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Epidemiological Study of *pap* Genes among Diarrheagenic or Septicemic *Escherichia coli* Strains Producing CS31A and F17 Adhesins and Characterization of Pap_{31A} Fimbriae

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The association of the *pap* operon with the CS31A and F17 adhesins was studied with 255 *Escherichia coli* strains isolated from calves, lambs, or humans with diarrhea. The three classes of PapG adhesin with different receptor binding preferences were also screened. The *pap* operon was associated with 50 and 36% of human strains that produced CS31A and ovine strains that produced F17, respectively. Among the bovine isolates, the *pap* operon was detected in 61% of the CS31A-positive isolates and 72% of the strains that produce both CS31A and F17. The class II adhesin gene was present in bovine (20%) and ovine (71%) isolates. Both class II and III adhesins were genetically associated with 36% of the human strains. The highest prevalence of the *pap* operon was observed among *E. coli* strains that produce additional adhesins involved in the binding of bacteria to intestinal cells. Among the bovine isolates, the reference strain for CS31A and F17c was found to be positive for the *pap* operon. Phenotypic and genotypic characterizations were undertaken. Pap_{31A} appeared as fine and flexible fimbriae surrounding the bacteria but did not mediate adhesion to calf intestinal villi. Pap_{31A} production was optimal with bacteria cultured on minimal growth media and repressed by addition of exogenous leucine. The deduced amino acid sequence of the $PapA_{31A}$ structural subunit showed 57 to 97% identity with the different P-related structural subunits produced by *E. coli* strains isolated from pigs with septicemia or humans with urinary tract infections. None of the three *papG* allelic variants was detected, but a homologous *papG* gene was present in the chromosome of strain 31A.

The reference strain *Escherichia coli* 31A, isolated from the feces of a diarrheic calf, causes experimental septicemia in gnotobiotic calves and lambs (9). This strain produces the plasmid-encoded CS31A antigen, which is closely related to the K88 fimbriae produced by porcine enterotoxigenic *E. coli* (18, 35). More recently, a new CS31A-related adhesin was described in *Klebsiella pneumoniae* strains involved in nosocomial infections (12). CS31A binds to an *N*-acetylneuraminic acid-containing receptor present in human Int-407 cells (12). However, the CS31A antigen clearly differs from typical fimbriae and appears as a capsule-like material surrounding the bacteria (18). Strain 31A also produces the F17c fimbria (formerly called 20K), which is responsible for in vitro *N*-acetyl-D-glucosamine-dependent adhesion of bacteria to calf and lamb brush border villi (4, 10). The F17c fimbria belongs to the F17 family, which includes fimbriae expressed by bovine diarrheic and septicemic *E. coli* strains and by human uropathogenic *E. coli* strains (4, 5, 30, 37). Pathogenic F17-producing *E. coli* strains represent a significant part of the bacterial strains isolated from diarrheic calves in France and Belgium and from lambs with nephrosis in Scotland (4, 5).

P fimbriae are mannose-resistant hemagglutinins detected predominantly at the cell surface of *E. coli* strains associated with human urinary tract infection (UTI) (25). These fimbriae are closely associated with upper urinary tract colonization and pyelonephritis and bind to the kidney vascular endothelium (28). P-related fimbriae are also associated with UTIs in dogs and septicemia in pigs (16, 33). The adhesin subunit, termed PapG, is located at the tip of the P fimbriae and mediates the binding of the bacteria to α-D-galactosyl-(1-4)-β-galactopyranose (Gal-Gal)-containing receptors present in host tissues (31). Three allelic variants of the gene that encodes the P or Prs adhesin were described previously (39). All recognize glycolipids in the globoseries but differ in their binding to GbO_3, GbO_4, and GbO_5 globosides (31, 39).

In the study described in this report, 255 pathogenic *E. coli* strains isolated from human, bovine, and ovine intestinal contents were screened for the *pap* operon that codes for the P-related fimbriae. The allelic variants that code for the three classes of P adhesins were also investigated. The association of the P-related fimbriae with CS31A antigen, F17c fimbriae, and other virulence factors was analyzed. In addition, we demonstrate that a 17.5-kDa protein produced by the 31A reference *E. coli* strain was a fimbrial structural subunit closely related to those of the Prs-like fimbria $F165_1$ produced by *E. coli* strains isolated from piglets with septicemia and to P or Prs fimbriae produced by *E. coli* strains isolated from humans with UTIs.

MATERIALS AND METHODS

Bacterial strains. *E. coli* 31A was isolated from the intestinal contents of a calf with diarrhea (9, 18). *E. coli* 31A/O6 is a plasmid-cured strain obtained from 31A (4, 18). Strain 31A/O6(20K−) is a spontaneous mutant defective in F17c production (4). Strain 5131 was isolated from the intestinal contents of a septicemic piglet and expresses $F165_1$ fimbriae (21). Wild-type *E. coli* strain IA2 was isolated from a human with a UTI and produces P fimbriae (F11 serotype) (41). Strains 5131 and IA2 were kindly provided by J. Harel (Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, Quebec, Canada) and M. Svensson (Department of Medical Microbiology, Lund, Sweden), respectively. *E. coli* HB101 strains that harbored recombinant plasmid pHRU845, pPILL110-35, or

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TABLE 1. Characteristics and origins of the bacterial strains

