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Adhesive Factor/Rabbit 2, a New Fimbrial Adhesin and a Virulence Factor from *Escherichia coli* O103, a Serogroup Enteropathogenic for Rabbits

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Enteropathogenic *Escherichia coli*-like *E. coli* strains belonging to serovar O103:K⁻:H2 and rhamnose-negative biotypes are highly pathogenic diarrhea-inducing strains for weaned European rabbits. We describe here the cloning and sequencing of the major subunit gene of a new fimbrial adhesin, adhesive factor/rabbit 2 (AF/R2), which confers on these strains the ability to attach to rabbit enterocytes and to HeLa cells in a diffuse manner and which is associated with in vivo virulence. The chromosomal operon that encodes functional AF/R2 has been cloned from strain B10. The major subunit gene *afr2G*, as well as an adjacent open reading frame, *afr2H*, has been sequenced. The Afr2G protein shows homologies with FaeG and ClpG, which are the respective major subunits of fimbrial adhesin K88 (F4) and afimbrial adhesin CS31A. Plasmid carrying the operon transcomplements an AF/R2-negative Tn*phoA* mutant for its ability to express AF/R2. As a whole, AF/R2 is a new member of the *E. coli* K88 adhesin family which is associated with virulence and which may serve in the design of vaccines.

Escherichia coli strains belonging to serovar O103:K⁻:H2 and to rhamnose-negative biovars are responsible for severe diarrheas in weaned rabbits, with considerable economical involvement in industrial fattening farms from Western Europe (4, 5). Previous data have suggested that these strains are analogous to enteropathogenic *E. coli* (EPEC) (13): (i) they adhere in vitro to rabbit ileal villi and to HeLa cells in a diffuse pattern by means of a specific adhesin (18), (ii) they induce attachment-effacement lesions in ileal enterocytes of infected rabbits (14, 21), (iii) they possess a gene that is homologous to the *eaeA* gene of EPEC and/or enterohemorrhagic *E. coli* strains (12, 22), and (iv) they do not produce Shiga-like toxins and/or do not have sequences analogous to *slt* genes (12, 16, 17). The specific adhesin, which is presumably involved in the first step of interaction between bacteria and enterocytes, enables the bacteria to attach to ileal villi of 8-day-old and 6-week-old rabbits, as well as to HeLa cells with a diffuse pattern and in a D-mannose-resistant way (18). A major component of this adhesin may be purified from surface extracts of the strain as a protein with an apparent molecular weight (MW) of 32,000 (32K). This component, as well as antibodies raised against it, inhibits adhesion of bacteria to cells in a competitive way (18). This adhesin is called adhesive factor/rabbit 2 (AF/R2) to distinguish it from AF/R1 (3), the adhesin expressed by the Rabbit Diarrheal *E. coli* 1 (RDEC-1) strain (6). Recently, we produced an AF/R2-negative Tn*phoA* mutant derived from the wild-type strain B10 (20). This mutant shows a significantly decreased pathogenicity compared to the wild-type strain when it is administered orally to weaned rabbits, indicating that AF/R2 is an important (although not unique) virulence factor. We also demonstrated that *afr2* genetic determinants are carried by the chromosome of O103:K⁻:H2 *E. coli* strains from rabbits (20).

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In this work, we describe the cloning of the whole operon that encodes AF/R2. The 32K major-subunit open reading frame (ORF), *afr2G*, as well as an adjacent ORF, *afr2H*, has been sequenced. Sequence analysis indicates that AF/R2 is a new fimbrial adhesin of *E. coli* belonging to the *fae* (K88 or F4) family.

