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Plasmid-Controlled Colonization Factor Associated with Virulence in *Escherichia coli* Enterotoxigenic for Humans

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An enterotoxin-producing strain of *Escherichia coli* isolated from a case of cholera-like diarrhea (*E. coli* strain H-10407) was found to possess a surface-associated colonization factor. Colonization was manifested as the ability of small inocula (10^5 bacteria) to attain large (10^9) populations in the infant rabbit intestine with a concomitant diarrheal response. A laboratory-passed derivative of *E. coli* H-10407, designated H-10407-P, failed to exhibit an increase in population in the infant rabbit and also failed to induce diarrhea. Cell-free culture supernatant fluids of *E. coli* H-10407 and H-10407-P produced equivalent enterotoxic responses in infant and in adult rabbits. Specific anti-colonization factor antiserum was produced by adsorbing hyperimmune anti-H-10407 serum with both heat-killed and living cells of *E. coli* H-10407-P. This specific adsorbed serum protected infant rabbits from challenge with living *E. coli* H-10407 although the serum did not possess bactericidal activity. The anti-colonization factor serum did not agglutinate a strain of *E. coli* K-12 possessing the K88 colonization factor peculiar to *E. coli* enterotoxigenic for swine. By electron microscopy it was demonstrated that *E. coli* H-10407, but not H-10407-P, possessed pilus-like surface structures which agglutinated with the specific adsorbed (anti-colonization factor) antiserum. *E. coli* H-10407 possessed three species of plasmid deoxyribonucleic acid, measuring 60×10^6, 42×10^6, and 3.7×10^6 daltons, respectively. *E. coli* H-10407-P possessed only the 42×10^6- and the 3.7×10^6-dalton plasmid species. Spontaneous loss of the specific H-10407 surface-associated antigen was accompanied by loss of the 60×10^6-dalton species of plasmid deoxyribonucleic acid and loss of colonizing ability. Thus, it is concluded that the *E. coli* colonization factor described here is a virulence factor which may play an important and possibly essential role in naturally occurring *E. coli* enterotoxic diarrhea in man.

It is characteristic of *Escherichia coli* enterotoxigenic for man that the bacteria attain a large population in the small intestine and there produce enterotoxin which specifically affects that part of the bowel (15, 16). Although ingestion of *E. coli* cannot be considered an uncommon event, the organisms rarely colonize the human small intestine. Thus, it may be speculated that enterotoxigenic *E. coli* possess an adhesive factor which would overcome normal intestinal clearing mechanisms and facilitate colonization. While evidence for intestinal adhesiveness in *E. coli* enteropathogenic for man is very sparse (6, 35), there is considerable evidence that the K88 antigen (24, 25) peculiar to *E. coli* enterotoxigenic for swine is adhesive for swine intestinal epithelium (1, 19, 32, 33). The K88 antigen is a protein in the form of numerous fine filaments covering the surface of the bacterial cell (36, 37). This antigen is an essential virulence factor of K88-positive enterotoxigenic *E. coli* in conventionally reared piglets (19). It is also of interest that the K88 gene is carried by a transmissible plasmid which is spontaneously lost upon subculture of K88-positive cultures in the laboratory (23, 33).

A prerequisite for the study of virulence factors is the availability of a suitable animal model. We chose 4-day-old infant rabbits on the basis of previous work which had demonstrated the high sensitivity of these animals to enterotoxins of *E. coli* and *Vibrio cholerae* and to challenge with viable cultures of these bacteria (9, 17, 30). In this paper we are reporting that enterotoxigenic *E. coli* isolated from man

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(strain H-10407) possesses a plasmid-associated heat-labile surface antigen which is similar to but not identical with the K88 antigen. The *E. coli* H-10407 antigen functions as a virulence factor in that it facilitates colonization of the small intestine.

MATERIALS AND METHODS

Organisms and stock culture conditions. *E. coli* strain H-10407, serotype O78:K80:H11, was isolated from the liquid stool of a patient with severe cholera-like, *Vibrio*-negative diarrhea in Dacca, Banladesh (11). Studies concerning the enterotoxigenicity of this isolate have been described in several previous reports (10, 12–14). The strain employed in the present study was a recently opened lyophile culture derived from the original isolate in Dacca in 1971. The strain designated H-10407-P here refers to the culture of *E. coli* H-10407 after approximately 4 years of maintenance and passage on artificial media in the laboratory. Stock cultures were maintained on slants composed of 2.0% peptone (Difco), 0.5% NaCl, and 2.0% agar.

E. coli strain H-10405 (untypable) was a non-enterotoxigenic human isolate from Dacca (11, 12) which served as a negative control. A strain of *E. coli* K-12 possessing the K88 antigen (K88 colonization factor) originating from an isolate of *E. coli* enterotoxigenic for swine was kindly supplied by Stanley Falkow.

Enterotoxin preparations. Crude enterotoxin preparations consisted of cell-free culture supernatant fluids. Eighty milliliters of a medium consisting of 2.0% Casamino Acids (Difco), 0.6% yeast extract (Difco), 0.25% NaCl, and 0.871% K_2HPO_4 (0.05 M) plus 0.005% $MgSO_4$, 0.005% $MnCl_2$, and 0.0005% $FeCl_3$ (final pH 8.5) in a 500-ml Erlenmeyer flask was inoculated directly from a peptone agar stock culture and incubated for 18 h at 37 C with shaking. Details concerning enterotoxin production in this medium have been described elsewhere (12). Cells were removed by centrifugation at 16,000 × *g* for 20 min followed by membrane (0.45 μm, Millipore Corp.) filtration of the supernatant fluid. The crude enterotoxin preparations employed here were not concentrated or otherwise treated except for refrigeration at 4 C.

Preparation of inocula for challenge of infant and adult rabbits. Five milliliters of brain heart infusion broth (BHIB, Difco) was inoculated directly from a peptone stock culture and incubated without shaking for 18 h. This culture (0.05 ml) was employed as inoculum of a second 5-ml BHIB culture that was incubated for 5 to 6 h and then diluted with sterile BHIB to approximately 10^5 bacteria per ml for intestinal challenge of infant rabbits. For challenge of the adult rabbit ligated intestine, the second BHIB culture was incubated for 7 to 8 h at 37 C and then diluted with sterile BHIB to approximately 10^8 bacteria per ml.

