INFECTION AND IMMUNITY, June 2002, p. 3094–3100 Vol. 70, No. 6
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.6.3094–3100.2002

Characterization of a Novel Type IV Pilus Locus Encoded on the Large Plasmid of Locus of Enterocyte Effacement-Negative Shiga-Toxigenic *Escherichia coli* Strains That Are Virulent for Humans

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Received 3 December 2001/Returned for modification 6 February 2002/Accepted 10 March 2002

The majority of Shiga-toxigenic *Escherichia coli* (STEC) strains isolated from humans with gastrointestinal disease carry large (approximately 90-kb) plasmids. We have been analyzing the megaplasmid (designated pO113) from an O113:H21 STEC strain (98NK2). This strain lacks the locus for enterocyte effacement (LEE) and yet was responsible for an outbreak of hemolytic uremic syndrome. In the present study, we demonstrate that pO113 carries a novel type IV pilus biosynthesis locus (*pil*) related to those of the IncI plasmids R721, R64, and ColIb9. The pO113 *pil* locus consists of 11 closely linked genes (*pilL* through *pilV*) with an additional separately transcribed upstream gene (*pilI*). It directs the expression of long thin pili on the 98NK2 surface and the hemagglutination of guinea pig erythrocytes. We also demonstrate that pO113 can be transferred by conjugation. However, the type IV pilus encoded by pO113 does not appear to be involved in the adherence of 98NK2 to HEp-2 or Hct-8 cells in vitro. Homologues of the pO113 *pil* locus were present in several other LEE-negative STEC strains but not in LEE-positive STEC strains belonging to serogroup O26, O111, or O157.

Shiga-toxigenic *Escherichia coli* (STEC) are an important cause of gastrointestinal disease in humans, particularly since infections with these bacteria may result in life-threatening sequelae, such as hemolytic uremic syndrome (HUS) (14, 20, 25). The production of Shiga toxin (Stx) is associated with over 200 *E. coli* O:H serotypes (20), but epidemiological data indicate that not all of these are highly virulent for humans. Thus, although Stx is a sine qua non of virulence, additional STEC properties, including the capacity to adhere to the intestinal epithelium and colonize the gut, undoubtedly contribute to the pathogenic process. Indeed, STEC strains with the capacity to form attaching and effacing lesions on intestinal mucosae appear to be responsible for the majority of serious cases (those complicated by HUS) (14, 25). This property is encoded by a pathogenicity island termed the locus of enterocyte effacement (LEE) (26), which is present in several important STEC serogroups, most notably O157 and O111. These two serogroups have been responsible for the vast majority of recorded outbreaks of STEC disease complicated by HUS. However, the presence of LEE is not essential for pathogenesis, and a proportion of sporadic cases of HUS, as well as occasional outbreaks, are caused by LEE-negative STEC strains (23, 25).

The mechanism whereby LEE-positive strains adhere intimately and generate attaching and effacing lesions has been the subject of intensive study in recent years (for reviews, see references 8 and 20), but it seems likely that additional (non-LEE-encoded) adherence mechanisms also operate in STEC. For example, O157:H7 STEC have been reported to produce a chromosomally encoded 67-kDa homologue of *Vibrio cholerae* IrgA, termed Iha, which mediates the adherence of O157:H7 STEC to HeLa cells (33). Other putative STEC adhesins include fimbriae, which were initially thought to be encoded by pO157, the 93-kb virulence-related plasmid of O157:H7 STEC. Karch et al. (13) found that the presence of the plasmid correlated with the expression of fimbriae and adherence to Henle 407 cells but not to HEp-2 cells. However, recent genome sequence analyses of two O157:H7 STEC strains indicated that fimbrial loci are not present on pO157 (6, 17), although several such loci are located on the chromosome (11, 27).

Adherence phenotypes have been examined for several LEE-negative STEC strains (7, 9, 30), but virtually nothing is known of the actual mechanisms involved. A gene has recently been isolated from the megaplasmid (designated pO113) of a LEE-negative O113:H21 STEC strain responsible for an outbreak of HUS (23), which encodes an autoagglutinating adhesin designated Saa (STEC autoagglutinating adhesin) (22). The introduction of *saa* cloned in pBC resulted in a 9.7-fold increase in adherence of *E. coli* JM109 to HEp-2 cells and a semilocalized adherence pattern. Mutagenesis of *saa*, or curing the wild-type strain of pO113, resulted in a significant reduction in adherence. Homologues of *saa* were found in several unrelated LEE-negative STEC serotypes (including additional isolates from patients with HUS) but were not present in LEE-positive STEC strains (22). These findings underscore the fact that there are fundamental differences in the genetic compositions of the megaplasmids of LEE-positive and LEE-negative STEC. In the present study, we have continued our examination of pO113 and describe a novel type IV pilus biosynthesis locus encoded thereon.

