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Participation of Pili and Cell Wall Adhesin in the Yeast Agglutination Activity of *Escherichia coli*

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Escherichia coli strain 2699 (O6:K13) which had been isolated from a case of urinary tract infection exhibited pili during the stationary phase (24 to 40 h), but not during the exponential phase (4 h), when grown in static broth culture. The bacteria were also piliated when grown for 24 h on agar. They agglutinated *Saccharomyces cerevisiae* (bakers' yeast) in the piliated as well as in the nonpiliated state. The agglutinations were mannose sensitive, i.e., they could be inhibited with 50 mM methyl-α-mannoside. The bacteria were first depiliated by shearing and then used for the isolation of outer membrane vesicles with an Omnimixer. Purified pili and outer membranes were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electron microscopy. The pili could be demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis only after treatment at low pH or in saturated guanidine hydrochloride which is typical of the common type 1 pili. Depiliated bacteria, purified pili, and purified outer membranes gave mannose-sensitive agglutination of *S. cerevisiae*. The findings are discussed with respect to possible mechanisms of cell agglutination.

The adherence of bacteria to host tissue is an early event in host-parasite interactions leading to bacterial colonization and infection of the host (8, 12, 19, 23). It is often paralleled by the ability of the bacteria to agglutinate erythrocytes or yeast cells (6, 13, 17, 24). Adherence and agglutination may or may not be inhibited by α-mannose or α-mannosides and are then termed mannose sensitive or mannose resistant (1, 6, 20). It has been shown that adherence and agglutination are frequently caused by bacterial pili (1, 6, 11, 22, 26). Mannose-sensitive adherence of *Escherichia coli* is mostly due to common type 1 pili (1, 2, 18, 22), although bacterial flagella have also been implicated in the adhesion of *E. coli* to human buccal cells, mouse spleen lymphocytes, and yeast cells (4). We have found that *E. coli* 2699, isolated from a case of urinary tract infection (L. Å, Hanson, Göteborg, personal communication), exhibited mannose-sensitive adherence to a number of tissue culture cells, to erythrocytes, and to yeast cells, and that the binding occurs not only with piliated but also with nonpiliated bacteria (11). In this communication we show that *E. coli* 2699 (O6:K13) exhibits agglutinins not only in the pili but also in the outer membrane. A putative role of these adhesins and their role in adhesion of the bacteria are discussed.

MATERIALS AND METHODS

Materials. Tris(hydroxymethyl)aminomethane and sodium dodecyl sulfate (SDS) were obtained from Roth, Karlsruhe. Ultrapure guanidine hydrochloride, methyl-α-mannoside, methyl-α-glucoside, and trypsin were purchased from Serva, Heidelberg, and phenylmethylsulfonyl fluoride was from Sigma, München. Other chemicals, purchased from commercial sources, were of the highest purity available and were used without further purification.

Bacteria and media. *E. coli* 2699 (O6:K13:H$^-$) was isolated from a case of urinary tract infection and was obtained from L. Å. Hanson, University of Göteborg. It agglutinated cells of *Saccharomyces cerevisiae* (bakers' yeast), guinea pig erythrocytes, and human erythrocytes independent of ABO blood group. The agglutinations could be inhibited with methyl-α-mannoside (11). The bacteria were grown either in Luria broth (containing 1% tryptone, 0.1% yeast extract, and 0.5% sodium chloride) or on Loeb agar (containing 1% tryptone, 0.1% yeast extract, 0.1% glucose, 0.8% sodium chloride, 0.028% calcium chloride, and 1.5% agar).

Yeast agglutination assay. Quantitative assays were done in duplicate in microtiter plates, each well containing 10^9 cells of *S. cerevisiae* per ml and dilutions of the samples to be tested in a final volume of 50 μl of phosphate-buffered saline. Methyl-α-mannoside (50 mM) and methyl-α-glucoside (50 mM) were used as putative inhibitors (3, 11, 17).

