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Impaired Colonization by and Full Invasiveness of *Escherichia coli* K1 Bearing a Site-Directed Mutation in the Type 1 Pilin Gene

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A type 1 pilus-deficient mutant of a systemically invasive *Escherichia coli* K1 strain was constructed by directed mutagenesis of *pilA*, the gene that codes for the major structural subunit of type 1 pili. By comparing this mutant with an isogenic *pilA*$^+$ strain, we were able to assess the role of type 1 piliation in alimentary tract colonization and bloodstream invasion in neonatal rats. Intestinal colonization was not significantly affected by the *pilA* mutation; in contrast, loss of type 1 piliation correlated with a dramatic decrease in oropharyngeal colonization. Nevertheless, development of bacteremia after oral administration of *E. coli* K1 was not diminished by the mutation in *pilA*. Thus, loss of type 1 piliation correlated with a site-dependent effect on colonization within the alimentary tract while not interfering with bloodstream invasion.

Escherichia coli strains of capsular serotype K1 are a major cause of sepsis and meningitis in human infants (29). Since the K1 capsule itself is poorly immunogenic (15) being cross-reactive with the developing human brain (7), efforts to produce an effective vaccine against K1 strains have focussed on the identification of other bacterial cell surface components which might be required for pathogenicity (9, 10, 16, 31). It has been proposed that prevention of type 1 pilus-mediated adherence might interrupt the development of K1 invasion (9). This study was designed to address the role played by type 1 pili in the pathogenesis of *E. coli* K1 neonatal sepsis.

Studies on the role of type 1 piliation in animal pathogenesis models using piliated and nonpiliated *E. coli* K1 variants have been difficult to interpret because nonpiliated (Pil$^-$) *E. coli* variants selected on the basis of phenotype are only metastable (4, 6, 34); they revert to the piliated (Pil$^+$) form in vitro at a rate of $\sim 10^{-3}$ to 10^{-4} per cell division (4, 6, 26) and in the rodent alimentary tract (9) at an undetermined rate. Cloning of the genes that determine type 1 piliation in *E. coli* (11) and recent adaptation of DNA transfer methods to a systemically invasive clinical isolate of *E. coli* K1 (3) allowed us to construct a stably nonpiliated mutant by site-directed mutagenesis of the *pil* region. The hypothesis that type 1 pili might be important for invasion of the bloodstream from the gastrointestinal tract (9) was tested by assessing the behavior of this strain in a neonatal-rat model of bacteremia after oral inoculation.

Our experimental strategy proceeded as follows. We insertionally mutagenized a cloned segment of *E. coli* DNA containing the *pilA* gene (24, 25) at either of two sites. A restriction fragment containing the tetracycline resistance determinant from Tn*10* (14) was cloned into the *pilA* locus in the first case and into an adjacent site which did not affect piliation in the second (Fig. 1). The wild-type *pil* DNA sequences were then replaced with these mutant sequences, referred to as *pilA*$^-$::*tetR* and *pilA*$^+$::*tetR*, respectively, in both the *E. coli* K-12 and *E. coli* K1 genomes. Southern analysis confirmed the generation of the predicted chromosomal insertions depicted in Fig. 1 (data not shown). As expected, the *pilA*$^-$::*tetR* mutants of both *E. coli* K-12 and *E. coli* K1 were stably Pil$^-$ (Fig. 1). In contrast, both of the *pilA*$^+$::*tetR* mutants exhibited the predicted Pil$^+$ phenotypes (Fig. 1). The phenotypes were confirmed by electron microscopy (data not shown). The electron microscopic studies also confirmed the presence of a second pilus type in the K1 strain of *E. coli* used in these studies. This pilus, presumably the S pilus—which has been previously demonstrated in our parental K1 strain, RS218 (27)—was found on about 1 in 100 cells of *E. coli* K1 *pilA*$^-$::*tetR* from a culture grown with aeration in L broth. Its role in alimentary tract colonization and/or induction of bacteremia was not examined in this study.

