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Transcriptional Organization Within an *Escherichia coli* Cell Division Gene Cluster: Direction of Transcription of the Cell Separation Gene *envA*

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A cluster of at least 14 genes, each concerned with some aspect of cell envelope growth, morphogenesis, or function, is located at 2 min on the genetic map of *Escherichia coli*. We located the *envA* cell division gene and its promoter within the cluster and determined the direction of transcription of the gene by constructing fusions between its promoter and the *galK* coding sequence. In addition, we identified the promoter of a possible new gene lying between *envA* and the *secA* gene. We also present evidence from gene fusion studies which shows the direction of transcription of the *ftsZ*(*sulB*) division gene. The direction of transcription is the same for all three promoters and is the same as that of all other cluster genes for which this is known. We discuss the significance of this observation, together with the fact that every gene examined in sufficient detail within the cluster appears to have its own promoter and to be able to be expressed from isolated cloned fragments. Using a novel variable-copy plasmid vector, we demonstrate that the DNA fragment containing the *envA* gene is not stably maintained in multiple copies. The construction of two independent, nonoverlapping deletions allows us to conclude that the *envA* product itself is responsible for this effect.

One particularly interesting aspect of genome organization in both procaryotes and eucaryotes is the existence of gene clusters (i.e., closely linked groups of separately transcribed genes of related function). In *Escherichia coli* one such cluster lies between *leu* and *azi* at 2 min on the genetic map (1) and contains 14 identified genes, each concerned with cell envelope growth or division. We would like to know how gene expression within the cluster is controlled and whether neighboring genes and operons interact. To do this we must first locate these genes precisely and then determine promoter locations and directions of transcription to identify possible regulatory regions.

We have studied a small part of this cluster, which includes the septation-specific gene *ftsZ* (11, 27) and the cell separation gene *envA* (19). We report here the location and direction of transcription of the *envA* gene in relation to its neighbors and show, contrary to previous indirect evidence (12), that the *envA* gene, like all other genes studied in this cluster, is transcribed clockwise relative to the *E. coli* genetic map.

One *envA* mutant has been isolated (19). This mutant is nonconditional but causes *E. coli* to form chains of cells during fast growth in rich media (19), presumably because it requires longer than the wild type does to complete a separation-specific step. The mutation is associated with low levels of *N*-acetylmuramyl-L-alanine amidase (28), an enzyme involved in splitting peptidoglycan molecules between the *N*-acetylmuramic acid residue and the pentapeptide side chain (26), and results in increased permeability to several antibiotics, both hydrophobic and hydrophilic (18, 20). We show here that excess *envA* protein is lethal to *E. coli*.

The *ftsZ* gene has been mapped counterclockwise of and adjacent to *envA* (11). A study of the *ftsZ84*(Ts) allele led Walker et al. (27) to suggest that the *ftsZ* gene product was required throughout septation. The *ftsZ* gene is allelic to the *sulB* locus, and its product is the probable target for *sulA* action in the *sulA*-dependent pathway of division inhibition which operates after the induction of the "SOS" pathway of DNA repair (9). By studying protein synthesis directed by λ phages carrying the *ftsZ* gene, it has been shown that the gene is transcribed in the clockwise direction (11). We report here direct gene fusion evidence for the direction of transcription of *ftsZ* which supports this result. In addition we identified the promoter of a probable new gene between *envA* and *secA* and show that this gene is also transcribed clockwise on the *E. coli* map.

MATERIALS AND METHODS

Bacterial strains and media. All bacterial strains used are listed in Table 1. For all cloning and galactokinase assays strain NFS6 was used. For copy number determinations JC411 *polA214*(Ts) was used. The strain NFS1 was a spontaneous nalidixic acid-resistant derivative of C600K$^-$ (*gyrA*; resistant to 20 μg of nalidixic acid per ml). NFS1 was mated with JC10-240, giving NFS6, a *recA* derivative of C600K$^-$. Media were as previously described (10).

DNA techniques. Restriction endonucleases and other enzymes were purchased from either Boehringer, New England Biolabs, Inc., or Bethesda Research Laboratories and used at 1 U/μg per h. All restriction digests were carried out in universal buffer, 33 mM Tris-acetate (pH 7.9)–10 mM magnesium acetate [$Mg(OAc)_2$]–66 mM potassium acetate (KOAc)–0.5 mM dithiothreitol at 37°C and terminated either by heating to 65°C for 10 min or by phenol extraction followed by ethanol precipitation. For cloning, 5′ extensions were filled in by using the large fragment of DNA polymerase I (Klenow fragment). Restricted or Bal 31-digested DNA was phenol extracted and ethanol precipitated. Reactions were performed in universal buffer and contained Klenow fragment (1 U), spermidine (to 1 mM), unlabeled deoxynucleoside triphosphates (as needed, 2 nmol), and [α-^{32}P]

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TABLE 1. Bacterial strains

Strain	Markers	Source
C600K$^-$	*thr leu thi galK lac tonA supE*	R.Hayward
NFS1	As C600k$^-$ but *gyrA*	This paper
NFS6	As NFS1 but *recA srl*::Tn*10*	This paper
JC411 *pol*Ats.214	*arg his lac leu mal met rps thy xyl polA*(Ts)	D. Helinski
ED5040	*lac his trp lys gal rpsL* F'*lac*::Tn*A*	J. Maule
Ken10	As C600K$^-$ but *leu*$^+$*envA1*	K. Begg

