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The 987P Fimbrial Gene Cluster of Enterotoxigenic *Escherichia coli* Is Plasmid Encoded

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A clone containing the 987P fimbrial gene cluster was selected from a cosmid library of total DNA of the prototype *Escherichia coli* strain 987 by using 987P-specific antiserum. A subclone of 12 kilobases containing all of the genes required for fimbrial expression on a nonfimbriated K-12 strain of *E. coli* and a DNA fragment internal to the fimbrial subunit gene were used to probe the prototype strain and various isolates of 987P-fimbriated enterotoxigenic *E. coli*. All strains had several plasmids, as shown by agarose gel electrophoresis, and each of five strains which expressed 987P fimbriae showed a plasmid of 35 to 40 megadaltons (MDa) hybridizing to both 987P-specific probes. Hybridization to restricted DNA of strain 987 supported a plasmid origin for the cloned 987P gene cluster. Moreover, an isogenic strain which had lost its 35-MDa plasmid was no longer capable of synthesizing fimbrial subunits, but regained fimbrial expression after reintroduction of the Tn*phoA* (Tn*5* IS*50*$_L$::*phoA*)-tagged 35-MDa plasmid. Absence of fimbrial subunit synthesis in K-12 strains transformed with the 35-MDa plasmid alone suggested the requirement of regulatory elements existing in strain 987 but missing in K-12 strains. A probe for the heat-stable enterotoxin STIa hybridized in each of the 987P-fimbriated strains to the plasmid containing the 987P genes and in most of these strains to an additional plasmid which contained the gene for the heat-stable enterotoxin STII. Occurrence of the 987P and STIa genes on the same replicon correlates with epidemiological observations, STIa being the most prevalent toxin produced by 987P-fimbriated *E. coli*.

Human and animal strains of enterotoxigenic *Escherichia coli* (ETEC) have been shown to cause diarrhea by direct delivery of their toxins to host enterocytes after binding to brush borders by means of adhesive proteins (21). The best-documented adhesive structures on the cell surface of ETEC are fimbrial organelles (7). Ligand activity of fimbriae has been shown to be specific for complementary receptors on the mucosal surfaces of the susceptible host(s) (19). For example, K88 and 987P fimbriae mediate the attachment of ETEC strains to receptors on pig enterocytes (18). The resistance of older pigs to 987P-mediated diarrhea has recently been proposed to be due to the release of receptors in older pigs promoting the clearance of 987P-fimbriated ETEC strains (4). The dual effect of receptor-fimbria interaction permitting bacterial colonization as well as dissemination may be instrumental in optimizing virulence and contagiousness of 987P-fimbriated ETEC strains.

In most cases studied, the genes required for enterotoxin production and fimbrial expression of ETEC strains reside on large plasmids (36). Exceptions included the 987P and F41 fimbrial genes which were thought to be encoded on the chromosome (25). Although chromosomal location was confirmed for F41 (29), no precise studies were undertaken to locate the 987P fimbrial genes.

We traced the 987P fimbrial genes in five ETEC strains and in each case located them on a plasmid of at least 35 megadaltons (MDa). Moreover, we found that these same plasmids contained the gene for the heat-stable enterotoxin STIa, a transposon-encoded toxin (37, 38).

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MATERIALS AND METHODS

Bacterial strains and media. The *E. coli* strains used in this study are listed in Table 1. The seven 987P ETEC strains studied were isolated from cases of neonatal diarrhea of pigs in Hungary and kindly donated by B. Nagy. All strains were maintained at −70°C in L broth (24) containing 33% (vol/vol) glycerol. Cultures for colony isolations or plasmid purification were routinely grown in Luria agar or broth with appropriate antibiotics used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; gentamicin, 30 μg/ml. For experiments involving the evaluation of fimbriation, bacteria were cultured in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) under static conditions for several days (31).

Plasmid preparation and analysis. Plasmids used in this study are listed in Table 1. Plasmids were routinely prepared by a modification of the alkaline extraction procedure (35). In some cases, plasmids from 987P strains were isolated by ultracentrifugation to equilibrium in cesium chloride gradients with ethidium bromide (35). Plasmids of the various strains were separated by electrophoresis in 0.5 to 1% agarose and TBE buffer (22). Covalently closed circular (CCC), open circular, and linear forms of each plasmid were determined by two-dimensional agarose gel electrophoresis with a UV light irradiation step between the two runs (15). Plasmid size was determined by using the CCC form of plasmids pLAFR2, R68.45, and pMAR2 as molecular-weight standards (Table 1). DNA restriction and modification enzymes were purchased from New England BioLabs, Beverly, Mass. [^{32}P]dATP (800 Ci/mmol) was purchased from Du Pont Co., Wilmington, Del.

