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Adherence of an Enteropathogenic Strain of *Escherichia coli* to Human Intestinal Mucosa Is Mediated by a Colicinogenic Conjugative Plasmid

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The capacity of a human enteropathogenic *Escherichia coli* (EPEC) strain, serotype O26:K60:H11, to adhere to the mucosa of the human fetal small intestine was shown to be plasmid mediated. Adherence was transferred at a high frequency in a long-term conjugal mating experiment to *E. coli* K-12 and was lost by treatment of the EPEC strain with the curing agent ethidium bromide. Analysis of radioactively labeled DNA from lysates of the EPEC, transconjugant, and cured strains indicated that adherence was correlated with the presence of plasmid DNA species with an approximate average molecular weight of 56×10^6. Resistance to the antibiotics spectinomycin, streptomycin, sulfonamides, and tetracycline and production of colicin Ib were all transferred in long-term mating and lost upon curing coordinately with the property of adherence. In conjugal mating experiments of limited duration between *E. coli* K-12 strains, however, segregation of colicin production and mucosal adherence from multiple drug resistance was observed. Analysis of plasmid DNA of segregant transconjugant strains confirmed the presence in the 56×10^6-dalton plasmid species of two previously unresolved components, pLG101 designating the ColIb plasmid which also carries the determinant for mucosal adherence and pLG102 representing the slightly smaller multiple drug resistance plasmid.

Certain strains of *Escherichia coli* are enteropathogenic (EPEC) and can cause infectious diarrhea in humans and domestic animals (35) in at least two ways. Some strains produce soluble enterotoxins that, like cholera toxin, stimulate fluid and electrolyte secretion into the bowel (12). Other strains penetrate the intestinal mucosa and induce a dysentery-like inflammatory response (18). Still other strains are implicated in pathogenesis by epidemiological studies but have an unknown pathogenic mechanism (17). This group would include many classical EPEC serotypes (34).

Several aspects of *E. coli* pathogenicity, including enterotoxin production, have been shown to be plasmid controlled, and in experimental animals a summation of virulence has been observed in bacterial strains harboring various combinations of such plasmids (32). In strains enterotoxigenic for young pigs the capacity of bacterial cells to adhere to the intestinal mucosa is an important additional determinant of virulence (32). Adherence is mediated by a plasmid-specified antigenic protein (K88) on the bacterial cell surface (33). An analogous surface antigen (K99) has been identified in bovine EPEC (25), and a human EPEC strain has been reported to have a plasmid-determined surface antigen allowing colonization of rabbit intestine (14).

To study the importance of the adherence property in the virulence of human EPEC, McNeish et al. (23) developed an assay for clinical isolates by using human fetal small intestine. In a preliminary communication it has been reported that, for one of the strains identified in this way, the property of adherence is specified by a transmissible plasmid (37). Data that confirm the earlier work and extend it to include a fuller genetic characterization of the plasmid that is responsible for adherence are presented in this paper.

MATERIALS AND METHODS

Bacterial strains. Strains used in this investigation are listed in Table 1. The strain designated O26 has been referred to previously as H19 (31).

Mucosal adherence. The assay for mannose-independent bacterial adherence to human fetal intestinal tissue has been described previously in detail

TABLE 1. *Bacterial strains*

Designation	Characteristics	Source, references
O26	Clinical isolate, serotype O26:K60:H11 Adh^{+a}	H. Williams Smith (23, 31)
LG1223	Derivative of O26, Adh	EtBr curing
711	K-12 *nalA*	(19)
LG1243	Derivative of 711, ColI^r	Spontaneous mutation
LG1122	Derivative of 711, Adh$^+$	O26 × 711 mating
LG1273	Derivative of LG1243 carrying ColIb-P9*drd-1*	Conjugation
LG1272	Derivative of LG1122 carrying pLG1^b	Transformation
GB20	Derivative of AB1157 (20), ColI^r	Spontaneous mutation
LG1247	Derivative of GB20, Cib$^+$	LG1272 × GB20 mating
LG1249	Derivative of GB20, Tc^r	LG1272 × GB20 mating
JK16	Derivative of W3110 (2), *rpsL*, carrying ColIa-CA53	J. Konisky (5)
LG1245	Derivative of JC411 (6), carrying ColIb-P9*drd-1*	Conjugation
LG1244	Derivative of JC411 (6), carrying ColV-K94	Conjugation
LG2253	Derivative of JC411 (6), carrying ColE2-P9	Transformation
LG1117	Derivative of C600 (1), carrying ColE1-K30	Transformation
K235	Wild isolate, carrying ColK-K235	P. Fredericq (9, 10)
B6	Wild isolate, carrying ColB-B6	I. B. Holland (28)
H-10407	Clinical isolate, serotype O78:K80:H11, CFA producing	L. LeMinor (14)
H-10407-P	Derivative of H-10407, CFA nonproducing	L. LeMinor (14)
NCTC10418	Standard *E. coli* strain	D. White

[a] We suggest the terminology Adh for the plasmid-mediated phenotypic character adherence to intestinal mucosa.

[b] Purified plasmid pLG1 DNA for transformation was generously supplied by R. Diaz. pLG1 is a derivative of ColE1 having a fragment carrying ampicillin resistance inserted at the single *Eco*RI restriction site.

