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Isolation and characterization of the α-sialyl-β-2,3-galactosyl-specific adhesin from fimbriated *Escherichia coli*

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ABSTRACT **The α-sialyl-β-2,3-galactosyl-specific adhesin (S adhesin) was isolated from cells of a recombinant *Escherichia coli* K-12 strain expressing the S-fimbrial adhesin complex. A crude cell extract was partially dissociated into fimbriae and an adhesin-enriched fraction by heating to 70°C. From the latter, adhesin was purified to apparent homogeneity (by fast protein liquid chromatography, immunoblot, and NaDodSO$_4$/PAGE) by differential ammonium sulfate precipitation, dissociation in 8 M guanidine hydrochloride, and high-resolution anion-exchange chromatography in 8 M urea. The purified adhesin formed an aggregate of $M_r \approx 10^6$ that was made up of one type of 12-kDa polypeptide (fimbrillin is 16.5 kDa). It had pI value of 4.7 (fimbriae has a pI value of 6). Adhesin and fimbrillin had different amino acid compositions. The purified adhesins agglutinated human and bovine erythrocytes with the same specificity as the whole bacteria; purified fimbriae were not adhesive. Monoclonal anti-adhesin and anti-fimbriae antibodies were obtained. Monoclonal anti-adhesin, but none of the anti-fimbriae, antibodies inhibited the agglutination of erythrocytes. The anti-adhesive antibodies were used in immuno-gold electron microscopy to localize adhesin exclusively on the fimbriae, with a possible preference to their tips.**

Infections caused by pathogenic *Escherichia coli* are often initiated by their adherence to host cell surfaces. This cell interaction is mediated by lectin-like adhesive proteins associated with the bacterial surface (1). In many cases the adhesiveness of *E. coli* is correlated with the expression of supermolecular, extracellular structures, the fimbriae or pili. Since isolated fimbriae often cause the agglutination of erythrocytes (RBC), which is used as an *in vitro* assay for the adhesiveness of bacteria, they were recognized as adhesive bacterial appendages (2–8). Bacterial adhesins specifically recognize certain carbohydrate moieties of glycolipids or glycoproteins such as α-mannose (common type 1 fimbriae), α-galactosyl-1,4-β-galactose (P fimbriae), α-sialyl-2,3-β-galactose (S fimbriae), or galactose/N-acetylgalactosamine/serin (M fimbriae) (2, 5, 9–12).

Molecular analysis of the cloned determinants coding for common type 1 (13, 14), P (15–17), and S (18) fimbriae revealed that distinct regions of these direct fimbriation and adhesiveness. Transposon-insertional mutants and subclones were found that still expressed fimbriae but were no longer adhesive or that lacked fimbriae but were adhesive. These results indicated that adhesin and fimbriae subunits may be different molecules. In spite of the wealth of information on fimbriae, to our knowledge the nature of the adhesins and their correlation to fimbriae had not yet been defined.

During our studies on fimbriae and adhesins of *E. coli*, we were able to physically separate adhesin from the fimbriae of a pyelonephritogenic *E. coli* O6 strain. The adhesin was characterized *inter alia* with the help of monoclonal anti-adhesin antibodies that do not cross-react with the fimbrial subunit.

MATERIALS AND METHODS

Bacteria. The strains used are listed in Table 1. *E. coli* wild-type strain 536 (serotype O6:K15:H31) was isolated from a patient with a urinary tract infection (18). It expresses common type 1 fimbriae and the S-fimbrial adhesin complex (Sfa, previously termed X; see refs. 18 and 19) consisting of S fimbriae and S adhesin. The phenotype for S fimbriae is termed Fim$^+$ and that for S adhesive capacity (indicated by mannose-resistant hemagglutination) Adh$^+$. The bacteria were grown at 37°C for at least 18 hr on Loeb agar containing 0.1% glucose and appropriate antibiotics (100 μg/ml; see Table 1). Before use the bacteria were monitored for hemagglutination. With the exception of strain HB101 (pANN801-13/Tn 5-38) the strains agglutinated human and bovine RBC; agglutination was more pronounced at 4°C.