Cloning 987P fimbrial genes. Total DNA was extracted from the prototype 987P-fimbriated strain 987 and partially restricted with *Sau*3A (22). Fragments of 25 to 40 kilobases (kb) were ligated into the *Bam*HI site from cosmid pHC79

TABLE 1. Bacterial strains, plasmids, and bacteriophage

Strain, plasmid, or phage	Genotype or relevant characteristics	Reference(s) or source
E. coli		
987	O9:K103:987P (prototype F6)	18, 31
S-15/85	OX, 987P, STa	B. Nagy
S-17/85	OX, 987P, STa	B. Nagy
S-103/85	O101:K$^+$, 987P K99, STa	B. Nagy
S-278	OX, 987P, K99, STa	B. Nagy
S-285	OX, 987P, K99, STa	B. Nagy
S-224	O141:987P, STa	B. Nagy
S-289	O141:987P	B. Nagy
LE392	K-12 F$^-$ *hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*	22, 42
ORN103	K-12 F$^-$ *thr-1 leuB6 thi-1 lacY1 malA1 xyl-7 ara-13 mtlA2 gal-6 rpsL tonA2 minA minB* Δ*pil* Δ(*argF lac*)*U169 recA13*	32
MC1061	K-12 F$^-$ *araD139* Δ(*araABC leu*)*7679 galU galK* Δ*lacX74 rpsL hsdR*	3
MM294	K-12 F$^-$ *endA1 hsdR17 pro supE44 thi-1*	14
Plasmid		
pHC79	pBR322 with λ*cos*	17
pBR322	*ori* ColE1 Amp^r Tet^r	1
pDMS6	pBR322 with *Bam*HI-*Sph*I fragment of 987	This study
pLAFR2	13.7 Mda; pLAFR1 with polylinker in *Eco*RI site	9
R68.45	39.5 Mda	13
pMAR2	55 Mda	29
pDAS101	pUC8 with *Hin*fI fragment (STIa gene) of pRIT10036	29, 40
pUD1	pT7-5 with *Hin*dIII-*Mnl*I fragment (STII gene) of pCHL6	L. Dreyfus; 41
pPH1JI	*tra* IncP1 Cam^r Spc^r Gen^r Str^r	16
Bacteriophage		
λTn*phoA*	*b*221, *c*I857, *P*am3 Tn*phoA* in or near *rex*	11

(17). DNA constructs were introduced in lambda bacteriophage heads by using an in vitro packaging kit (Promega, Madison, Wis.). *E. coli* LE392 was transduced with packaged phages, and expression of 987P fimbrial antigen was detected by colony immunoblotting on nylon filters (Schleicher & Schuell, Inc., Keene, N.H.), using anti-987P fimbrial rabbit polyclonal antiserum (34), peroxidase-conjugated anti-rabbit antibodies (Organon Teknika, Malvern, Pa.), and 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, Calif.) as chromophore. 987P fimbrial expression on the bacterial surface was determined by seroagglutination with the polyclonal antiserum and by electron microscopy after transforming the nonfimbriated *E. coli* ORN103 with isolated cosmid from strain LE392. A 12-kb *Sph*I-*Bam*HI fragment was further subcloned in pBR322(pDMS6). Similar to a previously described 987P fimbrial gene cluster of another ETEC isolate (5), the subcloned *Sph*I-*Bam*HI fragment of the prototype 987P-fimbriated ETEC strain 987 present on pDMS6 contains all of the genes required for fimbrial expression, as determined by seroagglutination and electron microscopy (manuscript in preparation).

Hybridization probes. The following probes were used for 987P fimbrial genes: a 12-kb *Sph*I-*Bam*HI fragment of pDMS6 containing the whole gene cluster and a 175-base-pair (bp) *Bgl*II-*Spe*I fragment of pDMS6 containing an internal portion of the fimbrial subunit gene (6). Heat-stable enterotoxin probes were prepared from plasmids pDAS101 (40) and pUD1, which contains the *Hin*dIII-*Mnl*I fragment of the STII gene fragment of pCHL6 (20) inserted into the *Hin*dIII-*Sma*I polylinker sites from pT7-5 (41; L. Dreyfus, personal communication). pDAS101 contains the 157-bp *Hin*fI fragment from the STIa (STp) gene of pRIT10036 (30). The STIa DNA fragment was obtained by restriction of pDAS101 with *Eco*RI-*Hin*dIII, whereas an STII DNA fragment was obtained by digesting pUD1 with *Eco*RI-*Hin*dIII. All probes were linear DNA fragments isolated by gel electrophoresis and electroelution (22) and were labeled by random priming (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). A 20-mer oligonucleotide which served as a probe for *estA2*, the STIc toxin gene (12), was end labeled with T4 polynucleotide kinase (22). The two enterotoxin plasmids and the oligonucleotide were kind gifts from S. Moseley, L. Dreyfus, and Y. Kupersztoch, respectively.

Southern blot hybridization. Southern transfer to nylon membrane and hybridization were performed by conventional techniques (22).

Transposon tagging. The large plasmids of the 987P-fimbriated prototype strain were labeled with a kanamycin resistance determinant by transposition of Tn*phoA* (Tn*5* IS*50*$_L$::*phoA*) (23). The transposon was delivered by transduction of strain 987 with λTn*phoA* as described previously (11). Successfully transduced cells were selected on L agar containing low levels of kanamycin (20 μg/ml). Tn*phoA* transposition in plasmids was monitored by agarose gel electrophoresis of plasmid preparations of independently transduced cells of strain 987.

Transformation and conjugation. Plasmids were introduced in recipient cells by either transformation (35) or conjugation (24). The presence of the respective plasmids was confirmed by agarose gel electrophoresis.

Seroagglutination. Slide agglutinations were performed with a rabbit anti-987P fimbrial antiserum (34) preadsorbed with a nonfimbriated mutant of strain 987 (see Results).