deoxynucleoside triphosphate (2 pmol; specific activity of >400 Ci/mmol; Amersham Corp.) in a total volume of 10 μl. Before use, the labeled nucleotide was dried, washed in 70% ethanol, and redried. After reaction (37°C, 30 min) unincorporated label was removed by precipitation with ammonium acetate (200 μl of 2.5 M ammonium acetate [NHOAc] plus 800 μl of ethanol; 70°C, 15 min), followed by ethanol precipitation. For Bal 31 digestion the enzyme was used so as to remove 20 base pairs (bp) per min from each end of a linearized plasmid at 30°C. The plasmid DNA was restricted, phenol extracted, ethanol precipitated, and resuspended in 50 μl of buffer containing 12 mM CaCl, 12 mM MgCl, 600 mM NaCl, 20 mM Tris-hydrochloride (pH 7.9), and 1 mM EDTA. Bal 31 enzyme was added, and 10-μl samples were removed after 0, 2, 4, 10, and 20 min to a tube containing 2 μl of 0.5 M EGTA [ethylene-bis(oxy-ethylenenitrile) tetraacetic acid] to stop the reaction. The time sample giving the required deletions was phenol-chloroform-ether extracted and ethanol precipitated before an "end filling" reaction with Klenow fragment. Prephosphorylated synthetic polynucleotide linkers were then attached. DNA used for ligation was ethanol precipitated and dried before use. All ligations were performed in 66 mM Tris-hydrochloride (pH 7.2)–1 mM EDTA–10 mM MgCl–10 mM dithiothreitol–0.1 M ATP. To obtain from agarose gels DNA fragments which were suitable for cloning, the slice of gel containing the DNA was placed in a dialysis bag with 0.5 TE buffer (10 mM Tris-hydrochloride [pH 7.6], 1 mM EDTA [pH 8.0]) and sealed. The DNA was eluted for 2 to 3 h at 100 V and then purified by phenol extraction and ethanol precipitation.

Enzyme assays. Galactokinase assays were performed as described by McKenney et al. (13). For β-lactamase assays a 2-ml sample of a log-phase culture (in L broth) was frozen and kept overnight at 20°C. The samples were thawed and sonicated (in three 20-s bursts with an MSE sonicator at a 5-μm peak to peak amplitude). Cell debris was removed by centrifugation, and the supernatant fluids were assayed for β-lactamase by measuring the initial rate of substrate utilization in a Perkin-Elmer 320 recording spectrophotometer. To 3 ml of 0.1 M phosphate buffer (pH 6.8) was added 10 μl of substrate; (87/312, 1 crystal in 0.1 ml DMS), and the instrument was zeroed before adding 10 μl of sample. From the initial rate of reaction, activity was calculated as the change in optical density at 600 nm (ΔOD_{600}) per minute per OD_{540} (ΔOD_{600}/min per OD_{540}). To obtain an estimate of absolute copy number, results (ΔOD_{600}/min per OD_{540}) were normalized to values obtained from a strain (ED5040) carrying a single chromosomal copy of Tn*A*, and hence of the *bla* gene.

Restriction mapping. A restriction map was prepared by the technique of Smith and Birnstiel (24).

In vitro transcription. To prepare DNA templates for in vitro transcription, the desired band was excised from a 5% polyacrylamide gel and ground to fine particles with a siliconized glass rod in a siliconized Corex tube. Gel particles were suspended in 1 ml of gel elution buffer (0.5 M ammonium acetate, 10 mM Tris-hydrochloride [pH 7.9], 1 mM EDTA) and left overnight at room temperature. The mixture was spun through siliconized glass wool (3,000 rpm; 5 min), and the glass wool was washed with a further 1 ml of gel elution buffer. To the resultant clear liquor was added 3 volumes of cold ethanol, and the DNA was precipitated at −70°C for 15 min before centrifugation (10,000 rpm; 25 min; 0°C). The DNA was resuspended in 200 μl of 0.3 M sodium acetate (NaOAc), phenol extracted twice, ether extracted twice, ethanol precipitated, washed with 70% (vol/vol) ethanol, and dried. For transcription the reaction mix contained (in 10 μl) 20 mM Tris-acetate (pH 7.9), 0.1 mM EDTA, 0.1 mM dithiothreitol, 4 mM Mg (OAc), 200 μM each of ATP, CTP, and GTP, 5 to 10 μCi of [α-^{32}P]UTP (specific activity of >400 Ci/mmol; Amersham), 0.1 to 0.2 pmol of DNA template, 0.1 pmol of RNA polymerase (New England Biolabs), and KCl at 10, 50, 100, 150, or 200 mM. After incubation for 20 min at 37°C, the reaction was quenched with 100 μl of 0.3 M NaOAc–1 mM EDTA–carrier tRNA (0.5 mg/ml; Sigma Chemical Co.) and precipitated with 250 μl of ethanol. RNA was dried and resuspended in 6 μl of Sanger dye. Of this, 3 μl was heat treated at 90°C for 5 min and loaded onto a 5% acrylamide–7 M urea gel. Gels were run until the bromophenol blue marker had reached three-fourths the length of the gel, fixed in 10% glacial acetic acid (HOAc)–1% glycerol (5 min), and washed in water (5 min) before drying down onto Whatman 3-mm paper. Autoradiography was at −70°C with flash-sensitized X-ray film (Cronex 4; DuPont Co.) and an intensifying screen (Fast Tungstate; Ilford). Two transcription templates were used to produce mRNA of known size: (i) the spot 42 gene of *E. coli* cloned into a pBR325 derivative to give pCJ32 (the gene is isolated as a 302-bp *Hin*dIII fragment which gives a 109-base transcript [7]) and (ii) the *lac* promoter-operator region cloned into pBR325 to give pGL101, (the region is isolated as a 95-bp *Eco*RI-*Pvu*II fragment and gives a 38-base mRNA [2]). Both plasmids were kindly provided by Barbara Newman.

