

## Molecular Cloning of the *Escherichia coli* O75X Adhesin

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**The uropathogenic strain *Escherichia coli* IH11128 (O75:K5:H<sup>-</sup>) exhibits a mannose-resistant O75X adhesin. The genes encoding the O75X adhesin were cloned from a clinical strain and transferred to *E. coli* K-12 derivatives. The recombinant plasmids were found to express a 15-kilodalton fimbrial subunit protein, a fimbrialike extracellular structure, and mannose-resistant hemagglutination. An indirect immunofluorescence assay was used to study attachment of the clone and purified adhesin to frozen sections of human kidney. The clone bound selectively to the interstitial areas and notably to Bowman's capsule. The purified adhesin bound to the basement membrane of the tubules and to Bowman's capsule.**

Fimbriae or fimbrialike structures serve as bacterial binding factors (4, 5). A particular strain may produce more than one fimbrial type at the same time (7, 9, 10, 28); however, different fimbrial types mostly occur on separate cells and show a rapid phase variation both in vitro and in vivo (1, 22-25, 29). Fimbriae are characterized by their hemagglutination (mannose resistant or mannose sensitive) (4, 26), the serological properties of the strains carrying them (10), tissue tropism (for example to bowel and kidney) (4, 14-16, 21), and associated clinical conditions (diarrhea, pyelonephritis, and meningitis) (4, 5, 25, 32).

A variety of adhesins have been identified on *Escherichia coli* strains associated with human extraintestinal infections. Of the adhesins characterized, P fimbriae recognize  $\alpha$ Gal-(1-4) $\beta$ Gal, a moiety of human P blood group glycosphingolipids (11, 18, 31). They are considered a major bacterial virulence factor in childhood pyelonephritis. Other mannose-resistant fimbriae constitute a heterogeneous group and have been termed X adhesins. One group of X adhesins, S fimbriae, recognizes sialyloligosaccharides and may be important in the pathogenesis of neonatal meningitis caused by *E. coli* O18ac:K1:H7 strains (6, 14, 23, 27). Recently, another X adhesin occurring on *E. coli* O75 strains has been described by Väisänen-Rhen (34). The O75X adhesin is chemically very similar to but morphologically different from typical *E. coli* fimbriae. By electron microscopy, the purified proteins were shown to be arranged in a coillike structure which consists of subunits with an apparent molecular mass of about 15 kilodaltons (kDa). The O75X adhesin was found to be serologically related in all of the O75X-positive strains tested. In a recent study by Nowicki et al. (21), it was shown that fluorochrome-labeled *E. coli* BN53 expressing the O75X adhesin bound to substructures of the human kidney, specifically to the interstitial elements and Bowman's capsule. However, characterization of the genes encoding this adhesin and its function in urinary tract infection remained to be determined.

In this report we describe the construction of a recombinant plasmid derived from *E. coli* IH11128 (O75:K5:H<sup>-</sup>) which encodes mannose-resistant hemagglutination (MRHA). This hemagglutination is associated with the O75X adhesin

and with specific adherence to the basement membrane and Bowman's capsule.

### MATERIALS AND METHODS

**Bacteria and media.** The bacterial strains used are listed in Table 1. Bacteria were grown on Luria agar, Luria broth, or liquid minimal medium (3). Media were supplemented with ampicillin (100  $\mu$ g/ml), chloramphenicol (20  $\mu$ g/ml), or kanamycin (20  $\mu$ g/ml) if required. For hemagglutination assays and immunological tests, the strains were subcultured twice on Luria agar plates. Before DNA isolation the strains were cultured overnight in Luria broth on a rotary shaker at 37°C.

**Agglutination tests and detection of fimbriation.** Hemagglutination tests with human OP<sub>1</sub> and O<sub>p</sub> erythrocytes were performed as previously described (21). All tests were done over crushed ice. Bacteria were suspended in phosphate-buffered saline (PBS) to an optical density at 600 nm of 0.5. Twofold dilutions of bacteria were mixed with 3% (vol/vol) erythrocytes and observed for hemagglutination. Bacterial agglutinations with antisera were done by mixing equal volumes of the suspension of bacteria and twofold dilutions of antisera prepared against the purified O75X adhesin as described previously (34).