Analytical methods. Polyacrylamide gel electrophoresis in the presence of SDS was performed in

slabs by the method of Laemmli and Favre (14) with 15% running gels as described previously (10). Samples of pili were treated with saturated guanidine hydrochloride before their application to the gel (Y. Eshdat, F. J. Silverblatt, and N. Sharon, submitted for publication). Protein determinations were done by the method of Markwell et al. (15) with bovine serum albumin as a standard. The concentrations of bacterial and yeast cell suspensions were determined by counting in a Petroff-Hausser chamber.

Electron microscopy. Preparations were stained with 0.1% phosphotungstic acid on copper grids coated with Formvar and carbon. The samples were examined in a Phillips EM 400 microscope.

Isolation of pili and membranes. *E. coli* 2699 was harvested from 25 agar plates (14-cm diameter) in 30 ml of ice-cold phosphate-buffered saline and centrifuged at 7,500 × *g* for 10 min. Unless otherwise stated, all manipulations were performed at 4°C. The washed bacteria (7 g, wet weight) were suspended in 35 ml of phosphate-buffered saline containing 850 mM sodium chloride, 100 mM methyl-α-mannoside, and 30 nM phenylmethylsulfonyl fluoride, and the suspension was agitated first with a Vortex mixer (setting 2, 3 × 5 min) and then in a sonication bath (room temperature, 5 min). Centrifugation at 8,000 × *g* for 10 min resulted in a pellet of depiliated bacteria which was used for the preparation of outer membranes and a supernatant from which the pili were isolated by differential centrifugation and precipitation with magnesium chloride.

From the above supernatant residual bacteria were first removed by two centrifugatons at 6,000 × *g* (10 min each), and the supernatant was then centrifuged at 40,000 × *g* to remove membrane vesicles. From the supernatant the pili were obtained either by ultracentrifugation at 150,000 × *g* for 2 h or by precipitation with magnesium chloride (0.1 M final concentration). The pili were suspended in phosphate-buffered saline and purified by a repeated centrifugation at 150,000 × *g* or by repeated precipitations with magnesium chloride. Both preparations were equally pure, as determined by electron microscopy and SDS-polyacrylamide gel electrophoresis.

For the preparation of outer membranes, the bacterial pellet obtained after depiliation was subjected to a second treatment with the Vortex mixer, centrifuged at 7,500 × *g* for 10 min, and then washed by centrifugation of the pellet in 0.5% NaCl (7,500 × *g*, 10 min). The pellet was suspended in 35 ml of 20 mM sodium acetate buffer (pH 4.4) containing 3 nM phenylmethylsulfonyl fluoride, and the suspension was agitated for 20 min in a Sorvall omnimixer at position 5. The bacteria were removed by repeated centrifugation at 10,000 × *g*, and the membranes were then obtained by centrifugation at 40,000 × *g* for 2 h.

Proteolytic digestion with trypsin. Bacteria (5 × 10^8/ml), bacterial membranes, or purified pili (1 mg of protein per ml) were suspended in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8), and trypsin (1 mg/ml in 1 mM hydrochloric acid) was added to a final concentration of 100 μg/ml. After 2 h at room temperature, samples were taken for the yeast agglutination assay and electron microscopic examination. The trypsin incubation of membranes in samples taken for SDS-polyacrylamide gel electrophoresis was terminated by the addition of an equal volume of the SDS disintegration mixture (10) followed by boiling for 5 min. To the trypsin-treated pili, guanidine hydrochloride (saturation) and mercaptoethanol (final concentration, 4%) were added. The mixtures were then incubated at 37°C for 2 h, dialyzed against 5 mM tris(hydroxymethyl)aminomethane-hydrochoride buffer (pH 8), and added to an equal volume of the SDS disintegration mixture (10).

