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emr, an *Escherichia coli* locus for multidrug resistance

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ABSTRACT **An *Escherichia coli* chromosomal DNA fragment cloned on a multicopy plasmid conferred resistance to carbonylcyanide *m*-chlorophenylhydrazone, nalidixic acid, and a number of other toxic compounds. The sequence of the cloned *emr* locus located at minute 57.5 of the chromosome revealed two open reading frames, *emrA* and *emrB*. *emrB* encodes a highly hydrophobic 56.2-kDa peptide, with 14 potential α-helices to span the inner membrane. The peptide is homologous to QacA, a multidrug-resistant pump from *Staphylococcus aureus*, and belongs to a gene family that includes tetracycline-resistant pumps of Gram-positive bacteria and the galactose/H⁺ symporter of *E. coli*. *emrA* encodes a putative 42.7-kDa peptide containing a single hydrophobic domain and a large C-terminal hydrophilic domain. An active pho-fusion to the C domain suggested that EmrA is a membrane protein. Disruption of *emrB* significantly increased sensitivity of cells to uncouplers. The cellular content of uncoupler increased in the order: overexpressed *emrB* cells > wild type > *emrB*⁻.**

Multiple resistance of a bacterial cell to several different antibiotics is a well-studied phenomenon. The resistance is often due to the presence of plasmids containing several genes, each encoding resistance to a specific antibiotic. A different case of multidrug resistance (mdr) is when the same mechanism protects the cell from various noxious substances. The best-studied example of this case comes from eukaryotic cells that possess the mdr pump P180, which is largely responsible for the resistance of tumors to chemotherapy (1). In Gram-positive bacteria, a number of pmf-dependent translocases that pump out various hydrophobic cations such as ethidium bromide and acriflavine were cloned and sequenced (2–7). Active efflux of hydrophobic cations was also demonstrated in *Escherichia coli* (8), but the pump has not been characterized. Porins in the outer membrane of Gram-negative species restrict the passage of large molecules and smaller hydrophobic substances, including antibiotics. Weak permeant acids, apparently acting through the acidification of the cytoplasm, repress the synthesis of OmpF (9, 10), a porin with a larger channel than in OmpC, the other major porin of *E. coli*. A cryptic *mar* locus in *E. coli* that provides antibiotic mdr upon activation also represses OmpF (11). Mutants that grow in the presence of uncouplers of oxidative phosphorylation have been isolated from a number of bacterial species, and this seems to be an especially interesting case of resistance, since the mechanism by which a cell can protect itself from uncouplers is not at all obvious (12). In this paper, we describe the cloning of a chromosomal locus, *emr* (*E. coli* mdr), that protects the cell from some uncouplers of oxidative phosphorylation and from a number of unrelated hydrophobic compounds.†

MATERIALS AND METHODS

Strains and Plasmids. RP437 [*thr-1*(am) *leuB6 hisG4 metF159*(am) *rpsL136 thi-1 ara-14 lacY mtl-1 xyl-5 tonA31 tsx-78*] was kindly provided by J. S. Parkinson (University of Utah). W3100 wild-type *E. coli* K-12 was kindly provided by F. Neidhardt (University of Michigan). Phage λ 1205 and plasmid pNK2882 were kindly provided by Nancy Kleckner (Harvard University). Strain CC118 [*araD139* Δ(*ara, leu*)7697 Δ(*lacX74*) Δ(*phoA20*) *galE galK thi rpsE rpoB argE recA1*] and phage λ Tn*phoA* were kindly provided by C. Manoil. pUC18 was purchased from New England Biolabs.

Bacterial Growth. For testing resistance to a number of drugs, cells were grown overnight in LB medium (1% tryptone/0.5% yeast extract/0.5% NaCl), diluted, and mixed with 0.3% agar in T broth (1% tryptone/0.5% NaCl) containing the tested compound. The number of colonies appearing after overnight growth at 35°C was counted.