(23). Pieces of intestinal tissue (approximately 15 mm^2), washed with phosphate-buffered saline (pH 7.2), were incubated at 37°C with gentle agitation in 1-ml volumes of bacterial suspensions (5×10^8 cells per ml) in phosphate-buffered saline containing 0.5% D-mannose. Tissue pieces were removed after 1 s or 30 min and washed thoroughly before being homogenized. The number of viable bacteria in the homogenates was determined. The increase in the number of viable organisms in the 30-min samples compared with the 1-s samples was taken as a measure of the level of adherence to the intestinal mucosa.

Analysis of plasmid DNA. Bacterial strains were grown in M9 medium (27) supplemented with glucose (0.2%) and Casamino Acids (0.5%) at 37°C with aeration for two to three mass-doublings to a cell density of 2×10^8 cells/ml. The growth medium contained deoxyguanosine (200 μg/ml) and either [^{3}H]thymidine (1 μCi/ml) or [^{14}C]thymidine (0.1 μCi/ml). Radioactively labeled cells were lysed by the lysozyme-ethylenediaminetetraacetic acid detergent procedure (7), using the detergent Sarkosyl (1% wt/vol, final concentration). Total cell lysates were centrifuged to equilibrium in neutral cesium chloride (CsCl) gradients (average density, 1.55 g/cm^3) containing ethidium bromide (EtBr, 300 μg/ml) (26). Centrifugation was at 15°C for 40 h at 35,000 rpm in a Beckman type 65 rotor. Plasmid-containing bands from gradients were treated with isopropyl alcohol to remove EtBr and dialyzed against TES buffer [50 mM tris(hydroxymethyl)aminomethane, pH 7.0–5 mM ethylenediaminetetraacetic acid–50 mM NaCl] to remove CsCl. Purified plasmid material was analyzed by velocity sedimentation through neutral sucrose density gradients as described in the legends of appropriate figures.

Plasmid curing. Portions (5 ml) of nutrient broth (pH 7.4) supplemented with trypsin (1 mg/ml) and ethidium bromide (50-175 μg/ml) were inoculated with approximately 1,000 cells of the bacterial strain to be cured (4). Trypsin was added to inactivate colicin produced by colicinogenic strains. Cultures were incubated overnight at 37°C without agitation and then plated out for well-separated colonies that were subsequently tested for loss of plasmid-determined characters.

Tests for extracellular proteins. To test for hemolysin production, bacterial strains were streaked on horse blood agar plates and incubated overnight at 37°C. Plates were checked for zones of partial or complete hemolysis around bacterial streaks. Heat-labile enterotoxin production was determined by the Y1 adrenal cell monolayer system as described by Donta et al. (11). Colicinogenicity was demonstrated by a soft agar overlay method (9, 10).

Drug resistance. Qualitative determination of drug resistance of bacterial strains involved antibiotic sensitivity disks (16). Subsequently, quantitative estimates of minimal inhibitory concentrations (MICs) of several antibiotics to various bacterial strains were made in liquid peptone-water medium and on solid nutrient agar or diagnostic sensitivity test agar (16).

UV light irradiation. Bacterial strains were grown in Luria broth at 37°C with aeration for two to three mass doublings to a cell density of 2×10^8 cells/ml. A portion (2 ml) of culture was diluted with 8 ml of phosphate buffer in a sterile glass petri dish (9 cm diameter) and irradiated with successive doses of UV light from a Hanovia germicidal lamp. Cultures were swirled gently during treatment to ensure uniform irradiation. Samples (0.1 ml) were taken after each dose and plated at appropriate dilutions on nutrient

agar to determine survivors. The radiation dose rate was adjusted with a Latarjet UV dosimeter (22). Comparative qualitative estimates of UV sensitivity were made by irradiation of bacterial streaks on nutrient agar plates.

Reagents and chemicals. Nutrient media and agar were purchased from Oxoid Ltd. Spectinomycin dihydrochloride pentahydrate (Trobicin) was obtained from Upjohn Ltd., streptomycin sulfate was obtained from Glaxo Ltd., sulfathiazole and tetracycline hydrochloride were obtained from Sigma Chemical Co. Ltd., and nalidixic acid was obtained from Winthrop Laboratories. Oxoid Multodisks code 30-44K carried chloramphenicol (50 μg), colistin sulfate (10 μg), nitrofurantoin (200 μg), sulfafurazole (500 μg), kanamycin (30 μg), ampicillin (25 μg), streptomycin (25 μg), and tetracycline (50 μg). Oxoid Multodisks code 6646E carried nalidixic acid (5 and 30 μg) in place of colistin sulfate and nitrofurantoin in 30-44 K. Mastring-S code PHL/L/275/5 (Mast Laboratories Ltd.) carried nalidixic acid (30 μg), nitrofurantoin (200 μg), ampicillin (25 μg), colistin sulfate (10 μg), cephaloridine (25 μg), tetracycline (30 μg), gentamicin (10 μg), and cotrimoxazole (25 μg). Horse blood (defribrinated, supplied without preservative) was purchased from Difco Laboratories and trypsin was obtained from Sigma Ltd. The Radiochemical Centre, Amersham, provided [*methyl*-^{3}H]thymidine (code TRK. 418, 40 to 60 Ci/mmol) and [2-^{14}C]thymidine (code CFA. 219, 50 mCi/mmol). Deoxyguanosine and lysozyme (egg white) were purchased from Sigma Ltd., Sarkosyl NL-97 was purchased from Geigy Ltd., ethidium bromide was purchased from Calbiochem Ltd., and cesium chloride was purchased from Fisons Ltd.

