Characterization of the *Escherichia coli* AF/R1 Pilus Operon: Novel Genes Necessary for Transcriptional Regulation and for Pilus-Mediated Adherence

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We isolated the genetic determinant of AF/R1 pilus production in attaching/effacing *Escherichia coli* RDEC-1 and identified seven genes required for pilus expression and function. DNA sequence analysis of the structural subunit gene afrA corrected an error in the published sequence and extended homology with the F18 pilus subunit of pig edema *E. coli* strains. AfrB and AfrC, encoded downstream from AfrA, were required for pilus expression. AfrB was related to the usher protein PefC of *Salmonella typhimurium* and encoded a chaperone protein and AfrC was related to PefD, a chaperone protein. AfrD and AfrE, encoded downstream from AfrC, were not necessary for the expression of AF/R1 pilus but were required for ileal adherence as assayed by ileal brush border aggregation. Thus, the adhesive subunit of the AF/R1 pilus is distinct from the structural subunit, as is the case for Pap pili and type 1 pili. AfrD was related to FedE of the F18 fimbrial operon of the *E. coli* strain that causes edema disease in pigs. AfrE was a novel protein. AfrR and AfrS are encoded upstream from AfrA, in the opposite orientation. AfrR is related to the AraC family of transcriptional regulators, and AfrR and AfrS interact to function in a novel mode of transcriptional activation of afrA. AF/R1 pilus mediates the adherence to Peyer’s patch M cells, ileal mucosa, and colonic mucosa in a rabbit model of diarrhea caused by enteropathogenic *E. coli*. Our observations will facilitate the further study of the phenomena of M-cell adherence.

*MATERIALS AND METHODS*

Bacterial strains, plasmids, and media. RDEC-1 is an *E. coli* O155K−:H− strain that expresses AF/R1 pilus. *Shigella* D15 and D12 were used as positive and negative strains for pilus expression, respectively, as strain D15 is an exconjugate containing the 86-MDa plasmid encoding AF/R1 pilus expression (11). Strain M129, an RDEC-1 strain cured of the 132-kb plasmid (reference 62 and unpublished observations), and RDEC-1 42-2-37-8, a strain resulting from a temperature-sensitive mutation that does not express AF/R1 pilus (27), both served as negative controls for pilus expression. DH5α [F−* lacZD515 metA1 recA1 bsdR17 supE44 thi-1 lacY1 galK1 gyrA96 relA1 80d−] (Gibco BRL, Gaithersburg, Md.) was used as a host for recombinant plasmid constructions and transposon mutagenesis. Strain JM101 (59) was used as a host for bacteriophage M13 derivatives in nucleotide sequence analysis (59). Multicopy plasmids pUC18 and pUC19 were used as cloning vectors, pKI100, a pUC derivative (29) compatible with pUC vectors, was used as a vector for complementation experiments with pUC constructs. A KpnI fragment internal to afrA was cloned into pBluescript II KS(+) phagemid (Stratagene Inc., La Jolla, Calif.) and used to prepare the probe for the Northern blots.

Organisms were grown in Luria broth or on Luria agar unless otherwise noted. Penassay broth was used to induce AF/R1 pilus expression in the wild-type RDEC-1 strain (11). Ticarcillin-clavulanic acid (100 mg/ml) was used to select for the presence of pUC derivatives, and kanamycin (25 mg/ml) was used for the selection of pKI100 derivatives. Strain DM15 was used as a positive control for pilus expression. A strain that expresses AF/R1 pili.

**Brush border preparation and bacterial adherence assay.** Rabbit ileal brush borders were purified according to the method of Cheney et al. (10). Bacterial strains were grown overnight and examined for their ability to adhere to the purified brush borders (10). Bacteria adhering to 50 brush borders were counted, and results were calculated as the average number of bacteria/brush border. It was difficult to count more than 10 bacteria/brush border with any accuracy. For that reason, these brush borders were considered to have 10 bacteria when the average number of adherent bacteria was calculated. Adherence assays were performed on four separate occasions with different brush border preparations each time. Adherence was considered to be present if the results were significantly different from negative controls.

