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Self-Transmissible R Plasmids Encoding CS31A among Human *Escherichia coli* Strains Isolated from Diarrheal Stools

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The CS31A antigen was first described for septicemic and enterotoxigenic bovine *E. coli* strains. In our study, of 597 human *Escherichia coli* strains isolated from diarrheagenic stools of hospitalized patients, 30 (5%) hybridized with the CS31A DNA probe. These CS31A-positive *E. coli* strains diffusely adhered to Caco-2 and/or HEp-2 cells and produced a major surface protein of either 30 or 30.5 kDa according to the strain. These proteins were antigenically related to the two forms of the CS31A antigen, namely, CS31A-L and CS31A-H. Genes encoding CS31A were located on 140-kb conjugative R plasmids. *E. coli* transconjugants expressed major surface proteins similar to those of the wild-type strains and adhered to Caco-2 and/or HEp-2 cells. An association of CS31A and another adhesive factor of the Dr family was found in 70% of wild-type strains, since 21 strains hybridized with the diffuse adhesion DNA probe corresponding to the accessory gene (*daaC*) of the F1845 adhesin. Comparison of the restriction patterns of the 140-kb R plasmids of the CS31A-positive *E. coli* strains showed these plasmids to be similar. Hybridization experiments indicated that the genes encoding CS31A and resistance to penicillin were located together on either of two 20- or 27-kb *Eco*RI restriction fragments in four *E. coli* strains. We reported a similar linkage between these genes in *Klebsiella pneumoniae* strains which produced CF29K, a CS31A-like antigen. These results suggest a horizontal transfer between *E. coli* and *K. pneumoniae* strains.

The ability to adhere to eucaryotic epithelial cells through bacterial adhesins is essential in the pathogenesis of diarrheal diseases and urinary tract infections. Human diarrheagenic *Escherichia coli* strains are classified in six pathogenic groups on the basis of their interactions with human enterocytes or cultured epithelial cells or both. The adhesins involved are group specific. Moreover, *E. coli* strains which belong to the pathogenic group harbor adhesins specific to particular animal species, e.g., K88, and colonization factor antigens are characteristic of porcine and human enterotoxigenic *E. coli* strains, respectively (16, 32).

In a recent study (23), we showed that 20% of *E. coli* strains isolated from patients' diarrheal stools belong to the diffusely adhering *E. coli* (DAEC) group. Diffuse adhesion is the ability of bacteria to bind to the entire available surface on a eucaryotic cell. Two different adhesins have been identified so far among the DAEC strains: a plasmid-encoded afimbrial adhesin, termed AIDA-I (2), and a chromosomally encoded fimbrial adhesin, termed F1845 (3). Both are involved in diffuse adhesion to HEp-2 cells, and the F1845 adhesin also mediates adhesion to Caco-2 cells (26). Two DNA probes have been described: a 450-bp fragment specific for AIDA, and a 390-bp fragment corresponding to the *daaC* gene of the F1845 operon. However, the latter detects not only the F1845 operon but also the operons encoding the Dr family adhesins. These adhesins, which include the F1845 adhesin of diarrheagenic *E. coli*, the afimbrial adhesins AFA-I and AFA-III, and the Dr hemagglutinin of uropathogenic *E. coli* strains, share numerous homologies in their genetic determinant and particularly in their accessory genes. Recently, a third adhesin, termed CF16K, was found in 9.8% of DAEC strains isolated in France (22). It is a major surface protein of 16 kDa, which was shown to be involved in diffuse adhesion to HEp-2 and Caco-2 cell lines. Finally, we showed that some DAEC strains produced a major surface protein identified as CS31A. CS31A (for coli surface-associated antigen) was first observed in septicemic and enterotoxigenic bovine *E. coli* strains and described as a 29-kDa K88-related protein organized in very fine organelles forming a wide capsule-like structure around bacterial cells (19). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis shows two different electrophoretic patterns for CS31A subunits referred to as CS31A-H (heavy), with an apparent molecular mass of 29.5 kDa, and CS31A-L (light), with an apparent molecular mass of 29 kDa (7). These two forms differ only by one amino acid in position 89 of the mature protein: asparagine for CS31A-H and lysine for CS31A-L (18). The genes encoding CS31A are located on high-molecular-weight self-transmissible R plasmids. A CS31A-related protein termed CF29K has been described in *Klebsiella pneumoniae* strains involved in nosocomial infections. The genes encoding this protein were also located on high-molecular-weight self-transmissible R plasmids (13).

In this study, we looked for CS31A-positive strains among *E. coli* strains isolated from sporadic diarrheal stools of patients hospitalized in the Centre Hospitalier Régional Universitaire of Clermont-Ferrand, France, over a 3-year period. Our aim was to determine the adhesive properties of CS31A-positive strains and to characterize the adhesive factors associated with CS31A. The self-transmissible R plasmids encoding CS31A were analyzed and compared to determine whether a single epidemic plasmid was involved.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Five hundred ninety-seven *E. coli* strains were obtained from infants, chil-

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TABLE 1. Sources and relevant characteristics of DNA probes used in this study

DNA probe specificity	*E. coli* positive control	Plasmid	Restriction endonuclease fragment and probe size	Reference
CS31A adhesin	DH5α/pAG315	pSP15a	950-bp *Hin*dIII-*Eco*RI	18a
AIDA-I adhesin	K-12 C600/pIB6	pIB6	450-bp *Eco*RI	2
F1845 (*daaC* gene)	C1845	pSLM852	390-bp *Pst*I	3
TEM	K-12 C600/pBR322	pBR322	560-bp *Pst*I-*Ssp*I	20
Aerobactin	HB101/pABN1	pABN1	4.8-kb *Hin*dIII-*Bgl*II	4
Aerobactin receptor	HB101/pABN1	pABN1	2.3-kb *Pvu*II	4

dren, and adults whose diarrheagenic stools contained no other recognized bacterial, viral, or parasitic pathogens. The strains were examined in previous studies by colony hybridization assays with DNA probes specific for enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroaggregative *E. coli* (22, 23).

The *K. pneumoniae* CF504 strain was isolated from blood samples from a patient hospitalized in an intensive care unit in a hospital of Clermont-Ferrand, France (13). *E. coli* Orne 6 and Orne 8 are bovine septicemic strains producing CS31A-H and CS31A-L, respectively (19). The strains used as positive controls in colony hybridization assays are listed in Table 1. *E. coli* K-12 C600 was used as the negative control in colony hybridization assay.

Depending on the experiments, strains were grown either in Luria-Bertani broth or in Mueller-Hinton media (broth or agar) (Institut Pasteur Production, Marnes-la-Coquette, France) at 37°C for 18 to 24 h.