Adult rabbit intestinal ligated loop assay. The adult rabbit intestinal loop assay (5) was performed as previously described (14) employing albino rabbits weighing 1.5 to 2.0 kg. Each 4- to 5-cm ligated segment received a 1.0-ml intraluminal injection of test material, taking special precaution against contamination of the peritoneal cavity. Loops were injected in a randomized sequence in a blind fashion employing a minimum of three animals per study. An 18-h gut exposure time (14) was employed; fluid accumulation was expressed as milliliters of fluid per centimeter of intestine. Challenge of ligated loops was carried out with bacterial suspensions prepared as described above employing sterile BHIB as a negative control. Appropriate dilutions of the crude enterotoxin preparations were assayed employing the phosphate-buffered saline (PBS) diluent as negative control.

Infant rabbit intestinal assay. Four-day-old infant rabbits, freshly separated from the mothers, were employed; animals from several litters were pooled and separated into groups of six to eight animals at random. A 2- to 3-cm midline incision was made under local anesthesia and the duodenum was located with the aid of a blunt curved glass rod. The inoculum (1.0 ml) was injected directly into the lumen of the gut taking care not to contaminate the peritoneum. The incision was closed with silk sutures. The animals were sacrificed 18 h later by intracardiac injection of Nembutol.

For the measurement of fluid accumulation the entire gut was dissected out after ligation of the anterior and posterior ends. The intestines were weighed in a preweighed container. The entire contents of the intestines were extruded out after dissecting of the intestines into short lengths. This was accomplished by firmly pressing the intestine against a glass plate covered with thick filter paper. The remaining tissue was weighed as before and the weight of the contents was determined by difference. Fluid accumulation was expressed as grams of content per gram of intestinal tissue.

For bacterial counts the intestines were excised as above and the uppermost section of the small intestine was removed, weighed, minced with sterile instruments, and homogenized with a glass homogenizer with an appropriate measured volume of sterile cold water. All of the procedures were carried out at 4 C. Tenfold dilutions were promptly prepared and plated on Tergitol-7 and MacConkey medium (Difco). Bacterial recovery was expressed as colony-forming units (CFU) per gram of intestine. Colonies were also selected at random and serotyped for identity; no background contamination was detected in the infant rabbits employed in this work.

Preparation of antiserum against E. coli H-10407. Rabbits were immunized with *E. coli* H-10407 as follows. Peptone agar plates were inoculated for confluent growth and harvested after 18 h at 37 C by suspension in PBS. The suspension was diluted to approximately 10^9 bacteria per ml, 0.5% formalin was added, and the mixture was maintained at 4 C for 48 h. Ten rabbits were inoculated intravenously with 0.1 ml of PBS containing 10^6 formalized bacteria. The dosage was doubled for four additional injections administered at 3-day intervals. A sixth dose consisted of 2 × 10^9 bacteria in PBS. The rabbits were bled 14 days after the sixth dose.

Adsorption of anti-H-10407 serum with E. coli H-10407-P cells. *E. coli* H-10407-P cells were harvested in PBS from the confluent growth obtained by growing 18-h peptone-agar cultures in 8-in. (ca. 20.3 cm) Roux bottles. The thick suspension was autoclaved for 1 h. Ten milliliters of anti-H-10407 serum was added to approximately 1 ml of packed H-10407-P cells, mixed, and centrifuged at 10,000 × *g* for 20 min. The serum was recovered and re-exposed to H-10407-P cells as before. This adsorption procedure was repeated until no agglutination of H-10407-P cells could be detected with the serum. The serum was then twice adsorbed, as above, with living H-10407-P cells derived from fresh young BHIB cultures. The final preparation is referred to as specific adsorbed serum.

Bacterial agglutination with antiserum. The antibody agglutination test was performed with young (4- to 5-h) BHIB-grown cells prepared as above and suspended in PBS. For example, the specific adsorbed serum was tested for bactericidal activity against H-10407 by mixing equal volumes of twofold dilutions of the serum and 4- to 5-h BHIB-grown cells (10^8 bacteria per ml). A second series was prepared to contain fresh guinea pig serum as a source of complement. A BHIB control and a control containing normal rabbit serum was also prepared. The tubes were mixed and incubated for 1 h at 37 C. After incubation, appropriate dilutions were prepared from each tube and plate counts performed.

Neutralization of colonization factor with specific adsorbed serum. *E. coli* H-10407 cells were prepared at 2×10^5 bacteria per ml in PBS and mixed with an equal volume of a 1:60 dilution of the specific adsorbed serum (final dilution 1:120). A similar mixture was prepared in a 1:60 dilution of pooled normal rabbit serum as control. The mixtures were incubated for 30 min at 37 C and administered to respective groups of infant rabbits by the intraluminal route described above.

Electron microscopy. Cells from 18-h, 37 C cultures grown on peptone agar plates were harvested and suspended in PBS at approximately 10^8 bacteria per ml. Negative staining for electron microscopy was accomplished as follows. Carbon-coated collodion grids were floated on a droplet of cell suspension and the excess was removed by adsorption into filter paper. The grids were then rested, for 1 min each, on droplets of bacitracin (wetting agent, 50 μg/ml), two changes of distilled water and finally 1.0% uranyl acetate. Cells were exposed to the specific adsorbed (anti-H-10407) serum, prepared as described above, by floating the grids on droplets of the serum (1:32 dilution) for 15 min before further treatment. After drying, the grids were examined in a Philips EM 200 electron microscope operated at 80 kV.

