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MdfA, an *Escherichia coli* Multidrug Resistance Protein with an Extraordinarily Broad Spectrum of Drug Recognition

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Multidrug resistance (MDR) translocators recently identified in bacteria constitute an excellent model system for studying the MDR phenomenon and its clinical relevance. Here we describe the identification and characterization of an unusual MDR gene (*mdfA*) from *Escherichia coli*. *mdfA* encodes a putative membrane protein (MdfA) of 410 amino acid residues which belongs to the major facilitator superfamily of transport proteins. Cells expressing MdfA from a multicopy plasmid are substantially more resistant to a diverse group of cationic or zwitterionic lipophilic compounds such as ethidium bromide, tetraphenylphosphonium, rhodamine, daunomycin, benzalkonium, rifampin, tetracycline, and puromycin. Surprisingly, however, MdfA also confers resistance to chemically unrelated, clinically important antibiotics such as chloramphenicol, erythromycin, and certain aminoglycosides and fluoroquinolones. Transport experiments with an *E. coli* strain lacking F_1-F_0 proton ATPase activity indicate that MdfA is a multidrug transporter that is driven by the proton electrochemical gradient.

The simultaneous emergence of resistance of eukaryotic and prokaryotic cells to many unrelated hydrophobic chemotherapeutic drugs is termed multidrug resistance (MDR). Recently, it has become evident that a variety of MDR efflux systems are widely distributed among prokaryotic microorganisms, including pathogenic bacteria (18, 29, 30). They belong to three different families of transport proteins: the major facilitator superfamily (MFS) (22), the resistance nodulation division (RND) family (28, 32), and the small multidrug resistance (SMR) family of small translocases (31). Broad substrate specificity and the ability of the prokaryotic MDR transporters to extrude a variety of unrelated drugs from the cell are similar to P-glycoprotein-mediated MDR in mammalian systems (9, 10). Importantly, however, unlike mammalian P glycoprotein, which belongs to the ATP binding cassette (ABC) superfamily of ATP-dependent transport proteins (15), the known bacterial MDR transporters probably utilize the membrane potential ($\Delta\psi$) and/or the proton gradient (ΔpH) as the driving force for drug export (6, 12, 38). There is also experimental evidence for putative ATP-dependent MDR systems in bacteria (5), but these proteins have not been characterized. Overall, it is likely that in the future, strategies for antibacterial chemotherapy will confront an increasing number of nonspecific mechanisms of resistance (27).

In addition to their potential clinical importance, the nonspecific MDR proteins pose intriguing and challenging questions concerning the mechanism of action of transport systems. While the substrate specificity of the mammalian P glycoprotein is probably limited to lipophilic compounds, most of which are protonated at physiological pH, there are bacterial MDR proteins that recognize antibiotics which are uncharged or zwitterionic at a neutral pH, in addition to the lipophilic drugs (25, 26).

Many of the bacterial drug translocators belong to the MFS of transport proteins (11, 22). They probably share a general secondary structural motif of 12 transmembrane segments (TMS) as determined by gene fusions in the case of the tetracycline pump (TetB) (17). However, a number of MFS drug exporters do not obey this consensus and probably contain 14 TMS (QacA and QacB) (30).

Two active MDR transport systems of the MFS superfamily have also been identified in *Escherichia coli*. These proteins, EmrB (20) and EmrD (24), confer resistance to uncouplers such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and in addition, EmrB extrudes the unrelated compound nalidixic acid. Both proteins probably operate in conjunction with periplasmically exposed accessory proteins proposed to form a linking pathway through which the drug is secreted across the outer membrane of the gram-negative bacterium (19). In addition, *E. coli* contains other drug translocators including proteins of the RND family (for a review, see reference 27) and of the SMR family of small translocases (for a review, see reference 31).

In this study we describe the cloning and characterization of MdfA, a new multidrug transporter from *E. coli* which exhibits multidrug efflux activities with an unusually broad pattern of drug specificities.

MATERIALS AND METHODS

Bacterial strains and plasmids. The outer membrane permeability mutant *E. coli* UTL2 (1) was used in tetraphenylphosphonium bromide (TPP) transport experiments. *E. coli* HB101 [*hsdS20* ($r_B^-m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44* λ^-/F^-] was used for preparation of various plasmid constructs, for preparation of chromosomal DNA, and for drug resistance assays. *E. coli* BL21(DE3) (34) was used for certain drug resistance assays as described below. Plasmid pT7-5 (35) was used in these studies in order to allow expression from either the T7 promoter or the native promoters of the cloned genes (see below).

Growth conditions and drug resistance assays. Cultures were grown at 37°C in Luria Bertani (LB) medium (33) supplemented with ampicillin (100 μg/ml) when necessary. Resistance of cells without plasmids or of cells harboring the different DNA constructs was assayed in either solid or liquid medium as specified. When tested on solid medium, *E. coli* HB101 was transformed with the appropriate plasmid and plated over LB plates (1.5% agar) supplemented with ampicillin (100 μg/ml) and various concentrations of the tested compound. (Chloramphenicol, kanamycin, rifampin, ethidium bromide [EtBr], rhodamine 6G, tetracycline, TPP, erythromycin, daunomycin, puromycin, and norfloxacin were obtained from Sigma. Benzalkonium and neomycin were from Calbiochem.) The ability of the transformants to form single colonies was recorded after 36 h at 37°C. When tested in liquid medium, with rhodamine 6G and daunomycin, overnight cultures were diluted into fresh LB broth containing ampicillin (100 μg/ml) and grown to an optical density at 600 nm (OD_{600}) of 0.6. Cells were then diluted again and

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aliquoted (50 μl) into 96-well microplates containing 50 μl of various concentrations of the drugs. Plates were incubated at 37°C in a shaker, and cell density was monitored by measuring the absorption at 600 nm every 6 h in a microplate autoreader (BIO-TEK, EL309). The drug concentrations which inhibit 50% of the growth were determined.

