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Identification of Plasmid and Chromosomal Copies of 987P Pilus Genes in Enterotoxigenic *Escherichia coli* 987

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A DNA probe derived from the subunit gene of a cloned 987P determinant was used to characterize the locations of the 987P genes in several *Escherichia coli* strains. We examined *E. coli* 987, a nonpiliated mutant of strain 987 (I36) that does not express 987P in vitro, and a variant of I36 that expressed 987P following growth in vivo. We found that plasmid and chromosomal copies of the 987P subunit gene could be differentiated in strain 987 by restriction endonuclease analysis and Southern blot hybridization. The nonpiliated mutant I36 had lost the plasmid copy but retained the chromosomal copy of the 987P gene. A 987P-piliated variant of I36, which did not contain the 987P plasmid, colonized and caused diarrhea in neonatal pigs similarly to wild-type 987. The plasmid that hybridized with the 987P probe was transferred from strain 987 to an *E. coli* K-12 strain by conjugation. We were unable to demonstrate expression of 987P by these transconjugants. The data suggest that the chromosomal and plasmid copies of the 987P genes may interact to influence 987P expression.

Enterotoxigenic *Escherichia coli* causes diarrhea by adhering, colonizing, and producing enterotoxins in the small intestine. The filamentous surface structures which mediate adherence of enterotoxigenic *E. coli* in the intestine are called fimbriae, pili, or adhesins. Enterotoxigenic *E. coli* strains that express 987P pili frequently cause diarrhea in neonatal swine. Expression of 987P pili is subject to spontaneous, rapid, and reversible on-and-off switching called phase variation. Phase variation of 987P has been reported to occur in response to growth under specific environmental conditions (13, 14).

The genes required for 987P expression have been cloned and characterized by several groups by using total bacterial DNA from 987P$^+$ strains, but the location of the 987P genes is controversial (3, 4, 6, 7, 12, 15–17). Klaasen et al. (7) reported that the 987P genes are chromosomally encoded in a number of 987P$^+$ isolates because plasmid DNA from these strains did not hybridize with a DNA probe for the 987P subunit gene. In contrast, Schifferli et al. (15) reported that the genes for 987P are located on a plasmid in strain 987 and several other 987P$^+$ strains.

In this study, we used a probe derived from the 987P subunit gene and found both plasmid and chromosomal copies of the 987P subunit gene in strain 987. Furthermore, we identified a mutant of strain 987 that lacked the plasmid 987P genes but expressed 987P pili, colonized, and caused diarrhea in neonatal pigs.

A 540-bp *Hpa*I-*Bgl*II fragment from pPM200, which encodes expression of 987P pili (12), was subcloned and used as a subunit probe for 987P. The DNA sequence of this fragment was determined (not shown) and found to be identical to the sequence of an *Hpa*I-*Bgl*II fragment from the 987P subunit gene sequence reported by de Graaf and Klaasen (4). By colony blot hybridizations as previously described (8), the 987P subunit probe hybridized with strain 987 and strain I36, a spontaneous mutant of strain 987 which is stably nonpiliated in vitro (5).

Although strain I36 does not express 987P pili in vitro, even under growth conditions that promote piliation (5), piliated phase variants of strain I36 have been isolated following growth in vivo (10). We isolated a piliated I36 variant (I36P$^+$) from a neonatal pig orally inoculated with nonpiliated strain I36. A colony immunoblot assay and slide agglutination with 987P-specific antiserum were used to differentiate piliated and nonpiliated bacterial cultures (2, 13, 14). We confirmed that the piliated variant, I36P$^+$, was identical to the nonpiliated parental strain, I36, by comparison of total cellular DNA restriction enzyme digestion patterns for seven different enzymes (data not shown). Plasmid DNA from strain I36P$^+$ was isolated by the alkaline lysis method (1) and compared with nonpiliated strain I36 and with wild-type piliated and nonpiliated strain 987. Plasmid DNA was separated by agarose gel electrophoresis, blotted, and hybridized with the 987P subunit probe by standard methods (9). Strain 987 contained four large plasmids ranging from 50 to 150 kb in size (Fig. 1A). In strain 987, the 50-kb plasmid hybridized with the 987P subunit probe (Fig. 1B, lanes 1 and 2). This plasmid was missing in strain I36 (Fig. 1B, lanes 3 and 4), indicating that I36 was a