Antibiotics. Antimicrobial resistance was determined by disk susceptibility. Disks for agar diffusion tests were purchased from Diagnostics Pasteur (Marnes-la-Coquette, France).

Adhesion to HEp-2 and Caco-2 cell lines. HEp-2 cells, derived from a human larynx carcinoma, were obtained from Flow Laboratories Inc. (McLean, Va.). Caco-2 cells, derived from a human colon carcinoma (17), were kindly provided by A. Zweibaum (Institut National de la Santé et de la Recherche Médicale, Villejuif, France). Monolayers of HEp-2 or Caco-2 cells were grown at 37°C in modified eagle medium or Dulbecco modified eagle medium (Flow Laboratories, Les Ulis, France), respectively, containing 10 or 20% (vol/vol) fetal bovine serum, 1% nonessential amino acids, 200 U of penicillin per liter, and 50 mg of streptomycin per liter in 24-well Falcon tissue culture plates (Becton Dickinson Labware, Oxnard, Calif.). The adhesion of *E. coli* to HEp-2 cells was performed by a modification of the HEp-2 cell assay of Cravioto et al. (10), and the adhesion to Caco-2 cells was performed as described previously (12). Briefly, monolayers of HEp-2 cells were grown to 50% confluence, whereas Caco-2 cells were used at postconfluence after 15 days of culture. Before the adhesion test, cells were washed once with phosphate-buffered saline (PBS; pH 7.2). A suspension of about 10^8 bacteria per ml in the cell line culture medium containing 2% (wt/vol) D-mannose was added to the tissue culture and incubated for 3 h at 37°C. After three washes with PBS, the cells were fixed in methanol, stained with 20% Giemsa stain, and examined microscopically under oil immersion. An adhesion index representing the average number of bacteria per cell was determined by examining 100 cells.

Transfer of R plasmids. Mating experiments were carried out in broth, as described by Lesage et al. (29). The 140-kb plasmid from wild-type *E. coli* strains was transferred to nalidixic acid-resistant *E. coli* K-12 C600. Transconjugants were selected on Mueller-Hinton agar containing nalidixic acid (150 mg/liter) and either ampicillin (20 mg/liter), tetracyclines (12.5 mg/liter), streptomycin (12.5 mg/liter) or kanamycin (20 mg/liter).

Extraction of bacterial surface proteins. The extraction of bacterial surface proteins was performed as previously described (30). Bacterial cultures grown overnight on Mueller-Hinton agar (two plates of 15 by 15 cm) were harvested in PBS (pH 7.2). The bacterial surface proteins were separated from bacterial cells by heating the suspension at 60°C for 20 min with gentle agitation. After centrifugation at 12,000 × *g* for 10 min, the resulting supernatant was analyzed by electrophoresis.

Electrophoretic study. Bacterial surface proteins were analyzed by SDS-PAGE as described by Laemmli (28), with vertical slab gels (Bio-Rad Laboratories apparatus). Proteins were stacked with 4.5% (wt/vol) acrylamide and separated with 10 or 15% (wt/vol) acrylamide. Electrophoresis was performed in a solution of 0.025 M Tris (Sigma Chemical Co., St. Louis, Mo.), 0.28 M glycine (Prolabo, Paris, France), and 0.1% (wt/vol) SDS (pH 8.6; Sigma Chemical Co.). Samples were denatured in 1.5% (wt/vol) SDS–1.5% (vol/vol) β-mercaptoethanol in 0.50 M Tris hydrochloride buffer (pH 6.8) for 5 min at 100°C, just before being loaded onto the gel. The molecular mass standards were phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and β-lactalbumin (14.4 kDa) (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Immunoblotting. The antiserum CS31A raised against the native H and L forms of CS31A antigen was obtained as previously described (19). Antiserum raised against purified CS31A antigen from the wild-type *E. coli* Orne 6 strain reacted against H and L forms of CS31A. This antiserum, which was absorbed twice by CS31A-L antigen, consisted of a specific anti-CS31A-H serum which reacted only against the native CS31A-H antigen.

The method for obtaining the antiserum CF16K raised against the denaturated CF16K protein has been described elsewhere (22).

Western blotting (immunoblotting) was performed by the technique of Towbin et al. (35) with minor modifications. Bacterial surface proteins were first subjected in duplicate to electrophoresis in the presence of SDS (28). Part of the gel was stained with Coomassie blue, and the rest was electroblotted onto nitrocellulose paper (Schleicher & Schuell, Inc., Dassel, Germany). A dilution of CS31A antiserum (dilution of 1:500) was applied to the nitrocellulose filter, which was incubated for 1 h at room temperature. After a thorough washing, the filter was incubated for 1 h at room temperature with peroxidase-labeled goat anti-rabbit immunoglobulin G (Nordic Immunological Laboratories, Tilburg, The Netherlands) at a dilution of 1:2,000. The blot was washed and soaked in a solution composed of 0.6 mg of 4-chloro-1-naphthol (Sigma) per ml, 0.1% H_2O_2, and 16.5% methanol in 10 mM Tris–0.9% NaCl (pH 7.5).

Colony immunoblotting was performed as follows. Bacterial strains were grown overnight at 37°C in Mueller-Hinton broth.