The neonatal-rat model used to assess *E. coli* K1 colonization and translocation from the alimentary tract to the bloodstream has been described (8) and modified (3, 32) previously. Briefly, newborn rat pups were fed 1-μl samples of a bacterial suspension at 1 day of age. After 72 h, they were sacrificed and their tissues were cultured. During the interval between inoculation and sacrifice, they received normal parental care and feeding.

Comparisons between *E. coli* K1 *pilA*$^-$::*tetR* and *E. coli* K1 *pilA*$^+$::*tetR* with respect to bloodstream invasion, intestinal colonization, and oropharyngeal colonization were made at two doses, 10^7 and 10^4 CFU. Blood cultures drawn from the rats at 72 h after oral inoculation with *E. coli* K1 *pilA*$^-$::*tetR* revealed that this strain was capable of invading the bloodstream (Table 1). Moreover, the prevalence of bacteremia induced by *E. coli* K1 *pilA*$^-$::*tetR* did not differ significantly from that induced by *E. coli* K1 *pilA*$^+$::*tetR*. Both of these rates were equivalent to the bacteremia prevalence induced by the orally inoculated parental K1 strain. As expected, *E. coli* K-12 *pilA*$^+$::*tetR* was completely unable to induce bacteremia. Further assessment of the two *E. coli* K1 *pil* region::*tetR* strains included concurrent measurement of intestinal colonization.

The parental K1 strain was previously shown to colonize neonatal rat colons at a density of $\sim 2 \times 10^9$/g of stool-filled bowel (3). At both doses, 10^7 and 10^4 CFU, *E. coli* K1 *pilA*$^-$::*tetR* and *E. coli* K1 *pilA*$^+$::*tetR* also colonized the descending colon at densities of $\sim 10^9$ CFU/g of stool-filled bowel (Table 2). The *pilA*$^+$::*tetR* strain achieved slightly

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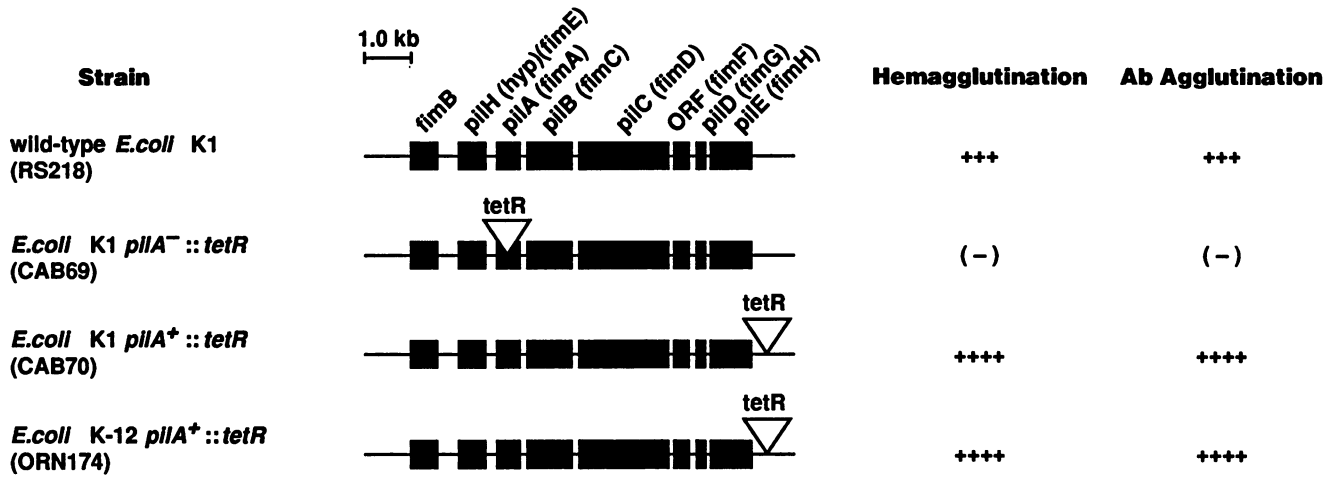

