JOURNAL OF BACTERIOLOGY, Dec. 1987, p. 5408–5415
0021-9193/87/125408-08$02.00/0

Vol. 169, No. 12

Sequence Analysis, Transcriptional Organization, and Insertional Mutagenesis of the *envA* Gene of *Escherichia coli*

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Received 29 June 1987/Accepted 2 September 1987

The *Escherichia coli* cell permeability-cell separation gene *envA* and the region immediately downstream were sequenced. The *envA* gene consisted of 305 codons which encoded a 34-kilodalton polypeptide that lacked a signal sequence and hydrophobic membrane-spanning regions. The *envA1* mutation was determined to be a missense mutation in codon 19 resulting in a change in the amino acid sequence from histidine to tyrosine. Located 299 base pairs downstream of the *envA* gene was an unidentified open reading frame consisting of 147 codons. This open reading frame was followed by an additional open reading frame starting 59 base pairs further downstream and corresponded to the *secA* gene. A transcription terminator was located just downstream of *envA* on a fragment that contained a sequence corresponding to a typical *rho*-independent terminator. Transcription of *envA* and the upstream *fts* genes terminated at this terminator and was probably uncoupled from the downstream genes, including *secA*. Gene disruption experiments indicated that the *envA* gene was an essential gene.

The *envA* gene maps within a large cell envelope-cell division gene cluster located at 2 min on the *Escherichia coli* genetic map (1). This region has received considerable attention because it contains several *fts* genes, including the *ftsZ* gene, which apparently has a pivotal role in cell division (31). In addition, this region contains the *secA* gene, which plays an essential role in protein export across the cytoplasmic membrane (22), and a number of peptidylglycan biosynthetic genes (1). These genes are very tightly clustered and are transcribed in the same direction (13, 14, 28). Nonetheless, at least one or more promoters have been associated with each gene, and no transcription terminators have been located in the region (23, 36).

The *envA* gene is immediately downstream of the cell division genes *ftsQ*, *ftsA*, and *ftsZ* and is separated from the 3′ end of the *ftsZ* gene by 100 base pairs (bp) (35, 36). Downstream of the *envA* gene is the *secA* gene, which is transcribed in the same direction as *envA* (22). It has been noted that there are approximately 900 bp of unassigned DNA sequence between *envA* and *secA* which could code for a moderately sized protein. Promoter analysis suggests that if a gene does exist, it would be transcribed in the same direction as *envA* and *secA* (28). Immediately upstream of the *fts* genes lie a number of murein biosynthetic genes. Two of these genes, *murC* and *ddl*, which are located immediately upstream of *ftsQ*, are transcribed in the same direction as *ftsQ* (13, 14). Sequence analysis has revealed that *ddl* and *ftsQ* are separated by only 2 bp (24) and that the *ftsQ* and *ftsA* genes actually overlap by 2 bp (23, 36). In contrast, *ftsA* and *ftsZ* are separated by 60 bp which include a sequence that could form a hairpin structure but is only a terminator in the reverse direction (35, 36).

The *fts* genes were identified through temperature-sensitive mutations that result in filamentous growth and eventual cell death at the nonpermissive temperature (2, 13, 29). In addition, mutations that render cells resistant to the SOS-inducible cell division inhibitor *sulA* have been located within the *ftsZ* gene (8, 11).

The *envA* gene was identified on the basis of a single non-temperature-sensitive mutation that results in a pleiotropic phenotype (18, 19). The phenotype associated with the *envA1* mutation includes increased sensitivity to hydrophobic and hydrophilic antibiotics and a defect in cell separation resulting in chain growth. This latter phenotype has been correlated with decreased *N*-acetylmuramyl-L-alanine amidase activity (33). The antibiotic hypersensitivity is due to hyperpermeability and is associated with a lower lipopolysaccharide/protein ratio in the outer membrane (5). One extragenic suppressor of antibiotic sensitivity, *sefA*, results in an increase in outer membrane protein content and cells with incomplete septa (5, 20).

To aid in determining the function of the *envA* gene product and the analysis of gene expression in this region, we have completed the sequence of the *envA* gene and the only known *envA* mutation. We also demonstrated through gene disruption experiments that *envA* is an essential gene. Further sequence analysis revealed an additional short open reading frame starting 299 bp downstream of *envA*, which is followed by the start of the *secA* gene. A strong transcriptional terminator was located within this 299-bp intergenic region downstream of *envA*.

MATERIALS AND METHODS

Bacterial and phage strains. All bacterial strains used in this work are derivatives of *E. coli* K-12 and are listed in Table 1. Strain GIA86 was used in complementation tests to assess the *envA* allele carried by plasmids. The transducing phages λ16-2 and λ16-3 have been described previously (12). They are identical except that λ16-2 carries the wild-type *envA* allele and λ16-3 carries *envA1*. In addition, these phage carry the *ftsZ*, *ftsA*, *ftsQ*, *murC*, and *ddl* genes.