Measurement of Tetrachlorosalicylanilide (TSA) Fluorescence. Cells were incubated in the presence of TSA, and 0.5-ml samples were withdrawn and pelleted in an Eppendorf centrifuge for 20 sec. Supernatant was aspirated, and cells were resuspended in 50 mM phosphate buffer (pH 7). The content of TSA was 25:1 pellet:supernatant. An aliquot of resuspended cells was transferred to a fluorometer cuvette (Perkin–Elmer LS-5B) at OD_{550} = 0.02 in 50 mM phosphate buffer (pH 7), and fluorescence was recorded at 442 nm, with a 382-nm excitation.

Recombinant DNA Methods. Chromosomal DNA was isolated from *E. coli* according to ref. 13. The DNA was partially digested by *Bam*HI and restriction fragments were cloned into the *Bam*HI site of multicopy pUC18 plasmid. The ligation mixture was transformed into RP437 and transformants were plated on VB minimal medium containing succinate and 40 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP). Plasmid DNA was isolated by the alkali lysis procedure (13). PCR was performed according to ref. 14 with the use of a GeneAmp kit (Perkin–Elmer/Cetus). The *emr* fragments from RP437 were amplified by PCR. Primers with *Eco*RI and *Bam*HI restriction sites were prepared. The PCR fragments were cloned into pUC18 digested with *Eco*RI and *Bam*HI. Sequencing by the dideoxy method was performed in both directions using a USB Sequenase Version 2.0 kit. Standard primers for pUC18 and 18-base-pair (bp) synthetic primers (synthesized by Biopolimers Laboratory, Massachusetts Institute of Technology) complementary to already determined sequence were used. Sequences were compared with the Protein Identification Resource data base using the BLAST program. Alignments were created by the PILEUP program. Kyte–Doolittle hydropathy plots were created by the PEPTIDESTRUCTURE program. All programs came from the GCG (Madison, WI) package. Restriction enzymes and DNA ligase were purchased from New England Biolabs.

Gene Disruption. Mini-Tn*10*kan-*lacZ* was delivered into strain RP437 with pEMR2.1 on λ 1205 (15). A plasmid with

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; mdr, multidrug resistance; *emr*, *E. coli* multidrug resistance; TSA, tetrachlorosalicylanilide; ʳ, resistant.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M86657).

a transposon insertion into *emrB* was isolated and used to replace chromosomal *emrB* (16).

Generation of PhoA Protein Fusions. Phage λ Tn*phoA* carrying a *phoA* gene (lacking promoter and ribosome binding sequences) linked to Tn*5kan*^{r} was used to infect CC118 (*phoA*) containing the recombinant plasmid (17). Plasmid DNA was isolated from Kan^r transductants and was used to retransform CC118. Kan^r transformants that were blue on 5-bromo-4-chloro-3-indolyl phosphate plates were further analyzed.

Primer Extension. The general approach was in accordance to ref. 14. RNA was isolated from 10 ml of logarithmic cells by acidic phenol extraction (14). The primer used was complementary to the 5′ end of *emrA* and started 46 bp downstream of *emrA* translational start.

***In Vitro* Transcription-Translation.** Plasmid DNA was isolated and used for *in vitro* transcription-translation according to the manufacturer's instructions (Amersham, prokaryotic DNA-directed translation kit). The resulting [^{35}S]methionine-labeled proteins were separated by SDS/15% PAGE electrophoresis (18).

RESULTS

Cloning a CCCP-Resistance Locus. To obtain a resistant mutant, wild-type W3110 was grown in liquid Vogel–Bonner (VB) minimal succinate (20 mM) medium in the presence of 100 μM CCCP overnight at 37°C, diluted, and then plated on an LB plate containing 80 μM CCCP. A colony (KLE110) was picked and was found to be resistant to 80 μM CCCP on a TB plate, whereas the parent strain was sensitive to 40 μM CCCP.

