AggR, a Transcriptional Activator of Aggregative Adherence Fimbria I Expression in Enteroaggregative *Escherichia coli*

JAMES P. NATARO,* DENG YIKANG, DENG YINGKANG, AND KIMBERLY WALKER

Center for Vaccine Development, Departments of Pediatrics and Medicine,
University of Maryland School of Medicine, Baltimore, Maryland

Received 21 October 1993/Accepted 13 May 1994

Enteroaggregative *Escherichia coli* (EAggEC) has been associated with persistent pediatric diarrhea in the developing world, yet the pathogenetic mechanisms of EAggEC infection are unknown. Our previous data have suggested that aggregative adherence of some EAggEC strains to HEp-2 cells is mediated by flexible, bundle-forming fimbriae, which we have termed aggregative adherence fimbriae I (AAF/I). Genes sufficient to confer expression of AAF/I are located on the 60-MDa plasmid of EAggEC 17-2; AAF/I genes are present as two unlinked plasmid regions (regions 1 and 2), separated by 9 kb of DNA. Here we report the complete DNA sequencing of region 2 and the identification of an open reading frame which is involved in the expression of AAF/I. One open reading frame of 794 bp encodes a protein (designated AggR) with a predicted molecular size of 29.4 kDa, which shows a high degree of amino acid sequence identity to CfaR and other members of the AraC class of gene regulators. The cloned *aggR* gene (or, alternatively, a cloned *cfaR* gene) was sufficient to complement a region 1 clone to confer AAF/I expression. To further substantiate the role of *aggR* in the regulation of AAF/I, we constructed a 289-bp in-frame *aggR* deletion and replaced the native gene in 17-2 by allelic exchange, using the temperature-sensitive vector pIB307. The resulting *aggR* deletions were negative for AAF/I expression, but expression was restored when the *aggR* gene (cloned into pBluescript II SK) was reintroduced into the *aggR* mutant. RNA slot blot experiments using a probe for the putative AAF/I pilin subunit (*aggA*) revealed that *aggR* operates as a transcriptional activator of *aggA* expression. *aggA::phoA* fusions were constructed in 17-2 and in 17-2Δ*aggR*. AggR was found to promote expression of the *aggA* gene under a variety of conditions of temperature, osmolarity, oxygen tension, and medium. At acid pH, *aggA* expression was maximal and was regulated by both AggR-dependent and AggR-independent mechanisms.

Enteroaggregative *Escherichia coli* (EAggEC) is a class of diarrheagenic *E. coli* associated with diarrhea in both epidemiologic and volunteer studies (2, 3, 13, 33, 34). EAggEC is defined by its characteristic aggregative adherence (AA) to HEp-2 cells in cell culture (33). AA in EAggEC 17-2 is associated with the presence of plasmid-encoded, bundle-forming fimbriae with diameters of 2 to 3 nm (termed aggregative adherence fimbriae I [AAF/I]) (32, 35).

In a previous publication, we reported the use of transposon mutagenesis and subcloning to localize genes necessary for the expression of AAF/I in 17-2 (32). AAF/I-encoding (*agg*) genes are present on the 60-MDa plasmid as two unlinked plasmid regions, separated by 9 kb of DNA (35). This 9-kb plasmid segment also encodes the putative enterotoxin EAST1 (40). We have cloned region 1 as a 6.8-kb *Cla*I fragment which contains several open reading frames (ORFs) encoding the AAF/I structural and processing genes (41). We previously defined region 2 as the smallest subclone (a 3.0-kb *Kpn*I-*Xba*I fragment) capable of complementing region 1 DNA to confer AAF/I expression (35).

Here, we report the complete sequencing of the 3.0-kb region 2 and the identification of several potential ORFs. One of these encodes a predicted protein of 29.4 kDa which bears homology to the AraC family of DNA binding proteins. Members of this family have been shown to be required for the expression of several other fimbriae in *E. coli* (9-11, 21). Our data suggest that *aggR* is a transcriptional activator necessary for the expression of AAF/I in 17-2.

MATERIALS AND METHODS

Strains and plasmids. All plasmid constructions used in this study are described in Table 1. EAggEC 17-2 (serotype O3:H2) was isolated from the diarrheic stool of a child in Santiago, Chile, in 1988. Cloning was performed using *E. coli* HB101 (5) as the host strain. Routine culture of strains was performed on Luria (L) agar or in L broth at 37°C. Counterselection against the *sacB* gene was done on L agar without sodium chloride, containing 6% sucrose as described elsewhere (4). All adherence assays were performed using L-broth cultures grown without shaking at 37°C. Media were supplemented as appropriate with the following concentrations of antibiotics: tetracycline, 15 µg/ml; ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; and chloramphenicol, 30 µg/ml.

Basic genetic techniques. Plasmid extraction, restriction digestion, and DNA cloning were performed by standard methods (1). Isolation of restriction enzyme fragments was performed using Prep-A-Gene (Bio-Rad Laboratories, Richmond, Calif.). Transformation was performed by the calcium chloride method (20). Electroporation of 17-2 was performed using 0.2-cm cuvettes in a Gene Pulser apparatus (Bio-Rad Laboratories) at settings of 2.5 kV, 25 µF, and 200 Ω.

DNA sequence analysis. DNA sequencing was performed by the Sanger dideoxy chain termination procedure, using Sequenase polymerase (version 2.0; U.S. Biochemical, Cleveland, Ohio) according to the manufacturer's protocols. Reactions were performed using sequence-directed oligonucleotide primers synthesized in the Department of Microbiology and Immunology, University of Maryland School of Medicine.

DNA sequence analysis was performed using Genepro sequence analysis software (version 5.00; Riverside Scientific, Bainbridge Island, Wash.) and the Wisconsin Genetics Com-

* Corresponding author. Phone: (410) 706-5328. Fax: (410) 706-6205.

