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Two novel genes, *fanA* and *fanB*, involved in the biogenesis of K99 fimbriae

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ABSTRACT

The nucleotide sequence of the region located transcriptionally upstream of the K99 fimbrial subunit gene (*fanC*) was determined. Several putative transcription signals and two open reading frames, designated *fanA* and *fanB*, became apparent. Frameshift mutations in *fanA* and *fanB* reduced K99 fimbriae expression 8-fold and 16-fold, respectively. Complementation of the mutants *in trans* restored the K99 expression to about 75% of the wild type level, indicating that *fanA* and *fanB* code for transacting polypeptides involved in the biogenesis of K99 fimbriae. The *fanA* and *fanB* gene products FanA and FanB were not detectable in minicell preparations, indicating that both polypeptides are synthesized in very small amounts. However, in an *in vitro* DNA directed translation system FanA and FanB could be identified. The deduced amino acid sequences of FanA and FanB showed that both polypeptides contain no signal peptides, indicating a cytoplasmic location. Furthermore, the polypeptides are very hydrophilic, mainly basic, and exhibit remarkable homology to each other and to a regulatory protein (papB) encoded by the *pap*-operon (1). Some of these features are characteristics of nucleic acid binding proteins, which suggests that FanA and FanB have a regulatory function in the synthesis of FanC and the auxiliary polypeptides FanD-H.

INTRODUCTION

Expression of K99 fimbriae on enterotoxigenic *E. coli* strains is subject to several regulation mechanisms. It is affected by growth rate and pH (2), the presence of alanine (3), and temperature (4). Strains harboring the recombinant plasmid pFK99, which contains the K99 genetic determinant (5,6), also exhibit the effect of temperature and alanine on the expression of K99 fimbriae. At present, it is not known whether in these strains the expression of K99 fimbriae is also affected by growth rate and/or pH.

Expression of K99 fimbriae from pFK99 is under control of its natural promoter (6). Thus, at least part of the regulatory sequences for the expression of K99 fimbriae are contained within pFK99. The location of the K99 fimbrial subunit gene (*fanC*, fan: fimbrial adhesin K ninety-nine) on pFK99 and its nucleotide sequence have been determined (7). It was anticipated that the region upstream of *fanC* contains sequences and/or genes, which regulate the expression of *fanC*. Characterization of this region was initiated with the determination of the nucleotide sequence. Several putative regulatory sequences and two open reading frames, allowing the synthesis of two 11 Kd polypeptides, became apparent. In this paper the

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nucleotide sequence of the regulatory region is presented, together with the analysis of the two putative genes.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli K12 C600 (*thr leu*-6 *thi*-1 *SupE*44 *lacY*1 *tonA*21), HB101 (8), and JM101 (9) were used as bacterial hosts. Minicells were isolated from *E. coli* K12 DS410 (10). The plasmid analyzed was pFK99 (5,6). The M13mp10 cloning vector was used for DNA sequencing (11).

Culture conditions

For isolation of pFK99, bacteria were grown in Trypticase Soy Broth (BBL, Microbiology Systems). Strain JM101 was grown in YT medium (5 g NaCl, 5 g yeast extract, and 8 g bactotryptone in 1 l). For isolation of minicells, determination of K99 fimbriae expression and heamagglutination, bacteria were grown in Minca medium (12), supplemented with 0.2% yeast extract. Cells, containing derivatives of pBR322, were cultured in the presence of ampicillin (100 μg/ml).

DNA manipulations

Plasmid isolation, DNA gel electrophoresis, transformations, and enzymatic manipulations of DNA were carried out by standard methods (13). SacI linkers (Boehringer Mannheim) were inserted, according to the instructions of the supplier. DNA restriction fragments were extracted from agarose slab gels by electroelution using an electrophoretic sample concentrator (ISCO).

DNA sequence determination

For DNA sequence determination the dideoxy-termination procedure of Sanger et al. (14) was used, combined with the M13 cloning system (9).

Digestion of DNA fragments with exonuclease BAL 31

Since the available restriction sites in the region upstream from *fanC* were not sufficient to determine the nucleotide sequence of both strands completely, appropriate restriction fragments were treated with BAL31 exonuclease. DNA fragments were resuspended in 300 μl BAL31 reaction buffer, containing 12 mM $CaCl_2$, 12 mM $MgCl_2$, 200 mM NaCl, and 1 mM EDTA in 20 mM Tris HCl-buffer, pH 8.1. Two μl of a freshly diluted BAL 31 solution (0.5 U/ml, Bethesda Research Laboratories) was added and samples of 60 μl were taken at 2, 4, 6 and 10 min. After phenol extraction and ethanol precipitation, the DNA was ready for ligation.