FIG. 1. Physical and genetic correlates in the *pil* regions of the *E. coli* strains used in this study. Two *pil* region alleles, one bearing a *tetR* insertion in the *pilA* structural gene and the other bearing the same *tetR* insertion in an adjacent sequence, were constructed as follows. Insertion of *tetR* in the *pilA* gene of RS218 was accomplished by insertion of the *Xho*I-linked *Bgl*II fragment from Tn*10* containing *tetR* (14) into the *pilA* gene on pORN117 (26), followed by use of linear transformation (26, 34) and P1 transduction (22) to introduce the lesion into *E. coli* K-12 strain ORN115 (26), creating strain ORN151. P1 transduction was then used to transfer the lesion from ORN151 to the RS218 parent strain (3), creating CAB69 (*E. coli* K1 *pilA*⁻::*tetR*). Analogous methods (insertion of the *tetR* determinant into a plasmid, linear transformation, and P1 transduction) were used to create CAB70 (*E. coli* K1 *pilA*⁺::*tetR*) and ORN174 (*E. coli* K-12 *pilA*⁺::*tetR*). Both CAB70 and ORN174 have the *tetR* determinant inserted at a site, formerly a *Pvu*II site, approximately 0.4 kilobase (kb) pair downstream from the 3′ end of the *pilE* gene (P. W. Russell and P. E. Orndorff, manuscript in preparation). Strains RS218, ORN151, CAB69, CAB70, and ORN174 were then subjected to Southern analysis (3) with probe pSH2 (24) and to tests for mannose-sensitive guinea pig erythrocyte hemagglutination and agglutination by anti-type 1 pilus antibody (Ab) as previously described (24). (ORN151 was omitted for clarity, since it was not used in the animal studies.) At the top are the *pil* region genes of the parental K1 strain, RS218 (inferred by Southern analysis). They are labeled with the mnemonics of both Maurer and Orndorff (20) and Klemm and Christiansen (in parentheses) (18). ORF, Open reading frame; tetR, tetracycline resistance determinant of Tn*10*. ++++, strong; +++, distinguishably weaker; (−), no agglutination.

higher growth than did the *pilA*⁻::*tetR* strain. In contrast, *E. coli* K-12 *pilA*⁺::*tetR* was undetectable in the colon at 72 h (data not shown). Whereas the data indicated that type 1 piliation was not an important determinant of either bacteremia (Table 1) or intestinal colonization (Table 2), the results were very different in the oropharynx.

Screening of the oropharyngeal microbiota in rats that received a dose of 10^7 CFU suggested that *E. coli* K1 *pilA*⁻::*tetR* was unable to colonize the oropharynx (data not shown). This phenomenon was examined systematically at the lower dose of 10^4 CFU. These data indicated that indeed oropharyngeal colonization was dramatically reduced with *E. coli* K1 *pilA*⁻::*tetR* compared with *E. coli* K1 *pilA*⁺::*tetR* (Table 3). All of the 40 rat pups fed the *pilA*⁺::*tetR* strain were colonized. Their semiquantitative oropharyngeal cultures yielded heavy growth. In contrast, approximately half (19 of 40) of the rat pups fed the *pilA*⁻::*tetR* strain yielded no detectable oropharyngeal growth. Importantly, even the positive cultures from this group had only scant growth (with one exception; Table 3). Bacteremia was found without

TABLE 1. Prevalence of *E. coli* bacteremia at 72 h after oral inoculation

Dose (CFU)[a]	Bacteremia rate [SD] (no. positive/total)[b]			
	Wild-type K1	K1 *pilA*⁻::*tetR*	K1 *pilA*⁺::*tetR*	K-12 *pilA*⁺::*tetR*
10^7	0.57 [16] (30/53) {4}[c]	0.66 [13] (35/53) {9}	0.53 [14] (39/73) {3}	0.0 [0] (0/43)
10^4		0.40 [7] (16/40)	0.53 [4] (21/40)	

[a] The test strains were stored in 50% L broth–50% (vol/vol) glycerol at −20°C. On evenings before individual challenges, lawns streaked onto L agar from the stored cultures were grown overnight at 37°C. These were suspended and diluted in iced phosphate-buffered saline before inoculation. Three experiments using seven or eight litters each were conducted at a dose of 10^7 CFU, and two experiments with four litters each were done with a dose of 10^4 CFU. The individual litters were adoptive litters constituted for each experiment by systematic distribution of the pups from seven or eight time-synchronized litters among postpartum dams as described earlier (3). All members of each adoptive litter were given the same inoculum.