**Purification of AF/R1 pilin.** AF/R1 pilin were purified by a modification of the technique of Isaiahcon (28). Pili were sheared from bacteria cultured overnight in static Penassay broth by using an Omnimixer (Omni International, Inc., Waterbury, Conn.). The pili were precipitated in an ammonium sulfate solution, re-suspended in phosphate-buffered saline, pH 7.5, dialyzed against water, and eluted from a DEAE column in a discontinuous salt gradient. Pili preparations were confirmed as pilus structures by electron microscopy of negatively stained
Transformants were screened for AF/R1 pilus expression with AF/R1 MAb. The thick lines on the boxes indicate the presence and positions of signal peptides. Arrows above the boxes indicate directions of transcription. The numbers in the boxes are the predicted molecular masses (in kilodaltons) of the mature polypeptides. Sites of transposon insertions in pRKB18 are indicated as follows: Tn5 insertions not affecting the expression of AF/R1 pilus (white triangles) and Tn5 insertions which abolished the expression of AF/R1 pilus (black triangles). All transposon mutagenesis was performed on pRKB18. p598 is described in Materials and Methods. Its construction required mutagenesis to insert an lambda affinity tag (anti-M-tag) at the carboxy-terminal end of afrS. The new site allowed the cloning of an Spnl-KpnI fragment that expressed AfrR.

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probe was generated with α-[35S]UTP, T3 RNA polymerase, and reagents provided in kit form (Maxiscript transcription kit; Ambion, Austin, Tex.). Hybridization conditions were as previously described (2). Following hybridization, the nylon membranes were washed in 300 mM sodium chloride or 30 mM sodium citrate at 75°C for 4 h and then incubated at room temperature in the same buffer with 25 μg of RNase A per ml and 10 μl of RNase T1 per ml. The membranes were then exposed to X-ray film.

**Results**

**Isolation of the afr gene cluster encoding the production of AF/R1 pili.** DNA isolated from *E. coli* RDEC-1 was partially digested with *Sal*3A1 and size fractionated by agarose gel electrophoresis. Fragments of 15 to 20 kb were ligated into *Bam*HI-digested pUC18, and the resulting plasmids were transformed into DH5α. Transformants were examined by colony hybridization for sequences homologous to a 68-base oligonucleotide probe, the sequence of which was derived from a partial amino acid sequence of purified AF/R1 pili. Three of 600 colonies examined hybridized with the probe, and each of these bound the antibody specific for AF/R1 (data not shown). One of these was selected for further study and designated DH5α(pJRCl0).

Deletion and subcloning experiments revealed that an 8,314-kb *Spf*I fragment of pJRCl0 was sufficient for the expression and function of AF/R1. A pUC18 derivative containing this fragment was designated pRKB15.

**Sequence analysis of the afr gene cluster.** The complete nucleotide sequence of pRKB15 was determined, and open reading frames preceded by sequences encoding potential ribosome binding sites (54) were identified corresponding to each of these genes (GenBank accession no. AF050217). Genes were designated *afrBCDE* (Fig. 1). Each gene encoded a predicted product of a size consistent with maxicell gel electrophoretic mobility and a signal peptide cleavage site predicted between amino acids 27 and 28. *AfrS* was predicted to be a mature protein of 18,804 Da. This gene is related to *afr* genes encoding fimbrial subunits and is predicted to be 23,196 Da and has significant relatedness to the F1 envelope protein of *Salmonella typhimurium* (17). *AfrD* is predicted to be 23,196 Da and has significant relatedness to the F1 envelope protein of *Salmonella typhimurium* (17). *AfrD* is predicted to be 23,196 Da and has significant relatedness to the F1 envelope protein of *Salmonella typhimurium* (17). **AfrD** encoded a predicted signal sequence, and a signal peptide cleavage site is predicted between amino acids 24 and 25. The mature protein is predicted to be 86,126 Da. *AfrB* is related to a number of usher proteins associated with bacterial pilus biogenesis. The most closely related protein was the F18 fimbrial subunit of *E. coli* associated with edema disease in swine (23). *AfrB* is predicted to encode an amino-terminal signal sequence, and a signal peptide cleavage site is predicted between amino acids 23 and 24. The mature protein is predicted to be 23,196 Da and has significant relatedness to the PeF D chaperone of *Salmonella* plasmid-encoded fimbriae (17). *AfrD* encoded a predicted signal sequence with a signal peptide cleavage site predicted between amino acids 21 and 22. The mature protein was predicted to be 15,652 Da and was related to *fedE* of the F18 fimbrial operon. *FedE* is thought to be involved in determining fimbrial length and binding specificity (24). The last open reading frame was *afrE*, which encoded a predicted signal sequence with a signal peptide cleavage site predicted between amino acids 32 and 33. The mature protein was predicted to be 30,259 Da. No significant homology exists with proteins entered in sequence databases as determined by BLASTP comparisons (1).