RESULTS

Conjugal transfer of mucosal adherence. To determine if the capacity of strain O26:K60:H11 (referred to, for simplicity, as O26) to adhere to human fetal intestinal mucosa is a plasmid-specified phenomenon, an attempt was made to transmit the character by conjugal mating to a laboratory strain of *E. coli* K-12 (strain 711, which carries chromosomally located resistance to nalidixic acid). Exponentially growing nutrient broth cultures of strains O26 and 711 were mixed in equal cell numbers and incubated without agitation at 37°C to late stationary phase to maximize the possibility of plasmid transfer. The mating mixture was diluted and plated out for single colonies on nutrient agar containing nalidixic acid to select recipient organisms. No selection was made for donor characters. Individual transconjugant clones were purified and tested for their capacity to adhere to human fetal small intestinal tissue in vitro (23). Of 25 clones tested, four initially gave a positive reaction in the assay system, and one of these which showed a consistent strong positive reaction (Fig. 1) was selected for further study and was designated LG1122. The high level of transmission of the adherence phenomenon empirically observed in the experiment is strongly suggestive of a plasmid-mediated characteristic.

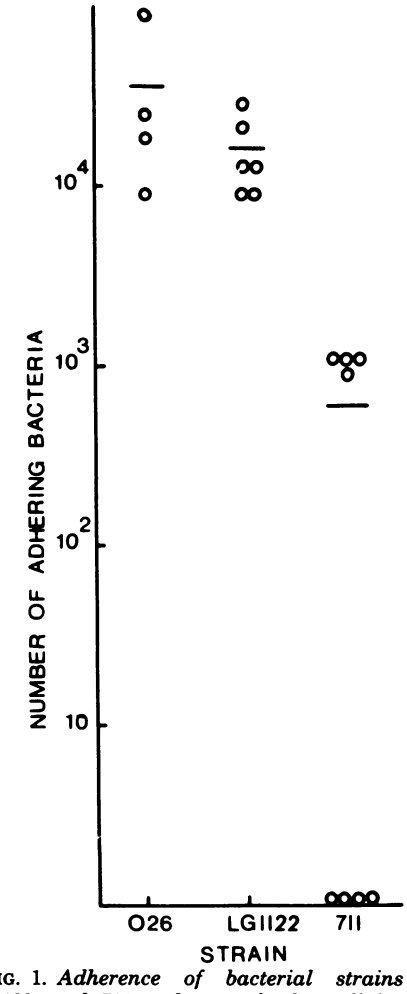

FIG. 1. *Adherence of bacterial strains O26, LG1122, and 711 to human fetal small intestinal mucosa in the presence of D-mannose (23). Each point is the mean of duplicate determinations using tissue from eight fetuses. Variation in number of adhering bacteria for each strain probably reflects differences in tissue maturity.*

Analysis of plasmid DNA. Physical evidence for the involvement of plasmids was obtained by analyzing radioactively labeled covalently closed circular plasmid DNA purified by CsCl equilibrium density gradient centrifugation in the presence of EtBr as described above. The plasmid DNA purified from strain O26 separated into various size classes when it was analyzed by velocity centrifugation in neutral 5 to 20% (wt/wt) sucrose density gradients. Short runs (Fig. 2A) revealed the presence of a fast sedimenting band of plasmid material representing approximately 30% of the radioactivity in super-

coiled material and a slower sedimenting band which separated further into approximately equal peaks when the centrifuge run time was increased (Fig. 2B). Plasmid DNA purified from strain LG1122, the transconjugant isolate which had acquired the capacity for adherence, comprised only material which cosedimented precisely with the faster sedimenting plasmid species of strain O26 in short neutral 5 to 20% sucrose density gradients (Fig. 2A). Preliminary studies (37) indicated that the average molecular weight of the fast sedimenting plasmid band is approximately 56×10^6.

Co-inheritance of adherence with antibiotic resistance, colicinogenicity, and resistance to UV light. The in vitro assay for mucosal adherence (23), although qualitatively reliable, is laborious to perform, somewhat subject to quantitative variation, and not readily adaptable to selection for plasmid-bearing strains. Tests were made, therefore, to detect other selectable characteristics of strain O26, which are plasmid mediated, and which were cotransferred to strain 711 with the adherence property in the initial conjugation experiment.