Construction of mutant plasmids

1. pFK99 (*fanA*$^-$). pFK99 was partially digested with HincII in the presence of ethidium bromide (15). Linear plasmid DNA was isolated and an 8 basepairs SacI-linker was inserted in the restriction site. After religation and transformation, the clone containing the desired insertion in *fanA* was identified by HincII digestion..

2. pFK99 (*fanB*$^-$). A 1500 basepairs BamHI/BglII fragment (Fig. 1), containing *fanA*, *fanB*

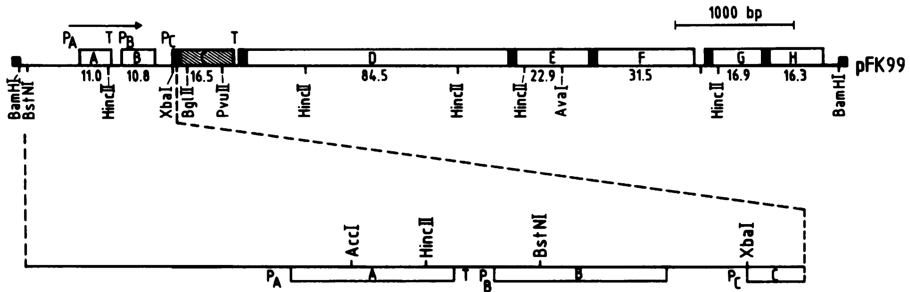

Fig. 1. Genetic map of pFK99. Blocks indicate the position and size of structural genes. Dark blocks at the left part of the genes indicate signal peptides. The numbers refer to the molecular mass of the gene product in kilodaltons (Kd). P and T refer to putative promoters and terminators of transcription. The arrow indicates the direction of transcription. The region upstream *fanC* is shown in detail. bp: basepairs.

and part of *fanC*, was cloned in M13mp10. ssDNA of this clone was used for synthetic oligonucleotide mutagenesis (see below). The HincII/XbaI fragment, containing *fanB*⁻ was isolated and used to replace the HincII/Xba fragment from pFK99. Therefore, pFK99 was partially digested with HincII, followed by a complete digestion with XbaI. The proper mutant was screened by labeled primer hybridization, followed by HincII digestion of mini DNA preparations.

3. pACYC (*fanA*) and pACYC (*fanB*). A 900 basepairs BstNI fragment, containing *fanA*, and a 560 basepairs HincII/XbaI fragment containing *fanB*, were treated with T4 DNA-polymerase (Boehringer) to create blunt ends. pACYC184 was digested with HincII and subsequently treated with alkaline phosphatase (CIAP, Boehringer). By blunt end ligation the BstNI and HincII/XbaI fragments were inserted in pACYC184.

4. pINIIIA (*fanB*). A 560 basepairs HincII/XbaI fragment containing *fanB*, and the pINIIIA vector (16) digested with BamHI, were treated with T4 DNA-polymerase. Subsequently, the HincII/XbaI fragment was inserted in the pINIIIA vector by blunt end ligation. The proper orientation of *fanB*, with respect to the vector promoter, was determined by digestion of plasmid DNA preparations of the obtained recombinants.

Synthetic oligonucleotide directed mutagenesis (Mutation in *fanB*)

Single stranded DNA (ssDNA) from the clone containing the BamHI/BglII fragment of pFK99 (Fig. 1) was isolated, using the procedure of Sanger et al. (9). In addition, to prevent extensive self-priming of RNA, the phage preparation was treated with RNase (free of DNase) for 15 min. A 14-mer synthetic oligonucleotide, complementary to position 965-979 (Fig. 2), with a 1 basepair-deletion at position 972, was obtained from Pharmacia Nederland B.V., Woerden. For mutagenesis the double primer approach without selection was used. Ten pmol M13 universal primer and 10 pmol mutagenic primer were kinased and, after annealling to 1 µg ssDNA in 20 µl, extended, using T4 DNA-polymerase and T4 DNA-ligase (Pharmacia).

After transformation to strain JM101, the mutant was selected by colony screening, using the mutagenic oligonucleotide for hybridization. The HincII/XbaI DNA fragment (Fig. 1), containing the one basepair deletion, was used for further studies and therefore sequenced completely. The DNA showed the desired deletion, while no other alterations, compared to the authentic fragment, were observed.

SDS-polyacrylamide gelelectrophoresis and autoradiography

Linear 14 to 20% gradient gels (24 cm) were prepared and autoradiographed, as described by Mooi et al. (17), except that the gels were treated with Amplify (Amersham Radiochemical centre) prior to autoradiography.

