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envM Genes of *Salmonella typhimurium* and *Escherichia coli*

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Conjugation and bacteriophage P1 transduction experiments in *Escherichia coli* showed that resistance to the antibacterial compound diazaborine is caused by an allelic form of the *envM* gene. The *envM* gene from *Salmonella typhimurium* was cloned and sequenced. It codes for a 27,765-dalton protein. The plasmids carrying this DNA complemented a conditionally lethal *envM* mutant of *E. coli*. Recombinant plasmids containing gene *envM* from a diazaborine-resistant *S. typhimurium* strain conferred the drug resistance phenotype to susceptible *E. coli* cells. A guanine-to-adenine exchange in the *envM* gene changing a Gly codon to a Ser codon was shown to be responsible for the resistance character. Upstream of *envM* a small gene coding for a 10,445-dalton protein was identified. Incubating a temperature-sensitive *E. coli envM* mutant at the nonpermissive temperature caused effects on the cells similar to those caused by treatment with diazaborine, i.e., inhibition of fatty acid, phospholipid, and lipopolysaccharide biosynthesis, induction of a 28,000-dalton inner membrane protein, and change in the ratio of the porins OmpC and OmpF.

We have a longstanding interest in a class of antibacterial agents, the diazaborines (Fig. 1), which have been shown to affect lipopolysaccharide (LPS) biosynthesis (10). In our attempt to identify the cellular target of these compounds, we generated diazaborine-resistant mutants in both *Escherichia coli* and *Salmonella typhimurium*. We found that the location of the resistant gene coincided with that of the *envM* gene of *E. coli*. The only previously described allelic form of this gene is a temperature-sensitive growth defect with an osmotically repairable phenotype affecting envelope structure (3). We cloned the DNA segment responsible for the resistant phenotype from *S. typhimurium*, sequenced it, and investigated its role in the bacterial physiology. The results of this investigation are reported in this communication.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids are listed in Tables 1 and 2. Dia^r designates diazaborine resistance.

Chemicals and enzymes. Diazaborine, compound 2b18, was described by Grassberger et al. (7). Ampicillin was from Bayer AG (Leverkusen, Federal Republic of Germany). Epicillin was from Biochemie GmbH (Kundl, Austria). Chloramphenicol was from Serva (Heidelberg, Federal Republic of Germany). Kanamycin and tetracycline were from Sigma Chemical Co. (St. Louis, Mo.). Radiochemicals were from Amersham International (Amersham, United Kingdom) or from Dupont, NEN Research Products (Frankfurt, Federal Republic of Germany). Enzymes for DNA work were from Boehringer GmbH (Mannheim, Federal Republic of Germany), Amersham International, and Bethesda Research Laboratories, Inc. (Gaithersburg, Md.).

Resistant mutants. Both *E. coli* D10 and *S. typhimurium* AG701i50 were grown to the mid-log phase, pelleted, and suspended in M9 minimal medium (16). The cells were irradiated with UV light for various periods to establish a killing curve. At 45 s, 1% of the cells survived, and this UV dose was used to generate mutants. Mutants were selected on agar plates containing 25 μg of diazaborine per ml, a concentration corresponding to at least 10 times the MICs. Resistant mutants were collected and analyzed further.

Mapping the diazaborine gene in *E. coli*. From diazaborine-resistant mutants of *E. coli* D10, P1 *vir* lysates were generated and used to transduce various Hfr strains. Selection was for diazaborine resistance. The transductants were crossed with *E. coli* Pa209a. Transconjugants were selected by plating the crosses on various selective media containing streptomycin. The sequence of transfer was established on the basis of viable counts.

Fine mapping was done by transduction experiments. P1 *vir* lysates were produced in the following *E. coli* strains: D10-1, D10-9, MY517, PAO183, CA198, and JP1111. These lysates were used to transduce test strains. One marker was selected in appropriate media. The cotransduction of nearby markers was assayed separately in a second selection step.

Construction of isogenic pair of *E. coli* strains differing in the *envM* allele. A bacteriophage P1 lysate was prepared from *E. coli* JP1111 and used to transduce *E. coli* MORSE2034. *trp*$^+$ transductants were selected and screened for temperature-sensitive growth characteristics. One temperature-sensitive mutant, BK80, was used for further work. As a wild-type control, *E. coli* BK41 was selected.

DNA manipulations. Recombinant DNA techniques were performed by the methods of Maniatis et al. (14) or by following the protocols of the manufacturer. Transformations were done by the standard $CaCl_2$ protocol.

Construction of recombinant plasmids. The general strategy for the construction of pKF400 through pKF404, pFT405, and pFT500 through 502 is described in the Results section. The plates for the selection of drug resistance contained 20 μg of diazaborine per ml and 50 μg of epicillin per ml. For the construction of pFT407, the DNA of the

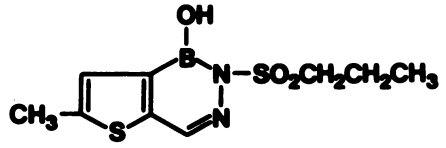

FIG. 1. Structure of the diazaborine derivative used in this work.

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promoter fusion vector pNM480 (17) was cleaved with *Eco*RI-*Sma*I and ligated with a 435-base-pair fragment obtained by *Eco*RI-*Pvu*II digestion of pKF403. pFT408 was obtained by ligating a *Sma*I-cleaved pNM480 vector DNA with the 426-base-pair *Pvu*II fragment from pKF403. Selection in both cases was for blue colonies on 5-bromo-4-chloro-3-indoyl-β-galactoside (X-Gal) plates containing epicillin. pFT407 carries the promoter region of *orf-1*, and pFT408 carries that of *envM*. pFT409 and pFT411 are derivatives of pT7-6 and pT7-5, respectively. pFT409 was constructed by ligating a 2.5-kilobase-pair (kbp) *Eco*RI-*Bam*HI fragment of pFT406 with the appropriately cleaved pT7-6 vector. pFT406 itself contains a 2.1-kbp *Acc*I fragment of pKF402 which, after the cohesive ends were filled, was ligated into the *Eco*RV site of pBR322. pFT409 contains only the *envM* gene. pFT411, which contains both *orf-1* and *envM*, was constructed by ligating the *Bam*HI fragment of pKF403 into the appropriately cleaved pT7-5 DNA. Recombinant plasmids with the correct transcription orientation were selected.