To clone the *afr2* operon, we prepared a genomic library of DNA from the O103:K⁻:H2 and rhamnose-negative *E. coli* strain B10. Briefly, high-MW DNA was obtained from B10 by lysozyme-proteinase K-Sarkosyl extraction and then by CsCl gradient centrifugation and dialysis (10). Large overlapping fragments were obtained by partial digestion of B10 DNA with *Sau*3A (Boehringer Mannheim, Meylan, France), size fractioned by a sucrose gradient, and analyzed by pulsed-field gel electrophoresis. Fragments of 35 to 45 kb were ligated to cosmid pHC79 (Boehringer Mannheim) digested by *Bam*HI, packaged in vitro into λ phage capsids (DNA packaging kit; Boehringer Mannheim), and transduced into *E. coli* HB101. A library comprising 863 ampicillin-resistant and tetracycline-sensitive clones was grown in Penassay broth plus 50 μg of ampicillin per ml and screened for the expression of AF/R2 by dot immunoblotting with anti-32K AF/R2 major subunit antiserum (19). Eight clones were found positive by immunoblotting, and their abilities to produce the 32K AF/R2 subunit were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting). Among these, four adhered firmly and in a diffuse manner to the HeLa cell line, as tested by a technique already described elsewhere (18), suggesting that they contained the entire genetic material for active AF/R2 production. The cosmid contained in one clone, named pFMBP5, was purified and further submitted to partial digestion by *Sau*3A, ligated into *Bam*HI-digested pUC19 (Gibco-BRL, Cergy, France), and transformed into *E. coli* XL1 Blue. Transformants were selected for their white phenotype on LB agar containing 100 μg of ampicillin and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml and then screened for expression of AF/R2 as described above. Thirteen independent

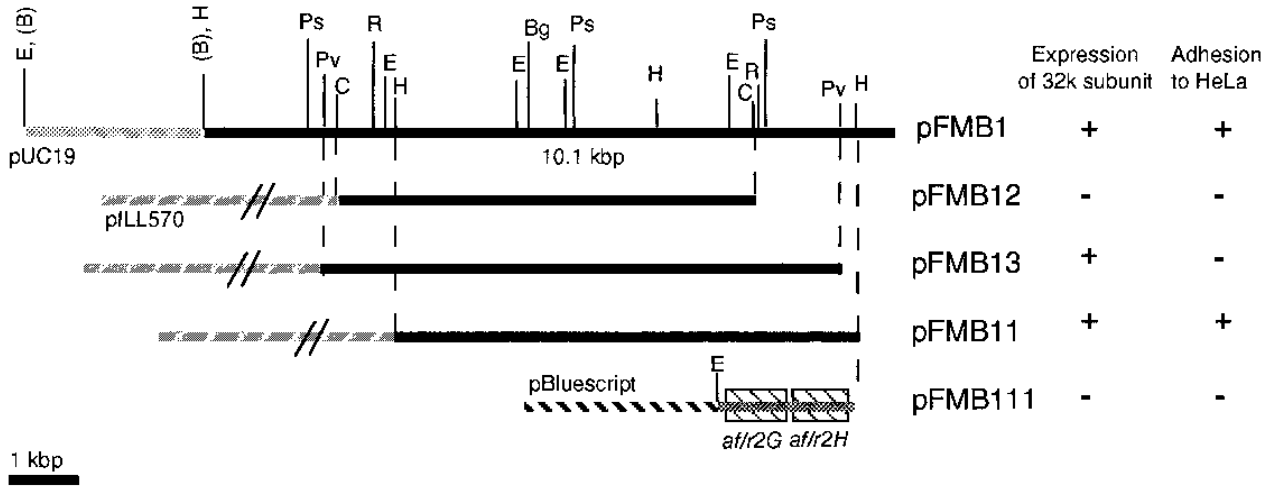

FIG. 1. Map of plasmid pFMB1 and its derivatives. Expression of the 32K AF/R2 subunit was checked in hot urea surface extracts of the strains by SDS-15% PAGE followed by Western blotting with a rabbit anti-32K antiserum (18). Adhesion to HeLa cells was tested as described elsewhere (18). Boxes in pFMB111 show location of ORFs *afr2G* and *afr2H*. The insert of pFMB111 was used as a probe to localize the Tn*phoA* insertion site in the AF/R2-negative B10/16E1 mutant (see Fig. 3). E, *Eco*RI; B, *Bam*HI; Ps, *Pst*I; Pv, *Pvu*II; C, *Cla*I; P, *Eco*RV; H, *Hin*dIII; Bg, *Bgl*II.