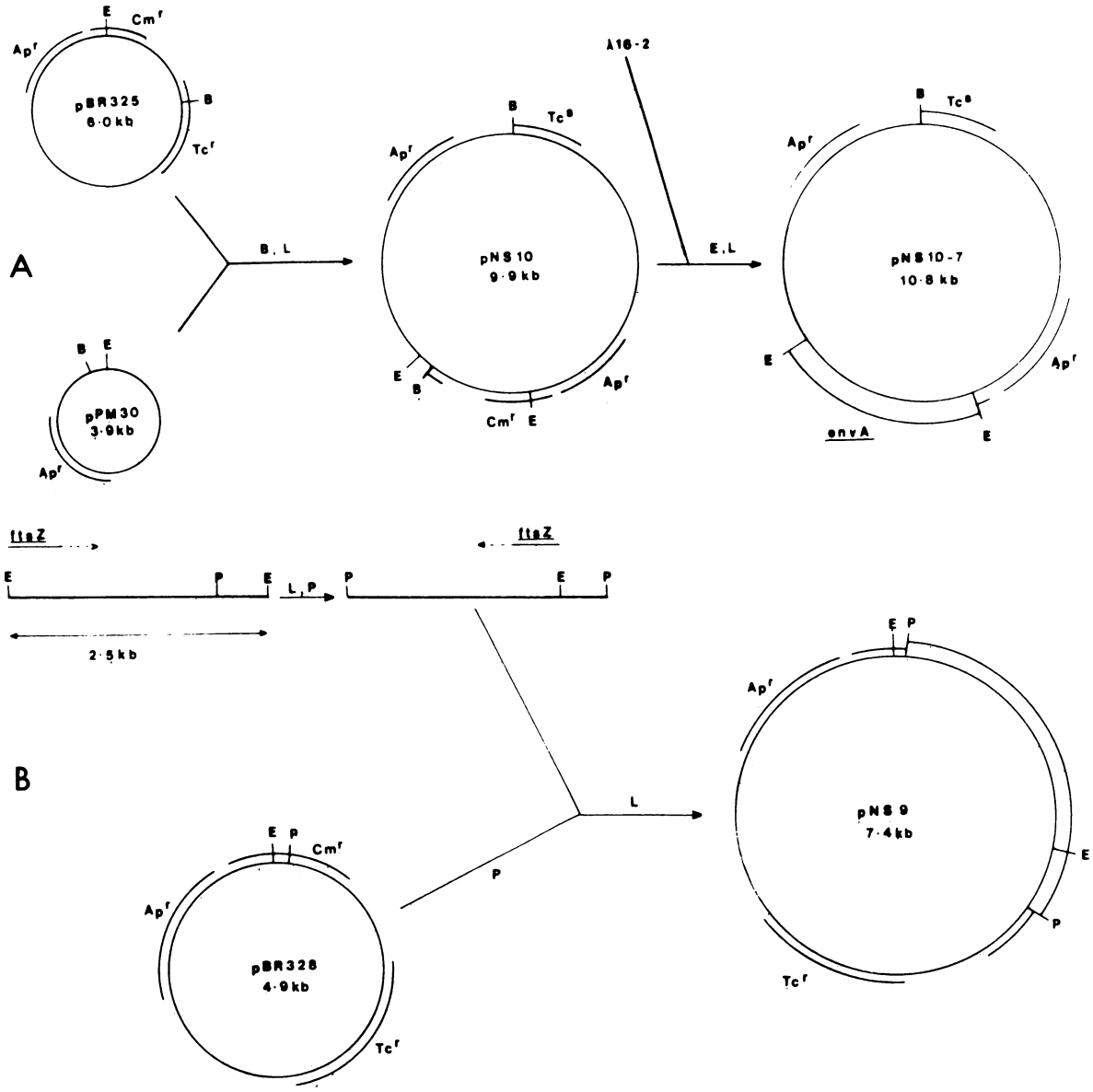
RESULTS

Cell killing by multiple copies of a 2.5-kb *Eco*RI fragment involved in cell division. A 2.5-kilobase (kb) *Eco*RI DNA fragment bearing the *E. coli envA* gene has been cloned on lambda transducing phages (λ*envA*, λ16-2, λJFL40 [10, 11]). Lysogens of these phages have a single extra copy of the cloned genes. We decided to clone this fragment on multiple-copy plasmids to investigate the structural organization of its genes and to obtain direct gene fusion evidence for the directions of transcription.

*Eco*RI-digested λ16-2 DNA (10) was ligated with *Eco*RI-digested pBR328 (4) and transformed into c600K$^-$. Transformant clones were screened for insertions into the chloramphenicol gene (Cm^s Ap^r Tc^r). More than one representative of each class of possible recombinant was obtained, with the exception of those carrying the 2.5-kb *Eco*RI fragment. Furthermore, repeating this experiment by using the 2.5-kb *Eco*RI fragment purified from an agarose gel failed to produce any pBR328 derivatives with the desired

insert. Since other *Eco*RI fragments from λ16-2 could be cloned, whereas the *envA* fragment could not be, this suggested that the 2.5-kb *Eco*RI fragment could not be maintained in the cell at high copy number.

To demonstrate this more clearly we made use of a novel plasmid vector in which we could vary the copy number and study the effect on the cell of high numbers of copies of cloned genes. Replication of ColE1 plasmids requires the product of the *polA* gene (8), whereas in pSC101-derived plasmids replication is *polA* independent (25). In a *polA*(Ts) strain, a ColE1 plasmid can replicate at 30°C but not at 42°C, whereas pSC101-derived plasmids can replicate at either temperature. Initiations from the pSC101 origin are inhibited at a lower copy number than those from the ColE1 origin, so that at 30°C the cointegrate should attain the copy number of ColE1 and be maintained as such (5). At 42°C the cointegrate should be maintained at a low copy number under the control of the pSC101 replication origin. The two plasmids chosen for construction of the cointegrate vector were the pSC101-derived replicon pPM30 (14) and the ColE1-related plasmid pBR325 (4). Figure 1A shows the construction of the cointegrate vector pNS10 and its *envA*-containing derivative pNS10-7. Beta-lactamase assays performed on both pNS10 and its parents in a *polA*(Ts) strain demonstrate that the copy number of the cointegrate does indeed increase as the temperature is lowered (Table 2). Figure 2 shows that the fraction of cells carrying the plasmid pNS10-7 (containing the 2.5-kb *envA* fragment) decreases over successive generations at both 30 and 42°C but at a much greater rate when this plasmid is being maintained at the higher copy number (30°C). The control plasmid pNS10 is not lost appreciably at either temperature. Thus, using a variable copy number plasmid, we demonstrated that a 2.5-kb *Eco*RI fragment containing the *envA* gene is not maintained in multiple copies, although it can be maintained at lower copy numbers (10). This might be attributed either to a deleterious effect of



excess *envA* protein or to titration of an essential regulatory molecule by the cloned DNA itself.

Initial cloning and restriction mapping of the 2.5-kb *Eco*RI fragment. With an *Eco*RI-*Pvu*II double digest of λ16-2 it was possible to show that the 2.5-kb *Eco*RI fragment was cut by *Pvu*II. The 2.5-kb *Eco*RI fragment was purified from an agarose gel and subsequently ligated on itself to form a circular molecule. This was then cut with *Pvu*II to form a 2.5-kb "inverted" fragment which was ligated into the *Pvu*II site of the pBR328 chloramphenicol gene to give pNS9 (Fig. 1B), a plasmid which fails to complement the *envA* mutation in Ken10, indicating that cutting at the *Pvu*II site disrupts the *envA* gene. An *Eco*RI digest of pNS9 indicates that this enzyme cuts ca. 500 bp from one end of the inverted fragment, so unless the *envA* and *ftsZ* genes overlap, this implies that *Pvu*II cuts 500 bp from the righthand end of the 2.5-kb *Eco*RI fragment. The 2.5-kb *Pvu*II fragment of pNS9 was transferred to the *Sma*I site of pK01 (13) to give pNS11, and a 3.3-kb *Hin*dIII-*Ava*II fragment containing this DNA was used to map restriction sites by the technique of Smith and Birnstiel (24). Figure 1C shows the restriction sites relevant to this paper, both within the 3.3-kb *Hin*dIII-*Ava*II fragment and within the reoriented 2.5-kb *Eco*RI fragment.