To clone uncoupler resistance, a DNA *Bam*HI library made from the CCCP-resistant KLE110 DNA in pUC18 was transformed into strain RP437 (*Materials and Methods*) and transformants were plated on VB minimal agar with 20 mM succinate and 40 μM CCCP. Plasmid DNA from two clones was isolated and retransformed into RP437. The transformants appeared to be substantially more resistant to CCCP than transformants with control pUC18 plasmid. Restriction maps of plasmids pEMR1 and pEMR2 are given in Fig. 1. The two plasmids appeared to share a common 3.5-kilobase (kb) *Bam*HI–*Xho* I fragment. This fragment was subcloned into pUC18. The resulting plasmid pEMR2.1 was found to confer resistance to CCCP.

Further restriction analysis was performed to locate the minimal locus conferring uncoupler resistance. By the approach used, the entire 3.5-kb segment appeared to represent the minimal fragment (Fig. 1).

Sequencing *emrA* and *emrB*. Sequencing of the entire insert in plasmid pEMR2.1 revealed two open reading frames (*emrA* and *emrB*, Fig. 2). From the deletion analysis of plasmid pEMR2 (Fig. 1), it followed that *emrA* and *emrB* were both necessary for uncoupler resistance. There is no promoter sequence upstream of *emrB*, which could suggest that the two genes are organized into an operon. However, typical −35 and −10 promoter sequences are not found upstream of *emrA*. Primer extension was used to obtain information about *emrA,B* transcription. Two primers complementary to the 5′ ends of *emrA* and *emrB* were prepared. The distance between primers and translational starts of *emrA* and *emrB* was 46 and 30 bp, correspondingly. The only product of primer extension was obtained with primer complementary to *emrA* sequence (not shown). The observed 5′ end of mRNA corresponds to A 103 (102 bp upstream ATG of *emrA*) (Fig. 2).

The sequence of the upstream region of *emrA* was identical to a known gene, *mprA*, with typical promoter sequence (19). It is possible that *emrA,B* actually form an operon with *mprA*. In this case, the *emrA,B* mRNA revealed by primer extension would have to be the product of processing a large mRNA carrying all three genes.

Since DNA of an uncoupler-resistant mutant was used for cloning, it was important to see whether the cloned fragment