subclones of pFMBP5 were found to express the AF/R2 32K subunit, 11 of which adhered to HeLa cells. The sizes of inserts in these plasmids ranged from 10 to ca. 33.6 kb. The smallest plasmid that expressed functional AF/R2, named pFMB1, was selected for further studies. A restriction endonuclease map of pFMB1 is given in Fig. 1. Different fragments of the insert of pFMB1 were subcloned into the pILL570 (*Not*I) vector (a derivative of pILL570 with an extra *Not*I site and an *Eco*RI site in the polylinker [a kind gift from A. Labigne-Roussel, Institut Pasteur, Paris, France]) (11). None of the *Eco*RI fragments obtained by total or partial digestions or of the individual *Hin*dIII fragments allowed the expression of AF/R2. However,

```
EcoR1                                                                                                                afr2G
gaattcttgc gaatagcact atgcctgctc aggagtgatt ttgtgataag tcctgaaggc ttatcaattg tacaaagtct ctttaaatat tgataaaaag gaaatcttt ATG AAA AAG
                                                                                                                     Met lys lys
AAA CTA ATT GCT ATT GCT GTA GCA ACA GCT TCC GTC ATG TCT GGA GTG GCG AAT GCT GAG TCA TCT TGG GTA GAG GCA TCC ACT GGC GGG ACT ATC GAT
lys leu ile ala ile ala val ala thr ala ser val met ser gly val ala asn ala glu ser ser trp val glu ala ser thr gly gly thr ile asp
ATC GGC GGA ACT ATT GAA GTC GAT AGC CAG TAC GAC GAT CTT TGG ACG TGG AAG TTA GGG GAC GCC ATT ACG GTG GCC AGT AAT GCT GCA GAT ATG AAT
ile gly gly thr ile glu val asp ser gln tyr asp asp leu trp thr trp lys leu gly asp ala ile thr val ala ser asn ala ala asp met asn
GCA GAA AAA ACT TCG CTG ACA ATT ACT ATG GAA CAG CGT AAG CCG CTT CTG GTA GGT AAG TCT GAG CCA TTT AAA GCT CCG TCC ACG GGT GTT GGT GCA
ala glu lys thr ser leu thr ile thr met glu gln arg lys pro leu leu val gly lys ser glu pro phe lys ala pro ser thr gly val gly ala
TCG CCG AGT ATT TCT TTC AGT GAC GCT GAC GGG AAT GAA GTT GCT CTT CAA ACC CCT GCC ACC CCA AGC CAG GGC AAG GCA AGT CTG ACG TTG CCA ATT
ser pro ser ile ser phe ser asp ala asp gly asn glu val ala leu gln thr pro ala thr pro ser gln gly lys ala ser leu thr leu pro ile
AAA GAT GAA TCA AAG GCT ACT ATC GGT TCT CTG ACA TTA AAC GTC CAG GCT ATC GGT GTA ATG TAT ACA CAA AGC ACC AAT CCC TCG TAT GGT GGT CCT
lys asp glu ser lys ala thr ile gly ser leu thr leu asn val gln ala ile gly val met tyr thr gln ser thr asn pro ser tyr gly gly pro
CAT GCT ATG ACG GCT AAT GAC GTA GGG AAT ATC TTC CAT GGC GGC TTA CCT ACT TCT GCT GCC GGT CTT AAT GGG TCT ACT GCG GTA AAC ATT GTG GCC
his ala met thr ala asn asp val gly asn ile phe his gly gly leu pro thr ser ala ala gly leu asn gly ser thr ala val asn ile val ala
AGC GAT GGC GGC TGG ACT GTC ACC GAA ATG ATG AAT AAA TGG AAT GAA TTT ACT GGT CAG AAT AAG TCC TGG ATT CAG ACT GTA TAC TCT GCC GGC TCC