Electron microscopy. Formvar-coated copper grids were placed on drops of bacteria for 3 min and subsequently on drops of 0.5% phosphotungstic acid for negative staining (2 min). For immunoelectron microscopy, grids with fimbriated bacteria were successively placed on drops of 0.5% bovine serum albumin (3 min), a 2×10^{-2} dilution of rabbit anti-987P fimbrial antiserum in phosphate-buffered saline (20

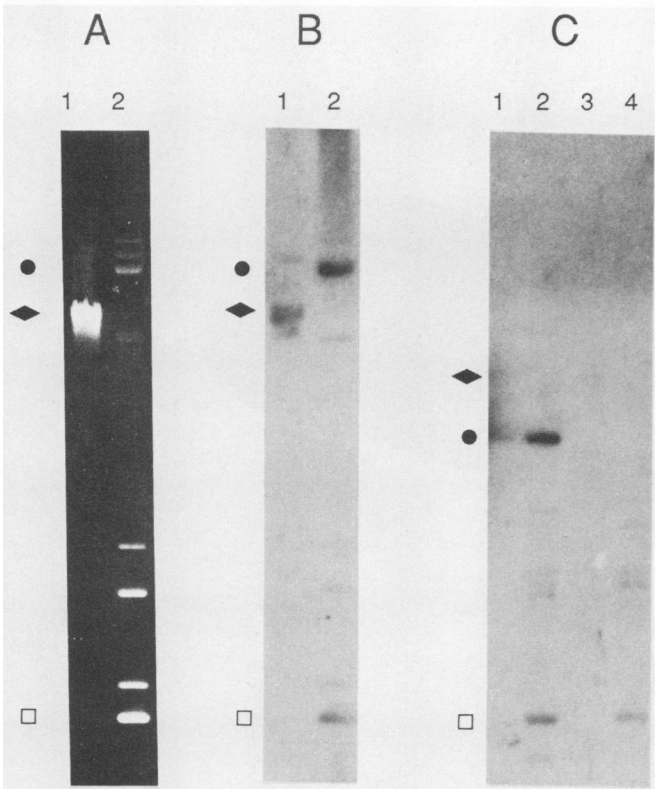

FIG. 1. Localization of the 987P fimbrial gene cluster in various DNA preparations of strain 987 and its isogenic mutant cured of the 35-MDa plasmid. (A) Agarose gel electrophoresis of unrestricted DNA; (B) Southern blot analysis of unrestricted DNA, using the 175-bp internal DNA fragment of the fimbrial subunit as probe; (C) Southern blot analysis of *Sph*I-*Bam*HI restricted DNA, using the same probe as in panel B. Lane 1, Total DNA of strain 987; lane 2, plasmid DNA of strain 987; lane 3, total DNA of the mutant; lane 4, plasmid DNA of the mutant. Symbols: (●) 35-MDa plasmid; (◆) randomly linearized total DNA; (□) small plasmid (see text).

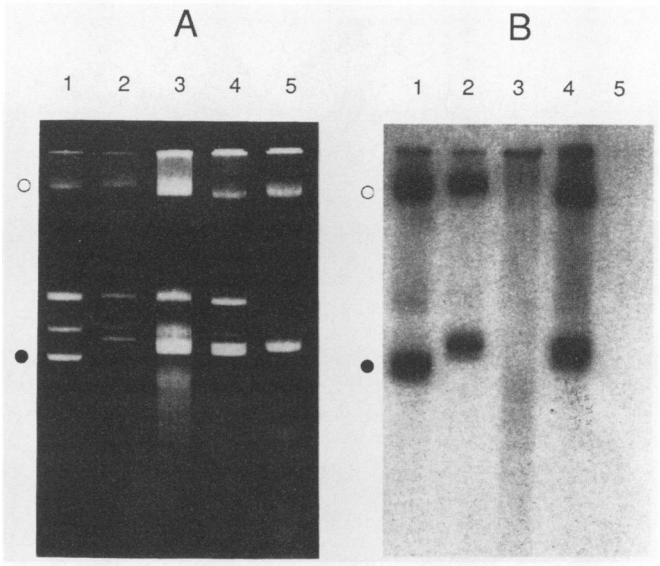

FIG. 2. Plasmid profiles of strain 987 and its mutants. (A) Agarose gel electrophoresis; (B) Southern blot analysis with the 175-bp internal DNA fragment of the fimbrial subunit. Lane 1, Strain 987; lane 2, mutant with Tn*phoA*-tagged 35-MDa plasmid; lane 3, mutant cured from the 35-MDa plasmid with pPH1JI; lane 4, cured mutant transformed with the Tn*phoA*-tagged 35-MDa plasmid; lane 5, pPH1JI. Symbols: (○) open circular and (●) CCC forms of the 35-MDa plasmid.

min), phosphate-buffered saline (six short washes), a 2.5×10^{-2} dilution of protein A-colloidal gold conjugates (10-nm gold particles; Structure Probe Inc., West Chester, Pa.) in phosphate-buffered saline (20 min), phosphate-buffered saline (six short washes), and 0.5% phosphotungstic acid. Electron micrographs were taken with a JEOL-1200EX electron microscope.

Western blotting. Total proteins of 0.5 ml of stationary phase-grown bacteria were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after boiling the pelleted bacteria for 5 min in 100 μl of sample buffer at pH 1.5 to optimize fimbrial dissociation. Western blotting (immunoblotting) of 987P fimbrial protein was performed as described previously (34), using rabbit anti-987P fimbrial antiserum and peroxidase-conjugated goat anti-rabbit immunoglobulin G.

