

An IncI1 Plasmid Contributes to the Adherence of the Atypical Enteroaggregative *Escherichia coli* Strain C1096 to Cultured Cells and Abiotic Surfaces

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Enteroaggregative *Escherichia coli* (EAEC) is defined by a characteristic “stacked-brick” aggregative adherence (AA) pattern to cultured cells. In well-studied EAEC prototype strains (called typical EAEC strains), the AA phenotype requires aggregative adherence fimbriae (AAF). However, previous studies suggest that known AAF alleles are not found in all EAEC strains. To define mechanisms contributing to adherence in an atypical strain, we studied EAEC strain C1096. An *E. coli* K12 derivative carrying two plasmids, designated pSERB1 and pSERB2, from C1096 adhered to cell lines and exhibited an AA pattern. Nucleotide sequence analysis of pSERB1 indicated that it is related to plasmids of the IncI1 incompatibility group. These plasmids encode genes involved in pilus-mediated conjugal transfer, as well as *pilL-V*, which encodes a second pilus of the type IV family. Insertional inactivation of the gene predicted to encode the major type IV pilin subunit (*pilS*) reduced conjugal transfer of the plasmid by 4 orders of magnitude. Adherence of the mutant strain to polystyrene and to HT29 cells was reduced by approximately 21% and 75%, respectively. In a continuous-flow microfermentor, the *pilS* inactivation reduced mature biofilm formation on a glass slide by approximately 50%. In addition, the simultaneous presence of both pSERB1 and pSERB2 plasmids promoted *pilS*-independent biofilm formation. We conclude that the IncI1 plasmid of EAEC C1096 encodes a type IV pilus that contributes to plasmid conjugation, epithelial cell adherence, and adherence to abiotic surfaces. We also observe that AA can be mediated by factors distinct from AAF adhesins.

Enteroaggregative *Escherichia coli* (EAEC) is an emerging diarrheal pathotype defined by its characteristic “stacked-brick” aggregative adherence (AA) pattern to semiconfluent HEp-2 cells (30). This pattern results from adherence to cultured cells, other bacteria, and surrounding abiotic surfaces. For the prototype EAEC strain 042, this phenotype is mediated by the aggregative adherence fimbria (AAF) designated AAF/II. Three other AAF alleles are defined, and current data suggest that others exist (2, 9, 31). AAF fimbriae are necessary *ex vivo* for adherence of EAEC 042 to intestinal epithelia, abiotic surfaces, and cultured cells (8, 31, 42). Following colonization, EAEC may release cytotoxins, resulting in cellular damage (14, 40), and elicit an intestinal inflammatory response, which is stimulated at least in part by the bacterial flagellum (44).

Distribution studies previously identified numerous plasmid and chromosomal genes that are well conserved in EAEC strains isolated from distinct geographic locations. These include genes for dispersin (*aap*), which is involved in dispersing bacterial aggregates (41); *ataA*, which encodes a TolC-related transport system for dispersin (33); an AggR-regulated chro-

mosomal gene (E. G. Dudley and J. P. Nataro, unpublished data); and the AraC-family transcriptional regulator *aggR* (32). These genes, including *aggR* itself (R. Sohoni and J. P. Nataro, unpublished data), are transcriptionally activated by AggR, and we hypothesize that the *aggR* regulon constitutes a package of linked virulence genes defining typical EAEC strains (30). Some clinical studies report that *aggR*-positive EAEC strains are isolated more frequently from individuals presenting with diarrhea and elevated fecal cytokine levels (19, 34, 35), supporting the hypothesis that the presence of the *aggR* regulon distinguishes pathogenic from nonpathogenic strains.

In contrast, atypical EAEC strains (30) lack all genes of the *aggR* regulon. It is suspected that the majority of these strains are nonpathogenic (6, 39). Atypical EAEC strains also lack genes for the currently defined AAFs, as do at least one-third of all EAEC strains (2). Therefore, either certain EAEC strains encode distantly related AAFs or there exist AAF-independent mechanisms for bacteria to adhere in an AA phenotype. Support for this latter hypothesis was recently reported for EAEC 236, which encodes an outer-membrane protein that confers AA upon *E. coli* K-12 (28).

Type IV pili are a group of well-studied extracellular structures defined by related phylogeny and a conserved pathway of pilus biogenesis (45). These pili are implicated in numerous phenotypes in gram-negative pathogens, including adherence (12, 47), twitching motility (26), DNA transformation (13), and

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TABLE 1. Strains and plasmids used in this study^a

Strain or plasmid	Description	Source or reference
<i>E. coli</i> strains		
C1096	Wild-type EAEC strain from diarrhea outbreak	5
C1096 <i>pilS::dfrA</i>	Strain C1096 with <i>dfrA</i> inserted into pSERB1-encoded <i>pilS</i> ; does not express visible type IV pili	This study
DH5 α - <i>λpir</i>	Strain for maintaining R6K-origin encoding plasmid pCVD442	Laboratory stock
SM10- <i>λpir</i>	Strain for mobilizing pCVD442 derivatives	Laboratory stock
Plasmids		
pCVD442	Ap ^r ; suicide vector encoding the counterselectable marker <i>sacB</i>	11
pKD3	Ap ^r Cm ^r ; template DNA for <i>cat</i> amplification by PCR	10
pKM208	Ap ^r ; temperature-sensitive vector encoding <i>red/gam</i> for one-step inactivation protocol	29
pPIL1096	Ap ^r ; pCVD442 encoding the <i>E. coli</i> C1096 <i>pilS</i> insertionally inactivated by replacing an internal sequence with a NotI site	This study
pPIL1096 <i>dfrA</i>	Ap ^r Tm ^r ; <i>dfrA</i> inserted into the NotI site of pPIL1096	This study
pSERB1	Plasmid from strain C1096 carrying <i>pilL-V</i> and conjugal transfer apparatus	This study
pSERB1: <i>aph</i>	Kn ^r ; pSERB1 with <i>aph</i> -marker in putative noncoding region	This study
pSERB2	Tm ^r ; 2.6-kb plasmid derived from a <i>dfrA</i> insert in the 1.6-kb plasmid of C1096	This study
pSERB3	Tm ^r Kn ^r ; pSERB1: <i>aph</i> with <i>dfrA</i> inserted into <i>pilS</i>	This study
pSERB4	Cm ^r Kn ^r ; pSERB1: <i>aph</i> with <i>cat</i> inserted into <i>pilS</i>	This study

^a Abbreviations: Ap^r, confers ampicillin resistance; Cm^r, confers chloramphenicol resistance; Kn^r, confers kanamycin resistance; Tm^r, confers trimethoprim resistance; *aph*, aminoglycoside phosphotransferase; *cat*, chloramphenicol acetyltransferase; *dfrA*, dihydrofolate reductase; *sacB*, levansucrase.

conjugation (20). The role of a type IV pilus in promoting conjugation is best described for the *Salmonella* plasmid designated R64 (17, 49), which is a member of the IncI1 plasmid incompatibility group. The IncI1 family encodes a large number of genes mediating the synthesis of two different pili, defined as the thick pilus and the thin pilus (3). The former is essential for conjugal transfer of R64, while the thin pilus, which is categorized as type IV, is needed only when conjugation occurs in liquid medium (3). The thin pilus locus encompasses 14 genes, designated *pilI* through *pilV*, and 12 of these genes are essential for pilus biogenesis (49). A number of studies define the role of the thin pili in mediating surface interactions between bacteria (16–18); however, the IncI1-encoded type IV pilus has not been implicated in phenotypes apart from conjugation.

Here, we report the first studies on a proven pathogenic atypical EAEC strain, targeting the multiple-antibiotic-resistant strain implicated in a 1995 Serbian neonatal ward outbreak (5). This strain, designated C1096, is probe negative for *aggR* and all currently known *AggR* regulon members (9). We found the following: (i) EAEC C1096 carries a large plasmid (pSERB1) that encodes a functional type IV pilus and is related to plasmids of the IncI1 family; (ii) type IV pilus synthesis is required for optimal conjugation of the large plasmid; (iii) the presence of pSERB1 mediates adherence and biofilm formation of C1096 via expression of type IV pili; (iv) pSERB1, in cooperation with the small cryptic plasmid pSERB2, also mediates type IV pilus-independent biofilm formation; and (v) the genes encoding the type IV pilus are found in approximately 10% of both typical and atypical EAEC strains. This paper therefore reports two previously undescribed phenotypes conferred by an IncI1 plasmid: adherence to epithelial cells in a type IV pilus-dependent manner and adherence to abiotic surfaces via a type IV pilus-independent mechanism.

MATERIALS AND METHODS

Strains, plasmids, and antibiotics. Strains and plasmids used in this study are listed in Table 1. Bacteria used in colony hybridization experiments were de-

scribed previously (9) or are clinical isolates from our laboratory stocks. Antibiotics, when used, were incorporated into solid and liquid media at the following concentrations: trimethoprim (TMP), 10 $\mu\text{g ml}^{-1}$; ampicillin, 60 $\mu\text{g ml}^{-1}$; chloramphenicol, 25 $\mu\text{g ml}^{-1}$; kanamycin (KAN), 20 $\mu\text{g ml}^{-1}$; streptomycin (STR), 50 $\mu\text{g ml}^{-1}$; and nalidixic acid, 10 $\mu\text{g ml}^{-1}$.

Cell culture techniques. All cell cultures were maintained in humidified 5% CO₂ at 37°C. HEp-2 cells were grown in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 U ml⁻¹), and STR (100 $\mu\text{g ml}^{-1}$) (all from Gibco-BRL). HT29/C1 human colonic cells were grown in high-glucose DMEM supplemented with 10% fetal calf serum, apotransferrin (10 $\mu\text{g ml}^{-1}$) (Sigma), STR (50 $\mu\text{g ml}^{-1}$), and penicillin (50 U ml⁻¹).