ser asp gly gly trp thr val thr glu met met asn lys trp asn glu phe thr gly gln asn lys ser trp ile gln thr val tyr ser ala gly ser
AGC GGT ACG ATT GAT GCG CTT TAC AAT TAC AGC CTG TCT ATC GCT CAA GGC CAA ACG CTT GAA GCT GAG TTC GAT TCA CCG GTA ACT ACG ACC ACT AAA
ser gly thr ile asp ala leu tyr asn tyr ser leu ser ile ala gln gly gln thr leu glu ala glu phe asp ser pro val thr thr thr lys
TGG AGT GCT CCG CTA ACT ATT TCA ATC GCA TAT AAC taa tttgtatgct ttagccccat tcctaatgga atggggcgtt aacggaatat tatacattga atagtattac
trp ser ala pro leu thr ile ser ile ala tyr asn ***
                                                afr2H
ccctgttaaa tgatgtgcct aaaaaaactt ttggggtgtt aaa ATG AAT GTG TTG AAA ATC TTA ATC CTA ATT TCA TCA GTT TTA TTT CCA GTT TGG CAT GTA AAG
                                                    Met asn val leu lys ile leu ile leu ile ser ser val leu phe pro val trp his val lys
GCT GAA ATA CTT GAT GGT GGT GAA ATA ATA TTC AAT GGT TTC GTT ACC GAT GAG GCA CCA AAA TGG ACA TGG CAA GTT GCT TCA CAT GAT CAA ACC TGG
ala glu ile leu asp gly gly glu ile ile phe asn gly phe val thr asp glu ala pro lys trp thr trp gln val ala ser his asp gln thr trp
AGA GTA GAT ACC GCC AAT GCG CGT ATT GAG AAC AAA GAA TTA GTG TTT AAT CTG CGC GAT AAA GGT TTT CTG CCT TTT CTT GAG GGG CAT TTA TTT GAG
arg val asp thr ala asn ala arg ile glu asn lys glu leu val phe asn leu arg asp lys gly phe leu pro phe leu glu gly his leu phe glu
ATA GCC GAA CGT GGC GGG CCT GGT TTT ACT CCG TAC ATT ACG TTC AGC AGT AAT GGA AAG CCT TTT ACG ATA CTC GAA GGG AAA GGT AGC AAT TCA CAA
ile ala glu arg gly gly pro gly phe thr pro tyr ile thr phe ser ser asn gly lys pro phe thr ile leu glu gly lys gly ser asn ser gln
CAT TTT CGC GCA ACA GTT CCG GTA AAC AAT CCT GAC AAT AAT GAA CTT ATT GGT CAG CTG GCG TTT GAT GGT GAT CAA GGT CTT GCT ATC AGT GTG GGC
his phe arg ala thr val pro val asn asn pro asp asn asn glu leu ile gly gln leu ala phe asp val asp gln gly leu ala ile ser val gly
AGT CAA GAA GAT AGT GTC GTC TTG CCA ACA GGT ATG TCA TTT ATA AGC GGA GAC AGT ATC ACC AAC GTC AAT ACA CAT AGT CTC CCA AAG GAG GTG GTT
ser gln glu asp ser val val leu pro thr gly met ser phe ile ser gly asp ser ile thr asn val asn thr his ser leu pro lys glu val val
AGC CGT CTG TCT GCA CTG TTA TTG ATG AAT AAT AAA TTT GGA AAT GGC ATG AGT TCA TCC AGT AAT GGC CAC GTA ATC AGT CAA GGT GTT CTG GCG AAT
ser arg leu ser ala leu leu leu met asn asn lys phe gly asn gly met ser ser ser ser asn gly his val ile ser gln gly val leu ala asn
GGC CAG GTT ACT AAT ATT GCT GCG GCA TAT GCA TCT AGT TTA TCA AAT TTT GAG CTT CGA TTA CCA GCG GAA AAC ACC CCG GCT CGA TGG GAA GCT AGT
gly gln val thr asn ile ala ala ala tyr ala ser ser leu ser asn phe glu leu arg leu pro ala glu asn thr pro ala arg trp glu ala ser
TTA AAT GTG ACA GTG ACT GTG CAT taa agctt
leu asn val thr val thr val his ***
                              HindIII
```