RESULTS

Localization of the 987P fimbrial gene cluster in the prototype strain. To determine the presence of one or more plasmids in ETEC strain 987, cesium chloride-isolated plasmid DNA was subjected to various conditions of agarose gel electrophoresis for small or large plasmids. Several plasmid bands could be detected (Fig. 1A, lane 2). The CCC forms of at least seven plasmids were distinguished from open circular forms (15) after two-dimensional gel electrophoresis (data not shown). The molecular masses of the four largest plasmids were estimated to be 35, 46, 51, and >55 MDa, respectively. The DNA band for the largest plasmid was faint and not discernible on all gels. Separated plasmids were hybridized to 987P fimbrial DNA by Southern blot analysis. The 12-kb probe including the complete fimbrial gene cluster, as well as the 175-bp probe specific for the fimbrial subunit (Fig. 1B, lane 2), hybridized mainly to the 35-MDa plasmid and to a lesser extent to one of the smaller plasmids. When total DNA was analyzed by Southern blot, in addition to the plasmid band, both probes hybridized also to the randomly linearized total DNA band (Fig. 1A and B, lane 1). To determine the origin of the cloned 987P fimbrial gene cluster in pDMS6, we evaluated hybridization of the 12-kb *Sph*I-*Bam*HI probe and the 175-bp fimbrial subunit probe (Fig. 1C, lanes 1 and 2) to *Sph*I-*Bam*HI-restricted total and plasmid DNA. Both probes hybridized to a band of 12 kb in restricted plasmid and total DNAs, indicating that the cloned gene cluster came from the 35-MDa plasmid. A second faint hybridizing band of larger size and only detectable in restricted total DNA suggested the existence of chromosomal DNA homologous to 987P fimbrial genes residing on the plasmid. Finally, we utilized the 175-bp fimbrial subunit probe to hybridize to restricted DNA from an isogenic nonfimbriated strain cured from its 35-MDa plasmid (see below) and were unable to detect the strong signal found with the 12-kb plasmid fragment of strain 987 (Fig. 1C, lanes 3 and 4). These experiments showed that the subcloned 987P gene cluster in pDMS6 originated from the 35-MDa plasmid. Because K-12 strains of *E. coli* with pDMS6 are 987P fimbriated, our data indicated also that the expressing 987P fimbrial genes are on the 35-MDa plasmid.

Curing the 35-MDa plasmid from strain 987 and reintroducing it into the cured strain. The 35-MDa plasmid is stably