Molecular biology protocols. DNA manipulations were performed using standard laboratory protocols (38). Total DNA was isolated from bacterial strains by the cetyltrimethylammonium bromide-NaCl method (1). PCRs were performed using either the Elongase Amplification System (Invitrogen) or *Taq* DNA polymerase (New England Biolabs).

Colony hybridizations were performed following the method of Sambrook and Russell (38), using high-stringency hybridization conditions (60°C; 5 \times SSC [1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% *N*-lauroylsarcosine, 0.5% blocking reagent, 0.02% sodium dodecyl sulfate in prehybridization and hybridization buffers) or low-stringency conditions (42°C; 10% formamide in prehybridization and hybridization buffers). Gene probes were synthesized using Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech, Inc.). Template DNA for *pilL*, *pilN*, *pilQ*, *pilS*, *pilT*, and *pilV* was synthesized by PCR, using the primer pairs 5'-CTTCTGCCGTCATTACTCA-3' and 5'-CATCGATGCTGTAGTTGACA-3', 5'-AGTTCTGACACGACACTTCC-3' and 5'-CTGGTTGATGGTTTTTCAGAT-3', 5'-ACGTCATAAAGGGAACACAG-3' and 5'-GACCTGACCATTGTTGATTT-3', 5'-ATGAGCGTCATAACCTGTTCC-3' and 5'-CTGTTGGTTTCCAGTTTGTAT-3', 5'-TGTTATGCTCACTTCTTCC-3' and 5'-CTAACGTCCTGCATGTAA-3', and 5'-CTTTGGGCAGAGTTATACC A-3' and 5'-TTAATTGAGCGTTACACACG-3', respectively. Amplification conditions were as follows: 95°C for 3 min and 30 cycles of 94°C for 45 s, 50°C for 30 s, and 72°C for 1 min, followed by a final extension of 72°C for 7 min.

Southern hybridizations were used to confirm successful construction of the *pilS* mutant of C1096 (see below), using the *pilS* gene probe described above. Total DNA was digested with *Afl*III (New England Biolabs), and fragments were separated on a 0.7% agarose gel. DNA transfer to a Hybond-N+ membrane (Amersham), prehybridization, hybridization, and radioactive detection were performed using standard methods (38). The prehybridization and hybridization temperature was 60°C. To construct derivatives of *E. coli* DH5 α carrying plasmids from strain C1096, an EZ::TN <DHFR-1> Insertion Kit (Epicenter Technologies) was used. A dihydrofolate reductase-encoding (*dfrA*) transposon was randomly inserted into CsCl-purified plasmids from strain C1096 in vitro, as recommended by the manufacturer. After the transposition reaction, plasmid

pools were transformed into DH5 α by electroporation and selected on LB plates containing TMP (LB+TMP).

Tagging of pSERB1 with a KAN resistance marker was achieved using a one-step recombination method (29). A PCR product encoding a KAN resistance marker and 50 nucleotides complementary to a putative noncoding region of pSERB1 on the 5' and 3' ends was generated using the primers 5'-AGACGATTGTTTCTCTCGTAGTTTTGTCCACTCTAAACAAAGTGTATCATATGAATATCCTCCTTAG-3' and 5'-GATCACAGGAAATGGAATAACA CTGTAAGTCAATGGAAAAGTGAACCGTGTAGGCTGGAGCTGCTT C-3'. The *aph*-encoding plasmid pKD4 (10) was used as template DNA, and the reaction conditions were 95°C for 2 min; 10 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 90 sec; and 15 cycles of 94°C for 45 s, 68°C for 30 s, and 72°C for 90 s. The PCR product was transformed into *E. coli* (pSERB1, pSERB2, and pKM208), and integrants were selected for on LB+KAN plates. Loss of pKM208 was confirmed by replica plating colonies onto LB+KAN and LB+KAN+ampicillin plates. Integration of the PCR product in the expected location was confirmed using PCR and the primers 5'-GCGTCAATAAGAGACGTTGT-3' and 5'-ATAAACATGAACGGGCATAC-3'. Reaction conditions were 95°C for 10 min and 30 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 2 min 15 s, followed by 72°C for 7 min. Template DNA consisted of 1 μ l of an overnight grown bacteria culture. A 0.4-kb or 2.0-kb PCR product was generated when the template was pSERB1 or pSERB1:*aph*, respectively. This method was also followed to construct pSERB3.

Quantification of the pSERB1-encoded genes *traN* and *traY* and chloramphenicol acetyltransferase (*cat*), which is not encoded on pSERB1, was accomplished using real-time PCR. Total DNA from strains *E. coli* C1096 and C1096*pilS::dfrA* was isolated using the Instagene matrix (Bio-Rad). Real-time PCR was performed in a Chromo4 Real-Time PCR Detector (Bio-Rad), using a Quantitect SYBR Green PCR kit (QIAGEN). The following primer pairs were used: *cat*, 5'-CCACTCATCGCAGTACTGTT-3' and 5'-TCACTGGATATACCACCGT T-3'; *traN*, 5'-GGAATGTGACGGTCTATCTC-3' and 5'-GACTGTCGATTT CTTGACTC-3'; and *traY*, 5'-ATGGGGATAGTCTGGTTTCT-3' and 5'-TCC ACTCAGACACTGAGTA-3'.

DNA sequencing and bioinformatic analysis. Plasmids from *E. coli* (pSERB1 and pSERB2) were isolated by CsCl density gradient centrifugation, sonicated using a Fisher Scientific 550 Sonic Dismembrator, and end repaired with T4 DNA polymerase, Klenow DNA polymerase, and T4 polynucleotide kinase (38). DNA was separated on a 0.6% agarose gel, and fragments between ~3 to 4 kb were isolated with a QIAquick Gel Extraction Kit (QIAGEN). Fragments were ligated into SmaI-digested pUC18 and transformed into DH5 α . Sequencing reactions were performed using BigDye Terminator Sequencing Reagents (Applied Biosystems) and were analyzed using an Applied Biosystems 3700 DNA analyzer.

DNA sequences were assembled and edited using a Lasergene software package (DNASTAR, Inc.). Assembled contigs were annotated using the Artemis package (37), and open reading frame identification was assisted by the GeneMark prediction program (<http://opal.biology.gatech.edu/GeneMark>) using the training set generated for enterohemorrhagic *E. coli* EDL933. Sequence alignments and amino acid motifs were identified using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) and the BLO SUM62 matrix. Nucleotide sequence overlaps between pSERB1 contigs and R64 were generated using BLASTALL from the NCBI website (<http://www.ncbi.nlm.nih.gov>) and were visualized using the Artemis Comparison Tool from the Sanger Centre (<http://www.sanger.ac.uk>).

Construction of pSERB1 derivatives carrying *pilS::dfrA* and *pilS::cat* mutations. A plasmid containing *dfrA* inserted into *pilS* was constructed by a modification of the overlap PCR protocol of Merlin et al. (27). Total DNA (~50 ng) from EAEC C1096 was used as a template in a 20- μ l reaction mixture. A PCR fragment encoding the 5' end of *pilS* and upstream DNA was generated using the primers 5'-AAAAAAGTCGACCGGATTTGGTAAAAAGAAA-3' (Nat1913) and 5'-CGCTCTTGGCGCGCTTGGAAACGGGTCATGCCTTTATCATCGT T-3' (Nat1916). A PCR fragment encoding the 3' end of *pilS* and downstream DNA was generated using primers 5'-AAAAAAGTCGACAGGCCTATGGTT TCTGTTTT-3' (Nat1914) and 5'-CCGTTCAAGCGGCGCAAGAGCGGG CAATGTGACGAATAAAGT-3' (Nat1915). The 3' end of the former product and the 5' end of the latter product overlap by 24 bp, and an NotI site is located within this overlap (underlined in the sequences of Nat1915 and Nat1916). Reaction conditions were 94°C for 30 s, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min. A second round of PCR was performed using approximately 20 ng of each PCR product from the prior reactions as template DNA and the primers Nat1913 and Nat1914. Reaction conditions were the same as before, except that an extension time of 2 min was used. The resulting PCR product was digested with Sall, ligated into similarly digested pCVD442, and

transformed into DH5 α -*lambda*pir. This plasmid was designated pPIL1096. Next, a PCR fragment encoding dihydrofolate reductase (*dfrA*) and 5' and 3' NotI sites was generated using primers 5'-AAAAAAGCGGCCGCGGATCACCGAAAT CTTCATA-3' and 5'-AAAAAAGCGGCCGCGCATGCACGATTTGTAATA A-3' and the EZ::TN <DHFR-1> Transposon (Epicenter Technologies) as template DNA. The cycling conditions were the same as those used with primers Nat1914 and Nat1915.

The PCR product was digested with NotI and ligated to NotI-digested pPIL1096, forming pPIL1096*dfrA*. This plasmid was transformed into *E. coli* SM10-*lambda*pir and was conjugated into EAEC C1096 (11). Sucrose-, chloramphenicol-, and TMP-resistant transconjugants were screened by PCR using primers Nat1913 and Nat1914; template DNA from wild-type C1096 generates a 2.7-kb product, whereas mutants containing the *dfrA* insert generate a 3.1-kb product. Putative mutants were further screened by Southern hybridization. The *pilS::dfrA* derivative of pSERB1 was tagged with *aph* as described above and was designated pSERB3.

