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Expression of the Gene Cluster Associated with the *Escherichia coli* Pilus Adhesin K99

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The biogenesis of the pilus adhesin K99 is dependent on the expression of eight contiguous genes, *fanA* to *fanH*. Transposon mutants were prepared by using Tn*lacZ* and Tn*phoA*, and selected transposon mutants were used to measure expression of each K99 gene. Expression of the K99 genes is likely controlled at the transcription level, since in general, there were no differences between the results obtained with the two transposons. *fanC* was the most highly expressed, and *fanD* was expressed at very low levels. The expression of Tn*lacZ* fusions in *fanA* and *fanB* fusions was high. Deletion of *fanA*, *fanB*, and part of *fanC* abolished the expression of *fanD* but had no effect on the distal genes *fanE* to *fanH*. To locate the DNA regions required for expression of *fanE* to *fanH*, deletion mutations were prepared and the effects on expression of *fanE* to *fanH* were determined. The deletion of a segment between *fanD* and *fanE* abolished *fanE* and *fanF* expression but did not affect *fanG* and *fanH*. The deletion of a portion of *fanF* (approximately 1 kb proximal to *fanG*) abolished the expression of *fanG* and *fanH*. These results indicate the presence of regulatory elements proximal to *fanE* and to *fanG*. Putative promoters were identified in these regions by DNA homology and by primer extension. A stem-loop structure that may act as a transcriptional attenuator of *fanF* was also found at the beginning of *fanF*. These data confirm our previous model of K99 transcriptional organization.

Enterotoxigenic *Escherichia coli* (ETEC) strains are an important cause of acute diarrhea in humans and young livestock animals (9, 22, 33). To cause disease, ETEC strains must colonize the mucosal surface of the small intestine and produce enterotoxins (5, 27). Colonization is facilitated by attachment to epithelial cells of the small intestine. Specific pilus adhesins on the bacterial surface are responsible for this attachment by interacting with specific receptors on the surface of epithelial cells. K99 is one pilus adhesin that mediates the attachment of ETEC strains to small intestines of neonatal calves, lambs, and piglets (14).

The genes encoding the biosynthesis of the K99 adhesin are present on an 87.8-kb nonconjugative plasmid (13). The K99 genes have been cloned and shown to reside on a 7.1-kb *Bam*HI fragment (7, 30). This fragment encodes eight gene products (*fanA* to *fanH*), and each is required for the biosynthesis of K99 (2, 7, 24, 30).

Previous studies have demonstrated that K99 expression is dependent on a variety of factors, including growth rate (31), growth phase (12), temperature (8), alanine (12), cyclic AMP (cAMP) and the cAMP receptor protein (CRP) (11, 12), and the leucine-responsive protein Lrp (4). These observations are consistent with the hypothesis that K99 is highly regulated. Since K99 must be expressed in vivo for disease to occur, its regulation is related to virulence. Our previous studies using Northern (RNA) blot analyses have shown that the transcriptional organization of K99 is complex. The data are consistent with the hypothesis that the K99 genes are divided into three separately regulated gene clusters, regions I to III (11). Using a transcription vector system and in vitro transcription, Roosendaal et al. (23) detected a promoter proximal to the 5′ end of *fanA* and a second promoter at the 5′ end of *fanB*. They presumed that these promoters were responsible for the transcription of *fanA*, *fanB*, *fanC*, and *fanD*, the region I genes. They also identified a strong transcription terminator between *fanC* and *fanD*. The results from our Northern blots suggest that additional promoters exist in other regions of the K99 genes and are responsible for the transcription of *fanE* to *fanH* (11).

In this study, we used gene fusions, deletion analysis, primer extension, and DNA sequence analysis to investigate K99 gene expression, to map the locations of the regulatory elements required for expression, and to corroborate the organization of the K99 genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. The bacterial strains used are listed in Table 1. The eight K99 genes (*fanA* to *fanH*) originate from ETEC strain B41 and were cloned in the vector pBR322 (13). Plasmid pIX12-1 was constructed from pIX12 by deletion of the 1.8-kb *Bam*HI and *Pvu*II fragment containing *fanA*, *fanB*, and the 5′ end of *fanC*. Bacteriophage λ::Tn*phoA′*-1 (λ::Tn*lacZ*), generated by Wilmes-Riesenberg et al. (32), was used to construct Tn*lacZ* fusions in K99 genes. Bacteriophage λ*c*I857 *b*221 *Pam3 rex*::Tn*phoA* (λ::Tn*phoA*) described by Manoil and Beckwith (19) was used to construct Tn*phoA* fusions in K99 genes.