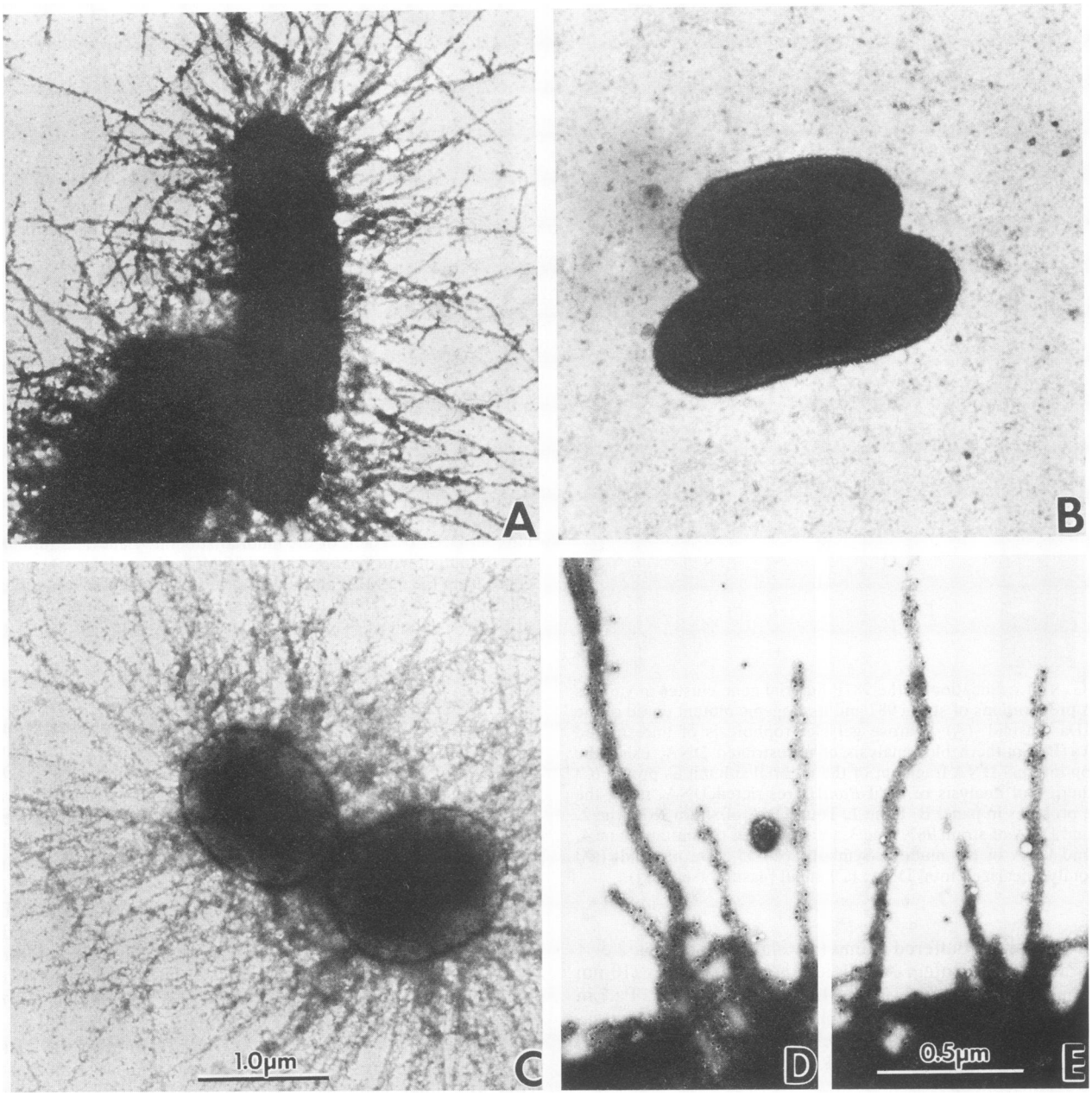

FIG. 3. Electron micrographs of negatively stained mutant of strain 987. (A) Mutant with Tn*phoA*-tagged 35-MDa plasmid; (B) mutant cured from the 35-MDA plasmid with pPH1JI; (C) cured mutant transformed with the Tn*phoA*-tagged 35-MDa plasmid. Bar, 1 μm for panels A to C. Immuno-gold-labeled fimbriae of (D) the mutant with the Tn*phoA*-tagged plasmid and (E) the cured mutant transformed with the Tn*phoA*-tagged plasmid. Bar, 0.5 μm for panels D and E.

maintained in strain 987. This strain kept its 35-MDa plasmid even after several culture passages on agar media which favor outgrowth of negative-phase variants. Neither heat nor subminimal inhibitory concentrations of ethidium bromide cured strain 987 of the 35-MDa plasmid at a detectable level (<1%). In contrast, we discovered some incompatibility between the 35-MDa and pPH1JI (Gm^r, IncP group) plasmids. Strain 987 displayed a strong tendency to lose the 35-MDa plasmid after introduction of pPH1JI by conjugation. Loss of this plasmid paralleled loss of plasmid-encoded 987P fimbrial DNA, as shown by Southern blot analysis (Fig. 2, lane 3; Fig. 1C, lanes 3 and 4). More importantly, genotypic loss correlated with phenotypic loss, as demonstrated by seroagglutination and electron microscopy of cured strain 987 grown in static broth (Fig. 3B). To prove that the lost phenotype was due only to the loss of the 35-MDa plasmid, we tested whether fimbrial expression could be regained by reintroducing the 35-MDa plasmid into the cured strain. For this purpose, the 35-MDa plasmid was first tagged with the kanamycin-resistant transposon Tn*phoA* (Fig. 2, lanes 1 and 2). By selecting a 987P-fimbriated kanamycin-resistant mutant (seroagglutination positive), we knew that Tn*phoA* had inserted outside of the fimbrial gene cluster (Fig. 3A). The 35-MDa plasmid with

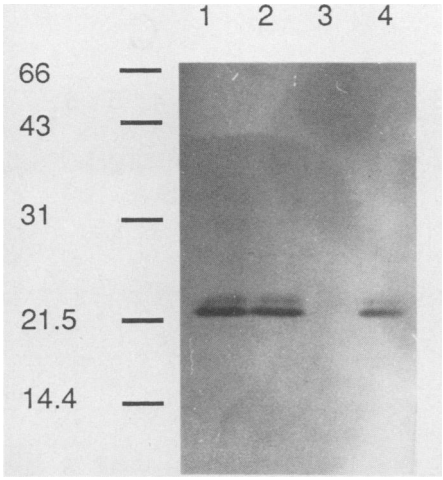

FIG. 4. Western blot of strain 987 and its mutants probed for 987P fimbrial subunit protein. Lane 1, Strain 987; lane 2, mutant strain with Tn*phoA*-tagged 35-MDa plasmid; lane 3, mutant strain cured from the 35-MDa plasmid with pPH1JI; lane 4, cured mutant strain transformed with the Tn*phoA*-tagged 35-MDa plasmid. Molecular weights (10^3) are given on the left. The fimbrial subunits were incompletely reduced, resulting in the separation of the reduced (upper band) and the unreduced (lower band) forms (34).

Tn*phoA* was isolated and introduced by transformation into strain 987 previously cured from the 35-MDa plasmid as described above (Fig. 2, lane 4). The resulting strain was cultured in static broth to promote 987P fimbrial expression, which was detected by seroagglutination. Electron microscopic examination showed no differences among strain 987, strain 987 with the Tn*phoA*-tagged plasmid (Fig. 3A), and cured strain 987 transformed with the Tn*phoA*-tagged plasmid (Fig. 3C). 987P-specific immunogold labeling of the fimbriated strains, using rabbit anti-987P fimbrial antibodies, also showed no differences (Fig. 3D and E). Therefore, even though previous Southern blot analysis suggested the presence of chromosomally encoded 987P fimbrial genes, these results confirmed that the 35-MDa plasmid contains all the genes responsible for 987P fimbriation.