DNA sequencing. The polynucleotide sequences of plasmids containing mutant and wild-type DNA were deter-

TABLE 1. List of strains

Strain	Genotype	Derived from:	Source
Escherichia coli			
JP1111	Hfr *galE45* λ⁻ *envM392*(Ts) *relA1 spoT1 thi-1*		B. Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.
5K	*tre thi rpsL hsdR hsdM⁺ lac*		W. Goebel, Universität Würzburg, Würzburg, Federal Republic of Germany
D10	F⁺ *met*		E. Küchler, Universität Wien, Vienna, Austria
D10-1	F⁺ *met dia-1*		This work
D10-8	F⁺ *met dia-8*		
D10-9	F⁺ *met dia-9*		
D10-10	F⁺ *met dia-10*	D10	
D10-11	F⁺ *met dia-11*		
D10-12	F⁺ *met dia-12*		
Pa209a	F⁻ *thr leu trp his gal thi rpsL*		J. Besemer, Sandoz Forschungsinstitut, Vienna, Austria
Hfr3000 Dia^r	Hfr *thi-1 relA1 spoT1* λ⁻ *supQ80 dia-1*	Hfr3000 (Hfr *thi-1 relA1 spoT1* λ⁻ *supQ80*)	This work
BW113 Dia^r	Hfr *met-31 relA1* λ⁻ *spoT1 dia-1*	BW113 (Hfr *met-31 relA1* λ⁻ *spoT1*)	This work
KL16 Dia^r	Hfr *thi-1 relA1* λ⁻ *spoT1 dia-1*	KL16 (Hfr *thi-1 relA1* λ⁻ *spoT1*)	This work
KL208 Dia^r	Hfr *relA1* λ⁻ *dia-1*	KL208 (Hfr *relA1* λ⁻)	This work
MY517	F⁻ *cysB trpE9829*		M. D. Yudkin, Oxford University, Oxford, England
MORSE2034	*trpE9851 leu-277* λ⁻ IN(*rrnD-rrnE*)*1*[a]		J. W. Payne, University College of North Wales, Bangor, United Kingdom
PAO183	*trpE9851 leu-277* λ⁻ IN(*rrnD-rrnE*)*1 tdk-tonB*		J. W. Payne, University College of North Wales, Bangor, United Kingdom
CA198	Hfr *galU106 relA1* λ⁻ *spoT1*		B. Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.
BK-80	*envM*(Ts) *trp⁺*	MORSE2034	This work
BK-41	*trp⁺*	MORSE2034	This work
MC1061	*araD139* Δ(*ara-leu*)*7697* Δ*lac74 galU galK hsdR hsdM rpsL*		N. D. Minton, Public Health Laboratory Service, Porton Down, United Kingdom
MV1190	Δ(*lac-proAB*) *thi supE* Δ(*srl-recA*)*306*::Tn*10* (Tet^r) (F′ *traD36 proAB lacI^{q}Z* ΔM15)		J. Vieira, Rutgers University, Piscataway, N.J.
X925	F⁻ *thr ara leu* Azi^r, *tonA*(R) *lacY* T6^s *minA gal* λ⁻ *minB rpsL malA xyl mtl thi sup*		S. Levy, Tufts University, Boston, Mass.
K38(pGP1-2)	HfrC		A. Böck, Universität München, Munich, Federal Republic of Germany
HMS174(pT7-5)	*recA1 hsdR*		A. Böck, Universität München, Munich, Federal Republic of Germany
HMS174(pT7-6)	*recA1 hsdR*		A. Böck, Universität München, Munich, Federal Republic of Germany
Salmonella typhimurium			
AG701	*galE uhp*(Con) *rpsL met trp his ilv pyrE*		Sandoz Forschungsinstitut, Vienna, Austria
AG701i50	*galE uhp*(Con) *rpsL met trp his ilv pyrE kdsA*	AG701	Sandoz Forschungsinstitut, Vienna, Austria
AG701i50-m2	*galE uhp*(Con) *rpsL met trp his ilv pyrE kdsA dia-2*	AG701i50	This work
AG701i50-m4	*galE uhp*(Con) *rpsL met trp his ilv pyrE kdsA dia-4*	AG701i50	This work

[a] IN, Inversion.

TABLE 2. List of plasmids used

Plasmid	Derivative of:	Diazaborine resistance[a]	Source
pBR322		−	J. Vieira
pUC118		ND[b]	J. Vieira
pUC119		ND	J. Vieira
pT7-5		ND	A. Böck
pT7-6		ND	A. Böck
pNM480		ND	N. P. Minton
pML31ts17	F	−	R. Eichenlaub
pKF400	pBR322	+	This work
pKF401	pBR322	+	This work
pKF402	pBR322	+	This work
pKF403	pBR322	+	This work
pKF404	pML31ts17	+	This work
pFT405	pBR322	+	This work
pFT406	pBR322	+	This work
pFT407	pNM480	−	This work
pFT408	pNM480	−	This work
pFT409	pT7-6	+	This work
pFT411	pT7-5	+	This work
pFT500	pBR322	ND	This work
pFT501	pBR322	±	This work
pFT502	pUC118	±	This work
pFT503	pUC119	ND	This work

[a] ±, Elevated MIC (25 μg/ml). For true resistant cells, the MIC is 200 μg/ml.
[b] ND, Not determined.

mined by the chain termination method of Sanger et al. (19). The mutant DNA was mainly sequenced in single-stranded phage M13mp10 and M13mp11 subclones with the universal primer (Amersham International) or with single-stranded recombinant plasmid DNA derived from pUC118 and pUC119 (22) by using specific oligonucleotide primers. Furthermore, the double-stranded DNA-sequencing method (2) was used to analyze both the mutant and the wild-type genes.

Gene expression studies. (i) Expression in minicells. *E. coli* X925 was transformed with a number of recombinant plasmids. The bacteria were grown and minicells were prepared by the method of Levy (12). The minicells were incubated with [^{35}S]methionine (800 Ci/mmol) and lysed with sodium dodecyl sulfate (SDS)-containing lysis buffer. The proteins were separated by SDS-polyacrylamide gel electrophoresis on a gradient gel ranging from 5 to 20% acrylamide.

(ii) T7 promoter expression. The recombinant plasmids pFT409 and pFT411 were introduced into *E. coli* K38 (pGP1-2) by transformation. The plasmid-containing strains were grown in 1 ml of 2× tryptone-yeast extract containing 40 μg of kanamycin and 20 μg of diazaborine. The cells were collected by centrifugation in the mid-log phase and transferred into 1 ml of M9 medium containing 2 mg of glucose, 20 μg of thiamine, and 100 μg of each of 18 amino acids. Methionine and cysteine were omitted. After shaking at 30°C for 1.5 h, the cell suspension was shifted to 42°C. After 10 min, rifampin was added to give a final concentration of 200 μg/ml and the cells were kept at 42°C for another 10 min. Subsequently, the cells were incubated at 30°C for 20 min, and then 13 μCi of [^{35}S]methionine (800 Ci/mmol) was added and the incubation continued for 5 min at 30°C. The cells were harvested by centrifugation, and the pellet was treated with a mixture containing 60 mM Tris hydrochloride (pH 6.8), 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue for 3 min at 95°C. This mixture was loaded onto a 15% SDS-polyacrylamide gel.

***lacZ* fusion experiments.** The fusions with the promoter probe vector pNM480, i.e., pFT407 and pFT408, were transformed into *E. coli* MC1061. Positive transformants appeared blue on X-Gal plates. Early-log-phase cells were tested for β-galactosidase production with *o*-nitrophenyl-β-D-galactoside as described by Miller (16). For testing anaerobic growth, the air in the culture vessel was displaced with argon. The cultures were incubated for 1 h without shaking.

Lipid A biosynthesis. *S. typhimurium* AG701i50 was grown in beef extract-Proteose Peptone (Difco Laboratories, Detroit, Mich.)-medium to the mid-log phase at 30°C (18), collected by centrifugation, resuspended in fresh medium, and incubated at 42°C for approximately 1 generation. This culture was labeled with 1 μCi of *N*-acetyl-D-[1-^{3}H]glucosamine (2.94 Ci/mmol), and at the same time various amounts of diazaborine were added. The cultures were subsequently incubated for 20 min at 42°C. The cells were harvested by centrifugation, dried, and dehydrated with acetone. The acetone powder was extracted with phenol-chloroform-light petroleum (2:5:8, vol/vol/vol). The organic phase was spotted onto Whatman no. 1 paper, and the lipids were separated by descending chromatography with *n*-butanol–isobutyric acid–1 M ammonia (10:28:15, vol/vol/vol) (18). The paper was cut into 1-cm-wide strips which were counted in a liquid scintillation spectrometer.