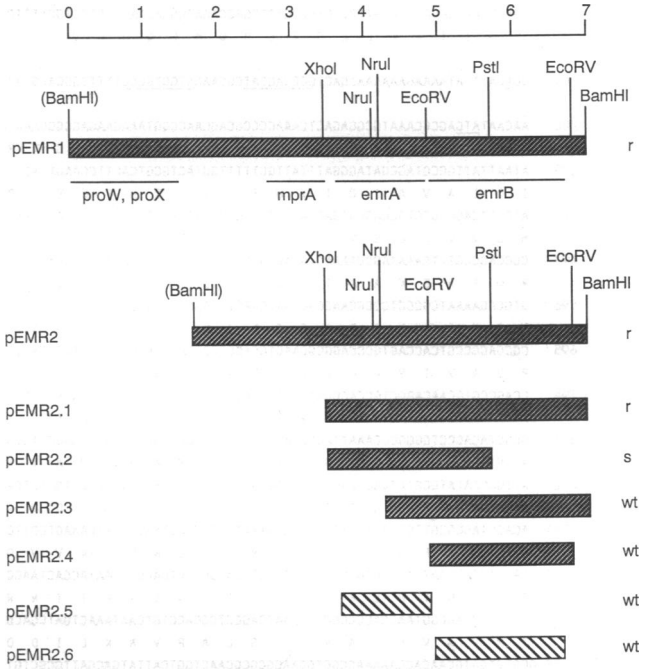

FIG. 1. Restriction maps of recombinant uncoupler-resistant plasmids and their derivatives. pEMR1 and pEMR2 are plasmids conferring uncoupler resistance that were obtained from a chromosomal *Bam*HI library in pUC18. The left *Bam*HI site of both plasmids was lost during cloning. The left part of pEMR1 contains part of *proW*, *proX*, and *mprA* genes. Plasmid pEMR2.1 was obtained by deleting an *Xho* I–*Eco*RI (*Eco*RI site is in the polylinker of pUC18) fragment of pEMR2; plasmid pEMR2.2 was obtained by deleting the *Pst* I–*Pst* I (the second *Pst* I is in the polylinker to the right of the insert) fragment from pEMR2.1; pEMR2.3 was obtained by deleting the *Eco*RI–*Nru* I (*Eco*RI is in the polylinker to the left of the insert) fragment from pEMR2.1; pEMR2.4 was obtained by cloning the *Eco*RV–*Eco*RV fragment of pEMR2 into the *Sma* I site of pUC18; plasmids pEMR2.5 and pEMR2.6 were obtained by cloning PCR products of *emrA* and *emrB*, correspondingly, into pUC18. r, CCCP resistance; wt, no difference from wild type; s, sensitivity greater than wild type.

was mutated or wild type. PCR fragments were prepared from RP437 DNA using appropriate primers. The PCR fragments, *emrA* and *emrB*, were cloned into pUC18 and sequenced. The sequence of the genes from the wild type was identical to that of the mutant, which means that a wild-type locus was cloned from strain KLE110. The resistance of the mutant to CCCP was somewhat higher than that of the strain with multicopy *emr*; it seems that the mutation in KLE110 might affect outer membrane permeability, as is the case with some other known uncoupler-resistant mutants of *E. coli* (12). It appears that cloning of uncoupler resistance in fact could have been done using DNA from a wild-type strain, and our finding suggests that other resistances might be cloned from wild-type *E. coli* by placing a locus on a multicopy vector.

Structure and Location of EmrA. The first frame is 1173 bp long and would encode a 390-amino acid peptide of 42.7 kDa. The Kyte–Doolittle hydropathy plot suggested a single 24-amino-acid-long hydrophobic domain near the N terminus (not shown). The structure of the putative peptide did not point to a possible cellular location. To locate the EmrA peptide, *phoA* fusions were prepared. The rationale is to make chimeric fusions of the protein of interest to alkaline phosphatase, which is only active when it is located in the periplasm (17). Therefore, only chimeras formed with membrane or periplasmic proteins have alkaline phosphatase activity. A *pho–emrA* fusion in plasmid pEMR2.1 was isolated (*Materials and Methods*), giving plasmid pEMR2.11, and the *phoA* insertion was localized by restriction mapping