Western blot analysis of 987 derivatives. Because 987P fimbrial antibodies have been shown previously to cross-react partly with one or more type 1 fimbrial epitopes (S. N. Abraham, D. M. Schifferli, D. Banks, and E. H. Beachey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, D109, p. 84), we needed to ensure that the previously described experiments were specific for 987P fimbriae. A clear size difference between the molecular mass of the type 1 fimbrial subunit (17,000 kDa) and that of the 987P fimbrial subunit (20,000 to 23,000 kDa) can be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We therefore analyzed strain 987, strain 987 with the Tn*phoA*-tagged plasmid, cured strain 987, and cured strain 987 transformed with the Tn*phoA*-tagged plasmid by Western blot, using rabbit anti-987P fimbrial antiserum and peroxidase-conjugated anti-rabbit anti-immunoglobulin G as second antibody (Fig. 4). The molecular mass of the reduced fimbrial subunit was 23,000 kDa for all three fimbriated strains, demonstrating that the seroagglutination-positive phenotype of these strains was indeed due to expression of 987P fimbriae.

Seroagglutination and hybridization profile of plasmids from various 987P fimbriated isolates. In addition to strain 987, seven ETEC isolates originally diagnosed as 987P fimbriated were grown under optimal conditions for fimbriation. Four of the seven isolates expressed 987P fimbriae, as demonstrated by seroagglutination (strains S-15/85, S-17/85, S-278, and S-224). All seven isolates had several plasmids, as shown by agarose gel electrophoresis, and the plasmid profiles differed from that of strain 987 (Fig. 5A). Plasmids were hybridized to the 175-bp or the 12-kb probe as described above. Each of the four strains positive by seroagglutination possessed a plasmid of 35 to 40 MDa which hybridized to both probes (Fig. 5B). Plasmids of similar molecular mass could not be observed for the three strains which did not express fimbriae. Moreover, none of the remaining plasmids of these three strains hybridized to the 987P DNA probes. Because these three strains had originally been isolated and determined to be 987P fimbriated, we suspect they have lost their 987P plasmid upon repeated in vitro subcultures. The complete agreement between the seroagglutination and hybridization results suggest that the genes responsible for 987P fimbriation of ETEC strains are plasmid encoded in most cases, if not always.

Hybridization of plasmids to DNA of various heat-stable toxins. Because enterotoxin genes of ETEC strains are usually present on plasmids, we wondered whether they were localized with the 987P genes on the same plasmids. We therefore used three different enterotoxin probes to analyze the hybridization pattern after Southern transfer of plasmid preparations of the eight ETEC strains examined in this study. The *Eco*RI-*Hin*dIII fragment of pDAS101 used as a probe showed that only the five strains which were positive with the 987P probes hybridized with the STIa probe (Fig. 5B and C). Moreover, all five strains possessed at least two copies of the STIa gene on each of two different plasmids. The STIc oligonucleotide probe did not hybridize to any of these strains even under conditions of reduced stringency. In contrast, the STII probe consisting of the *Eco*RI-*Hin*dIII fragment of pUD1 hybridized to four of the five strains positive with the 987P and STIa probes (Fig. 6). In these four strains, the plasmids hybridizing to the STII probe were the same ones that hybridized to the STIa probe but not to the 987P probes. These results indicated that enterotoxin genes are confined to two plasmids, one also containing 987P fimbrial genes.

Introduction of tagged 35-MDa plasmid into K-12 strains. We have shown above that the 35-MDa plasmid contains the complete fimbrial gene cluster present on pDMS6, which elaborates 987P on the surface of K-12 strains and therefore carries the specific genes required for 987P fimbrial expression (fimbrial subunit synthesis, transport, anchorage, and assembly) on K-12 strains of *E. coli*. To see whether the 35-MDa plasmid itself would confer the same 987P-specific fimbriation on K-12 strains as pDMS6, we introduced the Tn*phoA*-tagged 35-Mda plasmid into either MC1061 or ORN103 by transformation or conjugation. In contrast to pDMS6-transformed cells, we found that the two 35-MDa recipient strains were not 987P fimbriated, although both strains were cultured under optimal conditions for fimbrial expression and contained the plasmid as detected by agarose gel electrophoresis (data not shown). This result may suggest that the expression of 987P fimbriae is controlled by a regulatory pathway found only in the natural host of the 35-MDa plasmid. Because no fimbrial subunit synthesis was detectable by Western blot analysis of the two strains containing the Tn*phoA*-tagged 35-Mda plasmid (data not shown), regulation would act primarily on the production of fimbrial subunits.