Fatty acid biosynthesis in whole cells. Mid-log-phase cultures of *S. typhimurium* AG701i50 in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and its diazaborine-resistant mutant 2 were treated with various concentrations of diazaborine at 30°C and labeled with 4 μCi of [2-^{14}C]sodium acetate per ml (54 μCi/μmol) for 2 h. The cultures were chilled, and the cells were collected by centrifugation and treated with 4 M HCl at 100°C for 2 h. The liberated fatty acids were extracted with chloroform, and the solvent was evaporated. The residue was methylated at 4°C with diazomethane in ether. After evaporation of the ether, the methyl esters were dissolved in methanol and spotted on silanized silica thin-layer plates (E. Merck AG, Darmstadt, Federal Republic of Germany). The mixture was chromatographed with acetone-methanol-water-acetic acid (70:50:35:1, vol/vol/vol/vol) (9). The air-dried plates were autoradiographed.

To obtain a rough estimate of acetate incorporation into fatty acids of whole cells, we labeled mid-log-phase cultures with 2.5 μCi of [2-^{14}C]sodium acetate per ml (57 μCi/μmol) for 2 h in the presence or absence of diazaborine. The suspensions were treated with trichloroacetic acid and filtered through glass fiber filters. The filters were dried, and their radioactivity was counted.

Fatty acid synthesis in vitro. Acyl carrier protein and fatty acyl synthetase were prepared by established procedures (8, 13). The reaction mixture was a slight modification of that of Harder et al. (8). It contained 1.5 μg of acyl carrier protein, 15 μg of fatty acyl synthetase, 0.1 M potassium phosphate (pH 7), 0.002 M dithiothreitol, 30 μM acetyl coenzyme A (acetyl-CoA), 1 μCi of [2-^{14}C]malonyl-CoA, 0.2 mM NADPH, 5 mM glucose-6-phosphate, 1 U of glucose-6-phosphate dehydrogenase, and 0.2 mM EDTA. Diazaborine was added to give final concentrations of 50 and 125 μg/ml. The mixtures were incubated for 20 min at 37°C. The products were hydrolyzed by HCl treatment, and the free fatty acids were methylated as described in the previous paragraph. The thin-layer separation was also as described above.

Acetyl-CoA carboxylase. Acetyl-CoA carboxylase was prepared and tested as described by Alberts and Vagelos (1). Diazaborine concentrations of up to 14 μg/ml were tested.

Phospholipid biosynthesis. Various *E. coli* strains were grown in Luria broth to the early log phase. Cultures were either treated with 4 μg of diazaborine per ml at 30°C or shifted to 42°C for 30 min. For the same period, the cells were labeled with 10 μCi of $^{32}P_i$. After harvesting, the cells were extracted with chloroform-methanol (2:1) (6). The extract was mixed with 0.25 volume of water to separate the phases. The chloroform layer was removed and analyzed for phospholipids by two-dimensional thin-layer chromatography on silica plates (20). The first dimension was chloroform–methanol–25% ammonia (65:35:5, vol/vol/vol), and the second dimension was chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, vol/vol/vol/vol/vol). The dried plates were autoradiographed. The radioactivities of the chloroform and the aqueous phases were determined as well.

Membrane protein analysis. Various *E. coli* strains were grown in minimal M9 medium supplemented with glucose, thiamine, and Casamino Acids to the early log phase. Subsequently, either diazaborine was added to give a final concentration of 4 μg/ml or the temperature was shifted to 42°C and the incubation continued for 5 h. Then the cells were harvested and broken by sonication, and the membrane fraction was collected by centrifugation. The pellet was extracted with sodium lauroyl sarcosinate by the method of Filip et al. (5). The suspension was again centrifuged, and the supernatant containing the cytoplasmic membrane proteins was loaded onto an SDS-11% polyacrylamide gel after mixing with dissolving buffer and heating at 95°C. The pellet containing the outer membrane proteins was solubilized in dissolving buffer by heating at 95°C and also applied to the gel. After electrophoresis, the gels were stained with Coomassie brilliant blue.

RESULTS

Generation of diazaborine-resistant *S. typhimurium* and *E. coli* mutants. *S. typhimurium* AG701i50 and *E. coli* D10 were treated with UV light until 1% of the cells survived. The survivors were plated on media containing diazaborine. Several mutants were collected and further analyzed with respect to their resistance levels. One-step mutants of *S. typhimurium* which tolerated >200 μg of the drug per ml, an approximately 200-fold higher amount than that tolerated by the wild type, appeared with a frequency of about 1 in 10^8 to 10^9 cells. One of the *S. typhimurium* mutants (mutant 4) was selected for cloning the gene which causes drug resistance.

E. coli D10 mutants (mutants 1, 8, 9, 10, 11, and 12) for which MICs were >100 μg/ml (MIC for wild-type *E. coli* is 2 μg/ml) were selected for gene mapping experiments.

Cloning the *S. typhimurium* gene conferring diazaborine resistance. Chromosomal DNA from *S. typhimurium* AG 701i50-m4 was cleaved with the restriction enzyme *Cla*I. The fragments were ligated into a *Cla*I-cleaved pBR322 vector. *E. coli* 5K was transformed with this recombinant DNA mixture and streaked on diazaborine-containing agar plates. Several drug-resistant *E. coli* colonies appeared which were collected and used for plasmid DNA preparations. All resistant cells carried in the vector a 3.6-kbp *Cla*I fragment. The plasmid containing this 3.6-kbp insert from *S. typhimurium* was designated pKF401. The orientation relative to the vector did not influence the resistance phenotype. A physical map of the insert was established by using the restriction enzymes *Ava*I, *Acc*I, *Eco*RV, and *Hin*cII. The insert contains no site for *Bam*HI, *Hin*dIII, *Eco*RI, *Pst*I, and *Sal*I. A successful attempt to reduce the size of the insert and retain the resistance to the drug was made by eliminating 2.96 kbp of insert and vector DNA after *Cla*I-*Ava*I treatment of pKF401. Religation of a 1.65-kbp fragment with appropriately cut pBR322 DNA resulted in the smaller plasmid pKF402. Some additional size reduction was possible by *Bal* 31 exonuclease treatment of pKF402. Various deletions on the *Ava*I site of the insert were made, the ends were joined with a synthetic *Bam*HI linker, and the DNA was recircularized. The plasmid with the shortest DNA still able to confer the resistance phenotype was selected (pKF400). Subsequently, an *Eco*RV cut was made which removed a few nucleotides on the other end. The blunt ends were again joined with a *Bam*HI linker and recircularized. This resulted in a 4.25-kbp plasmid carrying a 1.45-kbp insert. The insert was flanked on both ends with artificial *Bam*HI sites. It still generated a diazaborine resistance phenotype in *E. coli* and *S. typhimurium* cells. This plasmid was designated pKF403. A different subcloning strategy was applied by cutting pKF403 with *Acc*I and *Bam*HI and ligating the larger part of the insert with a 2,490-nucleotide *Acc*I-*Bam*HI fragment of pBR322, giving the recombinant plasmid pFT405. It still conferred diazaborine resistance to its host cells. The maps of the clones are shown in Fig. 2.