TABLE 1. Plasmids used in this work

Plasmid construction	Description ^a	Source or reference
pJPN31	29-kb <i>Sau3AI</i> fragment encoding AAF/I cloned into cosmid vector pCVD301 (Tc ^r)	32
pJPN39	6.8-kb region 1 <i>ClaI</i> fragment of pJPN31 cloned into <i>ClaI</i> site of pACYC177 (Ap ^r)	This study
pJPN45	3.0-kb region 2 <i>KpnI-XbaI</i> fragment of pJPN31 cloned into the MCS of pRK415 (Tc ^r)	26, 35
pJPN45.1	pJPN45 deleted for 279-bp <i>ClaI</i> fragment internal to the <i>aggR</i> ORF (Tc ^r)	This study
pJPN52	1.2-kb <i>SspI-PvuI</i> <i>aggR</i> -encoding fragment of pJPN45 cloned into pBR328 (Tc ^r)	This study
pJPN54	1.7-kb <i>SstI-KpnI</i> fragment carrying mutated <i>aggR</i> gene of pJPN45.1 + 3.8-kb <i>SacB</i> -Neo ^r cassette of pIB279 both cloned into pIB307 (Ap ^r Ts)	This study
pIB307	Ts pSC101-derived plasmid including MCS (Ap ^r)	I. Blomfield (4)

^a MCS, multiple cloning site; Ap^r, ampicillin resistant; Tc^r, tetracycline resistant; Ts, temperature sensitive.

puter Group (GCG) sequence analysis package available through the Center of Marine Biotechnology, University of Maryland. The predicted amino acid sequence of each ORF product was compared with sequences of proteins listed in EMBL/GenBank by using the GCG TFASTA program.

Localization of *TnphoA* insertion sites. *TnphoA* insertion mutagenesis was performed on the AAF/I cosmid clone pJPN31 (carrying both regions 1 and 2) and was described in a previous publication (30, 32). Plasmid DNAs from the *TnphoA* insertion mutants were digested with *Bam*HI, and the kanamycin resistance-encoding fragment was cloned into pBlue-script II SK (Stratagene Cloning Systems, La Jolla, Calif.). The junction was then sequenced by the double-stranded sequencing method as described above, using a previously described primer homologous to the alkaline phosphatase (AP) gene (*phoA*) of *TnphoA* (23).

Tissue culture assays. The HEP-2 adherence assay was performed as originally described by Cravioto et al. and our group using a 3-h incubation (12, 44). Inoculum sizes were 50 μ l of L-broth culture for HB101 constructions and 20 μ l for wild-type constructions. Adherence was considered positive if there were at least two bacterial aggregates (of more than five bacteria) per high-power field. Caco-2 cells were propagated according to published methods (17). Before use in adherence assays, Caco-2 cells were grown to fully differentiated monolayers and were used in adherence assays after a total incubation time of 14 days. The Caco-2 assay was performed and interpreted in a manner identical to that used for HEP-2 cells.

Autoagglutination and HA. We have found that all AAF/I-expressing strains and clones exhibit autoagglutination in static L-broth cultures grown at 37°C. Autoagglutination was defined as the detection of any discernible aggregates of bacteria to the eye after gentle agitation of the broth culture. The hemagglutination (HA) assay was performed as described by Yamamoto et al. (45), using fresh human blood type A erythrocytes. HA was read after 20 min and was considered positive if agglutination was greater than that of the HB101 negative control by inverted microscopy.

Phenotypic characterization. Biotyping was performed with the API 20E system according to the manufacturer's instructions (Analytab Products, Plainview, N.Y.). The growth rate of mutated 17-2 was compared with that of the wild type by measuring the A_{600} with a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y.). Strains were freshly inoculated into L broth in triplicate, and the optical density was measured at 2-h intervals for 8 h. Optical densities were long transformed and subjected to linear regression analysis; resulting regressions were compared for quality of slope with the level of significance at $P < 0.05$.

Electron microscopy. Electron microscopy and immunogold electron microscopy were performed by standard methods

using a JOEL JEM 1200 EX II transmission electron microscope (27). AAF/I antiserum was derived from intravenous injection of rabbits with the AAF/I-positive clone HB101 (pJPN31) and absorption with a live AAF/I-negative *TnphoA* insertion derivative. The preparation and use of the antiserum have been previously described (32).

Allelic exchange. Allelic exchange in *E. coli* 17-2 was performed with the temperature-sensitive pSC101 system described by Blomfield et al. (4). DNA sequence analysis predicted that deletion of a 279-bp *ClaI* fragment near the N terminus of *aggR* would generate an in-frame deletion. Thus, region 2 clone pJPN45 was digested with *ClaI*, and the large fragment was religated and transformed into HB101 harboring the region 1 clone pJPN39; the *ClaI*-deleted construction was designated pJPN45.1. A 2.7-kb *SstI-KpnI* fragment containing the mutated *aggR* ORF from pJPN45.1 was then cloned into the multiple cloning site of the temperature-sensitive suicide vector pIB307 (to yield the intermediate pJPN45.2). All manipulations with pIB307 were carried out at 30°C. A 3.0-kb *Bam*HI fragment from pIB279 carrying the *Bacillus subtilis* *sacB* gene and the aminoglycoside phosphotransferase gene was also cloned into the *Bam*HI site of pJPN45.2 to yield pJPN54. A single kanamycin-resistant colony was selected, and the pJPN54 plasmid DNA was purified by alkaline lysis and introduced into *E. coli* 17-2 by electroporation and plating on ampicillin- and kanamycin-containing L agar at 30°C. Kanamycin-resistant colonies were confirmed to have acquired pJPN54 by alkaline lysis. A single such colony was selected, passaged overnight in L broth with kanamycin at 44°C, and plated on L agar with kanamycin at 37°C. Alkaline lysis confirmed the absence of pJPN54 from the plasmid profile and alteration of the restriction pattern of the wild-type plasmid p17-2, implying homologous recombination. The merodiploid state was resolved by passage of the cointegrate on 6% sucrose-L agar without sodium chloride or kanamycin. Colonies were picked and scored for kanamycin resistance and AA in the HEP-2 assay.

PCR. To screen for allelic exchange constructions, plasmid DNA from kanamycin-sensitive colonies was analyzed by PCR. DNA was extracted by alkaline lysis and amplified with various concentrations of two primers within ORF2, 5' to the *aggR* gene (CGT TCG TGA ATG CAA AG) and at the 3' end of *aggR* itself (GAG GCA TCA CCA ATA TG). Reactions were amplified in 100- μ l volumes using 200 nM (each) deoxynucleotide, 2.5 U of *Taq* polymerase, and buffer supplied by the manufacturer (Promega Inc., Madison, Wis.). Amplification was performed in a programmable thermal controller (MJ Research Inc., Watertown, Mass.) with 30 cycles of denaturation (30 s at 92°C), annealing (30 s at 45°C), and extension (1 min at 75°C). PCR products were analyzed by agarose gel (1.2%) electrophoresis.