Plasmid constructions. Plasmid pBL1 was constructed by ligating *Hin*dIII-digested λ16-2 and pBR322 DNAs and transforming JFL101 [*ftsZ84*(Ts)] to ampicillin resistance and temperature resistance. Plasmid pBL2 carrying the *envA1* mutation was constructed in the same manner by using λ16-3 DNA. Both plasmids contained a 3.5-kilobase (kb) *Hin*dIII fragment containing the entire *ftsZ* gene and the wild-type *envA* gene or the mutant *envA1* allele. These *Hin*dIII frag-

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TABLE 1. Bacterial and phage strains used

Bacterial strain	Relevant marker	Genotype	Source or reference
GIA86	*envA1*	*thr thi pyrF thyA ilvA his arg lac tonA tsx*	12
JFL101	*ftsZ84*(Ts)	*recA ilv deo ara*(Am) *lacZ125*(Am) *galU42*(Am) *trp*(Am) *tyrT* [*supFA81*(Ts)]	12
JC7623	*recB21 recC22 sbcB15*	*arg ara his leu pro thr*	31
NK6923	*leu*::Tn*10*	*thy*	M. Singer
BL7623(λ16-2)	*envA*::*kan*^{r} *leu*::Tn*10*	As JC7623	This study
W3110		Prototroph	Laboratory collection
JM101	Δ(*lac proAB*)/F′ *traD36 proAB*		16
NK5549	F′ Tn*9* *lacI*^{q}		Nancy Kleckner

ments were also cloned into pACYC184, yielding pBL3 (*envA*) and pBL4 (*envA1*).

Plasmid pBL5 consists of the 3.5-kb *Hin*dIII fragment of λ16-2 cloned into the low-copy-number plasmid vector pGB2 (4). Plasmid pBL5K, containing a disrupted *envA* gene, was constructed by replacing the 209-bp *Cla*I fragment of pBL5 with the *Acc*I fragment carrying the 1.5-kb kanamycin resistance cassette from pUC4K (see Fig. 6).

To test for possible transcription terminators, a pKO (16) derivative, pSR132 (Rockenbach and Lutkenhaus, unpublished), was used. This plasmid contains an *Eco*RI fragment containing a promoter located near the 5′ end of the *ftsQ* gene cloned upstream of the *galK* gene. The plasmid has several restriction sites located between the promoter and the *galK* gene in which fragments can be cloned to test for terminator activity (see Fig. 5).

Marker rescue of the *envA1* mutation. Strain GIA 86(F′::Tn*9* *lacI*^{q}) was infected in 2× YT medium (17) with recombinant M13 phage carrying different segments of the wild-type *envA* allele. Adequate time was allowed for recombination and expression of the wild-type *envA* allele. Rescue of the *envA1* mutation was scored by plating the infected cells onto L-agar plates containing rifampin (5 μg/ml).

DNA sequencing. DNA was sequenced by the dideoxy method with strain JM101 as the host for M13mp18 and M13mp19 derivatives (34). The orientations of cloned restriction fragments in these phages were determined with the C test as described previously (17). Products of the sequencing reactions were analyzed by use of buffer gradient gels (3).

Inactivation of the chromosomal *envA* gene. A strain containing a disrupted *envA* gene was constructed by the procedure of Winans et al. (32). First, strain JC7623 was lysogenized by λ16-2 to provide an additional copy of the *envA* gene. This lysogen was transformed with pBL5K that had been linearized by *Eco*RI digestion. A Kan^r Spc^s transformant was transduced to Tet^r by P1 grown on strain NK6923 (*leu*::Tn*10*). Among the Tet^r transductants, 53% had lost Kan^r, indicating that Kan^r was linked to *leu* and that the chromosomal copy of *envA* had been inactivated in this transformant. One of the Tet^r transformants that had retained Kan^r was designated BL7623(λ16-2) and used in further P1 transductions.