Cloning of *S. typhimurium* wild-type gene. An *Eco*RI gene library from *S. typhimurium* AG701 in plasmid pBR322 was screened by the colony hybridization technique with a radioactive DNA probe. One of three positive isolates was investigated further. Plasmid DNA was isolated and cleaved with *Cla*I-*Ava*I. A 1.65-kbp fragment gave a positive signal in a Southern hybridization experiment and was therefore isolated by cloning into the appropriately cut pBR322 DNA. The resulting plasmid was designated pFT501. The MIC for *E. coli* strains with recombinant plasmids carrying the wild-type gene was approximately 10-fold higher than that for plasmid-free bacteria (25 μg/ml). The high concentration of the EnvM protein in such bacteria, a consequence of the high gene dosage, probably lowers the intracellular concentration of free drug. Higher extracellular concentrations are therefore needed to block all the EnvM molecules.

Mapping of the gene in *E. coli* and identity with the *envM* gene. From six drug-resistant *E. coli* D10 mutants (mutants 1, 8, 9, 10, 11, and 12), phage P1 lysates were generated which were used to transduce *E. coli* Hfr strains. Drug-resistant transductants were isolated and crossed with *E. coli* K-12 Pa209a. The gradient of transmission indicated that the gene responsible for the diazaborine resistance phenotype was located near *trp* around minute 27 of the *E. coli* genetic map. All transductants originating from the various *E. coli* D10 mutants mapped in the same general region. Precise mapping in *E. coli* was done by measuring the cotransduction frequencies with P1 transducing particles from two of the drug-resistant mutants. We observed a >95% cotransduction frequency of the diazaborine resistance with the temperature-sensitive *envM* gene. To test for a possible identity of these genetic loci, we transformed plasmid pKF403 carrying the gene for diazaborine resistance into the *E. coli envM* mutant. pKF403 or pFT405 complemented the *envM* phenotype; thus, the *envM* gene and diazaborine resistance are either identical or very closely related. A deletion in *envM*, as in pFT408, was unable to complement the temperature-sensitive growth defect, again showing that the diazaborine resistance phenotype and the *envM* defect are two allelic forms of the same gene.

A single copy of the *Bam*HI fragment from pKF403 also

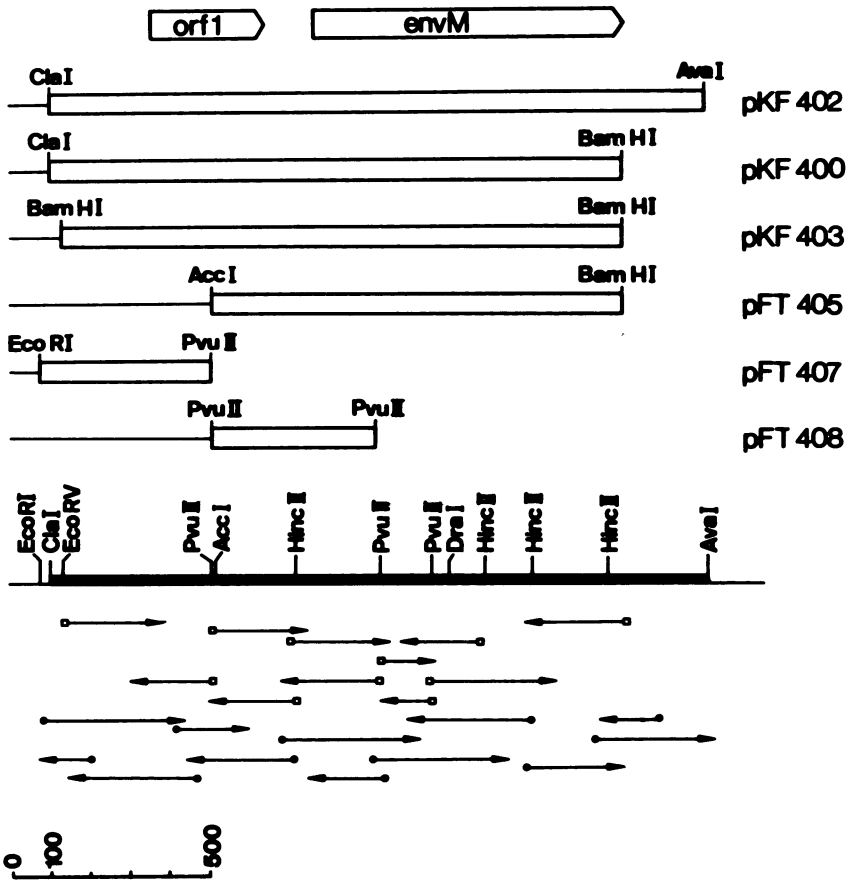

FIG. 2. Physical map, subclones, and sequencing strategy. The physical map (center) shows a selected number of restriction sites. The thick line represents the insert; the thin line to the left and the right is vector DNA. Above, the inserts of the relevant subclones and their designations are given. The artificial *Bam*HI site in plasmids pKF400, pKF403, and pFT405 is located after position 1469 of the sequence. Genes are represented as open arrows on the top. Below the physical map, the sequencing strategy is schematically represented. Arrows indicate the direction and length of each sequencing run. Squares represent universal priming of M13 subclones; dots show specific oligonucleotide priming of pUC subclones.

induced diazaborine resistance. The fragment was ligated with the artificial plasmid pML31ts17, a derivative of F and pSC101 which is temperature sensitive for replication (4, 23). pML31ts17 was digested with *Bam*HI. The larger of the two resulting fragments was linked with the *Bam*HI fragment from pKF403 to give pKF404, which is also replication defective at 42°C. After transforming pKF404 into *E. coli* Pa209a and heating it at 42°C, we selected diazaborine-resistant survivors. These cells had pKF404 integrated into their chromosome as was shown in one of these isolates by Southern hybridization.

Nucleotide sequence determination of DNA segment inducing diazaborine resistance. The *Bam*HI insert of pKF403 or fragments created by digestion with other restriction enzymes were cloned into the double-stranded DNA of the bacteriophage M13mp10 and M13mp11. The DNA was sequenced by the dideoxy-chain termination method (19) with the universal primer. To sequence the regions deleted by the *Bal* 31 treatment, DNA segments of pKF402 were cloned into pUC118 and pUC119. Single-stranded DNA was obtained with the aid of an M13 helper phage (22). It was sequenced with specific oligonucleotide primers.

The wild-type sequence of pFT501 was established by using double-stranded DNA and specific oligonucleotide primers. The sequencing strategy is shown in Fig. 2.

Analysis of polynucleotide sequence. The polynucleotide sequence of pFT501 (Fig. 3) contained two open reading frames (ORFs), a smaller one (*orf-1*) starting at nucleotide 259 and terminating at nucleotide 555 and a larger one starting at position 676 and terminating at position 1461. Because pFT405, which contains only the larger ORF, complemented the *envM* temperature-sensitive mutation, we infer that it represents the *envM* gene. When the sequence was analyzed by the algorithm of Staden based on the codon improbability method (15), both ORFs were recognized as genes. The *envM* gene was preceded by a sequence, arranged mainly in the intercistronic region, which showed all the characteristics of a procaryotic promoter. The smaller *orf-1* showed no such features. *orf-1* was not preceded by a ribosome-binding site. There were only two G residues at positions 247 and 248 which may constitute a fragmentary ribosome-binding site. We could not find a sequence which would act as a rho-independent terminator. In *envM* there were two potential translational start codons, one at nucleotide 655 and the second at nucleotide 676. Because only the second ATG was preceded by a ribosome-binding site, we propose that translation is initiated at the second of the two methionine codons. We partially sequenced the homologous *E. coli* gene. It contained only one ATG, which was congruent with the second of the two methionine codons in *S. typhimurium*. This supports our proposal that the second ATG is the initiation codon.

