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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}-\textit{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 (pUC19). 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 (TOYBO 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 using GENECLEAN\(^{\text{TM}}\) 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\textsuperscript{10}, using Genetyx software supplied from SDC Software Exproitation Co., Ltd. (Tokyo, Japan).

RESULTS

Primary structure of \textit{fanE} and \textit{fanF}: The nucleotide sequences of \textit{fanE} and \textit{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 \textit{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 \textit{HincII}, \textit{ClaI} and \textit{ AvaI}. The starting codon of \textit{fanE}, GTG (valine) was overlapped by the stopping codon of \textit{fanD}. Comparing fimbrial DNA sequences, \textit{fanE} had homology to \textit{fanA–fanB} (44.5%). The \textit{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 \textit{Sau3AI} and \textit{XmnI}. The starting region of \textit{fanF} was also overlapped by the terminal region of \textit{fanE} as observed at the \textit{fanD–fanE} region. \textit{fanF} showed homologies to \textit{fanD} (45.7%), \textit{papG} (47.8%) of \textit{P} fimbriae, \textit{fimH} (44.5%) of Type 1 fimbriae, and F41 fimbriae (45.1%). It is known that \textit{papG} and \textit{fimH} encode adhesin subunits of \textit{P} and Type 1 fimbriae, respectively.
The nucleotide sequence of the genes.

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CCT GCA GCT GCA AMG GGA GAT GGC GGT AGT CTG TCA CTG GCA ATT AAT GGA GTA AAA TTA ATA TAT GCA CGA 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  100 110 115

Ava
CTA AAA ATT GGT GCA GAT GAG GCA GAA AAT ATT AAG CTG ATT AGG GAT TCC GCT TCC GAA ATT ACA AGG
  Leu Lys Asn Gly Arg Asp 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

-35
CCA TAT TAC TTT GCA ATT AGT GAT GTT AAA ATT GGC AAA TCA ATT GAT TTA ATT TCT GAT GCA AAA AAT AGG GGA
  Pro Tyr Tyr Ala Ile Ser Asp Val Lys Ile Asn Gly Asp Arg Ala Asn Ser Asp Ala Gly Asn Lys Met Gly Met
  145 150 155 160 165

-10 S.D.
GAC GCA ACC AGC TAC ACT GTT GCA AAG AGT AAA TA ATG AAA AMT AAA TAT TAA TTA TTA TTA TTA TTA TTC TGT
  Gly Val Ala Thr Ser Tyr Thr Val Glu Arg Ser Lys
  170 175 180 185 190 195

fanF
GTC GAA GCA ACC AGC TAC ACT GTT GCA AAG AGT AAA TA ATG AAA AMT AAA TAT TAA TTA TTA TTA TTA TTA TTC TGT
  Gly Val Ala Thr Ser Tyr Thr Val Glu Arg Ser Lys
  200 205

-20 -15 -10
TGT TAT GGA GAT GTG GGC CTG GCA GCA TGC ACA GCG AAA CTG AAA ATC TCA CTT GGT TAT AGT GGCA ACT TAT TCA TTT
  Cys Tyr Gly Asp Val Ala Leu Ala Ala Cys Thr Gly Leu Lys Ile Ser Pro Gly Tyr Ser Gly His Thr Tyr Ser Phe Ser
  -5  1  5 10 15 20

GAT TCC AGT ATT CCA AAT AAT AGT ATT AAT GCA AAG AAG TAC CTG ATG GAG GAA ATT GTT TCT GAT GGA ATT ATT TAT
  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 GGT AAA GGT TAT GCT GAA TTA CAT TAT TCA TCA GGT GCC TTA TGA GCC AGT GTC AGT GGA GAT GGC
  Ser Gly Trp Asp Gly Lys Arg Tyr Ala Gln Leu His Leu Tyr Ser Ser Gly Ala Leu Cys Gin Ser Val Ser Gly Asp Gly
  50  55  60  65  70  75

ATT ACA TTT AGG TCA AAT GIG TGG CTG TCA TGG TCT TGG GCC AAT GGC ATA GCA TAC CAC TGT GCA GGC CAA ATA
  Ile Thr Phe Arg Ser Asn Val Ser Gly Leu Ser Trp Arg Phe Pro Asn Gly Ile Pro Tyr His Cys Ala Gly Gin Ile
  80  85  90  95 100

AAT CTA GCC GCT ATA AAA TAT GGC GAT AGA AAT GGT AAA GTT ACC TGG AAT CCT GGT GAA CTA GGA 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
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Fig. 2. Primary sequence of \textit{fanE} and \textit{fanF} genes. An arrow shows a position cleaved by digestion with signal peptidase I. –35 and –10 indicate promoter sequences. S. D. means ribosome-binding site. Stopping codon is indicated by ***.

\textbf{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 \textit{fanE} which periodically appeared.

\textbf{DISCUSSION}

Various kinds of fimbrial genes were cloned from \textit{Escherichia coli} or other bacterial cells. Among them, fimbrial genes of K2,3,4,11,12,14 and Type 17,8,9 were well
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 *fanC* encodes fimbrillin 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, *fanE* and *fanF* were sequenced for the first time. The starting codon of *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 *fanD*, *fanE*, and *fanF* overlapped each other in the structural gene regions. A similar overlapping was reported in *fanG* and *fanH* of K99 fimbriae\(^{18}\). In addition, the *fanF* had homologies to the genes encoding adhesin of P and Type 1 fimbriae. In contrast, *fanE* showed a homology only to the *fanA-fanB* connecting region. Thus, the E subunit seems a very unique subunit within the K99 fimbriae. Regarding the hydrophobic character of *fanE* and *fanF*, fifteen and fourteen peaks were detected, respectively. In addition, the peaks of *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 *fanF* had some homology to the adhesin gene of other fimbriae. Further analysis using mutants lacking *fanE* and *fanF* is necessary to determine the function of these subunits. Such experiments are now in progress.

**REFERENCES**


The nucleotide sequence of the genes.