Isolation and characterization of plasmid DNA. Cells were grown for several generations in 30 ml of M9 medium (21) with 0.5% glucose, 0.2% Casamino Acids, 250 μg of deoxyadenosine per ml and either [^{3}H]thymidine (1.0 μCi/ml, Schwartz/Mann; 18 Ci/mmol) or [^{14}C]thymine (0.2 μCi/ml, Schwartz/Mann; 58 mCi/mmol). Cells were harvested by centrifugation at 17,000 × *g* and lysed by the lysozyme-Brij 58 technique of Clewell and Helinski (4). The lysate was centrifuged at 48,000 × *g* for 25 min, a procedure which removes greater than 95% of the chromosomal deoxyribonucleic acid (DNA) while the plasmid DNA remains in the supernatant (cleared lysate). Material from the cleared lysate was further purified by centrifugation in cesium chloride (ρ = 1.625 g/cm^3) containing ethidium bromide (800 μg/ml) (26). The resultant plasmid mixture was centrifuged at 20 C for 40 h at 105,500 × *g* in a 50 Ti rotor in a Beckman L5-50 ultracentrifuge. The dense band of covalently closed circular (CCC) plasmid DNA was collected, the ethidium bromide was extracted with isopropanol, and the DNA was dialyzed. DNA samples (50 to 150 μl) were layered on 5 to 20% neutral sucrose gradients containing 0.5 M NaCl, 0.005 M ethylenediaminetetraacetate, and 0.05 M tris(hydroxymethyl)aminomethane-chloride buffer, pH 8.1, and then centrifuged at 149,000 × *g* for 60 min at 20 C in an SW50.1 rotor. After centrifugation, 6-drop fractions were collected onto circles (2.3-cm diameter) of Whatman no. 3 filter paper. The filters were dried and precipitated by immersion of the disks in cold 5% trichloroacetic acid containing thymine (100 μg/ml). The filters were washed once with cold 5% trichloroacetic acid and twice with 95% ethanol, dried, and counted in a Beckman LS-230 scintillation counter. Gradients contained either ^{14}C-labeled CCC Col D DNA (21*S*) (38) or ^{14}C-labeled CCC R1 DNA (75*S*) (28) and the *S* values of all ^{3}H-labeled DNA species were calculated relative to the reference DNA. The molecular weights of DNA species were calculated from the relationship $S = 0.034\ M^{0.428}$ (2).

RESULTS

Response of infant rabbits to intestinal challenge with E. coli H-10407 and its laboratory-passed derivative H-10407-P. It is a common observation that repeated subculture of originally virulent clones of pathogenic bacteria promotes a population shift in favor of variants lacking those characteristics which are advantageous only in the realm of the host-parasite relationship. We employed 4-day-old infant rabbits as the artificial hosts to compare the virulence of a lyophile-preserved culture of the enterotoxigenic *E. coli* strain H-10407 with its laboratory-passed derivative H-10407-P. Groups of infant rabbits were given intraluminal injections of fresh, young cultures of *E. coli* H-10407, H-10407-P, or H-10405 (non-enterotoxigenic control) or uninoculated culture medium. Only *E. coli* H-10407 elicited significant fluid accumulation (Table 1). The laboratory-passed derivative, H-10407-P, and the non-enterotoxigenic control strain, H-10405, failed to produce a positive reaction.

Response of infant rabbits to intraluminal administration of cell-free culture fluids of E. coli H-10407 and H-10407-P. It was apparent from the above results that *E. coli* strain H-10407-P was functionally avirulent for infant rabbits; that is, it did not produce fluid accu-

mulation when inoculated at a small dose (10^5 bacteria). We explored the hypothesis that this strain was deficient either in enterotoxin production or in its ability to colonize the infant rabbit intestine. The first possibility was tested by administering to groups of infant rabbits sterile supernatant fluids from freshly grown cultures of *E. coli* H-10407, H-10407-P, and H-10405, grown under conditions known to favor enterotoxin production. There was no demonstrable difference between the enterotoxin activities of the H-10407 and H-10407-P preparations, both of these eliciting a significant response in comparison to the preparation derived from the non-enterotoxigenic control *E. coli* H-10405 (Table 2).

Response of adult rabbit ligated intestines to E. coli H-10407 and its laboratory-passed derivative H-10407-P. In vitro enterotoxin production by *E. coli* H-10407 and H-10407-P and the effect of whole-cell, living preparations were next investigated by using the adult rabbit ileal loop assay (14). Ligated intestinal loops of adult rabbits were administered 1:5, 1:10, and 1:20 dilutions of crude enterotoxin preparations (cell-free culture fluids) derived from *E. coli* H-10407 and H-10407-P. There was no significant difference between the enterotoxin activities of the two cultures (Table 3).

The 18-h adult rabbit ileal loop assay was then employed to test viable cultures of H-10407 and H-10407-P, administering doses of 10^6, 10^7, and 10^8 bacteria per loop. All test samples were assayed at random in the same animals so as to minimize possible individual variations in responsiveness to *E. coli* enterotoxin. H-10407 produced fluid accumulation in the ileal loop when only 10^6 cells were administered (Table 4). On the other hand, a similar response by H-10407-P required a dose of 10^8 cells per loop.

Test for colonization of the infant rabbit intestine by E. coli H-10407 and H-10407-P. Results obtained with both the infant rabbit and the adult rabbit ligated intestinal loop assays showed that the difference between

TABLE 1. *Response of infant rabbit intestines to living E. coli strain H-10407 and its laboratory-passed derivative H-10407-P*

E. coli inoculum[a]	Wt of contents[b]	Wt of intestine	Activity ratio[c]
H-10407	8.34	1.34	6.23 ± 0.38[d], n = 9
H-10407-P	3.55	1.53	2.31 ± 0.16[d], n = 9
H-10405	3.02	1.11	2.73 ± 0.28, n = 8
None	2.78	1.18	2.35 ± 0.21, n = 8

[a] Inoculum was approximately 1×10^5 bacteria per ml in BHIB; H-10405 was the non-enterotoxigenic control; the medium controls were administered BHIB only.

[b] Mean values, in grams.

[c] Activity ratio: grams of contents per gram of intestine; values are means ± 1 standard error of the mean, and n = number of animals per group.

[d] Significantly different by pairwise comparison, $P < 0.001$.