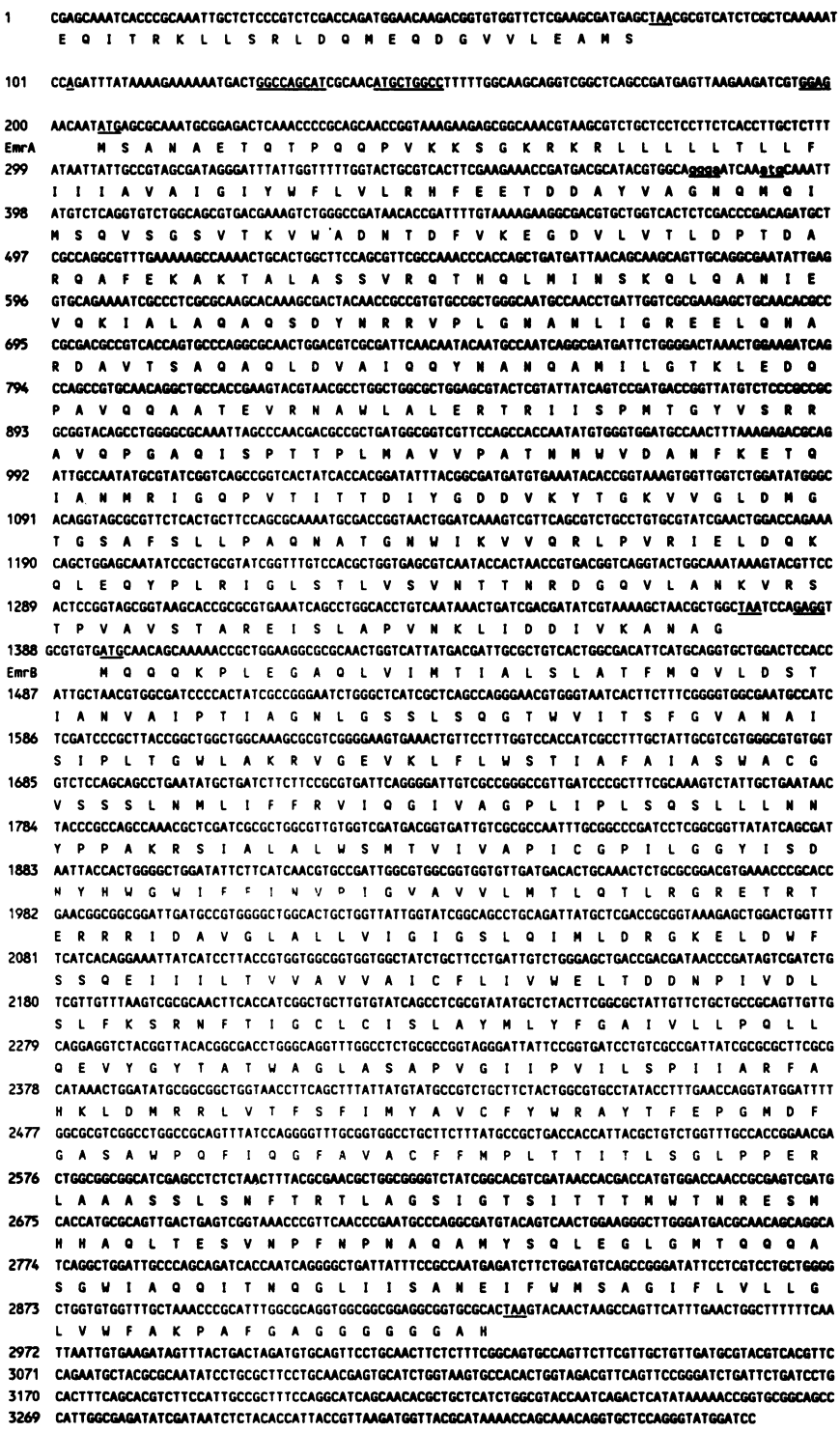

FIG. 2. Nucleotide sequences of genes conferring resistance to CCCP: *emrA* and *emrB*. The first two lines depict the 3′ sequence of an adjacent *mprA* gene with a stop codon and transcriptional terminator underlined. The 5′ end of *emrA,B* mRNA, start ATG and stop codons, and ribosomal binding sites of both genes are underlined. Alternative Shine–Dalgarno sequence and translation start of *emrA* are shown in lowercase letters.

to the 3′ end of *emrA*. The *phoA* insert was precisely located at Leu-380 at the C end of the *emrA* peptide by sequencing the distal part of the gene. The *emrA* gene lacks a sequence for a signal peptide that is necessary for the transport of proteins to the periplasm or to the outer membrane. On the other hand, the signal sequence is normally absent from integral inner membrane proteins. It seems that the short N-terminal end of EmrA is located in the cytoplasm, with the hydrophobic domain forming a single α-helix to span the membrane, and the large hydrophilic C-terminal domain is in the periplasm.

A search for homologies using the translated amino acid sequence of EmrA revealed homology to the CyaD protein (20) of *Bordetella pertussis* (28% identity and 49% similarity) that participates in the extrusion of cyclolysin. A limited homology was found to other members of the same family (21): to HlyD, a component of the *E. coli* hemolysin efflux pump (22), and to CvaA, a component of colicin V secretion (21).

