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Genetic Characterization and Virulence of Enterotoxigenic *Escherichia coli* Mutants Which Have Lost Virulence Genes In Vivo

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Loss of K99 and STaP genes from enterotoxigenic *Escherichia coli* 431 during infection occurred by either plasmid curing or plasmid deletion. These mutants expressed the F41 adhesin and colonized neonatal pigs, but only those mutants that retained STaP caused diarrhea with significant weight loss.

In vitro and in vivo loss of enterotoxigenic *Escherichia coli* virulence genes has been reported to occur by plasmid curing (5, 6, 8, 9, 11, 16–18). Pigs infected with enterotoxigenic *E. coli* 431 (O101:K30:NM, K99$^+$ F41$^+$ STaP$^+$) shed mutants which had lost both K99 and STaP or only K99 (11). Mutants which had lost STaP and retained K99 were not found. We examined these mutants and found that a single plasmid was lost in K99$^-$ STaP$^-$ mutants and that in K99$^-$ STaP$^+$ mutants the K99 genes were deleted from the plasmid. We found that both classes of mutants expressed F41 and colonized experimentally inoculated neonatal pigs. One of the K99$^-$ F41$^+$ STaP$^+$ mutants caused severe diarrhea comparable with that caused by wild-type strain 431.

Agarose gel electrophoresis of plasmid DNA isolated from strain 431 showed two large plasmid bands of approximately 115 and 85 kb and two smaller plasmid bands (Fig. 1A, lane 2). A K99$^-$ STaP$^-$ mutant (strain 518M) had a plasmid profile indistinguishable from the wild-type parent (Fig. 1A, lane 1). The 115-kb plasmid band from strain 431 hybridized with DNA probes for both K99 and STaP (Fig. 1B and C, lane 2). However, neither probe hybridized with the 115-kb plasmid band in the mutant (Fig. 1B and C, lane 1). Similar plasmid patterns for additional K99$^-$ STaP$^-$ mutants are shown in Fig. 2. Comparison of *Bgl*II restriction endonuclease fragment patterns of plasmids from strain 431 and a K99$^-$ STaP$^-$ mutant (Fig. 3, lanes 2 and 3) showed that several fragments were missing from the K99$^-$ STaP$^-$ mutants, suggesting the loss of a single plasmid coding for both of the traits.

The location of the STaP gene was determined by comparison of plasmid DNA isolated from four K99$^-$ STaP$^+$ mutants with plasmids isolated from strain 431 and from five K99$^-$ STaP$^-$ mutants (Fig. 2). The K99$^-$ STaP$^+$ mutants had an additional, 105-kb plasmid band which hybridized with the STaP probe (Fig. 2B). The same size *Bgl*II fragment of plasmid DNA from strain 431 and three different K99$^-$ STaP$^+$ mutants hybridized with the STaP probe (Fig. 3B, lanes 2, 4, and 5). These results suggest that the K99$^-$ STaP$^+$ mutants occurred by deletion of K99 genes from one of the 115-kb plasmids, leaving a 105-kb plasmid carrying STaP.

The additional plasmid bands in each of the K99$^-$ STaP$^+$ mutants shown in Fig. 2 were indistinguishable in size. Although the K99$^-$ STaP$^+$ mutants were from different animals, they may not be independent isolates because the animals were all from the same litter and were inoculated together in the same experiment. We identified three independent K99$^-$ STaP$^+$ mutants isolated from different animals inoculated in separate experiments to determine if a specific deletion of the K99 genes had occurred during infection. Figure 3 shows *Bgl*II fragments of plasmid DNA from the three independent K99$^-$ STaP$^+$ mutants. Two K99$^+$ STaP$^+$ mutants had identical *Bgl*II patterns (Fig. 3, lanes 2 and 4), while a third mutant has a different pattern (Fig. 3, lane 5), indicating a different, larger deletion. This larger deletion was also seen by electrophoresis of intact plasmid DNA from the third mutant (data not shown).

Mutants which had lost K99 or both K99 and STaP expressed the F41 adhesin when tested by enzyme-linked immunosorbent assay (13). Wild-type strain 431, a K99$^-$ STaP$^-$ mutant (strain 226M), and a K99$^-$ STaP$^+$ mutant (strain 234M) were each used to determine the effects of the loss of K99 and STaP on virulence by inoculation of hysterectomy-derived, colostrum-deprived neonatal pigs (Table 1). All three strains colonized the small intestine (>10^8 CFU/10-cm ileal segment), and adherent bacteria were seen in ileal sections from all inoculated animals. Immunofluorescence showed that adherent bacteria expressed F41 pili in the ilea of all inoculated animals but that only animals inoculated with strain 431 had adherent bacteria that expressed K99. Strain 234M caused diarrhea with weight loss that was not significantly different from the weight loss of animals inoculated with strain 431 ($P > 0.05$, Student's t test). Strain 226M caused mild diarrhea (three of five animals) and weight loss that was much less than with either strain 431 or the K99$^-$ STaP$^+$ mutant.