***envA* product is responsible for failure of cells to maintain the 2.5-kb fragment at a high copy number.** Since we demonstrated a copy number effect due to the cloned insert, it was important to determine its cause. Given the positions of the two *Cla*I sites and three *Pst*I sites within the fragment, it was possible to make two nonoverlapping deletions.

(i) *Cla*I deletion. The plasmid pNS10-7 contains two *Cla*I sites, both within the 2.5-kb *Eco*RI insert. This plasmid was restricted with *Cla*I, ligated in dilute solution, and trans-

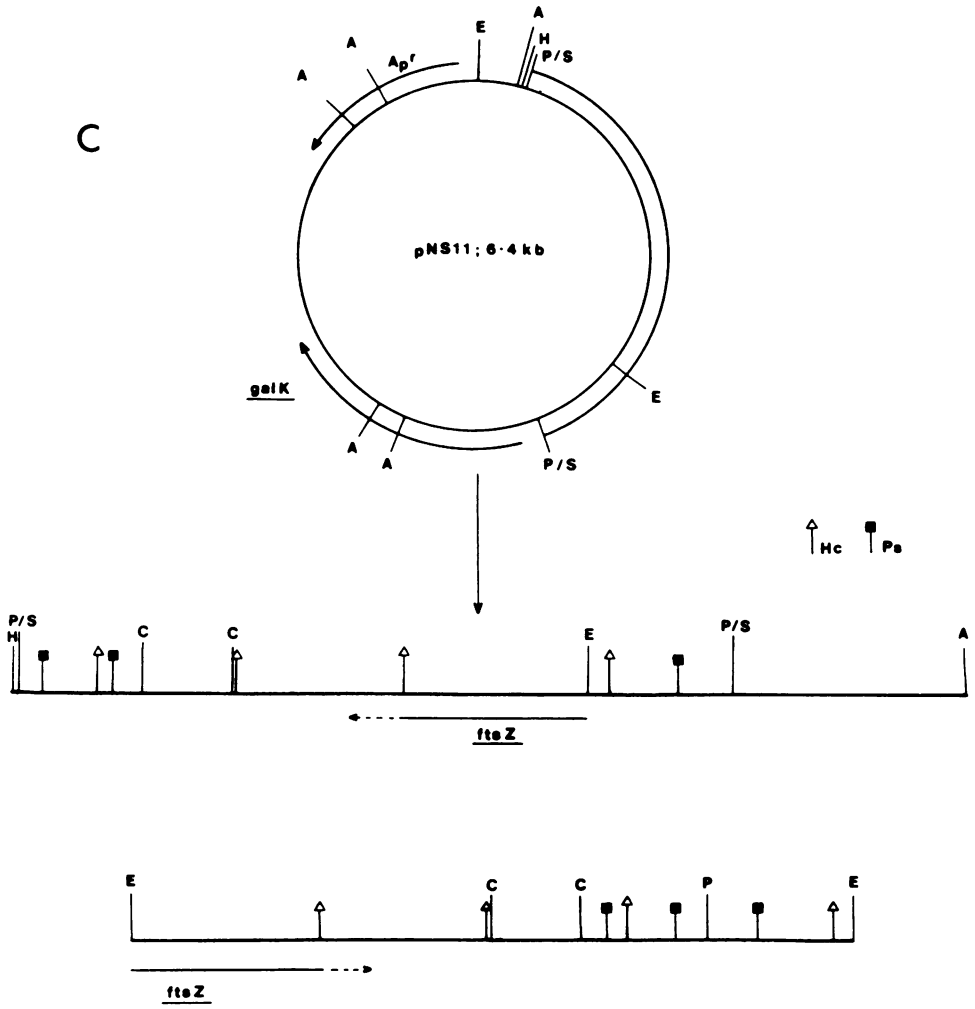


FIG. 1. The variable-copy-number vector pNS10 was constructed by ligating an equimolar mixture of *Bam*HI-digested pBR325 (4) and pPM30 (14). The mixture was transformed into JC411 *polA214*(Ts) selecting for growth on chloramphenicol (50 μg/ml) at 42°C. The 2.5-kb *Eco*RI fragment was subcloned from λ16-2(10) into pNS10, again transforming into JCHI *polA214*(Ts) but this time selecting for growth on ampicillin (50 μg/ml) at 42°C. Abbreviations: E, *Eco*RI; B, *Bam*HI; L, T4 DNA ligase. (B) The 2.5-kb *Eco*RI fragment from λ16-2 (10) was purified, ligated upon itself, restricted with *Pvu*II (P), and cloned into the *Pvu*II site of pBR328 (4) to give pNS9. (C) The plasmid pNS11 was constructed by cloning the 2.5-kb *Pvu*II fragment from pNS9 into the *Sma*I (S) site of pK01. pNS11 was restricted with *Hin*dIII (H) and treated with calf intestinal phosphate, and the 5′ ends were labeled with polynucleotide kinase (24). Subsequent *Ava*II (A) digestion allowed isolation of a 3.3-kb singly end-labeled fragment from a 20-bp labeled fragment and 1,861-, 902-, 222-, and 167-bp unlabeled fragments. The 3.3-kb fragment was used to map restriction sites by the technique of Smith and Birnstiel (24). Shown are restriction sites within the 3.3-kb *Hin*dIII-*Ava*II fragment. At the bottom the sites within the *envA* fragment are shown in its correct chromosomal orientation. Hc, *Hin*cII; C, *Cla*I; Ps, *Pst*I.

