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Characterization of the Plasmid from *Escherichia coli* RDEC-1 That Mediates Expression of Adhesin AF/R1 and Evidence that AF/R1 Pili Promote but Are Not Essential for Enteropathogenic Disease

MARCIA K. WOLF,[1]* GERARD P. ANDREWS,[1] DAVID L. FRITZ,[2] ROBERT W. SJOGREN, JR.,[1] AND EDGAR C. BOEDEKER[1]

Departments of Gastroenterology[1] and Ultrastructural Studies,[2] Walter Reed Army Institute of Research, Washington, D.C. 20307-5100

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RDEC-1, an *Escherichia coli* strain that adheres to rabbit mucosa and causes an attaching, effacing lesion, expresses the pilus adhesin AF/R1 which determines in vitro attachment to rabbit intestinal brush borders. In order to determine the role of AF/R1 pili in the pathogenesis of enteropathogenic diarrhea in rabbits, we localized the genes for AF/R1 expression, constructed an AF/R1$^-$ strain, and compared the virulence of the AF/R1$^+$ and AF/R1$^-$ strains with particular attention to the development of attaching, effacing lesions. We introduced Tn*5* into the 86-megadalton (MDa) conjugative plasmid known to mediate expression of AF/R1 pili and transferred the derivative plasmids into laboratory strain HB101. Transconjugant M5 was found to contain the 86-MDa plasmid from RDEC-1 and to express AF/R1 pili. Pilus expression on M5 was confirmed by reaction with antiserum raised against purified AF/R1 pili and allowed the bacteria to adhere to the rabbit ileum in an in vitro assay. Three Tn*5* insertions in the 86-MDa plasmid were obtained which resulted in loss of AF/R1 expression. Part of the plasmid was mapped, including a region necessary for AF/R1 pilus expression. AF/R1$^-$ mutant strain M34 was constructed, and its pathogenesis was investigated. M34 produced disease in rabbits but was less virulent than the parent. The characteristic effacing lesions of RDEC-1 and enteropathogenic *E. coli* developed in the intestine of rabbits infected with either M34 or RDEC-1, although with M34 they were much less frequent and did not involve the small bowel. We conclude that AF/R1 pilus expression is not essential for the attaching, effacing lesion but serves as an accessory virulence factor which promotes an initial interaction of RDEC-1 with normal epithelial cells.

Escherichia coli RDEC-1 causes diarrhea in rabbits (6, 7). RDEC-1 does not produce heat-labile or heat-stable enterotoxin and does not invade the intestinal epithelium. It is an attaching and effacing enteroadherent organism which appears to share many of the features of human enteropathogenic *E. coli* (EPEC). At the time of diarrhea (7 days postinoculation), adherence of the organisms is extensive in the colon, ileum, and especially the cecum. At this time, the microvillus border of affected epithelial cells is effaced. Bacteria, as visualized by electron microscopy, are associated closely with effacing lesions of the apical plasma membrane of absorptive epithelial cells on which microvilli are no longer present. Human EPEC strains cause lesions morphologically indistinguishable from those of RDEC-1 (16, 21).

Adherence to the rabbit intestinal epithelium is an important virulence property of RDEC-1. However, the determinants of the stages of in vivo adherence have not been defined. RDEC-1 expresses pili named AF/R1 (for adherence factor/rabbit 1), which serve as in vitro adhesins. These pili promote the adherence of bacteria to brush borders isolated from rabbits but not to rat, guinea pig, or human brush borders (11). Hence, in vitro adherence is species specific. AF/R1 pili also promote attachment of RDEC-1 to glycoproteins in the mucous layer (27). Despite these studies on the importance of AF/R1 pili to in vitro attachment, the role of AF/R1 pili in vivo is not completely defined. Cantey and co-workers have provided evidence that AF/R1 pili have a role in the attachment of RDEC-1 to specialized lymphoepithelial cells (M cells) over the Peyer's patch in the small intestine (13, 14). In their experiments, they used a *Shigella flexneri* derivative strain, D15, which contained an 85-megadalton (MDa) plasmid from RDEC-1 and expressed AF/R1 pili (10). D15 bacteria were found to adhere specifically to M cells hours after inoculation; however, they did not result in effacing lesions with elongation of microvilli characteristic of RDEC-1 (14). Hence, AF/R1 appears to mediate the attachment of bacteria to M cells early in infection but is not sufficient for attaching, effacing lesions.

In the present study, we characterized the 86-MDa plasmid of RDEC-1, constructed mutants in AF/R1 pilus expression, and tested the virulence of an AF/R1 mutant with particular emphasis on its ability to promote attaching, effacing lesions of the absorptive epithelial cells of the ileum, cecum, and colon.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* RDEC-1 is O15:H$^-$:K? and is resistant to nalidixic acid. Strains 640 and HS are commensal strains that were isolated from rabbits and humans, respectively. These were from the collection in the Department of Gastroenterology, Walter Reed Army Institute of Research. HB101 (5) and HU735, the strain containing the Tn*5* donor plasmid F'ts114 *lac*::Tn*5* (26) were obtained from D. Kopecko (Department of Bacterial Diseases, Walter Reed Army Institute of Research). Strain DP, a rabbit commensal strain (24), was obtained from J. Peeters (National Institute of Veterinary Research, Brussels, Belgium). L broth and L agar were prepared and antibiotics were added as described elsewhere (19). Antibiotic medium

* Corresponding author.

3 (pen assay broth [PAB]; Difco Laboratories, Detroit, Mich.), brain heart infusion broth (BHI; Difco), and MacConkey agar (Difco) were prepared per the directions of the manufacturer.

Preparation of antisera. AF/R1 pili were purified from strain RDEC-1 as previously described and used to produce antipilus antiserum (2). Pili (250 μg) were diluted in Freund complete adjuvant (Difco), and 2 ml of the mixture was inoculated subcutaneously into an adult male New Zealand White rabbit. The rabbit was boosted weekly with pili in Freund incomplete adjuvant. Blood (4 to 8 ml) was collected weekly by ear venipuncture, and 2 to 8 ml of antiserum was separated from erythrocytes by slow-speed centrifugation. Aliquots were stored at −20°C until use.

Antiserum against piliated whole cells was made by using the same regimen as that described above. Cells grown in 10 ml of PAB at 37°C overnight were harvested by centrifugation, washed in phosphate-buffered saline (PBS; 0.13 M NaCl, 0.01 M sodium phosphate [pH 7.4]), and then resuspended in PBS to 10^8 cells/ml.

Slide agglutination assay to detect AF/R1 pilus expression. Bacteria were grown overnight at 37°C in PAB or BHI medium and washed three times in PBS. The pellet was suspended in one-fourth of the original volume. Antiserum was diluted 1:100 in PBS and mixed on microscope slides at room temperature with an equal volume (15 μl each) of bacterial suspension. A +4 reaction was scored if there was immediate clumping upon rotation of the slide. A 0 was scored if there was no visible clumping after gentle agitation at room temperature for 2 min.

Adherence to brush borders. Intact apical brush borders were prepared from the ileum of adult male New Zealand rabbits and tested in the attachment assay as described elsewhere (9). In vitro adherence of bacteria to isolated brush borders was assayed as previously described (9). Adherence was expressed as the percentage of brush borders having more than 10 adherent bacteria.

Crossed-line immunoelectrophoresis. Heat-saline extracts containing bacterial surface components were prepared by the method of Ørskov et al. (23). RDEC-1 or HB101 cells were grown overnight in PAB at 37°C and harvested by centrifugation at a relative centrifugal force of 9,600 for 10 min. Cell pellets were resuspended in 1 ml of 0.85% sodium chloride per 0.6 g (wet weight), heated at 60°C for 20 min, sonicated on ice (two 1-min bursts), and centrifuged at a relative centrifugal force of 27,000 for 15 min. The supernatant was stored at −70°C.

Heat-saline extract (50 μl) was applied to a 1% agarose gel (type VI; Sigma Chemical Co., St. Louis, Mo.) on a 9 by 8.5 cm plate. Electrophoresis in one dimension was carried out in an LKB 2117 Multiphor apparatus, with cooling to 11°C by applying a voltage of 8 V/cm for approximately 2 h. The sample ran toward the anode. The buffer was 41.2 g of barbital sodium, 0.8 g of barbital, and 0.2 g of sodium azide per liter (pH 9.4). The part of the gel that did not contain the sample separated by electrophoresis was cut away and replaced with 1.0% agarose containing antiserum raised against RDEC-1 whole cells at a final dilution of 1:40. A 1-cm strip was removed between the electrophoresed sample and the gel containing antiserum and was replaced with agarose containing 50 μg of purified AF/R1 pili or 50 μl of the heat-saline extract from strain HB101. Electrophoresis in two dimensions was done at 2 V/cm for 20 h. The gel was blotted of excess buffer and unassociated protein and was stained with Coomassie blue. The procedure was basically as described by Axelson et al. (1).