Strain	Characteristics	Phenotype	Reference(s)
31A	*E. coli* strain from fecal flora of a diarrheic calf carrying plasmid p31A	CS31A, F17c, and Pap_{31A} positive	18
31A/O6	p31A-cured variant of 31A	F17c and Pap_{31A} positive	4, 18
31A/O6(20K−)	F17c-negative mutant from 31A/O6	Pap_{31A} positive	4
5131	*E. coli* strain from a pig with septicemia	$F165_1$ and $F165_2$ positive	21
IA2	*E. coli* strain from a human with a UTI	P (F11 serotype) positive	41
HB101(pHRU845)	Recombinant	Production of PapG class I adhesin	26
HB101(pPLILL110-35)	Recombinant	Production of PapG class II adhesin	26
HB101(pJFK102)	Recombinant	Production of PapG class III adhesin	26
HB101	*E. coli* K-12 recipient strain		

pJFK102, each of which contains the *pap* gene cluster that encodes the class I, class II, and class III adhesin, respectively, were used as positive controls. The recombinant strains were kindly provided by M. Svensson. The characteristics and origins of the *E. coli* strains are summarized in Table 1.

Genotypic detection was performed with human, bovine, and ovine intestinal *E. coli* strains. A total of 118 *E. coli* strains (included in our bacterial collection) were isolated from calves with septicemia and/or diarrhea in France and Belgium. The 77 human strains were isolated from sporadic diarrheal stools of patients in the Centre Hospitalier Régional Universitaire of Clermont-Ferrand, France (23). The 60 ovine *E. coli* strains described elsewhere (4, 5) were isolated in Scotland from lambs with nephropathy (1).

Genotypic detection of the *pap* operon and the related adhesin genes. The *pap* operon was detected by using a previously described PCR protocol (29). A 328-bp DNA fragment was amplified from the highly conserved *papC* gene. A multiplex-primer PCR assay was used to identify each of the three *papG* allelic variants that encode the Gal-Gal binding adhesin of P fimbriae (26). Use of the primer cocktail resulted in three DNA fragments of 461, 190, and 258 bp specific for the adhesin genes of classes I, II, and III, respectively. DNAs extracted from *E. coli* strains harboring recombinant plasmid pHRU845, pPILL110-35, or pJFK102 were used as positive controls. Amplification procedures were also performed to screen for the presence of the *papI*, *papE*, and *papF* genes located in different positions on the *pap* gene cluster (34, 43). A DNA extract from the IA2 reference strain was used as a positive control.

For all the amplification procedures, DNA extracted from the HB101 recipient strain was used as a negative control and was included in every PCR run. The PCR products obtained were electrophoresed on a 1.5% agarose gel and were visualized by staining with ethidium bromide.

Preparation of DNA probe and hybridization. The bacterial strains were cultured overnight at 37°C on Luria-Bertani (LB) broth, and total chromosomal DNA was extracted and purified with the Easy-DNA kit (Invitrogen). The chromosomal DNAs were plotted on a Hybond N^+ membrane (Amersham) and alkali fixed on the membrane according to the manufacturer's recommendations. A DNA probe was generated by PCR amplification as described previously (26). The amplified fragment was obtained from recombinant plasmid pHRU845 and was purified with the Wizard PCR Preps DNA purification system (Promega). The DNA probe was labeled with the Renaissance Random primer fluorescein kit under conditions recommended by the supplier (NEN Life Science Products) and then purified on a ProbeQuant G-50 microcolumn (Pharmacia). Membrane prehybridization, hybridization, and washes were performed in accordance with the manufacturer's recommendations (NEN). The enhanced chemiluminescence signal was detected on autoradiography film. Chromosomal DNAs extracted from recombinant strain HB101(pHRU845) and recipient strain HB101 were used as positive and negative controls, respectively.

Fimbrial purification and biochemical characterization. After growth on Minca agar medium (19), the bacterial suspension was mechanically sheared (2 min in a Top mix blender at maximal speed) and centrifuged (20,000 × *g* for 10 min at 4°C). The resultant supernatant was precipitated with 20% ammonium sulfate, and the precipitate was collected by centrifugation (10,000 × *g* for 10 min at 4°C). Sodium dodecyl sulfate (SDS) was added to a final concentration of 2%, and the SDS-insoluble material was collected by ultracentrifugation (110,000 × *g* for 200 min at 20°C). After dissociation of the polymers (2 h of incubation at 37°C in 8.5 M guanidine hydrochloride), 5 mM Tris hydrochloride (pH 7.8) was added to obtain 6 M guanidine hydrochloride. The suspension was subjected to gel filtration chromatography on a Sephacryl S300 (Pharmacia) column, and the fractions containing the major peak were pooled, dialyzed at 4°C against ammonium acetate buffer, and then lyophilized. SDS-polyacrylamide gel electrophoresis (PAGE) analysis revealed two copurified polypeptide bands of 20 and 17.5 kDa.

The lyophilized material was dissolved in Laemmli sample buffer and was loaded on a preparative acrylamide gel. After electrophoresis, the band corresponding to the 17.5-kDa polypeptide was cut, and the protein was electroeluted, dialyzed, and lyophilized as described elsewhere (4). A 15-μg sample of the purified 17.5-kDa polypeptide was transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) as described previously (4), and the first NH_2-terminal amino acid residues were determined by Edman degradation. The N-terminal amino acid sequence of the 17.5-kDa polypeptide was compared with the sequences of proteins listed in the National Biomedical Research Foundation (NBRF) protein sequence data bank by using the FASTA program.

Analysis of in vitro expression of the 17.5-kDa protein. In vitro production of the 17.5-kDa protein was analyzed by culturing the *E. coli* strains on different growth media. In addition to a complex agar medium (LB), different synthetic agar media were used: Minca medium (19) supplemented with 0.1% glucose (MG-1), minimal Davis agar medium (Difco) supplemented with 0.1% Casamino Acids and 0.1% glucose (MD-1) (15), and minimal M9 agar medium supplemented with 0.2% glucose or 0.2% glycerol.