TABLE 2. Characteristics of diarrheagenic *E. coli* strains producing CS31A

Strain	Antibiotic resistance[a]	CS31A expression[b]	Hybridization with DA probe[c]	Adhesion to cells[d]	
				Caco-2	HEp-2
CF147	AM, TIC, S, SPT, K, N, C, TE, SSS	H	+	+	+
CF1172	AM, TIC, S, SPT, TE, SSS	L	+	+	+
CF192	AM, TIC, S, K, N, C, SSS	L	−	+	+
CF224	AM, TIC, S, SPT, K, N, C, TE, SSS	H	+	+	+
CF228	AM, TIC, S, SPT, C, TE, SSS	H	−	+	+
CF246	AM, TIC, CF, S, SPT, K, N, C, TE, SSS	H	+	+	+
CF247	AM, TIC, S, K, N, TM, DKB, GM, SIS, C, TE, SSS	H	+	+	+
CF258	S, SPT, TE, SSS	L	−	+	+
CF285	AM, TIC, S, SPT, K, N, TM, DKB, GM, SIS, C, TE, TMP, SSS	L	+	+	+
CF290	AM, TIC, S, SPT, K, N, TM, DKB, GM, SIS, C, TE, TMP, SSS	L	+	+	+
CF294	AM, TIC, S, K, N, TE, SSS	L	+	+	+
CF371	AM, TIC, S, TE, SSS	L	−	+	+
CF375	AM, TIC, S, TE, SSS	L	+	+	+
CF454	AM, TIC, S, K, N, TE, SSS	L	+	+	+
CF458	AM, TIC, S, K, N, TE, SSS	L	+	+	+
CF474	S, SPT, C, TE, SSS	L	+	+	+
CF475	AM, TIC, S, SPT, K, N, C, TE, TMP, SSS	L	+	+	+
CF519	AM, TIC, S, SPT, K, N, C, TE, SSS	H	+	+	+
CF576	S, K, N, TE, SSS	L	−	+	−
CF585	AM, TIC, CF, S, SPT, K, N, C, TE, SSS	L	+	+	+
CF603	AM, TIC, S, K, N, C, TE, SSS	H	−	+	+
CF6261	AM, TIC, S, SPT, K, N, C, TE, TMP, SSS	L	+	+	+
CF6263	AM, TIC, S, SPT, TE, SSS	L	−	+/−	−
CF644	AM, TIC, S, SPT, C, TE, SSS	L	+	+	+
CF685	AM, TIC, S, K, N, C, TE, SSS	L	−	+	+
CF688	AM, TIC, S, SPT, C, TE, SSS	L	+	+	+
CF714	AM, TIC, S, SPT, K, N, C, TE, SSS	L	−	+	+
CF720	AM, CF, S, K, N, C, TE, TMP, SSS	H	+	+	+
CF739	AM, TIC, S, SSS	L	+	+	+
CF748	AM, TIC, S, SPT, C, TE, SSS	H	+	+	+

[a] Abbreviations: AM, ampicillin; TIC, ticarcillin; S, streptomycin; SPT, spectinomycin; K, kanamycin; N, neomycin; C, chloramphenicol; TE, tetracycline; SSS, sulfonamides; CF, cephalothin; TM, tobramycin; DKB, dibekacin; GM, gentamicin; SIS, sisomicin; TMP, trimethoprim.
[b] Two forms are described: H, heavy CS31A-H, and L, light CS31A-L.
[c] Refer to Table 1 for a description. DA, diffuse adhesion.
[d] Adhesion to Caco-2 and to HEp-2 cells: +, adhesion index superior to 1; −, adhesion index inferior to 0.5.

Samples (1 μl) of each broth were plated on Mueller-Hinton agar and incubated for 18 h at 37°C. Colonies were transferred to a nitrocellulose membrane. The membrane was dried and blocked overnight at room temperature with Tris-buffered saline (10 mM Tris–0.9% NaCl, pH 7.5) and 2% (wt/vol) bovine serum albumin (Sigma). Immunoblotting was performed as described above, with anti-CS31A (H and L) and with the specific anti-CS31A-H antisera.

Preparation of plasmid DNA. Plasmid DNA extractions of clinical isolates and transconjugants were performed by either the method of Kado and Liu (24) or that of Birnboim and Doly (5). The sizes of the plasmids were estimated by comparing their electrophoretic mobilities with those of known reference plasmids.

Restriction endonuclease analysis. Plasmid DNA was digested with restriction endonucleases according to the manufacturer's instructions (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). After digestion, the samples were run into horizontal 1% (wt/vol) agarose gels.

DNA probes and hybridization procedures. The specific DNA probes and fragments extracted from recombinant plasmids used in this study are listed in Table 1.

For colony blots, bacteria were grown overnight at 37°C in Mueller-Hinton broth. Five microliters of the culture was spotted onto Hybond nylon membranes (Amersham). After incubation for 4 h at 37°C, colony prints were lysed as described by Mass (31), air dried, and exposed for 5 min to UV light. For Southern blot experiments, restriction DNA fragments from agarose gels were denatured and transferred to Hybond N^+ nylon membrane by the method of Southern (34).

Colony and Southern blot hybridizations were performed with 50% formamide and at 42°C. The heat-denatured probes were used at a concentration of 10^6 dpm/ml overnight with gentle agitation. After washings with twice-concentrated SSC (15% NaCl plus 15 mM sodium citrate), the filters were exposed to X-Omat-AR X-ray film (Kodak, Rochester, N.Y.) for 12 h at −80°C. The film was developed according to the manufacturer's instructions.

RESULTS

Occurrence and characterization of strains hybridizing with the CS31A DNA probe. The occurrence of CS31A among diarrheagenic *E. coli* strains was determined by colony blot assays with the 0.95-kb *Hin*dIII-*Eco*RI DNA fragment corresponding to the structural gene of the CS31A antigen. Of 597 *E. coli* strains isolated from diarrheal stools, 30 (5%) hybridized with this probe: 8 strains isolated from infants, 6 from children, and 16 from adults. No seasonal association was reported for these isolates.

The 30 strains were tested to determine their abilities to adhere to Caco-2 and to HEp-2 cells, the presence of adhesive factors, and their antibiotic resistance patterns (Table 2). The 30 strains adhered to Caco-2 and HEp-2 cells diffusely, except

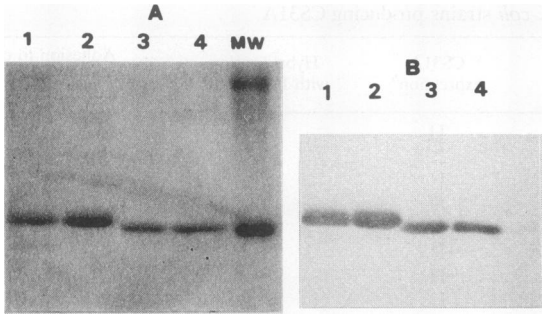

FIG. 1. SDS-PAGE (A) and Western blot (B) of crude bacterial surface components from *E. coli*. Lanes: 1, CF603; 2, Orne 6 (CS31A-H); 3, Orne 8 (CS31A-L); 4, CF1172. MW, molecular size standards (from the top, 43,000- and 30,000-Da bands). Antiserum against CS31A was used at a dilution of 1:500.

for two strains (CF576 and CF6263), which did not adhere to HEp-2 cells. The strains were then tested for the presence of the previously described adhesive factors involved in diffuse adhesion. Twenty-one of the thirty *E. coli* strains (70%) hybridized with a 390-bp *Pst*I fragment, corresponding to the *daaC* accessory gene of the F1845 adhesin operon, used as a diffuse adhesion DNA probe. None of the strains hybridized with the 450-bp *Eco*RI fragment used as a AIDA DNA probe. CF16K, an adhesin of human diarrheagenic DAEC, was sought by immunoblot experiments using the antiserum raised against the CF16K protein. None of the 30 strains produced CF16K.

