | 0.02 ($MIC_{50}$ = 100 mM)[b] |
| L-Threonine | 15 | 0.2 ($MIC_{50}$ > 100 mM) |
| L-Leucine | 15 | 0.005 |
| L-Glycine | 15 | 0.005 |
| *N*-Acetyl-D-glucosamine | >240 ($MIC_{50}$ = 10 mM)[c] | 0.005 |
| *N*-Acetyl-D-galactosamine | 15 | 0.005 |
| D-Glucose | 60 ($MIC_{50}$ = 20 mM) | 0.005 |
| D-Galactose | 15 | 0.005 |
| α-Methyl-D-mannopyranoside | 15 | 0.005 |

[a] Each inhibitor was tested at a concentration of 100 mM.
[b] $MIC_{50}$, Concentration of inhibitor needed to decrease the hemagglutination titer from 0.005 to 3.2 μg $ml^{-1}$.
[c] $MIC_{50}$, Concentration of inhibitor needed to decrease the hemagglutination titer from 15 to 120 μg $ml^{-1}$.

This conclusion is based on the following observations: (i) strains expressing only the M agglutinin were nonfimbriate (Table 1; Fig. 3), and (ii) no fimbrial structures were observed in M-agglutinin preparations (Fig. 3). Previously, nonfimbrial hemaglutinins have been purified from two other uropathogenic *E. coli* strains. These hemagglutinins were called O75X (54) and "AFA-1" adhesins (31). Neither of these was blood group M specific, and the apparent molecular mass of their subunit was 16 kDa, which is lower than the 21 kDa observed for the M agglutinin (Fig. 2). Also, the amino acid composition of the M agglutinin differed from those of O75X and AFA-1 (Table 3) (32, 54). Furthermore, the O75X hemagglutinin appeared to be immunologically unrelated to the M agglutinin (Table 4), and the $NH_2$-terminal amino acid sequences of the M agglutinin and AFA-1 adhesins appeared to be different (Fig. 4). It thus seems that *E. coli* strains can have many types of nonfimbrial hemagglutinins, as well as different types of fimbriae.

Neuraminic acid residues have been reported to be involved in the formation of M/N antigenicity (35). Treatment of MM erythrocytes with neuraminidase, a treatment that would remove α-(2-3)-bound neuraminic acid residues from O-glycosidic chains, increased rather than decreased the agglutinability of the erythrocytes (Table 2). The glycophorins $A^M$ and $A^N$ are known to differ in their $NH_2$-terminal portion; in glycophorin $A^M$ the five $NH_2$-terminal amino acids are Ser-Ser-Thr-Gly, and those of glycophorin $A^N$ are Leu-Ser-Thr-Glu (52). Precipitation of glycophorin $A^M$ by IH11165 bacteria is partially inhibited by L-serin (19). L-Serine also inhibited the hemagglutination caused by the purified M agglutinin (Table 5), suggesting that the $NH_2$-terminal serine residue, rather than the terminal α-(2-3)-bound neuraminic acid residues, is involved in the formation of the receptor structure for the M agglutinin. However, experiments made with the purified M agglutinin are more reliable since the wild-type *E. coli* IH 11165 expresses many hemagglutination specificities. Glycophorin A acts as a receptor for the influenza virus (52), and it is involved in the pathogenesis of *Plasmodium falciparum* infection (49). Recent studies indicate that at least two proteins of *P. falciparum* recognize *N*-acetyl-D-glucosamine present on glycophorin A (20).

We are now determining the biological function of the two new *E. coli* hemagglutinins (G fimbriae and M agglutinin) by testing adhesion of the recombinant strains to human tissue sections (58). Wheat germ agglutinin is known to bind to several human kidney structures (17) and one might expect a similar binding for G-fimbriate bacteria. Due to its high specific hemagglutination activity (Table 2), the M agglutinin could also be a useful reagent for determining the distribution of blood group M-like antigens in human tissue.

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