In vitro DNA-directed translation

For in vitro DNA-directed protein synthesis, a bacterial cell-free coupled transcription/ translation system (18, 19) was obtained from Amersham International. The polypeptides were labeled with ^{35}S-methionine and analyzed on SDS-polyacrylamide gels.

Isolation and labeling of *E. coli* minicells

Minicells were isolated and labeled with ^{3}H-amino acids, as described by Mooi et al. (17).

Enzyme-linked immunosorbent assay

K99 fimbriae in ultrasonic extracts and heat shock preparations from overnight grown cultures (37°C) were detected and quantitated by an enzyme-linked immunosorbent assay (ELISA), as described by Mooi et al. (20), using disposable polystyrene microtiter trays (Cooke). The trays were read with a micro ELISA reader (Organon Teknika) at 492 nm.

RESULTS

From the nucleotide sequence of the region located upstream of *fanC* two open reading frames and several putative regulatory sequences became apparent (Fig. 2). Translation of the first reading frame (*fanA*) initiates at position 546 and terminates at position 827, giving rise to a polypeptide with a molecular mass of 11.0 Kd. *FanA* is preceded by a sequence that shows homology to the consensus sequence of a ribosome binding site (Fig. 2) (21). Translation of the second reading frame (*fanB*) might initiate at three positions, being 901, 925 and 940. The reading frame terminates at position 1200. No discrimination can be made between the three initiation sites, because the N-terminal amino acid sequence of *fanB* gene product (FanB) has not been elucidated and all three initiation codons are preceded by sequences that show only poor homology to the consensus sequence for ribosome binding. Furthermore, none of the initiation codons is masked in a region with dyad symmetry. The largest reading frame (initiating at position 901) allows the synthesis of a polypeptide with a molecular mass of 11.7 Kd. The complementary strand of the sequence shown in Fig. 2 revealed an open reading frame between position 240 and 440. However, no suitable translation initiation codon in this reading frame was observed and therefore it will not be considered as a putative gene.

A sequence showing strong homology to the consensus sequence for *E. coli* promoters was

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            -10                              -35
GGCGCGTAGCATGCTAACAGAATAACACATCCCCGTCAATACGGTTCTCGCTGGACGCTT
BstNI     90        100       110       120       130       140

ACAGAAGTGAGGCGATTATGATGCTCTTGATAAAATTAGATTTGATTAAAACAGTGACTG
          150       160       170       180       190       200

TTGATTGCTGAAAAGTTAGCTGATTGGGGTTTTTATATTTTTTTTGTCTTTGTAACAAAG
          210       220       230       240       250       260

ATTGGTGTTTTTTGTATCTAAAGCCATACAAAAAACTAACAAAAAACTAACAATTAAAAC
          270       280       290       300       310       320

AATTTGAAATTTGAAGTAATGGTTTGAATTTGTTGTTTTTTGTGGCGTTTTTTGTTGTGA
          330       340       350       360       370       380

ATTTGATTTTTTAGCATAAAACTCTGGTTCTTCTTGGCTGTTTATTTTTTTTTTCTATAT
          390       400       410       420       430       440

GTTCAGTGTGTTATTTATACTCTTCCCTTTATTTTTGTTTTTTTTATGCCATATAATTCA
          450       460       470       480       490       500

 -35                     -10           S.D.     fanA →
                                               MET ARG SER PHE ASN
ATCAGCAGAGATGATTGGGATCATAAAAATGTCACTTGAGGGTATATGCGATCTTTTAAT
          510       520       530       540       550       560

LYS ASP GLU TYR LEU PHE ARG GLU LYS LEU GLY TYR LEU VAL LYS GLY MET VAL LYS ALA
AAAGATGAATACTTGTTCAGGGAGAAACTTGGTTATCTTGTGAAAGGAATGGTTAAAGCA
          570       580       590       600       610       620

ARG CYS PHE GLN LEU LEU VAL GLU LEU SER SER ILE ARG SER SER ARG GLY ILE TYR ALA
AGGTGCTTCCAATTATTAGTGGAGTTATCAAGTATACGTAGTTCTAGGGGTATTTACGCC
          630       640       650       660       670       680

LEU GLU ASP TYR PHE VAL LYS GLY ARG ASP LEU LYS GLU VAL CYS ASN GLU HIS ASP ILE
CTAGAAGATTACTTTGTTAAAGGCAGAGATTTAAAAGAAGTATGTAATGAGCATGATATA
          690       700       710       720       730       740

THR PRO SER TYR VAL THR SER LEU LEU ARG LYS LEU GLN GLU VAL ASN HIS LYS VAL ILE
ACACCATCATATGTCACTAGTTTATTGCGTAAGTTACAGGAAGTTAACCACAAAGTTATT
          750       760       770       780  HincII 790       800