The antibiotic resistance pattern of each strain is listed in Table 2. Most of the strains were resistant to more than five different antibiotics. All the *E. coli* strains were resistant to streptomycin and sulfonamides. Most were resistant to ampicillin and ticarcillin (27 of 30 strains) and to tetracyclines (28 of 30 strains).

Analysis of bacterial surface components. The bacterial surface components of the 30 *E. coli* strains hybridizing with the CS31A DNA probe were extracted by the method described by Martin et al. (30) and fractionated by SDS-PAGE. All these strains harbored a major surface protein with an apparent molecular mass of about 29 kDa revealed in a 15% polyacrylamide gel. In a 10% polyacrylamide gel, two different proteins were identified on the basis of their molecular mass. Nine strains harbored a major protein with an apparent molecular mass of 30.5 kDa similar to that of the reference strain Orne 6 producing CS31A-H (19), and 21 strains presented a major surface protein with an apparent molecular mass of 30 kDa similar to that of the reference strain Orne 8 producing CS31A-L (Fig. 1A). Western blot experiments showed that the antiserum raised against the native form of CS31A reacted with both the 30- and the 30.5-kDa proteins (Fig. 1B). Colony blot experiments revealed that the nine strains harboring the 30.5-kDa protein reacted with the specific antiserum anti-CS31A-H. Thus, 9 strains harbored CS31A-H and 21 strains harbored CS31A-L.

Transfer of R plasmids and analysis of the *E. coli* transconjugants. Plasmid content analysis of the 30 wild-type *E. coli* strains revealed the presence of at least one 140-kb plasmid (data not shown). Mating experiments of the 30 CS31A-positive *E. coli* strains with the *E. coli* K-12 C600 recipient strain were conducted. After selection on Mueller-Hinton agar supplemented with either ampicillin, tetracycline, streptomycin, or kanamycin (according to the strain), we obtained transconjugants for only 12 strains. As shown in Table 3, six transconjugants harbored exactly the same antibiotic resistance pattern as the donor strains (strains CF1172, CF258, CF454, CF475, CF576, and CF6263). Some antibiotic markers were not transferable for strains CF228, CF285, CF475, CF585, CF603, CF6261, and CF644. Two different transconjugants (CF475T1 and CF475T2) were obtained from *E. coli* CF475. The transconjugant CF475T2 showed the same antibiotic resistance pattern as the wild-type strain, and the transconjugant CF475T1 lacked resistance to chloramphenicol and tetracycline.

All 12 wild-type strains harbored a high-molecular-weight plasmid (around 140 kb), as found by electrophoresis of plasmid DNA extracted by the Kado-and-Liu procedure (Table 3). Four strains presented a second plasmid, of approximately 75 kb for strain CF644, 35 kb for strain CF576, 20 kb for strain CF6261, and 5 kb for strain CF1172. Only one strain (CF6263) harbored three extra plasmids, of about 55, 33, and 16 kb. All the transconjugants harbored the 140-kb plasmid. Two contained a second plasmid of 16 kb (transconjugant CF6263T) or 5 kb (transconjugant CF1172T). Comparison of antibiotic resistance patterns in *E. coli* transconjugants and the respective wild-type strains suggested the presence of a second plasmid of approximately 140 kb in the wild-type *E. coli* strains CF228, CF285, CF475, CF585, CF603, and CF6261. This hypothesis was confirmed for strain CF475, for which we obtained two transconjugants harboring a 140-kb plasmid but with different antibiotic resistance phenotypes.

Comparative abilities of wild-type *E. coli* strains and their transconjugants to adhere to Caco-2 and HEp-2 cells. The 12 *E. coli* strains and their corresponding transconjugants were compared for their abilities to adhere to both Caco-2 and HEp-2 cells (Table 3).

In the Caco-2 cell adhesion assays, all the *E. coli* strains adhered, even in the presence of 2% D-mannose, which prevents type 1 pili-mediated adhesion. The adhesion indices ranged from 1.2 to 3.3 bacteria per cell; one *E. coli* strain (CF6263) adhered poorly to Caco-2 cells (adhesion index of 0.5). All the *E. coli* transconjugants also adhered to Caco-2 cells, with adhesion indices close to those of the donor *E. coli* strains. *E. coli* K-12 C600, used as the recipient strain in mating experiments, did not adhere (adhesion index of 0.1).

In the HEp-2 cell adhesion assays, 10 of the 12 wild-type *E. coli* strains adhered at adhesion indices ranging from 1.2 to 6.4. Two strains (CF576 and CF6263) did not adhere; their adhesion indices were 0.2 and 0.4, respectively, similar to the adhesion index of the negative control *E. coli* K-12 C600 (adhesion index of 0.2). The highest adhesion indices (more than three bacteria per cell) were observed for the seven strains that hybridized with the *daaC* DNA probe (Table 3). Of the 12 transconjugants, 9 adhered to HEp-2 cells. Eight transconjugants obtained from the donor strains with adhesion indices of >3 (transconjugants CF1172T, CF285T, CF454T, CF475T1, CF475T2, CF585T, CF6261T, and CF644T) showed adhesion indices much lower than those of the corresponding wild-type strains (ranging from 1.1 to 2.6 instead of 1.2 to 6.4). Two transconjugants (CF228T and CF258T) showed adhesion indices close to those of wild-type strains. The transconjugant CF603T corresponding to the adhering strain CF603 did not adhere (adhesion index of 0.2) and neither did the two transconjugants CF576T and CF6263T corresponding to non-adhering wild-type strains (adhesion indices of 0.3 and 0.7, respectively).