The putative repression by the leucine was investigated after growing the bacteria on the M9 agar medium with or without 1 mM leucine supplemented with valine and isoleucine (each at 50 μg ml^{-1}). Overnight cultures were harvested in phosphate-buffered saline (pH 7.2) and adjusted to an optical density of 120 at 600 nm. The fimbrial suspensions were heated for 20 min at 60°C and centrifuged at 10,000 × *g* for 15 min. The resulting supernatants (2 μl) were plotted onto nitrocellulose membranes. Dot blotting assays were performed in triplicate with antibodies specific to the 17.5-kDa protein (dilution, 1:500). Images of the plots were obtained by using an Imager-Appligene, and density profiling was performed by using the Scion image-processing program.

Production of antisera. Two antisera directed against denatured and native 17.5-kDa protein, respectively, were produced in mice as described previously (4). The 31A/O6(20K−) strain cultured on MD-1 medium was chosen to produce the native fimbriae. The antiserum raised against the native protein was repeatedly adsorbed with the 31A/O6(20K−) strain cultured at 18°C on MD-1. Antibodies directed against $F165_1$ and P (F11 serotype) fimbriae were kindly provided by J. Harel and M. Svensson, respectively.

Electron microscopy. Bacterial cells were placed on carbon-stabilized collodion-coated copper grids, negatively stained with 1% phosphotungstic (pH 6.8), and observed with a transmission electron microscope (HU 12A; Hitachi) operated at 75 kV. Gold immunolabeling was performed with specific antibodies directed against the native 17.5-kDa polypeptide (dilution, 1:15) and 10-nm colloidal goat anti-rabbit immunoglobulin G (Nordic) as described previously (4, 18).

Assay of in vitro adhesion on calf intestinal villi. Adhesion to calf and lamb intestinal villi was tested as described previously (4, 17). Hemagglutination tests were performed in the presence or absence of 0.5% D-mannose. The villi were washed for 30 min in cold phosphate-buffered saline containing 1.5 mM 14-(2-aminoethyl)-benzensulfonyl-fluoride-hydrochloride to prevent proteolysis (4). Adhesion was scored by using a phase-contrast microscope at a magnification of ×1,000. A maximal attachment index was scored when 30 bacteria adhered to a 50-μm segment of a villus brush border.

Hemagglutination. Hemagglutination tests were performed on glass slides at 4°C in the absence or presence of 0.5% D-mannose as described previously (4, 18). A bacterial suspension (50 μl, 10^9 cells ml^{-1}) was mixed with an identical volume of a 3% (vol/vol) suspension of erythrocytes from group A negative human blood and from animals (calf, sheep, rabbit, goat, horse, and rat).

Nucleotide sequencing of the gene encoding the 17.5-kDa protein. DNA to be amplified was released from whole organisms by boiling. Bacteria were harvested from 1 ml of an overnight LB broth culture, resuspended in 1 ml of sterile water, and incubated at 100°C for 10 min. To amplify the gene encoding the 17.5-kDa protein, primers were deduced from the published sequence of the $f165_1$ operon (33). The sequences of the oligonucleotide primers located upstream and downstream of the $f165_1$-*A* gene were 5′-CTGTCGATAAATAACCTGCCCTG-3′ and 5′-GTAATGTACTCCAGAAATACATCA-3′, respectively. The Expand High Fidelity PCR system containing thermostable *Taq* DNA and *Pwo* DNA polymerases was used to amplify the $papA_{31A}$ gene as recommended by the manufacturer (Boehringer Mannheim). The bacterial extracts were subjected to 25 cycles of amplification at an annealing temperature of 63°C (GenAmp 2400 thermal cycler; Perkin-Elmer). The DNA fragment obtained (760 bp) was puri-

TABLE 2. Detection of *pap* operon and three allelic variants of PapG adhesin[a]

Host	Phenotype		No. of strains	No. (%) of strains with *pap* operon	No. of strains with *papG*-related adhesin gene		
	CS31A antigen production	F17 fimbria production			Class I	Class II	Class III
Calf	+	+	40	29 (72)	0	10	0
	+	−	21	8 (38)	0	0	0
Subtotal			61	37 (61)			
	−	+	32	7 (22)	0	0	0
	−	−	25	5 (20)	0	0	0
Total			118	49 (42)		10 (20)[b]	
Lamb	−	+	31	11 (36)	0	8	0
	−	−	29	6 (21)	0	4	1
Total			60	17 (28)		12 (71)	1 (6)
Human	+	−	26	13 (50)	0	2	1
	−	−	51	14 (27)	0	3	4
Total			77	27 (36)		5 (18)	5 (18)

[a] The genotypic detection of the *pap* operon and the three classes of PapG adhesin was performed by polymerase chain reaction as described previously (26, 29). The phenotypic detection of CS31A and F17 antigens was performed by Western blotting or dot blotting (4, 5, 18).
[b] Values in parentheses are percentage of strains with the *papG*-related adhesin gene among the *pap*-positive isolates.

fied by using the Prep-A-Gene DNA purification system (Bio-Rad) and was cloned into the Bluescript (KS^+) vector (Stratagene Ltd.). The nucleotide sequence was determined with double-stranded DNA by the dideoxy chain termination method with a model 373A automatic DNA sequencer (Applied Biosystems Inc.).