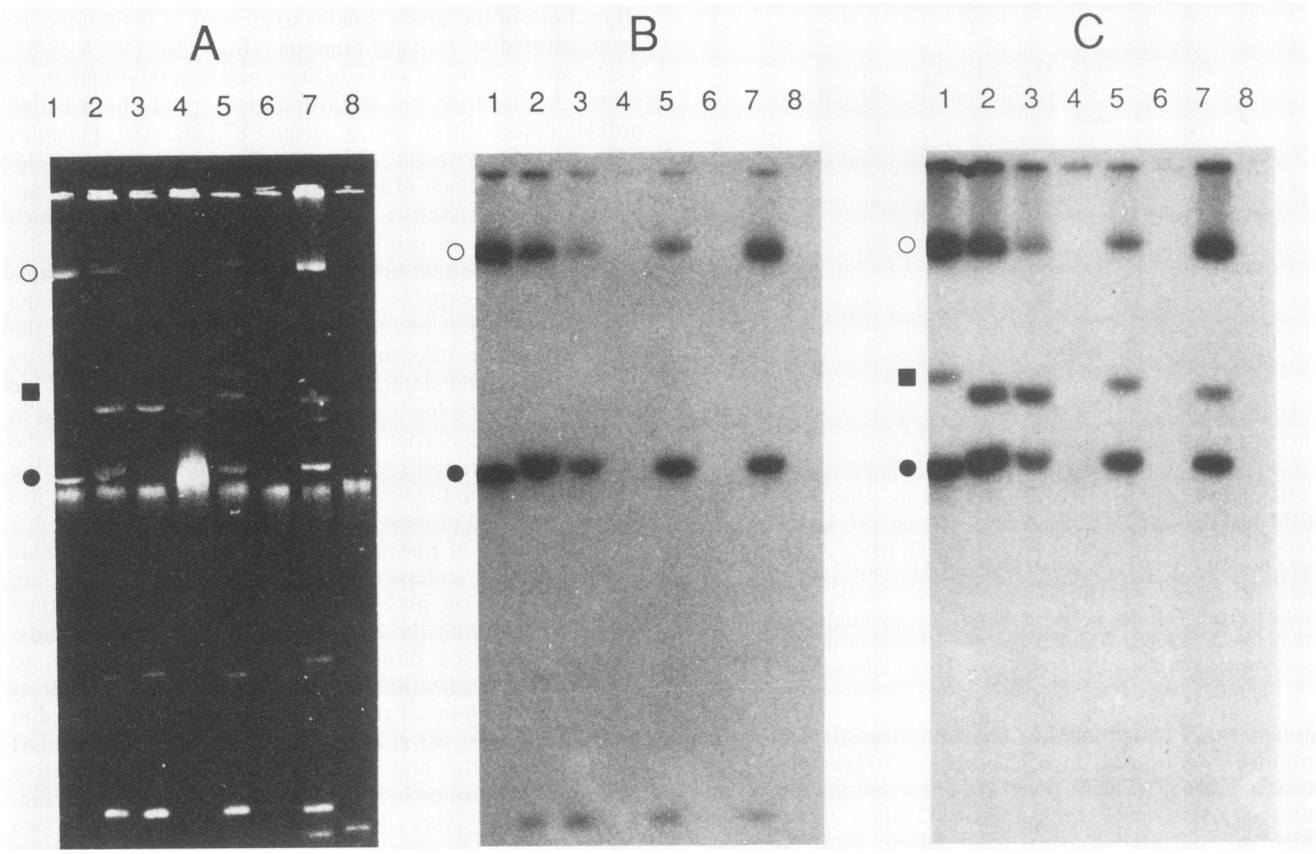

FIG. 5. Hybridization profile of plasmids of various 987P-fimbriated ETEC isolates. (A) Agarose gel electrophoresis of plasmid DNA preparations; (B) Southern blots of plasmid DNA with the 12-kb 987P fimbrial probe; (C) Southern blots of plasmid DNA with the STIa probe. Lane 1, Strain 987; lane 2, S-15/85; lane 3, S-17/85; lane 4, S-103/85; lane 5, S-278; lane 6, S-285; lane 7, S-224; lane 8, S-289. Symbols: (○) open circular and (●) CCC forms of the 35-MDa plasmid; (■) CCC form of the 51-MDa plasmid.

DISCUSSION

Molecular and genetic approaches were used to demonstrate that the expressed fimbrial gene cluster of the 987P-fimbriated prototype ETEC strain resides on a plasmid of 35 MDa. In addition, other 987P-fimbriated strains analyzed in this study were each shown to contain a large plasmid with genes for 987P fimbriae and heat-stable enterotoxin STIa.

Plasmid localization for 987P genes was originally suspected by Morrissey and Dougan, who mentioned having utilized a complete cosmid containing 987P genes as a probe (28). Because the observed hybridization could have arisen from homologous regions of the cosmid other than 987P DNA, the authors could draw no conclusions as to the significance of their findings. By using not only the complete 987P fimbrial gene cluster but also a section of the fimbrial subunit as probes, we could show that 987P genes in 987P-fimbriated ETEC strains were found on plasmids. Simultaneous studies undertaken by other authors described similar results (R. A. Schneider and T. A. Casey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, D116, p. 101) but called attention to possible chromosomal sequences homologous to 987P genes (T. A. Casey, R. A. Schneider, and E. A. Dean, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, D115, p. 101). Even though we observed hybridization signals with DNA from a small plasmid and the chromosome of strain 987, the diagnostic size of the hybridizing restriction fragment of plasmid DNA clearly situated the 987P fimbrial gene cluster on a plasmid of 35 MDa. Moreover, 987P-specific fimbriation depended on the presence of this plasmid in strain 987, as shown by loss and reappearance of fimbriation and fimbrial subunit synthesis after, respectively, removal of the plasmid and its reintroduction into the strain. The relevance of the hybridization signals shown by chromosomal DNA and the small plasmid remains to be clarified.