Colony hybridization, restriction pattern and Southern blot analysis of the R plasmids. Analysis of *E. coli* transconjugants by colony blot hybridization assays showed that all hybridized with the CS31A DNA probe. Since a single plasmid was

TABLE 3. Adhesion indices of diarrheagenic *E. coli* strains and their corresponding *E. coli* transconjugants for adhesion to Caco-2 and HEp-2 cells

Strain or transconjugant	Antibiotic resistance[a]	Plasmid size (kb)	Adhesion index[b]		Hybridization with DNA probe[c]			
			Caco-2 cells	HEp-2 cells	CS31A	*daaC*	Aerobactin	
							4.8 kb	2.3 kb
CF1172	AM, TIC, S, SPT, TE, SSS	140, 5	1.2 ± 0.2	6.4 ± 0.3	+	+	−	−
CF1172T	AM, TIC, S, SPT, TE, SSS	140, 5	1.5 ± 0.2	2.6 ± 0.3	+	−	−	−
CF228	AM, TIC, S, SPT, C, TE, SSS	140	1.2 ± 0.1	1.8 ± 0.4	+	−	−	−
CF228T	AM, TIC	140	1.6 ± 0.3	2.5 ± 0.1	+	−	−	−
CF258	S, SPT, TE, SSS	140	3.3 ± 0.3	1.2 ± 0.1	+	−	−	−
CF258T	S, SPT, TE, SSS	140	1.4 ± 0.2	1.6 ± 0.3	+	−	−	−
CF285	AM, TIC, S, SPT, K, N, TM, DKB, GM, SIS, C, TE, TMP, SSS	140	2.0 ± 0.1	5.1 ± 0.3	+	+	−	−
CF285T	AM, TIC, S, SPT, TE, TMP, SSS	140	1.4 ± 0.3	1.8 ± 0.3	+	−	−	−
CF454	AM, TIC, S, K, N, TE, SSS	140	2.2 ± 0.1	4.2 ± 0.2	+	+	+	+
CF454T	AM, TIC, S, K, N, TE, SSS	140	1.2 ± 0.2	1.6 ± 0.1	+	−	+	+
CF475	AM, TIC, S, SPT, K, N, C, TE, TMP, SSS	140	1.5 ± 0.2	3.1 ± 0.1	+	+	−	−
CF475T1	AM, TIC, S, SPT, K, N, TMP, SSS	140	1.5 ± 0.3	1.2 ± 0.3	+	−	−	−
CF475T2	AM, TIC, S, SPT, K, N, C, TE, TMP, SSS	140	1.4 ± 0.1	1.1 ± 0.1	+	−	−	−
CF576	S, K, N, TE, SSS	140, 35	1.2 ± 0.2	0.2 ± 0.1	+	−	+	+
CF576T	S, K, N, TE, SSS	140	1.2 ± 0.1	0.3 ± 0.1	+	−	+	+
CF585	AM, TIC, CF, S, SPT, K, N, C, TE, SSS	140	2.4 ± 0.2	3.5 ± 0.2	+	+	−	−
CF585T	AM, TIC, S, SPT, TE, SSS	140	1.8 ± 0.4	1.6 ± 0.4	+	−	−	−
CF603	AM, TIC, S, K, N, C, TE, SSS	140	1.7 ± 0.3	1.4 ± 0.1	+	−	−	+
CF603T	TE	140	1.3 ± 0.2	0.2 ± 0.1	+	−	−	+
CF6261	AM, TIC, S, SPT, K, N, C, TE, TMP, SSS	140, 20	1.4 ± 0.3	4.6 ± 0.3	+	+	−	−
CF6261T	AM, TIC, S, SPT, TE, SSS	140	1.9 ± 0.1	1.7 ± 0.1	+	−	−	−
CF6263	AM, TIC, S, SPT, TE, SSS	140, 55, 33, 16	0.5 ± 0.1	0.4 ± 0.1	+	−	−	−
CF6263T	AM, TIC, S, SPT, TE, SSS	140, 16	0.8 ± 0.1	0.7 ± 0.1	+	−	−	−
CF644	AM, TIC, S, SPT, C, TE, SSS	140, 75	2.6 ± 0.3	6.1 ± 0.4	+	+	−	−
CF644T	AM, TIC, S, SPT, TE	140	2.1 ± 0.3	2.3 ± 0.3	+	−	−	−
K-12 C600	NA		0.1 ± 0.1	0.2 ± 0.1	−	−	−	−

[a] Abbreviations: AM, ampicillin; TIC, ticarcillin; S, streptomycin; SPT, spectinomycin; TE, tetracycline; SSS, sulfonamides; C, chloramphenicol; K, kanamycin; N, neomycin; TM, tobramycin; DKB, dibekacin; GM, gentamicin; SIS, sisomicin; TMP, trimethoprim; NA, nalidixic acid.

[b] Values are means ± standard deviations. Each adhesion index is the mean number of bacteria per cell determined in two separate experiments.

[c] Refer to Table 1 for a description of the probes. +, hybridization; −, no hybridization.

transferred in most of the transconjugants (except for transconjugants CF6263T and CF1172T), the genes encoding CS31A were located on the large self-transmissible 140-kb R plasmid. The bacterial surface components of these transconjugants were extracted and analyzed by SDS-PAGE. All of them harbored a major surface protein with an apparent molecular weight the same as those from the corresponding donor strains, and all the major surface proteins were recognized by the antiserum raised against CS31A (21). Thus, 2 transconjugants harbored a 30.5-kDa protein corresponding to CS31A-H and 10 transconjugants harbored a 30-kDa protein corresponding to CS31A-L.

Colony hybridization experiments performed with the *daaC* DNA probe showed that none of the transconjugants hybridized. Since seven donor strains were positive with this probe, we assumed that the genes encoding the adhesin of the Dr family were located either on the chromosome or on one of the nontransferred plasmids harbored by the donor strains.

The 140-kb R plasmids harbored by transconjugants were compared by determining their endonuclease restriction patterns with *Eco*RI (Fig. 2) or *Eco*RI-*Hin*dIII (Fig. 3). Many of the plasmids shared common restriction fragments. This was especially noticeable after digestion with *Eco*RI-*Hin*dIII. The comparison of these plasmids with pCFF504 harbored by *K. pneumoniae* CF504 showed that some restriction fragments were of comparable sizes. The two plasmids from *E. coli* CF475 strain were very similar. It seems likely that the chloramphenicol resistance genes were rearranged from CF475T2 to yield CF475T1.

In previous work, we observed the presence of CS31A-related protein, β-lactams, aerobactin, and ferric aerobactin receptor-encoding genes on a self-transmissible R plasmid in *K. pneumoniae* strains (13). Therefore, we performed Southern hybridization experiments to look for these genes and to localize them. The hybridization patterns of the R plasmids with the CS31A DNA probe are shown in Fig. 2 and 3. In *Eco*RI digests of the 140-kb R-plasmid DNA, the CS31A probe recognized fragments of different sizes: a 20-kb fragment (transconjugants CF285T, CF454T, and CF644T), an 18-kb fragment (transconjugant CF576T), a 16-kb fragment (transconjugants CF285T, CF585T, CF6261T, CF475T, CF258T, and CF6263T), a 13-kb fragment (transconjugants CF1172T and CF603T), and a 27-kb fragment (transconjugant CF228T). In *Eco*RI-*Hin*dIII double digests of the same R plasmids, the hybridization of the CS31A DNA probe occurred within an 8.5-kb fragment for 11 of the 12 R plasmids analyzed and within a 14-kb fragment for the R plasmid of the transconjugant CF454T (Fig. 3).