A chloramphenicol acetyltransferase (*cat*) insertion into *pilS* was generated by amplifying *cat* using the primers 5'-AAAAAAGCGGCCGCGTGTAGGCTGG AGCTGCTTA-3' and 5'-ATATATGCGGCCCATATGAATATCCTCCTT AG-3' and pKD3 (10) as template DNA. Cycling conditions were 25 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 70 s. The product was digested with NotI and ligated into similarly digested pPIL1096. The resulting plasmid was used as template DNA in a PCR using primers Nat1913 and Nat1914 under the conditions described previously. The resulting PCR product, encoding *pilS::cat* and flanking DNA, was transformed into DH5 α (pSERB1:*aph*, pSERB2, pKM208) and integrated by the one-step recombination method (29). Proper integration was verified by PCR using primers 5'-TACGTTTTACGAGTCCCTTG-3' and 5'-TGTCGTGTACACCGCA GATA-3'. Reaction conditions were 94°C for 45 s, 55°C for 30 s, and 72°C for 4 min. A wild-type product generated a PCR product of 2.7 kb, while a mutant generated a 3.4-kb product. The resulting plasmid was designated pSERB4.

Scanning electron microscopy. Bacteria were grown overnight in LB with appropriate antibiotics and were inoculated to 2% in high-glucose DMEM and grown in 24-well polystyrene cell culture dishes containing a 1-cm² glass coverslip. After approximately 4 h of growth, the medium was removed, and nonadherent bacteria were removed by five washes with phosphate-buffered saline (PBS). Cells were fixed to the coverslips for 15 min with PBS containing 2% glutaraldehyde and 3 mM CaCl. Coverslips were sent to the Johns Hopkins School of Medicine Microscopy Facility for analysis.

Bacterial adherence assays. Adherence of bacteria to glass and polystyrene surfaces was performed using a previously described crystal violet-based method (42).

For qualitative adherence assays, cultured cells were grown on glass coverslips in 24-well tissue culture plates. T84 cells were grown to 100% confluence (8 to 10 days) and were subsequently incubated for an additional 2 to 3 days. HEp-2 and HT29 cells were grown to approximately 90% confluence. Overnight bacterial broth cultures were diluted 1:50 in culture medium supplemented to 1% methyl- α -D-mannopyranoside (to inhibit binding by type I fimbriae) and incubated with cells for 2.5 to 6 h at 37°C. After this period, cells were thoroughly washed with PBS to remove nonadherent bacteria, fixed in 70% methanol for 5 min, stained with 10% Giemsa for 15 min, and observed by light microscopy.

For quantitative adherence assays using plate counts, HT29 cells were grown to approximately 90% confluence. Cells were washed twice with PBS, and 1 ml of high-glucose DMEM supplemented to 1% methyl- α -D-mannopyranoside was added to each well. Overnight cultures of EAEC strains C1096 and C1096*pilS::dfrA* were diluted in PBS to an optical density at 600 nm (OD₆₀₀) of 0.08 (approximately 2×10^6 CFU ml⁻¹), and 10 μ l of each was added to wells. After a 2.5-h incubation at 37°C, half of the coverslips were moved to a new 24-well plate and washed with five 1-ml aliquots of PBS. After the last PBS aliquot was aspirated, 1 ml of 0.1% Triton X-100 was added, mixed thoroughly, and allowed to stand for approximately 5 min. Serial dilutions were made on LB and LB+TMP plates to determine the total number of adherent bacteria and *pilS* mutant cells, respectively. To the other half of the culture plate (the half for which the coverslips were not removed), 10 μ l of 10% Triton X-100 was added. Serial dilutions were made on LB and LB+TMP plates to determine the total number of bacteria and total number of *pilS* mutant cells, respectively, per well. The ratio of adherent wild-type to mutant bacteria was calculated by dividing the number of adherent wild-type C1096 bacteria by the number of adherent C1096*pilS::dfrA* bacteria and normalizing to the total CFU of each strain in the wells. In control experiments, no difference in CFU ml⁻¹ was found when serial dilutions of C1096*pilS::dfrA* were plated on LB or LB+TMP, indicating that differences in adherence calculated for this strain were not due to suppression of growth of C1096*pilS::dfrA* by TMP. Additionally, no difference in the growth rate was observed between C1096 and C1096*pilS::dfrA* in DMEM.

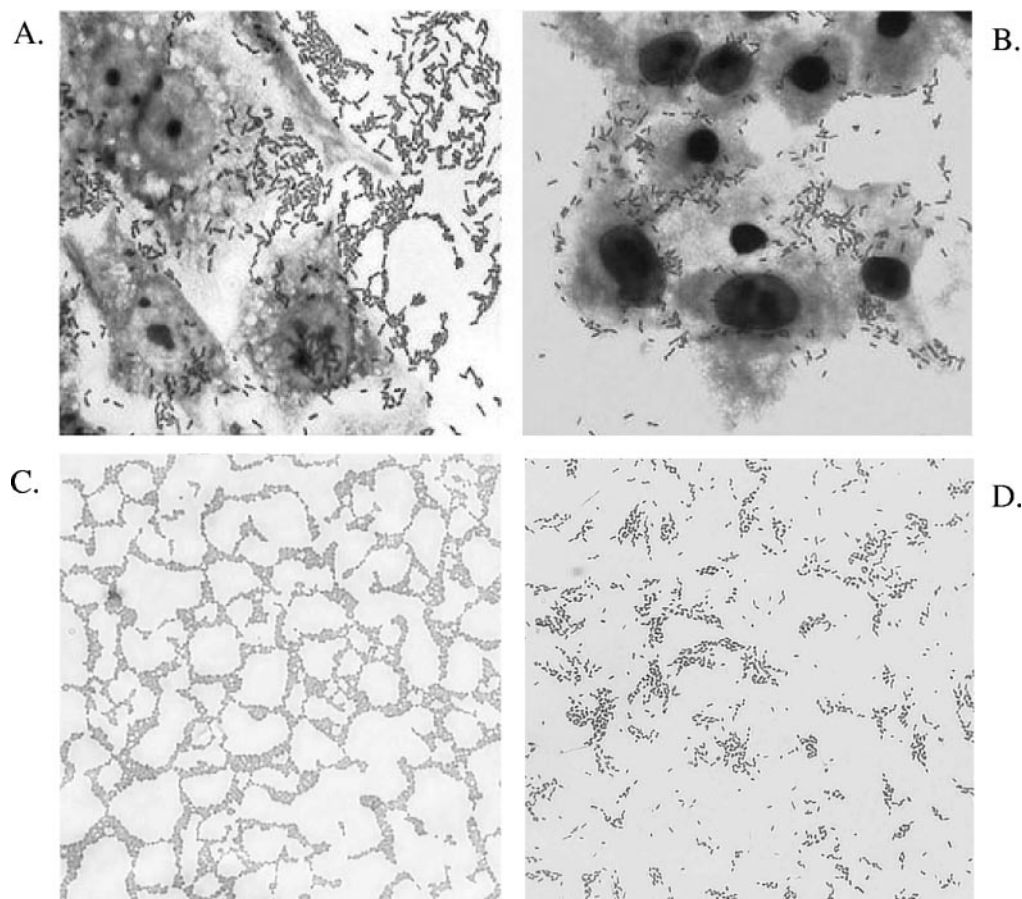


FIG. 1. EAEC C1096 adheres to cultured cells and glass by an aggregative adherence pattern. Overnight cultures of strain 042 (A) or C1096 (B) grown in LB were diluted 1:50 into 24-well cell culture plates containing high-glucose DMEM and HEp-2 cells. After 3 to 4 h of growth at 37°C, nonadherent bacteria were removed by washing with PBS. Cells were visualized by light microscopy after being fixed in methanol and stained with Giemsa. To observe adherence to glass, strains 042 (C) and C1096 (D) were grown and visualized as described for HEp-2 adherence assays; however, the 24-well cell culture plates contained a 12-mm diameter glass coverslip, and bacteria were stained with crystal violet.

For quantitative adherence assays using direct cell counting, HT29 cells and bacteria were grown, and infections were performed as described above. After the PBS washes, coverslips were fixed with 70% methanol for 5 min and stained with 10% Giemsa. Slides were visualized by light microscopy. A total of 100 HT29 cells were randomly chosen from each of five coverslips from the wild-type and mutant strain infections, and the number of bacteria adhering to each HT29 cell were counted. Data are reported as the number of wild-type bacteria adhering to 100 HT29 cells divided by the number of mutant bacteria adhering to 100 HT29 cells.

Biofilm formation assay in microfermentors. Sixty-milliliter microfermentors, each containing a removable Pyrex glass slide, were configured as continuous-flow culture bioreactors with a flow rate of 40 ml h⁻¹. This flow rate minimizes planktonic growth of the bacteria, and most of the observed growth occurs on the available surfaces inside the microfermentors and on the glass slide (15). (For a more detailed explanation, see <http://www.pasteur.fr/recherche/unites/Ggb/biofilmfermenter.htm>.) Overnight cultures grown in 0.4% glucose M63B1 minimal medium supplemented with appropriate antibiotics were used to inoculate microfermentors containing the same medium to 1 OD₆₀₀ equivalent. The bacteria were cultivated for 24 to 72 h. Biofilm biomass on the internal Pyrex glass slide was quantified by measuring the OD₆₀₀ after resuspension of the slide biofilm in 10 ml of M63B1 medium. Total biofilm was measured at OD₆₀₀ after resuspension of the total cell mass present within the microfermentor in 60 ml of M63B1 medium. All experiments were performed at least in triplicate.