Preparation of genomic library and DNA manipulations. Chromosomal DNA from *E. coli* HB101 was isolated as previously described (33). DNA was partially digested with *Sau*3AI and separated on an agarose gel (0.8%). DNA fragments (10 to 15 kb) were isolated and purified by GeneClene (Bio101). Plasmid pT7-5 (35) was digested with *Bam*HI and treated with alkaline phosphatase, and the isolated linear DNA was ligated with the chromosomal DNA fragments overnight at 16°C. *E. coli* HB101 was then transformed with the ligation mixture and plated onto LB plates containing ampicillin (100 μg/ml) and the selective drug. The drug resistance clone *P2* was subjected to restriction analysis and sequencing of the plasmid-insert junctions by using sense (5′CACCGAAACGCGCGAGG CCCAG) and antisense (5′GTCAGACCACGTTTACTC) deoxyoligonucleotides corresponding to up- and downstream sequences in plasmid pT7-5. (Oligodeoxynucleotides were synthesized by the Scientific Services Unit at the Weizmann Institute of Science.) The two DNA strands of the *Bsp*MI/*Nru*I DNA fragment (see Results) were sequenced by using sense deoxyoligonucleotides (5′AAGATGTCCCATCGTC and 5′CGATGAACGCGTATCTGGC) and antisense deoxyoligonucleotides (5′TGGTTCCTGATTCAGTC and 5′CAGCAG CGACCAATAGACC) in combination with deletion of DNA fragments which have been already sequenced. The *mdfA* gene was finally subcloned into the *Sma*I site in pT7-5 as a *Bsp*MI/*Acc*I fragment treated with Klenow (New England Biolabs). The gene encoding YjiO was amplified by PCR, as described in the Results section, by using Vent polymerase (New England Biolabs).

Transport experiments. [^{3}H]TPP (Amersham, 23.0 Ci/mmol) uptake assays were performed by rapid filtration essentially as described previously (3) but without the EDTA washing step because it is not required for permeabilization of the outer membrane of *E. coli* UTL2 (1, 2). Uptake of 1.3 μM [dichloroacetyl-1-^{14}C]chloramphenicol (Amersham, 55.0 mCi/mmol) was assayed also by rapid filtration with *E. coli* UTL2 as described before (23). EtBr efflux was monitored continuously by measuring the fluorescence (which is increased upon interaction of the compound with DNA and RNA inside the cell) in the SLM Instruments fluorimeter (SLM 8000), using excitation light at 545 nm and emission at 600 nm.

Nucleotide sequence accession number. The accession number of the *mdfA* open reading frame (ORF) is Y08743.

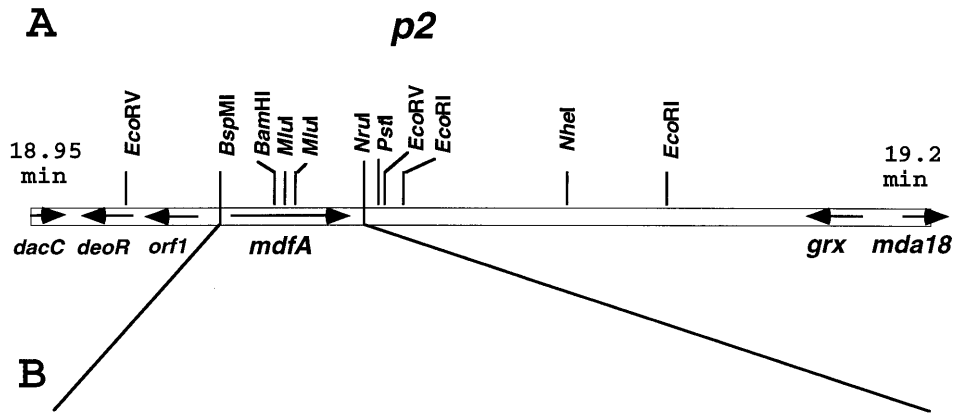
RESULTS

Cloning and sequencing of the multidrug resistance determinant *mdfA*. To identify an *E. coli* gene(s) that promotes cross-resistance to high concentrations of TPP and EtBr, *E. coli* HB101 was transformed with plasmid pT7-5 (35) harboring a chromosomal DNA library, and transformants were selected for growth on LB agar plates supplemented with 100 μg of ampicillin/ml, 0.4% glucose and 6 mM TPP. A number of TPP-resistant transformants were transferred to LB plates containing 100 μg of ampicillin/ml and 750 μg of EtBr/ml, and plasmid DNAs from the cross-resistant clones were analyzed by restriction enzymes, as shown for clone *P2* (Fig. 1A). Sequencing of the 5′ end of the chromosomal insert in *P2* revealed a DNA sequence identical to the 3′ end of *dacC* at minute 19 on the *E. coli* chromosome (Fig. 1A). Similarly, the DNA sequence at the 3′ end of *P2* is identical to the 5′ end of *mda18* at minute 19.2 (Fig. 1A). Most of this region of the chromosome flanked by *dacC* and *mda18* has not been sequenced. As expected, downstream to *dacC* we identified the *deoR* gene. In addition, two putative ORFs were identified, *orf1* of 198 codons in the antiparallel orientation and *orf2* of 410 codons. Deletion analysis and subcloning traced the MDR activity to a *Bsp*MI/*Nru*I DNA fragment (≈1.6 kb) harboring *orf2*, a putative promoter region (223 bp upstream to the initiation ATG codon) and a downstream sequence of 173 bp. This fragment was treated with Klenow and cloned into the *Sma*I site of pT7-5 (pT7-5 [*mdfA*]). Deletion of an internal *Mlu*I fragment of 400 bp from *orf2* (Fig. 1A) completely abolished the drug resistance phenotype. Therefore, *orf2* is responsible for resistance to TPP and EtBr. The gene is termed *mdfA* for multidrug facilitator A.