Conjugation and construction of Tn*5* insertions. Donor and recipient strains were grown overnight in PAB, mixed together in approximately equal portions and collected on a sterile filter (Falcon 7103; Becton Dickinson Labware, Oxnard, Calif.). Filters were placed onto PAB agar plates, incubated, and then flooded with 1 ml of sterile 0.9% saline. Ten-fold serial dilutions of the bacterial suspension were plated on L agar supplemented with the appropriate antibiotics. Resulting colonies were screened for other relevant characteristics. For HU735, the Tn*5* donor strain, and RDEC-1, cells were grown overnight at 32°C and the filter was incubated at 32°C for 5 h to preserve the F′ factor carrying Tn*5*. Selection was done at 32°C on L agar supplemented with kanamycin (50 μg/ml) and nalidixic acid (20 μg/ml), which select for the kanamycin resistance of Tn*5* and the nalidixic acid resistance of the RDEC-1 chromosome. Derivative M8 was obtained; it was resistant to both kanamycin and nalidixic acid and therefore presumably was RDEC-1 carrying F′ts114 *lac*::Tn*5*. To allow transposition of Tn*5* but promote loss of the F′ vector, M8 was passed three times at 42°C in LB while it was in the early exponential phase, which inhibits replication of the F′ts114 *lac*::Tn*5* plasmid (26). This treatment does not prevent transposition of the Tn*5* element. Ten-fold serial dilutions of the suspension were plated on L agar containing kanamycin and nalidixic acid to maintain selection for the presence of Tn*5*. A colony was chosen and conjugated with HB101 for 5 h at 37°C and plated with selection on MacConkey agar supplemented with kanamycin and streptomycin. This selection yielded HB101 derivatives carrying Tn*5* either on the original F′ts114 *lac* plasmid or on a conjugative plasmid from RDEC-1. To select HB101 derivatives carrying Tn*5* on an RDEC-1 plasmid, colonies that did not ferment lactose were chosen, i.e., colonies that lacked the phenotype conferred by F′ts114 *lac*. Colonies that were resistant to kanamycin and streptomycin, sensitive to nalidixic acid, and unable to ferment lactose were selected for further analysis. This selection resulted in strain M5, as well as others carrying RDEC-1 plasmids.

Conjugation of the Tn*5*-tagged 86-MDa plasmid from strain M5 into nalidixic acid-resistant derivatives of DP, HS, and 640 were performed with selection for the kanamycin resistance of Tn*5* and the nalidixic acid resistance of the recipient chromosomes. Nalidixic acid-resistant derivatives of the wild-type strains were obtained by sequential passage in increasing concentrations of the antibiotic.

Plasmid analysis. Bacteria were screened for plasmids by the alkaline lysis procedure of Birnboim and Doly (3) or White and Portnoy (12). Restriction enzymes were used according to the specifications of the manufacturer (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and RNase (Sigma) was added to samples after incubation. DNA was analyzed by agarose electrophoresis in TB buffer (89 mM Tris base [Sigma], 89 mM boric acid, 3 mM EDTA) in a high-resolution horizontal gel apparatus (International Biotechnologies, Inc., New Haven, Conn.) at 16°C. Plasmid DNA for construction of deletion derivatives and clones was prepared by a modification of the method of Helenski (19). Ligation with T4 ligase was performed according to the instructions of the manufacturer. Transformation was done as described by Crosa and Falkow (12).

Pathogenesis of an AF/R1 mutant in rabbits. M34, an AF/R1 mutant strain derived from RDEC-1 was obtained (see Results), and its virulence was tested in rabbits. RDEC-1 or M34 was grown overnight in PAB at 37°C, harvested by centrifugation, diluted into sterile PBS, and mixed with an

equal volume of 10% sodium bicarbonate. Inocula were 4×10^5 or 4×10^7 CFU of RDEC-1 and 5×10^5 or 5×10^7 CFU of M34. Inocula were quantitated by plating on MacConkey agar supplemented with nalidixic acid or with nalidixic acid and kanamycin. RDEC-1 was confirmed to be resistant to nalidixic acid, and M34 was resistant to nalidixic acid and kanamycin.

New Zealand White rabbits weighing less than 1.5 kg were inoculated by orogastric intubation. Fecal swabs from all animals failed to show growth on MacConkey agar prior to inoculation. There were five animals per dose. Following inoculation, the clinical status of all animals was determined daily by weighing, inspection for diarrhea, and semiquantitative rectal cultures. Diarrhea was graded by inspection of fecal contents in the cages; the presence of hard pellets was scored as 0, soft pellets as +1, mixed soft pellets and unformed stool was +2, and unformed stool as +3. Fecal shedding was scored by using semiquantitative rectal cultures as described by Cantey and Hosterman (8). The rectal area was swabbed with a sterile cotton swab, and the swab was rolled across a MacConkey agar plate supplemented with nalidixic acid. Scoring was based on the number of colonies, with 0 for no colonies, +1 for 1 to 50 colonies, +2 for 51 to 200 colonies, and +3 for confluent growth.

One rabbit from each group was sacrificed 2 days postinoculation, and another was sacrificed 7 days postinoculation. Segments (10 cm) of jejunum, ileum, cecum, and proximal and distal colon were excised for colony counts of bacteria and for histology. Contents were flushed with 10 ml of PBS, and the resulting suspension was mixed by vortexing and assayed for bacterial counts. Samples were diluted in PBS and plated on MacConkey agar supplemented with nalidixic acid. Results were expressed as CFU per milliliter for all segments except cecum segments. Results for the cecum were expressed as CFU per gram of cecal contents.

Colonization by the appropriate bacteria was confirmed on day 2 and the day of the last sampling before death. Colonies were picked off the plates from fecal swabs onto selective media to determine resistance to nalidixic acid and kanamycin. Random samples were checked for AF/R1 expression by the slide agglutination assay using the antiserum specific for AF/R1 pili and for plasmid composition.

Microscopy. Selected tissues were evaluated microscopically; for light microscopic evaluation, tissues were fixed in 10% buffered Formalin, embedded in paraffin, sectioned at 5 to 6 μm, and stained with hematoxylin and eosin and Giemsa stain. For ultrastructural examination, samples of intestine were diced into 1-mm sections, fixed in 1% glutaraldehyde–4% paraformaldehyde (20), postfixed in 2% aqueous osmium tetroxide, dehydrated in graded ethanols and propylene oxide, and then embedded in Poly/Bed 812 (Polyscience, Inc., Warrington, Pa.). Adherence was graded +1 for focal areas of attachment to single cells, +2 for diffuse attachment over the apical surface of several cells, and +3 for confluent attachment. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM-109 electron microscope.

RESULTS

Construction of transconjugants containing Tn*5* insertions in plasmids from RDEC-1. Plasmids of RDEC-1 were tagged with Tn*5* after conjugation of F'ts114 *lac*::Tn*5* from *E. coli* HU735 into RDEC-1. Selection for resistance to kanamycin carried on Tn*5* and nalidixic acid yielded strain M8, which is a derivative of RDEC-1 containing the F' factor carrying Tn*5*. Transfer of Tn*5*-tagged plasmids occurred upon mating M8 and HB101 at 42°C, a temperature which inhibits replication of the F' factor. Three derivatives which expressed the appropriate markers were selected and were designated M5, M6, and M7.

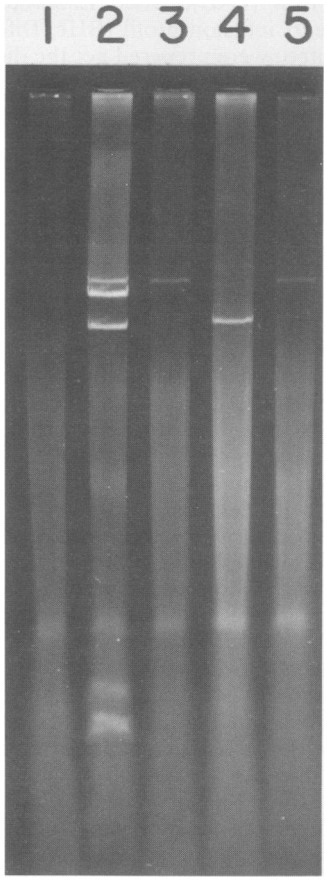


FIG. 1. Plasmid profiles of RDEC-1 and transconjugants. Lanes: 1, HB101; 2, RDEC-1; 3, M5; 4, M6; 5, M7.