TABLE 2. *Response of infant rabbit intestines to cell-free enterotoxin preparations of E. coli strain H-10407 and its laboratory-passed derivative H-10407-P*

E. coli enterotoxin prepn[a]	Wt of contents[b]	Wt of intestine	Activity ratio[c]
H-10407	8.26	1.34	6.15 ± 0.42, n = 8
H-10407-P	8.19	1.34	6.09 ± 0.21, n = 8
H-10405	3.12	1.30	2.41 ± 0.19, n = 8

[a] Cell-free crude culture supernatant fluids; H-10405 = non-enterotoxigenic control.

[b] Mean values, in grams.

[c] Activity ratio: grams of contents per gram of intestine; values are means ± 1 standard error of the mean, and n = number of animals per group.

TABLE 3. *Response of adult rabbit ligated intestines to cell-free enterotoxin preparations of E. coli strain H-10407 and its laboratory-passed derivative H-10407-P*

E. coli enterotoxin preparation[a]	Fluid accumulation (ml/cm)		
	1:5[b]	1:10	1:20
H-10407	1.69 ± 0.14[c]	1.46 ± 0.15	0.74 ± 0.19
H-10407-P	1.66 ± 0.09	1.28 ± 0.15	0.75 ± 0.18

[a] Cell-free crude culture supernatant fluids.

[b] Dilutions in PBS; negative control was PBS.

[c] Means ± 1 standard error of the mean; all samples were tested in seven ligated loops.

TABLE 4. *Response of adult rabbit ligated intestines to living E. coli strain H-10407 and its laboratory-passed derivative H-10407-P*

E. coli inoculum[a]	Fluid accumulation[b] (ml/cm)		
	10^8	10^7	10^6
H-10407	1.80 ± 0.18	1.02 ± 0.17[c]	0.45 ± 0.14[c, d]
H-10407-P	0.50 ± 0.14[d]	0.10 ± 0.03	0.06 ± 0.03

[a] Inoculum prepared and diluted in BHIB.

[b] Mean values ± 1 standard error of the mean; all samples were tested in 12 different ligated loops; negative control was BHIB.

[c] Significantly different by pairwise comparison, $P < 0.001$.

[d] Not significantly different by pairwise comparison.

TABLE 5. *Recovery of E. coli strain H-10407 and its laboratory-passed derivative H-10407-P from the infant rabbit after intestinal challenge*

E. coli inoculum[a]	CFU/g of tissue[b]
H-10407	9.31×10^7, $n = 12$[c]
H-10407-P	4.36×10^4, $n = 14$[c, d]
H-10405	9.90×10^4, $n = 8$[d]

[a] Inoculum was approximately 1×10^5 bacteria per ml, in BHIB; H-10405, non-enterotoxigenic control.

[b] Colony-forming units per gram of homogenized intestine.

[c] Significantly different by pairwise comparison, $P < 0.001$.

[d] Not significantly different by pairwise comparison.

H-10407 and H-10407-P involved a virulence factor other than enterotoxin. Infant rabbits were selected as a model system to test the possibility that H-10407-P was unable to colonize the intestine. This was accomplished by administering small doses (10^5 bacteria) of living H-10407, H-10407-P, and H-10405 to infant rabbits according to the procedures defined in Materials and Methods. H-10407 showed an increase in population to concentrations of 9.3×10^7 CFU/g of tissue. Both H-10407-P and H-10405 were able to survive in the small intestine, but their concentrations were approximately 3 logs less than H-10407 (Table 5). No *E. coli* were recovered in eight control animals administered only sterile culture medium.

Antigenic difference between E. coli H-10407 and H-10407-P. The results cited in Table 5 suggested that the difference in virulence between H-10407 and H-10407-P might be due to the loss of a factor responsible for the ability of H-10407 to colonize the intestine. The possibility that this colonization factor might be a specific surface antigen was investigated by preparing antiserum against *E. coli* strain H-10407, employing formalized cells as antigen. This antiserum initially agglutinated both H-10407 and H-10407-P cells; it was then exhaustively adsorbed with both living and heat-killed preparations of H-10407-P cells to remove the H, O, and other antigens common to the two strains. The adsorbed serum still agglutinated living but not heat-killed *E. coli* H-10407 cells, indicating that H-10407-P was lacking a heat-labile surface antigen that was present on H-10407 cells. A strain of *E. coli* K-12 possessing the K88 colonization factor peculiar to *E. coli* enterotoxigenic for swine failed to agglutinate with the anti-H-10407 antiserum (Table 6).

Neutralization of the colonization factor by specific adsorbed serum. Incubation of H-10407 cells with the specific adsorbed serum described above did not kill the bacteria even in the presence of added complement. Experiments were performed to test the possible relationship between the colonization factor and the antigen present on H-10407 but not on H-10407-P cells. In the first experiment the effect of the specific adsorbed serum on the response of infant rabbits to challenge with *E. coli* H-10407 was determined as a function of intraluminal fluid accumulation. There was a significant difference between the two experi-

TABLE 6. *Antigenic difference between E. coli strain H-10407 and its laboratory-passed derivative H-10407-P*

E. coli strain	Plus nonadsorbed anti-H-10407 serum	Plus adsorbed anti-H-10407 serum[a]
H-10407, living	1,024[b]	512[b]
H-10407, heat killed[c]	2,048	0
H-10407-P, living	1,024	0
H-10407-P, heat killed	2,048	0
H-10405, living	0	0
H-10405, heat killed	0	0
K-12 (K88+), living	32	0
K-12 (K88+), heat killed	0	0

[a] Anti-H-10407 serum adsorbed with both heat-killed and living cells of strain H-10407-P.

[b] Reciprocal of highest dilution producing complete agglutination.

[c] Heat killed denotes that agglutination was performed with autoclaved bacteria; incubation was for 18 h at 50 C.

TABLE 7. *Effect of specific adsorbed serum on the response of infant rabbits to challenge with E. coli H-10407*

Inoculum	Contents (g)	Intestine (g)	Activity ratio[a]
E. coli H-10407 plus normal rabbit serum	7.88	1.15	6.84 ± 0.67, $n = 8$[b]
E. coli H-10407 plus specific adsorbed serum[c]	3.29	1.33	2.48 ± 0.16, $n = 8$[b]

[a] Grams of contents per gram of intestine; mean values ± 1 standard error of the mean, and n = number of animals per group.