The product of *mdfA* (MdfA) is a putative membrane transport protein and belongs to the MFS superfamily of transporters. The *mdfA* gene is 1,233 bp and encodes a putative 410-amino-acid protein with a calculated molecular mass of 44,304 Da (Fig. 1B). Similarity search of protein data banks revealed a number of drug resistance proteins, all of which belong to the MFS superfamily of transport proteins (22), with a low but significant identity to MdfA, particularly in the consensus sequence of transport proteins (11, 14) (Fig. 2C, bold-faced letters in the first cytoplasmic loop). Among the more similar proteins are the *E. coli* uncouplers resistance protein EmrD (24) (24% identity) and the *Pseudomonas aeruginosa* chloramphenicol resistance protein CmlA (4) (25% identity). Interestingly, the most significant homolog of MdfA (≈40% identity) is a putative protein encoded by an uncharacterized ORF, *yjiO* (7), located at 98.3 min on the *E. coli* chromosome (Fig. 2A and B). A secondary structure model was constructed for MdfA (Fig. 2C) based on the hydropathy profile (Fig. 2B) with the N and the C termini on the cytoplasmic side of the membrane. The internal hydrophilic loop between TMS 4 and TMS 5 contains two net negative charges (QESFEEAVCIK), thus contradicting the positive-inside rule (36). Interestingly, the putative product of *yjiO*, which exhibits remarkable homology with MdfA (Fig. 2A and B), has one net positive charge in the same putative short loop (QEAFGQTKGIK).

The *yjiO* gene product also confers drug resistance. The striking homology between MdfA and the putative *yjiO* gene product raised the possibility that it is also a drug transporter. Using PCR, we amplified the gene with its 5′ putative promoter region and a downstream sequence of 200 bp by using a 5′ complementing deoxyoligonucleotide harboring a *Bam*HI site (underlined nucleotides) (5′-TTGGATCCGAGGCGCTT TCCAGG) and a 3′ complementing deoxyoligonucleotide also harboring a site for *Bam*HI (5′AAGGATCCAGCGGCGAT AAGC). The amplified gene was digested with *Bam*HI and ligated with *Bam*HI-linearized plasmid pT7-5 which was treated with alkaline phosphatase. *E. coli* BL21(DE3) cells transformed with the resulting plasmid (pT7-5/*yjiO*) are able to grow on LB plates supplemented with 100 μg of ampicillin/ml, 0.1 mM isopropyl-β-D-thiogalactopyranoside, and high concentrations of TPP (up to 3 mM) but only low concentrations of EtBr (up to 60 μg/ml). Both of the latter concentrations are higher than those tolerable by BL21(DE3) cells with vector alone, which are able to grow on 1 mM TPP or 30 μg of EtBr/ml.

MdfA exhibits an extraordinarily broad spectrum of drug specificities. As described above, MdfA exhibits moderate sequence similarities with other drug resistance transporters. To investigate the possibility that MdfA also shares functional properties with these homologs, we performed growth inhibition experiments to assay resistance to various antibiotics and cytotoxic drugs. *E. coli* HB101 transformed with pT7-5(*mdfA*), or pT7-5 with no insert, was plated on LB agar containing increasing concentrations of the test drugs in addition to ampicillin (100 μg/ml). The ability of the transformed cells to form single colonies after 36 h of incubation at 37°C served as an indicator for drug resistance. The results (Table 1) show that MdfA is an MDR protein with an exceptional pattern of drug recognition. The level of resistance is probably related to the level of expression of MdfA. The most intriguing observation is that MdfA confers resistance not only to an unusual ensemble of lipophilic compounds including EtBr, TPP, daunomycin, rhodamine, tetracycline, puromycin, benzalkonium, and rifampin but also to the uncharged antibiotic chloramphenicol, to the nonaromatic macrolide erythromycin, to the zwitterionic fluoroquinolones, and to a much lesser extent, to the nonaromatic and water-soluble aminoglycosides, neomycin and kanamycin. Many of these are prototypes of clinically important antibiotics that are used to treat infectious diseases.