Media. Growth of bacteria for the selection of transformants, transductants, and measurement of reporter gene activity was in LB (20). Terrific broth (17) was used to grow bacteria for preparation of plasmids. Selection of Tn*lacZ* mutants was on LB agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 20 μg/ml), and selection of Tn*phoA* mutants was on LB agar containing 5-bromo-4-chloro-3-indolyl phosphate (XP; 40 μg/ml).

Isolation of Tn*lacZ* and Tn*phoA* insertions in K99 genes. Lysates containing λ::Tn*lacZ* or λ::Tn*phoA* were prepared by using suppressor-positive *E. coli* LE392 or BW11397. *E. coli* KS300 containing pIX12 or pIX12-1 was grown in LB containing 10 mM Mg_2SO_4 and 50 μg of ampicillin per ml for 4 h. *E. coli* KS300 is a *phoA* and *lacZ* mutant. Lysates containing λ::Tn*lacZ* or λ::Tn*phoA* were added at multiplicities of approximately 1 and incubated at 30°C for 15 min. The cultures were plated onto LB agar containing ampicillin (50 μg/ml), kanamycin (300 μg/ml), and X-Gal (20 μg/ml) or XP (40 μg/ml) and incubated at 30°C for 48 h. The colonies were pooled and grown in LB for 7 h at 37°C, and plasmid DNA was prepared from the pool. Approximately 1 μg of plasmid was used to transform $CaCl_2$-treated KS300 cells (6). Transformants were selected by plating on LB agar containing kanamycin (30 μg/ml), ampicillin (50 μg/ml), and X-Gal (20 μg/ml) or XP (40 μg/ml). After incubation at 37°C for 24 h, cells producing white and blue colonies were picked. Plasmids were isolated from the cells, and

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TABLE 1. *E. coli* K-12 strains used

Strain	Genotype	Source or reference
KS300	F⁻ *galE galK* Δ*lac* (c74) *rpsL thi* Δ*phoA-pvuII rec-1* Str^r	K. Strauch
LE392	*hsdR514*(r_K^- m_K^-) *supE44 supF58 lacY galK2 galT22 metB1 trp55 mcrA* Δ*lacU169 proC*::Tn*5*	3
I297	*lac his trp proC phe* Nal^r, containing pIX12	
BW11397	Δ*lac-169* Δ*phoA8 creB510 supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1 tonA*	32
CA7900	F⁻ *thi crp*	J. Beckwith

the locations of the transposon insertions were mapped by using restriction endonucleases.

Isolation of plasmids. For restriction endonuclease mapping, plasmids were isolated by the method of Lee and Rasheed (17). Promega minipreparations (Promega, Madison, Wis.) and cesium chloride-ethidium bromide density gradient centrifugation (1) were used to prepare plasmid DNA for cloning, sequencing, and production of deletions. Plasmid DNA was introduced into *E. coli* strains by the $CaCl_2$ transformation method (6) or by electroporation (Invitrogen Co., San Diego, Calif.). As necessary, DNA molecules were purified by gel electrophoresis using low-melting-point agarose.

DNA sequencing. Nucleotide sequences were determined by the dideoxy-chain termination method, using Sequenase version 2.0 (United States Biochemical, Cleveland, Ohio). Primers (TCCCGACATGTATTA and AGATGGAAGGA TTAGC) were synthesized by the University of Illinois Biotechnology Center. The analysis of DNA sequences was performed with the following software: DNASIS-Mac version 2.0 (Hitachi, San Bruno, Calif.), Mac Targ search (Carnegie Mellon University, Pittsburgh, Pa.), and LOOPS (Dnastar).

Construction of deletions with exonuclease III. Restriction endonucleases, T4 DNA ligase, and S1 nuclease were used as instructed by the manufacturer (GIBCO-BRL, Gaithersburg, Md.). Plasmid DNA was digested with the restriction endonuclease *Spe*I or *Mlu*I to generate exonuclease III-sensitive 5′ overhanging ends. The DNA was then redigested with *Apa*I or *Kpn*I to generate exonuclease-resistant 3′ overhanging ends. *E. coli* exonuclease III (Pharmacia, Piscataway, N.J.) was added to the DNA. At various times, S1 nuclease was added to create blunt ends. These samples were mixed with T4 DNA ligase and incubated at room temperature for 2 h, and the ligated DNA was transformed into *E. coli* KS300. The extent of each deletion was determined by DNA sequencing.

Assays of β-galactosidase and alkaline phosphatase. Cells were grown to mid-logarithmic phase in LB. β-Galactosidase activity was assayed by hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (20). Alkaline phosphatase activity was assayed by measuring the rate of *p*-nitrophenyl phosphate (2 mg/ml; Sigma, St. Louis, Mo.) hydrolysis by permeabilized cells (21). One unit of activity was defined as one nanomole of substrate hydrolyzed per minute.