FIG. 1. Strategy for the construction of the *aggA*-AP reporter fusion. A 3.8-kb *PstI* fragment containing an *aggA::phoA* fusion was cloned into the *PstI* site of the temperature-sensitive vector pIB307. This construction was then introduced independently into both 17-2 and 17-2 Δ *aggR*, and the cultures were passaged at 44°C. Merodiploid fusions consisting of a complete region 1 cluster incorporating the *aggA::phoA* fusion were thus constructed. The chloramphenicol resistance gene of pIB307 provided selective pressure for maintenance of the merodiploid fusion. A' and C' represent truncated derivatives of *aggA* and *aggC*, respectively.

RNA slot blots. Static L-broth cultures were subcultured into fresh L broth, grown to for 2 h at 37°C with shaking, then pelleted by centrifugation, lysed with 0.2 ml of RNAzol B (Biotecx Laboratories, Inc., Houston, Tex.), and treated according to the manufacturer's protocol. Twenty micrograms of total bacterial RNA from each sample (determined spectrophotometrically) was denatured prior to application on a NYTRAN nylon transfer membrane (Schliecher & Schuell, Keene, N.H.). Alkaline RNA denaturation and fixation were performed as previously described (1) using a Bio-Dot SF microfiltration apparatus (Bio-Rad Laboratories). The transcript of interest was detected by hybridization with a 260-bp *HincII* DNA fragment derived from the *aggA* gene of region 1 (41); the probe was labeled by random priming using [α - 32 P]ATP and a random priming kit according to manufacturer's instructions (Gibco/BRL, Gaithersburg, Md.). Hybridizations were performed overnight at 37°C in 50% formamide according to standard methods (1) and visualized by autoradiography on Kodak X-Omat film after 24 h of exposure.

Tn*phoA* fusion constructions. In order to measure the effect of the *aggR* gene on *aggA* expression, we recombined an *aggA::phoA* fusion into the wild-type *aggA* locus in both wild-type and Δ *aggR* backgrounds. The source of the reporter fusion was a *phoA*-expressing Tn*phoA* insertion in the *aggA* gene constructed by Savarino et al. to be described in detail elsewhere (41). A 3.8-kb *PstI* fragment beginning within the *aggC* gene and including the entire *aggB* gene and the amino

terminus of *aggA* fused to *phoA* (Fig. 1) was cloned into the temperature-sensitive vector pIB307. This construction was then introduced by electroporation into 17-2 and 17-2 Δ *aggR*, and the cultures were passaged at 44°C. Chloramphenicol-resistant colonies resulting from these passages contained the merodiploid insertion into the homologous sites of the plasmids in either 17-2 or 17-2 Δ *aggR*. The region 1 gene cluster was thus reconstructed, yet with an in-frame, *phoA*-expressing fusion in the *aggA* gene. Scoring for the presence of chloramphenicol resistance allowed determination of persistence of the merodiploid construction including the *phoA* fusion.

All fusion experiments were performed at 37°C in L broth with shaking (150 rpm) with conditions modified as explicitly stated. AP expression was measured by the method of Brickman and Beckwith (7). M9 broth was prepared as described elsewhere (29), with or without the addition of 2.0 g of Casamino Acids per liter. Mueller-Hinton broth was obtained from Difco Laboratories, Detroit, Mich. L broth prepared for testing of pH effects was buffered with one of the following sulfonate buffers, each at a 100 mM concentration: morpholineethanesulfonic acid (MES) (pK_a at 20°C = 6.15), bis-Tris (pK_a = 6.50), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pK_a = 7.55), or morpholinepropanesulfonic acid (MOPS) (pK_a = 7.20). Each buffer was prepared at uniform osmolarity. To vary osmolarity, strains were grown in L broth containing NaCl at 170, 85, or 35 mM (high, medium, or low osmolarity, respectively). Low-iron medium consisted of

FIG. 2. ORFs of AAF/I region 2. The 3.0-kb *Xba*I-*Kpn*I fragment from region 2 clone pJPN45 was sequenced in its entirety. All potential ORFs greater than 5.0 kb are indicated.

L broth containing 50 μ g of ethylenediamine-*N,N'*-diacetic acid (EDDA; Sigma Chemical, St. Louis, Mo.) per ml. Anaerobic cultures were grown at 37°C in an anaerobic jar with the GasPak Anaerobic System (Becton Dickinson Microbiology Systems, Cockeysville, Md.). All reporter fusion experiments were performed with the bacterial culture at stationary phase, as determined by monitoring the optical density at 600 nm of the respective culture. Fusion experiments were performed by measuring the expression of AP in four constructions grown side by side under identical conditions: 17-2, 17-2 Δ *aggR*, 17-2*aggA::phoA* and 17-2 Δ *aggRaggA::phoA*. When AP expression was low, the experiment was repeated in the presence of 30 μ g of chloramphenicol per ml to ensure maintenance of the merodiploid construction.

Nucleotide sequence accession number. The GenBank accession number for the sequence of the 3.0-kb region 2 fragment is Z32523.

RESULTS

Sequence determination and characteristics of ORFs in region 2. The region 2 clone pJPN45 carries an insert of 3.0 kb; in our previous analyses, this construction was shown to carry the smallest insert capable of complementing region 1 clones to confer AAF/I expression in HB101. The 3.0-kb fragment was sequenced in its entirety.

Analysis of the DNA sequence revealed four potential ORFs (Fig. 2) in the rightward orientation, encoding predicted peptides of 13.5, 6.4, 29.4, and 14.9 kDa. One ORF (predicted molecular size of product, 10.9 kDa) was detected in the leftward orientation. With reference to the original AAF/I cosmid clones, the orientation of the first four potential ORFs was in the same direction as that of *astA* (encoding the toxin EAST1, 9 kb downstream) and in the direction opposite that of the AAF/I gene cluster in region 1 (35).