RESULTS

Influence of growth conditions on the development of bacterial pili. When *E. coli* 2699 was grown on agar at 37°C, the cells exhibited pili, but not flagella, as shown by electron microscopy (Fig. 1). The piliation was slightly denser after 40 h than after 24 h. Inspection of the bacteria grown in Luria broth showed that they were neither piliated nor flagellated after 4 h of growth and that they had acquired pili but not flagella after 24 h. The appearance of the cells had not changed after 40 h of growth. When grown on agar or in liquid culture at 20°C, only about 5% of the bacteria were slightly piliated.

Influence of growth conditions on yeast-agglutinating activity. The capacity of *E. coli* 2699 to agglutinate *S. cerevisiae* was tested after growth of the bacteria under the conditions described above. *E. coli* 2699 agglutinated the yeast cells after growth under all conditions. The agglutinations could be inhibited with 50 mM methyl-α-mannoside, but not with 50 mM methyl-α-glucoside. It is noteworthy that unpiliated bacteria also agglutinated the yeast cells. Thus, bacteria from 4-h cultures in Luria broth at 37°C as well as stationary cultures at 20°C were active. Since after growth at 20°C still about 5% of the bacteria were piliated, we wanted to know whether activity was due to the few piliated bacteria in the culture. The adherence of *E. coli* 2699 grown at 20 and 37°C to *S. cerevisiae* was therefore determined. To 10^9 yeast cells per ml 2.4 × 10^8 bacteria per ml were added. The mixture was incubated at room temperature for 10 min and then centrifuged at 750 × *g* to remove the yeast cells and their aggregates. When the bacteria in the supernatant were counted, the concentration of the bacteria grown at 20°C was 2.7 × 10^7/ml. When the experiment was performed in the presence of 10 mM methyl-α-mannoside, all bacteria were recovered from the supernatant. Methyl-α-glucoside did not interfere with the binding of the bacteria to *S. cerevisiae*. The same results were obtained with the bacteria grown at 37°C. This showed that 90% of the bacteria were attached to the yeast cells, also when only 5% of them

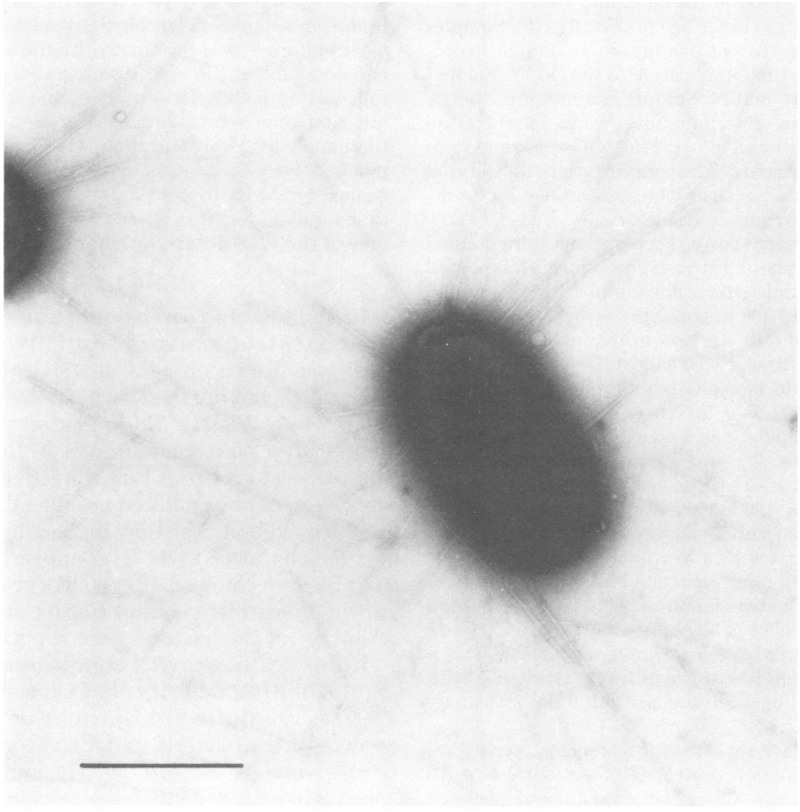

FIG. 1. *Electron micrograph of E. coli 2699 grown on Loeb agar at 37°C for 40 h. Bar, 1 μm.*

were piliated. Table 1 summarizes the influence of growth conditions on the agglutinating activity of *E. coli*. The results show that *E. coli* 2699, which is normally piliated, can be obtained in an unpiliated state in which it is still active.