Labeling proteins synthesized in maxicells. Strain JFL101 was transformed with the various plasmids and labeled by the maxicell system as described by Sancar et al. (26). The labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Sequence of the wild-type and mutant *envA* alleles. The *envA* gene has been located on a 2.5-kb *Eco*RI fragment that

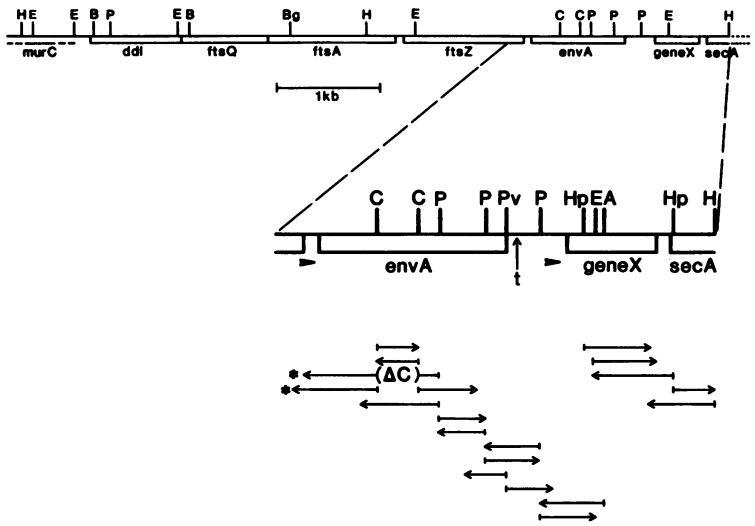

FIG. 1. Sequencing strategy for the *envA* region. The top line indicates the restriction map and gene order in the 2-min region of the *E. coli* genetic map. The expanded region is a restriction map of the DNA that was sequenced in this study. The positions of the consensus promoter sequences are indicated by arrowheads, and the terminator is indicated by t. The horizontal arrows indicate the lengths and directions of sequences determined by the dideoxy sequencing method. The fragments marked by the asterisks indicated those fragments that could rescue the *envA1* mutation. Abbreviations: A, *Asu*II; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; P, *Pst*I; Pv, *Pvu*II; ΔC, deletion of a 209-bp *Cla*I fragment.

```
                                [End FtsZ]
Phe Leu Arg Lys Gln Ala Asp ---                -35                    -10
TTC CTG CGT AAG CAA GCT GAT TAA GAATTGACTGGAATTTGGGTTTCGATTCTCTTTGTGCTAAACTGGCC  1571
                                                [Start EnvA]
                                                          Met Ile Lys Gln Arg Thr
CGCCGAATGTATAGTACACTTCGGTTGGATAGGTAATTTGGCGAGATAATACG ATG ATC AAA CAA AGG ACA   1642
                                                          Tyr
Leu Lys Arg Ile Val Gln Ala Thr Gly Val Gly Leu His Thr Gly Lys Lys Val Thr Leu
CTT AAA CGT ATC GTT CAG GCG ACG GGT GTC GGT TTA CAT ACC GGC AAG AAA GTC ACC CTG  1702
                                                T
Thr Leu Arg Pro Ala Pro Ala Asn Thr Gly Val Ile Tyr Arg Arg Thr Asp Leu Asn Pro
ACG TTA CGC CCT GCG CCG GCC AAC ACC GGG GTC ATC TAT CGT CGC ACC GAC TTG AAT CCA  1762

Pro Val Asp Phe Pro Ala Asp Ala Lys Ser Val Arg Asp Thr Met Leu Cys Thr Cys Leu
CCG GTA GAT TTC CCG GCC GAT GCC AAA TCT GTG CGT GAT ACC ATG CTC TGT ACG TGT CTG  1822

Val Asn Glu His Asp Val Arg Ile Ser Thr Val Glu His Leu Asn Ala Ala Leu Ala Gly
GTC AAC GAG CAT GAT GTA CGG ATT TCA ACC GTA GAG CAC CTC AAT GCT GCT CTC GCG GGC  1882

Leu Gly Ile Asp Asn Ile Val Ile Glu Val Asn Ala Pro Glu Ile Pro Ile Met Asp Gly
TTG GGC ATC GAT AAC ATT GTT ATC GAA GTT AAC GCG CCG GAA ATC CCG ATC ATG GAC GGC  1942
      ^ClaI                     ^HpaI
Ser Ala Ala Pro Phe Val Tyr Leu Leu Leu Asp Ala Gly Ile Asp Glu Leu Asn Cys Ala
AGC GCC GCT CCG TTT GTA TAC CTG CTG CTT GAC GCC GGT ATC GAC GAG TTG AAC TGC GCC  2002

Lys Lys Phe Val Arg Ile Lys Glu Thr Val Arg Val Glu Asp Gly Asp Lys Trp Ala Glu
AAA AAA TTT GTT CGC ATC AAA GAG ACT GTT CGT GTC GAA GAT GGC GAT AAG TGG GCT GAA  2062

Phe Lys Pro Tyr Asn Gly Phe Ser Leu Asp Phe Thr Ile Asp Phe Asn His Pro Ala Ile
TTT AAG CCG TAC AAT GGT TTT TCG CTG GAT TTC ACC ATC GAT TTT AAC CAT CCG GCT ATT  2122
                                    ^ClaI
Asp Ser Ser Asn Gln Arg Tyr Ala Met Asn Phe Ser Ala Asp Ala Phe Met Arg Gln Ile
GAT TCC AGC AAC CAG CGC TAT GCG ATG AAC TTC TCC GCT GAT GCG TTT ATG CGC CAG ATC  2182

Ser Arg Ala Arg Thr Phe Gly Phe Met Arg Asp Ile Glu Tyr Leu Gln Ser Arg Gly Leu
AGC CGT GCG CGT ACG TTC GGT TTC ATG CGT GAT ATC GAA TAT CTG CAG TCC CGT GGT TTG  2242
                                                ^PstI
Cys Leu Gly Gly Ser Phe Asp Cys Ala Ile Val Val Asp Asp Tyr Arg Val Leu Asn Glu
TGC CTG GGC GGC AGC TTC GAT TGT GCC ATC GTT GTT GAC GAT TAT CGC GTA CTG AAC GAA  2302

Asp Gly Leu Arg Phe Glu Asp Glu Phe Val Arg His Lys Met Leu Asp Ala Ile Gly Asp
GAC GGC CTG CGT TTT GAA GAC GAA TTT GTG CGT CAC AAA ATG CTC GAT GCG ATC GGT GAC  2362

Leu Phe Met Cys Gly His Asn Ile Ile Gly Ala Phe Thr Ala Tyr Lys Ser Gly His Ala
TTG TTC ATG TGT GGT CAC AAT ATT ATT GGT GCA TTT ACC GCT TAT AAA TCC GGT CAT GCA  2422

Leu Asn Asn Lys Leu Leu Gln Ala Val Leu Ala Lys Gln Glu Ala Trp Glu Tyr Val Thr
CTG AAT AAC AAA CTG CTG CAG GCT GTC CTG GCG AAA CAG GAA GCC TGG GAA TAT GTG ACC  2482
                    ^PstI                                          [End EnvA]
Phe Gln Asp Asp Ala Glu Leu Pro Leu Ala Phe Lys Ala Pro Ser Ala Val Leu Ala ---
TTC CAG GAC GAC GCA GAA CTG CCG TTG GCC TTC AAA GCG CCT TCA GCT GTA CTG GCA TAA  2542
                                                      ^PvuII
```