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161 tacaaaattcgctttctcgttagctgcgcttttattaaactctgcgcgattattattggcgaagaaattgc ATG CAA  237
  1                                                                          M   Q   2
238 AAT AAA TTA GCT TCC GGT GCC AGG CTT GGA CGT CAG GCG TTA CTT TTC CCT CTC TGT CTG  297
  3 N   K   L   A   S   G   A   R   L   G   R   Q   A   L   L   F   P   L   C   L   22
298 GTG CTT TAC GAA TTT TCA ACC TAT ATC GGC AAC GAT ATG ATT CAA CCC GGT ATG TTG GCC  357
 23 V   L   Y   E   F   S   T   Y   I   G   N   D   M   I   Q   P   G   M   L   A   42
358 GTG GTG GAA CAA TAT CAG GCG GGC ATT GAT TGG GTT CCT ACT TCG ATG AAC GCG TAT CTG  417
 43 V   V   E   Q   Y   Q   A   G   I   D   W   V   P   T   S   M   N   A   Y   L   62
418 GCG GGC GGG ATG TTT TTA CAA TGG CTG CTG GGG CCG CTG TCG GAT CGT ATT GGT CGC CGT  477
 63 A   G   G   M   F   L   Q   W   L   L   G   P   L   S   D   R   I   G   R   R   82
478 CCG GTG ATG CTG GCG GGA GTG GTG TGG TTT ATC GTC ACC TGT CTG GCA ATA TTG CTG GCG  537
 83 P   V   M   L   A   G   V   V   W   F   I   V   T   C   L   A   I   L   L   A  102
538 CAA AAC ATT GAA CAA TTC ACC CTG TTG CGC TTC TTG CAG GGC ATA AGC CTC TGT TTC ATT  597
103 Q   N   I   E   Q   F   T   L   L   R   F   L   Q   G   I   S   L   C   F   I  122
598 GGC GCT GTG GGA TAC GCC GCA ATT CAG GAA TCC TTC GAA GAG GCG GTT TGT ATC AAG ATC  657
123 G   A   V   G   Y   A   A   I   Q   E   S   F   E   E   A   V   C   I   K   I  142
658 ACC GCG CTG ATG GCG AAC GTG GCG CTG ATT GCT CCG CTA CTT GGT CCG CTG GTG GGC GCG  717
143 T   A   L   M   A   N   V   A   L   I   A   P   L   L   G   P   L   V   G   A  162
718 GCG TGG ATC CAT GTG CTG CCC TGG GAG GGG ATG TTT GTT TTG TTT GCC GCA TTG GCA GCG  777
163 A   W   I   H   V   L   P   W   E   G   M   F   V   L   F   A   A   L   A   A  182
778 ATC TCC TTT TTC GGT CTG CAA CGA GCC ATG CCT GAA ACC GCC ACG CGT ATA GGC GAG AAA  837
183 I   S   F   F   G   L   Q   R   A   M   P   E   T   A   T   R   I   G   E   K  202
838 CTG TCA CTG AAA GAA CTC GGT CGT GAC TAT AAG CTG GTG CTG AAG AAC GGC CGC TTT GTG  897
203 L   S   L   K   E   L   G   R   D   Y   K   L   V   L   K   N   G   R   F   V  222
898 GCG GGG GCG CTG GCG CTG GGA TTC GTT AGT CTG CCG TTG CTG GCG TGG ATC GCC CAG TCG  957
223 A   G   A   L   A   L   G   F   V   S   L   P   L   L   A   W   I   A   Q   S  242
958 CCG ATT ATC ATC ATT ACC GGC GAG CAG TTG AGC AGC TAT GAA TAT GGC TTG CTG CAA GTG 1017
243 P   I   I   I   I   T   G   E   Q   L   S   S   Y   E   Y   G   L   L   Q   V  262
1018 CCT ATT TTC GGG GCG TTA ATT GCG GGT AAC TTG CTG TTA GCG CGT CTG ACC TCG CGC CGC 1077
263 P   I   F   G   A   L   I   A   G   N   L   L   L   A   R   L   T   S   R   R  282
1078 ACC GTA CGT TCG CTG ATT ATT ATG GGC GGC TGG CCG ATT ATG ATT GGT CTA TTG GTC GCT 1137
283 T   V   R   S   L   I   I   M   G   G   W   P   I   M   I   G   L   L   V   A  302
1138 GCT GCG GCA ACG GTT ATC TCA TCG CAC GCG TAT TTA TGG ATG ACT GCC GGG TTA AGT ATT 1197
303 A   A   A   T   V   I   S   S   H   A   Y   L   W   M   T   A   G   L   S   I  322
1198 TAT GCT TTC GGT ATT GGT CTG GCG AAT GCG GGA CTG GTG CGA TTA ACC CTG TTT GCC AGC 1257
323 Y   A   F   G   I   G   L   A   N   A   G   L   V   R   L   T   L   F   A   S  342
1258 GAT ATG AGT AAA GGT ACG GTT TCT GCC GCG ATG GGA ATG CTG CAA ATG CTG ATC TTT ACC 1317
343 D   M   S   K   G   T   V   S   A   A   M   G   M   L   Q   M   L   I   F   T  362
1318 GTT GGT ATT GAA ATC AGC AAA CAT GCC TGG CTG AAC GGG GGC AAC GGA CTG TTT AAT CTC 1377
363 V   G   I   E   I   S   K   H   A   W   L   N   G   G   N   G   L   F   N   L  382
1378 TTC AAC CTT GTC AAC GGA ATT TTG TGG CTG TCG CTG ATG GTT ATC TTT TTA AAA GAT AAA 1437
383 F   N   L   V   N   G   I   L   W   L   S   L   M   V   I   F   L   K   D   K  402
1438 CAG ATG GGA AAT TCT CAC GAA GGG TAA aaaaatgcctgactgctttgtgcgatcaggcattctcgaattaa 1508
 403 Q   M   G   N   S   H   E   G   *                                              411
1509 tggtgatggtcgtcaatctggtgttcgataaccatcccttcacctacgctggcaagatggcgaacataaggatgcgggcg 1588
1589 gtaagcccggagctggcgcaggagccacatagacggtttgcggcacagtcg                           1639
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FIG. 1. (A) Organization of the chromosomal DNA clone *P2*. (B) Nucleotide sequence of *mdfA* and the predicted primary structure of MdfA.

Under the same experimental conditions, MdfA does not confer resistance to nalidixic acid, methyl viologen, spectinomycin or CCCP.