Primer extension analysis. RNA was purified from *E. coli* KS300 containing pIX12 by centrifugation through CsCl, using the procedure of Kenney et al. (16). DNA primers were end labeled by using 75 μCi of [γ-^{32}P]ATP (>5,000 Ci/mmol) and phage T4 polynucleotide kinase (GIBCO-BRL). The labeled primers were purified by using Bio-Spin 6 chromatography columns (BIO-RAD, Hercules, Calif.). Approximately 0.1 pmol of labeled primer was added to 10 μg of total RNA in 10-μl reaction volumes containing 0.05 M Tris-Cl (pH 8.3) and 0.1 M KCl. Reaction mixtures were incubated at 70°C for 10 min and then on ice for 3 min to allow annealing of primer to template. One microliter of 10 mM each dATP, dCTP, dGTP, and dTTP and 2 μl of reverse transcriptase buffer (0.25 M Tris HCl [pH 8.3], 0.2 M KCl, 0.036 M magnesium acetate, 0.01 M dithiothreitol) were added to the annealed reaction mixes along with 1 μl (3 U) of avian myeloblastosis virus reverse transcriptase (GIBCO-BRL). Reaction mixes were incubated at 50°C for 60 min, and then 5 μl of Sequenase stop buffer (United States Biochemical) was added to terminate the reactions. The primer extension mixtures were subjected to electrophoresis in 7% standard polyacrylamide gels followed by autoradiography.

RESULTS

Tn*lacZ* fusions. To investigate the expression of K99 genes, *lacZ* was inserted in each of the *fan* genes by using the transposon Tn*lacZ* and used as a reporter of gene activity. Plasmid pIX12 was mutagenized with Tn*lacZ*, and the sites of insertions were determined by restriction endonuclease mapping. Tn*lacZ* fusions in each of the *fan* genes were isolated, and the degree of expression of the fusions was determined by measuring β-galactosidase activity (Fig. 1). Since *lacZ* forms operon fusions by transposition (32), the enzyme activities represent transcriptional expression of each K99 gene. Of the fusions exhibiting reporter activity, *fanC* was the most highly expressed of the *fan* genes and the expression of the *fanD* fusion was very low. *fanA*, *-B*, *-E* and *-G* fusions each were expressed at relatively high levels. Expression of the *fanF* and *fanH* fusions was relatively low but was greater than expression of the *fanD* fusion. Interestingly, expression of some of the Tn*lacZ* fusions was dependent on their location in the *fan* genes. For example, expression of a *lacZ* fusion located at the 3′ end of *fanD* was three times greater than that of two other fusions in *fanD* and was similar to the expression of *fanE* (Fig. 1). One interpretation of this result is that a regulatory element for *fanE* may be present in *fanD* proximal to this insertion. Likewise, a fusion in the middle of *fanF* was more highly expressed than the fusions close to the 5′ end of *fanF* (Fig. 1).

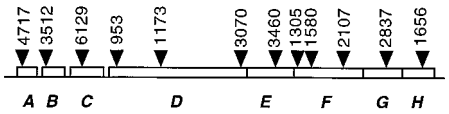

FIG. 1. β-Galactosidase activities of Tn*lacZ* fusions in *fan* genes in pIX12. Triangles indicate the locations of Tn*lacZ* insertions, and numbers represent units of β-galactosidase activity (average of at least five measurements).

Tn*phoA* fusions. Analyses using Tn*phoA* measure not only transcription but also translation and protein export (19). Thus, differences between gene activities measured by using Tn*lacZ* and Tn*phoA* should represent posttranscriptional events. To determine if posttranscriptional events modulate K99 expression, Tn*phoA* fusions were isolated, and expression was compared with the results obtained with Tn*lacZ*. Tn*phoA* insertions in *fanA*, *-C*, *-D*, *-E*, *-F*, and *-G* were identified, and the degree of expression of each *fan* gene was determined by measuring alkaline phosphatase activity. The activities and locations of representative fusions in the K99 genes are shown in Fig. 2a. Overall, the relative patterns of expression of the Tn*phoA* fusions were similar to those of the Tn*lacZ* fusions. Since *phoA* fusion proteins must be exported to yield active alkaline phosphatase, it was concluded that the gene products of *fanC*, *-D*, *-E*, *-F*, and *-G* were exported proteins. This conclusion is consistent with the results obtained by using minicells

