

## Sequences of the *envM* gene and of two mutated alleles in *Escherichia coli*

HELMUT BERGLER, GREGOR HÖGENAUER and FRIEDERIKE TURNOWSKY\*

Institut für Mikrobiologie, Karl-Franzens-Universität Graz, Universitätsplatz 2, A-8010 Graz, Austria

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The nucleotide sequence of the *Escherichia coli envM* gene was determined. It codes for a protein of 262 amino acids. The sequences of the *E. coli* and *Salmonella typhimurium* EnvM proteins are 98% identical. Gene *envM* is preceded in *E. coli* by a 43-nucleotide-long structural element, termed 'box C', which occurs in several *E. coli* operons between structural genes. This sequence element is totally absent in *S. typhimurium*. Gene *envM* was mapped at coordinate position 1366.8 kb of the physical map of Kohara *et al.* (Cell, 1987, 50, 495–508). As in *S. typhimurium*, a Gly for Ser exchange at position 93 of the amino acid sequence leads to a diazaborine-resistant *E. coli* phenotype. A Ser for Phe exchange at position 241 of the EnvM protein results in a temperature-sensitive growth phenotype. Comparison of the EnvM amino acid sequence with sequences available in databases showed significant homology with the family of short-chain alcohol dehydrogenases.

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### Introduction

The *envM* gene from *Salmonella typhimurium* has previously been cloned and sequenced (Turnowsky *et al.*, 1989). The gene product is essential for growth as it plays an important role in the biosynthesis of phospholipids and lipopolysaccharides by *Enterobacteriaceae* (Högenauer & Woisetschläger, 1981). The protein is the target of a group of antibacterial drugs, the diazaborines, the important structural element of which is a heterocyclic 1,2-diazine ring containing boron as a third hetero atom (Grassberger *et al.*, 1984).

One allelic form of *envM* with a temperature-sensitive (ts) growth phenotype in *E. coli* was described by Egan & Russell (1973). The effects on lipid biosynthesis and on cell morphology in this mutant at the nonpermissive temperature were comparable to those caused by drug treatment. The ts-phenotype in *E. coli* could be complemented by introducing the corresponding wild-type (wt) gene from *S. typhimurium* (Turnowsky *et al.*, 1989). Furthermore, wild-type *E. coli* cells became diazaborine-resistant after transformation with a plasmid carrying the resistant allelic form of the gene isolated from a drug-resistant *S. typhimurium* mutant.

We have cloned and sequenced the corresponding *E.*

*coli* wild-type gene from a recombinant phage of the collection described by Kohara *et al.* (1987), which represents the entire *E. coli* chromosome. The correct recombinant phage was chosen on the basis of P1 transduction experiments, which allowed the precise localization of the gene at min 28.5 (Turnowsky *et al.*, 1989).

When we analysed sequences isolated from either a drug-resistant *E. coli* mutant or from the *E. coli* ts-mutant of Egan & Russell (1973), we identified in each case single nucleotide exchanges which result in altered amino acid sequences. These analyses are described in the present communication.

### Methods

**Bacterial strains, phages and plasmids.** Strains, phage and plasmids are listed in Table 1. *E. coli* cells were grown in either LB-medium (Miller, 1972) or 2 × YT medium (Sambrook *et al.*, 1989), unless otherwise stated. Dia<sup>R</sup> designates diazaborine resistance. Antibiotics were added when needed at the following concentrations: diazaborine 20 µg ml<sup>-1</sup>, epicillin 100 µg ml<sup>-1</sup>.

**Chemicals and enzymes.** Epicillin was from Biochemie (Kundl, Austria). Radiochemicals were obtained from Amersham or from Dupont-NEN. Enzymes for DNA work were from Boehringer, BRL or New England Biolabs. For oligonucleotide synthesis, the reagents were from Applied Biosystems.