The plasmids present in RDEC-1 and the transconjugants are shown in Fig. 1. RDEC-1 contains plasmids of 86, 73, 42, 2.7, and 2.6 MDa. M5 and M7 each contain a plasmid of approximately 86 MDa, and M6 contains a plasmid slightly larger than 42 MDa. Tn*5* is 5.7 kilobase pairs (kbp) (25), so an insertion of Tn*5* into a large plasmid of RDEC-1 would cause a slight increase in size which might not be detectable in this gel. No transconjugants that contained plasmids of 73, 2.7 or 2.6 MDa were detected. The plasmids from M5, M6, and M7 were digested with the restriction enzymes *Eco*RI, *Bam*HI, *Hin*dIII, and *Pvu*II (data not shown). The plasmids in M5 and M7 contain many restriction fragments of similar size and thus seem to be derived from the same plasmid. The plasmid from M6, however, is distinct.

Expression of AF/R1 antigen by transconjugants. Antiserum raised against partially purified AF/R1 pili from RDEC-1 was used in a slide agglutination assay to detect AF/R1 antigen expression by the transconjugants. Derivative M5 was agglutinated by this antiserum, demonstrating that the AF/R1 antigen was expressed in this strain (Table 1). M6 and M7 were not agglutinated and, hence, do not appear to express the AF/R1 antigen. RDEC-1 expresses AF/R1 pili when grown in PAB but not when grown in BHI (10). Similar

TABLE 1. Expression of AF/R1 pilus in transconjugants

Strain	Growth medium	Results with slide agglutination[a]	Adherence to brush borders[b]
RDEC-1	PAB	+4	72
	BHI	0	0
HB101	PAB	0	0
M5	PAB	+4	32
	BHI	0	0
M6	PAB	0	0
M7	PAB	0	0
M42	PAB	+4	41
	BHI	0	
M34	PAB	0	0

[a] Using antiserum raised against purified AF/R1 pilus. +4, Immediate clumping upon rotation of the slide; 0, no visible clumping after gentle agitation at room temperature for 2 min.

[b] Percentage of brush borders with greater than 10 bacteria adhering.

regulation was observed for M5; cells grown in PAB expressed the antigen, but cells grown in BHI did not (Table 1).

Analysis of heat-saline extracts of M5 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a typical pattern for HB101, with the addition of a 19-kilodalton protein which is the size of the AF/R1 pilus subunit (data not shown).

Crossed-line immunoelectrophoresis using antiserum raised against whole cells of RDEC-1 expressing AF/R1 pili confirmed that M5 heat-saline extracts contain the AF/R1 antigen but HB101 characteristics. Figure 2A shows the results when a heat-saline extract of M5 was electrophoresed horizontally and then vertically through an intermediate gel containing purified AF/R1 pili and then into a gel containing antiserum raised against piliated RDEC-1 whole cells. A line of identity is apparent between the AF/R1 pilus antigen in the intermediate gel and an antigen in the heat-saline extract from M5 (arrow). The AF/R1 antigen was not present in the parent HB101 heat-saline extract (Fig. 2B and C). Placing an HB101 heat-saline extract in the intermediate gel resulted in lines of identity with many M5 antigens (Fig. 2B, arrowheads) but not with the AF/R1 pilus antigen (Fig. 2B, arrow). Conversely, an HB101 heat-saline extract run as the sample in the lower gel and then through an intermediate gel containing purified AF/R1 failed to develop any lines of identity and lacked an AF/R1 peak (Fig. 2C).

AF/R1 pili on transconjugant M5 function as adhesins. Adherence of the transconjugants to brush borders prepared from the distal ileum of rabbits was determined as a measure of the ability of the AF/R1 pili to promote attachment to epithelial cells. M5 cells grown in PAB adhere to brush borders, but M6 and M7 do not (Table 1). M5 cells grown in BHI do not adhere to brush borders. These data together with the data obtained with antiserum raised against AF/R1 indicate that transconjugant M5 expresses AF/R1 antigen on its surface and that these pili function as adhesins.

Mediation of AF/R1 expression by the 86-MDa plasmid in three wild-type strains. The tagged plasmid from M5 was transferred by conjugation into *E. coli* strains 640, a commensal strain isolated from a rabbit, HS, a commensal human isolate, and DP, isolated from a weanling rabbit. Each strain gained a plasmid of approximately 86 MDa and the ability to express AF/R1 pilus antigen. Pilus expression was inhibited by growth in BHI in all strains.

Characterization of Tn*5* insertion mutations in the 86-MDa plasmid. More transconjugants were selected after mating M8 and HB101, and those containing plasmids of approximately 86 MDa were analyzed for AF/R1 pilus expression by slide agglutination with the antiserum raised against AF/R1 pilus (Table 2). Transconjugants M7, M26, and M28 did not express AF/R1 pili. Plasmids of each transconjugant were characterized by restriction digestion with *Eco*RI and *Bam*HI. Tn*5* lacks *Eco*RI sites and contains one *Bam*HI site (25), so comparison of fragments from digestion by these enzymes allowed localization of the Tn*5* to specific *Eco*RI and *Bam*HI fragments and identified distinct insertions. Plasmids from the three transconjugants which did not react with the AF/R1-specific antiserum each contained Tn*5* insertions in the 2.4-kbp *Eco*RI and 12-kbp *Bam*HI fragments. The *Bam*HI restriction pattern revealed that Tn*5* was at a unique site in each of the three mutant strains. The Tn*5* insertion in strain M42 was located in the 12-kbp *Bam*HI fragment, but M42 expressed the AF/R1 pilus antigen, since it was agglutinated by the antiserum and adhered to brush borders (see Table 1). Hence, the entire *Bam*HI fragment was not essential for AF/R1 expression.

Partial map of the 86-MDa plasmid. Five deletion derivatives of the 86-MDa plasmid from transconjugant M5 were obtained after partial digestion of the plasmid with *Eco*RI and ligation. All contained the 9.5-kbp *Eco*RI fragment carrying Tn*5*, as well as 5.5- and 5.2-kbp fragments. One also contained a 3.0-kbp fragment; another contained 3.0- and 2.5-kbp fragments; another contained 3.0-, 2.5-, 3.0-, 2.4-, and 5.5-kbp fragments; and another contained fragments of 3.0, 2.5, 3.0, 2.4, 3.9, and approximately 21 kbp. Ligation of a similar *Eco*RI partial digest and vector pBR322 yielded clone M23, which contained the 9.5-kbp *Eco*RI fragment carrying Tn*5*, in addition to 5.5- and 3.9-kbp fragments. The sizes of the plasmid derivatives ranged from 28 to 60 kbp (up to half of the original plasmid). None of the derivatives mediated AF/R1 pilus antigen expression or was capable of conjugation. Analysis of these plasmids and the Tn*5* insertions resulted in the map shown in Fig. 3.

Construction of an RDEC-1 mutant not expressing AF/R1. The Tn*5*-tagged 86-MDa plasmid from M7, containing an insertion mutation in genes essential for AF/R1 expression, was transferred by conjugation into RDEC-1. The plasmid pattern of M34, the resultant derivative of RDEC-1, was indistinguishable from that of RDEC-1 (data not shown).

TABLE 2. Identification of transposon insertion mutations

Strain	AF/R1 expression[a]	Size of restriction fragment altered by Tn*5* insertion (kbp)	
		*Eco*RI	*Bam*HI
M5	+	9.5	19
M7	−	2.4	12
M25	+	5.5	19
M26	−	2.4	12
M28	−	2.4	12
M31	+	5.5	19
M32	+	8.2	40
M35	+	9.5	19
M36	+	21	40
M37	+	9.5	19
M38	+	9.5	19
M39	+	9.5	19
M40	+	9.5	19
M41	+	23	7.4
M42	+	3.0	12
M44	+	1.3	40
M45	+	21	40

[a] Determined by slide agglutination with antiserum raised in rabbits against purified AF/R1 pili.