A qualitative screening of drug resistance spectra of strains O26 and LG1122, using commercially available antibiotic sensitivity disks (as described above) and antibiotic-impregnated filter paper disks made in this laboratory, indicated resistance to tetracycline, streptomycin, sulfonamides, and spectinomycin of these strains as compared with the parental strain 711. MICs of the four antibiotics for various bacterial strains were determined (Table 2) in liquid medium as recommended for medical microbiological establishments (16) and on solid media for their immediate practical use as selective agents in genetics experiments. It is clear that acquisition of the property of adherence by strain LG1122 from the EPEC strain O26 during prolonged conjugal mating leads to simultaneous acquisition of resistance to the antibiotics streptomycin, spectinomycin, tetracycline, and sulfathiazole. The comparable levels of resistance to nalidixic acid, a chromosomal marker, of strains 711 and LG1122 confirm that the latter strain is a derivative of the recipient parental strain 711.

In a routine survey of extracellular protein production to which enteropathogenic bacterial strains are subjected in this laboratory (see above), strain O26 was found not to produce hemolysin or enterotoxin. However, it was found to be colicinogenic, that is, to produce a diffusible trypsin-sensitive product (a colicin) which inhibits the growth of sensitive *E. coli* strains such as NCTC10418. Furthermore, strain LG1122, which had acquired the capacity for

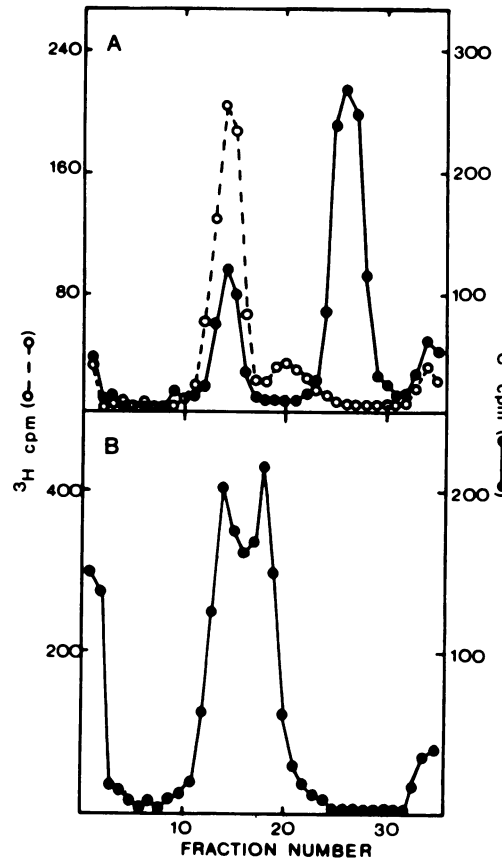

FIG. 2. *Neutral sucrose density gradient analysis of purified plasmid DNA from strains O26 and LG1122. Strain O26 was labeled with [^{14}C]thymidine (●) and strain LG1122 was labeled with [^{3}H]thymidine (○). Plasmid DNA was purified from total Sarkosyl lysates by CsCl-EtBr equilibrium density gradient centrifugation, and analyzed in neutral 5 to 20% (wt/wt) sucrose density gradients. Centrifugation was at 50,000 rpm at 15°C for (A) 40 min or (B) 120 min in a Beckman SW50.1 rotor. Gradients were collected dropwise from the bottom.*

TABLE 2. *MICs of antibiotics for enteropathogenic strain O26 and K-12 strains 711 and LG1122*

Antibiotic	Bacterial strain[a]		
	O26	LG1122	711
Nalidixic acid	4 (8)	64 (256)	64 (256)
Spectinomycin	128 (256)	64 (128)	1 (8)
Streptomycin	256 (32)	128 (16)	2 (1)
Sulfathiazole	>256 (>1,024)	>256 (>1,024)	1 (8)
Tetracycline	4 (32)	4 (16)	0.5 (2)

[a] Bacterial strains were grown in the presence of $\log_2$ series of antibiotic concentrations. MICs are defined as the lowest concentration of antibiotics (micrograms per milliliter) which inhibits bacterial growth. The first numbers in each column show MICs in peptone-water medium. Numbers in parentheses are MICs on nutrient agar (or diagnostic sensitivity test agar for sulfathiazole).

mucosal adherence through conjugation, was also found to produce colicin, unlike its parent strain 711. Identification of the colicin produced was achieved by performing cross-immunity tests with strains O26, LG1122, and a number of well-documented colicinogenic strains (Table 3). Strain O26, in common with other wild-type *E. coli* strains tested in this laboratory (P. H. Williams, unpublished data), was insensitive to all the colicins whose killing effect was tested. Strain LG1122, however, whose parent strain 711 was killed by all the colicins tested, was insensitive to colicins Ia, Ib, and V, a pattern characteristic of a number of colicin-resistant and -tolerant mutants having altered surface receptor proteins (9). It is probable that, in the mating mixture of strains O26 and 711 in which transfer of the adherence phenomenon was first detected, colicin produced by the former strain caused selection of colicin-resistant variants of the 711 strain as recipients. Colicinogenic strains of *E. coli* K-12 were constructed which were insensitive, due to plasmid-mediated immunity, only to the colicin they produced. The colicins produced by strains O26 and LG1122 were found to be identical in their ability to inhibit the growth of ColV and ColIa strains and of strains colicinogenic for group A colicins (10), but not of a ColIb strain.