Plasmid conjugation. Plasmid transfer for strain construction was performed by individually inoculating donor and recipient strains from an overnight culture to 1% in LB and growing them at 37°C for 1 h. Aliquots (250 μl) of donor and recipient strains were combined, centrifuged, and resuspended in 50 μl of LB. Cells were

spotted on LB agar and incubated at 37°C for 6 h, and transconjugants were plated on selective medium. When DH5α or HB101 was the recipient strain, selection was performed on LB+KAN+nalidixic acid or LB+KAN+STR plates, respectively. Plasmid transfer was confirmed by agarose gel electrophoresis. The conjugation frequency of pSERB1::aph and pSERB3 from DH5α to HB101 on a solid surface was assessed similarly, except that the resuspended cells were placed on a 0.22-μm-pore-size filter overlaying an LB plate. Serial dilutions were made onto LB+KAN+STR plates to quantify the number of transconjugants and on LB+STR plates to quantify the number of recipients. Conjugation frequency is reported as the number of transconjugants per recipient.

Quantitative broth conjugations were performed by centrifuging 500-μl aliquots of overnight cultures, washing them twice with 1 ml of LB broth, and inoculating each strain into 2 ml of LB broth to an OD₆₀₀ of approximately 0.03. Tubes inoculated with only one strain served as negative controls. Conjugations were carried out in 14-ml plastic tubes. Tubes were placed on their sides and rotated at 50 rpm for 6 h at 37°C. Plate counts were performed as described above for solid surface conjugation.

Nucleotide sequence accession numbers. The partial nucleotide sequence obtained for pSERB1 was deposited in the GenBank database under accession number AY686591. The full nucleotide sequence for pSERB2 was assigned accession number DQ269444.

RESULTS

EAEC C1096 adheres to epithelial cells and glass in an AA pattern. Previously, Cobeljic et al. implicated EAEC C1096 as

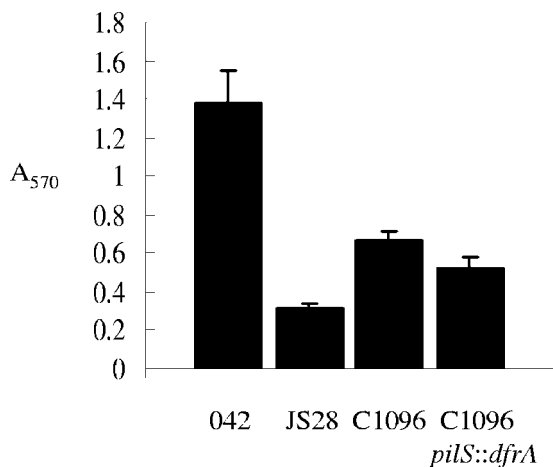


FIG. 2. EAEC C1096 forms biofilms on polystyrene, and adherence to polystyrene is augmented by type IV pili. Positive and negative control strains 042 and JS28, respectively, and C1096 and C1096*pilS::dfrA* were inoculated into DMEM at an initial OD₆₀₀ of 0.025 and allowed to adhere in 24-well polystyrene cell culture dishes for 7 h at 37°C. Adherent bacteria were stained with crystal violet and quantified by measuring the A₅₇₀ after solubilizing the stain in 95% ethanol. Values represent the average of four independent experiments (with four replicates per experiment), and error bars indicate experimental variation. The difference between C1096 and C1096*pilS::dfrA* adherence was significant ($P = 0.009$) by a Student's *t* test pairwise comparison.

the causative agent of a nonbloody diarrhea outbreak in Niš, Serbia. The authors reported that EAEC C1096 adhered mainly to the HEp-2 cells, with few bacteria adhering to the glass coverslips. Whereas prototype EAEC strain 042 requires AAF/II fimbriae for AA to abiotic surfaces and to human intestinal tissue (8, 42), C1096 does not encode genes for the expression of AAF/II or the distantly related fimbriae AAF/I (9). Additionally, total DNA from C1096 does not hybridize to a DNA probe (2) that identifies the usher subunit of AAF/I, AAF/II, AAF/III, and probably other as yet unidentified AAF alleles (data not shown). This suggests that AAF-independent determinants may be involved in C1096 adherence.

To characterize adherence factors of EAEC C1096, we began by confirming a previous report that this strain adhered to HEp-2 cells in an AA pattern, which is the defining characteristic of EAEC (Fig. 1A), and studying whether C1096 adhered to glass similarly to the prototype strain 042. In our hands, EAEC 042 and C1096 both displayed AA on HEp-2 cells (Fig. 1A and B, respectively). Strain C1096 also adhered to glass (Fig. 1D) in an AA pattern, although the pattern observed differed from the distinct honeycomb structure seen with EAEC 042 (Fig. 1C). Adherence to an abiotic surface was also measured quantitatively, with EAEC 042 and a biofilm-deficient derivative designated JS28 (42) serving as positive and negative controls, respectively. EAEC C1096 adherence was weaker than that observed with strain 042 (Fig. 2), although it was still significantly higher than that seen with strain JS28. Thus, EAEC C1096 adheres to HT29 cells and abiotic surfaces by the AA phenotype, confirming the classification of C1096 as EAEC.

Plasmids from C1096 confer adherence upon DH5 α . EAEC C1096 carries at least six plasmids (Fig. 3). To determine if any plasmids encode adherence factors, we used an *in vitro* trans-

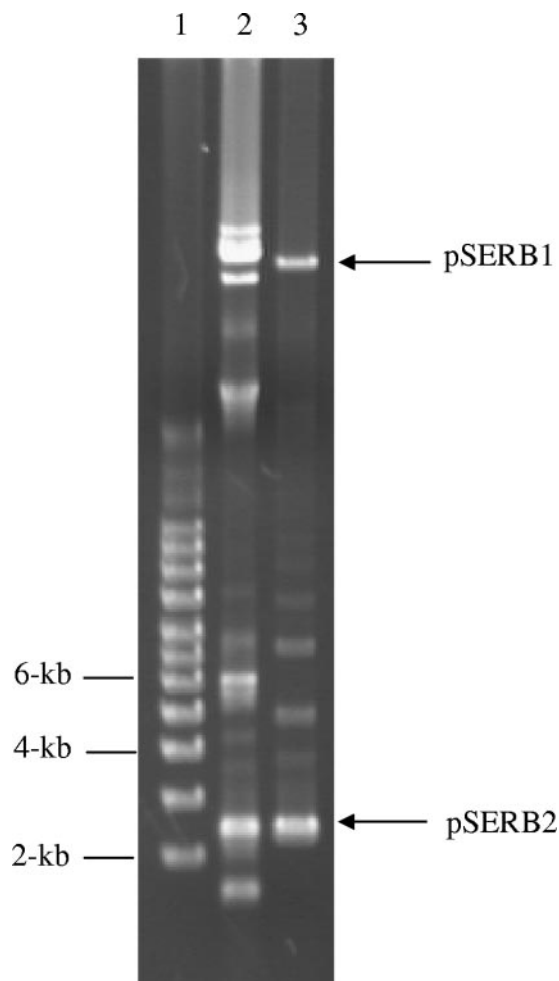


FIG. 3. DH5 α (pSERB1, pSERB2) carries two plasmids from EAEC C1096. Plasmids were isolated from strains by alkaline lysis and separated on a 0.6% agarose gel. Lane 1, supercoil size standard (Invitrogen); lane 2, plasmids of *E. coli* C1096; lane 3, plasmids of DH5 α (pSERB1, pSERB2). The sizes of the molecular mass markers are indicated to the left of the gel, and the positions of pSERB1 and pSERB2 are indicated to the right of the gel.

position reaction to randomly insert a *dfrA*-encoding transposon into the plasmids and transformed the tagged plasmids into *E. coli* DH5 α . All transformants screened carried at least one of the three lower-molecular-mass plasmids (1.6, 2.5, and 6.4 kb). One isolate was identified that carried one of the large plasmid species from C1096, in addition to a second plasmid that migrated in agarose gel electrophoresis at approximately 2.5 kb (Fig. 3, lane 3). The larger and smaller plasmid species from this strain were designated pSERB1 and pSERB2, respectively. Upon electroporation of plasmid pools from DH5 α (pSERB1, pSERB2) back into DH5 α , all TMP-resistant transformants carried pSERB2 only. This suggests that during strain construction, the 0.9-kb *dfrA*-encoding transposon inserted into the 1.6-kb plasmid from C1096, resulting in a 2.5-kb species, and this plasmid cotransformed with pSERB1 during electroporation of DH5 α .