**Protein and DNA analysis.** The amount of protein was estimated by the modified procedure of Lowry et al., using bovine serum albumin as a standard (19). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) was performed in 12 and 15% polyacrylamide slab gels by the method of Laemmli (17). The protein products of the cloned inserts were identified by using a bacterial-cell-free coupled transcription-translation system (Amersham Corp., Arlington Heights, Ill.) with [<sup>35</sup>S]methionine radiolabel. Immunoprecipitation of the in vitro-translated protein products with specific anti-O75X serum and *Staphylococcus aureus* was performed by the method of Kessler (12). Anti-O75X serum was preabsorbed with *E. coli* EC901 before use.

**Isolation of chromosomal and plasmid DNA.** Chromosomal DNA was isolated as described previously (7). Plasmid DNA from clones carrying recombinant DNA was purified by the cleared lysate procedure (30).

**Cosmid cloning procedure.** Genomic fragments of approximately 40 kilobases (kb) were obtained after size fractionation of whole-cell DNA partially digested with the restriction

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TABLE 1. *E. coli* strains used

Strain	Relevant genotype	Source or reference
IH11128	Clinical UTI <sup>a</sup> isolate	34
HB101	<i>hsdS20 recA13</i>	2
EC901	As AB1133, but <i>hsdR4 recA</i>	R. Gill
P678-54	<i>pil pap</i>	8
BN400	HB101(pBJN400)	This study
BN401	EC901(pBJN400)	This study
BN406	EC901(pBJN406)	This study
BN407	P678-54(pBJN406)	This study
BN409	EC901(pBJN409)	This study
BN410	EC901(pBJN410)	This study

<sup>a</sup> UTI, Urinary tract infection.

endonuclease *Sau3A* (8). The cosmid vector used was pHC79, which was digested with *Bam*HI. The two DNAs were mixed, ligated for 48 h at 4°C, and packaged in vitro into bacteriophage  $\lambda$ . The phage was then used to infect *E. coli* HB101.

**Preparation of fimbriae.** Fimbriae were isolated from bacteria grown on Luria agar by using deoxycholate and urea as described previously (13) except that the sucrose gradient purification step was omitted.

**Electron microscopy.** All samples were fixed in 3% buffered glutaraldehyde and negatively stained with 0.5% uranyl acetate by the pseudoreplication technique. Specimens were examined at 80 kV in a JEOL 100CX transmission electron microscope.

**Kidney samples.** Tissue samples were obtained from the macroscopically normal pole of a human kidney carrying renal adenocarcinoma at the opposite pole, frozen in liquid nitrogen, and stored at -70°C until use. Frozen sections were cut in a cryostat, and 4- $\mu$ m-thick sections were mounted on glass slides. The sections were fixed for 10 min at room temperature with ice-cold 3.5% (wt/vol) paraformaldehyde in PBS and washed three times with 100 ml of PBS.

**Labeling of bacteria and indirect IF assay.** Strains EC901 and BN406 were stained with fluorescein isothiocyanate as described previously (21). The labeled cells were then suspended in PBS and stored at -20°C in 100- $\mu$ l aliquots. The capacities of BN406 and purified adhesin to bind to human kidney were also tested by an indirect immunofluorescence (IF) method with specific anti-O75X serum and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins (21). Antiserum prepared previously (25) was preabsorbed with *E. coli* EC901.

**Adhesion assay.** Fluorochrome-stained bacteria were thawed and diluted in PBS containing 0.01% (vol/vol) Tween 20 and 1% (wt/vol) bovine serum albumin to give  $3 \times 10^{10}$  bacteria per ml. Fifty microliters of the bacterial suspension was pipetted onto tissue sections on a glass slide and incubated in a moist chamber at room temperature for 30 min; the sample was then washed in 100 ml of PBS three times for 5 min with gentle agitation. For purified fimbriae, 40  $\mu$ l of fimbriae (500  $\mu$ g/ml) was pipetted onto the fixed kidney sections and incubated in a moist chamber for 30 min at room temperature. After being washed, the samples were stained by the indirect IF method. Adherent bacteria or fimbriae were viewed by using a fluorescence microscope.