It has been reported that plasmids which specify colicin Ib protect the bacterial strain that harbors them from the lethal effects of UV radiation (21). To determine if the plasmid-coded colicin Ib production character that was co-inherited with mucosal adherence is linked to UV protection, survival curves were constructed for strains 711 and LG1122 and for strain 711 carrying the well-characterized plasmid ColIb-P9 (8) (Fig. 3). Clearly, the strains which were colicinogenic were also significantly more UV resistant than the corresponding Col^- strain at any particular dose of UV radiation.

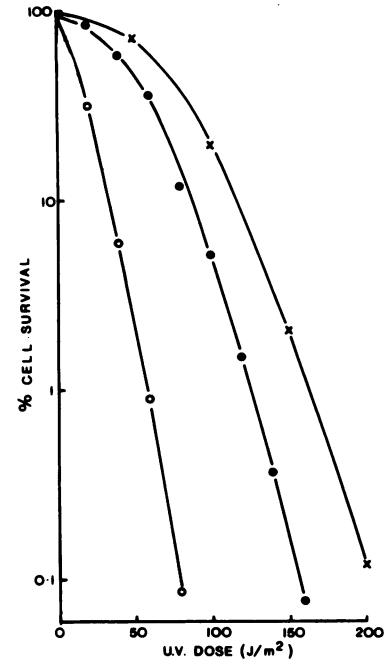

FIG. 3. *UV survival curves demonstrating protection by ColIb plasmids. Survival at a range of UV doses was determined for strains 711 (○), LG1122 (●), and LG1273 (×).*

TABLE 3. *Identification of the colicin produced by strains O26 and LG1122*[a]

Colicin-producing strain	Test strain						
	O26	LG1122	JK16 (ColIa)	LG1245 (ColIb)	LG1244 (ColV)	Col^s (Col^-)[b]	LG1243 (Col^-)
O26	+	+	−	+	−	−	+
LG1122	+	+	−	+	−	−	+
Type A colicins							
LG1117 (ColE1)	+	−	−	−	−	−	−
LG2253 (ColE2)	+	−	−	−	−	−	−
K235 (ColK)	+	−	−	−	−	−	−
B6 (ColB)	+	−	−	−	−	−	−
Type B colicins							
JK16 (ColIa)	+	+	+	−	−	−	+
LG1245 (ColIb)	+	+	−	+	−	−	+
LG1244 (ColV)	+	+	−	−	+	−	+

[a] Cultures of colicin-producing strains were inoculated in a broad streak across nutrient agar plates and grown overnight at 37°C. Bacterial growth was scraped off the streaks, and residual bacteria were killed by exposure to chloroform vapor. The plates were overlaid with soft nutrient agar, and bacterial strains were tested for immunity by cross-streaking over the position of the streak. Plates were incubated overnight at 37°C. Uninterrupted growth of the cross streak (+) indicates colicin immunity or resistance. Absence of growth of the cross streak at the position of the streak of colicin-producing bacteria (−) indicates colicin sensitivity.

[b] Col^S strains tested were W3110, JC411, and 711.

Plasmid curing of strain O26. After growth of strain O26 in the presence of ethidium bromide and nonselective plating for single colonies, individual clones were tested for the loss of their ability to produce colicin. Nonproducers were further tested qualitatively for their antibiotic resistance spectra by using antibiotic sensitivity disks. An isolate which had coordinately lost resistance to streptomycin, tetracycline, spectinomycin, and sulfonamides was purified and found to be precipitated by rabbit anti-O26 antiserum in a standard slide agglutination test. This isolate, designated LG1223, was therefore assumed to have been derived from strain O26 by curing of plasmid species responsible for colicinogenicity and drug resistance. The curing frequency was approximately 5×10^{-4} for these characters. The properties of strain O26 and its cured derivative LG1223 are summarized in Table 4.

Quantitative confirmation of the loss of drug resistance upon curing was obtained by measurements of MICs of the relevant antibiotics for strains O26 and LG1223 and for a standard control strain, NCTC10418. Levels of resistance to tetracycline and spectinomycin in the cured strain were similar to those in the control strain. Levels of resistance to streptomycin and sulfathiazole were lowered in the cured strain as compared with those of the original strain O26, but not to control levels. Concomitant with the loss of colicinogenicity in the cured strain was a loss of UV resistance such that a UV dose giving 80% survival of strain O26 allowed survival of fewer than 0.1% of cells of the cured strain LG1223. It is noticeable by comparison with Fig. 3 that the wild-type strain O26 in its colicinogenic form was more UV sensitive than the K-12 strain 711 in the non-colicinogenic form. The reason for this is not known. Finally, the cured strain LG1223 did not adhere significantly to human fetal small intestinal tissue in vitro.

TABLE 4. *Properties of strain LG1223, a derivative of enteropathogenic strain O26 cured for colicinogenicity and antibiotic resistance*

Property	Bacterial strain		
	O26	LG1223	NCTC10418
MIC (μg/ml)[a]			
Spectinomycin	128 (256)	2 (16)	2 (8)
Streptomycin	256 (32)	16 (4)	4 (2)
Sulfathiazole	256 (>1,024)	4 (64)	1 (8)
Tetracycline	4 (32)	0.5 (2)	0.5 (2)
Colicin production	+	–	
UV resistance (% survival at 20 J/m^2)	80	0.08	
Adherence	+	–	

[a] MICs were determined as described in footnote *a*, Table 2.