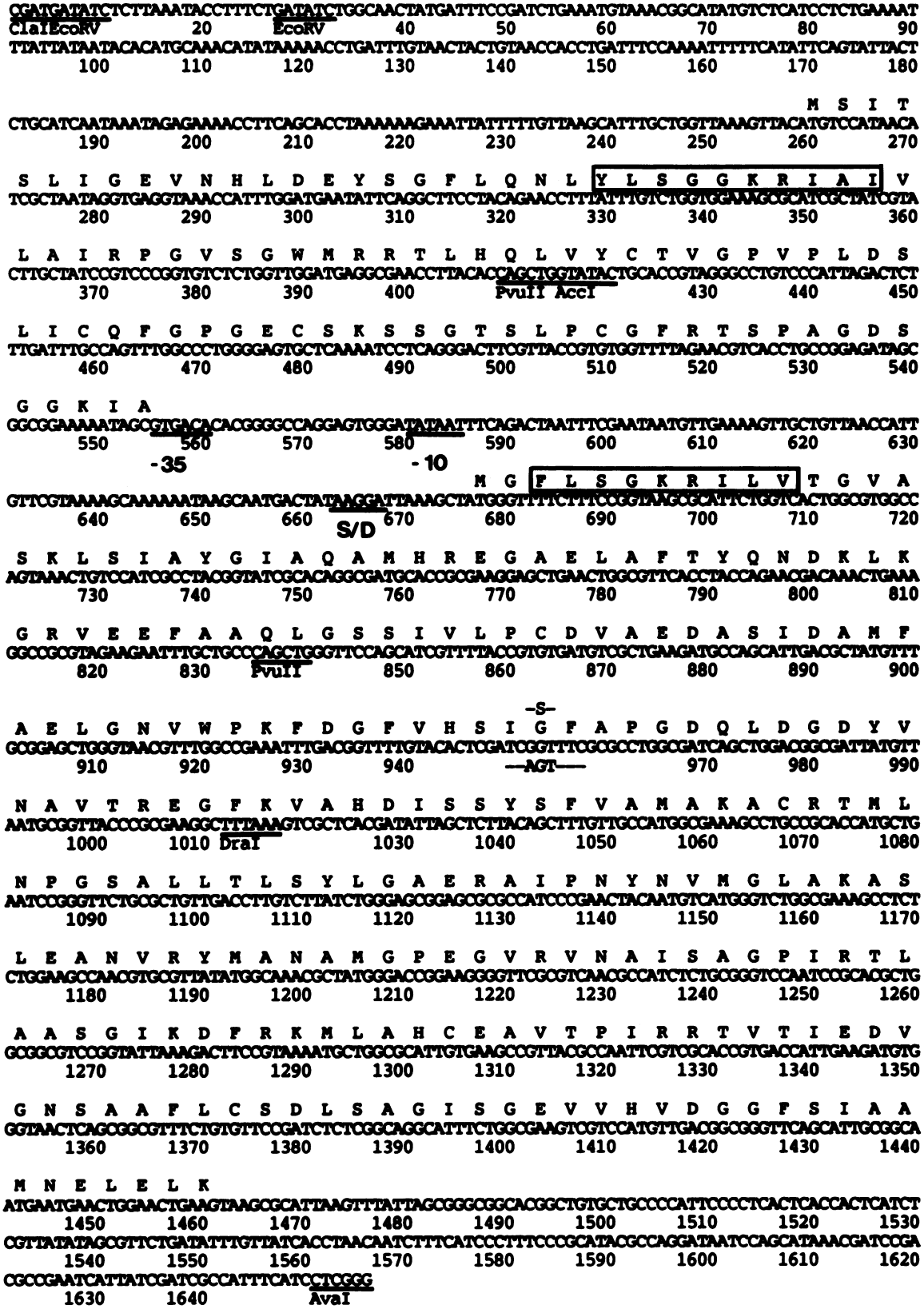

FIG. 3. Nucleotide sequence. The *S. typhimurium* wild-type sequence of the *Cla*I-*Ava*I fragment is shown. The mutation in position 952 leading to the resistance phenotype is indicated as an altered codon below the wild-type sequence. The altered amino acid, a serine, is shown above the amino acid sequence. The predicted amino acid sequence is represented by the one-letter code. A putative promoter of *envM* in the intercistronic region is underlined and designated with −10 and −35. A putative ribosome-binding site is also underlined and marked with the letters S/D. An amino acid motif common to both proteins is boxed. Restriction sites used for the construction of the subclones are appropriately designated and underlined.

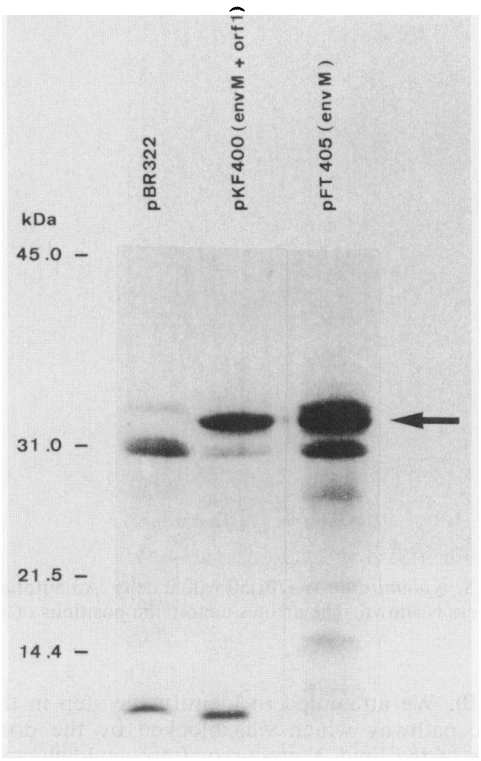

FIG. 4. Protein expression in minicells. An autoradiogram of an electrophoresis gel is shown. The arrow points to the position of the insert-specific EnvM protein. The band at the bottom of the gel in track pKF400 is a pBR322-coded protein, which is smaller than that appearing in the control lane because a *Bal* 31 deletion procedure giving pKF400 removed some of the vector DNA. The DNA segment coding for this protein is entirely missing in pFT405. kDa, Kilodaltons.