Analysis of ORF1 yields a predicted peptide of 116 amino acids (12.9 kDa; nucleotides 17 to 365). The predicted ORF1 peptide was not found to have any significant homology to known protein sequences in the GenBank library. We had previously published the N-terminal amino acid sequence of a 14-kDa protein which was the predominant species in supernatants of AAF/I-producing cosmid clones and which was apparently immunogenic to volunteers fed strain 17-2 (32). As part of these prior studies, the N-terminal amino acid sequence was determined from this protein and degenerate oligonucleotides were synthesized from published *E. coli* codon preferences; these oligonucleotides hybridized to region 2 DNA. In the current analysis, we found that the sequence of the 21 amino acids downstream from the first methionine in the ORF1 product is highly homologous (19 of 20 residues) to the previously determined amino terminus of the 14-kDa protein, suggesting that this previously described protein is in fact the product of ORF1. The 21 amino acids upstream from the determined N-terminal amino acid sequence meet require-

ments for a signal peptide cleaved during secretion (14). We had previously noted that the 3.0-kb region 2 subclone was capable of complementing region 1; current analysis, however, does not reveal a potential promoter upstream from ORF1 on the cloned 3.0-kb fragment, suggesting that expression of ORF1 is not required for AAF/I expression.

ORF3 encoded a predicted peptide of 265 amino acids (29.4 kDa; nucleotides 1208 to 2003). The ORF3 product, hereafter referred to as the *aggR* product, was 68% identical at the amino acid level over its entire length to CfaR, the regulator of CFA/I production in enterotoxigenic *E. coli* (11). The *aggR* product also revealed lesser homology with other members of the AraC family of DNA binding proteins. *aggR* was found to have a G+C content of 30%, a characteristic of some members of the AraC gene family (9).

ORF2 encodes a predicted peptide of 58 amino acids (6.4 kDa; nucleotides 697 to 871) which was found to have 56% identity to the *E. coli* IS2 product over a 57-amino-acid overlap. ORF4 encodes a predicted protein of 134 amino acids (14.9 kDa; nucleotides 2106 to 2508). The protein has 86.2% identity to the product of insertion element iso-IS1 from *Shigella dysenteriae* over a 65-amino-acid overlap. ORF5 spans nucleotides 675 to 381, is transcribed in the orientation opposite that of the other ORFs, and encodes a putative peptide of 98 residues (10.9 kDa). TFASTA analysis of the ORF5 product revealed 90.4% identity over 52 amino acids to the product of the IS3 element of *Escherichia fergusonii* (and 88.5% identity over the same span to the product of the IS3 element of *E. coli*).

Subcloning of *aggR*. We previously described the generation of *TnphoA* insertion mutations in cloned 17-2 genes which abolished expression of AAF/I (32). Four different *TnphoA* insertions were mapped to region 2; none expressed AP. *TnphoA*-flanking sequences were subcloned into pBluescript II SK, and sequence analysis was performed using a *phoA* primer. All insertions which abolished expression of AAF/I mapped within the *aggR* ORF.

TnphoA data, as well as the homology of AggR with the AraC family, suggested that this ORF alone might be sufficient to complement region 1 in the expression of AAF/I. *aggR* was subcloned by digesting pJPN45 with restriction enzymes *Ssp*I and *Pvu*I and ligating the 1.2-kb fragment (Fig. 3) into *Ssp*I- and *Pvu*I-digested pBR328. The resulting construction, designated pJPN52, was transformed into HB101 already harboring the region 1 clone pJPN39; expression of AAF/I was restored, as determined by autoagglutination, HA, and AA in the HEp-2 adherence assay. These experiments confirmed the lack of a requirement for the ORF1 product in AAF/I expression.

Homology of *aggR* with other AraC gene homologs. *aggR* exhibits several characteristics which are common to the genes encoding members of the AraC family of DNA binding proteins, including encoding a product with a molecular size of about 30 kDa and having a low G+C content (30% for *aggR*) (9). In addition, conserved regions have been identified within the amino acid sequences of AraC homologs (18, 37). Most prominently, this includes the helix-turn-helix DNA binding motif, which is at positions 197 to 216 of AraC itself. Inspection of the AggR amino acid sequence with the PEPTIDESTRUCTURE program of the GCG package revealed a helix-rich region meeting requirements for the helix-turn-helix motif (6) at amino acids 228 to 247 of AggR (Fig. 3).

Gallegos et al. (18) have identified 26 consensus amino acid residues in the carboxy termini of the AraC family. AggR exhibits residues identical to these consensus determinations at 19 of the 26 positions. At position 198, AggR analysis revealed a leucine residue instead of the phenylalanine proposed as the

CGATCGTGAAGCTATAGACTGGGCTGCAATTAAGATACAACCCCT	1009
<i>PvuI</i>	
GTCTGGAATAAGGGGCAAGAACACGATAAATAATTGCTTATTG	1054
TAATTATAAGCGTAAAAATTATATCTACATGACGATGTGGAAT	1099
TAATAAACGTATTTTATATGAGTTAAAAATTATCTTTTATTGA	1144
TAAGAGTTAGGTCACTCTAACGCAGAGTTGCCTGATAAAGACATT	1189
TTTTCATGTGAGAATGATATGAAATTAAAACAAAATATCGAAAA	1234
M K L K Q N I E K	
GAGATTATAAAATTAACAATATCAGAATACATCAGTACACTGTA	1279
E I I K I N N I R I H Q Y T V	
CTATATACATCTAATGTACAAATCGATGTATACACAAAAGAAGGA	1324
L Y T S N C T I D V Y T K E G	
AGCAATACATATCTTAGAAATGAACTCATATTTCTTGAGAGAGGA	1369
S N T Y L R N E L I F L E R G	
ATAAATATATCAGTAAGATTGCAAAAGAAGAAATCAACAGTAAAT	1414
I N I S V R L Q K K K S T V N	
CCATTTATCGCAATCAGATTAAGCAGCGATACATTAAGACGCCTA	1459
P F I A I R L S S D T L R R L	
AAGGATGCCCTGATGATAATATACGGAATATCAAAGTAGATGCT	1504
K D A L M I I Y G I S K V D A	
TGCAGTTGTCGCAATTTGGTCAAAAGGAATAATGTAGCTGATGCT	1549
C S C P N W N S K I I V A D A	
GACGATTCTGTATTAGATACATTCAAGAGTATCGATCATAATGAT	1594
D D S V L D T F K S I D H N D	
GATTCAAGAATTACTTCAGATTGATATATCTTATATCAAGATC	1639
D S R I T S D L I Y L I S K I	
GAAACAACAGGAAAATTATAGATCAATTTATATATCGGCTGTA	1684
E N N R K I I E S A I Y I S A V	
AGCTTCTTTTCCGATAAGGTGAGAAACACAATCGAAGGACTTA	1729
S F F S D K V R N T I E K D L	
TCAAAGAGATGGACTCTAGCTATTATGTCAGATGAATTTAATGTA	1774
S K R W T L A I I A D E F N V	
TCAGAGATACCAATTAAGGAAAGACTTGAGTCAGAGTATATTACT	1819
S E I T I R K R L E S E Y I T	
TTTAACCGATCCTTATGCAATCAAGAAAGAGCAAGCAGCATTG	1864
F N Q I L M Q S R M S K A A L	
CTGTGCTTGATAACTCATATCAGATATCAGATATCTAATATG	1909
L L L D N S Y Q I S Q I S N M	
ATAGGATTTTCCAGTACATCATATTTTCATTAGGCTTTTGTAAAA	1954
I G F S S S T S Y F I R L F V K	
CATTTTGGCATAACACCAAAAACATTTCTTGACTTATTTTAAAGC	1999
H F G I T P K Q F L T Y F K S	
CAATGAAAATATGTTTATATCATTATTTGAGATTGCTATAAACA	2044
Q *	
TATTGGTGATGCCTCTAATTAGTTGAATCTGATGTATAATACGGG	2089
<i>SspI</i>	
CTTTTGAGGTTATCTCATGTCCAGCGTTAATATT	2118