[b] Significantly different by pairwise comparison, $P < 0.001$.

[c] Inoculum was 1×10^8 cells per ml in each case; specific adsorbed serum was anti-H-10407 antiserum adsorbed with H-10407-P cells.

mental groups of rabbits in that fluid accumulation was abolished by the addition of the specific adsorbed serum to the inoculum (Table 7).

In the second experiment, the effect of the specific adsorbed serum on the recovery of *E. coli* H-10407 from infant rabbit intestines was determined. The specific serum did not prevent the survival of H-10407 in the intestine but did prevent the increase in population which occurred in the rabbits which were administered H-10407 plus normal rabbit serum (Table 8). These results demonstrate a definite relationship between the specific *E. coli* H-10407 antigen and the colonization factor.

Demonstration of the E. coli H-10407 colonization factor by electron microscopy. The negative staining technique was employed for an electron microscopic study *E. coli* H-10407 and H-10407-P. Cells were grown on the surface of peptone-agar plates to discourage production of common pili (7). In a comparative study of untreated H-10407 and H-10407-P cells (Fig. 1), it can be seen that cells of H-10407 possess numerous, delicate, pilus-like filaments 8 to 9 nm in diameter and of considerable variation in length. This variation in length could be attributable to the physical effects of suspension, as cell-free filaments can also be observed (Fig. 1A). The inset (Fig. 1B) shows a comparison of the pilus-like structure with a flagellum. The cells of *E. coli* H-10407-P prepared in an identical fashion do not possess pili or pili-like filaments, although flagella are present on H-10407-P (Fig. 1C). Figure 2 shows the results of a comparative study in which cells of *E. coli* H-10407 and H-10407-P were exposed to the specific adsorbed (anti-H-10407) antiserum immediately prior to negative staining. Both attached and unattached filaments were coated with antibody (Fig. 2A and B). Cells of *E. coli* H-10407-P did not react with the specific adsorbed serum (Fig. 2C). However, the antibody treatment is apparent because the proximal ends of dislodged flagella reacted with antibody which had not been fully removed by prior adsorption (Fig. 2C and D). This simply indicates that few proximal ends of flagella were exposed to antibody during the original adsorption procedure.

Characterization of plasmid DNA of E. coli H-10407 and H-10407-P. *E. coli* H-10407 and H-10407-P cells were uniformly labeled with [^{3}H]thymidine, and plasmid DNA was isolated as described in Materials and Methods. The sedimentation profile in a 5 to 20% neutral sucrose gradient of the CCC DNA isolated from strain H-10407 is shown in Fig. 3A. Three distinct species of circular DNA are present,

TABLE 8. *Recovery of E. coli strain H-10407 from infant rabbits after intestinal challenge in the presence of specific adsorbed serum*

Inoculum	CFU per g of tissue[a]
E. coli H-10407 plus normal rabbit serum	1.80×10^7, $n = 8$[c]
E. coli H-10407 plus specific adsorbed serum	3.13×10^4, $n = 8$[c]

[a] Colony-forming units per gram of homogenized intestine.

[b] Inoculum was 1×10^5 cells per ml in each case; specific adsorbed serum was anti-H-10407 antiserum adsorbed with H-10407-P cells.

[c] Significantly different by pairwise comparison, $P < 0.001$.

sedimenting at 73, 62, and 22*S* values. These *S* values correspond to molecular weights of approximately 60×10^6, 42×10^6, and 3.7×10^6 (Mdal), respectively. Analysis of this DNA by electron microscopy yielded contour length measurements which corresponded to the values obtained from velocity sedimentation data (unpublished data). These results also agree closely with those reported by Gyles et al. (18) for *E. coli* H-10407 plasmid DNA. On the other hand, sedimentation studies, as well as electron microscopy, of the CCC DNA isolated from strain H-10407-P indicated the presence of only the 42-Mdal and 3.7-Mdal DNA species (Fig. 3B). These results were confirmed by labeling the DNA of H-10407 with [^{3}H]thymidine and that of H-10407-P with [^{14}C]thymine. The plasmid DNA was then isolated from both strains and co-sedimented in a 5 to 20% neutral sucrose gradient. Only *E. coli* H-10407 contained the 60-Mdal species of DNA (Fig. 3C).

Spontaneous loss of colonizing ability and the 60 $\times$ 10^6-dalton plasmid by E. coli H-10407. As shown above (Table 6), strain H-10407, but not H-10407-P, agglutinated with the specific adsorbed anti-H-10407 serum. We found, however, that when individual clones derived from the H-10407 culture were tested for agglutination with the specific adsorbed serum, only 24% of these were positive. Three agglutinating and three non-agglutinating isolates were selected for further study. Cells were uniformly labeled with [^{3}H]thymidine, plasmid DNA was isolated by the cleared lysate technique, and sedimentation profiles were obtained by employing 5 to 20% neutral sucrose gradients (Table 9). Clones which were selected on the basis of their ability to agglutinate with the specific adsorbed serum (H-10407-5, -19, and -21) contained the three species of plasmid DNA observed in *E. coli* H-10407. On the other hand,