Isolation of pili and outer membrane vesicles. To elucidate the role of pili and cell walls in the adherence of *E. coli* 2699 to yeast cells, these bacterial components were isolated as indicated in Fig. 2. Agitation of the bacteria in a Vortex mixer followed by sonic irradiation in a sonication bath removed the pili from the bacterial cells, as evidenced by electron microscopy. The pili were purified from the supernatant either by differential centrifugation, where they sedimented at 105,000 × *g*, or by precipitation with magnesium chloride (0.1 M final concentration). Both methods yielded preparations of pili which appeared to be pure in electron microscopic observation (Fig. 3A).

Suspensions of the depiliated bacteria were agitated at pH 4.4 with an Omnimixer. This resulted in partial release of outer membranes which were purified by differential centrifugation. After the final centrifugation at 40,000 × *g* the membranes appeared as pure vesicles when inspected with the electron microscope (Fig. 3B).

TABLE 1. *Influence of growth conditions on the formation of bacterial appendages and on yeast agglutination activity of E. coli 2699*[a]

Growth medium	Growth conditions	Piliation	Yeast agglutinating activity (bacteria/ml)
Loeb agar	37°C, 24 h	pil$^+$	1.2×10^8
Loeb agar	37°C, 40 h	pil$^+$	5×10^7
Loeb agar	20°C, 24 h	(pil)	5×10^7
Luria broth	37°C, 4 h	pil$^-$	2.1×10^7
Luria broth	37°C, 5 × 24 h	pil$^+$	1×10^7

[a] Activity is expressed as the lowest bacterial concentration agglutinating 10^9 yeast cells at room temperature. Bacterial appendages were observed by electron microscopy: pil$^+$, all bacteria were densely piliated; (pil), only about 5% of the bacteria were piliated; pil$^-$, all bacteria were unpiliated.

Figure 4 shows the SDS-polyacrylamide gel electrophoresis of pili (Fig. 4B) and outer membranes (Fig. 4E). The bands in Fig. 4B could only be seen after pretreatment of the pili with saturated guanidine hydrochloride (Eshdat et al., submitted for publication) which seems to be

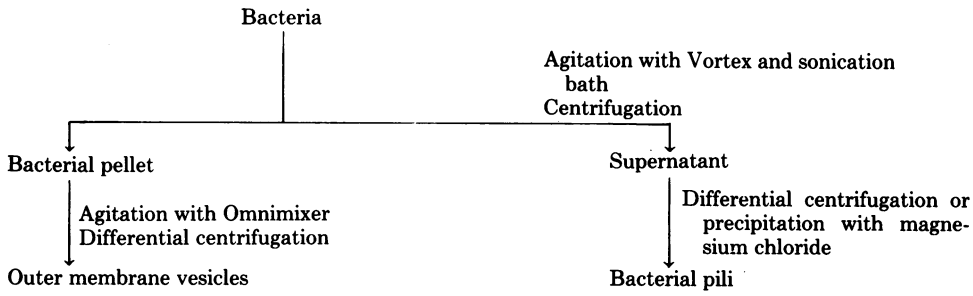

FIG. 2. *Isolation of bacterial pili and outer membrane vesicles from E. coli 2699.*

characteristic for type 1 pili (unpublished observations). It has been reported that untreated common type 1 pili cannot be demonstrated by SDS-polyacrylamide gel electrophoresis (16). The outer membrane preparation obtained by mixing the depiliated bacteria in an Omnimixer exhibited the same pattern as outer membranes isolated by the method of Uemura and Mizushima (25) from urinary tract-infective *E. coli* O6: K13 (10). When the membrane preparations were saturated with guanidine hydrochloride, no additional bands due to pili could be seen on subsequent SDS-polyacrylamide gel electrophoresis.