Nucleotide sequence accession number. The nucleotide sequence of $papA_{31A}$ has been deposited in GenBank under accession no. AF165981.

RESULTS

Genotypic detection of *pap* operon among bovine, ovine and human *E. coli* strains. In order to investigate the putative association of P-related fimbriae with CS31A and/or F17-related adhesins, 255 *E. coli* strains isolated from the intestinal contents of calves, lambs, and hospitalized patients were screened for the *papC* gene (which is highly conserved in *pap* operons). The results are summarized in Table 2.

Among the CS31A-positive isolates, the *pap* operon was detected in 61% of *E. coli* strains isolated from the intestinal contents of calves with diarrhea and/or septicemia and 50% of strains isolated from human diarrheic stools specimens. This percentage increased to 72% among bovine strains that produced both the CS31A antigen and F17-related fimbriae. The prevalence of the *pap* operon was greatly reduced among bovine and human CS31A-negative strains (20 and 27%, respectively).

The association of the *pap* operon with F17-related fimbriae was also investigated among *E. coli* strains isolated from the intestinal contents of calves with diarrhea or lambs with nephropathy (*E. coli* strains that produce F17-related fimbriae are not isolated from human feces [4]). CS31A-negative strains that produced or that did not produce F17 isolated from diarrheic calves were weakly associated with the *pap* operon (22 and 20%, respectively). PCR analysis showed that 36% of F17-producing strains from the intestinal contents of lambs with nephropathy were positive for the *pap* operon. This percentage decreased to 21% among ovine isolates which did not express F17-related fimbriae.

In order to compare the detection of a *pap* operon and the production of fimbriae, phenotypic detection of P-related fimbriae was performed with *pap*-positive *E. coli* strains grown on Davis medium (MD-1). Analysis of Western blotting assays results revealed that 89% of the *E. coli* strains that harbored the *pap* operon produced P-related fimbriae under these growth conditions, suggesting the presence of a complete *pap* operon.

Detection of the *pap*-related adhesin gene among bovine, ovine, and human *E. coli* strains. The PapG adhesin subunit occurs as three molecular variants (classes I to III) encoded by distinct allelic variants. Each class of the PapG adhesin exhibits subtly different receptor binding preferences (39). The 255 *E. coli* strains were investigated for the presence of the *papG* alleles by a multiplex PCR protocol. The results are summarized in Table 2.

The gene that encodes the class I adhesin was not detected among the human, ovine, or bovine strains tested, confirming that the class I adhesin is rare among *pap*-positive *E. coli* strains (24, 34).

The gene that encodes the class II adhesin gene was detected in 10 of the 49 (20%) strains isolated from calves that harbored the *pap* operon. Among the bovine *E. coli* strains, the class II allelic variant was exclusively associated with strains that produced both CS31A and F17 adhesins. The class III adhesin gene was not detected in DNAs extracted from the bovine isolates. Among the strains isolated from diarrheic stools of hospitalized patients, 10 of the 27 *pap*-positive *E. coli* strains (36%) exhibited a class II (18%) or a class III (18%) adhesin gene. Of the 17 *pap*-positive *E. coli* strains from lambs with nephropathy, 12 (71%) exhibited the class II adhesin gene (only 1 strain was found to have the class III adhesin gene).

Selection of bacterial strains. The *pap* operon was found to be highly associated with the bovine *E. coli* strains of this study that produce CS31A adhesins and F17-related fimbriae. Both F17c and CS31A have been characterized from reference strain 31A isolated in our laboratory from a calf with diarrhea (4, 18). In this report, *E. coli* 31A was found to be positive for the *pap* operon. In order to determine whether strain 31A produced a new P-related fimbria, biochemical and immunological characterization of the fimbriae and genotypic characterization of the structural and adhesin subunits were undertaken.

Purification and characterization of Pap_{31A} fimbriae. A 17.5-kDa protein was first detected during the F17c purification procedure described elsewhere (4). The purification was performed from a crude fimbrial extract of plasmidless strain *E. coli* 31A/O6 (CS31A negative, F17c positive, *pap* positive)

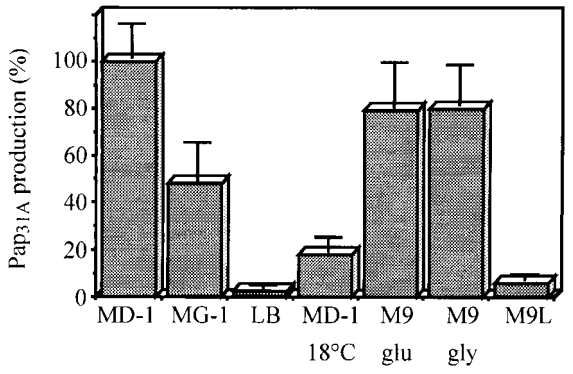

FIG. 1. In vitro expression of $PapA_{31A}$ structural subunit. $PapA_{31A}$ production was analyzed by culturing *E. coli* 31A on different growth media at 37°C: minimal Davis medium supplemented with glucose and Casamino Acids (MD-1), semisynthetic Minca medium supplemented with glucose (MG-1), and the complex media LB and M9 supplemented with glucose (M9 glu) or glycerol (M9 gly). The repression by leucine was investigated after growing the bacteria on M9 medium supplemented with glucose and 1 mM leucine (M9L). Pap_{31A} production was also determined on MD-1 at 18°C. Results were expressed as the percentages of $PapA_{31A}$ production related to maximal production (assigned as 100%). Fimbrial production was determined in triplicate by a quantitative dot blot assay.

cultured on Minca medium (MG-1). The surface structures were harvested from whole cells by mechanical shearing, precipitated with ammonium sulfate, and solubilized with SDS. After gel filtration chromatography, SDS-PAGE analysis of the fractions eluted from the S300 column revealed two major polypeptides bands of 20 and 17.5 kDa. The 20-kDa polypeptide represents the F17c-A fimbrial major subunit described previously (4, 37). The 17.5-kDa polypeptide was further purified by preparative gel electrophoresis.