**Insertion mutagenesis analysis.** Tn5 insertion mutagenesis was used to assess gene function in DH5α(pRKB18). Transductants were screened for the loss of AF/R1 pilus expression by using AF/R1 MAβ. The results are shown in Fig. 1. Insertions in *afrABC* resulted in a loss of pilus expression. Insertions in *afrS* and *afrE* did not affect pilus expression.

**AfrS and AfrR encode positive transcriptional regulators of the afr gene cluster.** The sequence relatedness of the predicted product of *afrR* with the AraC family of transcriptional activators suggested that *AfrR* may serve to regulate the expression of the *afr* gene cluster at the transcriptional level. We tested this hypothesis by complementation experiments on the expression of pilus and Northern blot analysis to ascertain the effects of complementation. We examined the expression of *afrA* in the presence and absence of *afrR*. We also examined the effect of *afrS*, the open reading frame immediately downstream of *afrR*, on *afrA* transcription. Subclones of pRKB15 were constructed which lacked the region encoding *afrS*.
pRKB18 (Fig. 1), which placed the Plac promoter upstream of afrA, mediated the production of AF/R1 pili as determined by a colony blot assay. In contrast, pRKB19 (Fig. 1), which placed Plac downstream of afrE, did not mediate the production of AF/R1 pili, suggesting that the production of pili by pRKB18 was due to the positioning of the vector promoter upstream of afrA. A plasmid was constructed using a vector compatible with pUC derivatives, pKI100 (29), which included afrA. A plasmid was constructed that encoded only afrS. Maxicell analysis of p501 confirmed the expression of afrS (data not shown). p501 did not complement AF/R1 pilus production. A plasmid encoding only afrR was constructed by using the pKI100 vector. This was designated p598, and afrR expression was confirmed by maxicell analysis (data not shown). p598 did not complement pRKB19 for AF/R1 expression. These results are summarized in Table 1.

Northern blot analysis confirmed that the results of expression analysis noted above involved transcriptional activation. When pRKB19 was complemented with p507 (Fig. 1), which encodes both afrS and afrR, an eightfold increase in the amount of afrA mRNA was observed (Fig. 2, lane 2) relative to the amount of afrA mRNA produced by E. coli harboring only pRKB19 (Fig. 2, lane 1), as determined by densitometric analysis. When pRKB19 was complemented with either p598 expressing afrR or p501 expressing afrS, no increase in the amount of afrA message was observed. These results are consistent with the hypothesis that AfrR and AfrS interact to activate the transcription of afrA.

AF/R1 pilus adherence is encoded in the afrD region. A subclone of pRKB15, p562, that deleted afrE (Fig. 1) was prepared. DHSa(pRKB15) and DHSa(p562) were examined for AF/R1 surface expression with MAbs and by rabbit intestinal brush agglutination. AF/R1 antigen was expressed in both cases. However, only DHSa(pRKB15) agglutinated rabbit brush borders (data not shown). This indicates that AfrA does not confer adhesiveness to AF/R1 fimbriae in the absence of AfrE. The role of afrD in AF/R1 pilus expression and function remains unknown. AfrD is highly homologous to FedE, one of two proteins reported to be essential for fimbrial adherence and fimbrial length of the F18 (24).

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* See Fig. 1 for details of plasmid constructs.

* ND, not done.

DISCUSSION

Sequence analysis, maxicell assays, complementation experiments, and Northern blots have defined seven genes associated with the expression and function of AF/R1 pili by the rabbit pathogen E. coli RDEC-1. Our sequence extends the homology displayed between AfrA and FedA, the major structural subunit of F107 (now called F18 [24] fimbriae) (23), through the C termini of the two peptides and is consistent with molecular size data. Comparison of the sequence of AfrA and the predicted sequence of mature FedA suggests that the two peptides have evolved from a common ancestor relatively recently, with an overall identity of 47% and a similarity of 62%.