Isolation of Fimbriae and Adhesin from the Recombinant *E. coli* Strain HB101 (pANN801-13). Agar-grown bacteria were collected and suspended in 50 mM Tris·HCl (pH 7.8) at a cell density of 10^{10} cells per ml and agitated twice for 5 min with an Omnimixer (setting 3) while cooling in an ice bath. The bacteria and debris were removed by centrifugation (27,000 × *g*, 40 min), and the fimbriae were pelleted from the supernatant by ultracentrifugation (140,000 × *g*, 120 min). The crude fimbriae were suspended to 1 mg of protein per ml in PBS/5 mM EDTA and heated to 70°C for 1 hr. (PBS = 10 mM phosphate buffer containing 0.2 g of KCl and 8 g of NaCl per liter, pH 7.4.) After a subsequent centrifugation (140,000 × *g*, 120 min), the pellet contained predominantly fimbriae, and the supernatant was enriched in adhesin. The adhesin-containing supernatant was dialyzed against 50 mM NH_4HCO_3, lyophilized, suspended to 500 μg of protein per ml in PBS, and precipitated with ammonium sulfate (10–40% saturation at 4°C).

Both the fimbriae and adhesin preparations were fractionated at room temperature by high-resolution anion-exchange chromatography [Pharmacia MonoQ HR 5/5 column under the following conditions: buffer A (20 mM Tris glycine, pH 9.0/8 M urea); buffer B (buffer A/0.2 M LiCl); flow rate, 0.5 ml/min; gradient slope, 2 mM/ml]. Fractions were collected and assayed for protein content and hemagglutination activity. Usually >80% of applied protein was recovered from the column. Pooled fractions were dialyzed against 50 mM ammonium bicarbonate and lyophilized.

Monoclonal Antibodies. Female BALB/c mice (8 weeks old) were immunized i.p. with crude fimbriae from *E. coli* HB101 (pANN801-13) (50 μg of protein per animal) three

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Abbreviations: RBC, erythrocyte(s); mAb, monoclonal antibody.

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Table 1. Bacteria and recombinant clones

E. coli strain	Recombinant DNA	Fimbriae		
		Type 1	S	S adhesin
536 (06:K15:H31)	—	+	+	+
HB101 (K-12)	—	−	−	−
HB101	pANN801-13(Ap^r)	−	+	+
HB101	pANN801-1 (Tc^r)	−	−	+
HB101	pANN801-13/ Tn5-38 (Ap^r,Km^r)	−	+	−

The recombinant DNAs were derived from the cosmid pANN801 carrying the S-fimbrial adhesin of strain 536 (18). Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance. +, Present. −, Absent.

times at weekly intervals, with the first injection in complete Freunds adjuvant, the second in incomplete Freunds adjuvant, and the third in PBS. Two weeks later the animals were given booster injections (i.p., 400 μg of protein in PBS per injection) on four consecutive days. One day after the last booster injection the animals were killed, and their spleens were excised. The spleen cells (10^8 cells) were fused (20) with 2×10^7 PAI nonproducer myeloma cells (ref. 21, obtained from T. Staehelin, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G.). For cloning, the cell suspension was distributed into the wells of 10 microtiter plates. Hybrid clones were screened for activity with purified fimbriae, adhesin, and bacteria (22) using the ELISA technique (23).

Monoclonal antibodies were obtained from cell culture supernatants by ammonium sulfate precipitation and high-resolution anion-exchange chromatography as described (24). Immunoglobulin subclasses were determined by the Ouchterlony technique. The reactivity of the antibodies to native proteins was assayed in dot blots in the absence of $NaDodSO_4$, and antibodies to denatured protein were tested in dot blots in the presence of $NaDodSO_4$ and in immunoblots (25).