Transport assays demonstrate that MdfA is an efflux protein driven by the proton motive force. Pleiotropic drug resistance may be caused either by a decreased drug permeability through the outer membrane of the gram-negative bacterium or by active drug extrusion through the cytoplasmic membrane. In either case, cellular accumulation of the drugs is significantly lower in resistant cells. Therefore, transport experiments were conducted with the outer membrane permeability mutant, *E. coli* UTL2 (1) harboring plasmid pT7-5(*mdfA*) or pT7-5 with no insert. [^{3}H]TPP uptake was assayed as described previously (3) by rapid filtration. As shown in Fig. 3A, cells harboring the vector without *mdfA* transport [^{3}H]TPP rapidly achieve a steady-state level of accumulation within about 2 min that is at least 1.8-fold higher than that observed in cells harboring pT7-5(*mdfA*). Similarly, a 2.5-fold difference is observed with [dichloroacetyl-1-^{14}C]chloramphenicol as the substrate (Fig. 3B). To test whether the low drug accumulation in cells expressing MdfA is due to active efflux, cells were loaded with EtBr in the presence of CCCP, essentially as previously described (38) but without glucose in the loading medium. Efflux of EtBr against a concentration gradient was initiated by

A

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MdfA  MqnklasgAr lgrqaLlFPl cLvLYeFstY ignDmIQPGm laVVeqyqAg idwvPtsmna YLAgGMfLQW LLGPLSDRIG RRPVmlaGvv wFivtClAil
YjiO  MprfftrhA. ...atLfFPm aLiLYdFaaY lstDlIQPGi inVVrdfnAd vslaPaavsl YLA.GMaLQW LLGPLSDRIG RRPVlitGal iFtlaCaAtm

MdfA  laqnieQFtl lRflQGiSlC FIgaVGYaai QEsFeeavcI KitAlmanva LIAPllGPLv GAAwiHvlpW egmFvlfAal aaISFfGLqr AMPETatRig
YjiO  fttsmtQFli aRaiQGtSiC FIatVGYvtv QEaFgqtkgI KlmAiitsiv LIAPiiGPLs GAAlmHfmhW kvlFaiiAvm gfISFvGLll AMPETvkRga

MdfA  eklSlKelgR DyklVlkNgr FvaGAlalgf vslPllaWiA qSPiIiItge qLssyeygll QVPiFGAlIa gNlllARlts rrTvrslIim ggwpimiGLl
YjiO  vpfSaKsvlR DfrnVfcNrl FlfGAatisl syiPmmsWvA vSPvIlIdag sLttsqfawt QVPvFGAvIv aNaivARfvk dpTeprfIwr avpiqlvGLs

MdfA  vaaaatviSs HayLWmtaGl SiYAFGIGLa nagLvRlTLF asdmsKGTVS AamgMlqmli ftVgiEIskh aWlNGGnglF nLfnlVnGil wlslmvifL.
YjiO  llivgnllSp HvwLWsvlGt SlYAFGIGLi fptLfRfTLF snklpKGTVS AslnMvilmv msVsvEIgrw lWfNGGrlpF hLlavVaGvi vvftlaglLn

MdfA  ..kdkQmgns hE  G
YjiO  rvrqhQaael vEeqG
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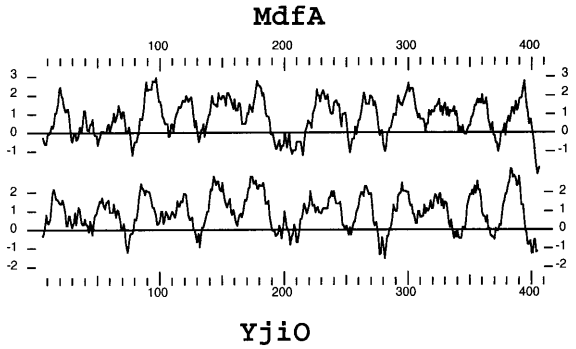
B



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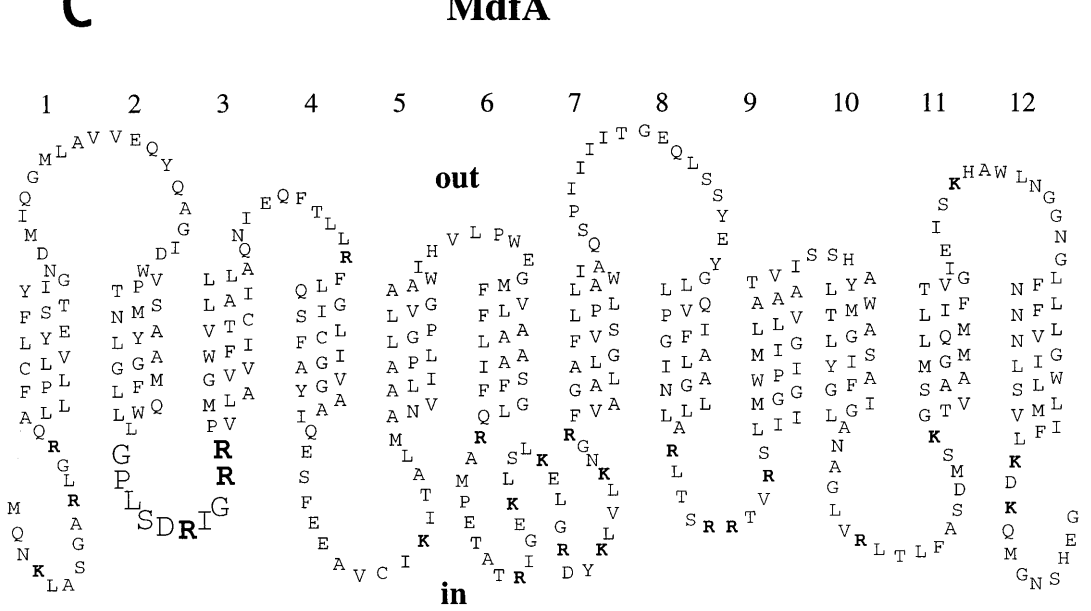