Physical evidence for the loss of plasmid material from strain LG1223 correlating with a loss of genetic characters is shown in Fig. 4. Covalently closed circular DNA could be purified from the strain, but analysis in neutral sucrose density gradients indicated that this cosedimented only with the slower sedimenting species observed in strain O26. Plasmid material with an average molecular weight of 56×10^6 was not present in the cured strain.

Segregation of plasmid characters during conjugal mating. The initial conjugation experiment designed to detect the transfer of mucosal adherence from strain O26 to 711 utilized an extended mating time to maximize the possibility of transfer. On the basis of physical and genetic characterization of the transconjugant LG1122 from this cross, we previously reported (24, 38) a plasmid, designated pLG101, which mediates mucosal adherence, colicin Ib production and immunity, and resistance to the antibiotics tetracycline, streptomycin, spectinomycin, and sulfonamides. However, in conjugation experiments conducted over shorter mating times, segregation of markers among transconjugants was observed, suggesting that what was previously designated pLG101 is in fact two plasmids of similar molecular size.

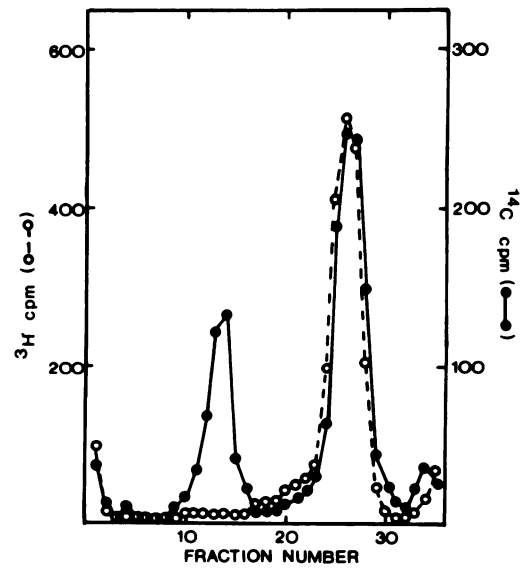

FIG. 4. *Neutral sucrose density gradient analysis of purified plasmid DNA from strain O26 and a cured derivative LG1223. Strain O26 was labeled with [^{14}C]thymidine (●) and strain LG1223 was labeled with [^{3}H]thymidine (○). Plasmid purification and centrifugation conditions were as described in the text and Fig. 2; the run time was 40 min.*

Strain LG1122 was transformed (36) with purified DNA of the nonconjugative plasmid pLG1, a derivative of plasmid ColE1 having a fragment carrying ampicillin resistance inserted at the single *Eco*RI restriction site (R. Diaz, personal communication). The transformant was designated LG1272. Exponentially growing nutrient broth cultures of this strain and of strain GB20, a derivative of the streptomycin-resistant strain AB1157 made resistant also to colicin Ib, were mixed in equal cell numbers and incubated without aeration at 37°C for 16 h. Samples of mating mixture were diluted and plated out on nutrient agar containing streptomycin and either tetracycline or ampicillin. Transconjugants were tested for unselected markers. It is clear from the results (Table 5) that drug resistance segregated from colicinogenicity and UV protection in the majority of the transconjugants when selection was made either for the multiple drug resistance characteristic of strain LG1122 or for the mobilized nonconjugative plasmid. The low incidence of transconjugants from short-term mating experiments which showed no segregation of markers suggests that the cotransfer of all markers in the initial conjugation experiment was due to continued plasmid transfer during the extended mating time.

Properties of segregant transconjugant clones. Two ampicillin-sensitive transconjugant clones were chosen for further characterization (Table 6). Strain LG1247 resembles the parental strain LG1122 in being colicinogenic and UV resistant and in having the capacity to adhere to human fetal intestinal tissue. This strain, however, was sensitive to the antibiotics spectinomycin, sulfonamides, and tetracycline for which tests could be conducted (the recipient strain GB20 is resistant to high levels of streptomycin because of a chromosomal marker). On the other hand, strain LG1249 was resistant to these antibiotics, but was non-colicinogenic, did not show enhanced UV protection compared with parental strain GB20, and did not adhere significantly in the mucosal adherence assay. We have therefore reassigned the notation pLG101 to the plasmid which mediates mucosal adherence, colicinogenicity, and UV protection and which is carried by strain LG1247. The drug resistance plasmid of strain LG1249 is designated pLG102.

TABLE 5. *Segregation of antibiotic resistance and colicinogenicity on conjugal mating of strains LG1272 and GB20*[a]

Genotypes of transconjugants		Selected donor marker	
Antibiotic resistance	Colicin Ib production	Tc^r	Ap^r
r	+	1	1
r	−	47	4
s	+	−	41
s	−	−	2

[a] Mating was performed as described in the text. A total of 48 transconjugants from each selective plate were tested for colicin production and UV resistance and for resistance to tetracycline (if not selected), spectinomycin, and sulfonamide. Plasmid-mediated resistance to streptomycin was masked by high-level chromosomal streptomycin resistance in the recipient strain. Transfer frequency of Tc^r was 10^{-4}, and transfer frequency of Ap^r was 10^{-5} donor bacteria in the mating mixture.