FIG. 2. Nucleotide sequence of the *envA* region. The deduced amino acid sequences for the end of *ftsZ*, *envA*, and gene X and the beginning of *secA* are given above the nucleotide sequence. The sequence of the *envA1* mutation is within the box. Putative promoter sequences have been underlined, and the location of the hyphenated dyad symmetry is indicated by arrows. The numbering is consistent with the previously published sequence of *ftsZ* (35).

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CGACATTTATACTGTCGTATAAAATTCGACTGGCAAATCTGGCACTCTCTCCGGCCAGGTGAACCAGTCGTTTTTTTTT  2621


GAATTTTATAAGAGCTATAAAAAACGGTGCGAACGCTGTTTTCTTAAGCACTTTTCCGCACAACTTATCTTCATTCGTG  2700

                                                        -35                  -10
CTGTGGACTGCAGGCTTTAATGATAAGATTTGTGCGCTAAATACGTTTGAATATGATCGGGATGGCAATAACGTGAGTG  2779
          ^PstI
                                                                  Met Val Ala Ala
GAATACTGACGCGCTGGCGACAGTTTGGTAAACGCTACTTCTGGCCGCATCTCTTATTAGGG ATG GTT GCG GCG   2853

Ser Leu Gly Leu Pro Ala Leu Ser Asn Ala Ala Glu Pro Asn Ala Pro Ala Lys Ala Thr
AGT TTA GGT TTG CCT GCG CTC AGC AAC GCC GCC GAA CCA AAC GCG CCC GCA AAA GCG ACA  2913

Thr Arg Asn His Glu Pro Ser Ala Lys Val Asn Phe Gly Gln Leu Ala Leu Leu Glu Ala
ACC CGC AAC CAC GAG CCT TCA GCC AAA GTT AAC TTT GGT CAA TTG GCC TTG CTG GAA GCG  2973
                                    ^HpaI
Asn Thr Arg Arg Pro Asn Ser Asn Tyr Ser Val Asp Tyr Trp His Gln His Ala Ile Arg
AAC ACA CGC CGC CCG AAT TCG AAC TAT TCC GTT GAT TAC TGG CAT CAA CAT GCC ATT CGC  3033
                  ^EcoRI^AsuII                                                  .
Thr Val Ile Arg His Leu Ser Phe Ala Met Ala Pro Gln Thr Leu Pro Val Ala Glu Glu
ACG GTA ATC CGT CAT CTT TCT TTC GCA ATG GCA CCG CAA ACA CTG CCC GTT GCT GAA GAA  3093