FIG. 2. (A) Primary sequence alignment of MdfA and the *yjiO* putative gene product (YjiO). Optimal alignment of the best region of similarity between the two sequences was performed by using the program BESTFIT (Genetics Computer Group, Version 8.1, UNIX). Identical residues are indicated by uppercase letters. (B) Hydrophobicity plot of MdfA and YjiO. The average local hydrophobicity at each residue of MdfA and YjiO, calculated by the method of Kyte and Doolittle (16), is plotted on the vertical axis and the residue number is plotted on the horizontal axis. Higher values represent greater hydrophobicity. The figure is adopted from the output of the program DNA Strider. (C) Secondary structure model of MdfA. TMSs of 19 to 21 hydrophobic amino acid residues were chosen based on the hydropathy profile. Positively charged residues are in bold-face type, and the transport protein's consensus footprint (11, 14) is emphasized in the loop between TMS 2 and TMS 3 (numbers at top of panel C indicate TMSs).

TABLE 1. Drug resistance of *E. coli* HB101 harboring pT7-5(*mdfA*) or pT7-5[a]

Drug	Drug concn (μg/ml) for cells transformed with:		Drug characteristics
	pT7-5	pT7-5 (*mdfA*)	
EtBr	75	750	Lipophilic, basic
TPP	839	2,517	Lipophilic, basic
Benzalkonium	50	400	Lipophilic, basic
Puromycin	82	217	Lipophilic, basic
Tetracycline	2	8	Lipophilic, basic
Daunomycin[b]	70	180	Lipophilic, basic
Rhodamine 6G[b]	70	160	Lipophilic, basic
Rifampin	10	20	Lipophilic, zwitterionic
Chloramphenicol	2	30	Aromatic, neutral
Erythromycin	50	200	Macrolide, basic
Neomycin	0.6	1.5	Aminoglycoside, hydrophilic
Kanamycin	1	2	Aminoglycoside, hydrophilic
Ciprofloxacin	0.002	0.007	Quinolone, zwitterionic
Norfloxacin	0.03	0.1	Quinolone, zwitterionic

[a] Drug resistance indicated by ability of transformed cells to form single colonies in the presence of the various test drugs at the indicated concentrations.
[b] Resistance assayed in liquid medium; all other determinations were made using solid medium.

addition of glucose and was monitored continuously by fluorescence spectroscopy. As shown in Fig. 4A, cells expressing MdfA rapidly extrude the drug relative to control cells. The slow efflux observed with control cells is probably due either to chromosomally encoded MdfA or to another efflux protein(s) (21). To verify functional interaction between MdfA and substrates other than EtBr and TPP, the effect of other substrates on EtBr efflux was determined. Addition of 100-fold excess of chloramphenicol, erythromycin, neomycin, or tetracycline inhibits EtBr efflux (Fig. 4B). As expected, spectinomycin, which is not a substrate for MdfA, does not affect EtBr efflux (Fig. 4B).

To investigate the driving force for the transport activity of MdfA, the effect of various energy sources on drug efflux was analyzed. MdfA is not an ABC protein, making it unlikely that ATP hydrolysis is the driving force for drug efflux. Nevertheless, to verify this notion, transport experiments were conducted using an *unc* mutant derivative of *E. coli* UTL2 which lacks a functional F_1-F_0 ATPase (2). As shown in Fig. 4D, glucose is unable to energize EtBr efflux in *unc* cells (compare with unc^+ cells, Fig. 4A) in the presence of iodoacetamide (IAA). Even without IAA, the effect of glucose on EtBr efflux from *unc* cells is marginal (see also Fig. 4C). By contrast, addition of the nonfermentable carbon source D-lactate (or succinate, data not shown) is sufficient to energize EtBr efflux (Fig. 4C and D), which is as expected if ATP is not required for active efflux by MdfA. Moreover, inhibition of respiration by cyanide (which blocks generation of the proton electrochemical gradient but does not affect ATP production in *unc* cells) abrogates efflux activity completely (Fig. 4C and D). Therefore, we conclude that MdfA is driven by the proton electrochemical potential.

DISCUSSION

As shown, the new MDR translocator, MdfA, displays a remarkably broad spectrum of drug recognition compared to known MDR proteins in the growing family of MFS-related drug transport systems. In addition to basic, aromatic, and lipophilic drugs, MdfA also confers resistance to neutral, nonaromatic, zwitterionic, or nonaromatic hydrophilic antibiotics. This pattern of drug specificity is unique even when compared to the most potent MFS-related MDR transporter identified so far, the *Bacillus subtilis* Bmr1 (14, 15), which is unable to confer resistance to tetracycline, erythromycin, and kanamycin (25a). Although previous models propose that MDR proteins remove lipophilic drugs directly from the lipid bilayer (9, 13, 27), MdfA is an example of an MDR transporter that may interact with at least some substrates from the aqueous environment. Notably, however, MdfA-associated resistance to aminoglycosides is reproducible but very low, and further studies are needed to demonstrate that these drugs are exported by MdfA. The level of resistance shown in Table 1 may reflect the concentration of MdfA in the membrane. This pattern of resistance was achieved only with cells harboring the *mdfA* gene on a multicopy plasmid under its native promoter. The amount of MdfA in these cells is probably not very high, since we have not been able to identify MdfA in membranes prepared from these cells by Coomassie or silver staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. The level of expression of MdfA from the chromosome or from a multicopy plasmid and