Immunoelectron Microscopy. Bacteria grown overnight at 37°C on agar were suspended in PBS (about 10^{10} cells per ml), transferred to copper grids coated with polyvinyl Formvar, and prepared for electron microscopy as described by Robinson *et al.* (26). Incubation was done by floating the inverted grids on drops of bovine serum albumin (1%), on monoclonal anti-adhesin antibodies (≈10 μg/ml of PBS, pH 8), and on protein A-coated gold spheres (10 nm diluted 1:25 in PBS/1% bovine serum albumin, adjusted to pH 8 with NaOH; Jansen Pharmaceutica, Beerse, Belgium). For contrasting the grids were floated on drops of 1% phosphotungstic acid in water, pH 8. After removal of excess fluid microscopy was done with a Phillips EM400 T/ST electron microscope. Photographs were processed on Rapitone P1-3 or P1-4 paper (Agfa Gevaert).

Hemagglutination and Hemagglutination Inhibition. The hemagglutinating activity of bacteria and adhesin preparations was determined with human or bovine RBC on ice as described (6, 8). RBC were treated with trypsin and periodate. For inhibition experiments bacteria or adhesin were preincubated with 0.1–10 mg of fetuin or of orosomucoid per ml for 15 min at 0°C.

Analytical Methods. $NaDodSO_4$/PAGE was performed according to Laemmli (27) in the presence or absence of 8 M urea. Molecular weight markers were bovine serum albumin, ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor, sperm whale myoglobin, lysozyme, and cytochrome *c*. For immunoblots, gels were incubated for 2 hr in transfer buffer/4 M urea prior to electrotransfer and immunochemical staining (25).

Isoelectric focusing gels (0.5% agarose gels, pH 3–10 Pharmalyte) were standardized with the Pharmacia isoelectric focusing calibration kit.

Protein concentrations were determined according to Bradford (28). Amino acid analyses were performed as described (29).

RESULTS

Isolation of Fimbriae and Adhesin. The uropathogenic *E. coli* strain expresses mannose-specific common type 1 fimbriae in addition to S fimbriae. We have evidence that not only S adhesins but also those of other receptor specificities can be extracted from the respective fimbriated *E. coli*, and the mannose-specific adhesin may thus interfere with our studies on the S adhesin. For separations and isolation of the S-adhesin and the S-fimbrial subunits, we, therefore, used the recombinant strain *E. coli* HB101 (pANN801-13) that expresses only the S-fimbrial adhesin complex.

For concomitant isolation of the adhesin and fimbrial proteins, we prepared a crude fimbrial preparation by mechanical treatment of the bacteria with an Omnimixer (30, 31).

Analogous to the isolation of adhesive proteins of nonfimbriated bacteria (22), a suspension of the crude preparation in PBS was heated to 70°C. After ultracentrifugation the supernatant was enriched in the adhesin and much reduced in fimbriae. In contrast to the crude preparation, the supernatant agglutinated RBC, so that subsequent purification steps could be monitored for the adhesin by a hemagglutinin assay.

The analysis of the fimbrial and adhesin fractions by gel filtration revealed that both proteins form supramolecular structures (M_r, $>10^6$) under nondenaturing conditions. Adhesin and fimbrial subunits could be separated by high-resolution anion-exchange chromatography in 8 M urea. The respective peaks contained additional proteins that $NaDodSO_4$/PAGE showed to be outer membrane proteins. These contaminants could not be removed on rechromatography under identical conditions. It was, however, possible to dissociate the complexes, which had been resistant to 8 M urea in 8 M guanidine hydrochloride and to purify fimbrillins and adhesin to apparent homogeneity (Fig. 1). After removal of the chaotropic agent, the fimbrillin units associated to fimbriae and the adhesin associated to biologically active aggregates. Purification of the proteins was monitored by

