Cloning of the Genes for AF/R1 Pili from Rabbit Enteroadherent
Escherichia coli RDEC-1 and DNA Sequence of the Major
Structural Subunit

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AF/R1 pilis on the surface of Escherichia coli RDEC-1 promote attachment of the bacteria to rabbit intestinal brush borders. In order to characterize AF/R1 pilis and manipulate their expression, we cloned the genes necessary for AF/R1 expression; determined the size of proteins produced in minicells; located the gene encoding the major structural subunit, named AfrA; and determined the DNA sequence of afrA as well as the sequence of 700 additional nucleotides upstream of afrA. Two contiguous EcoRI fragments spanning 7.9 kilobases were cloned from the 86-megadalton plasmid of RDEC-1 into vector pUC19 to make plasmid pW1. Bacteria carrying pW1 produced AF/R1 pilis that were recognized by AF/R1-specific antiserum and promoted adherence of bacteria to brush borders prepared from rabbit intestine. Proteins with a molecular weight of 17,000 (17K proteins), which was the size of AfrA, as well as 15K, 15.5K, 26K, 28K, and 80K proteins were detected in minicells carrying pW1. The gene afrA was located by using an oligonucleotide probe, and its DNA sequence was determined. The DNA sequence of 700 additional nucleotides upstream was determined because this sequence may be important in the regulation of AF/R1 expression.

AF/R1 pilis are surface antigens on Escherichia coli RDEC-1 that promote adherence of the bacteria to rabbit intestine. Mutants of RDEC-1 that do not express AF/R1 are virulent, but disease is less severe than that caused by the wild-type strain (7, 27). AF/R1 pilis promote adherence to brush borders prepared from the intestines of rabbits but not from those of other species (10) and are responsible for the initial adherence of RDEC-1 bacteria to epithelial cells in the gut, providing access to the surface of the epithelial cells, where unidentified bacterial virulence factors then efface the microvilli (6).

AF/R1 antigens are typical pilus structures on the bacterial surface (5; F. J. Cassels et al., manuscript in preparation). The AF/R1 pilis are composed of protein subunits, and the major subunit has an apparent molecular weight of 19,000 (5). The amino acid sequence of the N terminus of this major subunit has been determined and has features in common with type I, Pap, and K99 pilis (Cassels et al., in preparation). Expression of AF/R1 pilis is mediated by an 86-megadalton (MDa) plasmid (9), and a 2.4-kilobase (kb) EcoRI fragment from the 86-MDa plasmid has been shown to be important since three separate Tn5 insertions in this fragment resulted in the loss of AF/R1 expression (27).

We cloned the genes that are necessary for AF/R1 expression in a high-copy-number plasmid, mapped the cloned DNA, characterized the proteins encoded by the clone, and obtained the DNA sequence of the major subunit.

Frames from the 86-MDa plasmid that mediate expression of AF/R1 pilis were obtained after partial digestion of plasmid DNA with EcoRI and ligated to pUC19 (20, 27). Transformants of E. coli DH5α (purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were screened by using antiserum specific for AF/R1 pilis (27, 28), and an AF/R1 clone was obtained. The anti-AF/R1 antiserm recognized both the intact pilus structure and the major subunit that was obtained after dissociation of pili, so that transformants expressing either the intact pilus structure or the subunit on the surface would be detected.

The plasmid in this clone was designated pW1 and was composed of pUC19 plus an insert of 2.4- and 5.5-kb EcoRI fragments from the 86-MDa plasmid. AF/R1 antigen expression by DH5α carrying pW1 was confirmed by slide agglutination, using antiserum specific for AF/R1. A map of pW1 is given in Fig. 1. Only these contiguous 2.4- and 5.5-kb EcoRI fragments were necessary for expression of the AF/R1 antigen that functioned in adherence. This was somewhat unexpected, since a deletion derivative of the 86-MDa plasmid that retains these fragments does not express AF/R1 (27). It seems likely that the high copy number of pW1 may supplant a requirement of the native large plasmid.

Plasmid pW1 mediated expression of the AF/R1 antigen on the surface of both strains DH5α and HB101 (Table 1). The AF/R1 pili encoded by pW1 were shown to function in adherence, since they promoted attachment of the bacteria to intestinal brush borders isolated from rabbits (8). Significant numbers of DH5α isolates carrying pW1 adhered to rabbit brush borders (an average of 4.7 ± 2.8 bacteria per brush border) compared with DH5α isolates carrying the vector pUC19 that adhered to rabbit brush borders (an average of 0.3 ± 2.3 bacteria per brush border). Binding mediated by plasmid pW1 in DH5α was substantially less than that mediated by RDEC-1 grown in Penassay broth (PAB; Difco Laboratories, Detroit, Mich.) (greater than 10 bacteria per brush border).

Expression of AF/R1 from the native 86-MDa plasmid is inhibited by growth in brain heart infusion medium (BHI) and low growth temperature (9), so expression from pW1 was tested under these conditions. Strains DH5α and HB101 carrying pW1 expressed AF/R1 antigen whether they were grown in PAB or BHI (Table 1), but strains RDEC-1 and M5

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plasmids pW1, carrying pW1 to brush borders (3.0 ± 2.3 adherent bacteria per brush border) but reduced adherence of RDEC-1 to 0.2 ± 0.5 bacteria per brush border. This indicates that functional AF/R1 pili were expressed from pW1, even when the bacteria were grown in BHI.

