Enteropathogenic \textit{Escherichia coli}-like \textit{E. coli} strains belonging to serovar O103:K\textsuperscript{+}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 \textit{afr2G}, as well as an adjacent open reading frame, \textit{afr2H}, has been sequenced. The \textit{Afr2G} protein shows homologies with \textit{FaeG} and \textit{ClpG}, which are the respective major subunits of fimbrial adhesin K88 (F4) and afimbrial adhesin CS31A. Plasmids carrying the operon transcomplements an AF/R2-negative \textit{TnphoA} mutant for its ability to express AF/R2. As a whole, AF/R2 is a new member of the \textit{E. coli} K88 adhesin family which is associated with virulence and which may serve in the design of vaccines.

\textit{Escherichia coli} strains belonging to serovar O103:K\textsuperscript{+}H2 and to rhamnose-negative biotypes 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 \textit{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 \textit{eaeA} gene of EPEC and/or enterohemorrhagic \textit{E. coli} strains (12, 22), and (iv) they do not produce Shiga-like toxins and/or do not have sequences analogous to \textit{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 \textit{a}-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 \textit{E. coli} 1 (RDEC-1) strain (6). Recently, we produced an AF/R2-negative \textit{TnphoA} 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 \textit{afr2} genetic determinants are carried by the chromosome of O103:K\textsuperscript{+}H2 \textit{E. coli} strains from rabbits (20).

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

To clone the \textit{afr2} operon, we prepared a genomic library of DNA from the O103:K\textsuperscript{+}H2 and rhamnose-negative \textit{E. coli} strain B10. Briefly, high-MW DNA was obtained from B10 by lysosyme-proteinasic K-Sarkosyl extraction and then by CsCl gradient centrifugation and dialysis (10). Large overlapping fragments were obtained by partial digestion of B10 DNA with \textit{Sau3A} (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 phage capsids (DNA packaging kit; Boehringer Mannheim), and transduced into \textit{E. coli} HB101. A library comprising 863 ampicillin-resistant and tetracycline-sensitive clones was grown in Penassay broth plus 50 \textmu g of ampicillin per ml and screened for the expression of AF/R2 by dot immunoblotting with anti-32K AF/R2 major subunit antisem (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 cosmids contained in one clone, named pAMR5, was purified and further submitted to partial digestion by \textit{Sau3A}, ligated into \textit{BamHI}-digested pUC19 (Gibco-BRL, Cergy, France), and transformed into \textit{E. coli} XL1 Blue. Transformants were selected for their white phenotype on LB agar containing 100 \textmu g of ampicillin and 40 \textmu g of 5-bromo-4-chloro-3-indolyl-\textbeta-D-galactopyranoside (X-Gal) per ml and then screened for expression of AF/R2 as described above. Thirteen independent
subclones of pFMB5 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 (NotI) vector (a derivative of pILL570 with an extra NotI site and an EcoRI site in the polylinker [a kind gift from A. Labigne-Roussel, Institut Pasteur, Paris, France]) (11). None of the EcoRI fragments obtained by total or partial digestions or of the individual HindIII fragments allowed the expression of AF/R2. However,
FIG. 3. (A) Insertion site of the transposon TnpA1 in the genome of the AF/R2-negative mutant B10/16E1 (20). To localize insertion, the afr2G digoxigenin-labelled probe was used in Southern blots of digestions of B10 and B10/16E1 genomic DNA with EcoRI, HindIII, Clal, EcoRV, or PstI 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 HindIII 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 ClaI fragment (pFMB12) did not allow expression of the 32K subunit or adhesion of XL1 Blue to HeLa cells, while the 8-kbp PstI 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 EcoRI-HindIII 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 FacG 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 FacG and ClpG several features that assign them to class 3 fimbra 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 sequence in pathogenic strains and the molecular mass predicted from the sequence is well documented. For instance, the major subunit of K88, and F41 subunit genes. J. Bacteriol. 1981. The sequence analysis programs for the VAX. Nucleic Acids Res. 387–395.