ASP ILE LEU PRO TYR TYR ARG ASN ***                   -35
GATATTCTTCCGTATTATAGAAATTAAATTTAATTACATTTTATAACCCTGACAAAAACA
          810       820       830       840       850       860

         -10                       S.D.     fanB →
                                           MET TYR ILE ASP ASN TYR LEU
GGTGGATATTTATTATTGAATTTTTGTGTCAGTGTAGTTAATGTATATTGATAACTATTT
          870       880       890       900       910       920

ILE MET ARG LYS ILE ASN MET GLU GLU THR ILE LEU SER LEU THR SER TYR GLN LEU ARG
AATTATGCGTAAGATAAATATGGAAGAAACGATACTTTCCTTAACTTCTTATCAACTCCG
          930       940       950       960       970       980

PRO GLY LYS VAL ASP LYS LYS GLN PHE VAL LEU LEU ILE ASP VAL SER SER ILE ARG SER
ACCTGGGAAGGTAGATAAAAAGCAGTTTGTACTTCTAATTGATGTTTCCTCAATCAGAAG
BstNI     990       1000      1010      1020      1030      1040

TYR LYS VAL ILE ASN ALA LEU GLU ASP TYR PHE VAL ASN GLY LYS ASN ARG LYS GLU ILE
CTATAAAGTAATCAACGCATTAGAAGACTATTTTGTTAATGGTAAAAATAGAAAAGAAAT
          1050      1060      1070      1080      1090      1100

CYS ASP ASN HIS LYS ILE ASN GLN GLY TYR LEU SER LEU LYS ILE ARG GLU LEU GLN ASP
ATGTGACAACCATAAAATAAACCAAGGATACCTATCGCTAAAAATAAGGGAGCTACAAGA
          1110      1120      1130      1140      1150      1160

ILE SER LEU ARG ILE TYR ASN ILE SER HIS CYS ILE ***
CATTAGTTTGAGAATCTATAATATTTCACATTGTATTTAAATCAGTGCTCATGTTTATTG
          1170      1180      1190      1200      1210      1220

TACGTCATTATGATTTACTCTGTGGTATAAGCATAAAGCATATCTATTATTAATAGGGAA
          1230      1240      1250      1260      1270      1280

                -35                     -10           S.D.
TGGCTATGTTTTTCTGGTGATTCCACCGAACTAAAAAATAATATCGAACAATGGAGAATCT
          1290      1300      1310      1320      1330      1340

  fanC →
  MET LYS LYS THR LEU LEU
AGATGAAAAAAACACTGCTA
XbaI      1350      1360
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Fig. 2. Nucleotide sequence and deduced amino acid sequences of the region preceding the fimbrial subunit gene (*fanC*). Numbers beneath each line refer to nucleotide positions of the cloned K99 determinant contained in pFK99. The initiation sites of the genes have been indicated *fanA*, *B* and *C*. -35 and -10 refer to promoter sequences. S.D. denotes the Shine-Dalgarno sequences. Restriction sites mentioned in the text are indicated. Connected bars starting at position 662 indicate a region showing dyad symmetry.

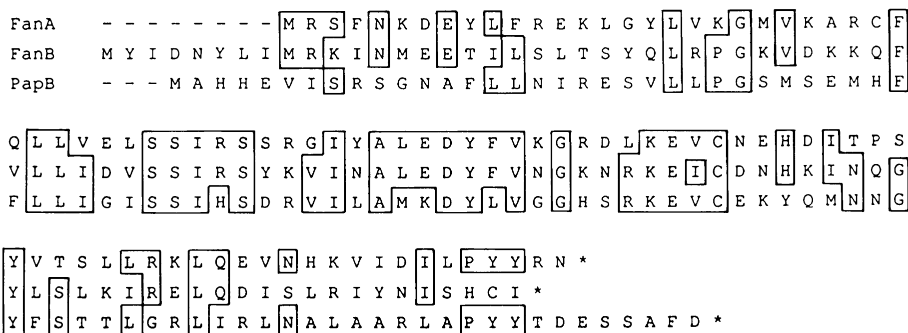

Fig. 3. Comparison of the primary structures of FanA, FanB, and PapB in standard one lettercode. Identical amino acids are boxed. The amino acid sequence of PapB was obtained from Båga et al. (1).

recognized at the intercistronic region upstream of *fanB* at position 860 (Fig. 2) (22). Possible promoter sequences upstream of *fanA* and *fanC* showed less homology to the consensus sequence, but are nevertheless indicated (Fig. 2). Whether the indicated sequences really act as promoters of transcription remains to be elucidated. A region showing dyad symmetry was observed within *fanA* at position 800 (Fig. 2). The free energy of the stem and loop structure, that could be formed by the mRNA from this region, is -16.6 kcal (23). Another promoter, directed leftward in Figure 2, was observed at position 120. Whether this promoter is implicated in K99 fimbriae expression is not known, since the putative gene downstream this promoter is not contained within pFK99.