[b] Cultures were taken as described earlier (3). Briefly, after sacrifice, 0.1-ml samples of heart blood were drawn by percutaneous cardiac puncture. These were plated onto L agar and incubated for 24 h at 37°C. Blood isolates were examined further by subculturing onto MacConkey lactose supplemented with tetracycline (12.5 μg/ml), as appropriate. Retention of the Pil⁺ or Pil⁻ phenotype was confirmed for some isolates, as was the nicotinate auxotrophy of the parental strain (cf. references 1 and 28). Bacteremia rates were calculated as the number of positive blood cultures divided by the total number of blood cultures.

[c] The number of rat pups missing (because of maternal cannibalism) or dead and from which cultures were therefore not taken is shown in braces.

TABLE 2. Colonization of the descending colon

Dose (CFU)	CFU/g of stool-filled bowel (no. of pups tested) [range][a]	
	K1 *pilA*⁻::*tetR*	K1 *pilA*⁺::*tetR*
10^7	5.7×10^8 (8) [1×10^8—2×10^9]	1.2×10^9 (4) [7×10^8–2×10^9]
10^4	6.1×10^8 (6) [7×10^7–2×10^9]	3.1×10^9 (6) [7×10^8–5×10^9]

[a] Measurements were made as described previously (3) at 72 h after oral inoculation of pups selected at random from the animals described in Table 1. Briefly, a 0.5-cm segment of stool-filled descending colon was resected and ground and then suspended and diluted in phosphate-buffered saline, plated in triplicate onto L agar supplemented with tetracycline (12.5 μg/ml), and counted after incubation overnight at 37°C.

TABLE 3. Oropharyngeal colonization[a]

Strain	Oropharyngeal colonization rate [SD] (no. positive/total)[b]	Density of oropharyngeal colonization (CFU) [SD][c]
K1 *pilA*$^-$::*tetR*	0.53 [11] (21/40)	15 [5][d]
K1 *pilA*$^+$::*tetR*	1.0 [0] (40/40)	10^4–10^5[e]

[a] Measurements were made 72 h after oral inoculation of 10^4 CFU into the animals in the 10^4-CFU inoculation group described in Table 1. Semiquantitative cultures of the oropharynx were performed as follows. A cotton-tipped applicator was inserted orally to the level of the pharynx and rotated ±~15° five times. Both the top and bottom surfaces of the applicator tip were then streaked onto L agar supplemented with tetracycline (12.5 μg/ml) over a surface area of 25 cm^2. By washing the oropharyngeal substance retrieved in this way from the cotton tip, we demonstrated that cultures taken at 1 min after feeding of 10^5 CFU of either *pil* region::*tetR* strain contained 24% (standard deviation, 6%) of the inoculum.

[b] Prevalence of oropharyngeal colonization was calculated as the number of positive oropharyngeal cultures divided by the total number of cultures.

[c] Average density of oropharyngeal colonization was calculated as the total number of CFU isolated divided by the number of positive cultures.

[d] This average excludes one pup fed *E. coli* K1 *pilA*$^-$::*tetR* from which 1,184 CFU were cultured. The excluded pup had a high density of bacteremia (10^4 CFU/ml) at the time of culture. We speculate that deteriorating clinical status induced by *E. coli* K1 bacteremia may have permitted post bacteremic oropharyngeal colonization by the *pilA* strain in this rat pup (cf. reference 36).

[e] All but three cultures yielded >10^3 CFU. In a separate experiment, a random *E. coli* K1::Tn*10* insertion mutant colonized the oropharynx with an average yield of 10^4 CFU per animal.

detectable oropharyngeal growth in five rat pups fed *E. coli* K1 *pilA*$^-$::*tetR*, a result which suggested that the anatomic site of entry to the bloodstream by orally inoculated *E. coli* K1 was distal to the oropharynx (cf. references 9 and 28). The fact that the rates of bacteremia did not differ significantly in rats fed either *E. coli* K1 *pilA*$^+$::*tetR* (heavy oropharyngeal colonization) or *E. coli* K1 *pilA*$^-$::*tetR* (scant or no oropharyngeal colonization) also supported this view.