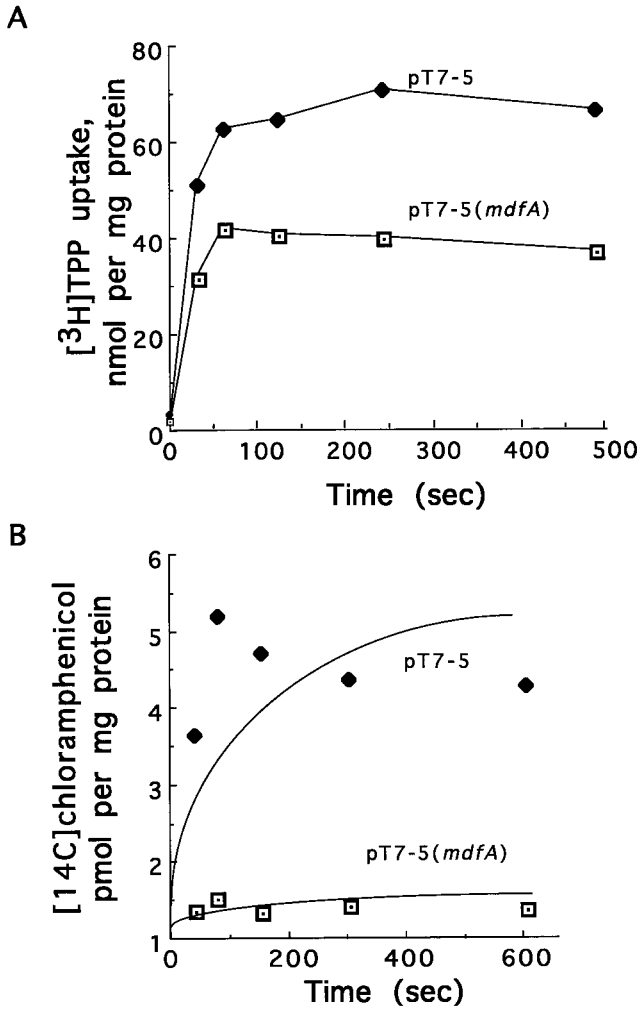

FIG. 3. Effect of MdfA on drug accumulation. (A) [^{3}H]TPP uptake assays (final concentration of TPP, 0.4 mM) were performed by rapid filtration with *E. coli* UTL2. (B) Uptake of 1.3 μM [dichloroacetyl-1-^{14}C]chloramphenicol was also assayed by rapid filtration with *E. coli* HB101.