The TEM DNA probe hybridized with *Eco*RI fragments of different electrophoretic mobilities for the transconjugants resistant to ampicillin and ticarcillin: a 20-kb fragment for transconjugants CF285T, CF454T, and CF644T; a 10.5-kb fragment for transconjugants CF1172T, CF585T, CF6261T, and CF6263T; a 27-kb fragment for transconjugant CF228T; and an 18.5-kb fragment for transconjugants CF475T1 and CF475T2.

Using aerobactin-specific DNA probes, we observed that the R plasmids harbored by two transconjugants, CF454T and

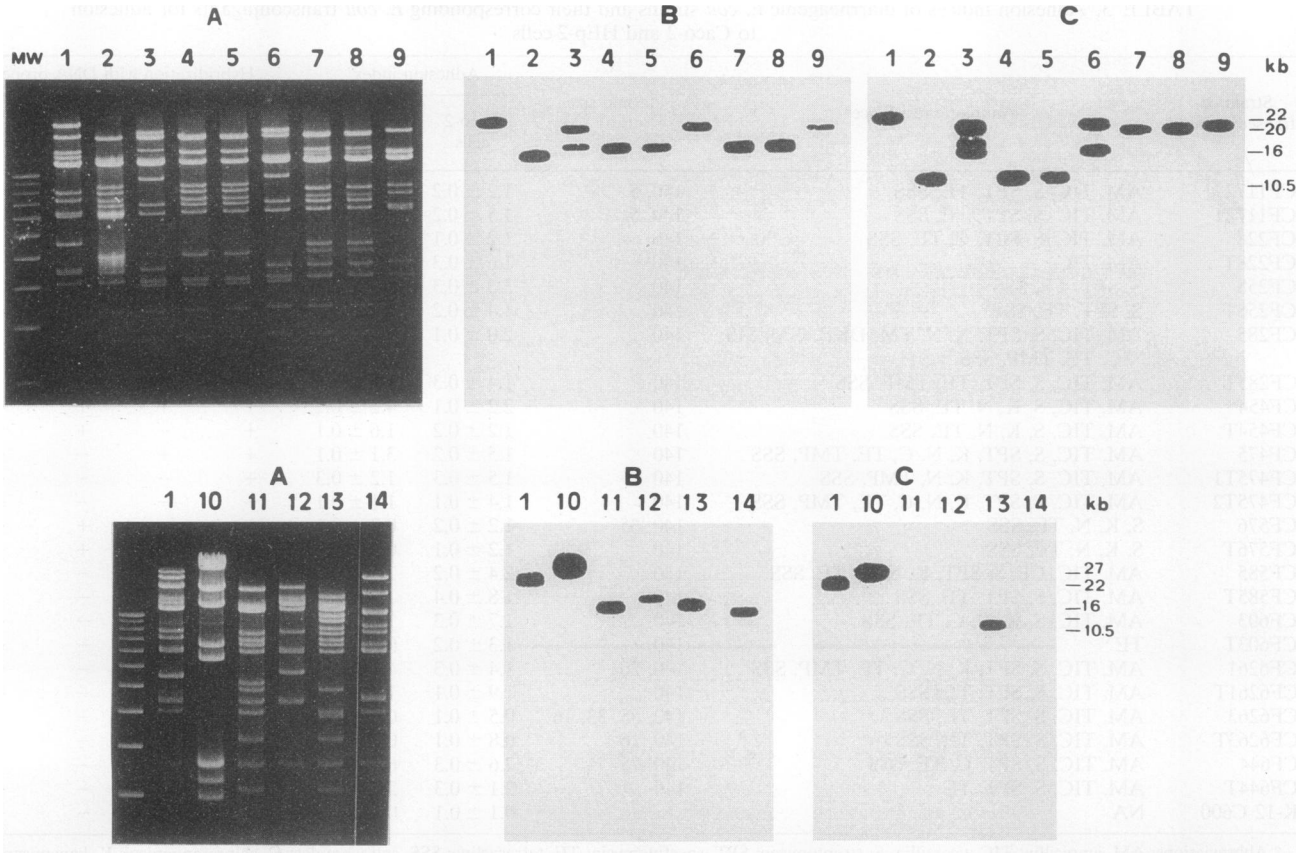

FIG. 2. Hybridization of specific DNA probes with *Eco*RI restriction fragments of the 185-kb R plasmid harbored by *K. pneumoniae* CF504 (lanes 1) and of the 140-kb R plasmid harbored by *E. coli* CF1172T (lanes 2), CF285T (lanes 3), CF585T (lanes 4), CF6261T (lanes 5), CF644T (lanes 6), CF475T1 (lanes 7), CF475T2 (lanes 8), CF454T (lanes 9), CF228T (lanes 10), CF258T (lanes 11), CF576T (lanes 12), CF6263T (lanes 13), and CF603T (lanes 14). (A) Agarose gel electrophoresis. MW, molecular size standards (1-kb ladder; Gibco BRL SARL, Cergy Pontoise, France). (B and C) Southern blots showing hybridization of probes with the *Hin*dIII-*Eco*RI 1-kb fragment which contains the structural gene of CS31A (B) and the *Pst*I-*Ssp*I 560-bp fragment specific for the TEM gene (C).

CF576T, possessed the genes encoding aerobactin and the ferric aerobactin receptor (21). The 4.8-kb *Hin*dIII-*Bgl*II fragment of pABN1 containing the genes encoding aerobactin hybridized with a 14- or 16-kb *Eco*RI fragment of the 140-kb R plasmid of transconjugant CF454T or CF576T, respectively. The 2.3-kb *Pvu*II fragment containing the genes encoding the ferric aerobactin receptor hybridized with the same 14- or 16-kb fragment and with an 8-kb *Eco*RI fragment of the two R plasmids of transconjugants CF454T and CF576T. Furthermore, 19- and 8-kb *Eco*RI fragments of the R plasmid from transconjugant CF603T were recognized only by the 2.3-kb aerobactin probe, indicating that this strain possessed only the gene encoding the ferric aerobactin receptor. The hybridization of two fragments within the same plasmid with this 2.3-kb aerobactin probe was expected, since the gene encoding the ferric aerobactin receptor possesses an intragenic *Eco*RI restriction site.