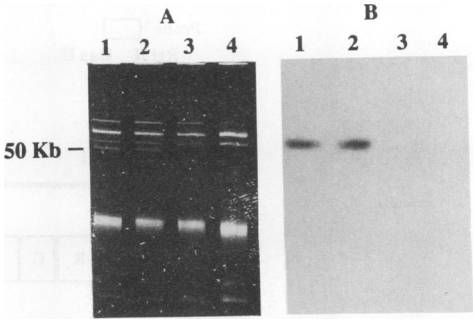

FIG. 1. Plasmid DNA isolated from strains 987 and I36 in the piliated and nonpiliated phases. (A) Agarose gel (1%) electrophoresis of intact plasmid DNA; (B) Southern blot of this gel hybridized with the 540-bp *Hpa*I-*Bgl*II fragment used as a 987P subunit probe. Lanes: 1, piliated strain 987; 2, nonpiliated strain 987; 3, nonpiliated strain I36; 4, piliated strain I36.

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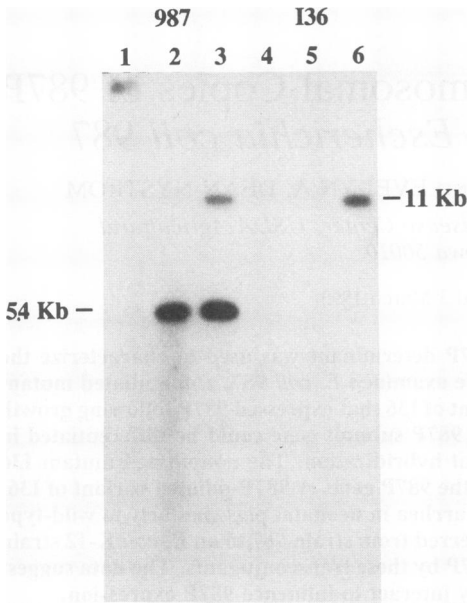

FIG. 2. Differentiation of the plasmid and chromosomal 987P genes by Southern blotting of plasmid and total cellular DNAs isolated from strains 987 and I36, separated by agarose gel electrophoresis, and hybridized with the 987P subunit probe. Lanes: 1, undigested plasmid DNA from strain 987; 2, plasmid DNA from strain 987 digested with *Cla*I; 3, total cellular DNA from strain 987 digested with *Cla*I; 4, undigested plasmid DNA from strain I36; 5, plasmid DNA from strain I36 digested with *Cla*I; 6, total cellular DNA from strain I36 digested with *Cla*I.

spontaneously plasmid-cured mutant of strain 987. There were no differences between plasmids from strain 987 in the piliated or nonpiliated phase (Fig. 1A, lanes 1 and 2) and no difference in plasmid content between piliated and nonpiliated strain I36 (Fig. 1A, lanes 3 and 4). We also examined plasmid DNA isolated from I35, an independent spontaneous mutant of strain 987 which, like I36, is nonpiliated in vitro (5). We found that strain I35 did not contain the 50-kb plasmid that hybridized with the 987P probe (data not shown).

To further characterize the location of the 987P subunit genes in strains 987 and I36, restriction endonuclease digestions of plasmid and total cellular DNAs isolated from these two strains were compared by Southern blot hybridization using the 987P subunit probe. The 987P subunit probe hybridized with a 5.4-kb *Cla*I fragment present in plasmid DNA from strain 987 and with an 11-kb *Cla*I fragment in total DNA from I36 (Fig. 2). Both of these fragments are present in total DNA isolated from strain 987. In similar experiments, the plasmid and chromosomal 987P genes could also be differentiated with *Eco*RI, *Bam*HI, *Sph*I, or *Sma*I. In each case, one band in plasmid DNA from strain 987 and a distinct band in total cellular DNA from I36 hybridized with the 987P subunit probe. Both of these bands were present in total cellular DNA from strain 987 (data not shown). Restriction endonucleases that did not differentiate the plasmid and chromosomal 987P genes were used to construct a restriction map of the common fragments that hybridized with the 987P subunit probe in plasmid DNA from strain 987 and total cellular DNA from strain I36 (Fig. 3A). Restriction sites for the enzymes we used, distal to those shown in Fig. 3A, diverged between plasmid and chromosomal fragments that hybridized with the 987P subunit probe. This map is compared with the restriction map of a cloned 987P determinant and the position of the 987P genes reported by Schifferli et al. (16, 17) (Fig. 3B). Although there are small differences in sizes of some of the fragments, all of the sites are in the same relative positions. The similarity of these restriction maps suggests that all genes associated with expression of 987P are present in both the plasmid and the chromosome of strain 987.