Further investigations with various 987P-fimbriated ETEC strains and enterotoxin probes allowed us to show that a copy of the STIa gene located always on the same plasmid as the 987P gene cluster. Interestingly, the structural link between STIa and 987P DNA correlated with the epidemiological observation of phenotypic linkage (8, 10, 26, 39, 43), STIa being the most prevalent toxin produced by 987P-fimbriated *E. coli*. In the strains analyzed in our study, additional STIa and STII genes were contained in another plasmid, allowing us to distinguish at least two plasmids each carrying genes for two virulence factors. Although we do not know whether the strains we studied produce both enterotoxins STIa and STII, strains with genes for both toxins are not unusual among ETEC strains (27) and production of both toxins is frequent, especially among 987P-fimbriated ETEC strains (39). The STIa gene has been described in many strains to be encoded by transposon Tn*1681*, which is flanked by IS*1* elements (37, 38). The occurrence of a transposition event preceding strain and plasmid dissemination could explain the presence of an STIa copy on each of

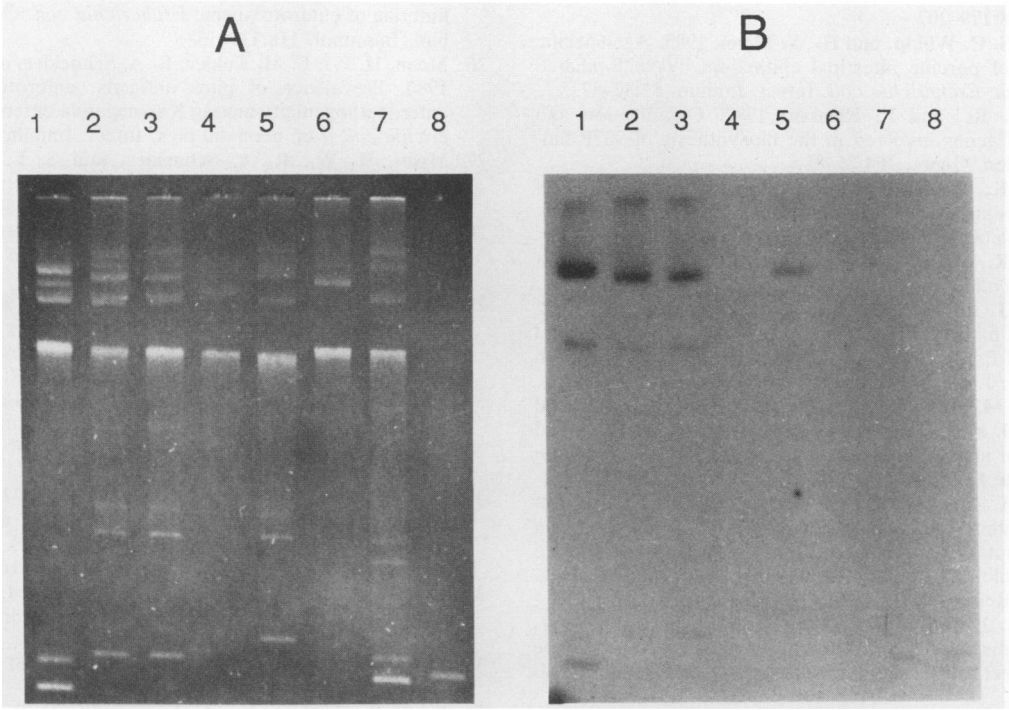

FIG. 6. Hybridization profile of plasmids of various 987P-fimbriated ETEC isolates with the STII probe. (A) Agarose gel electrophoresis; (B) Southern blot. Lane 1, Strain 987; lane 2, S-15/85; lane 3, S-17/85; lane 4, S-103/85; lane 5, S-278; lane 6, S-285; lane 7, S-224; lane 8, S-289.

two plasmids in all of the strains we studied. All of our strains were negative for STIc, an expected result for an enterotoxin isolated from a human ETEC strain (12, 27).

The stability of plasmids carrying 987P genes varies among ETEC strains. Although plasmids containing 987P and enterotoxin genes of isolates such as strain 987 appear to be stably inherited under in vitro conditions, plasmid loss may explain why we were unable to detect fimbriation after in vitro subcultures of three strains originally diagnosed as 987P fimbriated. Other authors have described nonfimbriated isolates that produce only STII and are unable to induce diarrhea (26, 39). As proposed by Söderling et al. (39), these strains may have lost a plasmid(s) encoding fimbrial adhesins. Maintenance of plasmids in these strains probably requires the selective pressure of an intestinal milieu, in which fimbria-mediated surface colonization assists bacterial multiplication.

Plasmid loss cannot explain why certain clinical isolates of 987P-fimbriated ETEC strains, which become nonfimbriated under in vitro conditions, regain 987P fimbriation upon inoculation of piglets (8, 26, 31). In these cases, environmental signals must regulate this phenotypic switch, either by interacting through the plasmid-encoded fimbrial gene cluster or, as recently postulated (Casey et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1989), by using chromosomal copies of 987P fimbrial genes. Obviously, the unique and specific conditions encountered in the pig intestines would be prerequisite for turning on fimbrial expression.

Fimbrial expression of K-12 strains containing plasmid pDMS6 with 12 kb of the 35-MDa plasmid of strain 987 is constitutive. On the other hand, the same K-12 strains with the complete 35-MDa plasmid neither express 987P fimbriae nor synthesize fimbrial subunits. Various regulatory elements for fimbriation of ETEC strains have been studied recently (2; A. Darfeuille-Michaud, C. Forestier, B. Joly, and R. Cluzei, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, D114, p. 101). Although many alternative regulatory circuits may be speculated for the 987P system in strain 987, one possibility may be the presence of a negative regulatory element encoded by the 35-MDa plasmid controlling fimbrial expression by acting in concert with one or more *trans*-active elements encoded by natural host DNA. Such an element would be missing in the cloned fimbrial gene cluster present in pDMS6. Another possibility is the presence of undetected DNA rearrangements that occur in the 35-MDa plasmid when it is introduced into *E. coli* K-12 strains, resulting in loss of fimbrial expression. We are currently evaluating control of 987P fimbrial expression in its natural host.

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