Southern hybridizations with the same probes (CS31A, TEM, aerobactin, and ferric aerobactin receptor) were performed with the conjugative plasmid from *K. pneumoniae* strains producing CF29K, a CS31A-related protein. Hybridization with the CS31A DNA probe occurred within an 8.5-kb *Eco*RI-*Hin*dIII fragment of the 185-kb R-plasmid DNA harbored by *K. pneumoniae* CF504. This fragment was of a size comparable to those of the R plasmids from the *E. coli* strains isolated from diarrheal stools. Likewise, the aerobactin DNA probes hybridized with *Eco*RI fragments of sizes similar to those of fragments detected in transconjugant CF454T R plasmid.

DISCUSSION

In this work, we studied *E. coli* strains hybridizing with the CS31A DNA probe to better define their virulence potential. The study was made on a collection of *E. coli* strains isolated from sporadic diarrheal stools of patients in the Centre Hospitalier Régional Universitaire of Clermont-Ferrand, France, during the 3-year period 1990 to 1992. These diarrheagenic *E. coli* strains were previously screened to classify them in the pathogenic *E. coli* groups described so far, i.e., enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroaggregative *E. coli* and DAEC (22, 23). We found a not inconsiderable incidence of CS31A-positive *E. coli* strains, since 30 of the 597 strains tested (5%) hybridized with the CS31A DNA probe. These strains did not belong to the enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, or enteroaggregative *E. coli* group, but 28 of them were classified in the DAEC group, since they hybridized with the diffuse adhesion DNA probe corresponding to the *daaC* gene of the F1845 adhesin operon, and/or adhered diffusely to

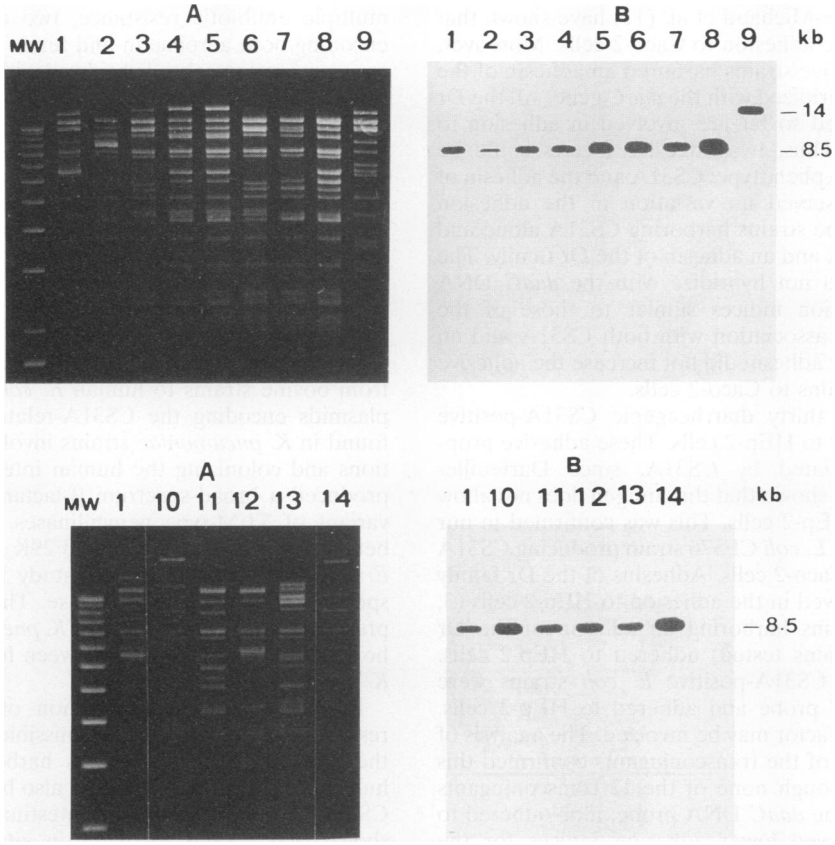

FIG. 3. Hybridization of specific DNA probes with *Eco*RI-*Hin*dIII restriction fragments of the 185-kb R plasmid harbored by *K. pneumoniae* CF504 (lanes 1) and of the 140-kb R plasmid harbored by *E. coli* CF1172T (lanes 2), CF285T (lanes 3), CF585T (lanes 4), CF6261T (lanes 5), CF644T (lanes 6), CF475T1 (lanes 7), CF475T2 (lanes 8), CF454T (lanes 9), CF228T (lanes 10), CF258T (lanes 11), CF576T (lanes 12), CF6263T (lanes 13), and CF603T (lanes 14). (A) Agarose gel electrophoresis. MW, molecular size standards (1-kb ladder; Gibco BRL SARL). Southern blots showing hybridization of probes with the *Hin*dIII-*Eco*RI 1-kb fragment which contains the structural gene of CS31A (B).

HEp-2 cells (22, 23). The presence of CS31A-positive *E. coli* strains in human diarrheal stools was surprising, since CS31A has been found in septicemic and enterotoxigenic bovine *E. coli* strains (8, 19). However, CS31A in septicemic human *E. coli* strains was recently described (6). Thus, unlike K88 or colonization factor antigen adhesins, which are specific for porcine and human *E. coli* strains, respectively (16, 32), CS31A is found not only in septicemic and enterotoxigenic bovine *E. coli* strains but also in *E. coli* strains isolated from human diarrheagenic stools.

Analysis of bacterial surface components from wild-type strains in SDS-PAGE showed large amounts of proteins with apparent molecular masses of 30 or 30.5 kDa, according to the strain. Of the 30 strains studied, 9 produced a 30.5-kDa protein and 21 strains produced a 30-kDa protein. Both the 30- and 30.5-kDa bands reacted with anti-CS31A serum, indicating that the two polypeptides corresponded to the two forms of CS31A described for septicemic and enterotoxigenic bovine *E. coli*: CS31A-H and CS31A-L (7). Most (70%) of the human *E. coli* strains described in this paper harbored CS31A-L, whereas 69% of septicemic and enterotoxigenic bovine *E. coli* strains produced CS31A-H (7). Thus, the two forms of CS31A, which differ only in one amino acid in position 89 of the mature protein, might have different host affinities.

The *E. coli* strains positive with the CS31A DNA probe showed multiple resistance to various antibiotics, including penicillins (ampicillin and ticarcillin), streptomycin, sulfonamides, tetracyclines, and kanamycin. Such an antibiotic resistance pattern has been observed in septicemic bovine and human CS31A-positive strains (6, 15). When the conjugative 140-kb R plasmids of 12 diarrheagenic *E. coli* strains were transferred to the *E. coli* K-12 C600 recipient strain, all the transconjugants hybridized with the CS31A DNA probe and all produced either the 30 or the 30.5 kDa protein. This indicates that CS31A is R plasmid encoded in human *E. coli* strains isolated from diarrheal stools, as has been shown for septicemic bovine and human *E. coli* strains (6, 15, 19).