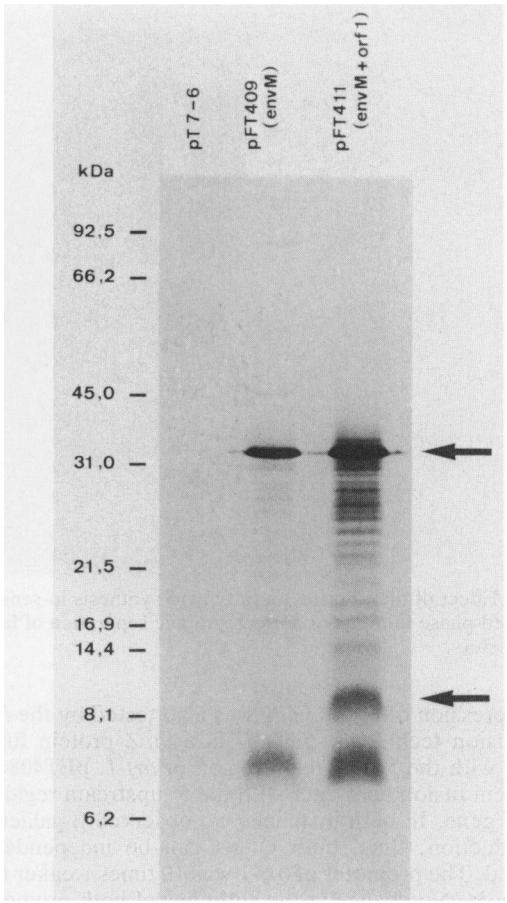

FIG. 5. Protein expression by the pT7 system. An autoradiogram of an electrophoresis gel is shown. The arrows point to the two main primary translation products of pFT411 which contains both *orf-1* and *envM*. pFT409 contains only the *envM* gene. Hence, it expresses only one protein with an apparent molecular weight of 33,000. kDa, Kilodaltons.

The smaller *orf-1* coded for a protein of 99 amino acids, and the *envM* gene coded for a protein of 262 amino acids, corresponding to molecular masses of 10,445 and 27,765 daltons, respectively. There was a common motif in both derived amino acid sequences, i.e., XLSG(G)KRIZZ. X is an aromatic amino acid, and Z is a hydrophobic amino acid. The sequence motif is boxed in both amino acid sequences of Fig. 3. Its function is unknown.

The sequence of the mutated *envM* gene leading to a drug resistance phenotype was identical to the wild-type sequence with one exception, a G-to-A transition in position 952, which changes a Gly codon to Ser.

Protein expression studies. The expression of the two genes was tested in several systems. Since *orf-1* was atypical in several ways, i.e., lack of a classical promoter and ribosome-binding structure, it was of particular importance to decide whether it could be translated into a protein. A number of expression studies were performed with the minicell system (12). By [^{35}S]methionine labeling, a protein corresponding to the *envM* gene product could be detected after separation by SDS-polyacrylamide gel electrophoresis with pKF400, pKF402, pKF403, or pFT405 as the DNA source in the minicell strain. The result with pKF400 and pFT405 is shown in Fig. 4. The protein migrated with an apparent molecular mass of 33,000 daltons. The protein could also be seen in whole-cell extracts separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue (data not shown). The protein appeared in the cytoplasmic fraction. In minicells the product of *orf-1* was never detected. However, when we linked the insert containing both ORFs to the bacteriophage T7 promoter present in plasmid pT7-5, *orf-1* was expressed in addition to the *envM* gene product. Its apparent molecular mass was 10,400 daltons (Fig. 5). The pT7 system (21) works with two plasmids, pGP1-2, which carries the gene for bacteriophage T7 polymerase under the control of the lambda p_L promoter, and a pT7 plasmid, which brings the gene to be investigated under the control of the T7 promoter ϕ10. Both plasmids are introduced into a host which produces a temperature-sensitive lambda repressor. At the nonpermissive temperature, transcription from the T7 promoter ensues. In the presence of rifampin, the formation of all other cellular proteins except those coded by the insert is blocked. The protein bands appearing between the two main translation products in Fig. 5 are believed to be proteolytic degradation fragments of the EnvM protein. They are not translational products of chromosomal origin because they were absent in a control experiment in which the genes were not expressed because of the inverse orientation of the insert and they were absent in the vector control. In the T7-polymerase-promoter system, *orf-1* was not expressed in amounts comparable to the EnvM protein. The reason is probably a tight translational control.

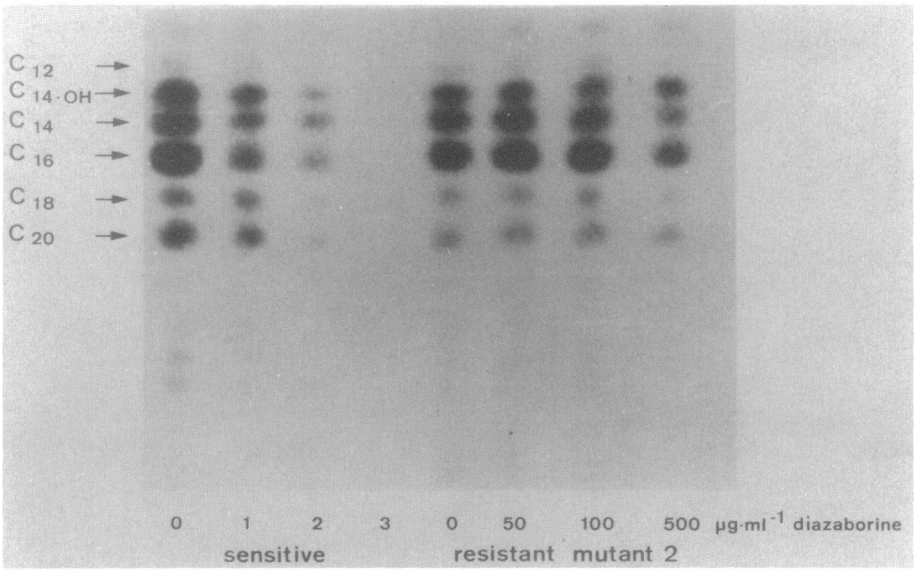

FIG. 6. Effect of diazaborine on fatty acid synthesis in sensitive and resistant *S. typhimurium* AG701i50 whole cells. An autoradiogram of a reversed-phase thin-layer chromatographic separation of fatty acid methyl esters is shown. The arrows denote the positions of standard fatty acyl esters.

The expression of both ORFs was also tested by the *lacZ* protein fusion technique. pFT407 is a *lacZ* protein fusion construct with the 5′ upstream region of *orf-1*. pFT408 is a *lacZ* protein fusion construct with the 5′ upstream region of the *envM* gene. In both instances we observed β-galactosidase production; thus, both ORFs can be independently transcribed. The promoter of *orf-1* was 10 times weaker than that of *envM*. No change in the activities of both promoters occurred during various growth conditions such as NaCl addition, anaerobic growth, or glycerol as the carbon source. Diazaborine was also without effect on the promoter activities.

Effects of gene *envM* on lipid biosynthesis, membrane composition, and cell morphology. (i) Effects on fatty acid biosynthesis. In whole *S. typhimurium* cells, diazaborine inhibited fatty acid formation. The composition of the fatty acids synthesized did not change when the cells were treated with the drug. The substance affected the amount of each individual fatty acid to the same extent as can be seen from the thin-layer chromatographic separation of fatty acids isolated from in vivo-labeled cells (Fig. 6). The fatty acid synthesis in a diazaborine-resistant mutant, *S. typhimurium* AG701i50-m2, was not inhibited by the drug at concentrations approximately 100 times higher than those effective in the wild type. Also, in *E. coli* BK80 *envM*, fatty acid biosynthesis was affected by diazaborine treatment as well as by shifting the cells to the nonpermissive temperature. This was demonstrated by incorporation of labeled acetate into whole cells (data not shown). Isolated components of the fatty acid synthesis enzymes of *E. coli* when tested in vitro were not affected by the drug, whereas positive controls with cerulenin showed the expected effects (data not shown). Acetyl-CoA carboxylase was separately tested and found not to be inhibited. These results imply that fatty acid biosynthesis enzymes per se were not targets of the compound. We interpret these results to mean that presumably the expression of one of the biosynthetic enzymes is affected.