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MATERIALS AND METHODS

Bacterial strains and cloning vectors. The O113:H21 STEC strain 98NK2 was isolated from a patient with HUS at the Women's and Children's Hospital, Adelaide, South Australia, Australia (23). A derivative of 98NK2 which had been cured of the megaplasmid (98NK2-Cu) and one in which a 1,402-bp internal portion of the *saa* gene carried by the megaplasmid had been deleted and replaced with a kanamycin resistance cartridge (98NK2-S) have also been described previously (22). All other STEC strains used in this study were also isolated at the Women's and Children's Hospital and have been described previously (24), except for the O157:H7 strain EDL933 (provided by R. Robins-Browne), the O91:H21 strain B2F1 (provided by A. Melton-Celsa), and the O113 strains 3848 and 1183 (provided by J. Bennet). *E. coli* K-12 strains DH1 and JM109 have been described previously (10, 35). The cosmid vector pHC79 has also been described previously (12). The phagemid pBC SK (encoding chloramphenicol resistance) was obtained from Stratagene (La Jolla, Calif.). All *E. coli* strains were routinely grown in Luria-Bertani (LB) medium (18) with or without l.5% Bacto Agar (Difco Laboratories, Detroit, Mich.). Where appropriate, ampicillin or chloramphenicol was added to growth media at a concentration of 50 or 40 μg/ml, respectively.

DNA manipulations. Routine DNA manipulations (restriction digestion, agarose gel electrophoresis, ligation, transformation, Southern hybridization analysis, etc.) were carried out essentially as described by Maniatis et al. (18).

Construction of cosmid gene bank. DNA from the megaplasmid (pO113) of 98NK2 was extracted by using a Qiagen (Hilden, Germany) plasmid mini kit and was digested partially with *Sau*3A1 to optimize the yield of fragments in the size range of 35 to 40 kb. This DNA was ligated with a fivefold molar excess of pHC79 DNA, which had been digested with *Bam*HI. Ligated DNA was packaged into lambda heads by using a Packagene kit (Promega Biotec, Madison, Wis.) and transfected into *E. coli* DH1, which had been grown in LB medium plus 2% maltose. The cells were then plated onto LB agar supplemented with ampicillin, and after incubation, the clones were stored in LB medium plus ampicillin plus 15% glycerol in microtiter plates at −70°C.

DNA sequencing. For DNA sequencing, the plasmid DNA template was purified by using a QIAPrep spin miniprep kit (Qiagen); alternatively, PCR products were purified by using an Ultraclean PCR clean-up kit (Mo Bio Laboratories, Solana Beach, Calif.). The sequences of both strands were then determined by dye terminator chemistry with either universal M13 sequencing primers or custom-made oligonucleotide primers on an Applied Biosystems model 3700 automated DNA sequencer.

Hemagglutination assay. Bacteria were grown overnight in LB medium supplemented with appropriate antibiotics, diluted 1:20 in 10 ml of fresh LB medium, and grown with aeration to an A_{600} of approximately 0.5. Cells were pelleted by centrifugation, resuspended in 10 ml of Dulbecco modified Eagle medium (DMEM) (supplemented with 20 μg of L-proline/ml for *E. coli* BH101), and incubated for 3 h at 37°C in 5% CO_2–95% air. Bacteria were then pelleted, washed once in phosphate-buffered saline (PBS), and resuspended in 100 μl of PBS containing 1% D-mannose. Guinea pig and chicken erythrocytes (obtained from the Institute of Medical and Veterinary Science, Adelaide, Australia) or human type A, B, and O erythrocytes (obtained from the Red Cross Blood Transfusion Service, Adelaide, Australia) were washed three times in PBS (pH 7.4) and suspended at a density of 0.3% (vol/vol) in PBS. Hemagglutination assays were carried out in 96-well round-bottom microtiter plates containing 50 μl of serial 10-fold dilutions of each bacterial suspension in PBS (pH 7.4) containing 1% D-mannose. Fifty microliters of washed erythrocyte suspension was then added to the appropriate wells, and the plates were examined for macroscopic hemagglutination after 2 to 4 h at room temperature. Complete hemagglutination involving all of the erythrocytes was scored as "+++"; progressively lesser degrees of hemagglutination were scored as "++" and "+". Trace hemagglutination was scored as "±", whereas reactions in which the erythrocytes formed a tight button indistinguishable from that seen in the presence of PBS were scored as "−".