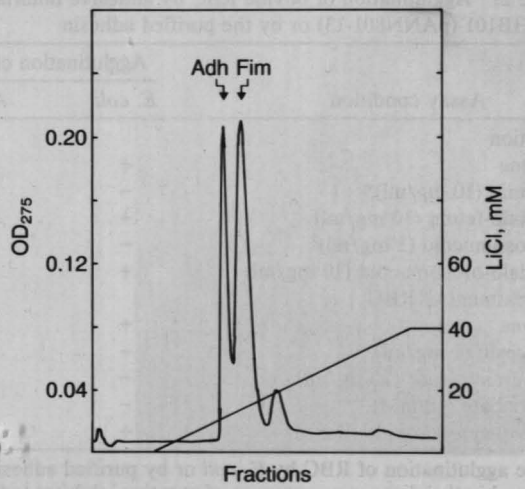

FIG. 1. Separation of the S-fimbriae-associated adhesin from the fimbrial subunits by ion-exchange chromatography on MonoQ HR5/5 in 8 M urea. The arrows indicate the elution positions of the adhesin (Adh) and the fimbrial subunits (Fim).

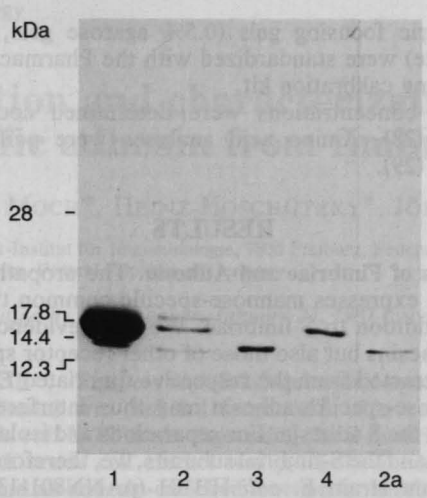

FIG. 2. $NaDodSO_4$/PAGE and immunoblot analysis of S fimbriae and adhesin. The Coomassie blue-stained lanes contain the following material: 1, crude fimbriae; 2, heat extract of crude fimbriae; 3, purified adhesin; 4, purified fimbrial subunits. Immunoblot of the heat extract of the crude fimbriae (proteins shown in lane 2) was performed with the adhesin-specific mAb A1 (lane 2a).

$NaDodSO_4$/PAGE (Fig. 2) and by immunoblotting with specific monoclonal antibodies (see below).

Characterization of the Adhesin. In crude extracts the fimbrial subunits are in a >100-fold molar excess over the adhesin (see Fig. 2, lane 1). The purified adhesin aggregate has a molecular size of $>10^6$ kDa as judged by gel filtration on a TSK 4000 column. $NaDodSO_4$/PAGE showed it to consist of one type of subunit (12 kDa), which is smaller than that of the fimbriae (16.5 kDa). While the fimbriae contain intramolecular but no intermolecular disulfide bridges, the adhesin contains no disulfide bridges as shown by $NaDodSO_4$/PAGE. The adhesin had pI value of 4.7, as compared to pI value of 6 of the fimbrillin. Comparative amino acid analysis showed that the adhesin contained one cysteine (two in the fimbrillin) and one methionine (none in the fimbrillin). Several other amino acids were also present in the two proteins in different amounts.

The RBC agglutination by the purified adhesin and by whole bacteria of strain HB101 (pANN801-13) exhibited the same pattern of sensitivity (Table 2). This suggests that the agglutinating capacity of the adhesive bacteria is due to the adhesin described in this paper.

Table 2. Agglutination of bovine RBC by adhesive fimbriated *E. coli* HB101 (pANN801-13) or by the purified adhesin

Assay condition	Agglutination of RBC	
	E. coli	Adhesin
Addition		
None	+	+
Fetuin (10 mg/ml)*	−	−
Asialo-fetuin (10 mg/ml)	+	+
Orosomucoid (1 mg/ml)†	−	−
Asialo-orosomucoid (10 mg/ml)	+	+
Pretreatment of RBC		
None	+	+
Trypsin (1 mg/ml)	−	−
Neuraminidase (20 μg/ml)	−	−
Periodate (10 mM)	−	−
Hydrogen peroxide (2 mM)	+	+

The agglutination of RBC by *E. coli* or by purified adhesion was assessed in the absence or presence of putative inhibitor (additions) and with or without pretreatments of the RBC. +, Agglutination. −, No agglutination.
*Corresponding to 10 mM *N*-acetylneuraminic acid.
†Corresponding to 1 mM *N*-acetylneuraminic acid.