Structure of EmrB. The 1542-bp open reading frame of *emrB* would encode a 513-amino acid peptide of 55.6 kDa. The hydropathy plot indicated a highly hydrophobic, integral membrane protein with 14 α-helices to span the membrane (not shown). The translated amino acid sequence appeared to be homologous to QacA (4), the pump for resistance to quaternary ammonium cations from *Staphylococcus* (23% identity and 54% similarity), to the *Streptomyces* Mmr putative peptide (23) conferring resistance to methylenomycin (26% identity and 50% similarity), and to genes conferring

resistance to tetracycline in Gram-positive bacteria (24, 25). The QacA and Mmr peptides have 14 putative transmembrane segments (23) as would the EmrB peptide.

mdr. Sequence upstream of *emrA* revealed identity to a known gene, *mprA* (19), placing *emr* at minute 57.5 of the *E. coli* chromosome. A chromosomal locus, *nalB*, is also located in the vicinity of 57.5 minutes (26). It confers low-level resistance to nalidixic acid and is believed to affect cellular permeability to nalidixic acid (27). We decided to see if *emr* might not have the properties of *nalB*. Strain RP437 carrying plasmid pEMR2.1 showed increased resistance to nalidixic acid. It did not protect cells from norfloxacin, a hydrophilic analogue of nalidixic acid toward which *norA* confers resistance (5). A survey of a number of toxic compounds (Table 1) showed that the cloned locus protects the cell from CCCP and its analogues; from TSA, a structurally unrelated hydrophobic uncoupler, but not from pentachlorophenol, a more hydrophilic uncoupler; and from organomercurials. There was no resistance to organic cations, such as tetraphenyl phosphonium and ethidium bromide, that are extruded by mdr of Gram-positive species. It appears that EMR only confers resistance to substances of high hydrophobicity.

Gene Disruption. The *emr* locus protected the cell from a number of drugs, when present on a multicopy plasmid. To see if *emr* is functional when present in a single copy, it would be necessary to compare resistance of wild-type and *emr*$^-$ cells.

A mini-Tn*10* was inserted into *emrB* in plasmid pEmr2.1 and disrupted *emrB* was then used to replace the chromosomal locus, giving strain KLE120. A maximal concentration of uncoupler TSA that did not inhibit growth of a strain with pEmr2.1 decreased the growth rate of a wild-type control and completely inhibited proliferation of the *emrB*$^-$ strain (Fig. 3). A parallel measurement of TSA following fluorescence showed that the cellular content of uncoupler declined in the order KL120 > RP437/pUC > RP437/pEMR2.1 (Fig. 3). When cells with TSA are diluted into a medium without TSA, the uncoupler leaks out rapidly, even from *emrB*$^-$ cells. There is no change in fluorescence between free and cell-bound TSA, further complicating measurements of efflux. Efforts to establish a convenient assay for uncoupler efflux measurement are necessary.

Protein Products of *emr*. An *in vitro* transcription-translation procedure was used to obtain the protein products of *emr* genes. Plasmid pEMR2.1 was used as a template, and the resultant peptide fraction was separated by SDS gel electrophoresis. Two prominent peptide bands (44 and 36 kDa) and one weak band (55 kDa) were present in the gel from pEMR2.1 carrying *emrA* and *emrB* as compared to the control pUC18 plasmid (Fig. 4). The prominent common band of β-lactamase present in all samples functions as a control for the extent of synthesis in the *in vitro* system.

Table 1. Resistance to toxic compounds conferred by plasmid pEMR2.1 carrying *emrA,B*

Inhibitory substance	Conc., μM	RP437, pEMR2.1
CCCP	40	+
CHH	80	+
TSA	40	+
PCP	200	−
Nal	20	+
Nor	1	−
Tet*	4	−, −
Cm	5	−
EtdBr	103	−
TPP$^+$	103	−
PMA†	1	+

Resistance was determined by growth of colonies in 0.3% agar/T broth medium with a given component. Resistance was scored as positive if there was a three to four order of magnitude difference in the number of RP437/pEMR2.1 colonies as compared to RP437/pUC18. CHH, 2-chlorophenylhydrazine hydrochloride; PCP, pentachlorophenol; Nal, nalidixic acid; Nor, norfloxacin; Tet, tetracycline; Cm, chloramphenicol; EtdBr, ethidium bromide; TPP$^+$, tetraphenyl phosphonium; PMA, phenylmercury acetate.
*pEMR2.1 made cells hypersensitive to tetracycline.
†PMA drastically decreased the size of the colonies in RP437/pUC18.