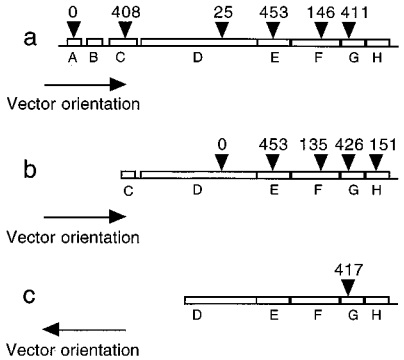

FIG. 2. Alkaline phosphatase activities of Tn*phoA* fusions in *fan* genes. (a) Fusions in pIX12; (b) fusions in pIX12-1 (*fanA*, *-B*, and *-C* deleted); (c) fusion in *fanG* with the direction of the vector opposite the direction of *fan* gene expression. Triangles indicate the locations of Tn*phoA* insertions, and numbers represent units of alkaline phosphatase activity (average of at least four measurements). Arrows indicate the direction of expression.

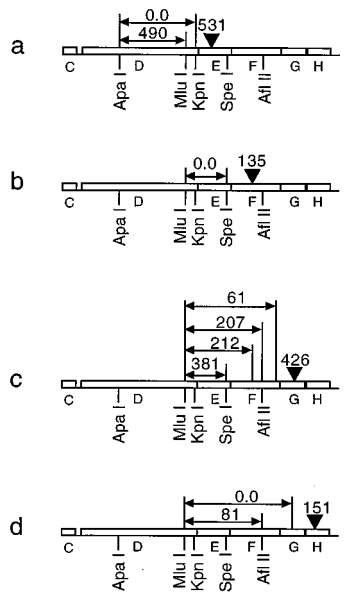

FIG. 3. Alkaline phosphatase activities of various deletions of pIX12-1. Tn*phoA* insertions are in *fanE* (a), *fanF* (b), *fanG* (c), and *fanH* (d). Arrows indicate the deletions. Numbers above the arrows represent units of alkaline phosphatase activity when the site was deleted; numbers above the triangles represent units of activity without deletions.

and DNA sequence analysis (2, 25, 26, 28, 29). As was the case with Tn*lacZ*, expression of the *fanD* fusion was very low. *fanC*, *-E*, and *-G* fusions were the most highly expressed, and expression of the *fanF* fusion was relatively low. Alkaline phosphatase activity was not detected in any of the *fanA* fusions isolated (five fusions were screened). This result was expected since the product of *fanA* has not been found in periplasm or total membrane preparations, and thus this product is not considered an exported protein (25, 28, 29).

Deletion analysis. To identify DNA regions required for expression of the various K99 genes, several deletion mutations were created. We used both Tn*phoA* and Tn*lacZ* fusions for this purpose. Initially a fragment containing *fanA*, *fanB*, and the 5′ end of *fanC* was deleted by removal of the *Bam*HI and *Pvu*II restriction fragments, yielding plasmid pIX12-1. Tn*phoA* insertions in *fanD*, *-E*, *-F*, *-G*, and *-H* were selected, and the expression of each of these genes was measured by assaying alkaline phosphatase activity (Fig. 2b). Fifteen fusions were found in *fanD*. None of them were expressed, indicating that *fanD* expression requires the region containing *fanA*, *-B*, and *-C*. However, expression of the *fanE*, *-F*, and *-G* fusions was the same in this deletion mutant as in pIX12, demonstrating that these genes were expressed independently of *fanA*, *-B*, and *-C*.

It is unlikely that the expression of *fanE*, *-F*, or *-G* was dependent on a vector-borne promoter because the strong terminator located between *fanC* and *fanD* was retained in pIX12-1. However, to verify this conclusion, the K99 DNA fragment from pIX12-1 containing Tn*phoA* in *fanG* was enzymatically cleaved with the restriction endonucleases *Bam*HI and *Eco*RV and religated. Clones in the opposite orientation were selected after restriction mapping. The change of orientation did not alter the expression of *fanG* (Fig. 2c). Therefore, it was concluded that the expression of *fanE* to *fanH* was dependent on K99-specific regulatory elements.

To define the regions required for the expression of *fanE* to *fanH*, additional deletions of pIX12-1 containing Tn*phoA* insertions in *fanE*, *-F*, *-G*, and *-H* were created. Deletion of the *Apa*I-*Mlu*I fragment in *fanD* (Fig. 3a) did not affect the expression of the *fanE* fusion. Therefore, this deleted region is not important for the expression of *fanE*. However, after sequential digestion with *Mlu*I and exonuclease III, deletion of the sequence adjacent to the 5′ end of *fanE* completely abolished *fanE* expression (Fig. 3a). This result suggests that a site between the *Mlu*I site and the 5′ end of *fanE* must contain a *cis*-acting regulatory element for the transcription of *fanE*. Expression of *fanF* was abolished when the *Mlu*I-*Spe*I restriction fragment spanning a region in *fanD-fanE* was removed (Fig. 3b). Since this deletion partially overlaps the exonuclease III-derived deletion that abolished the expression of *fanE*, it was assumed that both *fanE* and *fanF* were transcribed from the same regulatory element.