## RESULTS

**Isolation of recombinant plasmid expressing the O75X adhesin.** Bacteriophage  $\lambda$  transducing particles carrying

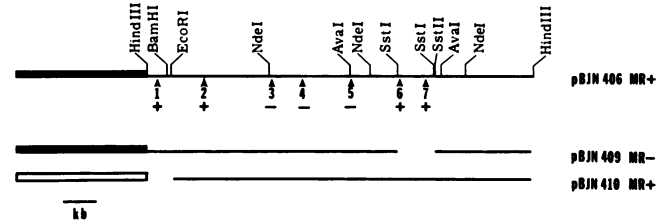


FIG. 1. Physical map of pBJN406 and its deletion derivatives. ■ and □, pACYC184 plasmid DNA and pBR322 DNA, respectively; —, insert DNA of IH11128; ▲, positions of transposon insertions; + and -, presence and absence, respectively, of MRHA activity.

recombinant cosmid molecules with portions of the strain IH11128 genome were prepared and used to transduce *E. coli* HB101. A total of 1,200 ampicillin-resistant transductants were screened for hemagglutination. One transductant, BN400, showed slow and weak granulation in the center of the bacterial-erythrocyte mixture. Since expression of the O75X hemagglutinin in *E. coli* HB101 was found to be poor, the recombinant cosmid in BN400, designated pBJN400, was isolated and used to transform *E. coli* EC901. The obtained transformant, BN401, showed positive and clearly stronger MRHA than did BN400. pBJN400 was digested with restriction endonuclease *Hind*III into five fragments. A 12-kb *Hind*III fragment was subcloned into the plasmid vector pACYC184 to form pBJN406; *E. coli* BN406 and BN407, transformed with pBJN406, exhibited strong MRHA and agglutination with anti-O75X serum.

**Analysis of the cloned DNA.** The subclone pBJN406 was digested with various restriction endonucleases to prepare a physical map (Fig. 1). The DNA insert was approximately 11.4 kb. To determine the limits of the O75X hemagglutinin-coding region, insertional inactivation mapping with the transposon Tn5 was used. Insertions 1, 2, 6, and 7 did not affect MRHA, whereas insertions 3, 4, and 5 abolished

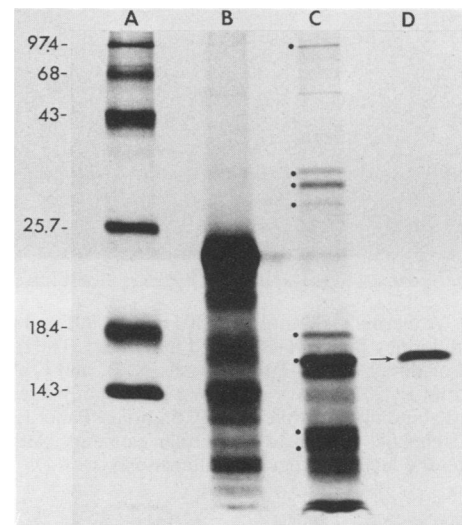


FIG. 2. Autoradiograph of 15% SDS-polyacrylamide gel of in vitro-translated pBJN406 polypeptide products. Lanes: A, standard marker proteins (molecular weights shown in thousands); B, polypeptide products of pACYC184; C, polypeptide products unique to coding region of pBJN406 (•); D, immunoprecipitated fimbrial protein (→).