TABLE 6. *Properties of strain LG1122 and two of its conjugal derivatives LG1247 and LG1249*

Property[a]	Bacterial strain		
	LG1122	LG1247	LG1249
Antibiotic resistance			
Spectinomycin	r	s	r
Sulfonamides	r	s	r
Tetracycline	r	s	r
Colicin production	+	+	−
UV resistance	r	r	s
Adherence	+	+	−

[a] Antibiotic resistance spectra were determined qualitatively with Multodisks. UV resistance was determined qualitatively by plate irradiation of bacterial streaks.

Physical confirmation of the genetic evidence for plasmid segregation is shown in Fig. 5. Neutral 5 to 20% sucrose gradients were run for a longer time than those shown in Fig. 2 to attempt resolution of presumptive components of the fast sedimenting plasmid material of strain LG1122. However, no clear resolution was observed (Fig. 5A), although slight skewing of the peak indicated that it might represent more than one component. It can be seen quite clearly, however, that covalently closed circular pLG101 DNA purified from strain LG1247 did not cosediment precisely with plasmid DNA from strain LG1122 under these conditions but formed a peak corresponding to the leading side of this material. Figure 2B shows that differentially labeled pLG101 and pLG102 were clearly distinguishable on the basis of sedimentation velocity even though as a mixture from strain LG1122 they were not. From preliminary estimates, we calculate the molecular weight of pLG101 at about 60×10^6. Experiments are in progress in this laboratory to determine accurately the molecular weights of plasmids pLG101 and pLG102 and to determine their homology with other plasmids of similar or analogous functions.

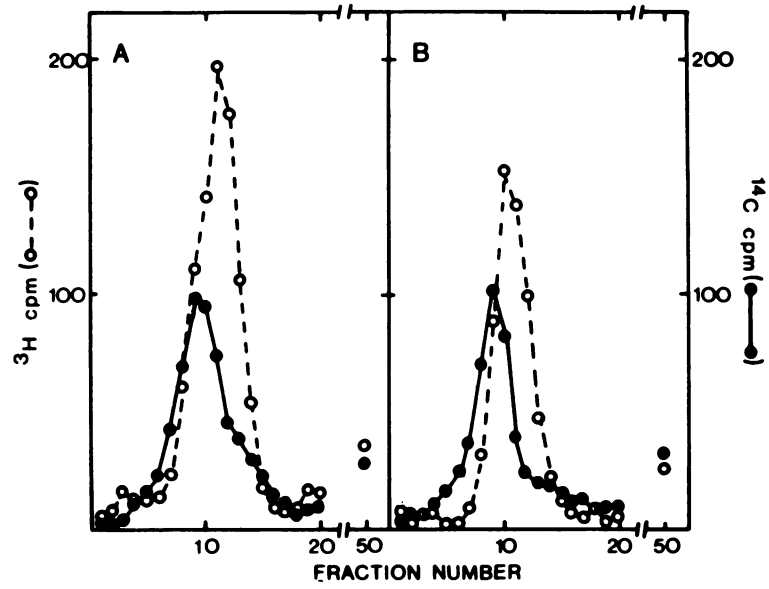

FIG. 5. *Neutral sucrose density gradient analysis of purified plasmid DNA from strains LG1122, LG1247, and LG1249. Strain LG1247 was labeled with [^{14}C]thymidine (●), and strains LG1122 and LG1249 were labeled with [^{3}H]thymidine (○). Plasmid purification was as described in the test. Centrifugation of ^{14}C-labeled LG1247 plasmid DNA with (A) ^{3}H-labeled LG1122 plasmid DNA and (B) ^{3}H-labeled LG1249 plasmid DNA was as described for Fig. 2, except that the run time was 50 min, and smaller fractions were collected.*

Antigenic properties of strains carrying pLG101 and pLG102. A human EPEC strain designated H-10407 has recently been shown to colonize the intestine of neonatal rabbits (13, 14). Spontaneous loss of a plasmid from this strain (the derivative being H-10407-P) resulted in loss of colonizing ability and concomitant absence of a surface antigen termed colonization factor antigen (CFA) (13, 14). We have tested monospecific anti-CFA antiserum prepared and kindly donated by C. Smyth and T. Wadström against strains O26, LG1122, LG1247, and LG1249. Growth from fresh overnight nutrient agar stocks of bacterial strains (conditions identical with those used in the human fetal mucosal adherence assay [23]) was suspended in saline on a glass slide and tested with anti-CFA antiserum. Positive agglutination of strain H-10407 was observed, but not of strain H-10407-P under these conditions. Furthermore, no agglutination was observed of any of the strains carrying pLG101 and pLG102.