Monoclonal Antibodies. Spleen cells from mice immunized with a crude S-fimbrial preparation were fused with PAI myeloma cells (21); hybrid cells were screened for activity in ELISA using purified fimbrillin, adhesin, and bacteria (22) as antigens. Purified monoclonal antibodies were obtained from the culture supernatants. Their properties are summarized in Table 3. Antibodies (mAb A1 and mAb A2) reacted only with the adhesin, and mAb F1–mAb F4 reacted only with the fimbriae. mAb A1 inhibited the adhesin-mediated agglutination of RBC whereas the other antibodies did not. The specificities of the monoclonal antibodies were corroborated by their capacity to agglutinate recombinant *E. coli* strains expressing fimbriae and/or adhesin (Table 3). mAb A1 and A2 were used to monitor the adhesin in bacterial extracts and to follow the course of purification.

To analyze the surface topography of the S adhesin, fimbriated adhesive *E. coli* HB101 (pANN801-13) were incubated with mAb A1 and then with protein A-gold. After contrasting the bacteria and their fimbriae with phosphotungstic acid, the samples were inspected with the electron microscope. The adhesin could be seen in close association with the fimbriae against a blank background (Fig. 3*A*). No gold label was seen in surface areas that did not contain fimbriae, including the outer membrane. Enlargements (Fig. 3*B*) showed that the gold label seems to be preferentially associated with the tips of the fimbriae, indicating a distal location of the adhesin. No gold label was seen on bacteria that did not contain the adhesin (data not shown). The adhesin could not be demonstrated on the adhesive unfimbriated *E. coli* HB101 (pANN801-1) probably due to a low concentration of adhesin on this strain or to a poor transport of the adhesin across the outer membrane as suggested for Fim^-/Adh^+ P-fimbriated strains (17). This is corroborated by the fact that the adhesin could be isolated from this strain only in very low yield, as compared to *E. coli* Fim^+/Adh^+ HB101 (pANN801-13).

DISCUSSION

The concept of a distinct localization of the genes for fimbriae and adhesin on the corresponding genetic determinant was emphasized for the various fimbrial adhesins common type 1, P, and S (13–19). For the latter the isolation of insertional mutants, the analysis of subclones, and molecular studies including the preparation of minicells and the determination of the DNA sequences (33) lead to the hypothesis that a distinct entity acted as the adhesin. The nature of this gene product and its association with fimbriae remained unknown. No protein of one of the defined (P, S, M or type 1) specificities could hitherto be isolated.

The present communication describes the isolation and characterization of the S adhesin from fimbriated adhesive *E. coli*. The fimbriae–adhesin complex, which could be easily isolated from the bacterial surface by shearing forces, could be partially dissociated by heating followed by ultracentrifugation into the mass of the fimbriae and a complex consisting of fimbrial subunits, adhesin, and some outer membrane proteins. This complex proved to be very stable and dissociated only in 8 M guanidine hydrochloride. Fimbrillin and adhesin could then be separated by high-performance anion-exchange chromatography in 8 M urea and purified to apparent homogeneity. After removal of the chaotropic agent, the fimbrillin molecules reaggregated and the adhesin aggregated to complexes of an apparent molecular weight of $>10^6$. The fimbrillin and the adhesin differed in molecular sizes and pI values. There were also differences in the amino acid compositions, the most drastic ones being in the adhesin in the presence of only one cysteine (as opposed to two