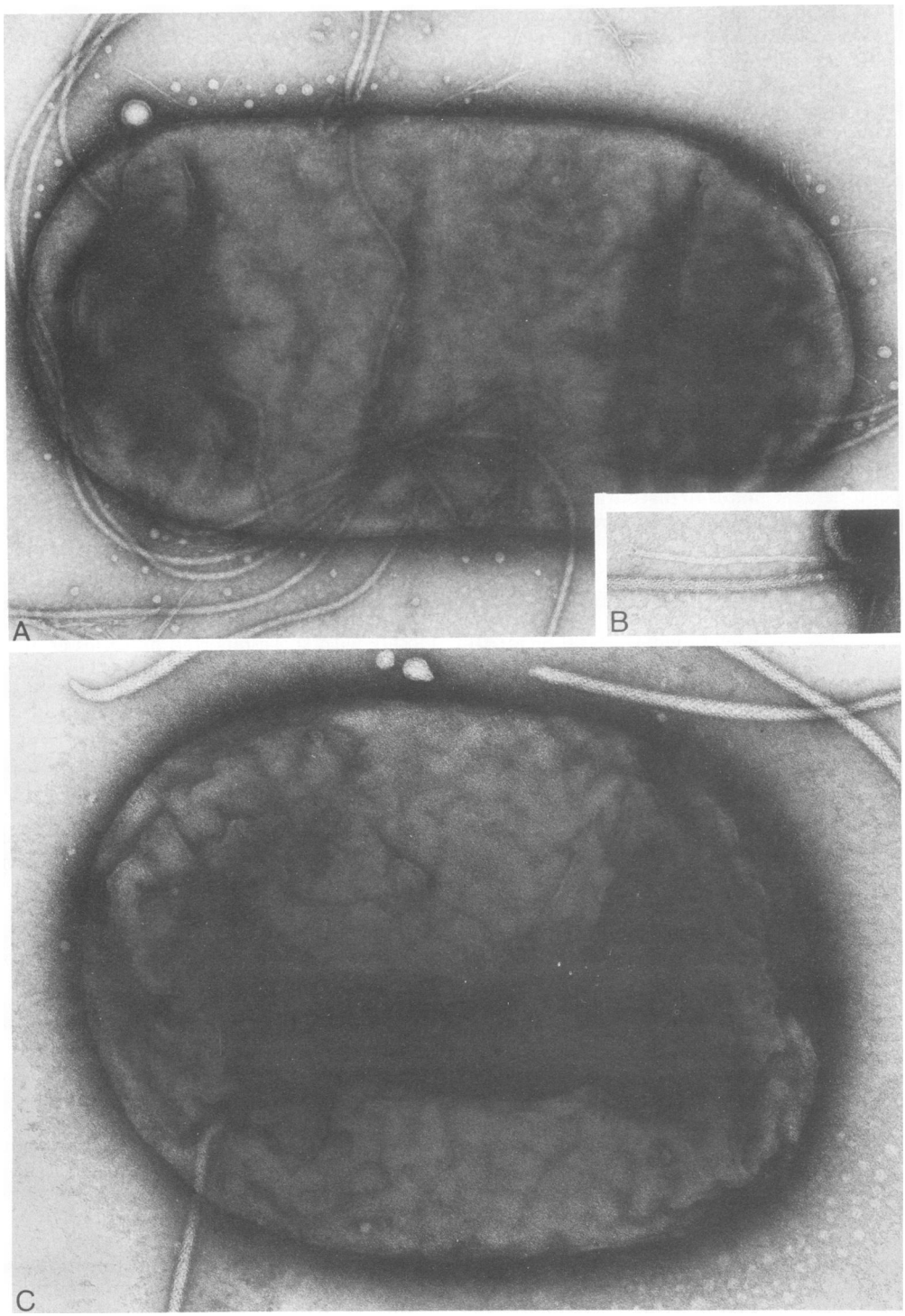

FIG. 1. *Electron micrographs of negatively stained cells of E. coli H-10407 and H-10407-P. The micrograph of E. coli H-10407 (A) shows delicate filaments which overlie and extend from the periphery of the cell. ×60,000. The inset (B) shows a thin filament lying next to a flagellum. ×100,000. The micrograph of E. coli H-10407-P (C) shows flagella as the only visible surface structure. ×100,000.*