FIG. 3. Nucleotide sequence of the *aggR*-encoding cloned insert in pJPN52. Nucleotide positions refer to the positions in the complete region 2 sequence. The predicted amino acid sequence of the AggR protein is illustrated below the corresponding nucleotide sequence. A potential ribosome binding site, identical to that proposed for the *E. coli fecE* gene (31, 38), is underlined. The dashed line represents the sequence homologous to the predicted DNA binding domain of AraC (16).

consensus by Gallegos et al. (18) and by Ramos et al. (37). According to data obtained from EMBL/GenBank, this leucine-for-phenylalanine substitution is also seen in the closest homologs to AggR, including CfaR (11, 42), Rns (9), CsrV (15), VirF (*S. dysenteriae*) (16), AppY (43), and EnvY (28).

Leucine is also present in this position in the TcpN (ToxT) protein of *Vibrio cholerae* (36).

The carboxy-terminal portion of the AraC family has been proposed to be involved in DNA binding, whereas the N terminus is thought to be responsible for interacting with activator molecules which confer specificity (8). We noted that AggR displayed greater amino acid homology with other proteins in the carboxy third of the molecule. Figure 4 shows alignment of the four AraC homologs with greatest homology to AggR (CfaD' is not included here, since it differs from CfaR mainly by the presence of a frameshift mutation) (22). The figure illustrates a very high level of conservation among the fimbrial regulators AggR, CfaR, Rns, and CsrV, suggesting the presence of a subfamily of AraC homologs. This conservation is especially marked in the first helix-turn-helix motif. VirF differs from this subfamily, although it also exhibits the RKRLESE motif at AggR positions 197 to 203. This motif is not found in more distant AraC homologs and is not a recognized consensus sequence of these homologs.

Construction of a deletion in *aggR*. Restriction analysis of the *aggR* gene revealed the presence of two *ClaI* sites; deletion of the intervening *ClaI* fragment was predicted to yield an in-frame deletion of 279 bp. The position of this deletion was in the amino one-third of the AggR protein, 53 amino acid residues upstream from the first helix-turn-helix region. The construction of the deletion and its introduction into 17-2 are described in Materials and Methods. Construction of the desired deletion was verified by sequencing of the deletion mutant (data not shown).

Properties of AggR deletion mutants. 17-2 Δ *aggR* was tested for expression of AAF/I. The mutant was negative for autoagglutination, HA, and AA in the HEP-2 assay and for the presence of AAF/I by immunogold electron microscopy. Growth curves and biochemical analysis of 17-2 Δ *aggR* were identical to those of the wild-type parent. As in the 17-2 background, the *aggR* deletion clone pJPN45.1 was unable to complement the intact region 1 clone pJPN39 in HB101 to confer AAF/I production, as assayed by AA, HA, and autoagglutination.

We hypothesize that the AAF/I may be responsible, at least in part, for adherence of EAggEC to the small-bowel mucosa. We thus tested 17-2 and its isogenic AAF/I-negative derivative for adherence to the intestinal cell line Caco-2. Adherence by 17-2 Δ *aggR* was markedly less than that of the parent strain. The Caco-2 adherence pattern of 17-2 revealed the characteristic stacked-brick phenotype similar to that seen in the HEP-2 assay.

Complementation of Δ *aggR*. To test for the ability to complement the *aggR* deletion, the *aggR* clone pJPN52 was electroporated into 17-2 Δ *aggR*. All transformants reacquired the properties of HA and AA in the HEP-2 assay. Since CfaR is the closest homolog of AggR, we attempted to complement Δ *aggR* with the cloned *cfaR* gene. In order to do this, we first cloned the *cfaR* gene on a 0.9-kb *EcoRI-HindIII* fragment into *EcoRI-HindIII*-digested pBR328. This construction was transformed into HB101 carrying region 1 clone pJPN36 (35) and also electroporated into 17-2 Δ *aggR*. Analysis of the transformants revealed that *cfaR* was capable of complementing both constructions to confer AAF/I production as assayed in the HEP-2 assay.