In this model, pigs inoculated with strain 431 consistently develop diarrhea (>20% weight loss in 18 h) and controls or those inoculated with nonpathogens remain normal and lose <15% of their body weight in 18 h (11, 12, 15; unpublished data). The response to strain 431 was indistinguishable from responses in previous experiments and to strain 234M. In contrast to nonpathogenic *E. coli*, strain 226M (STaP$^-$) caused mild diarrhea in three of five inoculated pigs. Others (3, 7, 20) have shown that *E. coli* which intensively colonizes but does not produce enterotoxin causes mild diarrhea in some individuals. Our results with a nontoxigenic F41$^+$ strain (strain 226M) are consistent with these reports. The results indicate that STaP is not required for the production of diarrhea but that STaP increases severity and consistency of diarrhea. It is possible that strain 431 is more virulent than strain 234M but that the difference was not detected because of the large numbers of enterotoxigenic *E. coli* inoculated. It would be useful to compare the virulence of these two strains by using much lower numbers of enterotoxigenic *E. coli* in the inocula.

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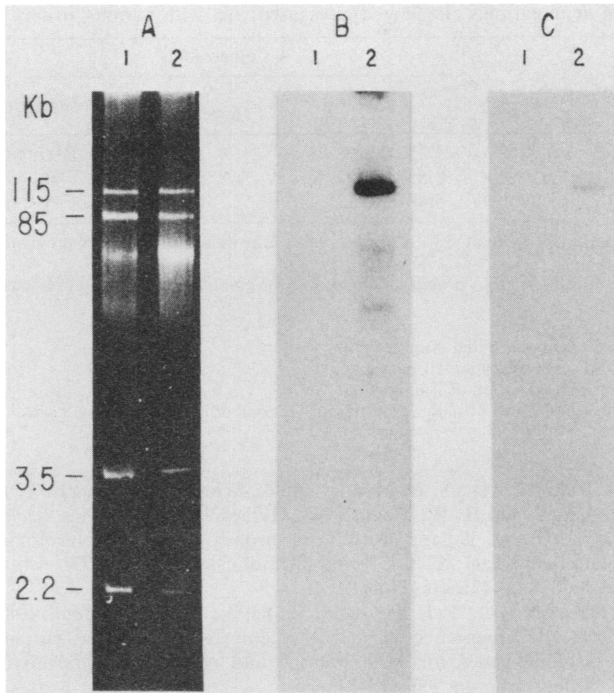

FIG. 1. (A) Plasmid DNA isolated by alkaline lysis (1), separated by electrophoresis in a 1% agarose gel. The sizes were determined by comparison with the sizes of reference plasmids. Kb, Kilobases. (B) Autoradiogram of a blot of the gel in panel A hybridized with a ^{32}P-labeled, 6.7-kb *Bam*HI fragment K99 probe derived from pFK99 (2, 13). (C) The blot was stripped and then hybridized with a ^{32}P-labeled STaP probe derived from pRIT10036 as a 157-bp *Hin*fI fragment (14, 21). The derivation of the probes and hybridization conditions have been described previously (10). Lanes: 1, strain 518M (K99$^-$ STaP$^-$); 2, strain 431.

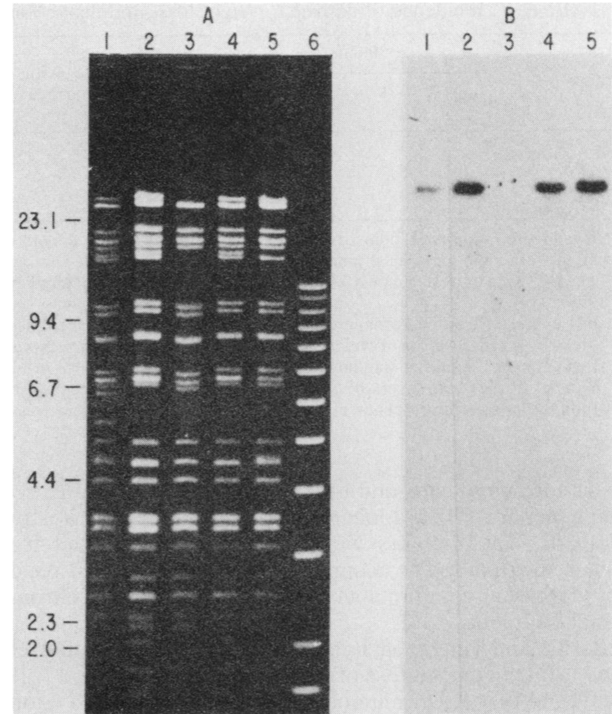

FIG. 3. Agarose gel (A) and Southern blot (B) of *Bgl*II restriction endonuclease digestion of plasmid DNA isolated from wild-type strain 431 (lanes 1), a K99$^-$ STaP$^-$ mutant (strain 85M) (lanes 3), and three independent K99$^-$ STaP$^+$ mutants (strains 82M, 267M, and 331M in lanes 2, 4, and 5, respectively). (B) The blot was hybridized with a ^{32}P-labeled probe for STaP. The sizes shown are for λ *Hin*dIII fragments separated in the same gel, and the 1-kb ladder (BRL) is in lane 6.