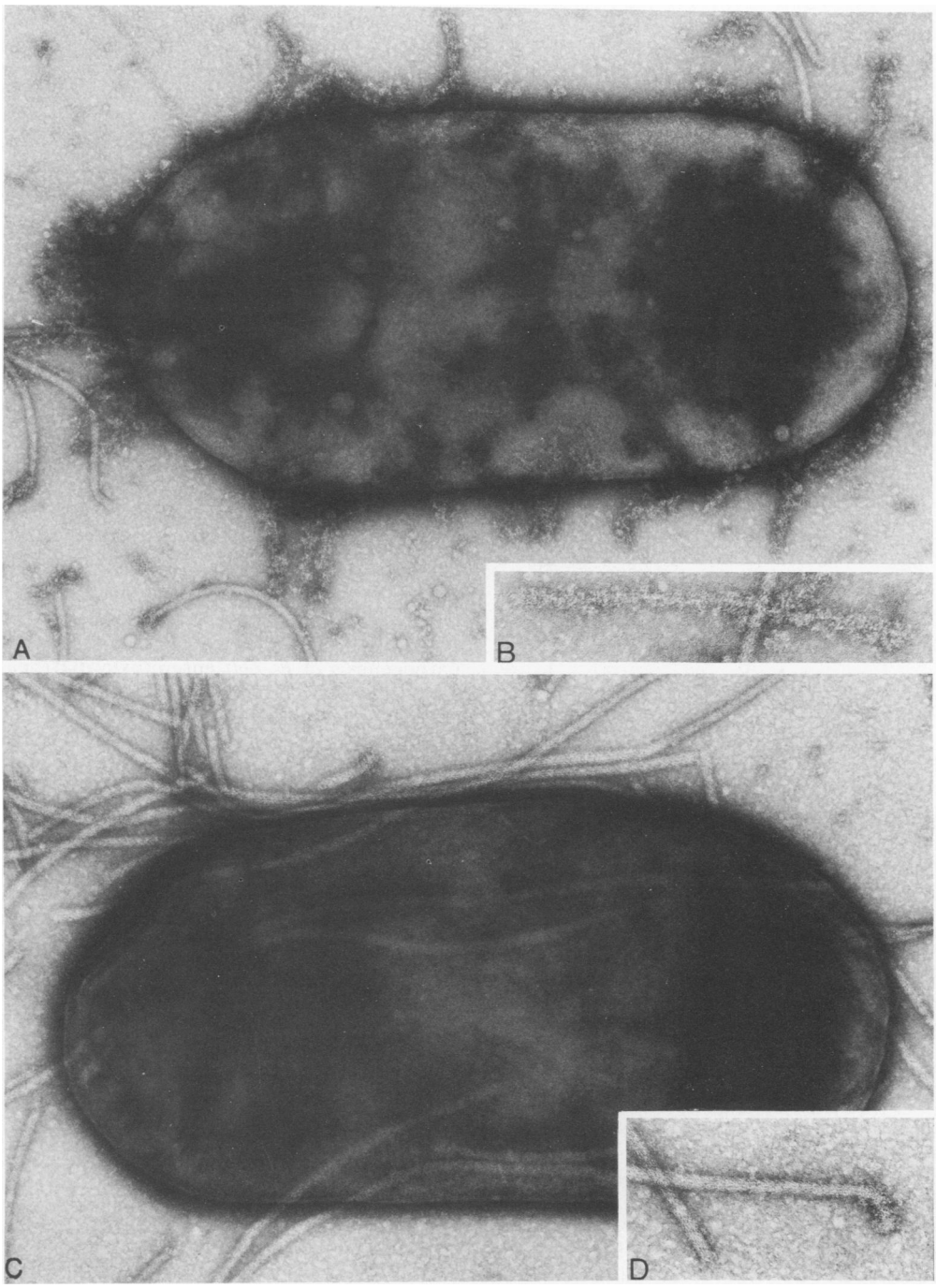

FIG. 2. *Electron micrographs of negatively stained cells of E. coli H-10407 and H-10407-P after treatment with specific adsorbed (anti-H-10407) antiserum. The micrograph of E. coli H-10407 (A) shows antibody adsorbed to the surface-associated filaments. ×60,000. The inset (B) shows a detached filament coated with antibody overlying the shaft of an antibody-free flagellum. The micrograph of E. coli H-10407-P (C) shows no antibody reaction with the cell surface. ×70,000. The inset (D) shows antibody reacted with the exposed proximal end of a dislodged flagellum. ×100,000.*

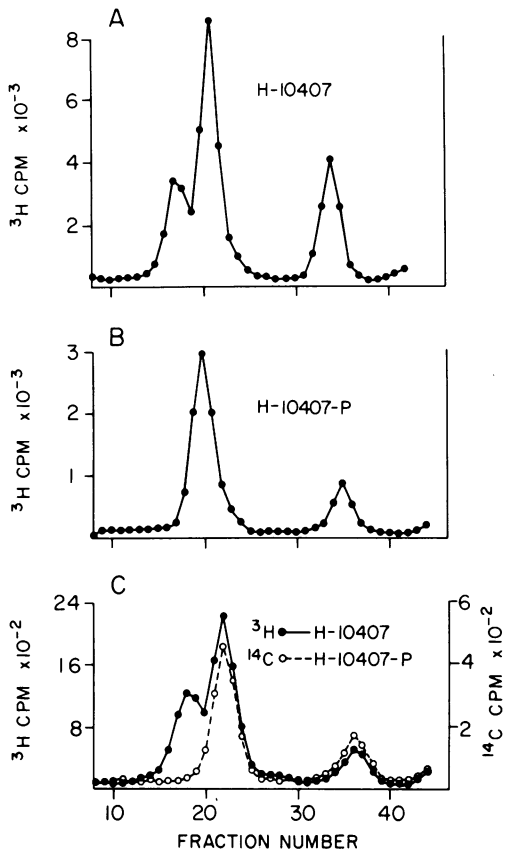

FIG. 3. *Neutral sucrose gradient analysis of the plasmid complement of E. coli H-10407 and H-10407-P. Plasmid DNA was uniformly labeled with [^{3}H]thymidine or [^{14}C]thymine and purified by cesium chloride-ethidium bromide density gradient centrifugation. DNA samples were layered onto 5 to 20% sucrose gradients and centrifuged at 149,500 × g for 60 min at 20 C. (A) ^{3}H-labeled H-10407 DNA; (B) ^{3}H-labeled H-10407-P DNA; (C) ^{3}H-labeled H-10407 DNA (—) and ^{14}C-labeled H-10407-P DNA (--).*

the non-agglutinating cultures (H-10407-9, -22, and -30) lacked the 60-Mdal plasmid although these did possess the 42-Mdal and the 3.7-Mdal species of DNA.

The colonizing ability of a non-agglutinating isolate, H-10407-22, was assayed in the infant rabbit model employing the same conditions as those described in Materials and Methods and in Table 5. *E. coli* H-10407-22 did not colonize the small intestine, as evidence by the fact that only 5.01 × 10^4 CFU per g of intestine (mean value, 10 animals) was recovered 18 h after challenge with 10^5 bacteria. H-10407-22 also failed to produce fluid accumulation (diarrhea) in infant rabbits when a group of seven animals was challenged with 10^5 bacteria according to the procedures illustrated in Table 1. The mean value (2.29 ± 0.12 g of contents per g of intestine) thus obtained with strain H-10407-22 was not different from that (2.31 ± 0.16) obtained with the noncolonizing laboratory-passed derivative strain H-10407-P. Thus, loss of the specific H-10407 surface antigen and loss of the 60-Mdal plasmid (Table 9) was also associated with loss of colonizing activity.

DISCUSSION

Severe *E. coli* enterotoxin diarrhea in man is analogous to Asiatic cholera in that the small bowel is heavily colonized by the pathogen and the symptomatology of the disease is entirely attributable to enterotoxin (3, 16). Two virulence factors are generally associated with enterotoxigenic *E. coli*: the ability to produce enterotoxin and the ability to colonize the small intestine in sufficient numbers to expose the mucosal tissue to biologically significant amounts of the toxin.