The first 15 N-terminal amino acid residues of the 17.5-kDa protein (APTTPQGQGRVTFNG) obtained by Edman degradation appeared to be identical to those of the major subunit of P-related fimbria $F165_1$ produced by *E. coli* strains responsible for septicemia in piglets (33). A high degree of homology was also observed with the first amino acid residues of the $PapA_{IA2}$ and $PapA_{J96}$ fimbrial subunits produced by the human uropathogenic *E. coli* strains IA2 (F11 serotype) and J96 (F13 serotype), respectively (3, 41). In view of these results, the 17.5-kDa polypeptide was termed $PapA_{31A}$.

A crude fimbrial extract from strain 31A was subjected to Western blot assay. A single 17.5-kDa band reacted with specific antibodies raised against the purified $PapA_{31A}$ structural subunit (data not shown). In addition, a high degree of cross-reactivity was observed with fimbrial extracts obtained from *E. coli* 5131 and IA2, which produce the $F165_1$ and the P (F11 serotype) fimbriae, respectively (data not shown).

In vitro expression of $PapA_{31A}$ structural subunit. To determine whether the production of $PapA_{31A}$ depends on the culture medium, strain 31A was grown on different media (LB, MD-1, MG-1, and M9), and $PapA_{31A}$ production was quantified by a dot blot assay. The results are summarized in Fig. 1.

The results were expressed as percentages of $PapA_{31A}$ production related to the maximal production (assigned value of 100%) when strain 31A was cultured on minimal Davis medium (MD-1) at 37°C. Fimbrial production decreased greatly when the bacteria were cultured on complex LB medium (2.5%) or on minimal MD-1 medium at 18°C (8%). Intermediate fimbrial production (48%) was obtained with bacteria cultured on semisynthetic Minca medium (MG-1) at 37°C.

A high level of production of $PapA_{31A}$ (79%) was observed when strain 31A was cultured on M9 medium supplemented with glycerol. A similar result was observed with bacteria grown on M9 medium with glucose as the carbon source, suggesting that $PapA_{31A}$ production was not subject to catabolic repression (Fig. 1).

Expression of some fimbriae (including $F165_1$) is repressed by exogenous leucine in the growth medium, whereas expression of other fimbriae (including P fimbriae from strains from humans with UTIs) is not (22). $PapA_{31A}$ production decreased greatly (6%) when 1 mM leucine was added to M9 medium (Fig. 1), suggesting that the regulation of Pap_{31A} clearly differs from that of P fimbriae produced by *E. coli* strains that cause UTIs in humans.

Morphological observations. Negative-staining electron microscopy of whole cells of *E. coli* 31A/O6(20K−) cultured on MD-1 at 37°C revealed fine and flexible fimbria-like filaments (diameter, approximately 8 to 10 nm) (Fig. 2A). Gold immunolabeling assays showed that antibodies directed against the native $PapA_{31A}$ protein completely decorated the fimbriae on *E. coli* 31A/O6(20K−) grown on MD-1 (Fig. 2B), indicating that the purified $PapA_{31A}$ protein was the structural subunit of the fimbria-like filaments. These fimbriae were termed Pap_{31A}.

The low level of production of the Pap_{31A} fimbriae quantified as described above when the bacteria were cultured on MD-1 at 18°C (8% of the maximal production) was confirmed since the bacteria were not decorated (Fig. 2C). A similar result was obtained with the bacterial strain cultured on complex LB medium (data not shown).

In vitro adhesion on intestinal villi and hemagglutination. In vitro adhesion to calf and lamb intestinal villi and agglutination of erythrocytes from different species were investigated in order to determine the putative adhesive ability of Pap_{31A} fimbriae. *E. coli* 31A/O6(20K−) cultured on MD-1 medium at 37°C was unable to adhere in vitro to calf or lamb intestinal villi. This bacterial strain did not agglutinate with human blood (type A negative) or with erythrocytes from a calf, sheep, rabbit, horse, or rat. These results indicated that Pap_{31A} fimbriae did not mediate hemagglutination or adhesion of the bacteria to calf or lamb intestinal villi.

Nucleotide sequence of gene encoding the structural subunit and amino acid comparison. The gene that encodes the $PapA_{31A}$ structural subunit was amplified with a high-fidelity PCR system from *E. coli* 31A, and the $papA_{31A}$ nucleotide sequence was determined. Sequence analysis revealed that a polypeptide of 182 amino acid residues could be translated from the ATG codon at position 79 to the TAA codon at position 625. The signal peptidase cleavage site deduced from the NH_2-terminal amino acid sequence of $PapA_{31A}$ was located between residues Ala-21 and Ala-22 (Fig. 3).

Comparison of the deduced $PapA_{31A}$ amino acid sequence with those listed in the NBRF and SWISS-PROT data banks revealed significant homologies with different PapA-related fimbrial structural subunits. The highest degrees of homology were observed with the Prs-like $F165_1A$ (98%) and the $PapA_{IA2}$ (F11 serotype) (96%) fimbrial structural subunits (Fig. 3). A high degree of homology (80%) was observed with the PapA fimbrial subunit produced by *E. coli* J96 (F13 serotype) from a human with a UTI (Fig. 3). A significant degree of identity was also observed with the sequence of the PapA subunit expressed by *E. coli* strains of serotypes $F7_1$ (61%), $F7_2$ (60%), and F9 (57%) from humans with UTIs (data not shown).