**DNA work.** Recombinant techniques were performed by the methods of Ausubel *et al.* (1987), Sambrook *et al.* (1989), or by following the protocols of the manufacturers. DNA from the recombinant phage 18B6 (Kohara *et al.*, 1987) was isolated according to Silhavy *et al.* (1984). Single-stranded DNA was prepared from recombinant

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\* Author for correspondence. Tel. 316 380 5620; fax 316 381548.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M97219.

Table 1. Bacterial strains, phage, and plasmids

Strain/phage/plasmid	Relevant genotype/characteristics	Source*/reference
<i>Escherichia coli</i>		
XL1-Blue2	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> (F' <i>proAB lac<sup>R</sup>ZΔM15 Tn10[Tet<sup>R</sup>]</i> )	Stratagene
MC1061	<i>araD 139 Δ(ara-leu)7697 ΔlacX74</i> <i>galU galK hsdR2 hsdM<sup>+</sup> rpsL</i>	Public Health Laboratory <sup>a</sup> Casabadan & Cohen (1980)
JP1111	Hfr <i>galE45 λ<sup>-</sup> envM392(ts) relA1 spoT1</i>	B. Bachmann <sup>b</sup> / Egan & Russell (1973)
D10-1	F <sup>+</sup> <i>met Dia<sup>R</sup></i>	Turnowsky <i>et al.</i> (1980)
BK 80	<i>leu-277 λ<sup>-</sup> IN(rmD-rmE)1 envM392(ts)</i>	
BK 41	<i>leu-277 λ<sup>-</sup> IN(rmD-rmE)1</i>	
Phage		
M13 K07	Km <sup>R</sup> p15A ori	J. Vieira <sup>c</sup> / Vieira & Messing (1987)
Plasmid		
pSP65	Ap <sup>R</sup>	Sandoz collection <sup>d</sup> / Melton <i>et al.</i> (1984)
pUC118	Ap <sup>R</sup> <i>lac'IPOZ'</i> M13 ori	J. Vieira <sup>c</sup> / Vieira & Messing (1987)
pBR322	Ap <sup>R</sup> Tc <sup>R</sup>	Sandoz collection <sup>d</sup>
pKF403	Ap <sup>R</sup> Dia <sup>R</sup> , 1.45 kb <i>Bam</i> HI fragment ( <i>orf1</i> + <i>envM</i> -Dia <sup>R</sup> from <i>S. typhimurium</i> ) in pBR322	Turnowsky <i>et al.</i> (1989)
pFT405	Ap <sup>R</sup> Dia <sup>R</sup> , 1 kb <i>AccI</i> - <i>Bam</i> HI fragment ( <i>envM</i> -Dia <sup>R</sup> from <i>S. typhimurium</i> ) in pBR322	
pHAP1	Ap <sup>R</sup> , 4.5 kb <i>Eco</i> RI- <i>Bam</i> HI fragment ( <i>envM</i> -wt from <i>E. coli</i> ) in pBR322	
pHAP2	Ap <sup>R</sup> , 4.5 kb <i>Eco</i> RI- <i>Bam</i> HI fragment ( <i>envM</i> -wt from <i>E. coli</i> ) in pUC118	
pHAP6	Ap <sup>R</sup> , 600 bp <i>Pst</i> I fragment from pHAP1 in pUC118	This work
pHAP16	Ap <sup>R</sup> , 1.6 kb <i>Pst</i> I fragment from pHAP1 in pUC118	
pFT617	Ap <sup>R</sup> , 4.5 kb <i>Eco</i> RI- <i>Bam</i> HI fragment ( <i>envM</i> -ts from <i>E. coli</i> ) in pUC118	
pFT632	Ap <sup>R</sup> , 1.9 kb <i>Eco</i> RV- <i>Sal</i> I fragment from pHAP1 in pSP65	
pFT650	Ap <sup>R</sup> Dia <sup>R</sup> , 4.5 kb <i>Eco</i> RI- <i>Bam</i> HI fragment ( <i>envM</i> -Dia <sup>R</sup> from <i>E. coli</i> ) in pUC118	

\* Addresses: *a*, Public Health Laboratory, Porton Down, UK; *b*, B. Bachmann, *E. coli* Genetic Stock Center, New Haven, CT, USA; *c*, J. Vieira, Waksman Institute of Microbiology, Piscataway, NJ, USA; *d*, Sandoz collection, Sandoz Forschungsinstitut, Vienna, Austria.

plasmids derived from pUC118 using the helper phage M13K07 (Vieira & Messing, 1987).