Expression of the *fanG* fusion was not reduced when almost all of *fanE* was removed (the *Mlu*I-*Spe*I restriction fragment). However, when additional regions within *fanF* were deleted by using exonuclease III, expression of the *fanG* fusion was decreased (Fig. 3c). Therefore, it was assumed that a transcriptional element for *fanG* was located within *fanF*. The reduction of *fanG* activity correlated with the removal of DNA approximately 1 kb from the 5′ end of *fanG*. The activity of *fanG* was sequentially reduced as the deleted region approached the 5′ end of *fanG*. *fanH* probably is expressed from the same regulatory region responsible for the expression of *fanG*, since there was a twofold decrease in expression of *fanH* when the *Kpn*I-*Afl*II fragment was deleted (Fig. 3d). No *fanH* was expressed when *fanF* was completely removed along with a portion of *fanG*.

Expression of the Tn*phoA* fusion in *fanD* was abolished when *fanA*, *-B*, and *-C* were deleted (Fig. 2b). One obvious explanation for this result is that the transcription of *fanD* originates from within the deleted region. However, it is possible that a protein(s) from the deleted region could be involved in the export of FanD. To clarify this possibility, Tn*lacZ* fusions in *fanD* were selected in pIX12-1, which lacked *fanA*, *fanB*, and part of *fanC*. Expression of the two *fanD* fusions was decreased approximately 10-fold (Fig. 4a). Since the Tn*lacZ* fusions measure only transcriptional events, this result indicated that the *fanA-fanC* region is involved in the transcription of *fanD*. Tn*lacZ* fusions in *fanE* to *fanG* also were selected, and the activities were the same as for similar fusions in pIX12, confirming that *fanE* to *fanH* do not require the *fanA-fanC* region for expression.

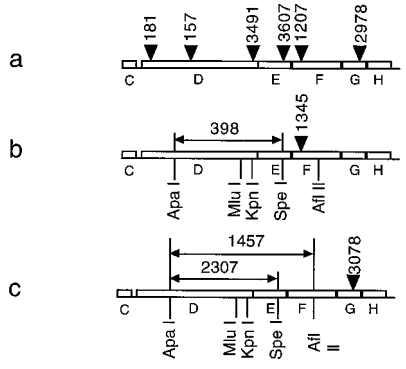

FIG. 4. β-Galactosidase activities of Tn*lacZ* fusions in *fan* genes in pIX12-1 (a) and various deletions (b and c). Triangles indicate the locations of Tn*lacZ* insertions, and numbers above the triangles represent units of β-galactosidase activity (average of at least five measurements). Arrows indicate the deletions, and numbers above the arrows represent units of β-galactosidase activity for the deletion mutants.

To confirm that some of the deleted regions identified by using the Tn*phoA* fusions in *fanE* to *fanH* were involved in transcription of these genes, deletions made by using the Tn*lacZ* fusions in the same genes were prepared. The overall activities of the deletions in Tn*lacZ* fusions were similar to those in Tn*phoA* fusions, confirming that the regulatory elements identified by using Tn*phoA* affected transcription. Deletion of the *Apa*I-*Spe*I restriction fragment in *fanD* and *fanE*, which was found by Tn*phoA* deletion analysis to contain the putative regulatory region for the expression of *fanF*, resulted in a 3.5-fold reduction of *fanF* expression (Fig. 4b). Using the *fanG* fusion, we prepared two deletions: the region between the *Apa*I site in *fanD* and the *Spe*I site in *fanE* and the region between the *Apa*I and *Afl*II sites in *fanF* (Fig. 4c). The expression of *fanG* was only slightly decreased by deleting the *Apa*I-*Spe*I restriction fragment, which demonstrates that *fanG* can be expressed regardless of whether the regulatory element for the expression of *fanE* and *fanF* is present. However, deletion of the *Apa*I-*Afl*II restriction fragment caused a large decrease of *fanG* expression.