DISCUSSION

The ability of an enteropathogenic bacterial strain to adhere to the gut wall of its victim is an important aspect of the establishment of infection and the induction of clinical diarrheal disease. In *E. coli* pathogenesis of pigs and cattle, plasmid-mediated surface antigens (K88 and K99) have been implicated in the promotion of bacterial adherence and colonization of the gut (25, 33). CFA has been purified from a strain (H-10407) presumed on epidemiological grounds to be human EPEC (15). CFA promoted colonization of the small intestine of neonatal rabbits and was not produced by a derivative of strain H-10407 from which a 60 × 10^6-dalton plasmid was spontaneously lost (13, 14). Another human *E. coli* isolate with the capacity of mucosal adherence, strain O26, has been reported (23). The strain was originally isolated in 1967 from a baby with diarrhea (31) and was selected for study in this laboratory specifically because of its ability to adhere to human fetal intestinal tissue (23). Antiserum raised in rabbits to strain H-10407 does not react with strain O26 in a standard slide agglutination test, suggesting that the molecular basis of adherence in these two strains may be different. In this paper we demonstrate unequivocally that mucosal adherence in strain O26 is a plasmid-mediated character by several lines of evidence: conjugal transfer, loss by curing, physical analysis of plasmid DNA, and genetic linkage to other characters.

Strain O26 is able to transfer the capacity for mucosal adherence by conjugal mating to a laboratory strain of *E. coli* K-12. Transfer was detected without the application of selection, a plasmid-bearing recipient being found upon testing a few individual transconjugant clones of the K-12 strain for their reaction in the in vitro

adherence assay (23). The high incidence of transfer is indicative of a plasmid-determined character. Furthermore, it was found that it is possible to induce the loss of the adherence property from strain O26 by treatment with ethidium bromide, a standard plasmid curing agent (4). The frequency of loss was significantly higher than could be expected for mutational loss of the character.

Physical analysis of radioactively labeled plasmid DNA indicates the presence of several plasmids in the enteropathogenic strain O26. A peak of fast sedimenting plasmid material, originally designated pLG101 (24, 38), is identical in its sedimentation properties in neutral sucrose velocity gradients to plasmid species present in the conjugal recipient LG1122 which had acquired the capacity to adhere, and is absent from the cured derivative LG1223 of EPEC strain O26 which has lost the capacity to adhere to human intestinal mucosa. This material has an average molecular weight of about 56×10^6 (37). We have not yet firmly assigned functions to smaller plasmid species present in strains O26 and LG1223, but their absence from strain LG1122 implies that they are not involved in adherence.

Further evidence for the plasmid nature of the adherence determinant, and an invaluable practical aid in the genetic analysis of plasmid-carrying strains, was the identification of a number of phenotypic traits of strain O26 that were coordinately transferred in prolonged conjugation and lost upon curing with the adherence property. The characters in question are resistant to the antibiotics tetracycline, streptomycin, spectinomycin, and sulfonamides, and the production of colicin Ib. This confirms and extends the observations of Smith and Linggood (31) who also suggested that drug resistance and colicinogenicity were carried on separate plasmids. It was our analysis of the segregation of these markers in conjugation experiments of limited duration that revealed that the peak of plasmid material with an approximate molecular weight of 56×10^6 comprises two plasmids, a colicinogenic plasmid which also carries the determinant of mucosal adherence (the newly designated pLG101) and a drug resistance plasmid (pLG102). The former is preliminarily estimated to be about 60×10^6 daltons, approximately the same size as ColIb-P9 (8) and as the plasmid which has been implicated in the promotion of colonization by strain H-10407 of the intestine of neonatal rabbits (14). It should be noted, however, that strain H-10407 is not colicinogenic (P. H. Williams, unpublished data).

A study of the MICs of spectinomycin and tetracycline suggests that the level of resistance of strain O26 to these antibiotics is wholly determined by plasmid pLG102 since strain LG1122 is approximately equally resistant to them, whereas the cured strain LG1223 is as sensitive as control strains. In the case of streptomycin, however, the resistance levels of strains O26 and LG1122 are sufficiently different that it can be postulated that a fraction of the resistance of the former is determined by plasmid species not transferred in the original conjugal mating. Furthermore, the levels of resistance to streptomycin and sulfathiazole are markedly reduced in cured strain LG1223 as compared to strain O26, but not to control levels, again indicating that a portion of the resistance of strain O26 to these drugs lies elsewhere than with pLG101 and pLG102. Possibly the small plasmid species in strain O26, which remain after curing in strain LG1223, mediate resistance to streptomycin and sulfathiazole.

Colicinogenicity has previously been shown to be common among pathogenic strains of *E. coli* (3). ColV plasmids are implicated in the virulence of invasive strains, but it is not known whether colicin V itself or some other plasmid-coded property is responsible for the selective advantage of $ColV^+$ over $ColV^-$ bacteria in the case of invasion (29, 30). In pLG101 we observe genetic linkage between colicin Ib production and the adherence phenomenon in an enteropathogenic *E. coli* strain. Experimental infections with the appropriate plasmid mutants would indicate whether colicin Ib itself actually enhances virulence in this situation, but it is possible that colicinogenicity confers an advantage in colonization of the gut by inhibiting the growth of competing bacterial strains. The fact that we find that many wild strains of *E. coli* are naturally resistant to several colicins in laboratory tests (P. H. Williams, unpublished data) does not preclude the possibility that colicins have an inhibitory effect on these strains in the natural environment. The significance in nature of the UV protective effect of ColIb plasmids (21), including pLG101, is not clear.

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