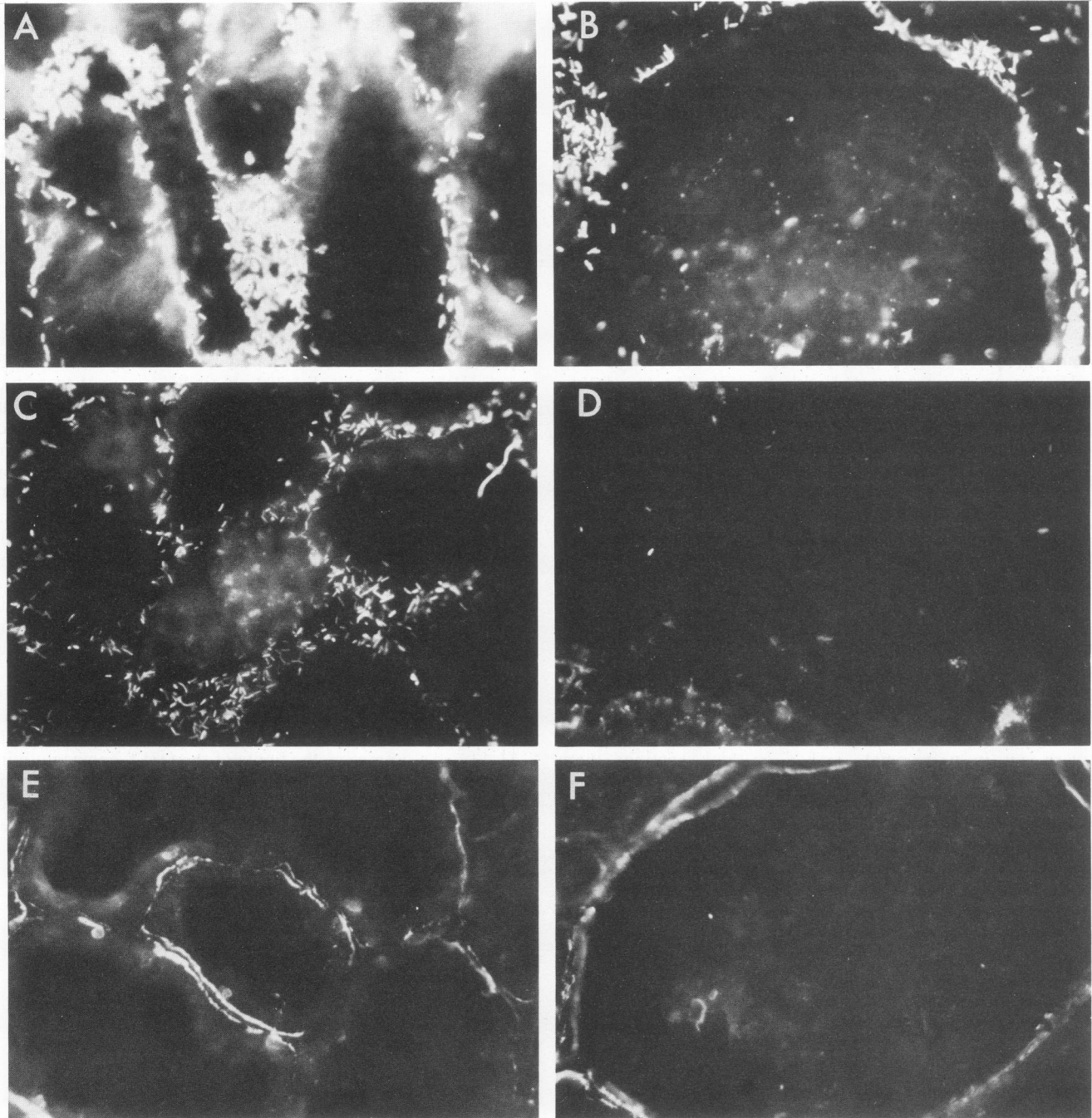


FIG. 3. Adherence of strains BN406 and EC901 and purified O75X fimbriae to human kidney sections. Bacteria or purified fimbriae were mixed with kidney tissue as described in the text. The tissue was washed, and the adherent material was visualized by either indirect (A, B, E, and F) or direct (C and D) IF. Panels A, B, and C show binding of the recombinant strain BN406, and panel D shows the nonadherent control strain EC901. Note very strong binding of bacteria to interstitial elements in panel A and to Bowman's capsule in panel B. Panels E and F show binding of purified O75X fimbriae. Panel E shows binding to the tubular basement membranes, and panel F shows binding to Bowman's capsule but not to glomerular elements. Although staining of two layers of Bowman's capsule is strong, it was not possible to photograph the layers in focus simultaneously.