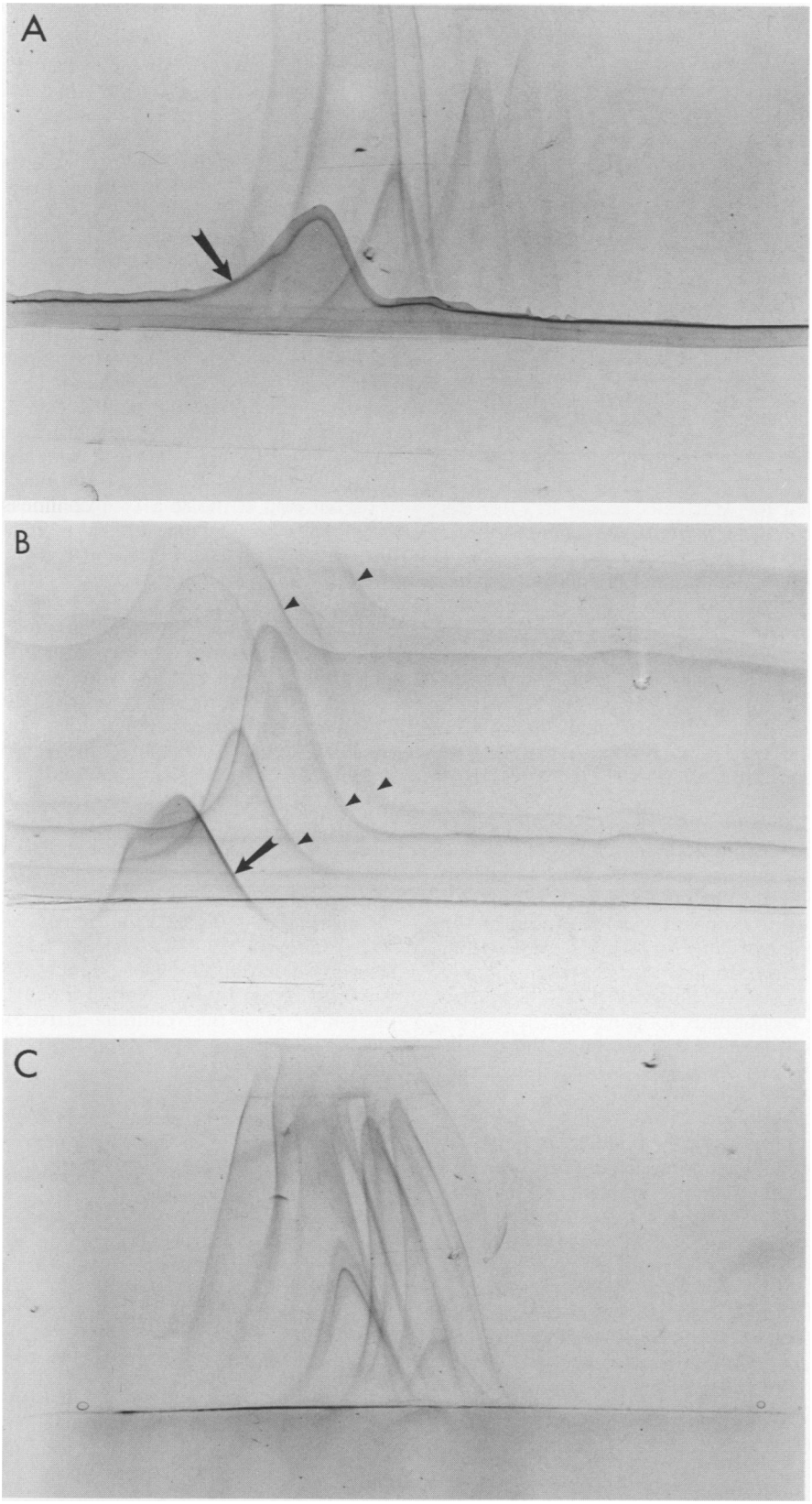


FIG. 2. Crossed-line immunoelectrophoresis of heat-saline extracts of transconjugant M5 or HB101 against piliated anti-RDEC-1 whole cells, with antiserum in the upper gel. (A) M5 extract run as sample in lower gel, AF/R1 pili in intermediate gel; (B) M5 heat-saline extract run as sample in the upper gel, HB101 extract in intermediate gel; (C) HB101 extract run as sample in the lower gel, AF/R1 pili in intermediate gel. Samples were placed to the left side of the lower gel. The anode was to the right in the first dimension and at the top in the second dimension. Arrows point to the precipitation line formed with AF/R1 pili, showing a line of identity with an M5 component in panel A. Arrowheads in panel B point to antigens that are shared between HB101 and M5.

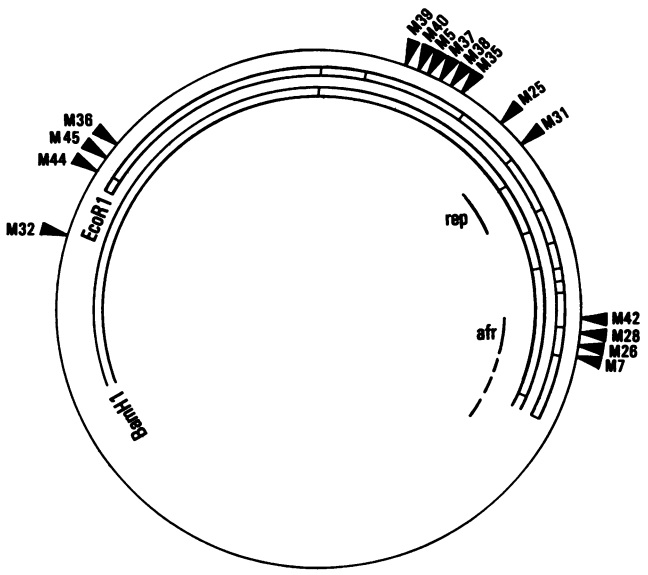


FIG. 3. Restriction map of the 86-MDa plasmid from RDEC-1 that mediates expression of AF/R1 pili. Arrowheads indicate sites of Tn*5* insertions. *rep*, Replication region; *afr*, region necessary for AF/R1 expression. The Tn*5* insertion in M7 is in a 2.4-kbp *Eco*RI fragment and a 12-kbp *Bam*HI fragment.

M34 did not react with the antiserum raised against AF/R1 pili and did not adhere to brush borders from rabbit ileum (Table 1); hence, it does not express AF/R1 pili. Crossed-line immunoelectrophoresis of a heat-saline extract prepared from M34 cells through an intermediate gel containing purified AF/R1 pili and into a gel containing antiserum raised against piliated RDEC-1 cells did not reveal any antigen forming a line of identity with the AF/R1 pili (data not shown); hence, the heat-saline extract from M34 did not contain AF/R1 pili. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the heat-saline extract lacked the 19-kilodalton protein that has previously been identified as the AF/R1 pilin subunit (data not shown). The surfaces of M34 cells were not hydrophobic, as measured by hydrophobic interaction chromatography; hydrophobicity is a property known to be associated with AF/R1 pilus expression (28). These data confirm that the 86-MDa plasmid mediates AF/R1 expression; when the native 86-MDa plasmid was replaced with a plasmid carrying an insertion mutation, AF/R1 expression was lost.

Pathogenicity of the RDEC-1 mutant lacking AF/R1 pili. The course of disease was followed in rabbits given RDEC-1 (4×10^5 or 4×10^7) or M34 (5×10^5 or 5×10^7). Five rabbits were inoculated at each dose; one animal from each group was sacrificed 2 days postinoculation, and another was sacrificed at 7 days postinoculation. Samples were obtained for colony counts and histological and electron microscopic analyses of intestinal segments. All animals that were allowed to survive past 2 days were colonized, as demonstrated by shedding of the inoculated bacteria in their feces. Animals fed RDEC-1 shed these bacteria beginning on day 1 postinoculation, except for one animal fed 4×10^7 RDEC-1, which did not shed until day 3. In contrast, shedding by animals fed 5×10^5 M34 was delayed by 2 to 3 days, so that these animals began shedding on days 2 to 4. Animals fed the high dose of M34 shed with a time course very much like that of RDEC-1; four of five animals shed beginning on day 1, and the other animal shed beginning on day 2. The antibiotic markers of shed bacteria were tested on all animals at 2 days postinoculation and on the day of the last sampling before death. In all cases but one (an animal fed 4×10^5 RDEC-1 shed a strain whose plasmid profile suggested a wild strain on the day before death), the markers of shed bacteria agreed with those of the inoculum, confirming that the rabbits were colonized with the appropriate bacteria. There was no evidence for reversion to expression of AF/R1 pili by the mutant, since all bacteria from rabbits inoculated with M34 retained kanamycin resistance, a property of the Tn*5* used to make the AF/R1$^-$ mutation. Furthermore, those isolates that were tested for AF/R1 expression by using the AF/R1-specific antiserum did not express AF/R1 pili.

All animals that were not sacrificed developed diarrhea. All animals that were fed either dose of RDEC-1 and allowed to survive past day 2 developed persistent diarrhea beginning by days 4 to 7. Two of three animals fed the low dose of M34 (5×10^5) did not develop clinical illness until day 14 after inoculation, and in this group illness was mild and only present for a few days. The other animal fed this low dose of M34 and all animals fed the high dose of M34 developed persistent diarrhea beginning between days 3 and 6. Weight gain was the best indicator of the course of infection (Fig. 4). All animals fed RDEC-1 lost weight. Those fed the high dose of RDEC-1 all died within 8 days postinoculation. Those fed the low dose of RDEC-1 also lost weight, but two animals in this group lived until days 13 and 27. Only animals inoculated with the low dose of M34 continued to gain weight. Animals fed the high dose of M34 showed a similar weight and survival time course as animals fed the low dose of RDEC-1.