P-related adhesin gene of *E. coli* 31A. *E. coli* 31A was negative by PCR for the three *papG* allelic variants. In order to determine whether strain 31A carries a *papG* gene on the chromosome, a nylon filter prepared with DNAs purified from

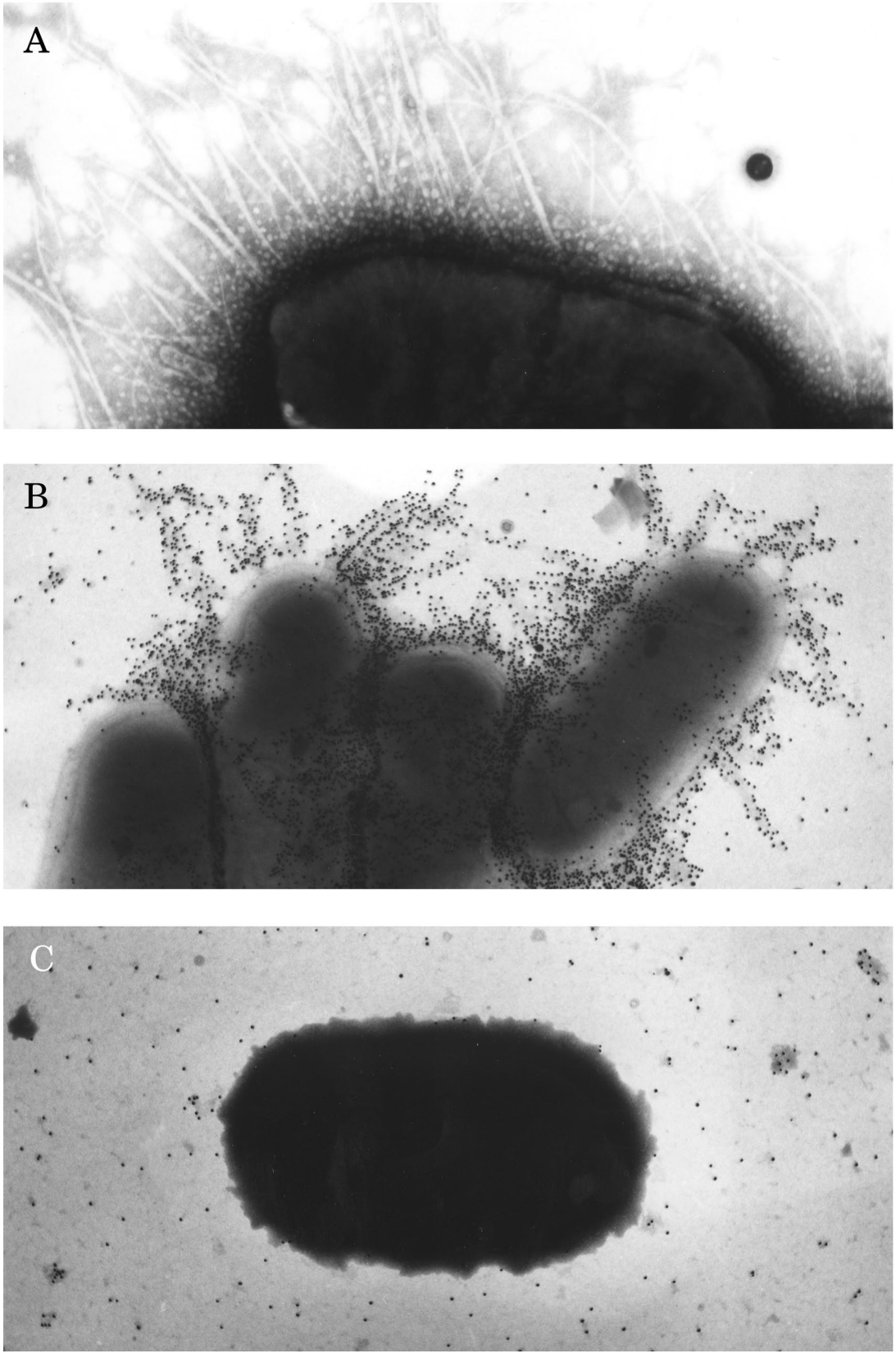

FIG. 2. Transmission electron micrographs of bacterial preparations. *E. coli* 31A/O6(20K−) cultured on minimal David medium (MD-1) at 37°C was negatively stained with 1% phosphotungstic acid (A) and labeled by the immunogold labeling technique with anti-$PapA_{31A}$ antibodies (B). (C) Strain 31A/O6(20K−) cultured on MD-1 at 18°C was labeled by immunogold labeling with anti-$PapA_{31A}$ antibodies. Magnifications: A, ×45,000; B, ×32,500; C, ×32,500.