TABLE 2. Copy number determinations[a]

Plasmid	ΔOD_{600}/min per $OD_{540} \times (10^2)$[b] at:		Estimated relative copy no. at	
	30°C	42°C	30°C	42°C
pBR325	1.575	ND[c]	21	ND
pPM30	0.31	0.365	4.1	4.9
pNS10[d]	1.81	0.65	12.1	4.3

[a] Cells were grown in LB broth plus ampicillin (50 μg/ml) at 42°C overnight (except pBR325, grown at 30°C) in the strain JC411 *polA*ts.214 and subcultured 1:100 into nonselective medium. Growth was continued for at least 20 generations when samples were taken. β-Lactamase assays were performed as described in the text.

[b] Values are normalized against the *bla* activity of ED5040 grown at 30°C to give the relative copy number. ΔOD_{600}/min per $OD_{540} = 0.075 \times 10^2$; copy number = 1. Standard error, ± 10%.

[c] ND, Not determined.

[d] Since pNS10 contains two copies of the *bla* gene, the ΔOD_{600}/min per OD_{540} values for this plasmid must be divided by two.

formed into JC411 *polA214*(Ts) at 42°C. One recombinant was designated pNS26 and was shown to have a 300-bp deletion within the *Eco*RI insert.

The plasmid pNS26 was able to be maintained stably in C600K$^-$ *polA*$^+$ and, in addition, did not complement the *envA* mutation in Ken10. The 2.2-kb *Eco*RI fragment was transferred to the *Eco*RI site within pK01 to give pNS31 and oriented such that *ftsZ* and *galK* transcribed in the same direction (Fig. 3).

(ii) *Pst*I deletion. Since pNS10-7 contained two *Pst*I sites within the vector, a similar strategy to that used for constructing pNS26 could not be used for the *Pst*I deletion. Instead, the purified 2.5-kb *Eco*RI fragment was ligated on itself and subsequently cleaved with *Pst*I. The inverted *Pst*I fragment was cloned into the *Pst*I site of the *bla* gene of pBR325 (4) to give pNS4. This fragment was shown to be 2 kb in length, indicating the loss of 500 bp of the original fragment. The 2-kb *Pst*I fragment of pNS4 was then purified and ligated on itself as before. This was cleaved with *Eco*RI

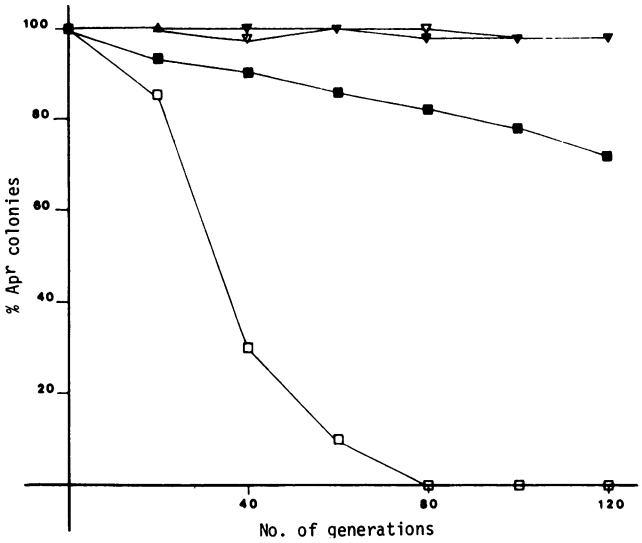


FIG. 2. JC411 *polA*ts.214 cells, freshly transformed with either pNS10 or pNS10-7, were grown overnight in L broth plus ampicillin (50 μg/ml) at 30°C and then subcultured 1:100 into nonselective media at both 30 and 42°C. After intervals of 20 generations samples were plated onto L broth and subsequently patched onto LB plates containing 50 μg of ampicillin per ml. Symbols: ▽, pNS10 at 30°C; ▼, pNS10 at 42°C; ■, pNS10-7 at 42°C; □, pNS10-7 at 30°C.

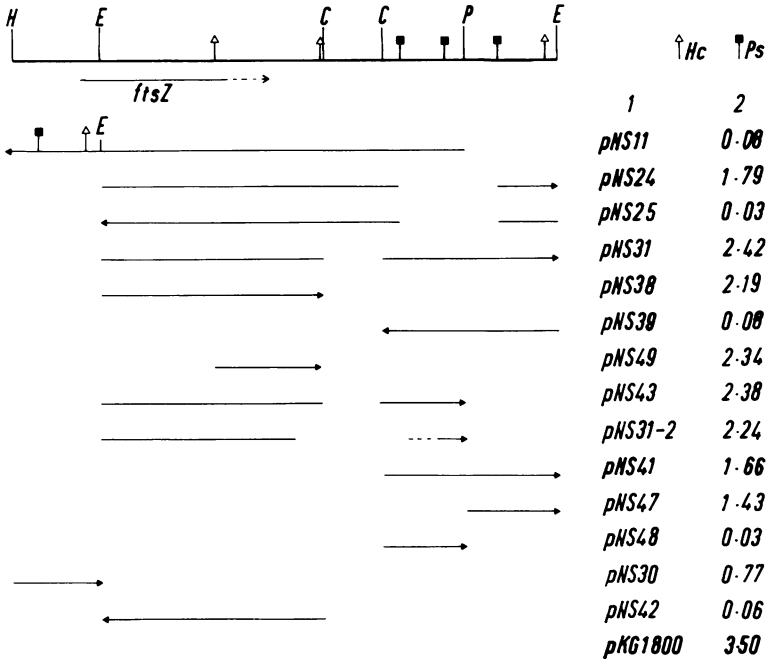


FIG. 3. Localization of the *envA* promoter and the extent of cloned fragments and their orientation relative to the galactokinase gene (arrowheads). Column 1 indicates plasmids as referred to in the text. The plasmid pKG1800 contains the *E. coli* galactose operon promoter (*galP*, [13]). Column 2 indicates relative galactokinase levels determined as previously described (13), initially expressed as nanomoles of galactose phosphorylated per minute per/OD_{650}. For each plasmid a minimum of four separate extracts were prepared and assayed twice, and the results were normalized. The observed variation was less than 10% in all cases. β-Lactamase assays showed no significant variation in plasmid copy number. (The plasmid pNS11 has the righthand *Pvu*II-*Eco*RI segment transposed to the left end as shown.) Hc, *Hin*cII; Ps, *Pst*I; H, *Hin*dIII; E, *Eco*RI; C, *Cla*I.