RDEC-1 or M34 bacteria were found in the ceca and

TABLE 3. Colonization of intestine

Inoculum		Tissue	Log_{10} viable count (CFU/ml) on day[a]:		Degree of adherence on day:	
Strain	Dose		2	7	2	7
M34	5×10^5	Jejunum	0[b]	0	0	0
		Ileum	0	0	0	0
		Cecum	3.3	7.8	0/+1	+1/+2
		Proximal colon	2.5	7.2	0	0
		Distal colon	2.2	7.8	0/+1	0
RDEC-1	4×10^5	Jejunum	0	6.4	0	+1
		Ileum	3.0	6.7	0	+1
		Cecum	5.2	7.9	0	+2
		Proximal colon	5.5	7.7	0	0/+1
		Distal colon	5.5	7.1	0	+2
M34	5×10^7	Jejunum	2.1	0	0	0
		Ileum	0	5.0	0	0
		Cecum	6.8	7.3	0	+2
		Proximal colon	7.0	7.5	0	0/+1
		Distal colon	5.5	7.9	0	+2
RDEC-1	4×10^7	Jejunum	0	3.5	0	0
		Ileum	0	5.9	0	+1
		Cecum	0	8.2	0	+2
		Proximal colon	0	7.3	0	+2
		Distal colon	0	7.3	0	+2

[a] Segments (10 cm) were flushed, and the CFU per milliliter was determined except for results for the cecum, which were expressed as CFU per gram of cecal contents.

[b] No bacteria detected.

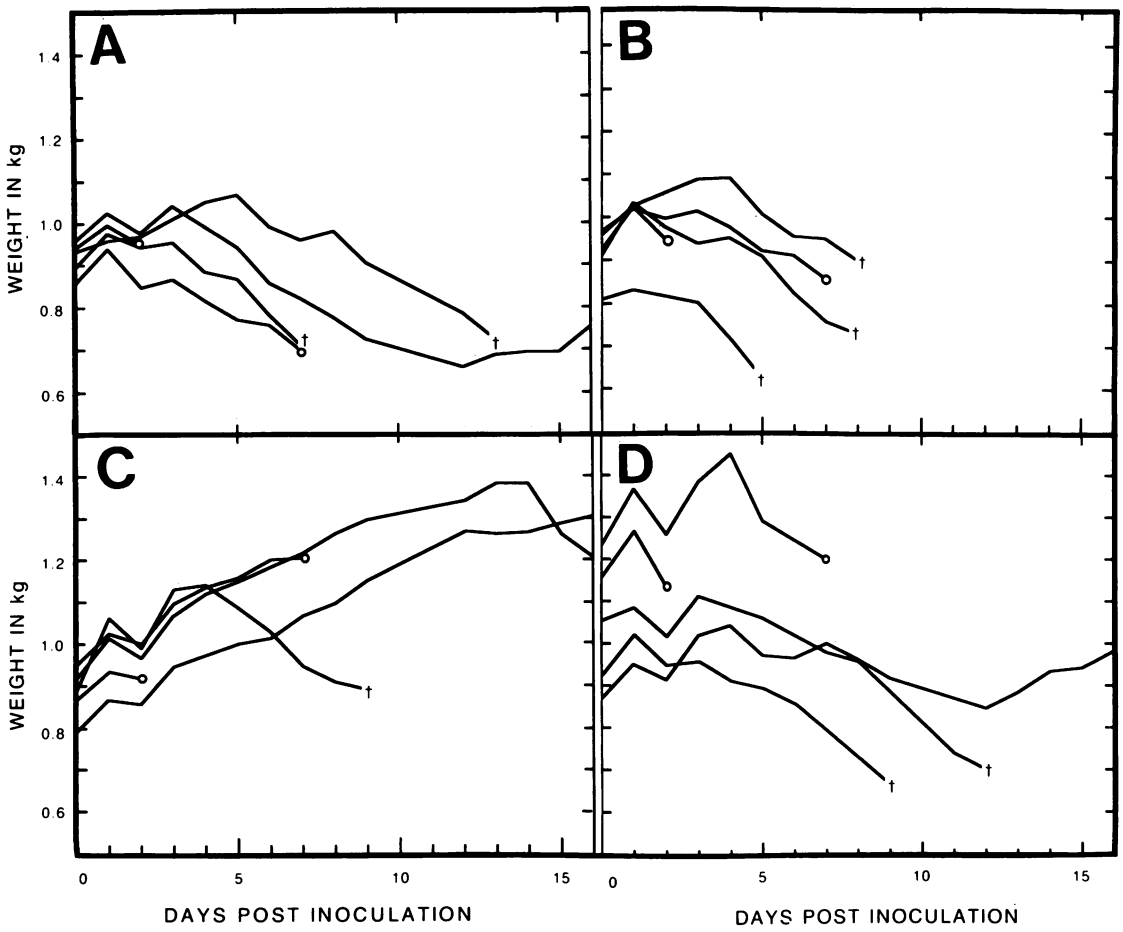


FIG. 4. Weight change. Symbols: ○, animals sacrificed at days 2 and 7; †, death. (A) RDEC-1 (4×10^5) inoculum; (B) RDEC-1 (4×10^7); (C) AF/R1$^-$ strain M34 (5×10^5); (D) M34 (5×10^7).

colons of all animals sacrificed except one animal inoculated with the high dose of RDEC-1 and sacrificed at day 2, a very early time when the course of RDEC-1 infection is variable (Table 3). At day 7, colony counts were similar for RDEC-1 and M34 in the cecum and colon, but many fewer M34 organisms than RDEC-1 were detected in the jejunum and ileum.

Light microscopy of tissues from rabbits sacrificed on day 7 revealed the absence of adherence of M34 in the jejunum and ileum and, at the low dose, only minimal adherence of M34 in the cecum (Table 3). In contrast, adherence of RDEC-1 was seen in the ileum at both doses. Thus, M34 resulted in less-widespread mucosal adherence. In areas of adherence, however, no major histological differences were seen in the lesions produced by RDEC-1 or M34 (Fig. 5). Generally, the mucosal architecture remained intact, and bacteria could be seen adhering to the surfaces of adjacent epithelial cells in the case of both M34 (Fig. 5A and B) and RDEC-1 (Fig. 5C and D). The data on luminal colonization from colony counts from the intestinal segments and mucosal adherence, as judged from stained tissue, did not always correlate. This was because luminal colonization was often evident from colony counts but no bacteria was seen on the surface of the epithelial cells by light microscopy, particularly with M34 (Table 3). Indeed, the recent report of Sherman and Boedeker (27) suggests that the bacteria may be expected to first colonize luminal mucus.

Transmission electron microscopy in areas of bacterial adherence demonstrated characteristic attaching, effacing lesions associated with close attachment of bacteria in the tissue of rabbits infected with either RDEC-1 or M34 (Fig. 6). Elongation and loss of microvilli (i.e., effacement) and condensation of apical cytoplasm were apparent in areas where bacteria were present on pedestals closely adhering to the apical epithelial membrane (Fig. 6, arrows). Neighboring cells lacking bacteria appeared normal.

DISCUSSION

We have confirmed that the 86-MDa plasmid of RDEC-1 mediates expression of the AF/R1 pilus and have characterized the plasmid. This plasmid was tagged with Tn*5* and transferred by conjugation into laboratory strain HB101. HB101 derivative M5 expressed AF/R1 pili that were recognized by antibody raised against purified AF/R1 pili. Crossed-line electrophoresis confirmed the identity of the AF/R1 antigen in M5. Strain M5 adhered to brush borders prepared from rabbit ileum, demonstrating that the AF/R1 pili on the surface of M5 function as adhesins.

The Tn*5*-tagged 86-MDa plasmid from M5 mediated AF/R1 expression when transferred into three natural isolates. In each strain, regulation by growth media was as reported by Cheney et al. (10); i.e., pili were expressed with PAB medium but poorly expressed with BHI. Cheney et al. found