Yeast agglutinating activity of depiliated bacteria, pili, and outer membranes. The depiliated bacteria, obtained by agitation with a Vortex mixer and low-energy sonication, were used in the yeast agglutination test. As shown in Table 2, they had about the same activity as the piliated bacteria. Outer membranes and pili, which were both pure preparations as shown by electron microscopy (Fig. 3) and SDS-polyacrylamide gel electrophoresis (Fig. 4), also agglutinated the yeast cells. The respective minimal agglutinating concentrations are included in Table 2. The agglutinating activity of all preparations was completely inhibited by 50 mM methyl-α-mannoside. Treatment of bacteria, pili, and outer membranes with trypsin abolished the agglutinating ability of the preparations. The SDS-polyacrylamide gel electrophoresis pattern of pili was unchanged when the action of trypsin was stopped before deaggregation of the pili into their subunits (Fig. 4C, as compared with Fig. 4B). When the action of trypsin was allowed to proceed with the pilus subunits, complete degradation was observed (Fig. 4D). Trypsinization of outer membranes resulted in cleavage of the 33K protein and in the appearance of a smaller split product (Fig. 4F). Trypsinization of the yeast cells before the agglutination test as well as the presence of trypsin during the agglutination test did not interfere with the agglutinating capacity of the preparations.

DISCUSSION

E. coli 2699, which had been isolated from a case of urinary tract infection, agglutinated cells of *S. cerevisiae*. The bacteria also agglutinated guinea pig and human erythrocytes and adhered to a number of tissue culture cells (11). All of these cell interactions could be inhibited by methyl-α-mannoside.

Since mannose-sensitive agglutination of yeast cells (1, 13, 21) and guinea pig erythrocytes is typically exhibited by bacteria with common type 1 pili, *E. coli* 2699 was subjected to electron microscopic examination. It was found that the bacteria which did not exhibit pili after 4 h of growth in Luria broth at 37°C were densely piliated after prolonged growth in liquid culture or on agar at 37°C. Growth at 20°C largely prevented the formation of pili. The bacteria had a smooth surface; structures indicating incomplete pili could not be seen. However, *E. coli* 2699 agglutinated yeast cells whether or not the bacteria were piliated. This raised the question whether or not the pili played a role in agglutination by or adherence of *E. coli* 2699. Pure isolated pili were also capable of agglutinating yeast cells; this agglutination could be inhibited by methyl-α-mannoside. The pili could be demonstrated by SDS-polyacrylamide gel electrophoresis only after subjection to treatments originally developed for common type 1 pili, viz., treatment at low pH (16) or with saturated guanidine hydrochloride (Eshdat et al., submitted for publication). SDS-polyacrylamide gel electrophoresis of the pili revealed a major and a minor peptide band of about 17K. The same pattern was obtained with common type 1 pili (data not shown). Therefore, 2699 pili resemble common type 1 pili with respect to the tightness of subunit assembly and the SDS-gel pattern of their subunits.