Analysis of the primary structures of FanA and FanB, as deduced from the nucleotide sequence (Fig. 2), revealed that both polypeptides are very hydrophilic with 30 and 28 charged amino acid residues, respectively. Most of these residues are basic, 18 and 17 residues, respectively. Furthermore, FanA and FanB contain no signal peptides, indicating that they reside in the cytoplasm. The more striking aspect, however, is that both polypeptides show considerable homology to each other (Fig. 3). About 40% of the amino acids is identical and several other residues, depending on the criteria used, are functionally alike. It is tempting to speculate that the genes originate from a gene duplication.

The involvement of FanA and FanB in the expression of K99 fimbriae was investigated by the introduction of frame shift mutations (Material and Methods). The mutation in *fanA* created a reading frame that terminates at position 844 and gives rise to a mutant FanA with a molecular mass of 12Kd, which is approximately 1 Kd larger than the wild type FanA. The one basepair deletion in *fanB* results in an almost immediate termination of translation at position 972. The obtained mutant plasmids were analyzed for K99 fimbriae expression by the enzyme-linked immunosorbent assay (ELISA) (Table I). Both mutants showed a reduced K99 expression, 12% and 6% of the wild type level for *fanA*$^-$ and *fanB*$^-$, respectively. Whether the

Table I. Effect of mutations in *fanA* and *fanB* on expression of K99 fimbriae[a]

pFK99-derived plasmids	Plasmids used for complementation	Relative amount[b] of K99 fimbriae
pFK99	-	100.0
pFK99 (fanA-)	-	12.5
pFK99 (fanB-)	-	6.2
pFK99	pACYC 184	100.0
pFK99 (fanA-)	pACYC (fanA)	75
pFK99 (fanA-)	pACYC (fanB)	12.3
pFK99 (fanB-)	pACYC (fanB)	72
pFK99 (fanB-)	pACYC (fanA)	6.4

[a] as measured by ELISA (Material and Methods)
[b] the amount of K99 fimbriae produced by strains containing pFK99 was arbitrarily set at 100%. The relative amounts of K99 fimbriae are average values of at least three independent assays of which the variation was no more than 10%.

observed reductions are due to the absence of diffusable products could be investigated by complementing both mutants *in trans* with their respective wild type genes. Therefore, *fanA* and *fanB* were inserted separately in the ColE1 compatible plasmid pACYC184 (Material and Methods). The subsequent complementation studies showed, that the expression of K99 fimbriae in both mutants was restored to about 75% of the wild type level (Table I), indicating that *fanA* and *fanB* code for *trans*-acting polypeptides involved in expression of K99 fimbriae. *FanB* was inserted in pACYC184 in two orientations. Both derivatives were used in complementation studies and showed similar levels of K99 fimbriae expression.

To specify the effects of the mutations in *fanA* and *fanB* on the synthesis of the fimbrial subunit (FanC) and the auxiliary polypeptides (FanD-H), the mutant plasmids and pFK99 were analyzed in minicells. An only slightly lower expression of FanC-H from the mutant derivatives, compared to pFK99, was observed (not shown). We also observed that, in contrast to the other FanC-H polypeptides, FanA and FanB could not be detected in minicell preparations.

The inability to detect these polypeptides would hamper their further characterization. Therefore, we tried to identify FanA and FanB, employing a DNA-directed in vitro translation system. A BamHI-BglII fragment, containing *fanA*, *fanB*, and part of *fanC* (Fig. 1), was isolated and used to direct protein synthesis *in vitro*. On SDS-PAGE one clearly visible band with an apparent molecular mass of 11 Kd could be observed (Fig. 4A), which was absent in control preparations of extracts without DNA (not shown). To characterize this component, the same DNA fragment was digested with AccI or BstNI (Fig. 1). This prevents protein synthesis from *fanA* or *fanB*, respectively. On SDS-PAGE the 11 Kd polypeptide was absent in the assay with the DNA digested with AccI (Fig. 4B) but not when the DNA was digested with

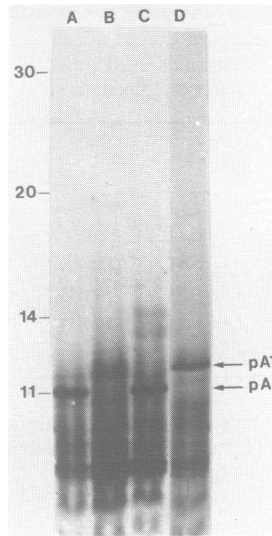

Fig.4. SDS-PAGE of polypeptides synthesized from the BamHI-BglII DNA-fragment (Fig. 2) in an *in vitro* DNA-directed translation system. DNA used in the system: A, untreated; B, digested with AccI; C, digested with BstNI; D, the BamHI-BglII fragment from the *fanA*⁻-mutant. Numbers indicate the molecular mass of the polypeptides in kilodaltons (Kd). pA refers to FanA.