Conjugation. Conjugation was carried out with 98NK2-S as the donor (the megaplasmid of this strain has a kanamycin resistance cartridge in lieu of the *saa* gene). The recipient was a streptomycin-resistant derivative of 98NK2-Cu (the megaplasmid-cured derivative of 98NK2). Donor and recipient strains were grown overnight at 37°C with shaking in LB medium supplemented with 50 μg of kanamycin/ml or 30 μg of streptomycin/ml, respectively. Donor and recipient strains were washed twice in PBS to eliminate antibiotic and then combined at ratios of 1:5, 1:10, and 1:20. The cells were pelleted by centrifugation, gently resuspended in 200 μl of LB broth, spread onto cellulose acetate filters (0.45-μm pore size, type HA; Millipore Corp., Bedford, Mass.) on LB agar, and incubated for 3 h at 37°C. The cells were resuspended in 10 ml of saline, and aliquots were plated onto LB agar plates supplemented with both 50 μg of kanamycin/ml and 30 μg of streptomycin/ml. The transconjugants were counted after overnight incubation at 37°C.

Electron microscopy. Electron microscopic examination for pili was performed essentially as described previously (32). Bacteria were grown overnight in LB medium supplemented with appropriate antibiotics, diluted 1:20 in 10 ml of fresh LB medium, and grown with aeration to an A_{600} of approximately 0.5. Cells were pelleted by centrifugation, resuspended in 10 ml of DMEM (supplemented with 20 μg of L-proline/ml for *E. coli* BH101), and incubated for 3 h at 37°C in 5% CO_2–95% air. Bacteria were then pelleted and resuspended in DMEM at a density of approximately 10^{10} CFU/ml. A 10-μl aliquot of each sample was spotted onto collodion-coated copper grids (300 mesh; TAAB). The grids were then washed twice in PBS and stained with 2% phosphotungstic acid (in PBS, pH 7.4). The grids were examined in a Philips CM 100 transmission electron microscope at an accelerating voltage of 80 kV.

Nucleotide sequence accession number. The DNA sequence described in this paper has been deposited with GenBank under accession number AF399919.

RESULTS

Cloning of the pO113 *pil* locus. In a previous study, it was demonstrated that a cosmid clone (designated pJCP561) derived from the megaplasmid pO113 of the O113:H21 STEC strain 98NK2 encoded a novel autoagglutinating adhesin, designated Saa (22). In the present study, we continued sequence analysis of this clone and found what appeared to be the 5′ portion of a novel type IV pilus biosynthesis locus approximately 11 kb downstream of *saa*. BLASTX analysis (1) indicated that the 3′ terminus of the pO113 insert in pJCP561 comprised open reading frames (ORFs) whose highest deduced amino acid similarities were with the PilL lipoproteins from two closely related IncI plasmids (ColIb-P9 from *Shigella sonnei* [GenBank accession number AB021078] and R64 from *Salmonella enterica* serovar Typhimurium [GenBank accession number AB027308]) (42% identity) and PilM from plasmid R721 (GenBank accession number AP002527) (36% identity). In order to isolate the complete locus, we constructed a cosmid gene bank of pO113 DNA (see Materials and Methods) and screened it by dot blot hybridization analysis for clones overlapping the 3′ end of pJCP561. One of these, designated pJCP575, was selected for further study. In order to determine the DNA sequence of the insert of pJCP575, a series of *Eco*RI and *Hin*dIII restriction fragments from pJCP575 were subcloned into pBC SK and transformed into *E. coli* JM109. The various subclones were then subjected to sequence analysis as described in Materials and Methods. Where subclones did not overlap, the sequence across the junction was determined with custom-designed oligonucleotide primers with pJCP575 DNA as the template.

The positions of ORFs within the compiled 13,585-bp sequence of part of the 98NK2 DNA insert in pJCP575 are shown in Fig. 1. Examination of the DNA sequence indicated that it contained 13 complete ORFs and 1 partial ORF, and some features of the genes and their predicted protein products are listed in Table 1. The sequenced region commenced with the 3′ end of an ORF with a high degree of similarity to YqiJ, a 23.1-kDa putative oxidoreductase located in the GLGS-WAAE region of *E. coli* K-12. The following ORF was similar to YqiK, a 60.7-kDa putative membrane protein encoded by the same region. Homologues of these two proteins are also present in the genome of *E. coli* O157:H7, although the degree of amino acid identity is slightly lower. The first apparently pilus-related gene, designated *pilI*, was sufficient to

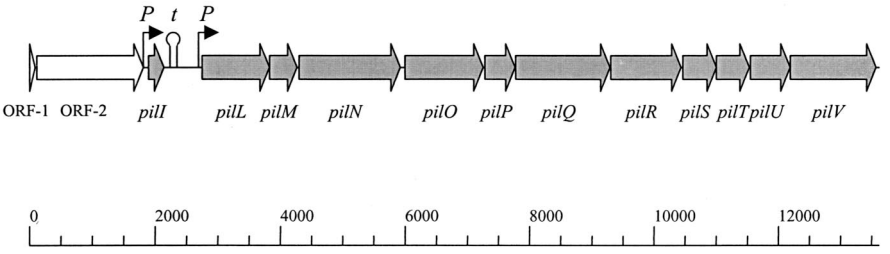

FIG. 1. Map of the portion of the 98NK2 DNA insert of pJCP575 subjected to sequence analysis, showing the location of ORFs (thick arrows) and putative promoter (*P*) and transcription termination (*t*) sites. The scale below the figure is in base pairs.