It was found that outer membranes isolated from depiliated *E. coli* 2699 agglutinated cells of *S. cerevisiae*, and that this agglutination could be inhibited with methyl-α-mannoside. This is in accord with the fact that unpiliated bacteria

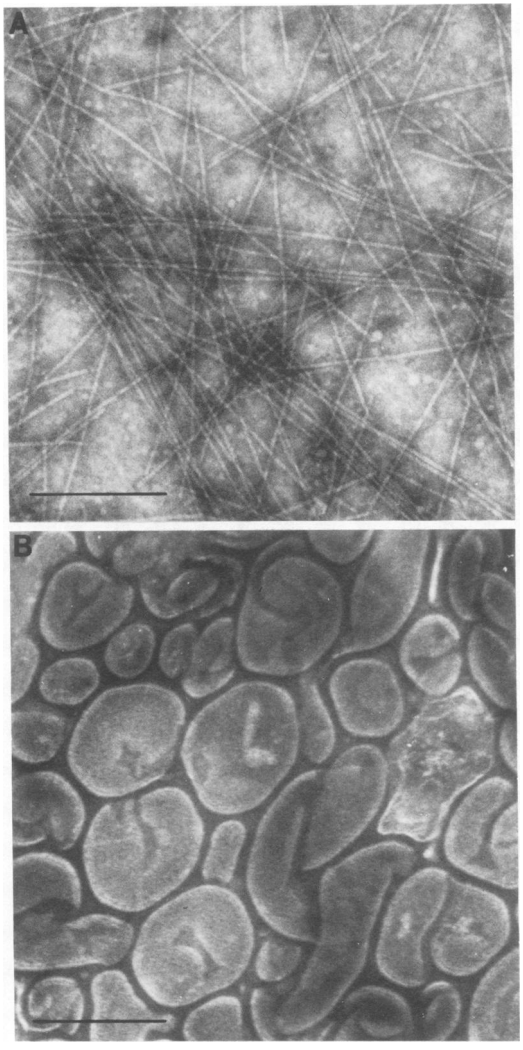

FIG. 3. *Electron micrographs of purified pili (A) and purified outer membranes (B) of E. coli 2699. Bar, 0.2 μm.*

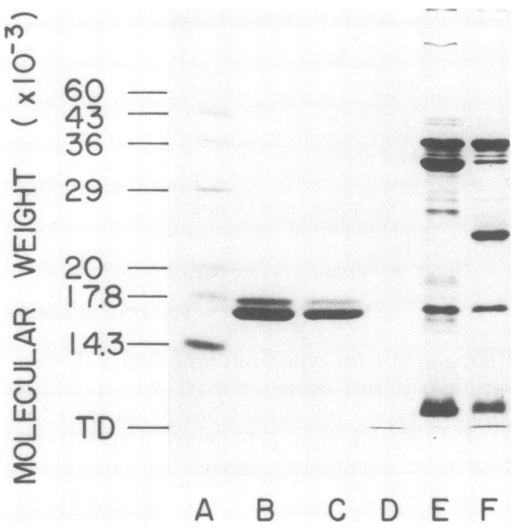

FIG. 4. *SDS-gel electrophoresis in 15% polyacrylamide separation gel of molecular weight markers (A), purified pili (B), purified pili incubated with trypsin followed by the addition of guanidine hydrochloride and mercaptoethanol (C), purified pili after the incubation with trypsin without subsequent addition of mercaptoethanol (D), purified outer membranes (E), and purified outer membranes after the incubation with trypsin (F). The molecular weight markers were lysozyme (14,300), sperm whale myoglobulin (17,800), soybean trypsin inhibitor (20,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), ovalbumin (43,000), and beef liver catalase (60,000). Tracking dye (TD) was bromophenol blue. The gel was stained with Coomassie brilliant blue R250.*

TABLE 2. *Agglutinating activities of piliated bacteria, depiliated bacteria, pili, and outer membranes of E. coli 2699 in the absence and presence of methyl-α-mannoside (αMM) and after trypsinization*[a]

Prepn	Minimal agglutinating concn (per ml)			
	Piliated *E. coli* 2699 (cells)	Depiliated *E. coli* 2699 (cells)	Purified pili (μg of protein)	Purified outer membranes (μg of protein)
With αMM	$>1 \times 10^{10}$	$>1 \times 10^{10}$	>500	>500
Without αMM	2×10^{7}	5×10^{7}	100	25
After trypsinization	$>1 \times 10^{10}$	$>1 \times 10^{10}$	>500	>500