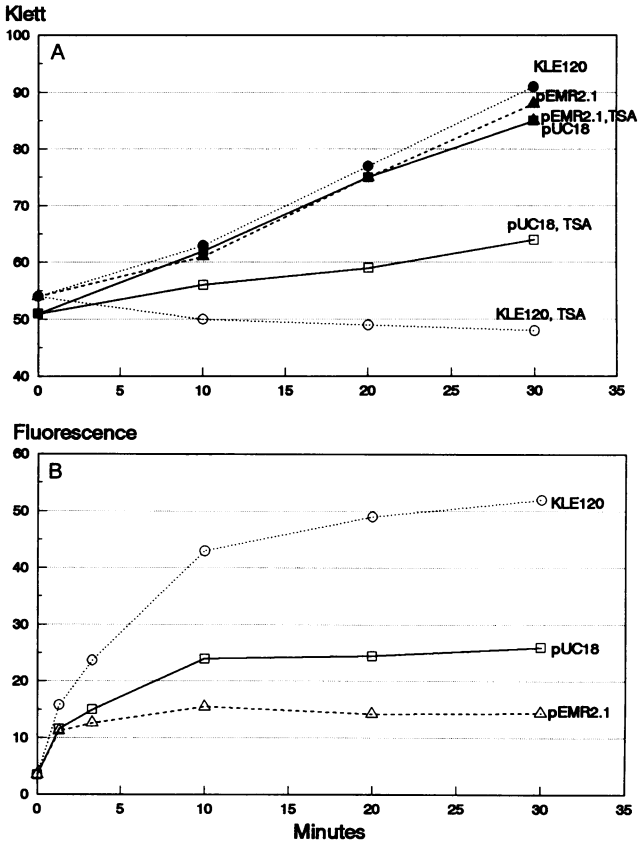

FIG. 3. (*A*) Cells were grown to Klett 50 (OD_{550} = 0.3) in LB medium, and TSA was added at 25 μM at time zero. KLE120 is the emrB strain in RP437 background; pEMR2.1 stands for RP437/pEMR2.1, and pUC18 is RP437/pUC18. (*B*) One-half milliliter samples were taken in duplicate from the cells of *A* at times indicated, and TSA content was determined.

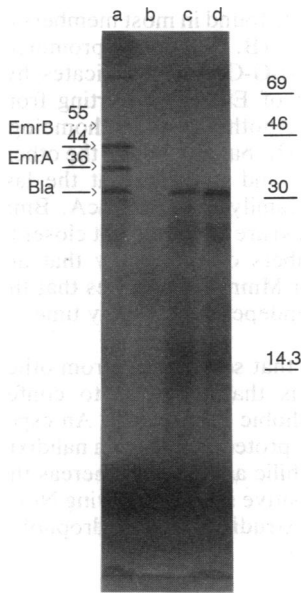

FIG. 4. Peptide products of *in vitro* transcription-translation. The products of coupled transcription-translation were separated by SDS/PAGE. Protein markers are indicated in kDa. Emr products and β-lactamase are indicated by arrows (the putative product of emrB, top dashed arrow, might be too faint for reproduction). Lane a, pEMR2.1 (emrA,B); lane b, no plasmid; lane c, pEMR2.6 (emrB); lane d, pUC18.