The piliated I36P$^+$ variant was compared with nonpiliated strain I36 and piliated wild-type strain 987 for the ability to adhere and cause diarrhea. Neonatal pigs were inoculated intragastrically as previously described (11, 13, 14). Table 1 shows that I36P$^+$ colonized and caused diarrhea with weight loss similar to that caused by the wild-type parent. In addition, strains 987 and I36P$^+$ caused a fatal infection in some animals because of dehydration and weight loss of more than 20%. The difference in weight loss between animals inoculated with strains 987 and I36P$^+$ was not significant, while the difference in weight loss between pigs inoculated with nonpiliated I36 and either I36P$^+$ or 987 was

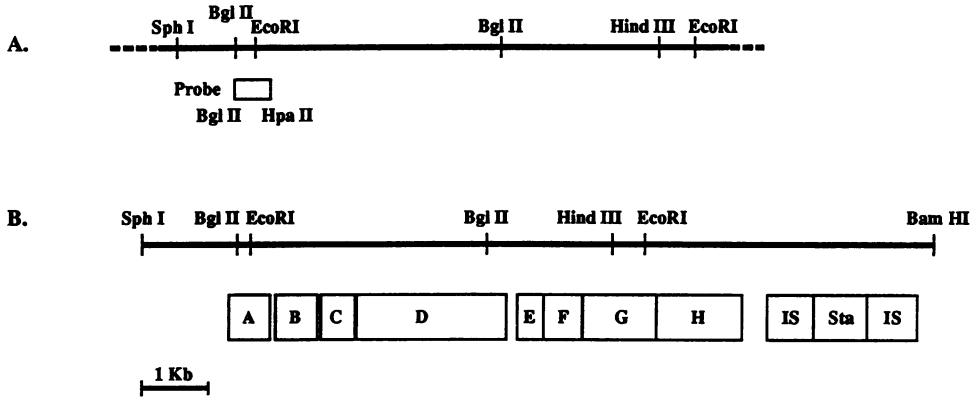

FIG. 3. Restriction endonuclease maps of the 987P genes. (A) Map of the common restriction fragments from plasmid and total cellular DNAs that hybridized with the 987P subunit probe. The positions of other restriction endonuclease sites on either side of those shown were distinct for plasmid and chromosomal fragments that hybridized with the 987P subunit gene. (B) Restriction map redrawn from the map of the cloned 987P determinant reported by Schifferli et al. (16, 17), showing the 987P gene products determined by these authors. The position of the 987P subunit probe used in this study is also shown.

TABLE 1. Experimental inoculation of neonatal pigs

Strain	No. of pigs			% Wt change[b]	Colonization[c]	
	Total[a]	With diarrhea	Dead		Log CFU (*n*)[d]	Adherence[e]
I36	4	0	0	−10.8 ± 4.0	5.8 ± 0.9 (4)	0/4
I36P$^+$	15	15	6	−22.5 ± 2.7	8.4 ± −0.7 (11)	8/11
987	4	4	2	−24.8 ± 2.7	9.7 ± 0.3 (3)	3/3

[a] Number of pigs inoculated, via gavage, with 10^{10} bacteria of the indicated strain.
[b] Mean weight change by 18 h postinoculation expressed as a percentage of initial body weight, ± standard deviation.
[c] Tissues from some animals that died were unacceptable for subsequent bacteriological or microscopic examination for colonization.
[d] CFU expressed as the mean total number of bacteria, ± standard deviation, in a 10-cm section of ileum removed at necropsy and processed as previously described. *n*, number examined.
[e] Number positive/number examined, determined by microscopic examination of hematoxylin-and-eosin-stained sections of ileum for layers of adherent bacteria.

significant ($P < 0.05$ by Student's *t* test). None of the animals inoculated with nonpiliated I36 were colonized or had adherent layers of bacteria in the small intestine. In contrast, strains 987 and I36P$^+$ adhered and colonized in the small intestines of nearly all of the animals inoculated with these strains.