encode an 80-amino-acid polypeptide with 32% identity over 62 amino acids with PilI from the *S. enterica* serovar Typhimurium plasmid R64. The *pilI* gene was preceded by a ribosome binding site, and the region immediately 5′ of this contained a putative promoter sequence, as predicted by the NNPP program (http://dot.imgen.bcm.tmc.edu) (28); transcription was predicted to start at T_{1878}. Immediately downstream of *pilI* (nucleotides [nt] 2152 to 2181) is a potential stem-loop element ($\Delta G = -18.4$ kcal/mol) which may function as a transcription terminator. There is a 610-nt noncoding region between *pilI* and the next gene, designated *pilL*. The *pilL* gene is also preceded by a weak ribosome-binding site, and a predicted promoter sequence is located approximately 150 nt upstream (the predicted transcription start site is nt 2602). The remainder of the *pil* locus comprises a further 10 genes (designated *pilM* through *pilV*), which appear to be

TABLE 1. Properties of pO113 *pil* genes and their products

Gene or ORF	Location in nucleotide sequence	% G+C	No. of amino acids	Similar protein(s); source (accession no.)	% Identical/% similar (no. of amino acids)	Putative function
ORF 1	1 to 93	50.6	29	YqiJ; *E. coli* K-12 (P76657)	62/82 (29)	Oxidoreductase
ORF 2	120 to 1814	47.3	564	YqiK; *E. coli* K-12 (P77306)	82/89 (541)	Membrane protein
pilI	1895 to 2137	44.8	80	PilI; plasmid R64 (AB027308)	32/51 (62)	
				PilI; plasmid ColIb-P9 (AB021078)	27/51 (61)	
pilL	2747 to 3820	41.5	357	PilL; plasmid R64	43/57 (355)	Lipoprotein
				PilL; plasmid ColIb-P9	42/56 (355)	
				PilL; plasmid R721 (AP002527)	28/44 (272)	
pilM	3825 to 4262	46.1	145	PilM; plasmid R721	36/65 (145)	
				PilM; plasmids R64 and ColIb-P9	29/52 (145)	
				PilM; *S. enterica* serovar Typhi (AF000001)	31/46 (145)	
				PilM; *S. enterica* serovar Dublin (AF247502)	31/47 (142)	
pilN	4293 to 5912	46.1	539	PilN; plasmid R721	64/77 (539)	Outer membrane lipoprotein
				PilN; plasmids R64 and ColIb-P9	39/58 (537)	
				PilNa; *S. enterica* serovar Typhi and *S. enterica* serovar Dublin	45/65 (253)	
				BfpB; enteropathogenic *E. coli* EAF plasmid (U27184)	26/45 (529)	
pilO	5988 to 7235	47.3	415	PilO; plasmid R721	40/52 (385)	
				PilO; *S. enterica* serovar Typhi and *S. enterica* serovar Dublin	25/37 (377)	
				PilO; plasmids R64 and ColIb-P9	21/37 (328)	
pilP	7225 to 7710	41.5	161	PilP; plasmid R721	51/69 (61)	
pilQ	7761 to 9269	48.3	502	PilQ; plasmid R721	66/80 (495)	Nucleotide-binding protein
				PilQ; *S. enterica* serovar Typhi and *S. enterica* serovar Dublin	45/60 (438)	
pilR	9271 to 10368	49.1	365	PilR; plasmid R721	45/67 (361)	Integral membrane protein
				PilR; *S. enterica* serovar Typhi and *S. enterica* serovar Dublin	29/46 (344)	
				BfpB; EPEC EAF plasmid	29/49 (337)	
pilS	10442 to 10978	43.3	178	PilS; plasmid R721	43/57 (180)	Major pilin subunit
pilT	10978 to 11505	46.6	175	PilT; plasmid R721	61/76 (149)	Transglycosylase
				PilT; plasmids R64 and ColIb-P9	54/67 (157)	
				PilT; *S. enterica* serovar Typhi and *S. enterica* serovar Dublin	54/70 (129)	
pilU	11524 to 12159	53.2	211	PilU; plasmid R721	30/51 (166)	Prepilin peptidase
				HopD; *E. coli* (leader peptidase)	28/43 (141)	
pilV	12164 to 13531	49.0	456	PilV (constant region); plasmid R721	52/65 (354)	Minor pilin subunit