Transcriptional activation by *aggR*. AraC homologs have been shown to be transcriptional activators (8, 24). Thus, we tested for the ability of *aggR* to increase the transcription of the *aggA* gene, suggested to be the structural subunit of AAF/I and contained within region 1 (41). Total cellular RNA was extracted from 17-2, 17-2 Δ *aggR*, and HB101 carrying region 1

FIG. 4. Amino acid identity of AggR with its closest homologs. The nucleotide sequence of *aggR* was translated by the TFASTA program of the GCG software package and compared with known predicted or actual protein sequences in GenBank/EMBL. Shaded areas represent amino acid residues identical to those of the predicted residues of AggR. Numbers refer to the residues of AggR, using the methionine at nucleotide position 1208 as the translational start point. Heavy bars indicate the areas of AraC family conservation as reported by Ramos et al. (37).

and/or region 2 clones (Fig. 5). A *HincII* fragment lying within the *aggA* ORF was labeled with [³²P]dATP and used as a probe to detect the corresponding transcript in RNA slot blots. At 37°C, 17-2Δ*aggR* revealed markedly less, but not absent, *aggA* expression. This same effect was seen in HB101: HB101 (pJPN39) produced low-level leaky expression of *aggA*. When

FIG. 5. *aggA* mRNA detected by RNA slot blot analysis. Twenty micrograms of total bacterial RNA was extracted from log-phase cultures of native and *aggR* deletion constructions, transferred to a nylon membrane, and hybridized with a DNA probe representing the *aggA* gene. Blots were exposed to Kodak X-Omat film for 24 h.

the *aggR* clone pJPN52 was introduced into either 17-2Δ*aggR* or HB101(pJPN39), expression of *aggA* was increased to wild-type levels. At 27°C, expression of *aggA* was reduced in both 17-2 and 17-2Δ*aggR*.

***aggA::phoA* fusion regulation by *aggR*.** In order to further assess the role of *aggR* in *aggA* expression, we constructed isogenic *phoA* fusions in the *aggA* gene of 17-2 and 17-2Δ*aggR*. Strains carrying these isogenic fusions were then grown side by side under various conditions to determine under which environmental conditions the *aggR* gene was involved in the regulation of *aggA* expression (Table 2). 17-2 and 17-2Δ*aggR* were included in each experiment to control for endogenous AP activity; these values were uniformly low and are therefore not reported. When AP expression of 17-2*aggA::phoA* was less than 100 U, the experiment was repeated in the presence of chloramphenicol to maintain selection for the merodiploid. In the presence of chloramphenicol, AP values were essentially unchanged, and these values are also not reported. A preliminary experiment revealed that AP activity was highest during

TABLE 2. AP activity of *aggA::phoA* fusion-containing strains under various growth conditions^a

Medium	Temp (°C)	Mean AP activity (U) $\pm$ SD of:	
		17-2 <i>aggA::phoA</i>	17-2 <i>ΔaggRaggA::phoA</i>
L broth	42	43.5 $\pm$ 4.5	44.4 $\pm$ 2.1
L broth	37	273 $\pm$ 3.6	45.5 $\pm$ 2.1
L broth	30	479 $\pm$ 8.8	75.6 $\pm$ 3.5
L broth	27	89.5 $\pm$ 5.9	107.4 $\pm$ 1.1
High osmolarity	37	247 $\pm$ 5.1	84.7 $\pm$ 26.8
Medium osmolarity	37	79.7 $\pm$ 12.2	51.3 $\pm$ 9.2
Low osmolarity	37	53.2 $\pm$ 7.5	58.8 $\pm$ 11.0
M9 (minimal)	37	91.1 $\pm$ 4.8	55.2 $\pm$ 0.8
M9-Casamino Acids	37	309 $\pm$ 3.3	58.0 $\pm$ 3.0
Mueller-Hinton	37	120 $\pm$ 3.7	46.1 $\pm$ 1.9
Nonshaking	37	391 $\pm$ 8.6	97.3 $\pm$ 8.8
Anaerobic	37	694 $\pm$ 30	147 $\pm$ 13.5
Low iron	37	888 $\pm$ 15	111 $\pm$ 3.5
MES, pH 5.5	37	2,555 $\pm$ 259	424 $\pm$ 36
Bis-Tris, pH 5.5	37	3,368 $\pm$ 131	465 $\pm$ 8.1
MOPS, pH 7.5	37	607 $\pm$ 15	99.3 $\pm$ 2.7
HEPES, pH 7.5	37	436 $\pm$ 3.9	100 $\pm$ 1.7

^a All assays were done in L broth with shaking at 150 rpm unless otherwise noted.

the stationary phase of growth in both 17-2*aggA::phoA* and 17-2*ΔaggRaggA::phoA* (not shown). Therefore, all further experiments were performed using stationary-phase cultures.

aggR was involved in up-regulation of the *aggA* gene at 37 and 30°C, under aerobic and anaerobic conditions, and under conditions of rich media and high osmolarity (sodium concentration). Even in the presence of a functional *aggR* locus, *aggA* expression was repressed at extremes of temperature, in minimal medium, and at low osmolarity. Expression of *aggA* was maximal in L broth buffered to pH 5.5 (in either MES or bis-Tris buffer). At pH 5.5, the *aggR* deletion mutant expressed the *aggA* fusion to a significantly greater degree than it did at pH 7.5, suggesting that *aggA* was responsive to pH by *aggR*-dependent and *aggR*-independent mechanisms.

DISCUSSION

EAggEC strains have been shown to be diarrheal pathogens in both epidemiologic and volunteer studies (2, 3, 12, 33, 34), yet the basic features of EAggEC pathogenesis are unknown. We have previously shown that the AA phenotype in EAggEC 17-2 is associated with the presence of a ca. 60-MDa plasmid. The AA phenotype itself is related to the presence of flexible-bundle-forming fimbriae 2 to 3 nm in diameter, which we have designated AAF/I (32). We have also shown that the AAF/I genes on the 17-2 plasmid exist as two unlinked regions separated by 9 kb of unrelated DNA (35).

AAF/I region 1 contains a cluster of genes involved in fimbrial expression, including that for the fimbrial subunit itself (*aggA*) (41). Region 2 was previously defined as the smallest subclone which complemented region 1 to confer AAF/I expression (35). Here, we report the complete sequencing of the 3.0-kb region 2 cloned fragment. Our sequencing data suggest five potential ORFs, four in one direction. An ORF herein designated *aggR* is sufficient by itself to complement region 1 for expression of AAF/I. *aggR* is flanked by ORFs with homology to known insertion sequences.

aggR encodes a 29.4-kDa protein with homology to the AraC family of DNA binding proteins. This family also includes AraC, CfaR, Rns, ToxT, VirF, and the recently described PerA of enteropathogenic *E. coli* (19). The AraC family has several

conserved features. All have revealed a helix-turn-helix motif, with highly conserved residues (18, 37). Members of this family often display a very low G+C content (9). This observation raises questions about the origin of this gene family, yet no definitive data are available to illuminate the source of these genes.