The plasmid that hybridized with the 987P subunit probe was transferred by conjugation to a nalidixic acid-resistant *E. coli* K-12 strain (strain 711). Mid-log-phase cultures of 987 and 711 were mixed, allowed to mate for 2 h at 37°C, plated on tryptic soy agar containing nalidixic acid (20 μg/ml), and screened for the presence of the 987P plasmid by colony blot hybridization with the 987P subunit probe. Plasmid DNAs isolated from representative transconjugants are shown in Fig. 4. A single plasmid band hybridized with the 987P subunit probe in these transconjugants (Fig. 4B). This plasmid was the same size as the 987P plasmid present in 987 and missing in I36. However, none of the transconjugants expressed 987P antigen.

We have attempted to identify variants of these transconjugants that express 987P by using growth conditions that have been reported to enrich for 987P$^+$ bacteria. These conditions included enrichment for piliated bacteria by growth in stationary broth cultures for pellicle formation, growth in vivo using ligated porcine intestinal loops, and growth under anaerobic conditions (5, 13, 14). Using these approaches, we have been unable to identify a 987P$^+$ piliated variant of the strain 711 transconjugants.

We do not know why we were unable to demonstrate expression of 987P in transconjugants that contain the 987P plasmid. Comparison of the restriction endonuclease maps for a cloned 987P determinant (3, 16, 17) and the chromosomal and plasmid fragments that hybridized with the 987P subunit probe suggested that all of the genes required for 987P expression were present. In addition, it appears that the 987P plasmid occurs frequently in 987P$^+$ strains. We have examined plasmid DNAs from 94 wild-type 987P$^+$ strains that were isolated from neonatal pigs with diarrhea. All of these strains had a large plasmid which hybridized with the 987P subunit probe (data not shown). We do not know if all of these strains have an additional chromosomal copy of the 987P genes.

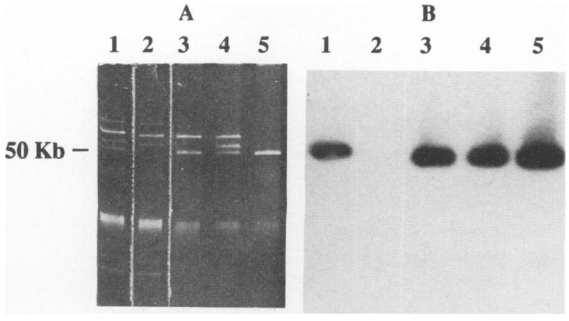

FIG. 4. Plasmid DNA isolated from strain 711 transconjugants containing the 987P plasmid. (A) Plasmids separated by agarose gel electrophoresis. (B) Southern blot of this gel hybridized with the 987P subunit probe. Lanes 1, strain 987; 2, strain I36; 3 through 5, independent strain 711 transconjugants containing the 987P plasmid.

Schifferli et al. (15) reported isolating a mutant of strain 987 which had lost the 987P plasmid and the ability to express 987P pili. Reintroduction of the 987P plasmid into this mutant restored 987P expression. They concluded that this plasmid encoded 987P pili. We examined a similar mutant, I36, a spontaneous mutant of 987 that no longer expressed 987P pili. We found that I36 had lost the 987P plasmid but contained chromosomal genes for 987P pili. We isolated a piliated variant of I36 (I36P$^+$) from a neonatal pig inoculated with nonpiliated I36. There was no difference between plasmids from piliated and nonpiliated I36, suggesting that the chromosomal copy was expressed. We found that I36P$^+$ expressed functional 987P pili, colonized, and caused diarrhea in neonatal pigs similarly to wild-type strain 987. We hypothesize that the defect in 987P expression by I36 may be related to the rate of switching to the piliated phase and not to pilus synthesis. However, it is possible that there are point mutations in the 987P structural or regulatory genes in I36 and that reversion to 987P expression occurred in vivo.