hemagglutination. Two deletion mutants of pBJN406 were also constructed to further delineate the O75X coding region. Plasmid pBJN409 contains a 1.1-kb *Sst*I deletion and no longer confers the MRHA<sup>+</sup> phenotype. Plasmid pBJN410 contains a 700-base-pair *Hind*III-*Eco*RI deletion and is MRHA<sup>+</sup>. The relative positions of the insertion and deletion

mutations is illustrated in Fig. 1. These results suggest a maximum size for the O75X coding region of 6 kb.

Polypeptides encoded by pBJN406 were analyzed by using a bacterial-cell-free coupled transcription-translation system. pBJN406 was digested with *Eco*RI to inactivate the chloramphenicol acetyltransferase gene and translated in

vitro. Radiolabeled proteins were separated by SDS-PAGE and visualized by autoradiography (Fig. 2). A total of eight proteins with approximate molecular masses of 90, 32, 29, 27, 17, 15.6, 12.5, and 11.5 kDa were found. Similar results were obtained when the *EcoRI-SstII* insert fragment was translated (results not shown). In addition, the in vitro-translated radiolabeled protein products were immunoprecipitated by using antisera specific for O75X fimbriae. The immunoprecipitated proteins were analyzed by SDS-PAGE (Fig. 2). The 15.6-kDa protein corresponds to the subunit protein of native O75X fimbrialike structures.

#### Adhesive properties of *E. coli* BN406, IH11128, and EC901.

*E. coli* BN406, IH11128, and EC901 were tested for their ability to hemagglutinate human OP<sub>1</sub> and O<sub>p</sub> erythrocytes. Dilutions of bacteria were mixed with erythrocytes to determine hemagglutination ability. The hemagglutination titers of *E. coli* IH11128 and BN406 were 1/32 and 1/256, respectively, with both erythrocyte types. EC901 was negative at all concentrations. Labeling with fluorescein isothiocyanate did not significantly alter the hemagglutination titers of the strains.

The attachment capacities of strains BN406 and EC901 were also tested. BN406 adhered to frozen sections of human kidney, binding to interstitial elements (Fig. 3C); the attachment and binding pattern of the clone was stronger and more specific than that of the clinical strain. Strain IH11128 showed weaker binding to interstitial tissue and Bowman's capsule and some additional attachment to glomerular elements (results not shown) (21). Very poor and nonspecific adhesion was observed with EC901 (Fig. 3D).

To ascertain the role of the cloned adhesin in adherence, we tested the attachment capacity of BN406 by using an indirect IF assay. The indirect IF method stains only cells expressing the O75X adhesin (Fig. 3A and B). Selective binding to interstitium and Bowman's capsule was observed. We conclude that the MRHA activity cloned from IH11128 into BN406 was responsible for the specific binding capacity of the clone.

**Characterization of the O75X adhesin.** The surface structures were purified from strains BN406 and IH11128 and characterized by SDS-PAGE. SDS-PAGE of the isolated surface structures revealed a peptide band with an apparent molecular weight of 15,000 for both IH11128 and BN406 (Fig. 4). Another intensely stained band with an apparent molecular weight of 27,000 was observed for BN406 but not for IH11128.

BN406 and EC901 cells were examined for extracellular structures in the electron microscope. Fimbrialike structures similar to those reported by Väisänen-Rhen (34) were seen on negatively stained intact cells of BN406 (Fig. 5B) but not on the recipient strain EC901 (Fig. 5A). An electron micrograph of purified extracellular elements extracted from BN406 revealed similar fimbrialike structures (Fig. 5C).