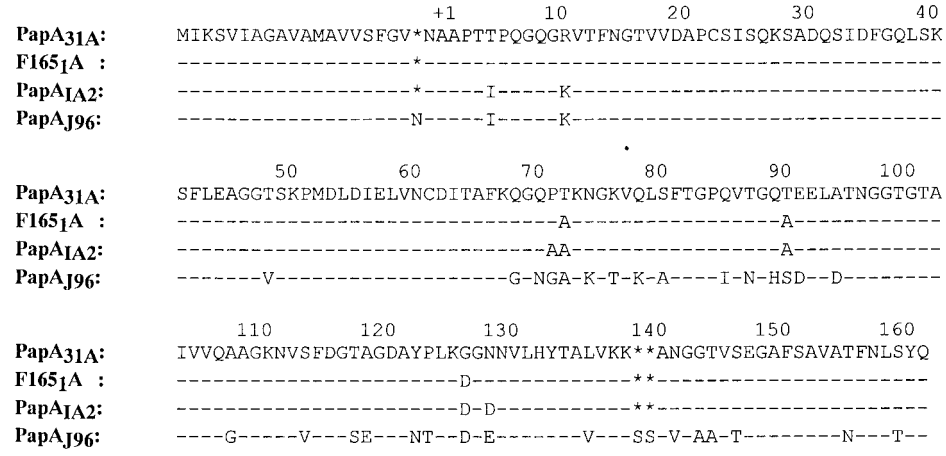

FIG. 3. Comparison of the deduced amino acid sequence of the structural subunit $PapA_{31A}$ with those of $F165_1$, Pap_{IA2}, and Pap_{J96} fimbriae. The first amino acid residue of the mature protein was numbered +1. Gaps (*) were introduced to obtain a maximal alignment. Identical amino acid residues are indicated by hyphens. The nucleotide sequence of the gene encoding the $PapA_{31A}$ protein is available from the GenBank data library under accession no. AF165981.

strain 31A and the plasmidless 31A/O6 strain was hybridized with a labeled probe specific for the $papG_{J96}$ adhesin gene. The DNA probe hybridized with *E. coli* 31A and 31A/O6, suggesting that a sequence homologous to the *papG* sequence was indeed present on the chromosomes of the strains and that the sequence probably represents a new *papG* variant.

In addition, the presence of genes located in different positions on the *pap* gene cluster was investigated. The DNA extracted from *E. coli* 31A was found to be positive by amplification for sequences specific for the *papI* gene located at the 5′ end of the *pap* gene cluster and for the *papE* and *papF* genes located immediately upstream of *papG* at the 3′ end of the *pap* gene cluster. These results strongly suggested that a complete *pap* gene cluster seems to be present on the bacterial chromosome.

DISCUSSION

Many microorganisms have the genetic capacity to express different adhesins, providing access to multiple receptors and therefore increasing their pathogenicities. In this report, the *pap* operon that encodes the P fimbriae and the *papG* genes that code for the three classes of PapG adhesin were investigated among human, ovine, and bovine *E. coli* strains that produce CS31A and/or F17 adhesins.

It is well documented that *pap*-positive strains are present in the fecal flora of 10 to 20% of healthy adults and children (2, 8, 42). We observed a similar percentage of *pap*-positive strains among the CS31A-negative *E. coli* strains isolated from hospitalized patients with sporadic diarrhea. This percentage increased significantly among diarrheagenic CS31A-positive strains (50%). However, only 23% of the *pap*-positive *E. coli* strains that produced CS31A were genetically associated with a PapG adhesin subunit of class II or class III. These results are in agreement with those of a study of human *pap*-positive strains which showed that fecal isolates carry new variants or unexpressed *papG* genes to a greater extent than urinary tract isolates (14). The human CS31A-producing strains in this study did not belong to the enterotoxigenic *E. coli*, enteropathogenic *E. coli*, enteroinvasive *E. coli*, or enterohemorrhagic *E. coli* bacterial groups generally associated with human diarrhea and do not possess the adhesive factors commonly associated with these pathogenic bacterial groups (23). However, CS31A promotes adhesion of the bacteria to the Caco-2 and Int-407 cell lines (12), suggesting a role for CS31A in bacterial adhesion to the bacteria to the human intestinal tract. Furthermore, human CS31A-positive isolates produced a Dr-related adhesin that recognizes the decay-accelerating factor present in both brush border enterocytes and urinary tract cells (27). We speculate that the simultaneous presence of CS31A, P, and Dr adhesins in human *E. coli* strains increases the pathogenicity of the strains first by allowing the colonization of the intestinal tract and then by permitting the spread of the bacteria to extraintestinal sites.

The ovine isolates were obtained from the intestinal contents of lambs with diarrhea for which a fatal acute renal failure was diagnosed (1). In accordance with the *E. coli* strains from humans with UTIs (24) but in contrast to the human and bovine intestinal isolates in this study, most *pap*-positive ovine strains (76%) were associated with an allelic variant of the PapG adhesin. Except for one strain, all the *pap*-positive ovine strains were genetically associated with a class II adhesin. The adhesin of class II preferentially binds GbO_4 globosides (the major isoreceptors on the human kidney) and is associated with acute pyelonephritis (24). It was of interest that the class II adhesin was associated with *E. coli* strains isolated both from lambs with severe renal tubular disease and from humans with acute pyelonephritis.