**Cloning procedures.** The general strategies for cloning the wt, the Dia<sup>R</sup> and the ts alleles of the *envM* gene are described in Results. Labelled DNA probes for Southern hybridization and colony hybridization experiments were prepared either by nick-translation of the *Bam*HI fragment of pKF403 (Turnowsky *et al.*, 1989), or by random priming using the 1.6 kb *Pst*I fragment of pHAP1 or the 1 kb *Bam*HI-*AccI* fragment of pFT405 (Turnowsky *et al.*, 1989) as templates. The recombinant plasmids carrying the wt, the Dia<sup>R</sup> and the ts alleles of *envM* were designated pHAP1, pFT650 and pFT617, respectively. Transformations were done by the standard CaCl<sub>2</sub> protocol.

**DNA sequencing.** The sequences of the DNA fragments containing the allelic forms of the *envM* gene were determined in the single-stranded form by the chain-termination method of Sanger *et al.* (1977), with both universal and specific oligonucleotide primers, using a Sequenase kit (United States Biochemicals).

**Growth curves and complementation of the ts-phenotype.** The isogenic strains *E. coli* BK80 and *E. coli* BK41 (Turnowsky *et al.*, 1989) were transformed with the recombinant plasmids pHAP2 and pFT617, as well as with the vector pUC118. The transformants were grown in Trypticase Soy Broth (Merck) at 30 °C to early exponential phase; the cultures were then shifted to 42 °C and the incubation continued for 4.5 h. The optical density at 600 nm was measured at various time points after the temperature shift. In separate experiments, *E. coli*

JP1111 was transformed with subclones of pHAP2, namely pFT632, pHAP6 and pHAP16 (Fig. 1), and the transformants tested for growth in LB medium at 42 °C in overnight cultures.

**Computer-assisted sequence analysis.** The GCG software package of the Wisconsin computer group (Devereux *et al.*, 1984) was used for all sequence analyses. Sequence databases (EMBL, GenBank and Swiss Prot) were screened using the 'wordsearch' program. Multiple sequence alignment was done with the program 'pile up'.

## Results and Discussion

### Cloning the *E. coli* wild-type *envM* gene

The recombinant phage 18B6 from the collection described by Kohara *et al.* (1987) carries the region around min 28.5 of the *E. coli* chromosome. Based on previous work (Turnowsky *et al.*, 1989), this region was expected to contain the *envM* gene. DNA from the phage was prepared and cleaved with various restriction endonucleases, either alone or in combination. The fragments obtained were separated by agarose gel electrophoresis and analysed by Southern blotting. As a