When the 86-MDa plasmid from strain M5 was conjugated into DH5α carrying pW1, normal regulation by the growth medium was restored (Table 1). This suggests that a repressor is produced by the 86-MDa plasmid that acts in trans to repress AF/R1 expression by pW1. This repressor is not expressed by the DNA cloned on pW1, and the gene may not be included in the clone.

In contrast, expression of AF/R1 was suppressed by growth at a low temperature whether it was encoded by pW1 or by the 86-MDa plasmid (Table 1). This temperature effect was specific for the AF/R1 genes and was not a feature of the lac promoter from the vector since bacteria carrying pUC19 expressed the Lac+ phenotype whether they were grown at 37 or 22°C (data not shown). It is not known whether the regulation is at the level of transcription, translation, or synthesis and assembly. All known E. coli pilus systems except type 1 are similarly controlled by growth temperature (18, 25). The mechanism of the control is largely unknown, but in those pilus systems that have been studied, temperature was shown to regulate transcription (14, 22, 26), so it seems likely that the cloned DNA includes sequences that can respond to transcriptional control.

Minicells were prepared from E. coli DS410 carrying plasmids pW1 and pUC19 (23), and plasmid-encoded proteins were labeled with [35S]methionine. A prominent protein with a molecular weight of 17,000 was expressed in minicells carrying pW1 but not in minicells carrying pUC19.

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**TABLE 1. Expression of AF/R1 pili in transformants**

<table>
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<th>Strain</th>
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<td>DH5α(pW1)</td>
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*Expression was determined by slide agglutination with AF/R1-specific antiserum. The reactions were scored from 0 to +4 as described previously (27).*

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**FIG. 1.** Map of plasmid pW1. The arrow indicates the direction of transcription from the lac promoter of pUC19. Abbreviations: E, EcoRI; B, BamHI; K, KpnI. The scale at the bottom indicates kilobase pairs of cloned DNA.

**FIG. 2.** Minicell analysis of proteins expressed by pW1. Proteins in minicells carrying pW1 or pUC19 were labeled with [35S]methionine, separated by polyacrylamide gel electrophoresis, and detected by autoradiography. Proteins were labeled with [35S]methionine (1.51 Ci/mmol; Du Pont, NEN Research Products, Boston, Mass.), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose (28), and detected by autoradiography.

(An HB101 derivative), which carry the entire 86-MDa plasmid, did not express AF/R1 when grown in BHI. Likewise, growth of bacteria in BHI did not affect adherence of DH5α carrying pW1 to brush borders (3.0 ± 2.3 adherent bacteria per brush border) but reduced adherence of RDEC-1 to 0.2 ± 0.5 bacteria per brush border. This indicates that functional AF/R1 pili were expressed from pW1, even when the bacteria were grown in BHI.

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FIG. 3. DNA sequence of the left part of the cloned plasmid DNA. The deduced amino acid sequence of AfrA, the major structural subunit of AF/R1, is given. -35 and -10 indicate putative promoters that matched consensus sequences for binding of RNA polymerase to initiate transcription. RBS indicates DNA that matched the consensus sequence for ribosome-binding sites to initiate translation. Underlined segments of DNA indicate an inverted repeat that may promote a stem-loop structure. Underlined and overlined segments of DNA indicate sequences homologous to the regulatory region of Pap DNA. The three asterisks indicate a stop codon for translation into amino acids. DNA was sequenced by Pan-Data Systems, Inc. (Rockville, Md.) by the dideoxy method. Analysis was performed by using software developed by the University of Wisconsin Genetics Computer Group (12).
molecular mass of 14,401 Da. We designated this gene afrA as the major structural gene for the AF/R1 pilus. The deduced amino acid sequence is given in Fig. 3.

The amino acid sequence of the AF/R1 major structural gene revealed that it belongs to a group of pilin related to type 1 pili, as described by Klemm (18), in which every other amino acid in a region near the N terminus is conserved (this report; Cassels et al., in preparation). The DNA sequence of the entire afrA gene was 43% homologous to the DNA encoding the FimA subunit of the type 1 pilus (17) and 42% homologous to the sequence encoding the PapA pilus (4). The 42 nucleotides in the region encoding the conserved amino acids were, likewise, 43% homologous to the DNA encoding similar areas of FimA and PapA, so were no more conserved than the rest of the protein was.

Although the cloned DNA had little homology with the pap pilus operon, 20 of 33 bases beginning at base 461 (including 11 of 13 bases beginning at base 475) matched a sequence from the pap pilus operon that has been implicated as a binding site for the catabolite repressor protein that is involved in catabolite repression (2). The match for the consensus sequence for the catabolite repressor protein-binding site described by Ebright et al. (13), however, was poor. Furthermore, there is no evidence that AF/R1 expression responds to catabolite repression (W. H. Cohen, M. S. thesis, The Catholic University of America, Washington, D.C., 1986). The significance of the homology with the pap DNA is unknown, but we speculate that this region may serve as a DNA-binding site for some regulatory protein (perhaps for temperature or growth medium regulation).

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LITERATURE CITED