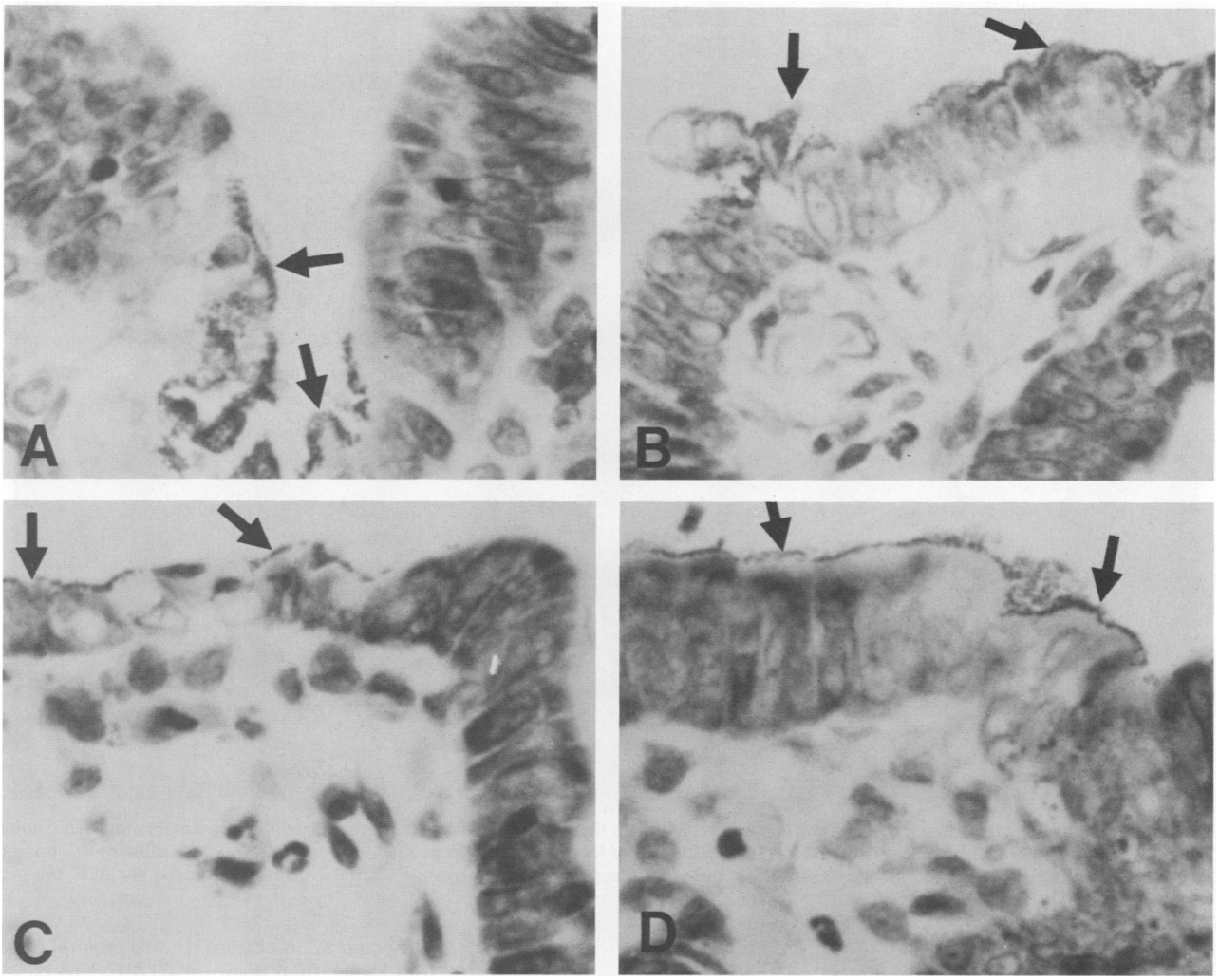


FIG. 5. Giemsa stain of ceca from animals sacrificed 7 days postinoculation. (A) AF/R1$^-$ strain M34 (5×10^5) inoculum; (B) M34 (5×10^7); (C) RDEC-1 (4×10^5); (D) RDEC-1 (4×10^7). Note bacteria adhering to apical surfaces of epithelial cells (arrows) and epithelial cell being extruded (panel B, upper left).

similar regulation in D15, a *Shigella flexneri* derivative carrying the 86-MDa plasmid. The nature of the regulation is not known, but the peptone component of PAB seems responsible (W. L. Houston, G. P. Andrews, and E. C. Boedeker, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B55, p. 27). Although it has not been established, the plasmid probably carries genes for the regulation, structure, and assembly of the pilus. Other plasmid-mediated pilus systems in *E. coli* have been shown to include regulatory genes, pilus subunit structural genes, and genes for synthesis and assembly on single plasmids (15).

Since the Tn*5* insertions in the 86-MDa plasmids of strains M7, M26, and M28 result in loss of the AF/R1 phenotype, the 2.4-kbp *Eco*RI fragment must be necessary for AF/R1 pilus expression. We have designated the genes for AF/R1 expression *afr*, and we have provisionally placed them in the area of the 2.4-kbp *Eco*RI fragment. The Tn*5* insertion in strain M42 does not eliminate AF/R1 pilus expression, but recovery of pili from M42 cells is variable (unpublished data); hence, the insertion lies within *afr* genes, perhaps those involved in stabilization of the pili on the cell surface. Since none of the deletion plasmids mediated expression of the AF/R1 pilus antigen, the *afr* genes must extend beyond the DNA encompassed by these derivatives. This area is indicated by hatched lines on the map shown in Fig. 3. This is consistent with other pilus systems (15) which require approximately 10 kbp for pilus expression. In these systems, genes necessary for the synthesis and assembly of pili account for much of the DNA. It is also possible that another distant site on the plasmid is involved in AF/R1 pilus expression, as has been found for CFA/I pilus expression (29). Cloning of the *afr* region is in progress and should determine the limits of the genes necessary for AF/R1 expression.

It is not known whether other virulence factors are encoded by the 86-MDa plasmid. Nataro et al. have reported that the 86-MDa plasmid shares much homology with plasmid pMAR2, isolated from EPEC, but the region of homology is not from the EPEC adherence factor of pMAR2, which has been suggested as a factor necessary for virulence in humans (22). Analyses of the deletion derivatives of the RDEC-1 plasmid and of Tn*5* insertion sites indicate that DNA sequences necessary for replication fall within the 5.5-kbp *Eco*RI fragment near the site of Tn*5* in strain M31. This area is designated *rep* on the map. Plasmids from strains M32 and M45 have lost the ability to conjugate (data not