Table 3. Properties of mAb against adhesin or fimbriae

mAb	Subclass	State of antigen	Inhibition of HA	Agglutination of *E. coli*		
				Fim$^+$ Adh$^+$	Fim$^-$ Adh$^+$	Fim$^+$ Adh$^-$
Anti-adhesin						
A1	IgG1	n,d	Yes	+	+	−
A2	IgM	n,d	No	+	+	−
Anti-fimbriae						
F1	IgG2a	n,d	No	+	−	+
F2	IgG1	n	No	+	−	+
F3	IgM	n	No	+	−	+
F4	IgG1	n	No	+	−	+

mAbs against fimbriae do not cross-react with adhesin and vice versa. HA, hemagglutination; Fim, fimbriae; Adh, adhesin; +, agglutination; −, no agglutination; n, native; d, denatured. The following *E. coli* strains were used: HB101 (pANN801-13) Fim$^+$, Adh$^+$; HB101 (pAN801-1) Fim$^-$, Adh$^+$; HB101 (pANN801-13/Tn5-38) Fim$^+$, Adh$^-$ (see also Table 1).

cysteines in the fimbrillin) and one methionine (absent in the fimbrillin). Adhesin, therefore, does not contain the disulfide loop characteristic of fimbrillins.

Adhesin is only a minor component of the adhesive complex, representing <1% of the fimbrillar mass. This, and the resistance to dissociation from the fimbriae, is probably the reason why this protein, although postulated on genetic evidence (32), has not been detected before to our knowledge.

In the genetic and functional studies of *E. coli* adhesins, the capacity of the bacteria to adhere to and to agglutinate RBC of different species was taken as an expression of the adhesive character of the bacteria. Human and bovine RBC were agglutinated by adhesive S-specific *E. coli* and by the purified adhesin. The agglutination was more pronounced at 4°C and inhibitable with sialoglycoproteins, such as fetuin and orosomucoid. Interestingly, the fimbriae–adhesin complex that was removed from the bacteria as a crude preparation by mixing with an Omnimixer did not agglutinate RBC. In pilot experiments, it was, however, found (data not shown) that the fimbriae–adhesin complex adhered to human tissue culture cells, as did the purified adhesin and the S-specific adhesive bacteria. This may be an indication that the fimbriae–adhesin complex (adhesive fimbriae) is monofunctional and unable to cross-link RBC, whereas the isolated and purified adhesin is polyfunctional and can thus cross-link RBC. These results confirm that the protein we have purified from adhesive sialic acid-specific *E. coli* is indeed S adhesin.

Crude extracts from *E. coli* HB101 (pANN801-13) were successfully used to produce monoclonal antibodies. Four antibodies reacted only with intact fimbriae and not with the adhesin, and two antibodies reacted only with the adhesin and not with fimbriae or with fimbrillin.

As indicated in Table 3 the monoclonal antibodies exclusively reacted with their corresponding recombinant strains. These data confirmed and extended observations (18, 19) on a distinct location of the genes coding for the fimbriae subunit and the adhesin on the S-fimbrial adhesin complex determinant. The fimbriae-specific gene (*sfaA*) is located near the 5′ end of the determinant, while the adhesin gene was found near its 3′ boundary with nearly 4 kilobases in between (ref. 33; J.H., unpublished data). One of the anti-adhesin antibodies inhibited the agglutination of RBC by the purified adhesin