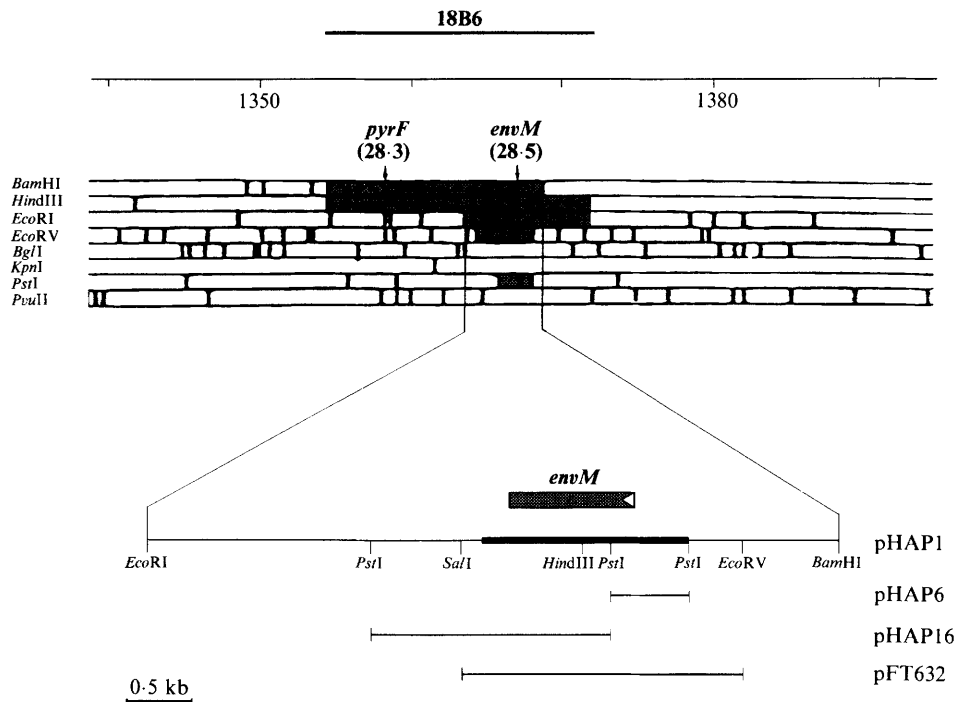


Fig. 1. Section of the physical map from 1340 to 1390 kb of the *E. coli* chromosome as described by Kohara *et al.* (1987). The locations of the *envM* and the *pyrF* genes are indicated: they map at min 28.5 and min 28.3, respectively. 18B6 designates the recombinant phage carrying the investigated region of the *E. coli* chromosome. The DNA restriction fragments that hybridized with a probe containing the *envM* gene from *S. typhimurium* are shown as hatched areas. The restriction enzymes *BglI*, *KpnI* and *PvuII* were not tested. Below, the restriction map of the 4.5 kb *EcoRI*–*BamHI* fragment in pHAP1 is shown. The sequenced region is marked as a thick line. The location of *envM* in the insert is shown as a hatched bar above the restriction map with the orientation of transcription indicated as an arrowhead. Underneath, the inserts of relevant subclones, i.e. pHAP6, pHAP16 and pFT632, are shown.

probe, a DNA fragment from *S. typhimurium* (pKF403) containing the entire *envM* gene was used. The fragments which gave signals after autoradiography are shown in Fig. 1 as hatched areas. The 4.5 kb *EcoRI*–*BamHI* fragment was isolated and partially sequenced. The recombinant plasmid was designated pHAP1.

Complementation assays showed that we had indeed cloned the *envM* gene: when pHAP1 was introduced into *E. coli* JP1111, the *envM*-ts mutant, temperature-insensitive growth was observed. The smallest subclone tested that was able to complement was pFT632, containing the 1.9 kb *EcoRV*–*SalI* fragment. The recombinant plasmids pHAP6 and pHAP16, which carry the two *PstI* fragments and which are shown shaded in Fig. 1, did not complement. Hence, the *envM* gene contains a single *PstI* site. On the basis of these mapping experiments we assign the location of gene *envM* to coordinates 1366.8 kb of the Kohara *et al.* (1987) map.

#### Nucleotide sequence

Part of the *BamHI*–*EcoRI* fragment was sequenced by the dideoxy method of Sanger *et al.* (1977) using the

vector plasmid pUC118. Both strands of appropriate subclones were independently sequenced in the single-stranded form using universal and specific oligonucleotide primers. Fig. 2 shows the sequence, covering 1366 nt (nucleotides) from the *PstI* site towards the *EcoRI* site of the original fragment. This sequence was searched for open reading frames (ORFs) by applying the 'positional base preference' and the 'testcode' methods using the SASP-software of the Genetic Computer Group, University of Wisconsin (Devereux *et al.*, 1984). One complete reading frame was found, from nt 404 through nt 1190, which showed an identity with the corresponding gene from *S. typhimurium* of 85% at the nucleotide level and 98% at the amino acid level, respectively. Thus, this ORF is the *envM* gene of *E. coli*. It codes for a protein of 262 amino acids with a calculated molecular mass of 27 868 Da. Only six amino acids differ from the wild-type *S. typhimurium* gene product. The sequence of the first 30 N-terminal amino acids of the EnvM protein was determined by Edman degradation. It was identical with that derived from the DNA sequence (not shown).