Ser Leu Pro Leu Gln Ala Gln His Leu Ala Leu Leu Asp Thr Leu Ser Ala Leu Leu Thr
TCT TTG CCT CTT CAG GCG CAA CAT CTT GCA TTA CTG GAT ACG CTC AGC GCG CTG CTG ACC  3153

Gln Glu Gly Thr Pro Ser Glu Lys Gly Tyr Arg Ile Asp Tyr Ala His Phe Thr Pro Gln
CAG GAA GGC ACG CCG TCT GAA AAG GGT TAT CGC ATT GAT TAT GCG CAT TTT ACC CCA CAA  3213

Ala Lys Phe Ser Thr Pro Val Trp Ile Ser Gln Ala Gln Gly Ile Arg Ala Gly Pro Gln
GCA AAA TTC AGC ACG CCC GTC TGG ATA AGC CAG GCG CAA GGC ATC CGT GCT GGC CCT CAA  3273
                                                                [Start SecA]
Arg Leu Thr ---                                                             Met
CGC CTC ACC TAACAACAATAAACCTTTACTTCATTTTATTAACTCCGCAACGCGGGGCGTTTGAGATTTTATT ATG 3349

Leu Ile Lys Leu Leu Thr Lys Val Phe Gly Ser Arg Asn Asp Arg Thr Leu Arg Arg Met
CTA ATC AAA TTG TTA ACT AAA GTT TTC GGT AGT CGT AAC GAT CGC ACC CTG CGC CGG ATG  3409
                  ^HpaI
Arg Lys Val Val Asn Ile Ile Asn Ala Met Glu Pro Glu Met Glu Lys Leu Ser Asp Glu
CGC AAA GTG GTC AAC ATC ATC AAT GCC ATG GAA CCG GAG ATG GAA AAA CTC TCC GAC GAA  3469

Glu Leu Lys Gly Lys Thr Ala Glu Phe Arg Ala Arg Leu Glu Lys Gly Glu Val Leu Glu
GAA CTG AAA GGG AAA ACC GCA GAG TTT CGT GCA CGT CTG GAA AAA GGC GAA GTG CTG GAA  3529

Asn Leu Ile Pro Glu Ala
AAT CTG ATC CCG GAA GCT T  3548
                  ^HindIII
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also contains most of the 3′ end of the *ftsZ* gene (Fig. 1) (13, 35). By promoter fusion experiments and nucleotide sequence analysis, its orientation of transcription was determined to be the same as that of the *ftsZ* gene (28). Previously the 5′ end of the *envA* gene was sequenced during the determination of the nucleotide sequence of the *ftsZ* gene (35). To complete the sequence of the *envA* gene, we used plasmids obtained by subcloning the *envA* gene from λ16-2 (see Materials and Methods). The strategy for sequencing the *envA* region is shown in Fig. 1, and the results are shown in Fig. 2.

Analysis of the sequence data revealed that the *envA* gene consisted of 305 codons yielding a predicted protein with a molecular weight of 34,000 (34K). The gene was preceded by a Shine-Dalgarno sequence (27), GAG, located 8 bp upstream of the initiation codon. Located 57 bp upstream of the initiation codon and just 14 bp beyond the 3′ end of the *ftsZ* gene was a sequence that showed strong homology to the consensus *E. coli* promoter sequence (6). Just downstream of the *envA* gene was a stretch of nine T residues preceded by a region of hyphenated dyad symmetry. Such sequences have been found to be *rho*-independent terminators (25).

The predicted *envA* gene product contained 22% charged residues which were distributed throughout the length of the protein. The protein lacked an amino-terminal signal sequence and long hydrophobic stretches (Fig. 3). Comparison of the predicted amino acid sequence of the *envA* gene product with protein sequences present in the Protein Identification Resource of the National Biomedical Research Foundation revealed no significant homologies.

Marker rescue of the *envA* mutation in strain GIA86 revealed that only phage containing DNA to the left of the first *Cla*I site within the *envA* gene were capable of rescuing the *envA* mutation (Fig. 1). These results were confirmed by the observation that pZAQ, which contained wild-type DNA to the left of this *Cla*I site inserted into pBR322, could also rescue *envA1* at a high frequency (data not shown). Sequence analysis of the mutant allele from the 5′ end to the *Cla*I site revealed a mutation in codon 19 resulting in a change in the amino acid sequence from histidine to tyrosine (Fig. 2). A missense mutation in *envA1* was not unexpected, because both *envA* and *envA1* code for proteins with the same molecular weight (Fig. 4).