**Characterization of binding specificity of purified O75X adhesin.** Fimbriae purified from BN406 were tested for hemagglutination and capacity to bind to sections of human kidney. This material exhibited MRHA activity and bound selectively to the basement membrane of the kidney tubules and to Bowman's capsule (Fig. 3E and F). Bowman's capsule showed a two-layer substructure intensely stained with the adhesin. No binding to the luminal domains of the tubules was observed. These experiments showed that the purified adhesin was active in hemagglutination and in specific binding to kidney substructures. The binding characteristics of purified recombinant O75X adhesin were similar to those of the native fimbriae purified from strain IH11128 (16).

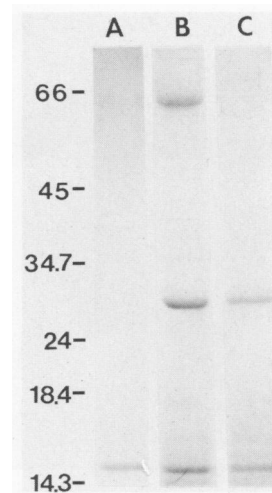


FIG. 4. SDS-PAGE of partially purified O75X adhesins from strains IH11128 and BN406. Partially purified adhesins from IH11128 (lane A) and from BN406 before (lane B) and after (lane C) urea treatment are shown. The numbers on the left are the apparent molecular weights in thousands of the marker proteins.

## DISCUSSION

One difficulty inherent in studying virulence-associated properties of bacteria is the lack of genetically defined strains. In vitro recombinant DNA techniques have been used to prepare strains expressing a single hemagglutinin so that its contribution to virulence may be studied; examples are P and S fimbriae (6, 8, 20, 28, 35). Recently, we found that *E. coli* BN53 is able to attach to and recognize specific elements in the renal interstitium (21). The specific attachment appeared to be mediated by the O75X adhesin. In this report we describe the preparation and characterization of a genetically defined bacterial derivative expressing the O75X adhesin of uropathogenic *E. coli* IH11128. A plasmid carrying genes for the mannose-resistant O75X adhesin was prepared in vitro and transferred to *E. coli* K-12 hosts. Both the bacteria carrying the recombinant plasmid pBJN406 and the adhesin purified from the recombinant strains were found to hemagglutinate human erythrocytes in the presence of D-mannose. The size of the O75X adhesin-coding region is about 6 kb and encodes eight proteins, including the 15,600-molecular-weight fimbrial protein, as shown by immunoprecipitation of in vitro-translated peptides with antifimbrial antisera. The in vitro-translated fimbrial subunit is slightly larger than the native form and presumably includes a signal peptide region. We observed very weak expression of the O75X adhesin with *E. coli* BN400 and good expression with BN406 and BN407. An unknown genetic lesion or lesions in HB101 may inhibit the synthesis, assembly, or transport of the O75X adhesin, resulting in poor expression of the cloned hemagglutinin.

Binding of the adhesin to frozen sections of human kidney appeared highly specific. The bacteria adhered selectively to the interstitial areas. A more detailed study with adhesin purified from the recombinant strain revealed that the substructures recognized by the adhesin were the tubular basement membrane and Bowman's capsule. It is of interest that Bowman's capsule showed two intensely labeled substructures, the inner and outer layers. By using a double-staining technique with fluorochrome-labeled bacteria and nephron site-specific markers, we recently showed that both O75X-positive and P-fimbriated *E. coli* strains adhere to sections of