The significance and pathogenic advantage of having two copies of the 987P subunit genes are not known. Our data indicate that the two copies may interact to affect 987P expression. The specific interaction between the plasmid and chromosomal 987P gene products and their role in phase variation remain to be determined.

REFERENCES

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
2. **Dean, E. A., S. C. Whipp, and H. W. Moon.** 1989. Age-specific colonization of porcine intestinal epithelium by 987P-piliated enterotoxigenic *Escherichia coli*. Infect. Immun. **57**:82–87.
3. **de Graaf, F. K., and P. Klaasen.** 1986. Organization and expression of genes involved in the biosynthesis of 987P fimbriae. Mol. Gen. Genet. **204**:75–81.
4. **de Graaf, F. K., and P. Klaasen.** 1987. Nucleotide sequence of the gene encoding the 987P fimbrial subunit of *Escherichia coli*. FEMS Microbiol. Lett. **42**:253–258.
5. **Isaacson, R. E., B. Nagy, and H. W. Moon.** 1977. Colonization of

porcine small intestine by *Escherichia coli*: colonization and adhesion factors of pig enteropathogens that lack K88. J. Infect. Dis. **135**:531–539.

6. **Klaasen, P., and F. K. de Graaf.** 1990. Characterization of FapR, a positive regulator of expression of the 987P operon in enterotoxigenic *Escherichia coli*. Mol. Microbiol. **4**:1779–1783.
7. **Klaasen, P., M. J. Woodward, F. G. van Zijderveld, and F. K. de Graaf.** 1990. The 987P gene cluster in enterotoxigenic *Escherichia coli* contains an STpa transposon that activates 987P expression. Infect. Immun. **58**:801–807.
8. **Mainil, J. G., S. L. Moseley, R. A. Schneider, K. Sutch, T. A. Casey, and H. W. Moon.** 1986. Hybridization of bovine *Escherichia coli* isolates with gene probes for four enterotoxins (STaP, STaH, STb, LT) and one adhesion factor (K99). Am. J. Vet. Res. **47**:1145–1148.
9. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Moon, H. W., and R. E. Isaacson.** 1981. Pili of enterotoxigenic *E. coli* as protective antigens in live oral vaccines, p. 335–341. *In* S. Kuwahara and Y. Zinnaka (ed.), Proceedings of the 16th Joint Conference of the U.S.-Japan Cooperative Medical Science Program on Cholera Panel. Toho University, Tokyo.
11. **Moon, H. W., E. M. Kohler, R. A. Schneider, and S. C. Whipp.** 1980. Prevalence of pilus antigens, enterotoxin types, and enteropathogenicity among K88-negative enterotoxigenic *Escherichia coli* from neonatal pigs. Infect. Immun. **27**:222–230.
12. **Morrissey, P. M., and G. Dougan.** 1986. Expression of a cloned 987P adhesion-antigen fimbrial determinant in *Escherichia coli* K-12 strain HB101. Gene **43**:79–94.
13. **Nagy, B., H. W. Moon, and R. E. Isaacson.** 1976. Colonization of porcine small intestine by *Escherichia coli*: ileal colonization and adhesion by pig enteropathogens that lack K88 antigen and by some acapsular mutants. Infect. Immun. **13**:1214–1220.
14. **Nagy, B., H. W. Moon, and R. E. Isaacson.** 1977. Colonization of porcine intestine by enterotoxigenic *Escherichia coli*: selection of piliated forms in vivo, adhesion of piliated forms to epithelial cells in vitro, and incidence of a pilus antigen among porcine enteropathogenic *E. coli*. Infect. Immun. **16**:344–352.
15. **Schifferli, D. M., E. H. Beachey, and R. K. Taylor.** 1990. The 987P fimbrial gene cluster of enterotoxigenic *Escherichia coli* is plasmid encoded. Infect. Immun. **58**:149–156.
16. **Schifferli, D. M., E. H. Beachey, and R. K. Taylor.** 1991. Genetic analysis of 987P adhesion and fimbriation of *Escherichia coli*: the *fas* genes link both phenotypes. J. Bacteriol. **173**:1230–1240.
17. **Schifferli, D. M., E. H. Beachey, and R. K. Taylor.** 1991. 987P fimbrial gene identification by T7 RNA polymerase-induced transcription and TnphoA mutagenesis. Mol. Microbiol. **5**:61–70.