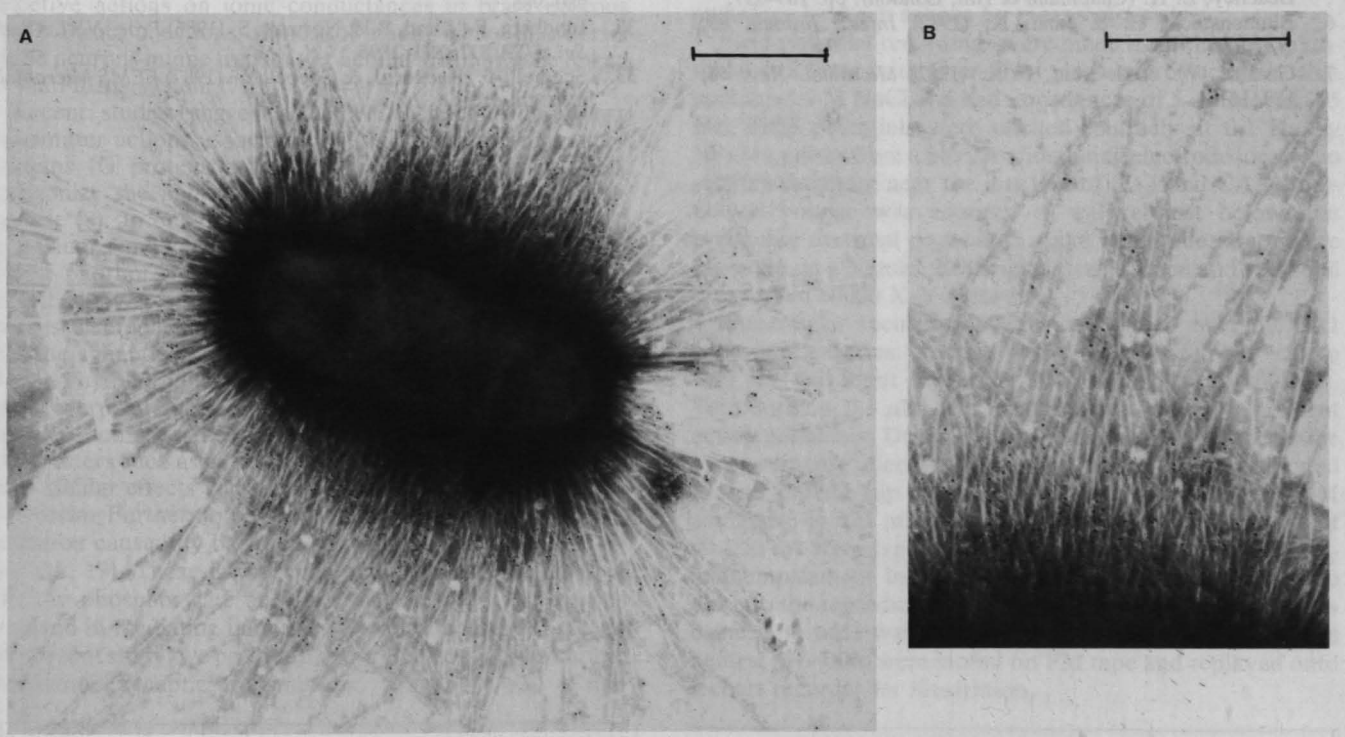

FIG. 3. (*A*) Electron micrograph of *E. coli* HB101 (pANN801-13) after reaction with the adhesin-specific mAb A1, followed by immuno-gold labeling and subsequent contrasting with phosphotungstic acid. (*B*) Enlargement of a section from *A*. (Bars = 0.5 μm.)

and by adhesive *E. coli*. With the use of the anti-adhesin (and especially the anti-adhesive) antibodies, it is now possible to screen bacterial populations for the presence of the adhesin. This will also be helpful in the study of adhesin biosynthesis and expression.

Recombinant strain HB101 (pANN801-13) was used for the adhesin preparation to circumvent an interference with type 1 fimbriae and their adhesin, especially since we have evidence that P, M, and type 1 adhesin can also be separated from their respective fimbriae. We have applied the isolation procedure also to wild-type S-specific *E. coli* not expressing type 1 fimbriae. The protein obtained (data not shown) has all the properties described here for S adhesin.

Although fimbriae and adhesin can be physically separated, they form a functional complex on the bacterial surface. An analysis of this problem became possible with the monoclonal antibodies now available. Immunoelectron microscopy with the anti-adhesive antibody showed that in fimbriated adhesive *E. coli* the adhesin is associated with fimbriae and suggests it is located at the tips of the fimbriae. The electron-microscopic picture shown in Fig. 3 does not give entirely convincing evidence for this. Further studies are necessary to clarify this point. The availability of pure adhesin and nonadhesive fimbriae from pathogenic *E. coli* as well as of an antiadhesive monoclonal antibody does not only help to understand the mechanism of bacterial adhesion but also allows a closer study of the role of adhesion in *E. coli* infections.

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