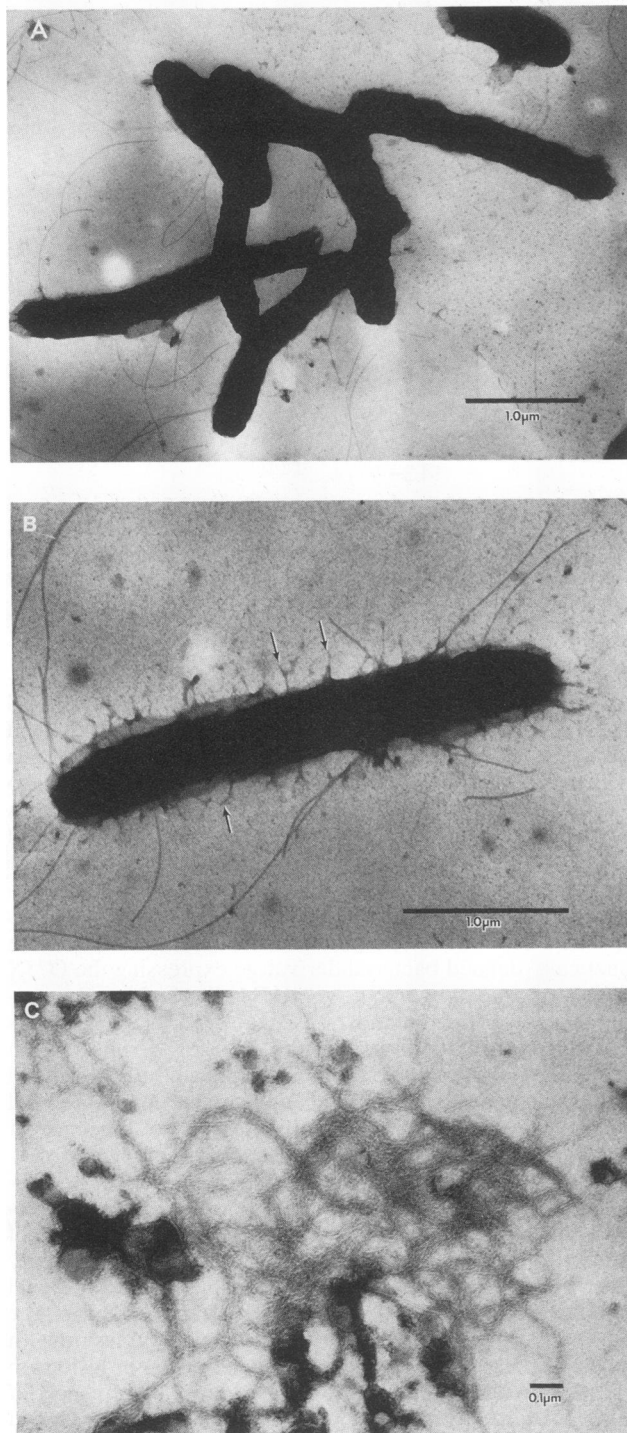


FIG. 5. Electron micrographs of recipient strain EC901 (A), strain BN406 (B) (arrows show fimbrial-like filaments), and partially purified extracellular structures of fimbrial-like morphology from BN406 (C).

human kidney (21). However, the P-fimbriated cells show specific adherence to the lumen of proximal and distal tubules, glomerular structures, and endothelium but do not adhere to renal interstitium (21). The lack of binding of BN406 to glomerular elements compared with that of IH1128 indicates that an additional binding factor is present

on the clinical strain which may mediate attachment to glomerular elements.

Both clinical and recombinant strains show fimbrial-like structures that have a subunit molecular mass of 15 kDa. We believe that extracellular fimbrial-like structures of the O75X adhesin are associated with specific attachment to the human kidneys and erythrocytes. However, the preparation from BN406 contained another distinct band with an apparent molecular mass of 27 kDa not sensitive to urea treatment. Whether this protein is associated with the fimbrial elements and attachment capacity remains to be determined (33). P and S fimbriae bind to digalactose and sialyloligosaccharides, respectively, and mostly to cells of epithelial origin (14, 15). The O75X adhesin recognizes other specific receptor molecules in the basement membrane and Bowman's capsule (B. Nowicki, J. Moulds, R. Hull, and S. Hull, submitted for publication). As a virulence determinant, the O75X adhesin may serve as a colonization factor in upper urinary tract infection by early interaction within the inner part of Bowman's capsule and then, after invasion, with interstitial tissue elements, leading to chronic nephritis. The possible role of the O75X adhesin in the pathogenesis of urinary tract infection and the molecular nature of receptor molecules on target tissues are under study.

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