Although the *envM* genes of *S. typhimurium* and *E. coli* show a very high degree of homology, the flanking DNA

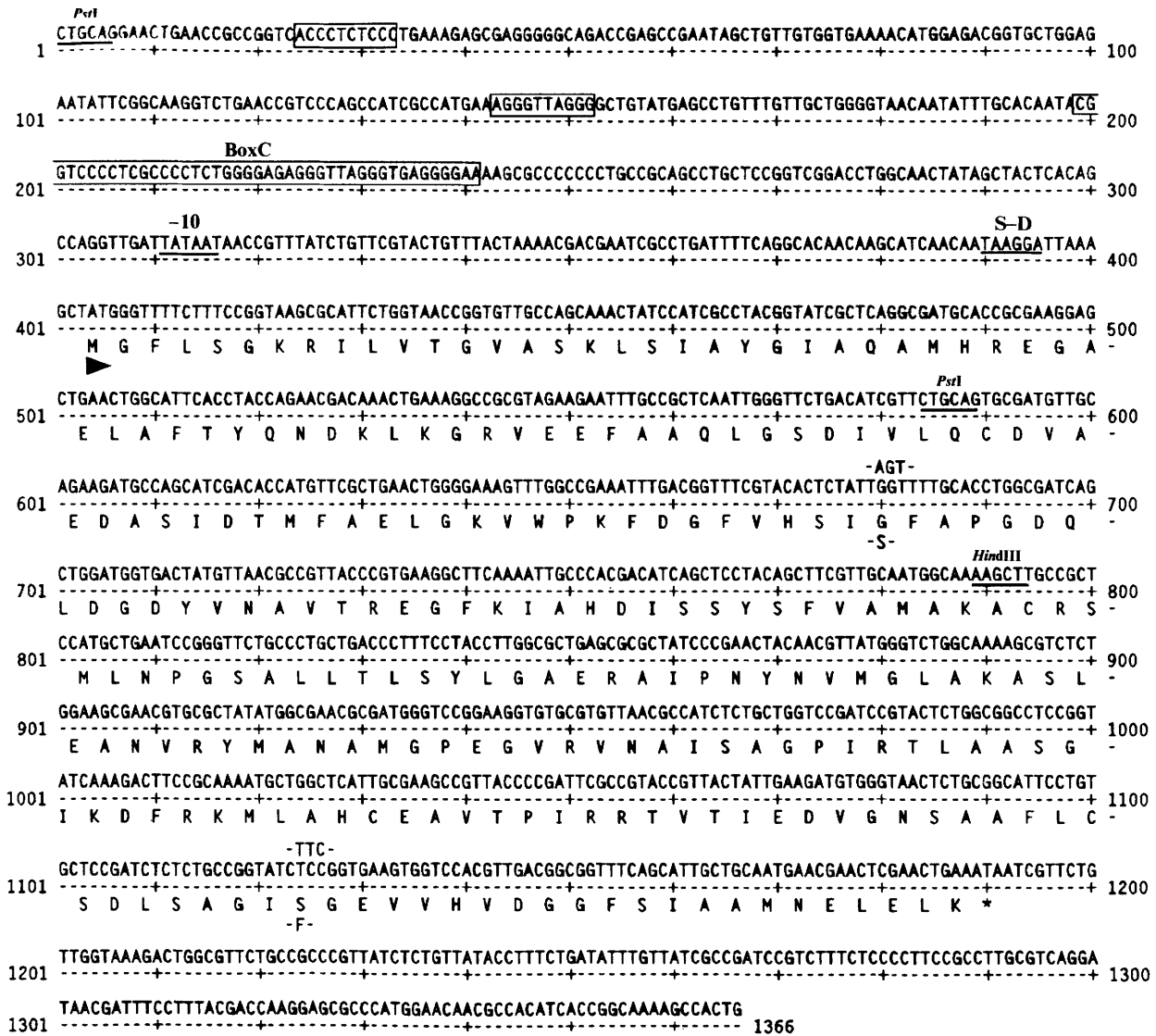


Fig. 2. Partial nucleotide sequence of pHAPI, starting at the *Pst*I site upstream of *envM* towards the *Eco*RI site of the original fragment, containing the complete wt-*envM* gene. Relevant restriction sites are indicated. A possible promoter sequence is underlined and marked -10. The putative ribosome binding site is also underlined and termed S-D. *envM* starts at nt 404 and ends at nt 1190; the direction of translation is indicated by an arrowhead below the sequence. The mutations in position 680 leading to the resistance-phenotype and in position 1125 leading to the ts-phenotype are indicated as altered codons above the sequence. The altered amino acids, a serine and a phenylalanine, respectively, are shown below the sequence. The deduced amino acid sequence is shown in the one-letter code. The highly conserved region 'box C', and partial sequences thereof in both orientations are boxed.

proved to be entirely different. In *S. typhimurium*, gene *envM* is preceded by a short ORF (*orf1*; Turnowsky *et al.*, 1989) coding for a polypeptide of 10445 Da. On the basis of sequencing data this gene is absent in *E. coli* both upstream and downstream of *envM*. In addition, Southern hybridization of *E. coli* chromosomal DNA cut with various restriction enzymes gave no signal when the *orf1* DNA was used as a probe in contrast to the control experiment with chromosomal DNA from *S. typhimurium*. Hence, we conclude that this gene is entirely absent