Sequence analysis of the region beyond the 3′ end of *envA*. The *secA* gene has been located downstream of the *envA* gene, with its 5′ end located on the 450-bp *Eco*RI-*Hin*dIII fragment (Fig. 1). In addition, at least one promoter has been located downstream of *envA* and upstream of *secA* (28). This promoter appears to be sufficiently upstream of *secA* that an additional gene may be located between the promoter and *secA*.

Analysis of the sequence data revealed an open reading frame starting at positions 3347 to 3349 and extending to the end of the fragment. The location of this reading frame is consistent with this being the beginning of the *secA* gene. Upstream of this reading frame was an additional open reading frame starting 299 bp downstream of *envA* and consisting of 147 codons. The predicted protein was 16K in size. A consensus promoterlike sequence occurred at positions 3747 to 3774.

Transcription termination. Earlier results had indicated that no transcription terminators existed in the region between *ddl* and *envA* and that this may extend to *secA*. Although a sequence existed between *ftsA* and *ftsZ* that could form a stable hairpin structure, it only acted as a terminator in the antisense orientation (unpublished observations). Previously, Sullivan and Donachie (28) used promoter fusion experiments to determine the position of promoters in the *envA* region. In those experiments they did not detect any transcription terminators immediately downstream of *envA*. However, inspection of the sequence in Fig. 3 raised the possibility that a terminator existed just downstream of *envA*, but due to the proximity of the promoter for gene X it would not be detected unless the two elements were separated.

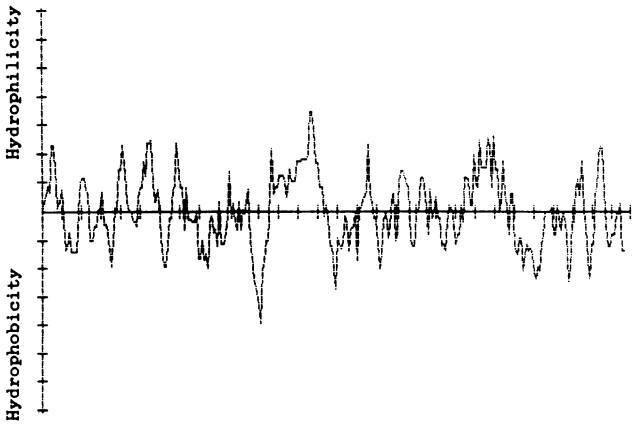

FIG. 3. Hydrophilicity-hydrophobicity plot for the EnvA protein. The abscissa is divided into increments of 10 amino acids. Hydrophilicity is indicated by a positive value on the ordinate, and hydrophobicity is indicated by a negative value. The values were determined by using a range of six residues (7).

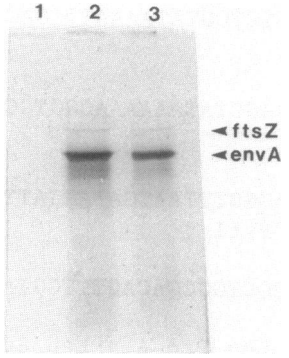

FIG. 4. Comparison of the gene products of the *envA* and *EnvA1* alleles. Maxicells containing plasmids were labeled with [^{35}S]methionine and analyzed by polyacrylamide gel electrophoresis and autoradiography. Lane 1, No plasmid; lane 2, pBL3 (*envA*); lane 3, pBL4 (*envA1*).

To test for the presence of a transcription terminator, the *Pst*I fragment containing the suspected terminator but not the gene X promoter (Fig. 1) was examined by cloning into a pKO vector, pSR132, that contained a promoter and therefore expressed *galK*. Since this *Pst*I fragment was already cloned into M13mp19 for sequencing, we were able to take advantage of flanking restriction sites to directionally subclone this fragment into pSR132 in both orientations (Fig. 5). The results clearly show that the expected orientation had a strong terminator, as pSR132 with the fragment in this orientation (pBL11) gave white colonies on MacConkey-galactose indicator plates. The opposite orientation (pBL10) did not significantly affect *galK* expression. Thus, transcription of *envA* would terminate at this terminator and not affect expression of the downstream *secA* gene.

