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

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(Received 24 April 1992; revised 25 June 1992; accepted 30 June 1992)

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.

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 & Woisetschlager, 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. DiaX designates diazaborine resistance. Antibiotics were added when needed at the following concentrations: diazaborine 20 μg ml⁻¹, epicillin 100 μg ml⁻¹.

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

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### Table 1. Bacterial strains, phage, and plasmids

<table>
<thead>
<tr>
<th>Strain/phage/plasmid</th>
<th>Relevant genotype/characteristics</th>
<th>Source*/*reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL1-Blue2</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F' proAB lacZΔM15 Tn10[tetR])</td>
<td>Stratagene</td>
</tr>
<tr>
<td>MC1061</td>
<td>araD 139 Δ ara-leu7697 ΔlacX74</td>
<td>Public Health Laboratory*</td>
</tr>
<tr>
<td>D10-1</td>
<td>F' met Dia*</td>
<td>B. Bachmann &amp; Egan &amp; Russell (1973)</td>
</tr>
<tr>
<td>BK 80, BK 41</td>
<td>lew-277 Δ-IN(rmd-dmE1)</td>
<td>Turnowsky et al. (1980)</td>
</tr>
<tr>
<td><strong>Phage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13 K07</td>
<td>KmR p15A ori</td>
<td>J. Vieira &amp; Vieira &amp; Messing (1987)</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSP65</td>
<td>Ap R</td>
<td>Sandoz collection*</td>
</tr>
<tr>
<td>pUC118</td>
<td>Ap R lac'P0Z' M163 ori</td>
<td>J. Vieira &amp; Vieira &amp; Messing (1987)</td>
</tr>
<tr>
<td>pBR322</td>
<td>Ap R Tc R</td>
<td>Sandoz collection*</td>
</tr>
<tr>
<td>pKF403</td>
<td>Ap R Dia*, 1-45 kb BamHI fragment (oru) + enuM-Dia* from S. typhimurium</td>
<td>Turnowsky et al. (1989)</td>
</tr>
<tr>
<td>pFT405</td>
<td>Ap R Dia*, 1 kb Accl–BamHI fragment (enuM-Dia* from S. typhimurium) in pBR322</td>
<td></td>
</tr>
<tr>
<td>pHAP1</td>
<td>Ap R, 4-5 kb EcoRI–BamHI fragment (enuM-wt from E. coli) in pBR322</td>
<td></td>
</tr>
<tr>
<td>pHAP2</td>
<td>Ap R, 4.5 kb EcoRI–BamHI fragment (enuM-wt from E. coli) in pUC118</td>
<td></td>
</tr>
<tr>
<td>pHAP6</td>
<td>Ap R, 600 bp PstI fragment from pHAP1 in pUC118</td>
<td></td>
</tr>
<tr>
<td>pHAP16</td>
<td>Ap R, 1-6 kb PstI fragment from pHAP1 in pUC118</td>
<td></td>
</tr>
<tr>
<td>pFT617</td>
<td>Ap R, 4-5 kb EcoRI–BamHI fragment (enuM-wt from E. coli) in pUC118</td>
<td></td>
</tr>
<tr>
<td>pFT632</td>
<td>Ap R, 1-9 kb EcoRV–SalI fragment from pHAP1 in pSP65</td>
<td></td>
</tr>
<tr>
<td>pFT650</td>
<td>Ap R Dia*, 4-5 kb EcoRI–BamHI fragment (enuM-Dia* from E. coli) in pUC118</td>
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</tbody>
</table>

* 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* and the ts alleles of the enuM gene are described in Results. Labelled DNA probes for Southern hybridization and colony hybridization experiments were prepared either by nick-translation of the BamHI fragment of pKF403 (Turnowsky et al., 1989), or by random priming using the 1-6 kb PstI fragment of pHAP1 or the 1 kb BamHI–Acl fragment of pFT405 (Turnowsky et al., 1989) as templates. The recombinant plasmids carrying the wt, the Dia* and the ts alleles of enuM were designated pHAP1, pFT650 and pFT617, respectively. Transformations were done by the standard CaCl₂ protocol.

**DNA sequencing.** The sequences of the DNA fragments containing the allelic forms of the enuM 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 enuM 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 enuM 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
**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
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.
**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 *mtlDI* 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>phnP</em></td>
<td>ATATCGAGCAGCAATCGTCGCCCTCCTGCGCCCTCTGTGGGAGAGGGTCTGGAGGGGAGGGCAAGCCG</td>
</tr>
<tr>
<td><em>phoS</em></td>
<td>AAAAGGCCGCTCGGTTCAGGCCTCTGCCCCTCTGTGGGAGAGGGTCTGGAGGGGAGGGCAAGCCG</td>
</tr>
<tr>
<td><em>adk</em></td>
<td>AAAGCGCCCGAGGACGCATGCAGGCCTCTGCCCCTCTGTGGGAGAGGGTCTGGAGGGGAGGGCAAGCCG</td>
</tr>
<tr>
<td><em>fepB</em></td>
<td>ACAGACGCGACGCGCTCCAGGCCTCTGCCCCTCTGTGGGAGAGGGTCTGGAGGGGAGGGCAAGCCG</td>
</tr>
<tr>
<td><em>envM</em></td>
<td>AAATTCAGACATACGGGCGCTCCCTGCCCCTCTGTGGGAGAGGGTCTGGAGGGGAGGGCAAGCCG</td>
</tr>
<tr>
<td><em>melR</em></td>
<td>TCTATGATACCTAGGGGAGACGGGGGGAGGGTCTGGAGGGGAGGGCAAGCCG</td>
</tr>
<tr>
<td><em>mtlDI</em></td>
<td>CTAGACGCGACGCGCTCCAGGCCTCTGCCCCTCTGTGGGAGAGGGTCTGGAGGGGAGGGCAAGCCG</td>
</tr>
</tbody>
</table>

**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 intercistronic 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 BamHI/EcoRI 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 BamHI–EcoRI 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); pketgran, polyketide dehydrogenase (orf6) from Streptomyces violaceoruber (Sherman et al., 1989); fixrrm, fixR gene from Bradyrhizobium japonicum (Thony et al., 1987); nodgrm, nodG gene from Rhizobium meliloti (Debelle & Sharma, 1986); ap27m, adipocyte protein 27 (Navre & Ringold, 1988); dihdiol, dihydrodiol dehydrogenase (bphB gene) from Pseudomonas pseudoalcaligenes (Furukawa et al., 1987); dienocoara, 2,4-dienoyl-CoA reductase from rat liver (Hirose et al., 1990); envmec, enuM gene from E. coli (this work); ohsterhu, human placental 17β-hydroxysteroid dehydrogenase (Peltoketo et al., 1988).
long PstI 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 JPl111 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+ alters the lipid composition. The identification of a substrate–product relationship would allow an assessment of the physiological function of this protein.

The expert help of Petra Wallner and Astrid Stecher is gratefully acknowledged. We are grateful to Yuji Kohara for providing the recombinant lambda phage and to Barbara Bachmann for supplying strains. We thank Günther Koraimann for synthesizing oligonucleotides and for his help with the computer work and Heinrich Aschauer for determining the N-terminal amino acid sequence. This work was supported by the Fonds zur Förderung der wissenschaftlichen Forschung, grant no. P7939.

References