DH5 α transformants carrying different plasmid combinations from C1096 were screened for the ability to adhere to

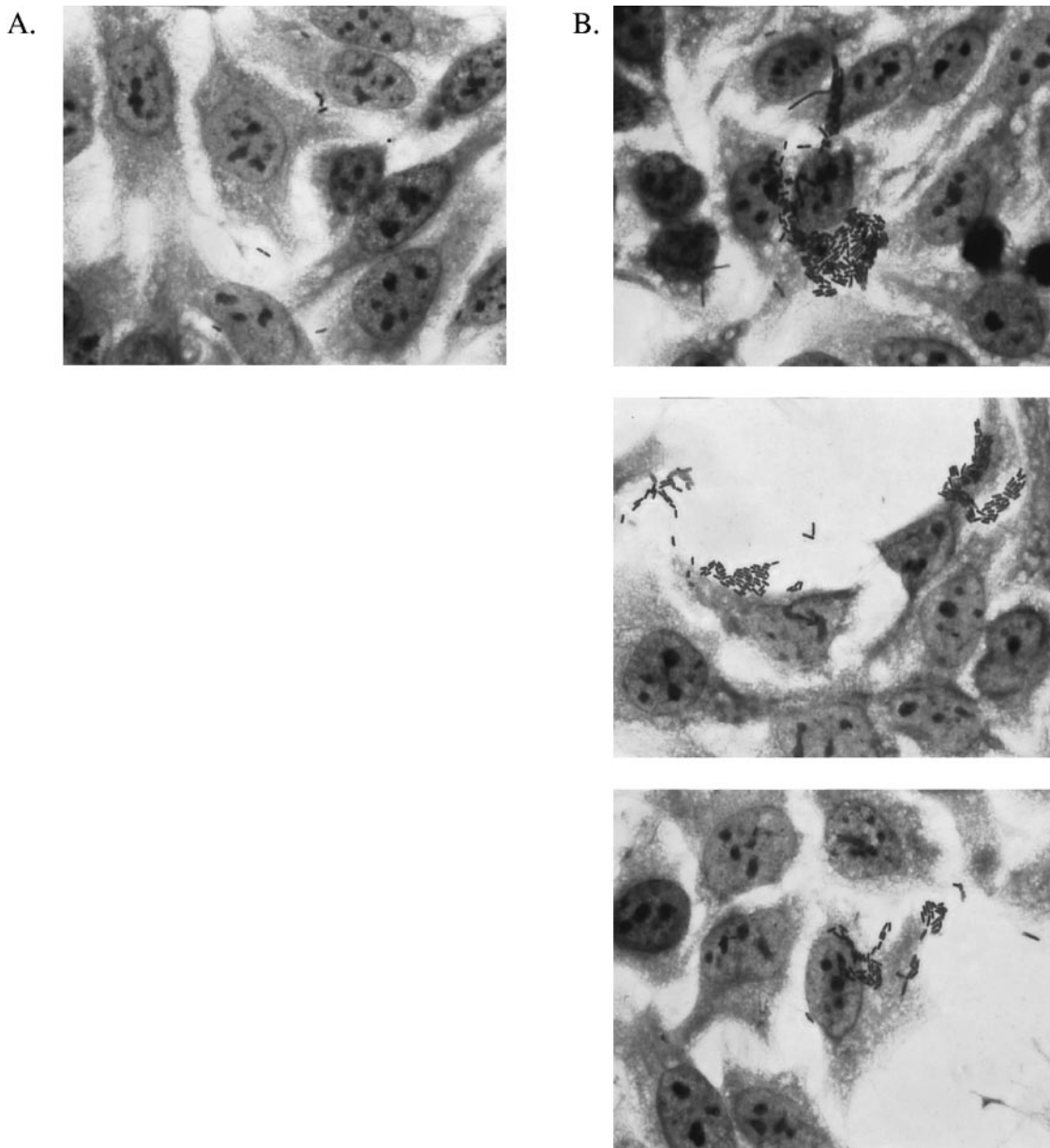


FIG. 4. DH5 α (pSERB1, pSERB2) adheres to cultured HEp-2 cells in an aggregative pattern. Bacteria from an overnight LB culture were diluted 1:50 in high-glucose DMEM in a 24-well cell culture plate containing semiconfluent HEp-2 cells. Bacteria were grown for 6 h at 37°C. Nonadherent bacteria were removed by PBS washes and were visualized after Giemsa staining. Adherence of DH5 α (A) and three different fields of adherent DH5 α (pSERB1, pSERB2) (B) are shown.

HEp-2 cells. DH5 α (pSERB1, pSERB2) adhered to cultured cells in the presence of 1% methyl- α -D-mannopyranoside (Fig. 4B). The bacteria were typically found in clusters, adhering to the cultured cells as well as to each other. DH5 α (pSERB1, pSERB2) adhered to a smaller number of HEp-2 cells than did C1096 and did not display the extensive adherence seen with strain C1096 (compare Fig. 4B with 1B). A much lower level of adherence was seen with wild-type DH5 α (Fig. 4A) and with derivatives carrying any of the three low-molecular-mass plasmids from C1096 (data not shown). These observations suggest that at least one factor that contributes to the adherence of

EAEC C1096 to HEp-2 cells is encoded on pSERB1 and/or pSERB2.

Adherence of DH5 α (pSERB1, pSERB2) to abiotic surfaces requires both plasmids. In order to test the adherence of DH5 α (pSERB1, pSERB2) on abiotic surfaces, we tested this strain for initial adhesion in microtiter plate assays and for mature biofilm formation in microfermentors. Although adherence of DH5 α (pSERB1, pSERB2) was not observed on polystyrene tissue culture plates (data not shown), DH5 α (pSERB1, pSERB2) formed a strong mature biofilm in a continuous-culture microfermentor, where the formation of a

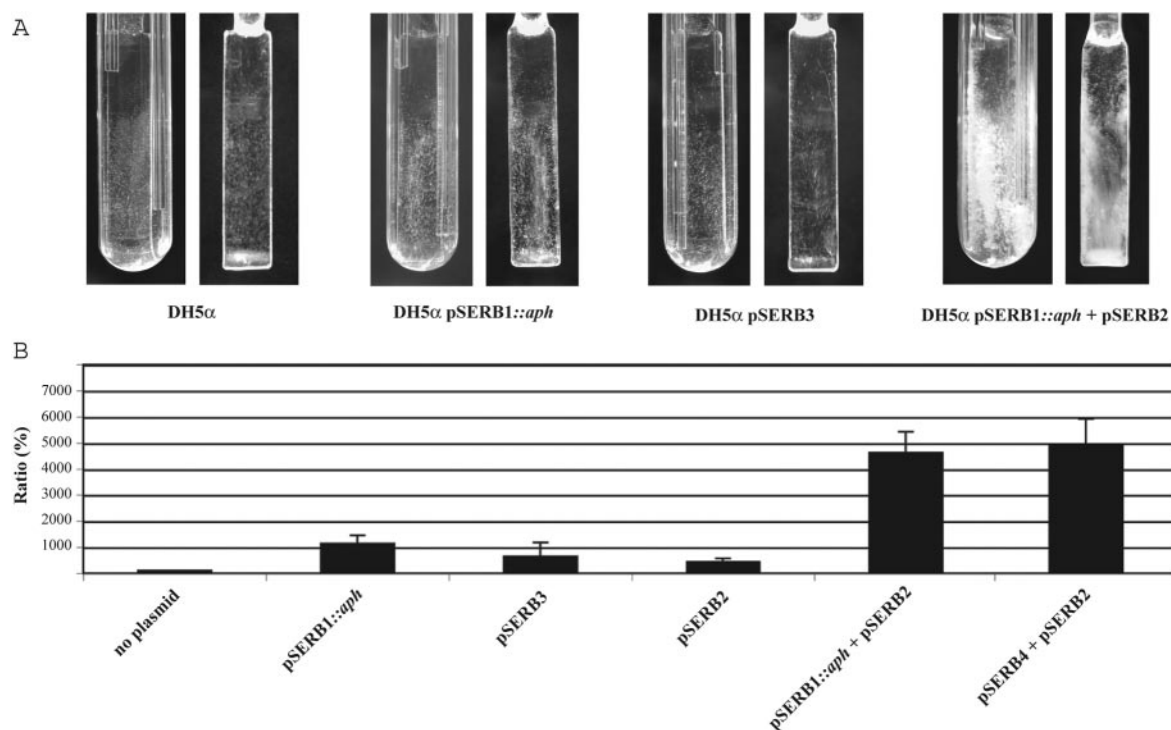


FIG. 5. *E. coli* C1096 plasmids pSERB1 and pSERB2 promote biofilm formation on an abiotic surface. Biofilm formation was analyzed in microfermentors culturing *E. coli* DH5 α strains carrying pSERB1::aph, pSERB2, pSERB3 (pSERB1::aph with a dihydrofolate reductase gene inserted into *pilS*), or pSERB4 (pSERB1::aph with a chloramphenicol acetyltransferase gene inserted into *pilS*). (A) Biofilm development was observed after 60 h of growth at 37°C in M63B1 glucose medium. Representative images of biofilms formed on the bottom of the microfermentors and on the Pyrex glass spatula are shown. (B) Biofilms formed on the glass spatula were resuspended in 10 ml of M63B1 medium and the OD₆₀₀ was measured. The plots represent the average of at least three independent experiments. The level of biofilm formed by *E. coli* DH5 α was set to 100%.

thick biofilm can be monitored on removable Pyrex glass slides between 48 to 72 h (Fig. 5A). To determine which plasmid was responsible for this phenotype, pSERB1 was tagged in a predicted noncoding region with the KAN resistance marker *aph*, creating pSERB1::aph, and pSERB2 was electroporated into both DH5 α and DH5 α (pSERB1::aph). Adherence of DH5 α (pSERB1::aph) and DH5 α (pSERB2) to the spatula was significantly decreased compared to DH5 α (pSERB1::aph, pSERB2) (Fig. 5A and B), although adherence of each was greater than DH5 α . These results demonstrate that both plasmids are required for fully mature biofilm formation under the conditions tested and that pSERB1 and pSERB2 promote biofilm formation in a synergistic manner.

pSERB1 is related to IncI1 plasmids, and pSERB2 is a cryptic plasmid. To identify adherence and other possible virulence factors of EAEC C1096, the plasmids from DH5 α (pSERB1, pSERB2) were subjected to nucleotide sequence analysis. A total of 742 shotgun sequences were assembled into five contigs of 67.2, 7.2, 7.1, 2.5, and 1.1 kb, with an estimated sequence coverage of 5.9-fold.