In addition, 70% of the CS31A-positive *E. coli* strains hybridized with the *daaC* DNA probe. This suggests that these strains produced an adhesin of the Dr family, such as F1845, AFA-I, AFA-III, or the Dr hemagglutinin. The Dr family adhesins AFA-I, AFA-II, and Dr hemagglutinin, are chromosomally encoded, while AFA-III and F1845 are either plasmid- or chromosome-encoded (3, 27). No transconjugant hybridized with the *daaC* DNA probe. Thus, the genes encoding the adhesins of the Dr family in diarrheagenic *E. coli* strains are located either on the chromosome or on a nontransferred plasmid harbored by the wild-type strains.

All the CS31A-positive *E. coli* strains isolated from patients with diarrhea adhered in vitro to the human intestinal Caco-2 cells. These cells exhibit structural and functional differentiation pattern characteristics of mature enterocytes in postcon-

fluent culture. Darfeuille-Michaud et al. (11) have shown that CS31A is involved in the adhesion to Caco-2 cells. Moreover, 70% of the CS31A-positive strains harbored an adhesin of the Dr family, since they hybridized with the *daaC* gene. All the Dr family adhesins described so far are involved in adhesion to Caco-2 cells (25, 26). Thus, two adhesive factors could be involved in this adhesion phenotype: CS31A and the adhesin of the Dr family. We observed no variation in the adhesion indices between wild-type strains harboring CS31A alone and strains harboring CS31A and an adhesin of the Dr family. The transconjugants that did not hybridize with the *daaC* DNA probe presented adhesion indices similar to those of the wild-type strains. Thus, association with both CS31A and an adhesin of the Dr family adhesin did not increase the adhesive ability of the *E. coli* strains to Caco-2 cells.

Twenty-eight of the thirty diarrheagenic CS31A-positive strains adhered diffusely to HEp-2 cells. These adhesive properties cannot be mediated by CS31A, since Darfeuille-Michaud et al. (11) have shown that this antigen does not allow bacteria to adhere to HEp-2 cells. This was confirmed in our study by the fact that the *E. coli* CF576 strain producing CS31A alone adhered only to Caco-2 cells. Adhesins of the Dr family are reported to be involved in the adhesion to HEp-2 cells (3, 27). The wild-type strains harboring an adhesin of the Dr family (70% of the strains tested) adhered to HEp-2 cells. However, 20% of the CS31A-positive *E. coli* strains were negative with the *daaC* probe and adhered to HEp-2 cells. Thus, another adhesive factor may be involved. The analysis of the adhesive properties of the transconjugants confirmed this hypothesis. Indeed, although none of the 12 transconjugants tested hybridized with the *daaC* DNA probe, nine adhered to HEp-2 cells. We observed lower adhesion indices for the transconjugants than for the donor strains producing an adhesin of the Dr family. The association of an adhesin of the Dr family with another adhesive factor may have increased the ability to adhere to HEp-2 cells of the wild-type strains. The presence of two or more adherence systems in a single bacterial strain is not rare and seems to be characteristic of strains isolated from urinary tract infections (1). Similar observations were made with diarrheagenic and septicemic *E. coli* strains, and CS31A has been reported to be associated with either the K99, F165, or FY adhesins in a single strain (9, 19). Furthermore, it has been shown that the 185-kb R plasmid of *K. pneumoniae* producing the CS31A-like antigen also encodes another adhesive factor involved in HEp-2 cell adhesion (14). The association of several adhesive factors might not only increase the ability to adhere to a given cell type but also mediate the colonization of several host tissues.

Endonuclease restriction analysis of patterns of the 140-kb R plasmids showed them to be similar. It seems likely that most of these plasmids are derivatives of a common ancestor. Hybridization experiments with the CS31A- and TEM-specific DNA probes showed that in four strains these genes were both located on the same *Eco*RI fragment of 20 or 27 kb, depending on the R plasmid. A linkage between the two genes was previously reported for the 185-kb R plasmid of *K. pneumoniae* producing CF29K (13). In *Eco*RI-*Hin*dIII double digests, hybridization with the CS31A DNA probe occurred within 8.5-kb fragments of both the 140-kb R plasmid from *E. coli* strains isolated in diarrheal stools and the 185-kb R plasmid from *K. pneumoniae* strains. Likewise, an 8.5-kb *Eco*RI-*Hin*dIII DNA fragment has been reported to carry the operon encoding CS31A in bovine septicemic *E. coli* strains (30). Aerobactin genes have been described for R plasmids encoding CS31A in 80% of *E. coli* strains involved in bovine septicemia (33). Analysis of the 140-kb R plasmids showed that, in addition to multiple antibiotic resistance, two of them harbored genes encoding both aerobactin and ferric aerobactin receptor. The two aerobactin probes hybridized with *Eco*RI fragments of the same size on R plasmids of human *E. coli* or *K. pneumoniae* strains. Hence, the R plasmids harbored by septicemic bovine *E. coli* strains, human *E. coli* strains isolated from diarrheal stools, and *K. pneumoniae* strains involved in human nosocomial infections are related. There is a great difference between the rates of occurrence of CS31A-positive *E. coli* strains in bovine and human isolates (38% for bovine strains and 5% for human strains). This suggests that the presence of *E. coli* strains harboring R plasmids encoding CS31A among human isolates could be due to contamination as a result of the ingestion of bovine strains or to horizontal plasmid transfer from bovine strains to human *E. coli* strains. Very similar R plasmids encoding the CS31A-related protein CF29K were found in *K. pneumoniae* strains involved in nosocomial infections and colonizing the human intestine (13). These strains produced a broad-spectrum β-lactamase, CAZ-1, which is a variant of TEM-type penicillinases. We observed a linkage between the genes encoding CF29K and CAZ-1. None of the *E. coli* strains tested in this study produced such a broad-spectrum CAZ-type β-lactamase. Thus, we assumed that the presence of such R plasmids in *K. pneumoniae* may result from horizontal transfer in vivo between human *E. coli* strains and *K. pneumoniae* strains.

In conclusion, the association of CS31A and antibiotic resistance genes on self-transmissible plasmids might explain the emergence of the strains harboring these plasmids in humans. This emergence could also be due to in vivo ability of CS31A to adhere to human intestinal cells, since it has been shown that CS31A mediates in vitro adhesion to cultured human intestinal Caco-2 cells. However, the exact role of CS31A remains uncertain, as this adhesin is more common in bovine than in human *E. coli* strains and does not mediate adhesion to bovine intestinal villi.

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