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K99 fimbriae of enterotoxigenic \textit{Escherichia coli} consist of eight different subunits. A major subunit called fimbrillin forms fimbrial structure and a minor subunit called adhesin localizes at the tip of fimbriae and recognizes host receptor ganglioside. Within this eight gene cluster, \textit{fanE} and \textit{fanF} have not yet been sequenced. In this study, \textit{fanE} and \textit{fanF} genes were sequenced by analyzing several DNA fragments produced by endonuclease or exonuclease digestion. The \textit{fanE} gene encoded 227 amino acids containing 20 amino acids of signal peptide starting from GTG (valine) and showed a homology to \textit{fanA-fanB}. The \textit{fanF} gene encoded 271 amino acids containing 20 amino acids of signal peptide starting from ATG (methionine) and showed homologies to the \textit{fanD} gene, fimbrillin gene of F41, adhesin gene of P fimbriae (\textit{papG}) and adhesin gene of Type 1 fimbriae (\textit{fimH}). E and F subunits had fifteen and fourteen hydrophobic domains, respectively, which periodically appeared possibly forming a hydrophobic region.

Key words: Enterotoxigenic \textit{Escherichia coli}, K99 fimbriae, \textit{fanE} and \textit{fanF} DNA sequence, hydrophobic domain.

INTRODUCTION

Enterotoxigenic \textit{Escherichia coli} possessing K99 fimbrial antigen are frequently isolated from newborn calves, piglets, and lambs suffering from diarrhea\textsuperscript{21).} It is known that fimbriae are important as an adhesive and colonization factor of bacteria on the mucosal surface of the small intestine in the first stage of infection\textsuperscript{1,5,6).} Fimbriae have been classified by their antigenicity and subunit molecular weight. The fimbriae are composed of fimbrillin which forms main part of fimbriae and adhesin which recognizes carbohydrate receptors on the surface of host cells\textsuperscript{13,23,24).}

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The K99 fimbrial gene cluster named fanA to fanH encodes eight different subunits\(^{15}\). The nucleotide sequences of fanA, fanB, fanC, fanD, fanG, and fanH have already been published\(^{16,17,19}\). The functions of these subunits were analyzed and it has been established that fanC encodes fimbrillin\(^{16}\), fanA and fanB encode regulatory proteins which control expression of fimbriae\(^{17,20}\), fanD encodes platform protein\(^{19}\) and fanG and fanH encode minor subunits\(^{18}\). However there is no available information about the gene encoding adhesin. Since it is highly possible that either fanE or fanF encodes this receptor protein, in this study, the complete gene structure of fanE and fanF were determined.

**MATERIALS AND METHODS**

*Host cells and plasmid vector:* As a host bacterial strain, JM109 or JPA101 was used, and as a vector, pUC19\(^{26}\) or BLUESCRIPT\(^{22}\) was prepared for isolation of single strand DNA. Isolation of plasmid DNA, agarose gel electrophoresis, and transformation were performed according to conventional methods\(^{22}\). Transformants were cultured in LB media or 2YT media\(^{22}\) with 50 \(\mu\)g/ml of ampicillin.

*Endonuclease and the other enzymes:* AvaI, BamHI, BglII, ClaI, HincII, KpnI, PstI, XbaI, exonuclease III, S1 nuclease, and E. coli DNA polymerase I (Klenow fragment) (TAKARA Co. Ltd., Kyoto, Japan) were used to prepare several DNA fragments from an original plasmid pFK99 (pBR322). T4 ligase (NIPPON GENE Co. Ltd., Toyama, Japan) was used for ligation.

*Sequence procedure:* Single strand DNA was prepared according to SEQUENASE protocol or Biochemical research products: Protocol and application committed to science worldwide (Promega protocol). 6% polyacrylamide gel containing 7M urea was used in the electrophoresis for sequencing. Samples for sequencing were prepared using SEQUENASE Ver. 2.0 kit (TOYOBO Co. Ltd., Osaka, Japan). pFK99 (pBR322) which contained K99 fimbrial gene cluster (fanA to fanH) were kindly supplied by Dr. de Graaf, Vrije University, Amsterdam, the Netherlands. BamHI–BamHI fragment encoding the whole K99 gene cluster of pFK99 (pBR322)\(^{25}\) was cloned into pUC19 vector (pFK99(pUC19)) and used for subcloning (Fig. 1).