Complementation. There is a possibility that the various regions deleted from pIX12 encode *trans*-acting factors required for the expression of K99 genes, which would explain why some deletions resulted in altered downstream activities. To clarify this, several constructs were prepared to determine if complementation in *trans* restored K99 gene expression. *fanA* to *fanH* were cloned from pIX12 into the compatible vector pACYC184, generating pIX15. A second plasmid containing *fanA* to *fanD* in pACYC184 also was constructed (pIX35). pIX15 was introduced into each of the deletion mutants described in Fig. 2 and 4, and pIX35 was independently introduced into the deletion mutant described in Fig. 2. When *fanA* to *fanC* were deleted from pIX12, neither pIX15 nor pIX35 restored expression of *fanD*. Similarly, pIX15 did not restore the expression of *fanF* when the putative regulatory sequence for *fanF* was removed, nor did it restore expression of *fanG* when the putative regulatory sequence for *fanG* was removed.

Mapping of transcription initiation of *fanE* to *fanH*. The deletion analysis (Fig. 3a, 3b, and 4b) indicated that the DNA region between the *Mlu*I site and 5′ end of *fanE* contains a regulatory element for the expression of *fanE* and *fanF*. The DNA sequence of this region was analyzed for the presence of putative regulatory elements, using commercially available software. A putative promoter and CRP binding site was found in this region. To determine if this promoter was active, primer extension was performed with a labeled primer within *fanE* (FEPEI). An initiation site was identified 17 bp upstream from the initiation codon (GUG) of *fanE* (Fig. 5). The −10 and −35 sequences of the promoter fit well with the *E. coli* consensus sequences (Fig. 6) (10) but were positioned slightly downstream of the putative promoter identified by the computer search.

The data described above suggest that the region in *fanF* regulates expression of *fanG* and *fanH* (Fig. 3c, 3d, and 4c). A computer search identified five to six putative promoters in this region. Using the primers FGPEI to FGPEIV, we identified three transcription initiation sites for *fanG* and *fanH* genes at 311, 541, and 980 bp downstream from the initiation codon of *fanF* (Fig. 7). Of the three transcripts, the one initiated at 311 bp appeared to be present in the highest concentration. The promoter regions did not match exactly the putative promoters identified by the computer search, but each was found in close proximity to the putative promoters. A possible stem-loop structure that could serve as an attenuator of *fanF* expression also was found at the beginning of *fanF* (Fig. 8).

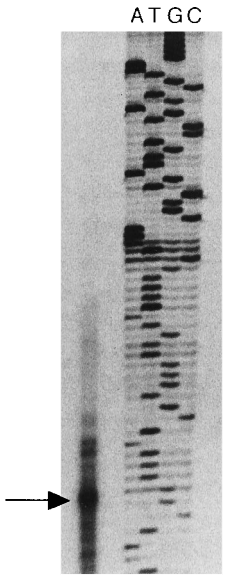

FIG. 5. Results of primer extension experiments to detect transcription initiation sites for *fanE* and *fanF*. The initiation site is indicated with an arrow. The two primers used were AAACATCACGGTAACAGCA and GAATGTTTC TATTTTACCAGT.

DISCUSSION

Expression of each of the genes in the cluster associated with the *E. coli* pilus adhesin K99 was measured by using LacZ and PhoA as reporters of gene expression. The transposons Tn*lacZ* and Tn*phoA* were used to insert the reporter genes into the K99 genes. The *lacZ* fusions measured transcription only, while the *phoA* fusions measured transcription, translation, and protein export. In general, there were no differences between the results obtained with the two transposons. *fanC* was the most highly expressed of the K99 genes, and *fanD* was expressed the least. These results are consistent with results obtained with minicells (25, 28, 29) and Northern blots, in which case *fanC*, the major pilin gene, was highly expressed and *fanD* was not (11). It is known that a transcription terminator is located between *fanC* and *fanD* (23), and this probably accounts for the variable expression of *fanC* and *fanD*, even though the two genes are transcribed from the same promoters. *fanE* and *fanG* were the next most highly expressed K99 genes. *fanF* and *fanH* were expressed at lower levels than these genes. There is a stem-loop structure located between *fanE* and *fanF*, and this structure probably is responsible for the decreased expression of *fanF* relative to *fanE*. No such structure was detected between *fanG* and *fanH* or anywhere else in the K99 genes (except for the known attenuator between *fanC* and *fanD*).