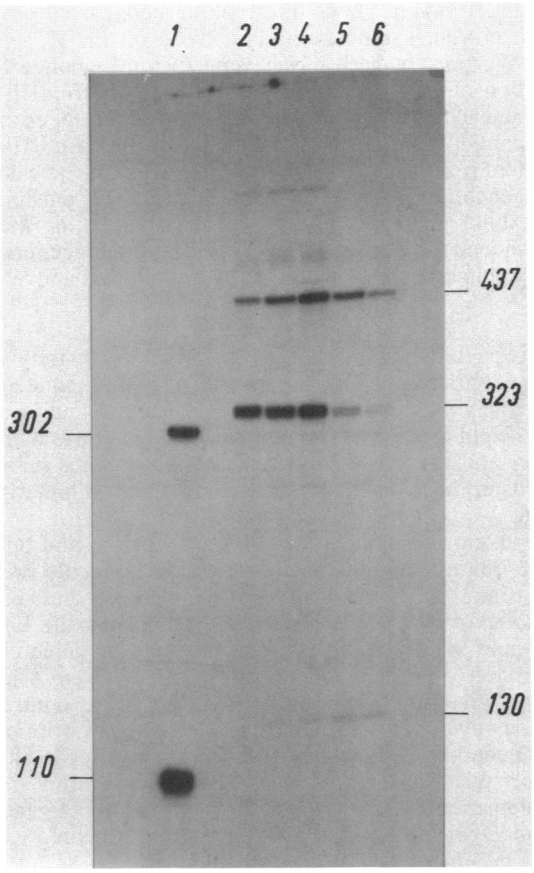


FIG. 4. Autoradiogram of an in vitro transcription of the 590-bp *Hin*cII-*Hin*cII fragment. Track 1 is a transcription primed with spot 42 DNA. (150 mM KCl). Tracks 2 through 6 are primed with DNA of the 590-bp fragment and contain 200, 250, 100, 50, and 10 mM KCl, respectively.

to produce a 2-kb *Eco*RI fragment, i.e., the 2.5-kb *Eco*RI *envA* fragment with a 500-bp *Pst*I deletion. This was inserted into the *Eco*RI site of pK01 in both orientations to give pNS24 and pNS25 (Fig. 3). pNS24 was oriented such that *ftsZ* and *galK* read in the same direction. Both *Pst*I deletions were stably maintained at multiple copies and did not complement the *envA* mutation in Ken10.

As already stated, the only complete gene known to exist on the 2.5-kb *Eco*RI fragment is the *envA* gene. That two independent, nonoverlapping deletions, a 300-bp *Cla*I deletion and a 500-bp *Pst*I deletion, are both able to be maintained in high copy number and do not complement the *envA* mutation suggests very strongly that overproduction of the *envA* protein itself is lethal to the cell.

Direction of transcription of *envA* and location of the *envA* promoter. If the *envA* gene is transcribed from right to left, as suggested by Lutkenhaus and Wu (12), then we might expect pNS11 to show promoter activity. None was detected (Fig. 3). The two 500-bp *Pst*I deletion derivatives pNS24 and pNS25 show the existence of a strong promoter in one orientation but not in the other (Fig. 3). If *envA* reads from left to right in pNS25, then we might expect that the promoter has been removed. The strong promoter(s) detected on pNS24 might be due to *envA* reading left to right, to a new gene between *envA* and *secA*, or to the sum total of both. A similar argument applies to pNS31. To locate the promoters within the 2.5-kb *Eco*RI fragment, the two *Eco*RI-*Cla*I fragments from pNS31 were cloned into the *Eco*RI and *Cla*I sites of pBR322, giving pNS34 and pNS35, which contain the 0.95- and 1.25-kb *Eco*RI-*Cla*I fragments, respectively. These fragments were then excised again as *Eco*RI-*Hin*dIII fragments and cloned in either orientation into pK01 or pK06 (13) to give pNS38 and pNS39 (Fig. 3). The net effect of this is to add a 6-bp linker to the *Cla*I site. If the *envA* gene transcribed from right to left, then we would expect pNS39 to exhibit promoter activity. None was observed (Fig. 3). This, taken together with the evidence presented for pNS11, shows that the *envA* gene does not initiate from within this 0.95-kb region. The plasmid pNS38 clearly shows promoter activity which can be attributed to the *envA* gene reading left to right (i.e., clockwise on the *E. coli* genetic map). From the approximate size of the *envA* product (12) and the probable extent of *ftsZ* (12), the *envA* promoter was expected to be on a 590-bp *Hin*cII-*Hin*cII fragment internal to the insert in pNS38. This piece of DNA was subcloned into the *Sma*I site of pK01 (13) and shown to have promoter activity (pNS49; Fig. 3). In vitro transcription of the 590-bp *Hin*cII-*Hin*cII fragment showed a specific mRNA of 323 bases with a high transcriptional salt optimum (Fig. 4). To confirm the location of the *envA* promoter, the exonuclease Bal 31 was used to generate deletions extending leftward from the *Cla*I site in pNS31. To prevent digestion of vector sequences and ensure that the inserted fragment only contained the *envA* promoter, pNS43 was constructed from

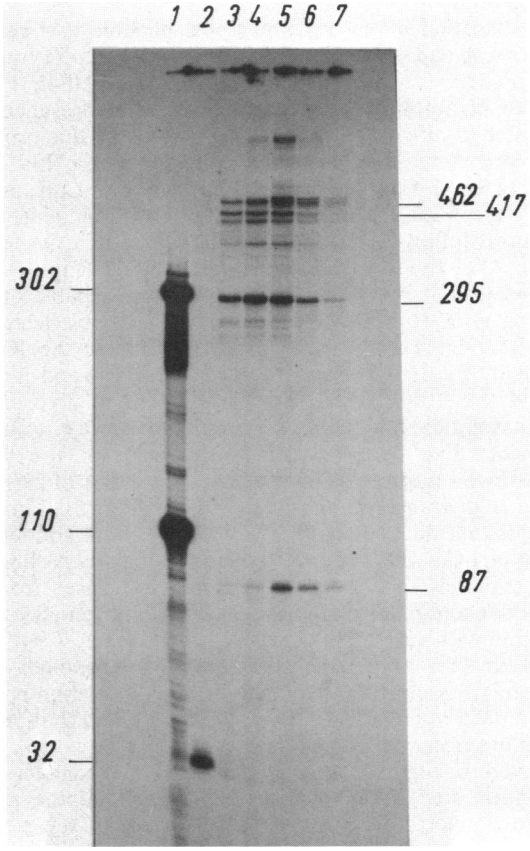


FIG. 5. Autoradiogram of an in vitro transcription of the 310-bp *Pvu*II-*Eco*RI fragment. Tracks 1 and 2 are primed with spot 42 and *lac* DNA, respectively, with 150 mM KCl. Tracks 3 through 7 are primed with the 510-bp fragment and contain 200, 150, 100, 50, and 10 mM KCl, respectively.