The highest prevalence of the *pap* operon (72%) was observed in bovine isolates that produced both CS31A and F17 adhesins. In contrast, 22 to 38% of the bovine *E. coli* strains that produced CS31A or F17 were associated with the *pap* operon. A high prevalence of P fimbriae has been described only among the strains that produced cytotoxic necrotizing factor type I (CNF1) and that were isolated from calves with septicemia or diarrhea (6, 32). The presence of a pathogenicity island (PAI) that included at least the *cnf1* and *pap* operons explains the high prevalence of P fimbriae among bovine CNF1-producing strains (J. P. Nougareyde, F. Herault, E. Jacquemin, J. De Ryckes, J. Mainil, and E. Oswald, Abstr. 96th Gen. Meet. Am. Soc. Microbiol. 1996, abstr. B-77, p. 168, 1996). However, the presence of a similar PAI could not explain the close association between CS31A, F17, and a *pap* operon among the bovine strains included in the study described in this report. Indeed, the genetic information necessary for the synthesis of CS31A is located on a large conjuga-

tive plasmid (18, 35), and the bovine strains tested do not produce CNF1. While the *papG* genes are not detected among fecal isolates collected from healthy cows (34), the class II adhesin was found to be genetically associated with the bovine isolates studied. These results suggested a role of class II adhesin in the bacterial infectious process. In this report, we demonstrated the exclusive association of the class II adhesin gene with bovine *E. coli* strains that produce both CS31A and F17. In contrast, it has been demonstrated that the class III variant was found to be associated with CNF1-producing strains isolated from pigs and calves with septicemia or diarrhea (6, 13). The different PapG allelic variants seemed to be associated with particular virulence factors produced by pathogenic *E. coli* strains in domestic animals.

In view of these results, it seemed of interest to undertake additional experiments to study the organization of the *pap* operon and the biochemical properties of putative P-related fimbriae produced by diarrheagenic *E. coli* strains. The bovine reference strain *E. coli* 31A has been chosen for this study because (i) *E. coli* 31A isolated in our laboratory from a calf with diarrhea was the reference strain for both CS31A and F17c adhesins (4, 5, 18, 35, 37), (ii) the highest prevalence of the *pap* operon was observed among bovine isolates that produced both CS31A and F17, and (iii) strain 31A was found to be genetically associated with the *pap* operon.

The gene that encodes the P structural subunit was amplified from *E. coli* 31A, and the nucleotide sequence of the $papA_{31A}$ gene was determined. When the deduced amino acid sequences were compared, $PapA_{31A}$ showed the highest degree of homology with the structural subunit of $F165_1$ and P (F11 serotype) fimbriae produced by *E. coli* strains from pigs with septicemia and humans with UTIs, respectively (33, 41). The presence of the *papA*, *papE*, *papF*, *papI*, and *papG* genes located in different positions in the gene cluster suggested the presence of a complete *pap* gene cluster on the 31A bacterial chromosome. However, the *papG* gene present on the chromosome of *E. coli* 31A was different from those of the three allelic variants described to date. This result strongly suggested the presence of additional *papG* variants different from those that have been described previously and that encode adhesin subunits able to recognize GbO_3, GbO_4, or GbO_5 isoreceptors. Characterization of the binding properties of *E. coli* 31A and nucleotide sequence analysis of this new *papG* variant would be of great interest.

Despite their genetic relatedness, the optimal conditions for the expression of Pap_{31A} were different from those for the expression of $F165_1$ and P fimbriae. In contrast to Pap_{31A}, the production of both P and $F165_1$ is controlled by catabolic repression (22, 36). Furthermore, as described for $F165_1$ (11) but not for P fimbriae of strains from humans with UTIs (7), Pap_{31A} production is reduced by addition of exogenous leucine. These results indicated that the regulation of Pap_{31A} production clearly differs from that of P-related fimbria production of strains from different hosts.

In addition to Pap_{31A}, *E. coli* 31A produces CS31A and F17 adhesins. However, expression of the three surface structures was subject to differential regulation. Indeed, in contrast to F17c, expression of both CS31A (36) and Pap_{31A} is regulated by addition of exogenous leucine and rich growth medium. Moreover, CS31A (but not Pap_{31A}) production is subject to catabolic repression by glucose (36). These results suggested different roles for the three fimbriae in different locations in the host tissues or in different physiological states.

Reference strain 31A produced flexible Pap_{31A} fimbria-like filaments. However, in vitro adhesion tests suggest the lack of functional receptors for P-related adhesins on bovine intestinal cells. Experimental oral infection of gnotobiotic calves with strain 31A causes septicemia with constant edema of the kidney and death of the animals in less than 48 h (9). These experiments demonstrate that strain 31A is able to persist in the bovine intestinal tract, colonize mucosal surfaces, and translocate to the mesenteric lymph nodes. It is well documented that $F165_1$ fimbriae are required for the full pathogenicity of *E. coli* strains in gnotobiotic pigs, not for initial colonization of the intestinal mucosa but for systemic bacterial persistence and resistance to phagocytosis (38). Furthermore, among human uropathogenic *E. coli* strains, P fimbriae are not only responsible for binding to and colonization of the urinary mucosa by *E. coli* (20) but also protect *E. coli* strains from the bactericidal activity of human polymorphonuclear neutrophils (40). We speculate that Pap_{31A} fimbriae could play a role in the resistance to phagocytosis or extraintestinal adherence of *E. coli* 31A.

In summary, the distribution of the *pap* operon among diarrheagenic *E. coli* strains that produce additional adhesive factors implicated in bacterial binding to intestinal cells suggested a role for P-related fimbriae in the spread of bacteria to extraintestinal sites. In addition, the *pap* operon was highly associated with bovine isolates that produce both CS31A and F17 adhesins. Therefore, a genotypic and phenotypic characterization of the P-related fimbriae produced by *E. coli* 31A assigned as the bovine reference strain for CS31A and F17c production was performed and demonstrated that (i) a complete *pap* gene cluster necessary for the synthesis of P-related fimbriae seems to be present in the bacterial chromosome, (ii) *E. coli* 31A contains a variant or a partial copy of the *papG* adhesin gene, and (iii) $PapA_{31A}$ is highly related to PapA and $F165_1$-A subunits but the environmental conditions for optimal production are different.

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