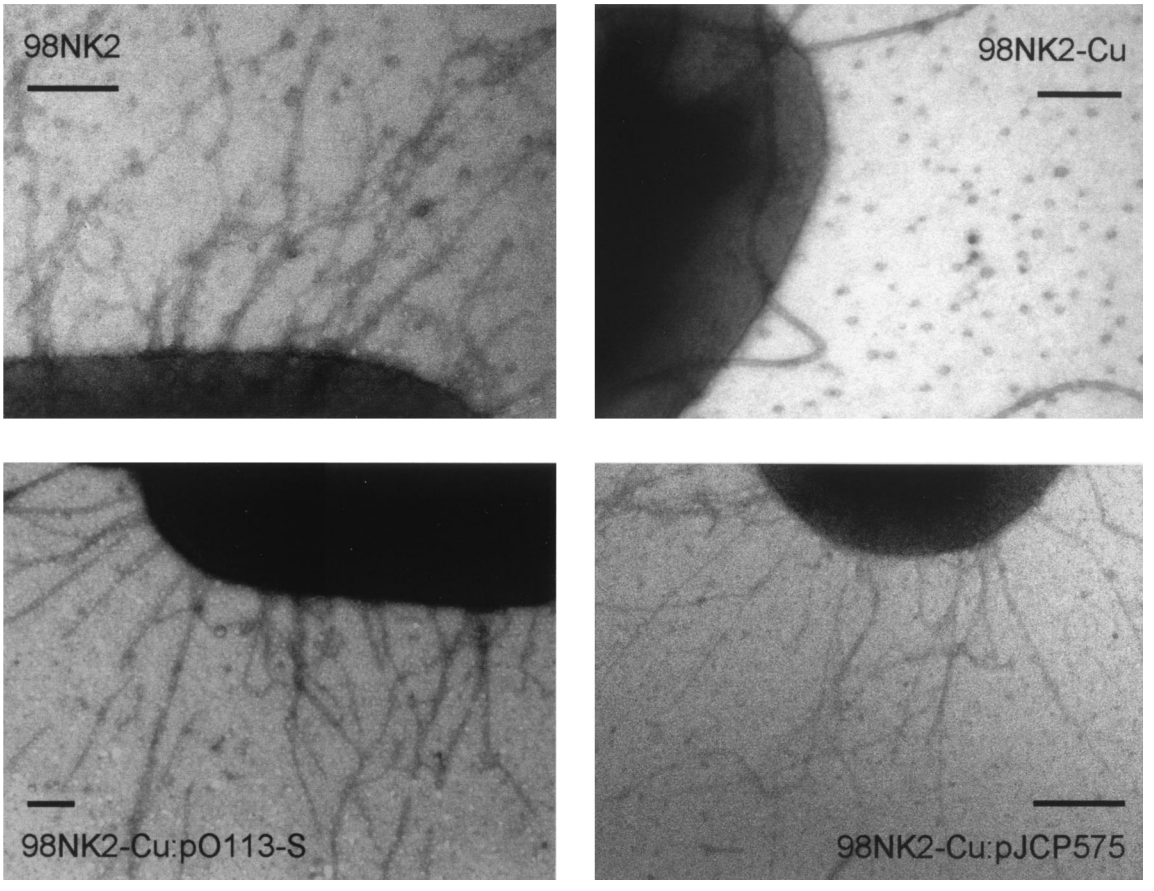

FIG. 2. Electron micrographs of STEC strains after staining with phosphotungstic acid. Bars, 0.2 μm.

linked. The intergenic gaps ranged from −11 to 75 nt (mean = 24 nt). Ribosome binding sites were present before each gene, with the exception of *pilR*, and there were no stem-loop elements within the *pilLMNOPQRSTUV* region, suggesting that the genes may form an operon. BLASTX analysis indicated significant similarity between each of the ORFs and the respective components of the *pil* operon from the IncI plasmid R721 (30 to 66% deduced amino acid sequence identity). Slightly lesser degrees of similarity were observed between the pO113 Pil proteins and their homologues from R64 and ColIb9 and those encoded by the *pil* locus on the large pathogenicity island of *S. enterica* serovar Typhi (38) (Table 1). Putative functions for some of the Pil proteins based on amino acid similarities also are also listed in Table 1. On this basis, *pilS* and *pilV* are predicted to encode the major and minor prepilin proteins, respectively.

Electron microscopy. To examine whether the *pil* locus carried by pO113 is expressed under in vitro conditions, 98NK2 and various derivatives thereof were examined by transmission electron microscopy after being stained with phosphotungstic acid (see Materials and Methods). Long thin pili were observed on the surface of 98NK2, but these structures were absent in 98NK2-Cu, a derivative of 98NK2 which had been cured of pO113 (Fig. 2). Thin pili were, however, observed on the surface of 98NK2-Cu after transformation with either a derivative of pO113 containing a kanamycin resistance cartridge inserted into the *saa* gene or the cosmid pJCP575 (Fig. 2). Collectively, these data indicate that the pili observed on the surface of 98NK2 are indeed encoded by the pO113 *pil* locus.