*Preparation of subclones containing fanE and fanF:* Subclones including fanE and fanF genes were prepared (Fig. 1). KpnI–AvaI fragment isolated after agarose gel electrophoresis containing GENECLEAN Kit (FUNAKOSHI Co. Ltd., Tokyo, Japan) was treated with Klenow enzyme and inserted again into pUC19 cleaved with KpnI and HincII, to make pFK99KA. pFK99AH (AvaI–HincII, fragment) and pFK99KC (KpnI–ClaI fragment) were prepared by using each particular restriction enzyme site. pFK99 (KK3.3R) was prepared from pFK99 (pUC19) by using the KpnI site. Digestion of pFK99(KK3.3R) with exonuclease III was performed at 37°C and at 30 sec intervals, samples of partially digested DNA were taken according to Promega protocol. After cloning, DNA length was checked by agarose gel electrophoresis and 15
The nucleotide sequence of the genes.

subclones were selected to sequence completely.

**Analysis of hydrophobic character of products**: The hydrophobic character of the gene products was analyzed by the method described by Kyte and Doolittle\(^{10}\), using Genetyx software supplied from SDC Software Exproitation Co., Ltd. (Tokyo, Japan).

**RESULTS**

**Primary structure of fanE and fanF**: The nucleotide sequences of *fanE* and *fanF* are shown in Fig. 2 with the corresponding amino acid sequences. Each structural gene had -35 and -10 promoter sequences upstream from the ribosome-binding site (S. D.). The *fanE* product had a signal peptide of 20 amino acids and a mature molecule consisted of 207 amino acids. There are 3 restriction enzyme sites for *HincII*, *ClaI* and *AvaI*. The starting codon of *fanE*, GTG (valine) was overlapped by the stopping codon of *fanD*. Comparing fimbrial DNA sequences, *fanE* had homology to *fanA-fanB* (44.5%). The *fanF* product however, had a signal peptide of 20 amino acids and a mature protein consisted of 251 amino acids. There are 2 restriction enzyme sites for *Sau3AI* and *XmnI*. The starting region of *fanF* was also overlapped by the terminal region of *fanE* as observed at the *fanD-fanE* region. *fanF* showed homologies to *fanD* (45.7%), *papG* (47.8%) of P fimbriae, *fimH* (44.5%) of Type 1 fimbriae, and F41 fimbriae (45.1%). It is known that *papG* and *fimH* encode adhesin subunits of P and Type 1 fimbriae, respectively.

![Nucleotide and amino acid sequences of *fanE* and *fanF*](image-url)
The nucleotide sequence of the genes.

CCT GCA GCT GCA AAG GGA CAT GGG GGT AGT CTG TCA CTG GCA ATT AAC GGA GTA AAA TTA ATA TAT GCA GCA ATT GCT
Pro Pro Ala Pro Lys Gly Asp Arg Glu Ser Leu Ser Leu Ala Ile Asn Asn Arg Val Lys Leu Ile Tyr Arg Pro Ile Ala
90 95 100 105 110 115

AvaL

CTA AAA ATT GCT GGA GAT GAG GCA GAA AAT ATT AAC CTG ATT AAC TTG GGC AAG GAT TCT GTC CTT GAA ATT ACA AGG
Leu Lys Asn Gly Arg Asp Glu Ala Glu Asn Asn Ile Lys Leu Ile Asn Ser Gly Thr Asp Ser Cys Leu Glu Asn Thr Thr
120 125 130 135 140

CCA TAT TAC TTT GCA ATT AGT GAT GTT AAA ATT GCC AAA TCA ATT GAT TTA ATT TCT GAT GCA AAA ATT AAG ATG GGA
Pro Tyr Tyr Phe Ala Ile Ser Asp Val Lys Ile Asn Gly Val Asn Ser Ile Asp Ala Lys Asn Lys Met Gly
145 150 155 160 165

GTA TIC TCC CCA TIC TCG GAA AAT GCA ATT ACT AGT GGA MA ATC ACG GTA ACA GCA TTT ATT GAC TAT
Val Phe Ser Pro Pro Ser Lys Val Cys Leu Gly Asn Val Asn Thr Ser Gly Asn Ile Thr Val Ala Phe Asn Asp Tyr
170 175 180 185 190 195

-35

GTT GCC GAA ACC AGC TAC ACT GTT GCA AAG AGT AAA TA ATG AAA ATT AAA TAT TAT TTA TTA TTA TTA CTG TGC
Gly Val Ala Thr Ser Tyr Thr Val Gin Arg Ser Leu Leu Leu Leu Leu Leu Leu Leu
200 205

S. D.

-10

Gly Val Ala Thr Ser Tyr Thr Val Gin Arg Ser Leu Leu Leu Leu Leu
-20 -15 -10

TGT TAT GGA GAT GGG CTC GCA GCA TCC ACA GAG AAA CTC AAA ATT TCA CTT GCT TAT AAT GGC CAT ACT TAT TCA TTT
Cys Tyr Gly Asp Val Ala Leu Ala Ala Cys Thr Gly Lys Leu Lys Ile Ser Pro Gly Tyr Ser Gly His Thr Tyr Ser Phe
-5 0 5 10 15 20