We report the sequence of a gene downstream of afrA which we have designated afrB. Analysis of the predicted amino acid sequence of the putative gene product revealed significant homology with a number of usher proteins associated with pilus biogenesis. The most closely related was PefC, the usher protein of S. typhimurium plasmid-encoded fimbriae (17). PefC has homology with PapC and FaeD, outer membrane proteins required for the biosynthesis of P and K88 fimbriae of E. coli, respectively (38, 42). These proteins are required for the extracellular localization of fimbrial proteins, and proteins of similar sizes (80 to 100 kDa) are encoded by all E. coli fimbrial determinants studied (15). We hypothesize that afrB encodes an outer membrane protein involved in the assembly of AF/R1 fimbriae. Transposon insertions in afrB prevent the expression of AfrA antigen on the bacterial cell surface, consistent with this hypothesis (Fig. 1).

afrC encodes a protein with homology to periplasmic chaperones required for fimbrial synthesis in a number of systems, the best characterized of which is the pap operon, for which PapD serves as the chaperonin protein. These proteins act to protect fimbrial subunits from proteolytic degradation in the periplasm prior to assembly at the outer membrane (15). Transposon insertions in afrC also prevent the expression of AfrA antigen on the bacterial cell surface (Fig. 1), consistent with this hypothesis. Transposon insertion mutagenesis revealed that only afrABC were necessary for the expression of the AF/R1 pilus.

The functions of the region downstream of afrC, afrDE, which encodes two proteins, appear to include the adhesive properties of AF/R1 fimbriae. When afrE is deleted, AF/R1 fimbriae are produced which are antigenic but which do not mediate the adherence of E. coli to rabbit intestinal brush borders. This indicates that the adhesive subunit is distinct from the major structural subunit of AF/R1 and is likely encoded by afrE. Several fimbrial adhesins are characterized by adhesive subunits distinct from the major fimbrial subunits,
including P pili (36), type I pili (34), and others. The characterization of the adhesive subunit of AF/R1 pili will be an important contribution to the understanding of the M cell-specific adherence mediated by these pili.

The regulation of several fimbrial adhesin operons of E. coli involves members of the AraC family of transcriptional activators. Examples include Rns, CfaD, CsvR, and FapR, which are regulatory proteins for the fimbrial adhesins CS1, CS2, CS5, CFA/I, CS5, and 987P, respectively (8, 9, 13, 33). Our observations indicate that the expression of AF/R1 pili requires a protein, AfrR, which displays homology with the AraC family of transcriptional activators (Fig. 3). The homology of AfrR with Rns is rather limited, in contrast with the other AraC-related activators of E. coli fimbrial adhesins, which form a highly homologous group. Rns, CfaD, CsvR, and FapR have extensive regions of similarity and are all related to AraC at the C terminus. AfrR does not have any homology with Rns outside the AraC-related region. Moreover, the region of homology with the C terminus of AraC is at the N terminus of AfrR. The close relationship of AfrR with the Y. pestis regulatory protein Caf1R is of interest. The caf1 operon of Y. pestis encodes the production of a proteinaceous capsule, the major component of which is the F1 antigen (19). Although this protein does not appear to form a fimbria-like structure, the operon encodes at least two proteins required for capsule assembly which have homology with fimbrial assembly and periplasmic chaperone proteins (19, 32). Capsule production requires the presence of Caf1R, and the truncated derivative of Caf1R consisting of the N-terminal 81 amino acids of the protein is capable of positively regulating the expression of the capsule (31). This is consistent with the observed homology of the N terminus of Caf1R with the DNA binding domain of AraC. The occurrence of these closely related regulatory proteins in two otherwise unrelated fimbria-like operons suggests that AfrR-mediated expression may represent a third general pattern of regulation of E. coli fimbriae associated with pathogenesis.

AfrS was related to two proteins of unknown function. Thus, the sequence information does not provide an indication of the function. Our data indicate that AfrS is required for the transcription of the afr operon. A transposon insertion in afrS did not affect pilus expression (Fig. 1). However, in this construct, afrA transcription was likely mediated by a vector promoter, relieving the requirement for afrS. Possible modes of action of AfrS include a direct interaction with AfrR, an indirect involvement through interaction with another unknown regulatory protein, or signal transduction, as suggested by the predicted presence of a signal sequence for secretion. Details of the role of AfrS in afr transcriptional regulation await further studies on the localization of the protein and identification of potential interactions with other proteins or with the afr promoter.

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REFERENCES


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