A large subpopulation of *E. coli* neonatal sepsis and meningitis isolates carries the K1 capsule (29). The parental K1 isolate from which the *pil*::*tetR* mutants described herein were constructed, strain RS218 (33), belongs to one of six temporally and geographically widespread, and probably clonal, groups of *E. coli* K1 commonly involved in newborn disease (1). We used isogenic *pilA*$^-$ and *pilA*$^+$ mutants of RS218 to assess the role of type 1 piliation in colonization and translocation from the alimentary tract to the bloodstream. This allowed us to circumvent the difficulties inherent in interpreting comparisons between wild-type phase variants or heterogenic strains (17).

These studies resulted in two important findings. (i) Stable loss of type 1 piliation exerted a site-dependent effect on alimentary tract colonization in our model system. With the nonpiliated K1 strain, oropharyngeal colonization was dramatically reduced, while colonization of the descending colon was not significantly affected. (ii) Type 1 piliation was not required for invasion of the neonatal rat bloodstream. Indeed, under our test conditions, stable absence of type 1 piliation did not significantly affect the prevalence of bacteremia achieved by *E. coli* K1 after oral inoculation.

Mannose-sensitive binding between *E. coli* and human oral epithelial cells has been demonstrated in vitro (23). Additionally, isolation of exclusively Pil$^+$ *E. coli* K1 from neonatal rat oropharynxes following administration of Pil$^-$ bacterium-enriched inocula suggested that mannose-sensitive pili may be involved in oropharyngeal colonization in vivo (9). The comparison presented here, of isogenic *pilA*$^+$ and *pilA*$^-$ mutants of *E. coli* K1 in neonatal rats, indicated that in fact, type 1 piliation was required for oropharyngeal colonization in vivo. Oropharyngeal colonization may contribute to pathogenesis in certain clinical settings (13). Our results are consistent with the possibility that under such conditions, type 1 piliation has a role in pathogenesis. Nonetheless, oropharyngeal colonization status did not affect virulence under the conditions examined here.

There have been conflicting studies regarding the possible importance of type 1 piliation in intestinal colonization. Mannose-sensitive binding by *E. coli* to intestinal epithelial cells has been shown (5, 12). But studies with intact segments of intestinal mucosa were unable to demonstrate adherence by an *E. coli* clinical isolate which expressed type 1 pili (21). It has been suggested that type 1 pili may not be uniformly expressed along intestinal mucosal surfaces on the basis of findings that exposure of the intestinal tract to orally inoculated *pil*$^+$ strains of enterotoxigenic *E. coli* did not consistently stimulate an immune response to type 1 pili in human volunteers (19) or livestock (35). Implicit in this suggestion has been the possibility that type 1 piliation is not necessary for the intestinal phase of enterotoxigenic *E. coli* and, perhaps, of *E. coli* in general. Supporting and extending that possibility, our results demonstrated that stable loss of type 1 pili in *E. coli* K1, a common intestinal isolate from healthy humans (30), did not significantly affect the density of colonization of neonatal rat large intestines. An analogous finding has recently been made by McCormick et al. (20a).

It has been proposed that prevention of pilus-mediated mucosal adherence might interrupt the development of bacterial pathogenesis (2). The invasiveness demonstrated by *E. coli* K1 *pilA*$^-$::*tetR* suggests that a strategy for interruption of *E. coli* K1 invasion in neonates based on prevention of type 1 pilus-mediated adherence (9) might not be generally effective. The role of type 1 pili in the pathogenesis of various *E. coli* infections has been unclear, partly because in vivo analyses of metastable phase variants have been difficult to interpret. Generation by directed mutagenesis of additional mutations in *E. coli* K1, some of which, like that at *pilA*, cannot be obtained by classical bacterial genetic techniques, will permit assessment of the roles played in enterobacterial pathogenesis by other putative virulence factors which have previously been difficult to study.

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