BstNI (Fig. 4C), suggesting that the 11 Kd polypeptide is FanA. The polypeptide was further characterized, using the BamHI-BglII fragment from the *fanA*-mutant. A band with an apparent molecular mass of 12 Kd could be observed, while the 11 Kd polypeptide was absent (Fig. 4D). These observations are in agreement with the DNA sequence data and indicate that the 11 Kd polypeptide is FanA.

Although in the above described experiments no expression of FanB was observed, it is possible that this polypeptide was masked by the FanA band on SDS-PAGE. However, when FanA synthesis was blocked by digesting the DNA or using *fanA*⁻ mutant DNA no FanB was observed. This indicates that either FanB was synthesized in undetectable amounts or requires FanA to be expressed. To circumvent this possible requirement, *fanB* was inserted downstream the strong promoter of expression vector pINIIIA (Material and Methods) (16). Using this constructed plasmid to direct polypeptide synthesis *in vitro* a band somewhat smaller than FanA could be observed (Fig. 5B). This component was absent in the control preparation with the pINIIIA vector (Fig. 5A). Since examination of the DNA sequences of both inserted fragment and vector demonstrated, that no other polypeptide with a molecular mass of 10.8 Kd could originate from the fusion sites, we concluded that the 10.8 Kd component is FanB. Considering the molecular mass of 10.8 Kd, this might indicate that translation of *fanB* is initiated at the AUG codon at position 925.

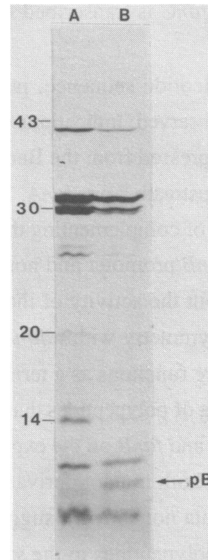

Fig. 5. SDS-PAGE of polypeptides synthesized from pINIIIA plasmids in an *in vitro* DNA-directed translation system. DNA used in the system: A, pINIIIA; B, pINIIIA containing *fanB* on fragment HincII-XbaI (Fig. 2). Numbers indicate the molecular mass of the polypeptides in kilodaltons (Kd). pB refers to FanB. The 20 Kd band in lane B becomes visible after prolonged exposure of the gel.

DISCUSSION

Two genes, designated *fanA* and *fanB* and located in the region upstream of the fimbrial subunit gene *fanC* have been implicated in K99 fimbriae synthesis. Mutations in *fanA* and *fanB* reduced K99 fimbriae expression 8 and 16-fold, respectively (Table I). The possibility cannot be excluded that complete destruction of *fanA* results in a more than 8-fold reduction in K99 expression, because the 12 Kd mutant FanA might have a residual activity. When mutations in *fanA* and *fanB* were complemented *in trans*, K99 fimbriae production was restored to 75% of the wild type level, indicating that *fanA* and *fanB* code for *trans*-acting polypeptides involved in expression of K99 fimbriae. It is not clear why complementation did not fully restore K99 fimbriae production. Several possibilities can be considered. First, FanA and FanB might be labile polypeptides. Since FanA and FanB in the complemented mutant strains have to diffuse over a longer distance, before they are able to practise their function, their activity might be reduced due to decay of the polypeptides. Second, the amount of FanA and FanB synthesized, might be smaller when expressed from the pACYC184 derivatives, due to a lower copynumber of these derivatives. Third, there might be a slight polarity effect on the expression of *fanC* due to premature termination of translation of *fanA* and *fanB*. This polarity

effect, however, can only occur when *fanC* is transcribed in a polycistronic messenger RNA together with *fanA* and/or *fanB*.