(ii) Effects on LPS biosynthesis. As shown earlier, the diazaborine derivative has a profound effect on LPS biosynthesis (10). We attempted to identify the step in this biosynthetic pathway which was blocked by the drug. The synthesis of the lipid A precursor (tetraacyldiglucosamine-1,4′-biphosphate) which accumulates in a temperature-sensitive *kdsA* mutant of *S. typhimurium* at the nonpermissive temperature (18) was severely inhibited at drug concentrations comparable to the MIC. We ruled out any inhibition of later metabolic steps, like that catalyzed by CMP-3-deoxy-D-*manno*-octulosonic acid transferase, because 3-deoxy-D-*manno*-octulosonic acid-containing metabolites appeared after temperature shift-down both in inhibited and uninhibited cells. Maturation of LPS after temperature shift-down, as analyzed by galactose incorporation, was also not inhibited (data not shown).

Although we could not detect the accumulation of an earlier metabolite, we conclude from this analysis that a step early in lipid A precursor synthesis is affected, possibly as a result of either the inhibition in fatty acid biosynthesis or the acyl transfer to the UDP-*N*-acetylglucosamine moiety.

(iii) Effects on phospholipid biosynthesis. Phospholipid biosynthesis was affected profoundly either by diazaborine treatment or by raising the temperature in a temperature-sensitive *envM* mutant. This was shown by incorporation of radioactive phosphate in whole *E. coli* cells, followed by extraction and analysis of the lipids. The total phospholipid fraction, which was enriched in the chloroform layer, was greatly diminished under these two conditions compared with controls (Table 3). The effect observed after shifting *envM* cells to the nonpermissive temperature could be complemented by the presence of pKF402. The methanol-water phase contained LPS. Here the inhibition was not as pronounced as in the phospholipid fraction. The qualitative composition of the phospholipids was not altered by either treatment. This was shown by two-dimensional chromatographic separations of the extracted phospholipids (data not presented).

Changes in membrane protein composition. The influence of diazaborine on the protein composition of inner and outer membranes of several *E. coli* strains was investigated and compared with the effect of a temperature shift in the

TABLE 3. Phospholipid (chloroform phase) and LPS (methanol-water phase) biosynthesis in an *envM*(Ts) mutant at 30 and 42°C and in the presence of drug[a]

Strain and condition(s)	Chloroform phase		Methanol-water phase	
	Radio-activity (cpm)	% Control	Radio-activity (cpm)	% Control
BK80 *envM* (30°C)	25,042	100	11,716	100
BK80 *envM* (42°C)	1,288	5	4,108	35
BK80 *envM* (4 μg of diazaborine per ml, 30°C)	993	4	3,957	34
BK80 *envM*(pKF402) (42°C)	23,196	93	10,530	90

[a] The incorporation of radioactive phosphate was measured.

temperature-sensitive *envM* mutant. Figure 7 shows an electrophoresis gel of inner membrane proteins prepared from the *E. coli envM* mutant that is temperature sensitive for growth. Diazaborine treatment at 30°C or shift to the nonpermissive temperature strongly induced the accumulation of a 28,000-dalton protein. The induction of this protein disappeared when the cells contained a plasmid carrying gene *envM* as in pKF403. pBR322-containing cells behave like the plasmid-free mutant (data not shown).

Concerning the outer membrane proteins, we observed changes in the ratio of porin proteins OmpC and OmpF. OmpC always exceeded OmpF in membranes prepared from the plasmid-free *envM* mutant grown at 30°C or from cells containing plasmid pKF403. However, when plasmid-free *envM* mutants were incubated at the nonpermissive temperature, OmpF and OmpC appeared to be present in approximately equal amounts, which means that the ratio shifted toward OmpF. This effect did not occur if the temperature-sensitive mutation was complemented by pKF403. In wild-type cells, we noticed little change in the ratio of these two proteins upon temperature elevation. Diazaborine treatment of plasmid-free cells at 30°C resulted in an effect comparable to the temperature shift.

Morphological effects of diazaborine treatment. When *E. coli* 5K cells in the early logarithmic phase were treated with diazaborine, characteristic morphological effects could be observed in the phase-contrast light microscope. The majority of the rod-shaped cells showed zones of enhanced transparency at the poles. In electron micrographs of drug-treated *E. coli* D10 cells, these light zones appeared as empty areas which seem to have been formed by retraction of the inner membrane. In many instances also, a detachment of the inner membrane at the cylindrical part of the bacterial cell could be seen, but this effect was not nearly as pronounced as the one appearing at the poles (Fig. 8).

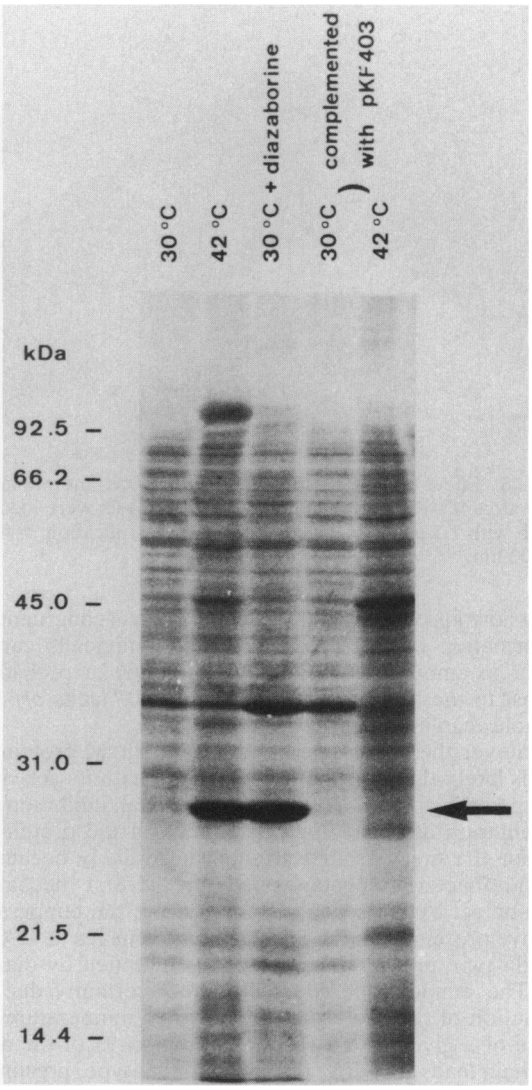

FIG. 7. Inner membrane proteins of *E. coli* JP1111. A stained electrophoresis gel is shown. The arrow points to a 28,000-dalton protein induced after inhibition of EnvM. kDa, Kilodaltons.