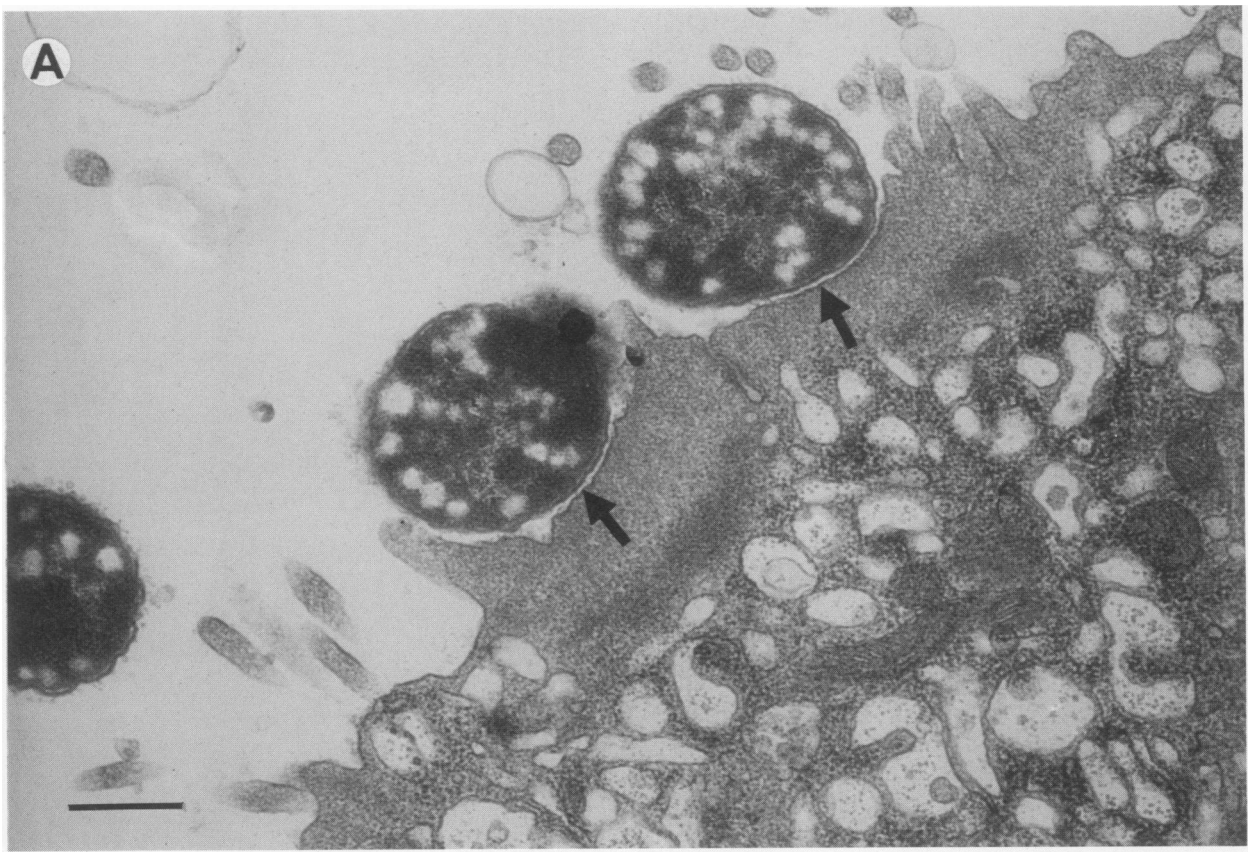


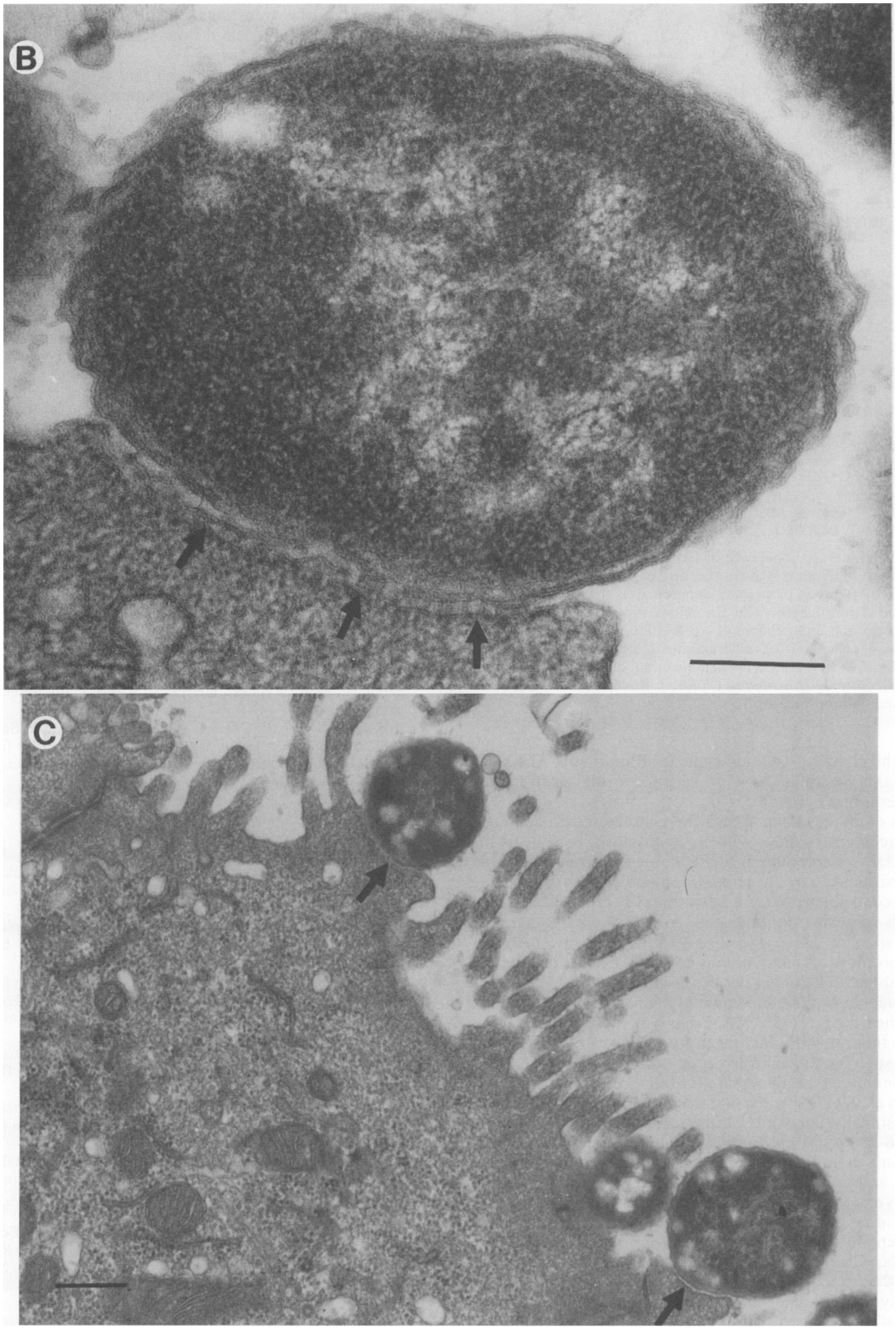
FIG. 6. Transmission electron micrographs of bacteria attaching to the mucosal surfaces of epithelial cells of proximal colons from animals sacrificed 7 days postinoculation. (A and B) M34; (C) RDEC-1. Note loss of microvilli, pedestal formation, and close effacing adherence of both M34 and RDEC-1 bacteria to apical membranes of absorptive epithelial cells (arrows). (A and C) Bars, 1 μm; (B) bar, 0.5 μm.

shown); hence, the area around these Tn*5* insertions is involved in conjugation. Part of the large span(s) of the 86-MDa plasmid lacking Tn*5* insertions are probably involved in conjugation of the plasmid. At present, no other functions are known to be associated with the plasmid.

A derivative of the 86-MDa plasmid carrying a Tn*5* insertion mutation eliminating AF/R1 pilus expression was transferred into RDEC-1 by conjugation. Presumably, the native 86-MDa plasmid and the mutant plasmid carrying Tn*5* were incompatible, and selection for the antibiotic resistance encoded by Tn*5* yielded strain M34, a derivative of RDEC-1 in which the native 85-MDa plasmid was replaced by the mutant plasmid. M34 no longer expressed the AF/R1 antigen or adhered to rabbit brush borders, whereas HB101, carrying the plasmid with Tn*5* inserted elsewhere (strain M5), did express the AF/R1 antigen and adhered to rabbit brush borders. These results confirm that the 86-MDa plasmid carries genes mediating expression of the AF/R1 pilus. Since the nature of the mutation in the plasmid from M7 has not been defined, it is possible that the expression of proteins other than those involved in AF/R1 expression may be affected in strain M34. Further studies on the nature of the Tn*5* insertion mutations should determine whether antigens other than AF/R1 pili are altered in M34, but at present we have no data to suggest that any other antigen is altered.

M34 is clearly capable of causing the characteristic effacement of microvilli associated with EPEC; hence, AF/R1 pilus expression is not essential for the lesions or for disease. However, the presence of AF/R1 pili on the surface of RDEC-1 seems to promote infection; it took inocula of M34 that were 100-fold larger than those of RDEC-1 to cause a similar degree of infection, as measured by weight loss, diarrhea, and luminal and mucosal colonization, and even then, M34 was not seen adhering in the small intestine. Our data show that M34 could be present in the lumen and perhaps the mucous layer without attaching to the absorptive epithelial cell surface. Our data are consistent with the model for RDEC-1 adherence shown in Fig. 7 (4). AF/R1 may play a role in initial adherence to intact microvilli. This attachment step, corresponding to in vitro attachment to intact brush borders, may give the bacteria an advantage in colonizing the mucosal surface with an increased opportunity to produce late adherence with elongation and shedding of microvilli, effacement of the apical surface, and pedestal formation. Symptoms of disease appear to occur when the mucosa is widely affected by close attachment (late adherence). In the absence of AF/R1 pili, late adherence can still occur but is less extensive unless large inocula are ingested. Type 1 pili may also promote attachment of RDEC-1 to the mucosa (28) and hence provide another route of increasing bacterial concentrations at the absorptive cell surface, where late adherence can occur. This could abrogate the absolute requirement for AF/R1 pili.

Knutton et al. (17) have reported experiments of interactions of human EPEC strains with human absorptive epithelial cells in explant cultures of biopsies that lead to similar conclusions. In their report, the loss of a plasmid mediating EPEC attachment to cultured HEp-2 cells did not result in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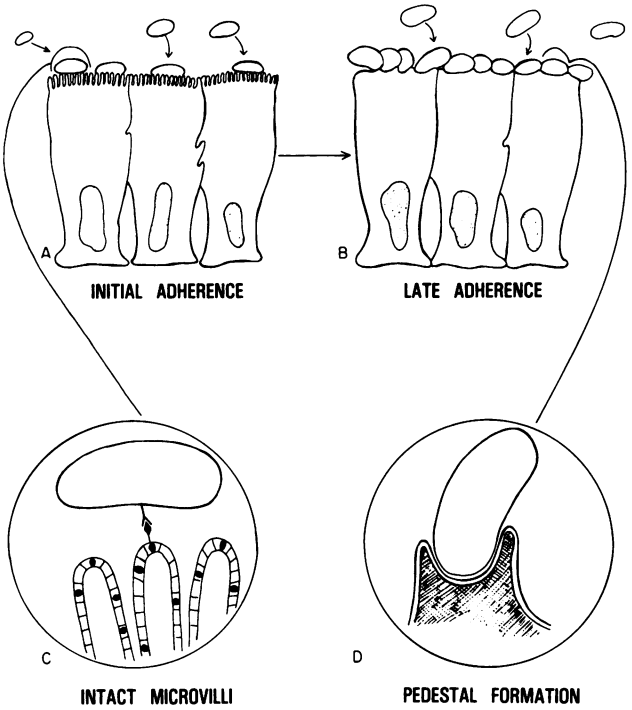

FIG. 7. Model for adherence of RDEC-1 to absorptive epithelial cells. Initial adherence is near the tips of the microvilli. This is mediated by AF/R1 pili. Late adherence is to the apical cell membrane after effacement of the microvilli with pedestal formation. This event is independent of AF/R1 pilus expression. (From E. Boedeker, Mechanisms of adherence of *Escherichia coli* to enterocytes: their possible role in intractable infant diarrhea, p. 336. *In* E. Lebenthal, *Chronic Diarrhea in Children*. Raven Press, New York, 1984. Used with permission.)

loss of the characteristic effacement of microvilli. The presence of this plasmid was necessary for virulence in human volunteers, however (18).