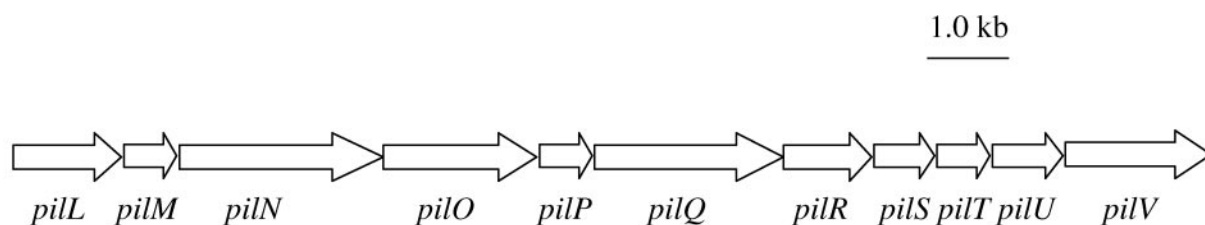
A BLAST analysis indicated that the 2.5-kb contig encoded *dfrA* and, therefore, is derived from pSERB2. This contig also encoded two plasmid replication protein homologs. Further analysis of the pSERB2 sequence did not provide any additional insights into the requirement of both plasmids for the

adherence phenotype observed with DH5 α (pSERB1::aph, pSERB2).

The remaining four contigs were all aligned by BLAST to plasmids of the IncI1 incompatibility group, including R64 (GenBank accession no. NC_005014) of *Salmonella enterica* serovar Typhimurium. EcoRI digests suggested that pSERB1 is approximately 82 kb in size and that the 7.2-kb, 1.1-kb, 7.1-kb, and 67.2-kb contigs (total, 82.6 kb) were aligned with nucleotides 48284 to 52639, 54932 to 56052, 56043 to 62909, and 63385 to 102094, respectively, of R64 (data not shown). These data strongly suggest that nearly all of the sequence of pSERB1 was obtained.

pSERB1 encodes a conjugal transfer locus and a type IV pilus system. R64 encodes a large number of genes responsible for the formation of two pilus types referred to as thin pili and thick pili (3). Thick pilus biosynthesis requires genes encoded on two distinct regions of R64: *traBC*, which encodes regulatory proteins, and a 31-kb region encoding 26 open reading frames, 22 of which are required for conjugal transfer in broth and on solid surfaces (23). Further analysis of the 67.2-kb contig revealed that homologs of these 22 proteins and TraBC were present on pSERB1, suggesting that pSERB1 may be a conjugative plasmid.

R64 thin pili are members of the type IV pilus family and are required for plasmid conjugation in broth only (22); biosyn-



Putative homolog	Length (aa)	Identity to protein from STEC O113:H21 ¹	Predicted function
<i>pilL</i>	356	91%	lipoprotein
<i>pilM</i>	145	75%	lipoprotein
<i>pilN</i>	539	90%	lipoprotein
<i>pilO</i>	431	65%	unknown
<i>pilP</i>	152	67%	unknown
<i>pilQ</i>	502	87%	nucleotide-binding protein
<i>pilR</i>	364	72%	integral membrane protein
<i>pilS</i>	178	68%	major pilin subunit
<i>pilT</i>	161	79%	membrane protein
<i>pilU</i>	208	48%	prepilin peptidase
<i>pilV</i>	405	80% over N-terminal 331 aa	minor pilin subunit

¹ identity is over full length of the translated ORF from Shiga toxin producing *E. coli* O113:H21, unless otherwise noted.

FIG. 6. The pSERB1 *pilL-V* region, which likely encodes all genes necessary for type IV pilus biogenesis, is most closely related by BLAST searches of deduced amino acid sequences to the type IV pilus locus of the Shiga toxin *E. coli* plasmid pO113 (24).

thesis requires 12 genes designated *pilK-V* (49). Genes similar to *pilL-V* are encoded on pSERB1 in the same order as on R64 (Fig. 6). Although no *pilK* was identified, characterization of type IV pili from *Yersinia pseudotuberculosis* and *S. enterica* serovar Typhi (7, 50) suggests that either some systems do not require *pilK* or certain type IV pilus-expressing organisms synthesize an unrelated protein with PilK activity. BLAST searches indicated that PilL-V are most closely related (48 to 91% amino acid identity) to a type IV pilus locus encoded on pO113, a plasmid isolated from a Shiga toxin-producing *E. coli* (STEC) strain (24).

Certain type IV pilus biogenesis systems encode two prepilin subunits, designated *pilS* (major subunit) and *pilV* (minor subunit). These pili are further divided into two classes based upon posttranslational processing events (25): type IVA, in which the prepilus has a short (usually 5 to 6 amino acids [aa]) leader sequence and the N-terminal amino acid residue of the mature protein is methylated phenylalanine; and type IVB, in which the prepilus has a longer (13 to 30 aa) signal sequence and the N-terminal amino acid residue is Met, Leu, Trp, or Ser. Based upon predicted signal sequence cleavage sites, PilS from EAEC C1096 is predicted to be type IVB (predicted leader sequence of 22 aa; mature PilS is predicted to have an N-terminal Met), and PilV is type IVA (predicted leader sequence of 8 aa; mature PilV is predicted to have an N-terminal Phe). The deduced amino acid sequence of PilS is aligned by

BLAST only with the PilS homologs from pO113 (Fig. 6) and plasmid R721 (accession no. NP_065349; 43% identical to *pilS* from R64 over 173 aa).

EAEC C1096 expresses surface pili absent in a *pilS::dfrA* mutant. To test whether the pSERB1-encoded *pil* locus is expressed, *dfrA* was inserted into the plasmid-encoded copy of *pilS* by homologous recombination, creating EAEC C1096*pilS::dfrA*. Such a mutation in plasmid R64 eliminates the assembly of functional type IV pili (49), and by analogy the same phenotype was expected with pSERB1. Under magnification of $\times 15,000$, numerous pili of approximately 1 μm in length were observed extending from the surface of most bacterial cells (Fig. 7). The pili appear to tether bacteria together over a distance, and direct interaction of the pili with the glass coverslip was also noted. In sharp contrast, similar surface pili were not seen on the surface of EAEC C1096*pilS::dfrA* cells, although a minority of these cells still expressed much shorter pili (Fig. 7B).

pSERB1 is a conjugative plasmid that requires the type IV pilus for optimal conjugal transfer in broth cultures. As noted, our sequence analysis suggested that pSERB1 is an IncI1-type plasmid. This finding implies that pSERB1 is conjugative, as are other IncI1 plasmids, and that efficient conjugation in broth, but not on solid surfaces, is promoted by the type IV pilus (22). To test this, the pSERB1-derivative from EAEC C1096*pilS::dfrA* was tagged with *aph*, creating pSERB3, and

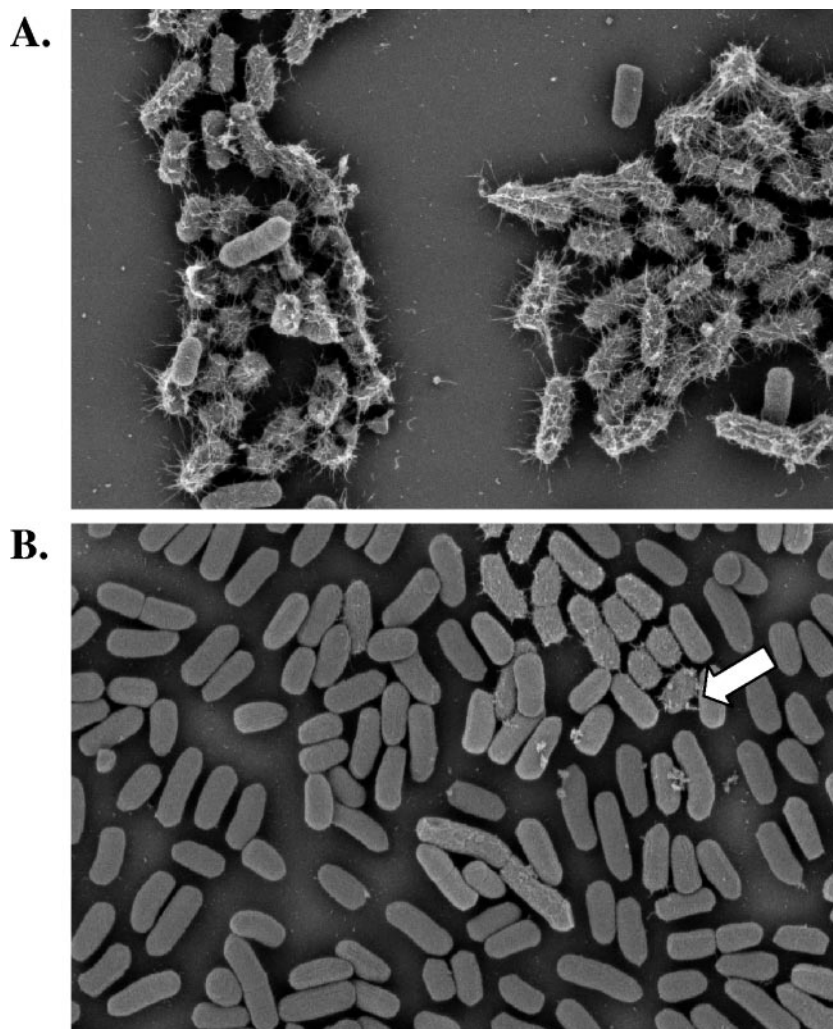


FIG. 7. Wild-type EAEC C1096 expresses surface pili that are absent in the C1096 *pilS::dfrA* mutant. Wild-type EAEC C1096 (A) and C1096 *pilS::dfrA* (B) strains were grown overnight in LB and were subsequently diluted 1:50 into 24-well cell culture plates containing high-glucose DMEM and a 12-mm glass coverslip. After approximately 4 h of growth, the growth medium was aspirated, and nonadherent bacteria were removed by five PBS washes. Bacteria were fixed with PBS containing 2% glutaraldehyde and 3 mM CaCl₂ and sent to the Johns Hopkins School of Medicine Microscopy Facility for scanning electron microscopy analysis. Pictures are at a magnification of $\times 15,000$. The arrow in panel B highlights one of the C1096 *pilS::dfrA* bacteria among the population that expresses a short pilus that appears to also mediate bacteria-bacteria interactions.

this construct was transferred to DH5 α . When matings were performed in LB broth at 37°C, the transfer frequency of pSERB1::*aph* and pSERB3 to *E. coli* HB101 after 6 h was 2.6×10^{-2} and 6.7×10^{-6} transconjugants per recipient, respectively. When matings were performed on a 0.22- μ M-pore-size filter overlaying LB agar, transfer frequencies were 1.3×10^{-3} and 3.2×10^{-3} , which was not a statistically significant difference ($P = 0.17$).