The widespread occurrence of *E. coli* enterotoxigenic for piglets, calves, and lambs has stimulated numerous investigations into the pathogenesis of *E. coli* enterotoxic diarrhea in these animals. The ready availability of homologous animal model systems facilitated these studies and has led to elegant descriptions of the role of the surface-associated K antigens as virulence factors in animal-associated enterotoxigenic *E. coli* diarrheal disease (19, 27, 31, 33, 34, and 40). Numerous lines of evidence indicate that the K88 antigen (24) is a colonization factor which plays an important, and possibly essential, role in the naturally occur-

TABLE 9. *Molecular nature of plasmids isolated from E. coli H-10407 and its laboratory-passed derivatives: correlation between presence of specific H-10407 antigen and the 60 Mdal plasmid*

Strain	Antigen[a]	Enterotoxin	Plasmids[b]		
			60	42	3.7
H-10407	+	+	+	+	+
H-10407-P	−	+	−	+	+
H-10407-5	+	+	+	+	+
H-10407-19	+	+	+	+	+
H-10407-21	+	+	+	+	+
H-10407-9	−	+	−	+	+
H-10407-22	−	+	−	+	+
H-10407-30	−	+	−	+	+

[a] Determined by agglutination with the specific adsorbed anti-(H-10407) serum.

[b] Molecular weight × 10^6.

ring disease in swine. There is also evidence for analogous surface-associated antigens on *E. coli* enterotoxigenic for cattle and sheep, and it appears that such antigens play a role in the host specificity of these pathogens (34, 40).

Although the pathogenic mechanisms of animal-specific enterotoxigenic *E. coli* are similar to those of *E. coli* enterotoxigenic for man, there is one major exception. Certain animal-specific strains of *E. coli* only produce a heat-stable enterotoxin, whereas all human isolates thus far have been found to produce both heat-stable and a potent heat-labile enterotoxin (12, 18, 30). The heat-labile enterotoxin produced by some animal strains of *E. coli* may be identical to that produced by the human-associated isolates (14, 18).

Heat-stable enterotoxin, heat-labile enterotoxin, and K88 production are all associated with plasmids harbored by *E. coli* (16, 18, 34). However, the occurrence in human isolates of *E. coli* of a plasmid associated with a surface colonization factor has hitherfore not been reported. In this report we present evidence that the 60-Mdal plasmid of *E. coli* H-10407 is associated with a heat-labile surface antigen which functions as a colonization factor. Gyles and co-workers (18) have reported the existence of this plasmid in *E. coli* H-10407 but assigned to it a role in enterotoxin production. However, here we show that loss of this plasmid does not alter enterotoxin production in H-10407. However, spontaneous loss of the 60-Mdal plasmid is accompanied by the ability to colonize the small intestine of infant rabbits and the loss of a heat-labile surface-associated antigen which possesses pilus-like morphology. This leads us to conclude that the 42-Mdal plasmid is the *ent* plasmid in H-10407. In fact, this has been confirmed in recent experiments in which we were able to transfer the 42-Mdal plasmid, and the ability to produce enterotoxin, from *E. coli* H-10407-P to *E. coli* K-12.

Since strain H-10407-P lacked the colonization-associated surface antigen of H-10407, it was possible to prepare antiserum against H-10407 and to exhaustively adsorb the serum with living and heat-killed H-10407-P cells. This resulted in a specific adsorbed (anti-H-10407) serum which agglutinated only H-10407 cells; that is, cells possessing the specific pilus-like antigen. This serum effectively neutralized the colonization activity of *E. coli* H-10407 although the serum had no bactericidal activity. Furthermore, the specific adsorbed serum was shown, by electron microscopy, to react with pilus-like structures on the surface of *E. coli* H-10407 cells, structures which were absent from H-10407-P cells. Clones isolated from the original H-10407 culture on the basis of their inability to agglutinate with the specific adsorbed serum were shown to have lost the 60-Mdal plasmid and colonization activity. It is evident that loss of the 60-Mdal plasmid, along with retention of the 42-Mdal plasmid (*ent*), is responsible for the observed differences between *E. coli* H-10407 and H-10407-P.

The results reported here indicate that adhesiveness of the *E. coli* H-10407 pilus-like surface antigen for the intestinal epithelium probably plays a role in the observed colonizing activity, as would be expected from its similarity to the K88 antigen. However, the data presented here also show that the mechanism of intestinal colonization involves an ability to overcome those intestinal factors which control bacterial populations in the gut. It is evident that *E. coli* such as H-10407-P and H-10405 which lack colonization activity do survive but do not attain very large populations in the intestine. This is not necessarily a surprising result. It indicates that colonization activity is not simply a matter of overcoming a physical clearance mechanism. Furthermore, since colonization initiated by adhesiveness would likely invoke a dynamic situation involving as yet ill-defined surface phenomena, death/survival rates, and differential growth rates, it would be premature to speculate on a simplistic mechanism for intestinal colonization by enterotoxigenic *E. coli*. Strains containing only the 60-Mdal plasmid will be useful in the investigation of the mechanism of colonization, but such strains are not yet available.

The present work leaves many questions to be answered. For example, how widespread is the occurrence of the H-10407 colonization factor? In preliminary studies we have found several other human-associated enterotoxigenic isolates from Dacca to be positive when tested for agglutination with the specific adsorbed anti-colonization factor antiserum. However, the multiplicity of surface-associated antigens in *E. coli* dictates that the question of antigenic identity should be approached with caution.

It would be of particular interest to define the host specificity of the H-10407 colonization factor. Infant rabbit intestines react rather nonspecifically to *V. cholerae* and to enterotoxigenic *E. coli* isolates from both human and animal sources (9, 30). Therefore it would be of value to carry out comparative studies employing piglets, pigs, calves, and lambs as reported by Smith and Linggood in 1971 (33). The ultimate test, of course, would be human volunteer studies carried out under carefully con-

trolled conditions; such studies having been carried out successfully with *E. coli* and other bacterial agents of severe diarrhea (3, 8).

Our results indicate that an essential role for antigenic virulence factors other than enterotoxin in human enterotoxic *E. coli* diarrhea can now be seriously contemplated. Specific serological tests for such surface-associated antigens might prove to be simple, rapid, and highly valuable tools for the diagnosis of enterotoxigenic *E. coli* diarrhea in man. Finally, recent studies regarding the possibility of artificially inducing immunological protection against enterotoxigenic *E. coli* causing diarrhea in piglets (19, 27, 29) and calves (20, 22, 39) deserve attention. It remains to be determined whether similar studies may be carried out with regard to human enterotoxigenic *E. coli* diarrhea.

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