from *E. coli*. The position where *orf1* appears in the *S. typhimurium* sequence is occupied in *E. coli* by another unusual structural feature. It is a 43-nt-long segment that we designate 'box C'. Southern hybridization experiments using a 43-nt-long synthetic oligonucleotide representing box C as a probe showed signals with at least five restriction endonuclease fragments from the *E. coli* chromosome. No signal was observed in a similar experiment with *S. typhimurium* DNA, where this sequence motif is probably absent. In *E. coli*, this

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phnrev ATATCGAGCACAAT.CGGTCCCCTCGCCCCTCTGGGGAGAGGGTTAGGGTGAGGGGGCA.GCGCG
phoSrev AAAAGGCGCTCGGT.CGGTCCCCTCGCCCCTCTGGGGAGAGGGTTAGGGTGAGGGGAAAA.CCGTG
adk AAAGCGCGCACGGT.CAGTCCCCTCGCCCC.CTCGGGAGAGGGTTAGGGTGAGGGGAACAGGCCCG
fepBrev ACAAAGCGCACAAT.CCGTCCCCTCGCCCCTTGGGGAGAGGGTTAGGGTGAGGGGAACA.GCCAG
envM AATATTTGCACAATA.CGGTCCCCTCGCCCCTCTGGGGAGAGGGTTAGGGTGAGGGGAAAAGCGCCC
glnHPQ GTAAAACGCACTG.GCAGTTCCCCTCTCCCCTATGGGGAGAGGATTAGGGTGAGGGGGCGCAACCCG
melR CTATGATACTCGGAGAGTCCCCTCGCCCCTCTGGGAAGAGGGT.....GAAAACCGTGCT
mtlD1 CTTACGCGCATCCGCCTGCCCCTCTCCTCAC...GGAGAGGGTTTGGGTGAGGGAAAAGCCTCAC
    
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Fig. 3. Multiple sequence alignment of the 43-nt-long box C within the *phn* (Makino *et al.*, 1991) and *phoS* (Surin *et al.*, 1985) operons, behind the *adk* gene (Brune *et al.*, 1985), in front of the *fepB* (Elkins & Earhart 1989) and the *envM* genes, in the *glnHPQ* operon (Nohno *et al.*, 1986) and preceding the *melR* (Webster *et al.*, 1987) and the *mtlD1* genes (GenBank accession number X51359). The appearance of box C in the direction opposite to the transcriptional orientation in *phn*, *phoS* and *fepB* is indicated as 'rev' following the gene designation.