Because of the cooperative role of pSERB1 and pSERB2 in biofilm formation, we also tested whether the transfer frequency of pSERB1::*aph* was altered by the presence of pSERB2. However, no detectable difference was noted in the transfer frequency of pSERB1::*aph* to *E. coli* HB101 when either DH5 α (pSERB1::*aph*, pSERB2) or DH5 α (pSERB1::*aph*) was used as the donor strain. This suggests that the biofilm enhancement phenotype is not the result of increased conjugal transfer of pSERB1.

The type IV pilus contributes to adherence of EAEC C1096 to HT29 cells and to abiotic surfaces. Next, we tested whether the type IV pilus mediates adherence of EAEC C1096 to cells and abiotic surfaces. Adherence to HT29 cells was quantified both as single-strain infections and as a coinfection. First, HT29 cells were coinfecting with equivalent numbers of C1096 and the *pilS::dfrA* mutant strains. We performed coinfections because we found that C1096 detaches epithelial cells from the glass coverslips during infection, and therefore differences seen in experiments performed as single-strain infections could be due to differences in the numbers of detached HT29 cells. Data from four independent experiments revealed 2.9-fold more CFU for the wild-type compared to the mutant strain, or a 65% decrease in adherence ($P = 0.02$ by a Student's pairwise *t* test). Direct counting of bacteria adhering to HT29 cells by microscopic examination of single-strain infections supported this result, as 5.4-fold fewer *pilS::dfrA*

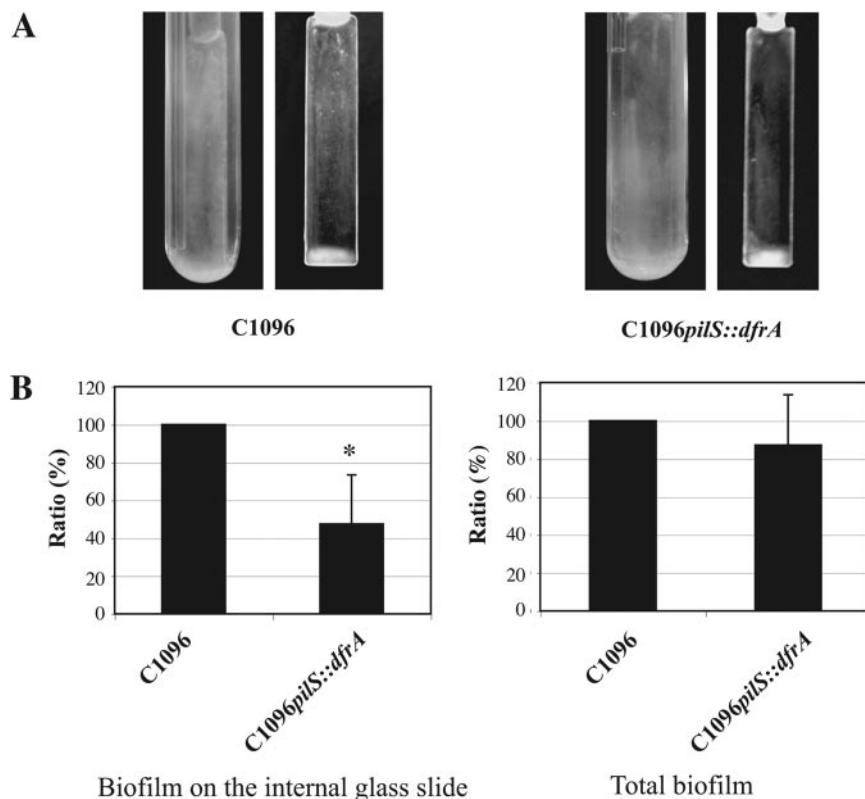


FIG. 8. Inactivation of *pilS* reduces adherence of *E. coli* C1096*pilS::dfrA* to a glass slide while not affecting the total amount of biofilm formed within a continuous fermentor. Biofilm formation was analyzed in microfermentors culturing *E. coli* C1096 and its *pilS* mutant, designated *E. coli* C1096*pilS::dfrA*. (A) Biofilm development was observed after 30 h of growth at 37°C in M63B1 glucose medium. Representative images of biofilms formed on the bottom of the microfermentors and on the Pyrex glass spatula are shown. (B) Biofilms formed on the glass spatula and total microfermentor biofilm were measured and quantified as indicated in Materials and Methods. The plots represent the average of six independent experiments. The level of biofilm formed by *E. coli* C1096 for both biofilm measurements was set to 100%. The asterisk indicates a significant difference between the mutant and wild-type strains ($P = 0.014$ by a *t* test).

bacteria (82% reduction) were counted adhering to HT29 cells than with the wild-type bacteria. Therefore, the two different methods of quantifying bacterial adherence to cultured cells were in agreement and suggested that EAEC C1096 adherence to epithelial cells is reduced by approximately 75% when the type IV pilus is inactivated.

Qualitative examination of adherence to glass of the mutant and wild-type strains indicated that the pattern of biofilm formation is not strongly affected by the *pilS* mutation. Nevertheless, quantitative assays revealed a slight (21%) but statistically significant reduction of biomass adhering in wells containing the mutant strain compared to the wild type (Fig. 2) ($P = 0.009$ by a Student's pairwise *t* test). In continuous-flow culture, the inactivation of *pilS* did not affect the total biofilm biomass in the microfermentor but reduced biofilm development on the vertical internal glass slide by approximately 50% (Fig. 8) ($P = 0.014$), indicating that a more fragile biofilm was formed by the C1096*pilS* mutant. Since the wild-type C1096 and its *pilS* mutant have similar growth rates in liquid culture, this suggests that the type IV pilus encoded on pSERB1 affects the initial stages of adhesion and/or promotes tight bacteria-bacteria interactions within a biofilm.

Many conjugative plasmids, including those of the IncI1 group, are known to mediate biofilm formation in bacteria via a conju-

gation-dependent mechanism (15). The contribution of the type IV pilus subunit was therefore further investigated in the microfermentor-biofilm model. As shown in Fig. 5, biofilm formation by strains DH5 α (pSERB3) and DH5 α (pSERB4, pSERB2) was not significantly different than that formed by strains DH5 α (pSERB1::aph) and DH5 α (pSERB1::aph, pSERB2), respectively. Therefore, adherence in the microfermentor assay does not depend upon efficient plasmid conjugation.

Lastly, we wanted to confirm that phenotypes observed with *E. coli* C1096*pilS::dfrA* were not due to an altered copy number of pSERB1 or to altered expression of the pSERB1-encoded thick pilus. Real-time PCR using total DNA from C1096 and C1096*pilS::dfrA* as a template indicated that the quantity of *traN* and *traY* (two pSERB1-encoded genes) relative to that of *cat* (a gene not encoded on pSERB1) was the same for both strains. This indicates that the *dfrA* insertion does not affect plasmid copy number. Additionally, as reported above, the transfer frequency to *E. coli* HB101, when conjugation was performed on a 0.22- μ m-pore-size filter, was comparable for DH5 α (pSERB1::aph) and DH5 α (pSERB3). This suggests inactivation of *pilS* did not affect the synthesis of the thick pilus, which is essential for plasmid conjugation.

Taken together, these results indicate that the type IV pili from EAEC C1096 play a role in adherence to cultured epi-

thelial cells and moderately contribute to both initial or late biofilm formation on abiotic surfaces. These data also suggest the presence of other adherence mechanisms in this strain.

Genes from the EAEC C1096 type IV pilus locus are found in both typical and atypical EAEC strains. Colony hybridizations were performed to determine whether other EAEC strains carry genes for type IV pilus biogenesis that are related to those found in EAEC C1096. A library consisting of 40 typical and 20 atypical EAEC strains was screened. Under both high-stringency (60°C hybridization) and low-stringency (42°C hybridization in 10% formamide) conditions, four of the typical EAEC strains were hybridization positive using the *pilL*, *pilN*, *pilQ*, *pilS*, *pilT*, and *pilV* probes (data not shown). These strains were previously designated H38-1, DS67-R2, 278-1, and 495-1 (9) and were isolated from Peru (H38-1), the Philippines (DS67-R2), and Thailand (278-1 and 495-1). Two atypical EAEC strains, designated strains "A" and 25-1, were also probe positive under high-stringency conditions with all six gene probes. Additionally, an atypical strain designated 6-1 was probe positive under high-stringency conditions only with *pilN*, *pilL*, and *pilV*, and a fourth strain designated DS61-R2 was only probe positive with *pilV* under low-stringency conditions. As expected, the prototype EAEC strain 042 was probe negative with all probes.