Nagy et al. (16) reported that antibody selected for K99$^-$ mutants in vitro but that these mutants revert to K99$^+$ phenotype when subcultured, suggesting that point mutations had occurred which prevented K99 expression. The K99 DNA probes used here and by Mainil et al. (11) would not have differentiated between the wild type and variants with point mutations affecting K99 expression, which might also occur in vivo.

It has been suggested that antibodies induce curing of virulence plasmids (8, 9, 17, 18) or, alternatively, select for mutants that have lost plasmids (16). We found that both plasmid loss and deletion of K99 genes from the plasmid also occurred. Hacker et al. (4) reported deletion of chromosomal

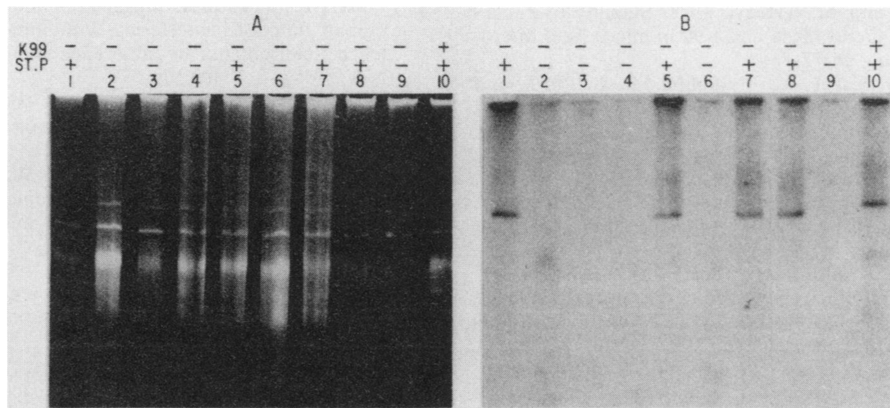

FIG. 2. (A) A 1% agarose gel of plasmid DNA isolated from K99$^-$ STaP$^+$ strains compared with plasmids from strain 431 and K99$^-$ STaP$^-$ mutants. The results of colony hybridization with a ^{32}P-labeled 460-bp probe for K99 (11) and the STaP probe (11) are shown for each strain above the lanes. (B) Autoradiogram of a blot of this gel hybridized with a ^{32}P-labeled STaP probe. Lanes: 1, strain 82M; 2, strain 85M; 3, strain 125M; 4, strain 226M; 5, strain 228M; 6, strain 396M; 7, strain 234M; 8, strain 468M; 9, strain 518M; 10, strain 431.

TABLE 1. Incidence of diarrhea, weight loss, and ileal colonization of neonatal pigs challenged intragastrically with various strains

Strain[a]	Phenotype			No. with diarrhea	% Wt loss[d]	Colonization		
	K99[b]	STaP[b]	F41[c]			CFU[e]	No. adherent[f]	Antigens[g]
431	+	+	+	5	27.1 ± 0.9	9.32 ± 0.085	5	K99, F41
234M	−	+	+	5	24.8 ± 2.5	8.83 ± 0.32	5	F41
226M	−	−	+	3	10.3 ± 1.9	8.48 ± 0.11	5	F41

[a] Hysterectomy-derived, colostrum-deprived pigs which were <8 h old were intragastrically inoculated with 10^{10} bacteria of the indicated strains as previously described (15). Five pigs per strain were inoculated.
[b] The presence or absence of K99 and STaP was determined by colony blot hybridization. The DNA probes and hybridization conditions have been previously described (10).
[c] F41 expression was determined by enzyme-linked immunosorbent assay (14).
[d] Mean ± standard error of weight loss 18 h following challenge, expressed as a percentage of initial body weight.
[e] Log geometric mean ± standard error of the number of bacteria present in a 10-cm section of ileum at necropsy.
[f] Number of pigs with layers of adherent bacteria on villi, determined by microscopic examination of ileal sections.
[g] Indirect immunofluorescence assay using specific rabbit antisera was used to detect K99 and F41 antigens in frozen sections of ilea from infected pigs.

genes for hemolysins and fimbriae in extraintestinal *E. coli*. The genes for F41 are located on the chromosome in strain 431 (13). The mutants we examined were isolated from piglets suckling sows vaccinated against F41 (11, 19). We did not identify any mutants which did not express the chromosomal F41 genes.

Porter and Linggood hypothesized that vaccination efficacy is due in part to loss of virulence plasmids (8, 9, 17, 18). We found that $K99^-$ mutants of strain 431 colonized neonatal pigs and that those mutants which retained STaP caused severe diarrhea. Therefore, immune induction or selection for the loss of virulence genes may not result in avirulent derivatives for strains that express more than one adhesin.

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