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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. The nucleotide sequences of fanA, fanB, fanC, fanD, fanG, and fanH have already been published. The functions of these subunits were analyzed and it has been established that fanC encodes fimbrillin, fanA and fanB encode regulatory proteins which control expression of fimbriae, fanD encodes platform protein and fanG and fanH encode minor subunits. 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, pHUC19 or BLUESCRIPT was prepared for isolation of single strand DNA. Isolation of plasmid DNA, agarose gel electrophoresis, and transformation were performed according to conventional methods. Transformants were cultured in LB media or 2YT media with 50 μg/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. 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) was cloned into pUC19 vector 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 GENE CLEAN 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\(^1\), using Genetyx software supplied from SDC Software Exploitation Co., Ltd. (Tokyo, Japan).

RESULTS

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

CCT CCA GCT GCA AMG GGA GAT GGG GGT AGT CTG TCA CTG GCA ATT AAT GGA GTA AAA TTA ATA TAT GCA CCA 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 CCA GAT GAG GCA GAA AAT ATT AAG CTG ATT AAT GAT TGT CTT GAC GTA AAA ATT GAA ACC GAG
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 GGC AAA TCA ATT GAT TTA ATT TCT GAT GCA AAA ATT AMG ATG GGA
Pro Tyr Tyr Phe Ala Ile Ser Asp Val Lys Ile Asn Gly Ser Ile Asp Leu Asn Ser Asp Ala Lys Asn Lys Met Gly
145 150 155 160 165 170 175 180 185 190 195

GTA TIC TCC CCA TCG AAA GTT CTT CTC GGA AAT GTA AAT ACT AGT GCA AAC ATC AGG GTA ACA GCA TTT ATT GAC TAT
Val Phe Ser Pro Phe Ser Lys Val Cys Leu Gly Asn Val Asn Thr Ser Gly Asn Ile Thr Val Thr Ala Phe Asn Asp Tyr
200 205 210 215 220 225

-35

GGC GTT GCA ACC AGC TAC ACT GTT GCA AGG ACT AAA ATG AAA ATT AAA TAT TAT TTA TTA TTT TTT TTT TTT CTG
Gly Val Ala Thr Ser Tyr Thr Val Gin Arg Ser Lys
230 235 240 245 250 255

-10 S. D.

Gly Val Ala Thr Ser Tyr Thr Val Gin Arg Ser Lys
260 265

Met Lys Asn Lys Tyr Asn Leu Leu Phe Leu Phe Leu Leu
-20 -15 -10

-5 TIS 10 15 20

TGT TAT GGA GAT GGT GGG CTG GCA GCA TGC ACA GAC AAA ATC TCA CCT GCT TAT AGT 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
1 5 10 15 20

GAT TGC AGT ATT CCA AAT AGT ATT AAT GCA AGA ACA TAC CTT GTC GTA ATT TCT GAG AAA ATT GTT GTT GAT GGG GAC CAG
Asp Ser Ser Ile Pro Asn Asn Ser Asn Ile Ala Arg Tyr Leu Val Ile Ser Glu Ser 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 CAT TAT TCA TCA CCT GGT GCC TTA TCT GAT GAC ACT GGA GAT GGG
Ser Gly Trp Asp Gly Lys Arg Tyr Ala Gin Leu His Leu Tyr Ser Ser Gly Asp Asp Ala Leu Cys Gin Ser Val Ser Gly Asp Gin
50 55 60 65 70 75

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

AAT CTT GCC GGT ATA AAA TAT GCG GAT AGA AAT GTT AAA ATT ACC TGC AAT CCT GGT GCA GTA CTA GAT GAA ATT TTT TTA
Asn Leu Gly Gly Ile Lys Tyr Ala Asp Arg Asn Gly Lys Val Thr Trp Asn Pro Gly Leu Arg His Gly 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 K2,5,11,12,14 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.