DISCUSSION

A cloned DNA segment from a diazaborine-resistant *Salmonella* mutant complements the temperature-sensitive *envM* phenotype in *E. coli* and at the same time renders the *E. coli* strain diazaborine resistant. Hence, the EnvM protein must be the target of the diazaborines. Nucleotide sequencing showed that this DNA fragment contains two ORFs. The larger one, which by itself confers resistance to diazaborine and complements the *envM* mutation, is translated readily in minicells into a protein of the expected size. It is this gene which is altered by a point mutation to give drug resistance. The expression of the smaller *orf-1* could be detected only in a forced expression system, i.e., if the gene was linked to a bacteriophage T7 promoter and transcribed by T7 RNA polymerase. Both ORFs contain their own promoters which, as *lacZ* fusion experiments suggest, are quite active. The reason why the small polypeptide coded by *orf-1* cannot be seen in labeled extracts from minicells is probably partly related to its lack of a well-established ribosome-binding site and to its codon composition. This results in a very low translational efficiency. The gene contains two AUA (isoleucine) and two AGG (arginine) codons which are practically absent in highly expressed proteins (11). Presently, we cannot decide whether the function of *orf-1* is linked in any way with *envM* or whether it belongs to another genetic system.

envM, however, is preceded by a classical promoter. The activity of this promoter was also measured by the protein fusion technique, and it proved to be stronger than that of *orf-1*. Because *orf-1* was absent in this construct, we can disregard any readthrough from the upstream gene. This observation indicated that *envM* is transcribed individually

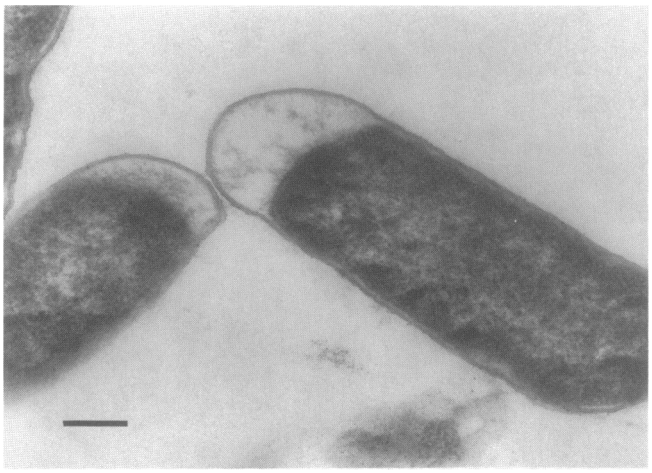

FIG. 8. Effect of diazaborine on *E. coli* D10 cell morphology. A thin section of treated *E. coli* cells is shown. Cells were incubated for 22 h with 1.5 μg of diazaborine per ml. Magnification, ×40,000. Bar, 200 nm.

from its own promoter, and this conclusion is congruent with the formation of the EnvM protein in minicells carrying pFT405 in amounts comparable to pKF400 or pKF403. In contrast to the latter two plasmids, pFT405 lacks *orf-1* and its whole regulatory upstream region.

Whatever the immediate effect of the EnvM protein may be, it is likely that diazaborine interferes with its activity by binding to the protein. This could result in an inhibition or an augmentation of the biological function of the protein. We consider the first possibility much more likely because all other antibacterial substances act by the first mechanism, and none act by the second. Furthermore, the temperature-sensitive protein from the *envM* mutant behaves at 42°C like the wild-type protein does when it is inhibited by diazaborine. The temperature effect is almost certainly due to a destruction of the protein at the elevated temperature. The change of a glycine to a serine in position 93 of the amino acid chain leads to a drug resistance phenotype, presumably by preventing the drug from binding to the protein. Further experiments with purified protein are needed to test this proposition. What can be concluded from our experiments so far is that EnvM induces several major changes in the membrane composition of *E. coli* or *S. typhimurium*. When inhibited by the drug or by temperature, EnvM leads to the massive accumulation of a 28,000-dalton inner membrane protein, and it shifts the OmpC/OmpF ratio in the outer membrane toward OmpF. Furthermore, when inhibited it reduces fatty acid, phospholipid, and LPS biosynthesis. The three latter effects may be interrelated because phospholipid and LPS biosyntheses depend on available fatty acids. However, since the quantitative responses of fatty acid, phospholipid, and LPS biosynthesis to diazaborine differ, the possibility must be considered that EnvM acts separately on these biosynthetic steps in a truly pleiotropic manner. The cytoplasmic membrane protein induction is not triggered by an inhibition of fatty acid synthesis because the 28,000-dalton protein did not appear in membranes from cerulenin-treated *E. coli* cells.

Diazaborine treatment of *E. coli* cells had a unique effect on cell morphology. A similar effect was observed in the *envM* mutant at nonpermissive conditions. A peculiar detachment of the cytoplasm, predominantly at the poles of the cells, was generally observed. The morphological alterations probably result from the effects on membrane component synthesis. The greatly reduced phospholipid content may contract the cytoplasmic membrane, giving it a shrunken appearance.

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LITERATURE CITED

1. **Alberts, A. W., and P. R. Vagelos.** 1968. Acetyl CoA carboxylase. I. Requirement for two protein fractions. Proc. Natl. Acad. Sci. USA **59**:561–568.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
3. **Egan, A. F., and R. R. B. Russell.** 1973. Conditional mutants affecting the cell envelope of *Escherichia coli* K-12. Genet. Res. **21**:139–152.
4. **Eichenlaub, R.** 1979. Mutants of the mini-F plasmid pML31 thermosensitive in replication. J. Bacteriol. **138**:559–566.
5. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115**:717–722.
6. **Folch, J., M. Lees, and S. G. Sloane.** 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**:497–509.
7. **Grassberger, M. A., F. Turnowsky, and J. Hildebrandt.** 1984. Preparation and antibacterial activities of new 1,2,3-diazaborine derivatives and analogues. J. Med. Chem. **27**:947–953.
8. **Harder, M. E., R. C. Ladenson, S. D. Schimmel, and D. F. Silbert.** 1974. Mutants of *Escherichia coli* with temperature sensitive malonyl coenzyme A-acyl carrier protein transacylase. J. Biol. Chem. **249**:7468–7475.
9. **Heusser, D.** 1968. Dünnschichchromatographie von Fettsäuren auf silanisiertem Kieselgel. J. Chromatogr. **33**:62–69.
10. **Högenauer, G., and M. Woisetschläger.** 1981. A diazaborine derivative inhibits lipopolysaccharide biosynthesis. Nature (London) **293**:662–664.
11. **Konigsberg, W., and G. N. Godson.** 1983. Evidence for use of rare codons in the *dna*G gene and other regulatory genes of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **80**:687–691.
12. **Levy, S. B.** 1974. R-factor proteins synthesized in *Escherichia coli* minicells: incorporation studies with different R-factors and detection of DNA-binding proteins. J. Bacteriol. **120**:1451–1463.
13. **Majerus, P. W., A. W. Alberts, and P. R. Vagelos.** 1969. Acyl-carrier protein from *Escherichia coli*. Methods Enzymol. **14**:43–50.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **McLachlan, A. D., R. Staden, and D. R. Boswell.** 1984. Measure of strength of codon preference. Nucleic Acids Res. **12**:9567–9575.
16. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Minton, N. P.** 1984. Improved plasmid vectors for the isolation of translational *lac* gene fusions. Gene **31**:269–273.
18. **Rick, P. D., and M. J. Osborn.** 1977. Lipid A mutants of *Salmonella typhimurium*. J. Biol. Chem. **252**:4895–4903.
19. **Sanger, T., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.

20. **Sperka-Gottlieb, C. D. M., A. Hermetter, F. Paltauf, and G. Daum.** 1988. Lipid topology and physical properties of the outer mitochondrial membrane of the yeast, *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **946**:227–234.
21. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1087.
22. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153**:3–11.
23. **Wehlmann, H., and R. Eichenlaub.** 1980. Plasmid mini-F encoded proteins. Mol. Gen. Genet. **180**:205–211.