Expression of *fanA* and *fanB* was detected only with the *lacZ* fusions, since the products of these genes are not exported proteins (25, 28, 29). Transcriptional expression of *fanA* and *fanB* is high. Using a *fanA* gene probe in Northern blotting, we have recently found that the amount of *fanA*-specific mRNA is roughly equivalent to the amount of *fanC*-specific mRNA (unpublished result). This finding is consistent with the results of assays described here using *lacZ* fusions; i.e., these two genes are expressed at similar levels. However, previous studies using minicells demonstrated that FanA and FanB are produced in small amounts (24). The reason for this discrepancy is unknown but may result from the processing and degradation of

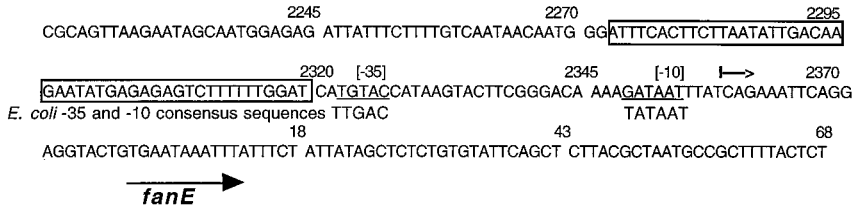

FIG. 6. Nucleotide sequence of the region upstream of *fanE* and the location (⟶) of the transcriptional initiation site as measured by primer extension. The −10 and −35 sequences are underlined. The *E. coli* −10 and −35 consensus sequences (10) also are indicated. Boxed areas correspond to a putative promoter identified by computer search.

the *fanA* and *fanB* mRNAs (15) or other translational control mechanisms.

To identify DNA regions required for expression of the various K99 genes, a variety of deletions in the Tn*phoA* and Tn*lacZ* fusions were created and their effects on gene expression were measured. The deletion of *fanA*, *fanB*, and part of *fanC* resulted in the loss of *fanD* expression. The loss of expression could not be reversed by complementation in *trans* using *fanA* to *fanD* or the entire K99 gene cluster (*fanA* to *fanH*). Previously, Roosendaal et al. (23) identified promoters proximal to the 5′ end of *fanA* and proximal to the 5′ end of *fanB*. These promoters are likely responsible for the expression of *fanA* to *fanD*. However, this deletion did not alter the expression of *fanE*, *-F*, *-G*, and *-H*. These results indicated that unlike *fanD*, *fanE* to *fanH* were expressed independently of this deleted region. Since altering the orientation of the vector did not change the expression of these genes, it was concluded that *fanE* to *fanH* were expressed by their own regulatory element(s). Additional deletions were selected to localize the regulatory regions required for the expression of *fanE* to *fanH*. Deletion of a DNA fragment from the *Mlu*I site in *fanD* to a location just proximal to *fanE* totally abolished the expression of *fanE*, whereas deletion of the fragment bounded by *Bam*HI near (*fanA*) and *Mlu*I did not alter *fanE* expression. These results suggested that a regulatory element for *fanE* expression was positioned in the region between the *Mlu*I site and the 5′ end of *fanE* (Fig. 3a). As was observed with the *fanA* to *fanD* gene cluster, expression of *fanE* could not be restored by complementation in *trans* with the entire K99 gene cluster. Expression of one of the *lacZ* fusions located in this region was three times greater than expression of two other fusions in *fanD* located closer to the 5′ end and was similar to the expression of *fanE*. This result supports our interpretation that a promoter for *fanE* is located in this region. The DNA sequence of this region was analyzed by computer, using Mac Targ Search, and a putative promoter sequence was found. By using primer extension, the presence of a promoter in this area was confirmed. The promoter for *fanE* is located 17 bp from the initiation codon of *fanE*. A putative CRP binding site also was adjacent to this promoter. Since *fanE* (region II gene) is dependent upon the CRP-cAMP complex (11), the presence of a CRP binding site was expected and strengthens the argument that this promoter is responsible for the expression of *fanE*. While sequences that match the *E. coli* −10 and −35 promoter