By examining the determined nucleotide sequence, putative promoter sequences and a region showing dyad symmetry were observed. Indications were obtained about the activity of two promoters. First, *fanA* could be expressed from the BamHI/BglII DNA fragment in vitro, suggesting that a promoter is located upstream from *fanA*. Second, both orientations of *fanB* inserted into pACYC184 were capable of complementing the *fanB* mutant *in trans*, indicating that *fanB* is expressed from the K99 *fanB* promoter and not from a vector promoter. Thusfar, no indications have been obtained about the activity of the promoter upstream of *fanC*. The location of the region showing dyad symmetry within *fanA* and its relatively low ΔG (-16.6 kcal) make it unlikely that this structure functions as a terminator of transcription. Structures like this have been implicated in binding of polypeptides that regulate gene expression (24).

The effects of the mutations in *fanA* and *fanB* on the expression of *fanC-H* were studied in minicells. Expression of *fanC-H* from both mutant derivatives was comparable and slightly lower than expression from pFK99 (data not shown), suggesting that mutations in *fanA* and *fanB* affect the expression of all K99 polypeptides to the same extent. It is not clear why the mutations affect expression of fimbriae in normal cells 8 and 16 fold, while only a slight reduction is observed on the synthesis of *fanC-H* in minicells. One explanation is that FanA and FanB do not function in minicells, which suggests that ancillary activating factors are required for the *fan* cluster to be expressed. Another explanation is that in normal cells containing the *fanA* and *fanB* mutant derivatives, not the synthesis of FanC-H, but the assembly of subunits into fimbriae is affected. This might lead to a rapid turnover of the subunits and a consequently reduced amount of fimbriae.

FanA and FanB were not detectable in minicell preparations, indicating that both polypeptides are synthesized in very small amounts. However, another attempt to identify both proteins, employing an *in vitro* DNA directed translation system, was successful. Employing this system FanA could be detected clearly on SDS-PAGE (Fig. 4A). FanB was detected as a faint band on SDS-PAGE, even though it was cloned downstream the strong promoter of the expression vector pINIIIA (Fig. 5B). This might indicate, that the poor homology of the sequences preceding *fanB* to the Shine-Dalgarno sequence results in a very inefficient translation of the *fanB* transcript. Hence, the ability to detect both proteins will further facilitate the characterization of their function, expression and eventually their isolation.

At present, we can only speculate about the functions of FanA and FanB. Considering their homology (Fig. 3), we consider that both genes originate from a gene duplication. *FanA*$^-$ and *fanB*$^-$ mutants showed a reduced K99 fimbriae expression, indicating that one protein, is not able to take over the function of the other. When the *fanA* mutant was complemented with pACYC (*fanB*) or the *fanB* mutant with pACYC (*fanA*) no stimulation of fimbriae expression was observed (Table I). The observations indicate that FanA and FanB have different functions.

Comparison of the K99 genetic determinant with determinants, encoding other fimbriae might give a clue to their function. One determinant, showing similarities in genetic organization, when compared to the K99 determinant, is the *pap* gene cluster. This gene cluster codes for polypeptides involved in the biogenesis of Pap fimbriae, which enable the *E. coli* bacteria to infect the urinary tract of man, causing pyelonephritis (25). The *pap* gene cluster contains a *papB* gene, which is located upstream of the fimbrial subunit gene and codes for a 13 Kd polypeptide. This resembles the situation of *fanB* in the K99 determinant. Comparison of the primary structures of the *papB* and *fanB* gene products showed a remarkable homology. About 35% of the amino acids are identical (Fig. 3) and several other residues, depending on the criteria used, are functionally alike. The *papB* gene product has been implicated in regulation of transcription of the *pap* gene cluster (1). This suggests that FanA and FanB also act at the level of transcription. This possibility was further investigated by comparing the primary structures of FanA and FanB with a database, using the FASTP program (26) on an IBM PC. Although we paid special attention to transcription regulatory proteins no convincing homology was observed.

Polypeptides known to regulate transcription often exist as dimers, which are active in DNA binding at regions showing dyad symmetry (24). Considering the homology between FanA and FanB, they might form a dimer as well. Since it is unlikely that the region showing dyad symmetry located within *fanA* functions as a terminator of transcription, it could be the attachment site for the putative FanA/FanB dimer. We might speculate that this complex is involved in the regulation of expression of K99 fimbriae with respect to growth rate, temperature, pH and alanine.

It was demonstrated that at least two α-helixes, with basic amino acid residues and linked by a tight turn are responsible for the binding of lambda cro, lambda repressor, and *E. coli* CAP to their attachment sites on the DNA (24). Therefore, we examined FanA and FanB for these structures. However, predictions of the secundary structures employing the Chou and Fasman method (27) do as yet not show these structures for FanA and FanB (data not shown).