[a] Serial dilutions of the preparations were incubated with 10^{9} yeast cells per ml in microtiter plates.

also agglutinated the yeast cells in a mannose-sensitive way—presumably via components of the outer membrane. Electron microscopic and gel electrophoretic examinations did not reveal pili in the outer membrane preparation. Short, rigid structures which could be interpreted as residues of pili broken during depiliation by shearing were also absent from the membranes. This and the lack of incomplete stubs of pili in unpiliated *E. coli* 2699 raised the possibility that the cell wall agglutinins are pilus monomers inserted into the outer membrane without polymerization. Alternatively, the cell wall agglutinin could be a protein different from pilin which contains the same mannose recognition site as pilin. SDS-polyacrylamide gel electrophoresis under the conditions which we have used cannot differentiate between these possibilities. The band seen in the outer membrane pattern

at about 17K may be due to pilin monomers; however, it could be another, unrelated protein such as protein III (7, 9).

The agglutinating activity of *E. coli* 2699 was lost after incubation of the bacteria with trypsin. Trypsinization also abolished the agglutinating activity of purified pili and outer membranes. The SDS-polyacrylamide gel electrophoretic pattern of trypsin-treated pili was the same as that of untrypsinized pili. When the pili were exposed to the action of trypsin under conditions which disaggregated the pili into subunits, complete enzymatic digestion occurred. One possible explanation for these findings is that enzymatic digestion occurred only at the terminal pilus subunit (tip), and the internal subunits were completely protected from the action of trypsin by their association. Only their disaggregation rendered them vulnerable to proteolysis. Since trypsin treatment of the complete pili abolished their agglutination activity, such an interpretation leads to the assumption that the strain 2699 pili have exposed mannose-binding sites only at the tip and that they are monovalent. Alternatively, although pilus subunits are protected from complete enzymatic digestion, each subunit may have an exposed mannose-binding site which is trypsin sensitive. In this case the pili would be polyvalent, and the destruction of their binding sites must have occurred by a shortening of the primary peptide chain too insignificant to be observed in SDS-polyacrylamide gel electrophoresis. When outer membranes were analyzed by SDS-polyacrylamide gel electrophoresis after incubation with trypsin, a major outer membrane protein had disappeared and a new smaller protein band was observed. It had been shown by Haller and Henning (9) that protein II*, one of the major outer membrane proteins of *E. coli* K-12, is split by trypsin with the formation of protein II. Both proteins II* and II have the same electrophoretic mobility in SDS-gel as the proteins of *E. coli* 2699 before and after trypsin treatment of the outer membranes. Since protein II* is also a major outer membrane protein in *E. coli* strains which do not agglutinate yeast cells, the effect of trypsin observed by SDS-polyacrylamide gel electrophoresis is probably unrelated to its effect on the cell wall agglutinin, especially since many outer membrane proteins seem to be trypsin sensitive (9).

Our results show that *E. coli* 2699 possesses agglutinins (adhesins) both in the pili and in the outer surface of the cell wall, and that both exhibit the same specificity for attachment. This may indicate the operation of a two-stage adhesion of *E. coli* 2699 to target cells, a long-distance interaction via pili and a short-distance interaction via the cell wall causing final adherence. Such an interpretation would favor the assumption of monovalent pili with the mannose-binding site only in the tip. There is of course the possibility that the mannose-binding protein in the cell wall has no function in binding, which is stabilized by multiple attachment of polyvalent pili to the target cell. In the latter case the cell wall adhesin would represent a portion of pilus subunits which had not or not yet assembled into pili. To study this phenomenon further we are attempting to isolate mutants which lack one or the other of the agglutinins. This will not only enable us to analyze the phenomenon of bacterial adherence further, but it may also be helpful in understanding the assembly of bacterial pili.

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