FIG. 2. DNA sequence of pFMB111. The deduced amino acid sequences of Afr2G, the major subunit of AF/R2, and of Afr2H are given. The three asterisks indicate stop codons for translation into amino acids. DNA was sequenced by Genome Express (Grenoble, France) by the dideoxy method.

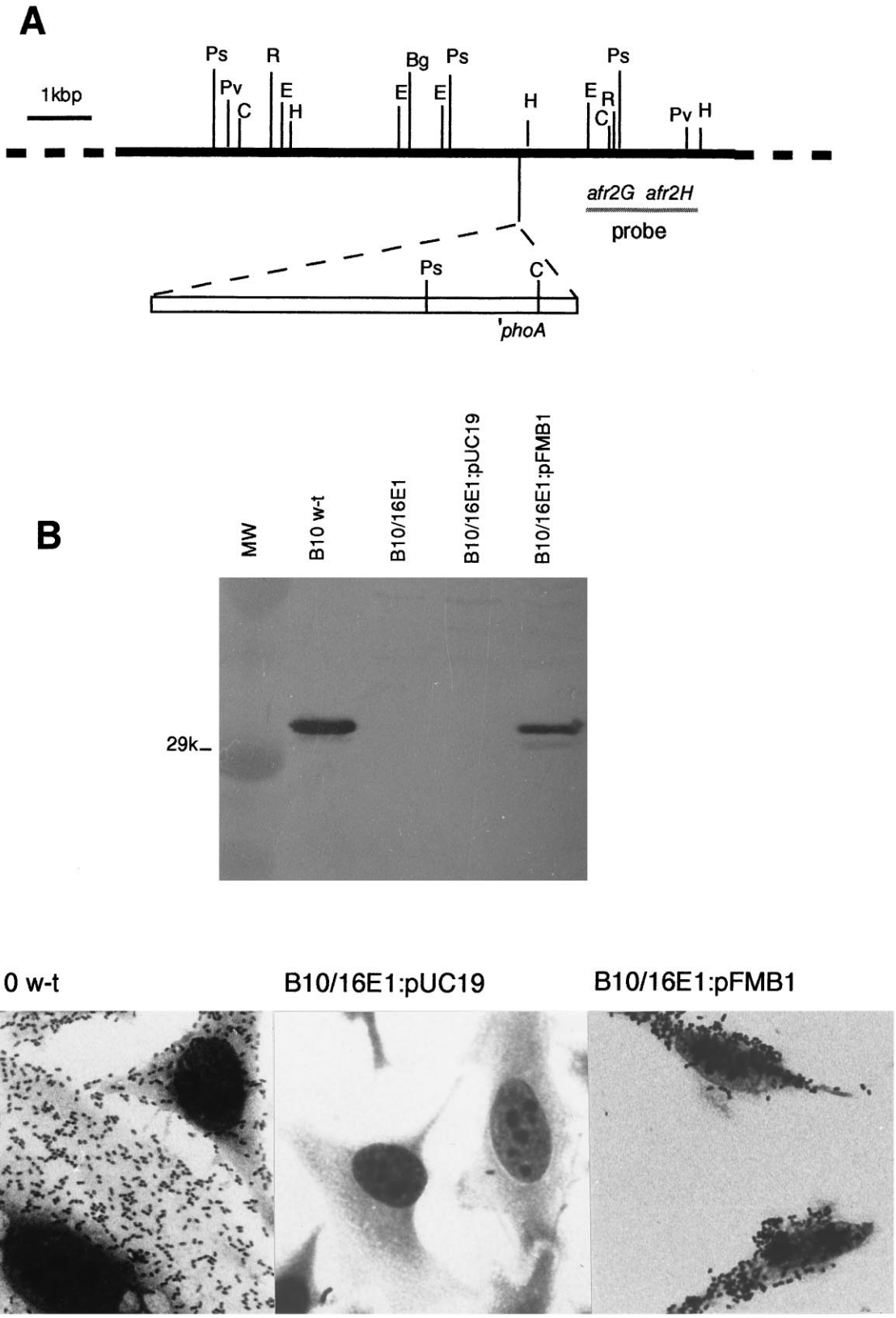

FIG. 3. (A) Insertion site of the transposon Tn*phoA* in the genome of the AF/R2-negative mutant B10/16E1 (20). To localize insertion, the *afr2GH* digoxigenin-labelled probe was used in Southern blots of digestions of B10 and B10/16E1 genomic DNA with *Eco*RI, *Hin*dIII, *Cla*I, *Eco*RV, or *Pst*I restriction endonuclease. (B) Transcomplementation of B10/16E1 with pFMB1. The plasmid pUC19 or pFMB1 was transferred into B10/16E1. Transformants were selected by resistance to ampicillin and were then tested for expression of the AF/R2 32K subunit (Afr2G) by SDS-PAGE (not shown) and immunoblotting (upper panel) and for adhesion to HeLa cells (lower panels). MW, prestained MW markers (Gibco-BRL 26041-020); w-t, wild type.