pNS31 by *Pvu*II-*Sma*I double digestion, followed by ligation in dilute solution (Fig. 3). The 450-bp-downstream *Cla*I-*Pvu*II fragment does not contain a promoter (see below). The plasmid pNS43 was linearized with *Cla*I, digested with Bal 31, calibrated to remove 20 nucleotides per min, and treated with Klenow fragment to increase the proportion of blunt ends. Prephosphorylated synthetic polynucleotide *Bam*HI linkers were then attached, and the DNA mixture transformed. The recombinant with the largest deletion (140 bp) was designated pNS31-2 (Fig. 3). This deletion failed to remove the *envA* promoter, which must therefore be located at least 140 bp leftward from the *Cla*I site in pNS43. A *Hin*cII-*Bam*HI digest of pNS31-2 allowed the isolation of a 450-bp *Hin*cII-*Bam*HI fragment which was used for in vitro transcription. Such a transcription gives an mRNA of 184 bases with a high transcriptional salt optimum (data not shown). This is consistent with the 323-base transcript obtained for the 590-bp *Hin*cII-*Hin*cII fragment and suggests that the 323-base transcript runs from left to right. Since this mRNA is the most likely candidate for an *envA* transcript and since in vivo fusions to *galK* indicate promoter activity, the *envA* gene must read clockwise on the *E. coli* genetic map and probably starts 323 bp leftward from the leftmost *Cla*I site.

The 437-bp transcript shown in Fig. 4 has a lower salt optimum but might also be a specific transcript. It is therefore interesting to note that a 214-bp transcript is also produced from the 450-bp *Hin*cII-*Bam*HI sequence in vitro. The possibility of a second *envA* promoter cannot therefore be excluded.

Identification of the promoter of a possible new gene between *envA* and *secA*. The 0.95-kb *Hin*dIII-*Eco*RI fragment from pNS34 was inserted into pK06 to give pNS41 (Fig. 3). In vivo galactokinase levels indicated the presence of a promoter on this fragment (Fig. 3). To locate this promoter more precisely, two deletion derivatives of pNS41 were constructed. First, pNS41 was restricted with *Cla*I, and the ends were filled in by using the Klenow fragment. *Pvu*II digestion, followed by blunt-end ligation, then deleted the 450-bp *Cla*I-*Pvu*II fragment to give pNS47 (Fig. 3). This plasmid was shown to contain a promoter capable of transcribing *galK* (Fig. 3). Second, pNS41 was restricted with *Eco*RI, and the ends were filled in by using the Klenow fragment. *Pvu*II digestion, followed by blunt-end ligation, deleted the 510-bp *Pvu*II-*Eco*RI fragment to give pNS48. This plasmid did not contain a promoter capable of transcribing *galK* (Fig. 3). In vitro transcription of the 510-bp *Pvu*II-*Eco*RI fragment shows a specific mRNA of 295 bases with a high transcription salt optimum (Fig. 5). This data suggests that the 510-bp *Pvu*II-*Eco*RI fragment contains the promoter of a new gene (*X*) which lies between *envA* and *secA* and that this promoter initiates 295 bases from the *Eco*RI site and reads clockwise on the *E. coli* genetic map in common with all other genes of this cluster. Oliver and Beckwith (21) first noted that there was enough space between *envA* and *secA* to code for a 30-kilodalton protein. Such a protein has not yet been identified, and the function of this promoter remains unknown.

Relative strengths of the *envA* and gene *X* promoters. The *envA* promoter is ca. 78% of the strength of the *E. coli* galactose operon promoter (*gal*P), whereas the gene *X* promoter is ca. 55% of *gal*P. The galactose promoter may be regarded as a medium-strength *E. coli* promoter (22)

Direct gene fusion evidence for the direction of transcription of *ftsZ*. The *ftsZ* gene has been located between *ftsA* and *envA* by the use of λ transducing phages (11). The direction of transcription has been inferred indirectly, from phage-encoded protein synthesis, as clockwise on the *E. coli* map (12). We present here direct gene fusion evidence for the direction of transcription of *ftsZ*. A 480-bp *Hin*dIII-*Eco*RI fragment containing an *ftsZ* promoter has been cloned upstream of *galK* (pNS30; (24a). The 1.25-kb *Hin*dIII-*Eco*RI fragment from pNS35 was cloned upstream of the galactokinase gene in pK06 to give pNS42 (Fig. 3). This plasmid does not exhibit promoter activity (Fig. 3), indicating that *ftsZ* does indeed transcribe clockwise on the *E. coli* genetic map, a finding in agreement with that of Lutkenhaus and Wu (12).

DISCUSSION

This paper reports a detailed transcriptional study of part of a remarkable *E. coli* gene cluster. We concentrated specifically on the cell separation gene *envA* and its immediate neighbors. Both the positions of these genes within the cluster and the precise locations of these genes within the subcluster have been determined and are summarized in Fig. 6.

By using a combination of gene fusion and in vitro transcription techniques, it was possible to locate precisely the promoters of the *ftsZ* and *envA* genes and also the promoter of a possible new gene which must lie between *envA* and *secA* (gene *X*). With the use of galactokinase expression vectors, we demonstrate that the *envA* gene is transcribed clockwise on the *E. coli* map, contrary to previous evidence (12). By using in vitro transcription, the *envA* gene has been shown to have a promoter located ca. 930 bp from the *Eco*RI site which lies within *ftsZ*. A complementation analysis indicates that the *envA* gene must extend leftward at least as far as a *Pvu*II site located 1,070 bp away. Assuming the average molecular mass of an amino acid in *E. coli* to be 110 daltons, then this is sufficient to code for a 40-kilodalton protein. This is larger than the molecular mass suggested for *envA* protein (31 kilodaltons [12]). A promoter reading left to right was detected within the 510-bp *Pvu*II-*Eco*RI fragment which overlaps with the end of *envA*. Between *envA* and *secA* there is ca. 900 bp of unassigned DNA, sufficient to code for an *E. coli* protein of average size. This promoter may belong to a hitherto unknown gene, designated gene *X*. As determined by in vitro transcription, gene *X* has a promoter which initiates 295 bases from the *Eco*RI site.