Moon et al. (21) have shown the characteristic adherence and effacement of microvilli in gnotobiotic pigs by using strain RDEC-1 and human EPEC strains. This suggests that EPEC strains share a common mechanism for effacing adherence to absorptive epithelia that is not species specific, although adhesins involved in early adherence may be species specific. This is the case with RDEC-1, in which AF/R1 pili promote attachment only to rabbit brush borders, not to human, rat, or guinea pig brush borders (11).

The mechanism of effacement of microvilli and production of diarrhea by *E. coli* RDEC-1 has yet to be elucidated. It appears that an additional property specific to RDEC-1 and EPEC (such as shigalike toxin or other as yet unidentified toxins or antigens) is necessary for full virulence. The data of Knutton et al. (17) suggest that the EPEC adherence factor is chromosomally encoded. Strains generated by the addition of the 86-MDa plasmid mediating AF/R1 pili to other *E. coli* may result in strains that are able to colonize the lumen or mucosa and that are perhaps pathogenic for rabbits. M5, a derivative of the laboratory strain HB101 that expresses AF/R1 pili, does not colonize or cause disease in rabbits (C. McQueen, W. Yonushonis, M. Wolf, and E. Boedeker, abstracts, Gastroenterology **90**:1547, 1986), but it is expected that HB101 would survive poorly in the intestine. Derivatives of strains 640, DP, and HS are especially interesting in this regard, since they are commensal strains isolated from the intestines of rabbits and humans that contain the 86-MDa plasmid from RDEC-1 and express AF/R1 pili.

Inman and Cantey have shown that AF/R1 pili promote attachment to Peyer's patch lymphoepithelial cells and specifically to M cells within hours following inoculation (13, 14). We did not study this phase of the infection in the present experiments. If AF/R1 pili are required for M-cell attachment as suggested (14), M-cell attachment should not be expected with M34 since M34 lack AF/R1 pili. However Inman and Cantey suggest that AF/R1 pili promote initial adherence to M cells, which is followed by loss of microvilli and close adherence (14). Thus M-cell attachment of RDEC-1 may also proceed via the stages indicated for absorptive cells, shown in Fig. 7. Strains generated here, particularly M34, the derivative of RDEC-1 not expressing AF/R1 pili, should allow more precise definition of the role of AF/R1 pili in M-cell attachment and a further definition of the stages of this attachment.

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LITERATURE CITED

1. **Axelson, N. H., E. Bock, and J. Kroll.** 1973. Comparison of antigens: the reaction of "identity." Scand. J. Immunol. **2**(Suppl. 1):91–94.
2. **Berendson, R., C. P. Cheney, P. A. Schad, and E. C. Boedeker.** 1983. Species-specific binding of purified pili (AF/R1) from the *Escherichia coli* RDEC-1 to rabbit intestinal mucosa. Gastroenterology **85**:837–845.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **6**:1513–1523.
4. **Boedeker, E. C.** 1984. Mechanisms of adherence of *Escherichia coli* to enterocytes: their possible role in intractable infant diarrhea, p. 329–345. *In* E. Lebenthal (ed.), Chronic diarrhea in children. Raven Press, New York.
5. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
6. **Cantey, J. R.** 1981. The rabbit model of *Escherichia coli* (strain RDEC-1) diarrhea, p. 39–47. *In* E. C. Boedeker (ed.), Attachment of organisms to the gut mucosa, vol. 1. CRC Press, Inc., Boca Raton, Fla.
7. **Cantey, J. R., and R. K. Blake.** 1977. Diarrhea due to *Escherichia coli* in the rabbit: a novel mechanism. J. Infect. Dis. **135**: 454–462.
8. **Cantey, J. R., and D. S. Hosterman.** 1979. Characterization of colonization of the rabbit gastrointestinal tract by *Escherichia coli* RDEC-1. Infect. Immun. **26**:1099–1103.
9. **Cheney, C. P., E. C. Boedeker, and S. B. Formal.** 1979. Quantitation of the adherence of an enteropathogenic *Escherichia coli* to isolated rabbit intestinal brush borders. Infect. Immun. **26**:736–743.
10. **Cheney, C. P., S. B. Formal, P. A. Schad, and E. C. Boedeker.** 1983. Genetic transfer of a mucosal adherence factor (R1) from an enteropathogenic *Escherichia coli* strain into a *Shigella flexneri* strain and the phenotypic suppression of this adherence factor. J. Infect. Dis. **147**:711–723.
11. **Cheney, C. P., P. A. Schad, S. B. Formal, and E. C. Boedeker.**

1980. Species specificity of in vitro *Escherichia coli* adherence to host intestinal cell membranes and its correlation with in vivo colonization and infectivity. Infect. Immun. **289**:1019–1027.
12. **Crosa, J. H., and S. Falkow.** 1981. Plasmids, p. 266–282. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
13. **Inman, L. R., and J. R. Cantey.** 1983. Specific adherence of *Escherichia coli* (strain RDEC-1) to membranous (M) cells of the Peyer's patch in *Escherichia coli* diarrhea in the rabbit. J. Clin. Invest. **71**:1–8.
14. **Inman, L. R., and J. R. Cantey.** 1984. Peyer's patch lymphoid follicle epithelial adherence of a rabbit enteropathogenic *Escherichia coli* (strain RDEC-1). Role of plasmid-mediated pili in initial adherence. J. Clin. Invest. **74**:90–95.
15. **Klemm, P.** 1985. Fimbrial adhesins of *Escherichia coli*. Rev. Infect. Dis. **7**:321–340.
16. **Knutton, S., M. M. Baldini, J. B. Kaper, and A. S. NcNeish.** 1987. Role of plasmid-encoded adherence factors in adhesion of enteropathogenic *Escherichia coli* to HEp-2 cells. Infect. Immun. **55**:78–85.
17. **Knutton, S., D. R. Lloyd, and A. S. McNeish.** 1987. Adhesion of enteropathogenic *Escherichia coli* to human intestinal enterocytes and cultured human intestinal mucosa. Infect. Immun. **55**:69–77.
18. **Levine, M. M., J. P. Nataro, H. Karch, M. M. Baldini, J. B. Kaper, R. E. Black, M. L. Clements, and A. D. O'Brien.** 1985. The diarrheal response of man to some classical enteropathogenic *Escherichia coli* is dependent upon a plasmid encoding an enteroadhesiveness factor. J. Infect. Dis. **152**:550–559.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook (ed.).** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **McDowell, E. M., and B. F. Trump.** 1976. Histologic fixatives suitable for diagnostic light and electron microscopy. Arch. Pathol. Lab. Med. **100**:405–414.
21. **Moon, H. W., S. C. Whipp, R. A. Argenzio, M. M. Levine, and R. A. Gianella.** 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect. Immun. **41**:1340–1351.
22. **Nataro, J. P., K. O. Maher, P. Mackie, and J. B. Kaper.** 1987. Characterization of plasmids encoding the adherence factor of enteropathogenic *Escherichia coli*. Infect. Immun. **55**:2370–2377.
23. **Ørskov, I., F. Ørskov, and A. Birch-Anderson.** 1980. Comparison of *Escherichia coli* fimbrial antigen F7 with type 1 fimbriae. Infect. Immun. **27**:657–666.
24. **Peeters, J. E., G. J. Charlier, and P. H. Halen.** 1984. Pathogenicity of *Escherichia coli* isolated from diarrheic suckling and weanling rabbits for newborn rabbits. Infect. Immun. **46**:690–696.
25. **Rothstein, S. J., and W. S. Reznikoff.** 1981. The functional differences in the inverted repeats of Tn*5* are caused by a single base pair nonhomology. Cell **23**:191–199.
26. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35**:852–860.
27. **Sherman, P. M., and E. C. Boedeker.** 1987. Pilus-mediated interactions of the *Escherichia coli* strain RDEC-1 with mucosal glycoproteins in the small intestine of rabbits. Gastroenterology **93**:734–743.
28. **Sherman, P. M., W. L. Houston, and E. C. Boedeker.** 1985. Functional heterogeneity of intestinal *Escherichia coli* strains expressing type 1 somatic pili (fimbriae): assessment of bacterial adherence to intestinal membranes and surface hydrophobicity. Infect. Immun. **49**:797–804.
29. **Smith, H. R., G. A. Willshaw, and B. Rowe.** 1982. Mapping of a plasmid, coding for colonization factor antigen I and heat-stable enterotoxin production, isolated from an enterotoxigenic strain of *Escherichia coli*. J. Bacteriol. **149**:264–275.