When *fanA*$^-$ and *fanB*$^-$ mutants are studied in a haemagglutination assay with horse erythrocytes the reduced expression of K99 fimbriae (8 and 16 fold, respectively) resulted in a similar decrease in haemagglutinating activity (Jacobs, unpublished results). The observations indicate that the attraction force between fimbriated bacteria and erythrocytes decreases proportionally with the amount of fimbriae on the bacterial cell surface and that FanA and FanB affect this attraction force.

When FanA and FanB are indeed polypeptides responsible for the regulation of K99 fimbriae expression, the 8 and 16 fold decrease in the respective mutants is not sufficient to explain the reduction of K99 expression to less than 1% obtained by variation in growth rate, temperature, pH and alanine. A few explanations can be considered. First, it is not known whether the effect of the *fanA* and *fanB* mutations on K99 expression are additional, since no double mutant is available. Second, the results discussed above are all observations from a

multicopy plasmid system, while the authentic K99 gene cluster is located on a low copy number plasmid. Mutations in *fanA* and *fanB* of this plasmid might affect K99 expression to a larger extent than the 8 and 16 fold measured with the multicopy system.

The results discussed above suggest, that several transcription regulation signals and two gene products are involved in the regulation of transcription of K99 fimbriae expression.

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REFERENCES

1. Båga, M., Göransson, M., Normark, S. and Uhlin, B.E. (1985) The EMBO J. 4, no. 13B, 3887-3893.
2. Van Verseveld, H.W., Bakker, P., Van der Woude, T., Terleth, C. and De Graaf, F.K. (1985) Infect. Immun. 49, 159-163.
3. De Graaf, F.K., Klaasen-Boor, P. and Van Hees, J.E. (1980) Infect. Immun. 30, 125-128.
4. De Graaf, F.K., Wientjes, F.B. and Klaasen-Boor, P. (1980) Infect. Immun. 27, 216-221.
5. De Graaf, F.K., Krenn, B.E. and Klaasen, P. (1984) Infect. Immun. 43, 508-514.
6. Van Embden, J.D.A., De Graaf, F.K., Schouls, L.M.and Teppema, J.S. (1980) Infect. Immun. 29, 1125-1133.
7. Roosendaal, E., Gaastra, W. and De Graaf, F.K. (1984) FEMS Microbiol. Lett. 22, 253-258.
8. Boyer, H.W. and Roullard-Dussoix, D. (1969) J. Mol. Biol. 41, 459-465.
9. Sanger, F., Coulson, R., Barrell, B.G., Smith, J.H. and Roe, B.A. (1980) J. Mol. Biol. 143, 161-178.
10. Dougan, G. and Sherratt, D. (1977) Mol. Gen. Genet. 151, 151-160.
11. Messing, J., Crea, R. and Seeburg, P.H. (1981) Nucl. Acids Res. 9, 309-321.
12. Guinée, P.A.M., Jansen, W.H. and Agterberg, C.M. (1976) Infect. Immun. 13, 1369-1377.
13. Maniates, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory.
14. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Ac. Sci. USA 74, 5463-5467.
15. Mooi, F.R., Wouters, C., Wijfjes, A. and De Graaf, F.K. (1982) J. Bacteriol. 150, 512-521.
16. Lunn, C.A., Takahara, M. and Inouye, M. (1986) Cur. Top. Microbiol. Immunol. 125, 59-74.
17. Mooi, F.R., Harms, N., Bakker, D. and De Graaf, F.K. (1981) Infect. Immun. 32, 1155-1163.
18. Zubay, G. (1973) Ann. Rev. Genet. 7, 267-287.
19. Collins, J. (1979) Gene 6, 29-42.
20. Mooi, F.R., De Graaf, F.K. and Van Embden, J.D.A. (1979) Nucl. Acids Res. 6, 849-865.
21. Steitz, J.A. (1979) In Biological Regulation and development. Goldberger, R.F., Ed., Vol. I, 349-399, Plenum Press, New York.
22. Rosenberg, M. and Court, D. (1979) Ann. Rev. Genet. 13, 319-353.
23. Tinoco, I., Borer, P.N., Dengler, B., Levine, M.D., Uhlenbeck, O.C., Crothers, D.M. and Gralla, J. (1973) Nature 246, 40-41.
24. Pabo, C.O. and Sauer, R.T. (1984) Ann. Rev. Biochem. 53, 293-321.
25. Uhlin, B.E., Båga, M., Göransson, M., Lindberg, F.P., Lund, B., Norgren, M. and Normark, S. (1985) Cur. Top. Microbiol. Immunol. 118, 163-178
26. Lipman, D.J. and Pearson, W.R. (1985) Science 227, 1435-1441.
27. Chou, P.Y. and Fasman, G.D. (1978) Adv. Enzymol. 47, 45-148.