Again, by using the galactokinase expression vectors of McKenney et al. (13), the *ftsZ* gene has been shown to transcribe from left to right, in agreement with the indirect data of Lutkenhaus and Wu (12). In vivo determination of galactokinase levels in two different gene fusions (pNS30 and pNS54) indicates that *ftsZ* must have at least two promoters (24a). The upstream genes *ftsA* (12), *ftsQ* (D. J. Kenan, N. F. Sullivan, and A. C. Robinson, unpublished data), *murC* (12), *ddl* (12), and *ftsI* (16) all appear to transcribe clockwise on the *E. coli* map, as does the downstream gene *secA* (21). An unassigned 48-kilodalton polypeptide to the left of *murC* (12) may be the product of the *murG* gene (23), also transcribing in a clockwise direction.

This cluster exhibits a number of interesting transcriptional features. First, all genes from *murC* through to *envA* and probably *secA* are contiguous, with no intervening "spacer" DNA, i.e., this region (inclusive of the region covered by gene *X*) is greater than 90% coding. Neidhardt et al. (17) calculated that on the average 70% of the coding capacity was used in the inserted fragments in selected Clark-Carbon plasmids, so by comparison the 2-min region appears tightly packed. Second, all genes tested from *ftsI*

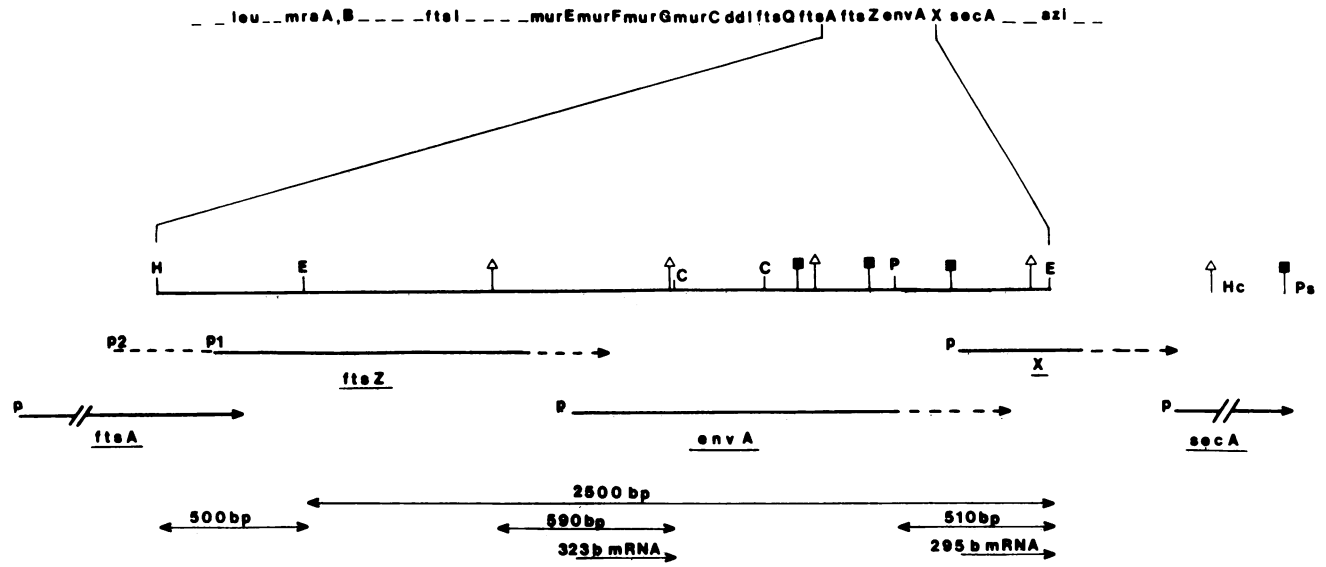


FIG. 6. Summary, showing the genomic organization in the neighborhood of the *envA* gene. Abbreviations are as for Fig. 1 and 3.

down to *secA* transcribe in the same direction, i.e., clockwise on the *E. coli* map. Third, all genes tested have their own promoters and can be independently expressed. As yet no strong transcriptional terminators have been found between genes (D. Kenan, unpublished data) and apart from an overlap of the *ftsZ* regulatory region with the coding sequence of *ftsA* (24a), there is no evidence that any of these genes interact in any way. Thus, the genes of this region do not conform to the classical definition of the operon, where genes coding for proteins of mutually dependent function are both cotranscribed and coregulated by transcription from a single promoter. An analysis of in vivo transcripts from this cluster will prove a most interesting study for the future.

Excluding a regulatory interaction, it is difficult to see why these genes should be clustered. As first noted by Miyakawa et al. (15), there appears to be no simple relationship between the sequence of reactions involved in peptidoglycan biosynthesis and gene location. A similar argument applies to *ftsQ*, *ftsA*, and *ftsZ*, where two genes required throughout septation, *ftsQ* (3) and *ftsZ* (27), sandwich a gene involved in late stages of septation (*ftsA*) (6). The order of genes within this cluster appears to be conserved, at least within the members of the family *Enterobacteriaceae* (unpublished data). This suggests that these genes may have arisen in the remote past by gene duplication and subsequent divergence of function in situ.

The 2.5-kb *Eco*RI fragment containing the *envA* gene could only be cloned in multicopy if the *envA* gene was disrupted, as in pNS9. This was demonstrated more clearly by inserting this fragment into a *polA*-dependent, variable-copy-number vector and showing increasing segregation with increasing copy number. Construction of two independent, nonoverlapping deletions of the 2.5-kb *Eco*RI fragment, both of which could be maintained in multiple copy and neither of which complemented *envA*, suggested that the *envA* protein itself (rather than some noncoding stretch of DNA) was deleterious to the cell. The *envA* gene is associated with the *N*-acetylmuramyl-L-alanine amidase enzyme, and overproduction of this enzyme may well affect the structural integrity of the sacculus.

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