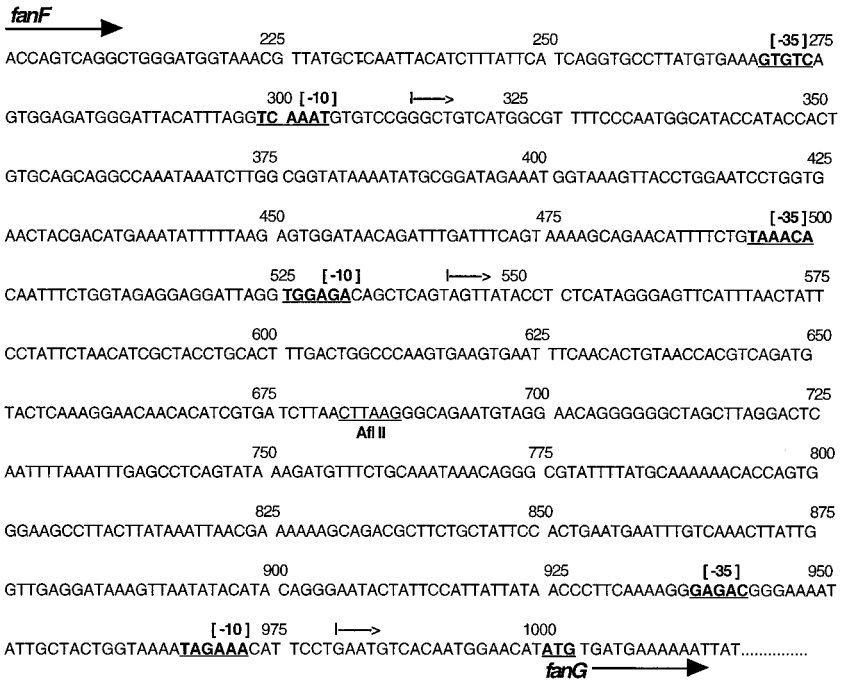

FIG. 7. Nucleotide sequence of the *fanG* upstream regions and the location (⟶) of transcriptional initiation sites determined by primer extension. The four primers used were TGAATACACAGAGAGCTATAATAGAAATAA, ACTTGTTATTGCTTTATATAATTTTTTCATC, GATTTATTTGGCCTGCTGCACAGTGGTA TG, and TTAATTTATAAGTAAGGCTTCCACTGGTGT.

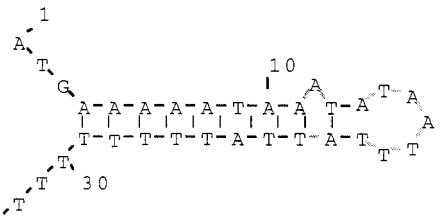

FIG. 8. The stem-loop structure that may be formed in the mRNA at the beginning of *fanF*. The nucleotide numbers indicate positions in *fanF*.

consensus sequences were found adjacent to the transcriptional initiation site, the spacing between the two elements is 3 bp longer than in the consensus sequence. It is likely that the same promoter is responsible for *fanF* expression, since the expression of *fanF* was abolished when a similar site was removed.

Deletion of the *fanE-fanF* regulatory sequence did not affect expression of *fanG* and *fanH*. On the other hand, when portions of *fanF* were deleted, the expression of *fanG* was decreased. The sequential deletion of DNA from approximately 1 kb distal to the start of *fanF* through the 3′ end of *fanF* caused decreased expression of *fanG* which could not be restored by complementation with the entire K99 gene cluster. One interpretation of this effect is that *fanG* transcription is initiated from several different promoters. Several putative promoters were found in *fanF* by a computer search. Using primer extension analysis, we found three potential transcription initiation sites in *fanF*. While mRNA degradation or premature termination of extension could also lead to this result, the proximity to putative promoters identified by the computer search suggested that those sites are important transcription start sites. These results are consistent with the hypothesis that transcription of *fanG* is initiated from multiple promoters within *fanF*. Expression from multiple promoters is not unique. Other bacterial genes are known to be transcribed from multiple promoter elements. For example, the *E. coli dam* gene utilizes multiple promoters (18, 34). The −10 and −35 regions show greater divergence from the *E. coli* consensus sequences compared with the promoters for *fanE* and *fanF*. Furthermore, the putative −10 and −35 elements are 24 to 25 bp apart, which suggests that if these are indeed the promoters for *fanG* and *fanH*, they may not be recognized by σ^{70}. It is likely that *fanH* is also expressed from the same regulatory elements in the *fanF* region, since the expression of *fanH* was decreased by the deletion of the *fanF* region.

The deletions described decreased the expression of some K99 genes and were used to help identify potential regulatory elements. Since the effects on transcription could be due to the requirement of *trans*-acting factors encoded within the deleted regions, as described above, wild-type sequences were introduced into strains with the deletions to determine if expression could be restored. Complementation did not restore the expression of any of the deletions, which suggests that *trans*-acting factors required for the expression of *fanE* and *fanF* or *fanG* and *fanH* are not encoded within the deleted areas, and thus these sequences are *cis* active.

The data presented here confirm our previous model derived from Northern blot analysis that K99 genes can be divided into three separately regulated gene clusters: region I, encoding *fanA* to *fanD*; region II, encoding *fanE* and *fanF*; and region III, encoding *fanG* and *fanH* (11). K99 expression is shown to be regulated by the global regulators cAMP-CRP and Lrp. Lrp and CRP-cAMP serve as positive regulators of region I, while only CRP-cAMP is required for expression of region II genes. The region III genes are independent of both global regulators (reference 11 and unpublished result). Deletion analysis confirmed that the regulatory elements of region I are not required for the expression of region II and III genes. Conversely, the expression of region I is not dependent on region II or III, since the expression of *fanC* was not altered when region II and III genes were removed (data not shown).

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