Construction of a null mutation in *envA*. Our strategy for obtaining a null mutation in the *envA* gene was to place a selectable marker within the *envA* gene on a plasmid and then cross this mutation onto the chromosome (Fig. 6). Strain JC7623 was lysogenized with λ16-2 to provide an additional copy of *envA*. This lysogen was transformed with pBL5K linearized by *Eco*RI digestion. To determine the location of the *kan* marker, phage were induced from several Kan^r Spc^s colonies and screened for the ability to transduce cells to Kan^r. If a phage was unable to transduce cells to Kan^r, the corresponding lysogen was a candidate for having the *kan* marker in the chromosomal *envA* gene and not in the λ16-2 *envA* locus. The location of the *kan* marker in one such transformant was tested by P1 transduction with a closely linked Tn*10* insertion in *leu*. Of the Tet^r transductants, 53% were Kan^s, demonstrating linkage of *kan* and *leu* (Table 2). One Kan^r Tet^r transductant, designated BL6723(λ16-2), was infected with P1 for transduction of strains having one (W3110) or two [W3110(λ16-2)] copies of the *envA* gene. When the selected marker was Kan^r, only the strain with two

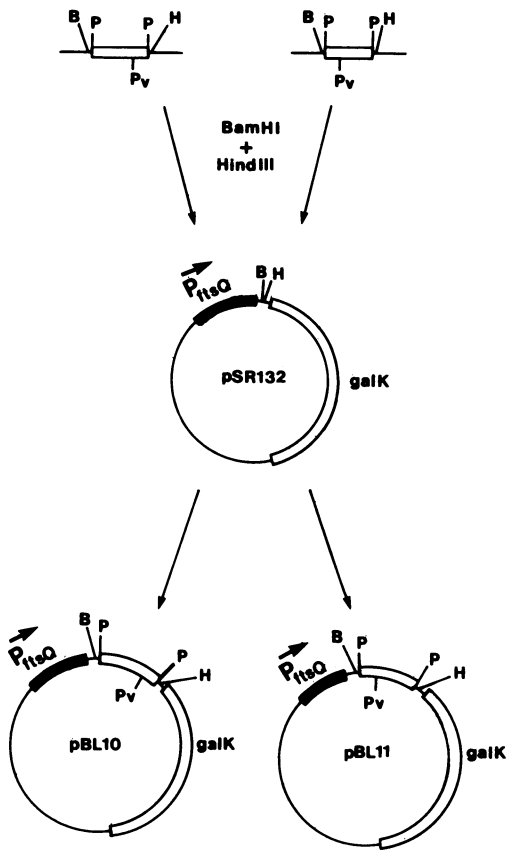

FIG. 5. Plasmid constructions for analysis of terminator activity. The *Pst*I fragment contained in both orientations in M13mp19 was cloned into pSR132 by using *Bam*HI (B) and *Hin*dIII (H) sites. The resultant plasmids were tested for *galK* expression. P_{ftsQ}, *ftsQ* promoters. See Fig. 1 legend for other abbreviations.

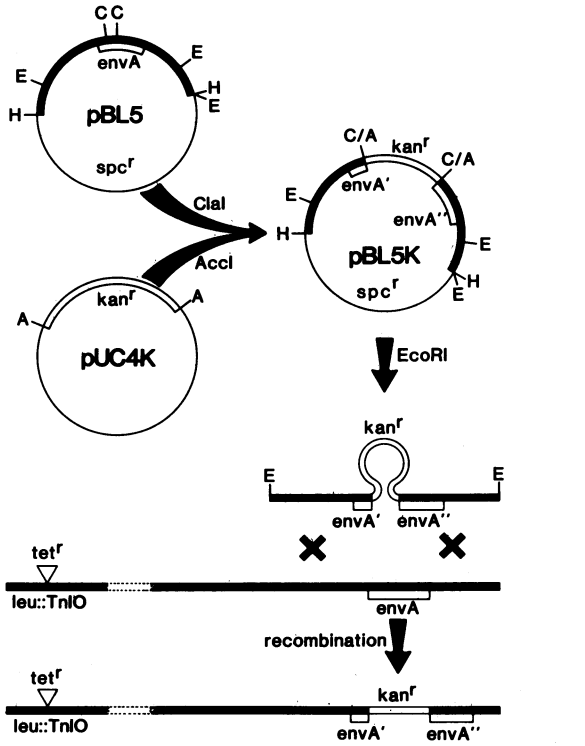

FIG. 6. Construction of a null allele of *envA*. The small *Cla*I fragment of pBL5 was replaced with the *Acc*I fragment containing the *kan* cassette from pUC4K. The resultant plasmid pBL5K was digested with *Eco*RI and used to transform JC7623(λ16-2) to Kan^r. Homologous recombination results in replacement of the chromosomal gene with the disrupted allele. The thick solid line represents chromosomal DNA, and the open box represents plasmid DNA. Abbreviations: H, *Hin*dIII; E, *Eco*RI; C, *Cla*I; C/A, *Cla*I-*Acc*I hybrid site; A, *Acc*I.

copies of *envA* yielded transductants, and it did so at a high frequency. In addition, in one of these transductants Kan^r was shown to be linked to Tet^r, as expected. In contrast, selection for Tet^r resulted in transductants at about equal frequency for both strains. However, only the strain with two copies of *envA* showed cotransduction of Kan^r, indicating that inactivation of the single copy of the *envA* gene was lethal (Table 2). These results indicate that *envA* is an essential gene.