Plasmid pEMR2.2 that carries a deletion in *emrB* only produced the prominent peptides (not shown). pEMR2.5 containing an *emrA* PCR fragment (nucleotides 182–1411, Fig. 2) also produced the two prominent peptides (not shown). No peptides (except β-lactamase) were produced from plasmid pEMR2.6 carrying the *emrB* gene alone (nucleotides 1224–3137, Fig. 2), confirming the conclusion that this gene lacks its own promoter. The theoretical molecular mass of *emrA*, 43 kDa, closely matched the larger 44-kDa peptide produced by the plasmid carrying only the *emrA* gene. The second, smaller 36-kDa peptide produced from the same plasmid might be due to alternative translation from an ATG located at position 396 (Fig. 2). There is indeed a good putative ribosomal binding site 5 bp upstream of this ATG, and the theoretical mass of this peptide would be 36 kDa. It is not clear if both peptides are synthesized from *emrA in vivo*. The molecular mass of the 55-kDa peptide matches the predicted mass of EmrB, 56 kDa. The small amount of EmrB production might be due to difficulties arising from *in vitro* translation of this very hydrophobic peptide.

DISCUSSION

In bacteria, a mdr pump was first reported in the Gram-positive *Staphylococcus aureus*. Plasmids from *S. aureus* carry genes *qacA*, *qacB*, *qacC*, *qacD*, and *qacE* (*smr*), each encoding an efflux translocase for hydrophobic cations such as ethidium bromide or acriflavine (2–4, 7). *norA*, a gene conferring resistance to a different class of compounds—hydrophilic quinolones, analogues of nalidixic acid such as norfloxacin—was identified in a staphylococcal plasmid (20) and was found to be homologous to *tetA*, a [tetracycline $Me^+]/H^+$ antiporter encoded by Tn*10* (28). A *Bacillus subtilis* chromosomal gene, *bmr*, conferring resistance to hydrophobic cations (6) and to quinolones (29) is homologous to *norA* and *tet*^{r} genes from other Gram-positive bacteria. No known ATP binding sites are present in the sequences of bacterial mdr pumps, and there is no homology to eukaryotic MDR (see ref. 31 for a review).

The cloned *emrB* gene of *E. coli* belongs to a family of membrane translocases that include multidrug-resistant proteins of Gram-positive bacteria and, to our knowledge, such a finding has not been reported previously in a Gram-negative species. This gene family also includes pumps that protect cells from individual antibiotics: the tetracycline (24, 25) and the methylenomycin pump (23) and the arabinose and galactose H^+/sugar symporters (see ref. 4 for a discussion). EmrB has a typical structure of an integral membrane translocase, with 14 putative α-helices spanning the membrane. There are four putative consensus sequences found in most members of this family (4) as well as in EmrB. The most prominent consensus is G-X-hy-hy-G-P-X-I-G-G ("hy" indicates hydrophobic), which, in the case of EmrB, is starting from Ala-150: A-X-I-C-G-P-I-L-G-G. Another region of homology is shared between QacA and EmrB, but not among the other, shorter proteins of this family, and is located at the last α-helix. Members of this gene family (EmrB, QacA, Bmr, NorA, and the other Qac peptides) are generally not closer to each other than to other members of the family that are specific pumps, such as TetA or Mmr. This implies that the phenomenon of mdr has arisen independently many times in different bacterial species.

An interesting feature of *emr* that sets it apart from other members of the gene family is that it seems to confer resistance only to fairly hydrophobic compounds. An especially telling example is that *emr* protects cells from nalidixic acid, but not from more hydrophilic analogues, whereas the resistance spectrum of Gram-positive species carrying NorA or Bmr is exactly the opposite. Extruding highly hydrophobic substances from the cell does pose a logistical problem, for they will rapidly reenter the cytoplasmic membrane. It seems possible that *E. coli* and other Gram-negative bacteria might extrude such components across both membranes at adhesion zones (30). Such a mechanism would take advantage of the low permeability of the outer membrane to hydrophobic substances. Our data show that the cell content of the uncoupler TSA is significantly lower in a strain carrying multicopy *emr* as compared to an *emrB*$^-$ mutant. However, further experiments will be necessary to observe possible Emr-dependent extrusion of uncoupler.

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