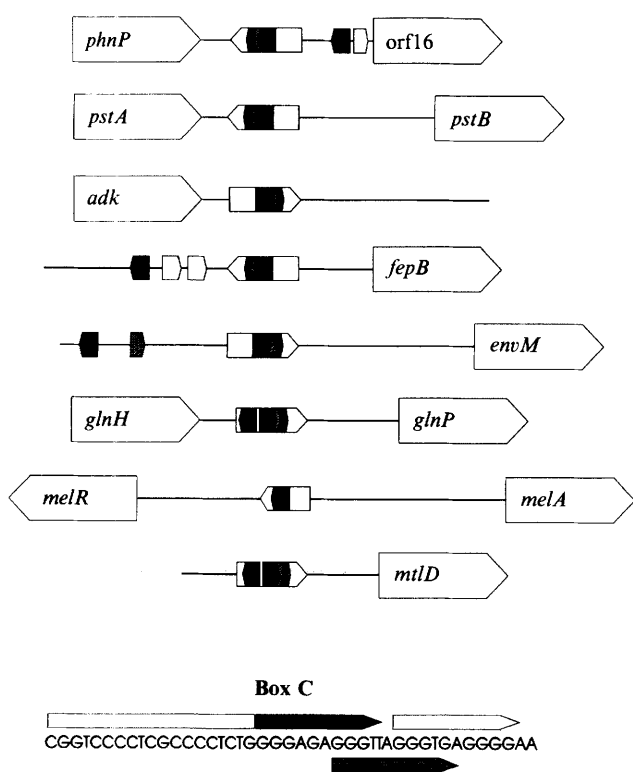


Fig. 4. Location of box C within the operons and genes listed in Fig. 3. Adjacent genes are indicated according to the transcription direction by arrowheads and the names of the genes. Box C is shown as a smaller arrowhead. At the bottom the graphical representation of box C is related to sequence details. The open, shaded and filled bars denote those specific sequence motifs which appear within box C and outside of it. The arrowhead of box C in the graphical representation designates the orientation of the sequence as it occurs upstream of *envM*.

sequence or a slightly modified version thereof appears in the intergenic region of various operons in both possible orientations. Partial sequences typical for box C sometimes appear detached from the central region. An example is given in the sequence bordering *envM*. Here, two such sequence motifs occur upstream of box C. They are appropriately marked in Fig. 2. The sequence of box C seems to be highly conserved as the multiple sequence

alignment in Fig. 3 shows. The arrangement of box C in various operons is shown in Fig. 4. This sequence and its occurrence in the *fepB*, *adk*, and *pst* operons of the *E. coli* chromosome was also observed by Elkins & Earhart (1989). Its function is unknown. However, a possible folding of the transcribed RNA into a hairpin, which this sequence permits, could influence either transcription of the downstream gene(s) or the stability of the entire mRNA.

#### Cloning and sequencing the diazaborine-resistant *envM* allele

Chromosomal DNA was prepared from the diazaborine-resistant mutant *E. coli* D10-1. The DNA was digested with *Bam*HI/*Eco*RI and the fragment mixture ligated into appropriately cleaved pUC118 DNA. After transformation, colonies were screened by hybridization with the DNA from pFT405, a recombinant plasmid containing the *envM* gene from *S. typhimurium* (Turnowsky *et al.*, 1989). The DNA from positive colonies was prepared in the single-stranded form and sequenced. At position 680, a G to A transition was observed that caused a Gly for Ser exchange in the amino acid sequence (Fig. 2). In the diazaborine-resistant *S. typhimurium* mutant, the equivalent codon was altered by the same type of transition in comparison to the wild-type gene (Turnowsky *et al.*, 1989). We therefore conclude that the glycine at position 93 of the amino acid chain is important for binding the drug to the protein. Preliminary binding studies with the labelled compound using the equilibrium dialysis technique confirm this proposition (unpublished).