DISCUSSION

The *envA* gene is of interest because of the pleiotropic phenotypes of the only known *envA* mutation and its location within the large cell envelope-cell division gene cluster occurring at 2 min on the *E. coli* genetic map.

The nucleotide sequence we have obtained along with our other results and other published results have helped elucidate the organization and expression of genes in the *envA* region. From the sequence analysis, there appear to be two consensuslike promoters within the region, one just upstream of *envA* and one upstream of gene X. This agrees with the promoter fusion experiments of Sullivan and Donachie (25), in which they located two promoters within this region. By using runoff transcription experiments, they were able to position promoters relative to restriction sites. Our location for the *envA* promoter agrees exactly with their results; however, the positioning of the gene X promoter is different. From their results, they indicated that this promoter was located 295 bp upstream of the *Eco*RI site (approximately position 2680), just upstream of the proximal *Pst*I site. However, we observed that the *Pst*I fragment

TABLE 2. P1 transduction of *leu*::Tn*10* with *kan*

Donor	Recipient	Selected marker	Unselected marker	Cotransduction (%)
NK6923 (*leu*::Tn*10*)	Kan^r transformant of JC7623(λ16-2)	*leu*::Tn*10*	Kan^s	53
BL7623(λ16-2) (*leu*::Tn*10* *envA*::*kan*)	W3110	*leu*::Tn*10* Kan^r	Kan^r —[b]	0[a]
BL7623(λ16-2) (*leu*::Tn*10* *envA*::*kan*)	W3110(λ16-2)	*leu*::Tn*10* Kan^r	Kan^r *leu*::Tn*10*	34 74

[a] 500 Tet^r colonies were screened for cotransduction of Kan^r.
[b] —, No transductants.

containing this region had no promoter activity and in fact had terminator activity. Therefore, we believe the position just downstream of the *Pst*I site at positions 2747 to 2774 to be a better estimate for the gene X promoter. It also appears from the sequence that this promoter would also serve for expression of *secA*, since there does not appear to be a terminator between gene X and *secA*.

The open reading frame corresponding to gene X indicated in Fig. 2 is not the longest possible open reading frame. There are two possible GTG initiation codons in phase and upstream of the open reading frame we have indicated; however, we favor the ATG codon at positions 2842 to 2844. The major argument is that this promoter sequence overlaps one of the GTG codons and is downstream of the other one, precluding their presence in any transcript. The initiation codon we have chosen is the next possible in-frame initiation codon even though it lacks an easily recognizable Shine-Dalgarno sequence. We were unable to detect a corresponding gene product in maxicells (Fig. 3), and so we have no proof that it is actually translated.

The location of a transcription terminator downstream of *envA* was revealed by the use of a terminator-probe vector. Recent S1 analysis has confirmed the location of the transcription terminator as the region of hyphenated dyad symmetry followed by a run of T's located just downstream of the *envA* gene (Corton and Lutkenhaus, unpublished). This transcription terminator is the first terminator discovered in the 2 min region, and it has several implications for expression of the genes in this area. First, the expression of *envA* and genes further upstream is probably uncoupled from gene X and *secA*. Second, transcription initiating as far upstream as *murC* probably terminates at this terminator, since no terminators have been found between *murC* and *envA*. Thus, *envA* could be expressed from a variety of different transcripts starting at different upstream promoters. Interestingly, though, the *envA* promoter appears to be the strongest promoter in this region, and complementation tests with single-copy vectors have shown that it can provide sufficient *envA* gene product.

The *envA* gene product lacks the amino-terminal signal sequence typical of periplasmic and outer membrane proteins (21) and lacks long hydrophobic stretches characteristic of an integral membrane protein (10). These factors are consistent with the apparent distribution of the EnvA protein between the cytoplasm and the cytoplasmic membrane in maxicells (9). It is therefore unlikely that the *envA* gene product is the *E. coli* *N*-acetylmuramyl-L-alanine amidase, since this activity has been located to the periplasmic space and the outer membrane (30).

Since it appeared possible that the *envA1* gene product had residual activity, we set out to construct a null allele of *envA* and test its effect on the cell. The results indicate that inactivation of *envA* when present in single copy is lethal, and therefore the *envA1* allele must have residual activity. Isolation of temperature-sensitive and nonsense mutations in *envA* will allow characterization of the effects on the cell of depleting this gene product.

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