Hemagglutination. The capacity of the pO113 *pil* locus to mediate hemagglutination was examined by using guinea pig, chicken, and human group A, B, AB, and O erythrocytes, as described in Materials and Methods. For 98NK2, the strongest hemagglutination activity was observed with guinea pig cells. Maximal hemagglutination was observed at a bacterial cell density of 10^8 CFU/ml, with weak hemagglutination also detectable at 10^7 CFU/ml (Table 2). 98NK2 also agglutinated chicken erythrocytes at bacterial cell densities greater than 10^9

TABLE 2. Hemagglutination of guinea pig erythrocytes

Strain	Hemagglutination score[a] at indicated bacterial cell density (CFU/ml)					
	10^{11}	10^{10}	10^9	10^8	10^7	10^6
98NK2	+++	+++	+++	+++	+	−
98NK2-Cu	±	−	−	−	−	−
98NK2-Cu:pO113-S	+++	+++	+++	+	−	−
98NK2-Cu:pJCP575	+++	+++	+++	−	−	−

[a] Hemagglutination was scored after incubation of the various dilutions of bacteria with guinea pig erythrocytes for 2 to 4 h at room temperature (see Materials and Methods, "Hemagglutination assay").

CFU/ml, but there was no agglutination with any of the human erythrocyte suspensions, even when undiluted bacterial suspensions were used (data not shown). Hemagglutination of guinea pig erythrocytes mediated by various 98NK2 derivatives is also shown in Table 2. Only trace hemagglutination was observed with undiluted suspensions of the megaplasmid-cured derivative 98NK2-Cu. However, hemagglutination activity was largely reconstituted in this strain after transformation with the *kan*-tagged megaplasmid pO113-S or the *pil*-carrying cosmid pJCP575. Thus, hemagglutination of guinea pig erythrocytes by 98NK2 appears to be mediated by the pO113 *pil* locus.

In vitro adherence to epithelial cells. It has previously been shown that 98NK2-Cu has a significantly reduced level of in vitro adherence to HEp-2 cells compared with that of wild-type 98NK2 (22). To determine whether the *pil* locus carried by pO113 contributes to this adherence, 98NK2, 98NK2-Cu, and 98NK2-Cu:pJCP575 were tested in the HEp-2 adherence assay as previously described (22). However, the presence of pJCP575 did not significantly increase the adherence of 98NK2-Cu, which remained roughly 50% of that of the wild-type strain (data not shown). The introduction of pJCP575 also had no effect on the adherence of 98NK2-Cu to a human colonic cell line (Hct-8) (data not shown).

Conjugation. The similarity of the pO113 *pil* locus to those of R721, R64, and ColIb9 raised the possibility that this plasmid could also be mobilized by conjugation. To examine this, a streptomycin-resistant derivative of 98NK2-Cu was mated with 98NK2-S, which carries pO113 with a kanamycin resistance cassette inserted into the *saa* gene (see Materials and Methods). Transconjugants resistant to both streptomycin and kanamycin were obtained at all donor-to-recipient ratios tested (1:5, 1:10, and 1:20). However, there was no growth in the presence of streptomycin plus kanamycin when either donor or recipient cells were omitted from the mating mix. The transconjugants were all positive by PCR for a variety of genes present in the donor plasmid but absent in 98NK2-Cu, including the *saa*::*kan* locus, *pilS*, *espP*, *iha*, and *ehxA* (data not shown).

Presence of the pO113 *pil* locus in other *E. coli* strains. The presence of related *pil* loci in other *E. coli* strains was examined by Southern hybridization analysis with a digoxigenin-labeled *pilS*-specific probe as well as by PCR with primers specific for *pilS* (Table 3). None of the LEE-positive STEC strains tested (including representatives of serogroups O157, O111, and O26) contained *pilS*-related sequences. Among the LEE-negative STEC strains, *pilS*-related sequences were detected in 5 of 5 O113 strains as well as in 11 of 14 strains belonging to other serogroups. Interestingly, the presence of *pilS* did not necessarily correlate with the presence of the other putative virulence genes carried by the megaplasmid, *saa* and *ehxA*, a result which further underscores the heterogeneity of these plasmids in STEC.