#### Cloning and sequencing the *envM-ts* allele

Chromosomal DNA was prepared from *E. coli* JP1111 (Egan & Russell, 1973), digested with *Bam*HI-*Eco*RI and ligated into pBR322. Transformants were screened by the colony hybridization technique using the 1.6 kb-



Fig. 5. Sequence homology between some members of the short-chain alcohol dehydrogenase family and the EnvM protein. Amino acids identical to those in EnvM and conservative substitutions are shaded black and grey, respectively. The putative nucleotide-binding region is marked with a bar above the sequences. The amino acid residues in positions 93 and 241, which lead to the diazaborine-resistance and the ts-phenotype, respectively, are marked with open arrowheads below the sequences. Abbreviations used are: act3, actIII gene from *Streptomyces coelicolor* (Hallam et al., 1988); pktetgran, polyketide dehydrogenase (orf6) from *Streptomyces violaceoruber* (Sherman et al., 1989); fixrrm, fixR gene from *Bradyrhizobium japonicum* (Thöny et al., 1987); nodgrm, nodG gene from *Rhizobium meliloti* (Debelle & Sharma, 1986); ap27m, adipocyte protein 27 (Navre & Ringold, 1988); dihdliol, dihydriol dehydrogenase (bphB gene) from *Pseudomonas pseudoalcaligenes* (Furukawa et al., 1987); dienocoara, 2,4-dienoyl-CoA reductase from rat liver (Hirose et al., 1990); envmec, envM gene from *E. coli* (this work); ohsterhu, human placental 17β-hydroxysteroid dehydrogenase (Peltoketo et al., 1988).

long *Pst*I fragment from pHAP1 as a probe. DNA was prepared from a positive transformant, the insert subcloned into sequencing vectors and sequenced as described above. A single point mutation was observed, i.e. a C to T transition at position 1125 (Fig. 2). This mutation causes a Ser for Phe exchange at amino acid 241 of the amino acid sequence. The electrophoretic mobility of the EnvM-ts protein in partially purified samples isolated from overexpressing strains was consistently faster than that of the wild-type protein. The apparent molecular mass of the ts-protein was approximately 2000 Da smaller. The mutation could generate a proteolytic cleavage site which would lead to a protein lacking the 21 C-terminal amino acids. This size reduction would explain the observed change in electrophoretic mobility. Alternatively, the mutation could directly influence the electrophoretic mobility of the protein.

Overproduction of the ts-protein in the ts-mutant *E. coli* JP1111 partially restored the growth defect at the nonpermissive temperature (data not shown). The exposure of the ts-protein at the nonpermissive temperature probably does not result in a quantitative destruction of its activity. Hence, the overproduction of this protein yields enough residual activity in order to sustain growth at the nonpermissive temperature.

#### Comparison of amino acid sequences

Multiple alignment of the amino acid sequence of EnvM with sequences from databases showed significant homologies of EnvM with the so-called short-chain alcohol dehydrogenases (Jörnvall *et al.*, 1984, Persson *et al.*, 1991, Baker 1990a, b), an example being 27% identity and 46% similarity, respectively, between EnvM and the dienoyl-CoA reductase from rat liver. The alignment is shown in Fig. 5. The nucleotide-binding domain of this family of proteins is assumed to be located in the N-terminal part of the sequence (Baker 1990b; Navre & Ringold, 1988; Jörnvall *et al.*, 1984). The glycine residue at position 93 of the EnvM protein, whose exchange for serine results in diazaborine resistance, appears to be highly conserved. The serine at position 241, whose exchange for phenylalanine leads to a ts-phenotype, is at a location where hydrophilic amino acids, i.e. threonine or serine, appear. The introduction of an aromatic amino acid at this position obviously has a dramatic effect on the structure of the protein.

The relationship of EnvM, a protein which plays a crucial role in lipid biosynthesis, with this class of dehydrogenases is surprising, because it shows that knowledge about metabolic pathways of lipids is still incomplete. Presently, we are testing whether addition of purified EnvM to a lipid extract in the presence or

absence of NAD<sup>+</sup> alters the lipid composition. The identification of a substrate-product relationship would allow an assessment of the physiological function of this protein.

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