The closest homologs to *AggR* are all positive regulators of fimbrial expression in *E. coli* (CfaR, Rns, and CsrR). These proteins reveal remarkable conservation of amino acid residues in the C terminus, the proposed DNA binding portion of the molecules. Amino acids 187 to 201 of *AggR*, including the helix-turn-helix motif, are identical to the other fimbrial regulators, except for one substitution of an alanine for a valine in Rns. Thus, these fimbrial regulators (with Rns as the prototype) represent a subfamily of AraC homologs adapted to the regulation of fimbriae. Notably, all four proteins feature a leucine at *AggR* position 198, which differs from the phenylalanine consensus residue at this position proposed by both Ramos et al. (37) and Gallegos et al. (18). VirF of *Shigella* spp. and ToxT of *V. cholerae* also feature this substitution. This leucine-for-phenylalanine change may represent a branch point in the evolution of AraC proteins.

Our initial experiments suggested that *aggR* was required for expression of AAF/I in HB101. In order to demonstrate a requirement for *aggR* in the native background, we constructed an in-frame deletion of the N-terminal region of *aggR* by allelic exchange. 17-2*ΔaggR* did not express AAF/I, as evidenced by a lack of autoagglutination, HA, and AA in the HEP-2 assay and the lack of fimbriae by immunogold electron microscopy. These phenotypes were restored by the reintroduction of the cloned *aggR* gene. We recognize that some members of the AraC family (such as VirF of *Shigella* spp. [25, 39]) are regulators of multiple bacterial genes. Thus, although we have shown a dependency of AAF/I expression on the presence of *aggR*, the lack of adherence phenotype may not necessarily be due to lack of AAF/I expression alone.

Experiments using an *aggA::phoA* fusion introduced into the wild-type plasmid as well as into the isogenic *aggR* deletion mutant allowed us to assess conditions under which *aggR* was active in the regulation of *aggA* expression. *aggR* functioned as an activator of *aggA* under a wide variety of conditions, including variations in growth medium, temperature, and oxygen tension, but was inactive at extremes of temperature and in minimal medium. The effect of *aggR* on *aggA* expression was most marked at pH 5.5. Moreover, *aggA* expression in the absence of a functional *aggR* gene was high at pH 5.5, suggesting that the expression of *aggA* was regulated by pH in an *aggR*-independent manner. *aggA* expression was maximal when both *aggR*-dependent and *aggR*-independent mechanisms were functional.

Our data for both HB101 and 17-2 suggest that *aggR* exerts its effect on AAF/I expression by acting as a positive regulator of fimbrial gene transcription. Temperature regulation of CFA/I expression requires intact *hns* and *cfaR* genes and may be related to the linking number of the plasmid DNA (24). Like the CFA/I system, transcription of AAF/I genes also exhibits temperature dependence: at 27°C, *aggA* is repressed even in the presence of *aggR*. Whether linking number is relevant to AAF/I expression is the focus of research in our laboratory at this time.

ACKNOWLEDGMENTS

This work was supported by PHS grant R29-AI-33096 and by the Norwich-Eaton/Infectious Disease Society of America Young Investigator Award.

We thank Stephen J. Savarino and David Hone for critical reading of the manuscript and Ian Blomfield for providing plasmid vectors.