TABLE 3. Presence of *pilS* homologues in other STEC strains

E. coli strain	Serotype[a]	Source[b]	Presence of:			
			pilS[c]	*saa*[d]	LEE[e]	*ehxA*[e]
98NK2	O113:H21	HUS	+	+	−	+
97MW1	O113:H21	BD, MHA, T	+	+	−	+
3848	O113:H21	HUS	+	+	−	+
1183	O113:H21	HUS	+	+	−	+
MW10	O113:H21	Food	+	+	−	+
94CR	O48:H21	HUS	+	+	−	+
B2F1	O91:H21	HUS	−	+	−	+
MW13	O98	Food	+	+	−	+
MW2	Ont	Food	+	+	−	+
MW14	ND	Food	+	+	−	+
MW8	Ont	Food	+	+	−	+
MW6	OR	Food	+	+	−	+
MW3	O82:H8	Food	+	+	−	+
MW7	Ont:H11	Food	+	+	−	+
95HE4	O91	D	+	+	−	+
MW15	O141	Food	+	−	−	−
MW4	Ont	Food	+	−	−	−
MW11	ND	Food	−	−	−	−
MW12	O159	Food	−	−	−	−
EDL933	O157:H7	BD	−	−	+	+
96GR1	O157:H$^-$	HUS	−	−	+	+
95ZG1	O26	BD	−	−	+	+
96RO1	O111:H$^-$	HUS	−	−	+	+
95NR1	O111:H$^-$	HUS	−	−	+	+

[a] Ont, O nontypeable; OR, O rough; ND, serotype not determined.
[b] STEC strains were isolated either from foods or from the feces of patients with uncomplicated diarrhea (D), bloody diarrhea (BD), microangiopathic hemolytic anemia (MHA), thrombocytopenia (T), or hemolytic uremic syndrome (HUS).
[c] Determined by Southern hybridization analysis with a digoxigenin-labeled PCR product corresponding to the complete *pilS* open reading frame as probe and by PCR.
[d] Determined by Southern hybridization analysis (22).
[e] Determined by PCR for *eae* and *ehxA* (21, 24).

DISCUSSION

The majority of STEC strains isolated from humans with gastrointestinal disease carry large (approximately 90-kb) plasmids (25). These encode a number of putative accessory virulence proteins, such as the enterohemolysin EhxA (29) and the serine protease EspP (5). The complete DNA sequences of pO157 plasmids from two distinct O157:H7 STEC strains have been determined and found to be very similar to each other (6, 17). However, PCR, Southern hybridization, and partial sequence analyses of the megaplasmids from STEC strains belonging to different serotypes suggest that there are major differences in both the genes present and their organization (4). We have been analyzing the megaplasmid pO113 from an O113:H21 STEC strain (98NK2), which lacks the LEE locus and yet was responsible for an outbreak of hemolytic uremic syndrome. It has previously been demonstrated that this plasmid encodes a novel adhesin (Saa) which is unique to LEE-negative STEC strains (22). In the present study, we have shown that pO113 also carries a novel type IV pilus biosynthesis locus comprising 11 closely linked genes (*pilL* through *pilV*) with an additional, separately transcribed gene (*pilI*) upstream. This pilus locus is most closely related to those of the IncI plasmids R721, R64, and ColIb9, but there are several differences in organization. In R721, *pilL* and *pilM* are separated from *pilNOPQRSTUV* by a 10.6-kb region containing 12 genes (*ygeA*, *traB* through *traK*, and *yfdA*) and there is also no homologue of *pilI*. On the other hand, the R64 and ColIb9 loci contain two additional genes (*pilJ* and *pilK*) between *pilI* and *pilL*, and both loci appear to form a single 14-gene transcriptional unit. However, in R64, *pilI* and *pilJ* have been shown not to be essential for pilus biogenesis (37). The organization of the *pil* loci from the major pathogenicity islands of *S. enterica*

serovar Typhi (38) and *S. enterica* serovar Dublin (AF247502) is essentially the same as that of the *pilL*-through-*pilV* region of pO113. The pO113 *pilI* and *pilL* gene products are more similar to their respective R64 homologues than they are to those of R721, whereas the reverse is true for the products of *pilM* through *pilV*; this also suggests the possibility of recombination events during the evolution of the pO113 *pil* locus.

Type IV pili have been shown to be involved in the adherence of a number of pathogenic bacteria to host epithelial cells. Among the better characterized of these are the bundle-forming pilus of enteropathogenic *E. coli*, which has been implicated in human virulence, autoaggregation, and localized adherence (2, 31, 32), and the toxin-coregulated pilus of *V. cholerae*, which is essential for gut colonization and virulence in an infant mouse model (19). The type IV pilus encoded on the major pathogenicity island of *S. enterica* serovar Typhi, which is even more closely related to the pO113 pilus, has also been shown to be important for adherence to and invasion of intestinal epithelial cells (38). In the present study, we demonstrated that 98NK2, but not the plasmid-cured derivative 98NK2-Cu, expressed long thin pili visible by electron microscopy after negative staining. Pili were also present on the surfaces of derivatives of 98NK2-Cu after electroporation with pO113-S or the cosmid pJCP575, both of which carry complete *pil* loci. Both of these 98NK2-Cu derivatives were also capable of hemagglutinating guinea pig erythrocytes. However, the introduction of pJCP575 into 98NK2-Cu did not increase the in vitro adherence to HEp-2 cells. It has previously been reported that 98NK2-Cu exhibits a level of adherence to HEp-2 cells similar to that seen for 98NK2 carrying pO113-S, which lacks the *saa* gene (in both cases, roughly 50% of that for wild-type 98NK2) (22). Thus, the HEp-2 cell adherence conferred by pO113 appears to be largely attributable to *saa*. It is possible, of course, that the pO113-encoded pili may mediate adherence to receptors on human intestinal epithelial cells that are not present on HEp-2 cells, although in the present study, the introduction of pJCP575 did not increase the adherence of 98NK2-Cu to Hct-8, a colonic cell line of human origin.