GAT TCC AGT ATT CCA ATT AAT AGT ATA GCA AAG ATC TCA CTT GCT ATT TAA ATT GAT TGG GAG CAG
Asp Ser Ser Ile Pro Asn Asn Ser Asn Ile Ala Arg Tyr Leu Val Glu Ile Ser Gly Lys Ile Val Cys Asp Ala Asp Gin
25 30 35 40 45

TCA GGC TGG GAT GGC AAA GCT TAT GCT CAA TTA CAT TAT TCA TCA GGG GCC TGA TTA GAT GTC AGT GAT GGC TCA
Ser Gly Trp Asp Gly Tyr Ala Gin Leu His Leu Tyr Ser Ser Gly Ala Leu Cys Gin Ser Val Ser Gly Asp Gin
50 55 60 65 70 75

ATT ACA TGY AGC CTA AAT GGG TCG CTG TCA TCG CTG TCT GCC TGA TCC AGC ACA TAC CAC TGG GCA GCC CAA ATA
Ile Thr Phe Arg Ser Asn Val Ser Gly Leu Ser Trp Arg Phe Pro Gly Asp Ile Pro Tyr His Cys Ala Alg Gly Gin Ile
80 85 90 95 100

AAT CTT GCC GAT ATT TTT AGG GAT AGA AAT GGT AAA TTA ACC TCG ATT GGT GAA CTA GCA CAT GAA ATA TTT TTA
Asp Leu Gly Ile Lys Tyr Ala Asp Arg Asn Gly Lys Val Thr Trp Asn Pro Gly Leu Arg His Glu Ile Phe Leu
105 110 115 120 125
Characterization of E and F subunits: According to computer analysis, the E subunit had partial homologies to the B, C, and D subunits of K99 fimbriae in its amino acid Sequence. The F subunit was partially homologous to the G adhesin subunit of P fimbriae, C and H subunits of K99 fimbriae. The hydrophobic character of these proteins are shown in Fig. 3. Fifteen and fourteen hydrophobic domains appeared in the E and F subunits, respectively. Particularly notable are the hydrophobic peaks of fanE which periodically appeared.

DISCUSSION

Various kinds of fimbrial genes were cloned from *Escherichia coli* or other bacterial cells. Among them, fimbrial genes of P2,3,4,11,12,14 and Type 17,8,9 were well
The nucleotide sequence of the genes.

Fig. 3. Hydrophobic character of E and F subunits. Signal peptide is removed at a position indicated by an arrow. Hydrophobic peaks are indicated by boxes.
analyzed. It is known that the fimbrial gene and amino acid sequence of the products have some homologies.

Roosendaal et al. have reported on the K99 gene cluster and indicated that \( \text{fanC} \) encodes fimbriillin of K99 antigen (16.5 KDa)\(^{16}\). The A subunit (11.0 KDa)\(^{17}\), B subunit (10.8 KDa)\(^{17}\), D subunit (84.5 KDa)\(^{19}\), G subunit (16.9 KDa)\(^{18}\), and H subunit (16.3 KDa)\(^{18}\) have been reported to be homologous in their nucleotide sequences. In the analysis of hydrophobicity, the periodic appearance of hydrophobic domain was detected.

In this study, \( \text{fanE} \) and \( \text{fanF} \) were sequenced for the first time. The starting codon of \( \text{fanE} \) was GTG, coding for valine. This starting codon is unique among the fimbrial genes of which nucleotide sequences have been reported. It was found that \( \text{fanD}, \text{fanE}, \) and \( \text{fanF} \) overlapped each other in the structural gene regions. A similar overlapping was reported in \( \text{fanG} \) and \( \text{fanH} \) of K99 fimbriae\(^{18}\). In addition, the \( \text{fanF} \) had homologies to the genes encoding adhesin of P and Type 1 fimbriae. In contrast, \( \text{fanE} \) showed a homology only to the \( \text{fanA-fanB} \) connecting region. Thus, the E subunit seems a very unique subunit within the K99 fimbriae. Regarding the hydrophobic character of \( \text{fanE} \) and \( \text{fanF} \), fifteen and fourteen peaks were detected, respectively. In addition, the peaks of \( \text{fanE} \) appeared periodically, indicating that those formed the hydrophobic part of the E subunit.

At present it is not known which gene encodes K99 adhesin. However, it was found that the \( \text{fanF} \) had some homology to the adhesin gene of other fimbriae. Further analysis using mutants lacking \( \text{fanE} \) and \( \text{fanF} \) is necessary to determine the function of these subunits. Such experiments are now in progress.

REFERENCES


The nucleotide sequence of the genes.