REFERENCES

- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl. 1989. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
- Bhan, M. K., V. Khoshoo, H. Sommerfelt, P. Raj, S. Sazawal, and R. Srivastava. 1989. Enterotoxigenic *Escherichia coli* and salmonella associated with nondysenteric persistent diarrhea. *Pediatr. Infect. Dis. J.* 8:499–502.
- Bhan, M. K., P. Raj, M. M. Levine, J. B. Kaper, N. Bhandari, R. Srivastava, R. Kumar, and S. Sazawal. 1989. Enterotoxigenic *Escherichia coli* associated with persistent diarrhea in a cohort of rural children in India. *J. Infect. Dis.* 159:1061–1064.
- Blomfield, I. C., M. S. McClain, and B. I. Eisenstein. 1991. Type 1 fimbriae mutants of *Escherichia coli* K12: characterization of recognized afimbriae strains and construction of new *fim* deletion mutants. *Mol. Microbiol.* 5:1439–1445.
- Boyer, H. W., and R. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J. Mol. Biol.* 41:459.
- Brandon, C., and J. Tooze. 1991. DNA recognition by proteins with the helix-turn-helix motif, p. 87–111. In *Introduction to protein structure*. Garland Publishing Inc., New York.
- Brickman, E., and J. Beckwith. 1975. Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and ϕ 80 transducing phages. *J. Mol. Biol.* 96:307–316.
- Bustos, S. A., and R. F. Schleif. 1993. Functional domains of the AraC protein. *Proc. Natl. Acad. Sci. USA* 90:5638–5642.
- Caron, J., L. M. Coffield, and J. R. Scott. 1989. A plasmid-encoded regulatory gene, *ms*, required for expression of the CS1 and CS2 adhesins of enterotoxigenic *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 86:963–967.
- Caron, J., D. R. Maneval, J. B. Kaper, and J. R. Scott. 1990. Association of *ms* homologs with colonization factor antigens in clinical *Escherichia coli* isolates. *Infect. Immun.* 58:3442–3444.
- Caron, J., and J. R. Scott. 1990. A *ms*-like regulatory gene for colonization factor antigen I (CFA/I) that controls expression of CFA/I pilin. *Infect. Immun.* 58:874–878.
- Cravioto, A., R. J. Gross, S. Scotland, and B. Rowe. 1979. An adhesive factor found in strains of *Escherichia coli* belonging to the traditional enteropathogenic serotypes. *Curr. Microbiol.* 3:95–99.
- Cravioto, A., A. Tello, A. Navarro, J. Ruiz, H. Villafan, F. Uribe, and C. Eslava. 1991. Association of *Escherichia coli* HEp-2 adherence patterns with type and duration of diarrhoea. *Lancet* 337:262–264.
- Creighton, T. E. 1993. Proteins: structures and molecular properties, 2nd ed., p. 66–68. W. H. Freeman & Co., New York.
- de Haan, L. A., G. A. Willshaw, B. A. van der Zeijst, and W. Gastra. 1991. The nucleotide sequence of a regulatory gene present on a plasmid in an enterotoxigenic *Escherichia coli* strain of serotype O167:H5. *FEMS Microbiol. Lett.* 67:341–346.
- Dorman, C. J. 1992. The VirF protein from *Shigella flexneri* is a member of the AraC transcription factor superfamily and is highly homologous to Rns, a positive regulator of virulence genes in enterotoxigenic *E. coli*. *Mol. Microbiol.* 6:1575.
- Finlay, B. B., and S. Falkow. 1990. *Salmonella* interactions with polarized human intestinal Caco-2 epithelial cells. *J. Infect. Dis.* 162:1096–1106.
- Gallegos, M.-T., C. Michan, and J. L. Ramos. 1993. The XylS/AraC family of regulators. *Nucleic Acids Res.* 21:807–810.
- Gomez, O., and J. B. Kaper. Unpublished data.
- Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:557–580.
- Hibberd, M. L., M. M. McConnell, G. A. Willshaw, H. R. Smith, and B. Rowe. 1991. Positive regulation of colonization factor antigen I (CFA/I) production by enterotoxigenic *Escherichia coli* producing the colonization factors CS5, CS6, CS7, CS17, PCF09, PCF0159:H4 and PCF0166. *J. Gen. Microbiol.* 137:1963–1970.
- Hone, D., and J. Xu. Unpublished data.
- Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper. 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc. Natl. Acad. Sci. USA* 87:739–743.
- Jordi, B., B. Dagberg, L. de Haan, A. Hamers, B. van der Zeijst, W. Gastra, and B. Uhlin. 1992. The positive regulator CfaD overcomes the repression mediated by histone-like protein H-NS (H1) in the CFA/I fimbrial operon of *Escherichia coli*. *EMBO J.* 11:2627–2632.
- Kato, J.-I., K.-I. Ito, A. Nakamura, and H. Watanabe. 1989. Cloning of regions required for contact hemolysis and entry into LLC-MK2 cells from *Shigella sonnei* form I plasmid: *virF* is a positive regulator gene for these phenotypes. *Infect. Immun.* 57:1391–1398.
- Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollingier. 1988. Improved broad host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* 70:191–197.
- Levine, M. M., P. Ristaino, G. Marley, C. Smyth, S. Knutton, E. Boedeker, R. Black, C. Young, M. L. Clements, C. Cheney, and R. Patnaik. 1984. Coli surface antigens 1 and 3 of colonization factor antigen II-positive enterotoxigenic *Escherichia coli*: morphology, purification, and immune responses in humans. *Infect. Immun.* 44:409–420.
- Lundrigan, M. D., M. J. Friedrich, and R. J. Kadner. 1989. Nucleotide sequence of the *Escherichia coli* porin thermoregulatory gene *envY*. *Nucleic Acids Res.* 17:800.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual, p. 440–441. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Manoil, C., and J. Beckwith. 1985. *TnphoA*: a transposon probe for protein export signals. *Proc. Natl. Acad. Sci. USA* 82:8129–8133.
- Nataro, J. P. Unpublished observations.
- Nataro, J. P., Y. Deng, D. R. Maneval, A. L. German, W. C. Martin, and M. M. Levine. 1992. Aggregative adherence fimbriae I of enterotoxigenic *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. *Infect. Immun.* 60:2297–2304.
- Nataro, J. P., J. B. Kaper, R. Robins-Browne, V. Prado, and M. M. Levine. 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. *Pediatr. Infect. Dis. J.* 6:829–831.
- Nataro, J. P., M. M. Levine, S. Savarino, G. Losonsky, L. Guers, and C. O. Tacket. 1993. Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993, abstr. B315.
- Nataro, J. P., D. Yikang, J. Giron, S. J. Savarino, M. H. Kothary, and R. Hall. 1993. Aggregative adherence fimbria I expression in enterotoxigenic *Escherichia coli* requires two unlinked plasmid regions. *Infect. Immun.* 61:1126–1131.
- Ogierman, M. A., and P. A. Manning. 1992. Homology of TcpN, a putative regulatory protein of *V. cholerae*, to the AraC family of transcriptional activators. *Gene* 116:93–97.
- Ramos, J. L., F. Rojo, L. Zhou, and K. N. Timmis. 1990. A family of positive regulators related to the *Pseudomonas putida* TOL plasmid XylS and the *Escherichia coli* AraC activators. *Nucleic Acids Res.* 18:2149–2152.
- Rudd, K. E., and T. D. Schneider. 1992. Compilation of *E. coli* ribosome binding sites, p. 17.19. In J. H. Miller (ed.), *A short course in bacterial genetics*. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
- Sakai, T., C. Sasakawa, and M. Yoshikawa. 1988. Expression of four virulence antigens of *Shigella flexneri* is positively regulated at the transcriptional level by the 30 kiloDalton *virF* protein. *Mol. Microbiol.* 2:589–597.
- Savarino, S. J., A. Fasano, J. Watson, B. M. Martin, M. M. Levine, S. Guandalini, and P. Guerry. 1993. Enterotoxigenic *Escherichia coli* heat-stable enterotoxin 1 represents another subfamily of *E. coli* heat-stable toxin. *Proc. Natl. Acad. Sci. USA* 90:3093–3097.
- Savarino, S. J., P. Fox, D. Yikang, and J. P. Nataro. Unpublished data.
- Savelkoul, P. H., G. A. Willshaw, M. M. McConnell, H. R. Smith, A. M. Hamers, B. A. van der Zeijst, and W. Gastra. 1990. Expression of CFA/I fimbriae is positively regulated. *Microb. Pathog.* 8:91–99.

43. Tove, A., A. Nielsen, and F. Hansen. 1989. Isolation, characterization and nucleotide sequence of *appY*, a regulatory gene for growth phase-dependent gene expression in *Escherichia coli*. J. Bacteriol. **171**:1683–1691.
44. Vial, P. A., J. J. Mathewson, H. L. DuPont, L. Guers, and M. M. Levine. 1990. Comparison of two assay methods for patterns of adherence to HEp-2 cells of *Escherichia coli* from patients with diarrhea. J. Clin. Microbiol. **28**:882–885.
45. Yamamoto, T., S. Endo, T. Yokota, and P. Echeverria. 1991. Characteristics of adherence of enteroaggregative *Escherichia coli* to human and animal mucosa. Infect. Immun. **59**:3722–3739.