The R64 pilus was the first member of the type IV family to be implicated in bacterial conjugation, and several genes within this locus have been shown to be essential for liquid mating, presumably by facilitating the attachment of donor cells to recipient bacteria (16, 37). The minor pilin subunit protein PilV appears to play a key role in recipient cell recognition. Interestingly, the C-terminal portion of this protein and its homologues encoded on ColIb9 and R721 undergo structural rearrangements under the control of shufflons (15, 36, 37). The shufflons comprise a *pilV* constant region followed by several DNA segments flanked and separated by inverted repeat sequences. These are followed by the *rci* gene, the product of which promotes site-specific recombination between any two inverted repeat sequences, resulting in the in-frame fusion of one of the alternative segments to the constant *pilV* ORF. In R64, the resultant variation in the C-terminal portion of PilV determines the recipient cell specificity in liquid mating (16). However, pO113 *pilV* does not appear to contain such repeat elements. In the present study, we have demonstrated that like R721, R64, and ColIb9, the *kan*-tagged megaplasmid derivative pO113-S can be transferred by conjugation into 98NK2-Cu. Confirmation that the *pil* locus is essential for this will require construction of pO113 derivatives with defined *pil* knockout mutations. Nevertheless, the fact that pO113 is transferable distinguishes it from pO157, the megaplasmid of O157:H7 STEC strains, which lacks *pil* loci or *tra* genes and is presumed to be nonconjugable (6, 17).

High-stringency homologues of pO113 *pilS* were found in a wide range of LEE-negative, but not in any LEE-positive, STEC strains. The presence of *pilS* sequences did not always correlate with the presence of other presumptive megaplasmid genes. For example, the O91:H21 strain B2F1 contains both *saa* and *ehxA* but lacks *pilS*, underscoring the likelihood of further differences in the genetic composition of LEE-negative STEC megaplasmids. Conversely, two other STEC strains tested contained a *pilS* homologue but lacked *ehxA* or *saa*. It is not yet known whether these two strains actually harbor large virulence plasmids, but Brunder et al. (4) have reported that some STEC megaplasmids lack *ehxA* as well other genes carried by pO157, such as *espP* and *katP*. Interestingly, although pO157 from O157:H7 STEC lacks *pil* loci, Brunder et al. (3) have recently described a six-gene operon on the megaplasmid of sorbitol-fermenting O157:H$^-$ STEC strains which encodes the synthesis of pili distantly related to uropathogenic *E. coli* P-pili. This operon was present in all sorbitol-fermenting O157:H$^-$ strains tested but not in sorbitol-negative O157 or any of the other STEC or other pathogenic *E. coli* strains tested. However, although this operon directed the expression of pili when cloned in *E. coli* K-12, the expression appeared to be repressed in wild-type sorbitol-fermenting O157:H$^-$ strains in vitro.

The conjugal transfer of STEC megaplasmids may help to explain the broad range of STEC strains in which they are found. However, it does not account for the fact that *ehxA*, which has been commonly used as a marker for the presence of an STEC megaplasmid, is rarely found in non-STEC strains (29). Since Shiga toxins are almost invariably phage encoded, this suggests that there is a tight association between the presence of a megaplasmid and the susceptibility to lysogeny by Stx-converting phages. Other closely related members of the type IV pilus family provide ample precedent for this possibility. TcpA, the major toxin-coregulated pilus subunit of *V. cholerae*, is known to be the receptor for Ctxϕ, the phage which encodes cholera toxin (34). Similarly, phages Iα and PR64FS specifically adsorb to *E. coli* via the shaft (PilS) and tip (PilV), respectively, of the thin pili encoded by plasmid R64 (37). Thus, the very presence of the *pil* locus may have contributed to the diversity of *E. coli* strains carrying *stx* genes and capable of causing disease in humans.

ACKNOWLEDGMENT

This work was supported by a grant from the National Health and Medical Research Council of Australia.

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Editor: A. D. O'Brien