DISCUSSION

EAEC strains are defined as strains that adhere to HEp-2 cells in an aggregative pattern (30). While the majority of well-characterized strains adhere to abiotic surfaces (42), cultured cell lines (8, 31), and human intestinal tissue (8) via AAFs, it is clear that alternative mechanisms are also responsible for aggregative adherence patterns (2, 9, 28). While studying the adherence factors of EAEC strain C1096, we discovered that one of the large plasmids carried by this strain is related to members of the IncI1 incompatibility group and encodes genes for two previously described pili designated thick and thin (type IV) pili. We suggest here a model where the IncI1 plasmid-encoded type IV pili carried by EAEC C1096 contribute to, but are not essential for, adherence of this strain to cells and surfaces (Fig. 9). Additionally, this pilus mediates an increase in adherence of the bacteria to abiotic surfaces in a manner that is further enhanced by an unknown interaction with a small cryptic plasmid designated pSERB2 (Fig. 9A).

It is well established that IncI1-encoded type IV pili mediate conjugal transfer (Fig. 9A) of these plasmids (21). We are unaware, however, of any prior reports implicating the thin pilus from conjugative plasmids as a factor mediating adherence to intestinal epithelial cells (Fig. 9B); in fact Sriramanote et al. (43) previously reported that the STEC plasmid pO113, which belongs to the IncI1 family, does not play a role in adherence to intestinal tissue. Some conjugative plasmids, including IncI1 members, also catalyze adherence to abiotic surfaces as the result of host-donor contact during DNA transfer (15, 36). Therefore, it is possible that the decreased adherence to HT29 cells observed with the C1096 *dfrA::pilS* mutant is the result of decreased mating aggregate formation. However, given that C1096 *dfrA::pilS* demonstrated a 65 to 82% decrease in adherence to HT29 cells, while only a 21% decrease in

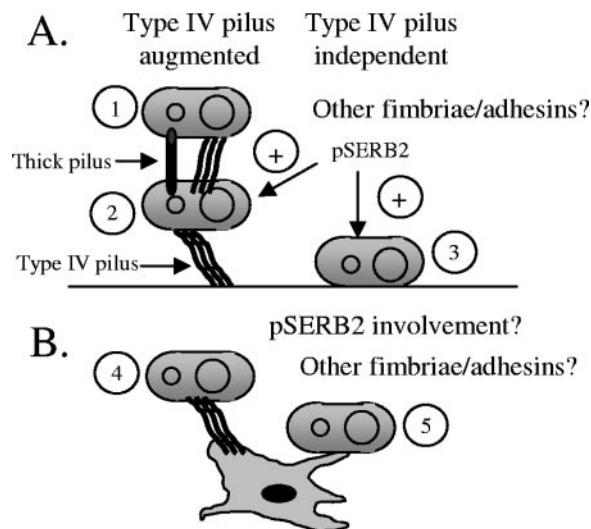


FIG. 9. Working model for adherence of EAEC C1096 to abiotic surfaces and to human cell lines. (A) Efficient conjugation (no. 1) of pSERB1 from *E. coli* C1096 requires the presence of both the thick pilus and the type IV pilus. Adherence to surfaces (no. 2 and 3) is augmented by the presence of the type IV pilus, and the presence of the small cryptic plasmid pSERB2. Adherence to surfaces also involves other currently unidentified factors. (B) Adherence of C1096 to HT29 cells is augmented by the pSERB1-encoded type IV pilus (no. 4), and a type IV pilus-independent mechanism also exists that involves other unknown factors (no. 5).

adherence to polystyrene, we favor a model in which the type IV pilus mediates adherence through direct contact with the eukaryotic cell membrane or other surface structure. The decreased adherence of the *pilS* mutant on polystyrene suggests that the pSERB1-encoded *pilL-V* may also augment biofilm levels or may accelerate biofilm formation, as suggested for the *Vibrio cholerae* type IV pilus (48). In summary, our data indicate that type IV pili encoded on some IncI1 plasmids may be multifunctional, serving roles in adherence to both host cells and abiotic surfaces and in plasmid conjugation.

We are interested in determining whether this observation can be extended to other IncI1 plasmids found in gram-negative bacterial pathogens. We would also like to further characterize the role of individual genes from the *pilL-V* locus and those encoding the thick pilus. However, given the antibiotic resistance profile of C1096 (including ampicillin, chloramphenicol, KAN, tetracycline, gentamicin, neomycin, and STR) (reference 5 and data not shown), the large number of plasmids present in this strain, and the current lack of knowledge concerning genetic manipulations in this strain, further studies are needed before investigators can use sophisticated genetic techniques that combine gene knockouts with complementation in *trans* using expression vectors. We are pursuing the development of such vectors rather than studying a more genetically tractable host, as C1096 is currently the only atypical EAEC strain that is a proven human pathogen.

Strains carrying IncI1 plasmids were previously shown to form biofilms in continuous-flow microfermentors (15). This phenotype required the presence of recipient bacteria, presumably because the expression of the plasmid-encoded conjugation apparatus and surface pilus is repressed unless bacteria

lacking the IncI1 plasmid are present in the population. We found that pure cultures of C1096 form a type IV pilus-mediated biofilm in the microfermentor, suggesting that this pSERB1-encoded pilus is expressed regardless of whether recipient bacteria are present. The continuous-flow culture experiments also showed that while both C1096 and C1096*pilS::dfxA* biofilms initially form on the internal vertical surfaces, the *pilS* mutant falls to the bottom of the microfermentor to a greater extent than the wild type during later stages of biofilm development. This suggests that the *pilS* mutant may form weaker bacteria-bacteria interactions, resulting in a more fragile biofilm under these conditions. Interestingly, biofilm formation by DH5 α (pSERB1::*aph*, pSERB2) in the microfermentor was more than fourfold greater than that of DH5 α (pSERB1::*aph*) (Fig. 9A), and this adherence did not depend upon the pSERB1-encoded type IV pilus or efficient conjugal transfer of pSERB1. Given the scarcity of genetic information found in the pSERB2 sequence, it is difficult to speculate as to the nature of this effect. While pSERB2 is mobilizable in the presence of pSERB1 (E. G. Dudley and J. P. Nataro, unpublished results), the frequency of pSERB2 conjugation is approximately 3 log units less than that of pSERB1. Therefore, it seems unlikely that the conjugative nature of pSERB2 alone contributes to increasing the number of mating aggregates. Plasmids related to pSERB2 from putative uropathogenic *E. coli* have been described by others (4), and thus studies concerning whether these plasmids modulate adherence in other species would be of interest.

Colony hybridization experiments indicated that approximately 10% of the EAEC strains screened encode genes related to *pilL*, *pilN*, *pilQ*, *pilS*, *pilT*, and *pilV* and that these genes are often inherited together. This distribution frequency is similar to that reported for the well-studied alleles of the AAF fimbriae, in which the AAF/I, AAF/II, and AAF/III are also detected only in a minority of EAEC isolates (2, 9). It is possible that type IV pili are more widely distributed in this EAEC library, assuming that some probe-negative strains encode distantly related genes. The prototype EAEC strain 042 was probe negative with all gene probes tested, and genes similar to *pilL-V* from pSERB1 are not found in the completed genome sequence of prototype EAEC strain 042 (<http://www.sanger.ac.uk>). As four of the probe-positive strains were also probe positive for *aggR* and other members of the *aggR* regulon (9), these data suggest that type IV pili related to those encoded on pSERB1 are not a defining characteristic of either typical or atypical EAEC but may serve as an accessory adhesin of EAEC. These genes are not as widely distributed as the related type IV pili of STEC, which were found in 16 of 19 strains that lack the locus of enterocyte effacement (43). Future work is directed at identifying additional adherence factors of atypical EAEC in order to determine whether this pathotype adheres to surfaces and cells via a common mechanism.

This study and previous work highlight a difficulty with the current method of defining the EAEC pathotype. The HEp-2 adherence assay is the current "gold standard" for identification; however, it has become clear in recent years that many strains exhibiting the EAEC "stacked-brick" pattern on cultured cells are distinct from the prototype strain 042 as determined by probe hybridization and/or multilocus enzyme electrophoresis (2, 9). While the HEp-2 adherence assay is a

proven and reliable method for distinguishing EAEC from other *E. coli* pathotypes, genetic heterogeneity of isolates and variability of adherence patterns (2, 43, 46) highlight the need for molecular methods of identifying and characterizing EAEC strains. A growing body of evidence suggests that the EAEC 042 plasmid-encoded regulator AggR is a global regulator of virulence determinants (9, 35; E. G. Dudley and J. P. Nataro, unpublished data) and that genes of this regulon are detected more frequently in EAEC isolates from stools of patients with diarrhea than from healthy individuals (19, 35, 39). Therefore, the nomenclature of typical EAEC and atypical EAEC was recently proposed to differentiate EAEC strains carrying and lacking members of the *aggR* regulon, respectively (30), and genes of the *aggR* regulon were designated as markers defining typical EAEC. While the majority of atypical EAEC strains are believed to be nonpathogenic (39), outbreaks such as the one involving EAEC C1096 demonstrate that some atypical strains cause diarrhea and that AAFs are not the only factors that confer AA. The virulence factors of atypical EAEC are largely undefined, and this work represents the first study directed at defining the molecular pathogenesis of an AggR regulon-negative EAEC strain. Further studies to understand the pathogenic factors of EAEC C1096 and to characterize the similarities and differences between atypical EAEC strains are under way.

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