the 6.9-kb *Hin*dIII fragment obtained by partial digestion in pFMB11 permitted expression of the 32-kDa subunit as well as adhesion to HeLa cells. Interestingly, the 6.4-kbp *Cla*I fragment (pFMB12) did not allow expression of the 32K subunit or adhesion of XL1 Blue to HeLa cells, while the 8-kbp *Pvu*II fragment in pFMB13 allowed expression of the 32K subunit but not adhesion to HeLa cells (Fig. 1). This suggested that the gene encoding the major subunit was located near the right end of pFMB11. Therefore, we subcloned the 1.8-kbp *Eco*RI-*Hin*dIII fragment into pBluescript (pFMB111 [Fig. 1]), and both DNA strands were sequenced by the dideoxynucleotide technique. It reveals ORFs, the sequences of which are shown in Fig. 2. Sequence analyses and comparisons were performed on line by use of software developed by the University of Wisconsin Genetics Computer Group (7). The first ORF is 840 bp long (Fig. 2) and encodes a precursor protein of 279 amino acids, with a predicted MW of 28,984 and with a hydrophobic signal sequence of 19 amino acids, giving a mature protein of 260 amino acids with a predicted MW of 27,014. This protein shows an overall identity of 34.3% with the protein FaeG and of 35% with ClpG, which are the respective major subunits of the fimbria K88 (F4) (1, 8) and of the adhesin CS31A (9). It shares with FaeG and ClpG several features that assign them to class 3 fimbria major subunits, as proposed by Low et al.: lack of cysteine residues, penultimate tyrosine at the carboxy terminus, and four conserved prolines in aligned sequences (15). The second 780-bp ORF starts 114 bp downstream of the stop codon of *afr2G* (Fig. 2). It encodes a precursor protein of 259 amino acids, with a predicted MW of 28,232 and a signal sequence. This protein shows 63.9% identity with the proteins FaeH and ClpH, which are minor components of the adhesins cited above (2, 6a [accession no. M96152]). On the basis of these structural homologies, we called these proteins, respectively, Afr2G and Afr2H.

Recently, an AF/R2-negative mutant called B10/16E1 was produced by single insertion of Tn*phoA* into the wild-type strain B10 (20). This mutant has lost its ability to produce the 32K AF/R2 subunit and to adhere to HeLa cells. It colonizes the digestive tracts of rabbits poorly and is significantly less virulent than the B10 wild-type strain (20). The 1.8-kbp *Eco*RI-*Hin*dIII fragment that contains *afr2G* and *afr2H* ORFs was used as a probe, after labelling with digoxigenin (DIG UTP labelling kit; Boehringer Mannheim) as described by the manufacturer, to localize the Tn*phoA* insertion in the mutant B10/16E1. In B10/16E1, the transposon is inserted ca. 1.4 kb upstream from the *Cla*I-*Eco*RV-*Pst*I sequence inside *afr2G* (Fig. 3). When it was transformed by plasmid pFMB1, the ability of B10/16E1 to produce AF/R2 and to adhere to HeLa cells was restored (Fig. 3).

Based on the homologies to FaeG and ClpG, it seems very likely that Afr2G is the major component of the AF/R2 adhesin, which, once isolated from surface extract of wild-type O103 strains, migrates in SDS–15% PAGE gels with an apparent molecular mass of 32 kDa (18). The discrepancy between the apparent molecular mass of this kind of protein isolated from wild-type strains and the molecular mass predicted from the sequence is well documented. For instance, the major subunit of AF/R1 was first reported to be 19 kDa (3) but ran to 17 kDa when the operon was expressed in minicells, while the molecular mass deduced from the sequence was calculated to be 14,401 Da (23). The role of Afr2H remains putative, but it may be involved in the adhesive activity of the fimbrial structure, since its absence of production in XL1 Blue:pFMB13 (in which its ORF is truncated) correlates with an absence of adhesion to HeLa cells. The corresponding FaeH protein has been shown to be important in the biogenesis but not in the adhesive properties of the K88 fimbriae (2). As a whole, our results indicate that AF/R2 is a new member of the K88 family of *E. coli* adhesins and give new tools to study its involvement in the pathogenesis of EPEC-like strains from rabbits as well as to analyze the regulation of its synthesis and to rationally design vaccines.

Nucleotide sequence accession number. The complete sequence reported in this paper has been assigned GenBank accession no. U77302.

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