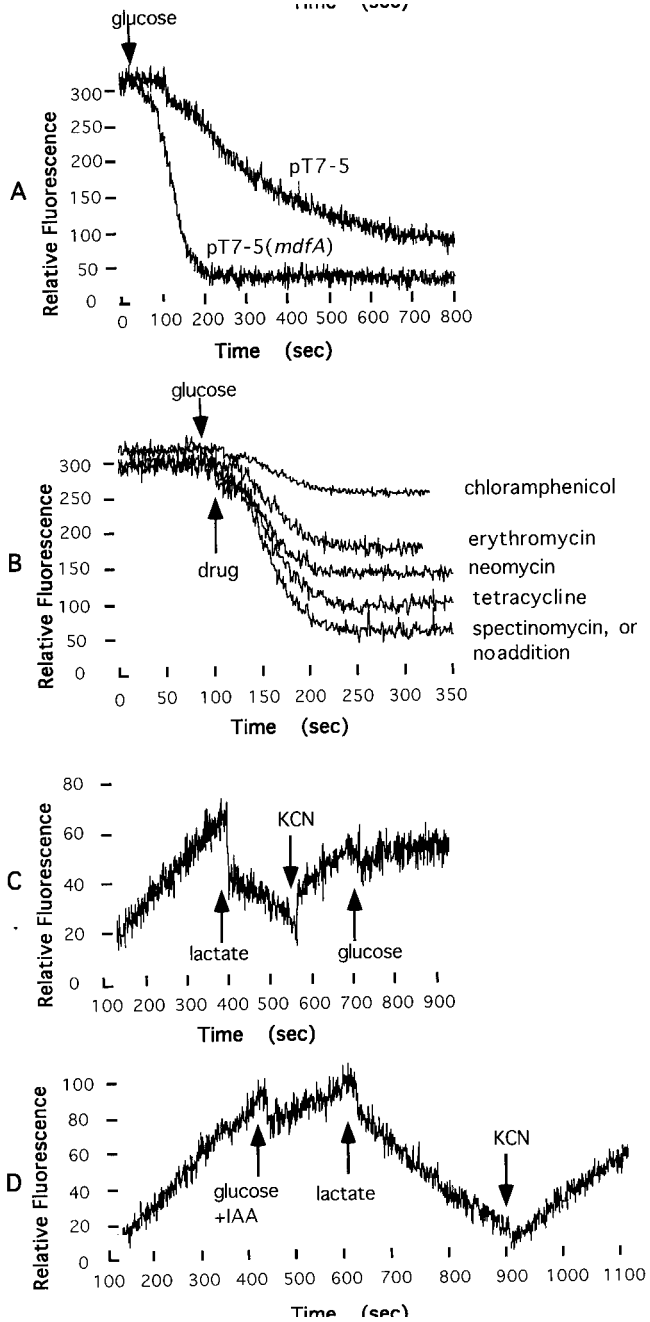

FIG. 4. Active extrusion of EtBr from cells expressing MdfA. EtBr efflux was monitored continuously by measuring the fluorescence as described in Materials and Methods. Exponential cultures of *E. coli* UTL2 (A and B) or UTL2*unc* (C and D) were collected and washed in M9 minimal medium containing $MgSO_4$ (1 mM). To enable maximal accumulation of EtBr (panels A and B), the cells (OD_{600} = 0.1) were incubated in M9 and supplemented with EtBr (2.5 μM) and the proton ionophore CCCP (40 μM) at 37°C for 60 min (38) but without glucose. Cells were then centrifuged (13,000 rpm for 3 min in an Eppendorf microcentrifuge), resuspended in the same medium containing only EtBr (2.5 μM), and subjected to fluorescence measurements. (A) After approximately 0.5 min in the fluorimeter, glucose was added (final concentration, 0.4%) to energize the cells, and the efflux was monitored continuously. (B) Cells were prepared as described for panel A and where indicated the respective drug was added (final concentration, 250 μM). (C) Cells were incubated with 40 μM CCCP at 37°C for 5 min, mixed with EtBr (2.5 μM), and placed immediately in the fluorimeter. Where indicated the following reagents were added: D-lactate (20 mM), KCN (10 mM), and glucose (25 mM). (D) Cells were prepared as described for panel C (C), and where indicated the following reagents were added: glucose (25 mM), IAA (5 mM), D-lactate (20 mM), and KCN (10 mM).

its specific activity will be characterized in the future by using specific antibodies.

Extensive sequence alignment did not reveal strong similarity between MdfA and other proteins except for the *E. coli* putative protein YjiO. The only significant similarity found is in the consensus sequence signature of the MFS superfamily of transport proteins (14). MdfA is highly hydrophobic, having only one charged amino acid residue inside a putative membrane-spanning segment, glutamate at position 26, which is located in the middle of putative TMS 1. YjiO (and also CmlA) contains aspartate at exactly the same position, and this is also the only charged residue inside its putative TMS. Interestingly, many other drug transporters also contain an acidic group at a similar position (29). The most striking example is that all proteins of the SMR family (31) contain glutamate in a similar position as in MdfA, and it is the only charged residue inside their membrane domain. Moreover, site-directed mutagenesis studies have demonstrated that this glutamate is essential for transport activity (12). Site-directed mutagenesis is currently being used to analyze the importance of this glutamate in MdfA.

It seems likely that MdfA is responsible for the drug transport observed in direct transport assays. However, it is not clear whether MdfA is solely responsible for drug resistance or requires helper proteins. In gram-negative bacteria many transport systems require accessory proteins. For example, ABC exporters involved in protein secretion require two accessory envelope proteins in the inner and in the outer membrane (37). In addition, some of the non-ABC drug translocators in gram-negative bacteria (especially those which belong to the RND superfamily) also function in conjunction with two helper proteins (8, 27). In these cases, export of the drugs from the cytoplasm would not produce resistance unless the helper proteins excrete the drugs from the periplasm. Although not shown, sequence analysis of the chromosomal fragment *P2*, which flanks *mdfA*, did not reveal putative ORFs with similarity to any of the known helper proteins.

The MDR properties of MdfA provoke further questions. What is the widest possible spectrum of drug selectivity for a single transporter? What is the origin of such a nonspecific transporter? Is MdfA an evolutionary precursor of more specialized proteins, or is it the product of an evolutionary loss of specificity? In any case, it appears that the prokaryotic microorganisms have acquired a system likely to present a rapidly growing clinical threat. It also presents an obvious and challenging target for antibacterial chemotherapy planned in anticipation of expanding mechanisms of microbial drug resistance.

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ADDENDUM IN PROOF

A gene (*cmr*) which has been isolated recently is almost identical to *mdfA* (I. W. Nilsen, I. Bakke, A. Vader, O. Olsvik, and M. R. El-Gewely, J. Bacteriol. **178:**3188–3193, 1996).

REFERENCES

1. **Béjà, O., and E. Bibi.** 1996. Functional expression of mouse Mdr1 in a membrane permeability mutant of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **93:**5969–5964.

2. **Bibi, E., R. Edgar, and O. Béjà.** Expression of eukaryotic ABC proteins in *Escherichia coli*. Submitted for publication.
3. **Bibi, E., P. Gros, and H. R. Kaback.** 1993. Functional expression of mouse multidrug resistance protein in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **90:**9209–9213.
4. **Bissonette, L., S. Champetier, J.-P. Buisson, and P. H. Roy.** 1991. Characterization of the nonenzymatic chloramphenicol resistance (*cmlA*) gene of the In*4* integron of Tn*1696*: similarity of the product to transmembrane transport proteins. J. Bacteriol. **173:**4493–4502.
5. **Bolhuis, H., D. Molenaar, G. Poelarends, H. W. van Veen, B. Poolman, A. J. M. Driessen, and W. N. Konings.** 1994. Proton motive force-driven and ATP-dependent drug extrusion systems in multidrug-resistant *Lactococcus lactis*. J. Bacteriol. **176:**6957–6964.
6. **Bolhuis, H., G. Poelarends, H. W. van Veen, B. Poolman, A. J. M. Driessen, and W. N. Konings.** 1995. The lactococcal lmrP gene encodes a proton motive force-dependent drug transporter. J. Biol. Chem. **270:**26092–26098.
7. **Burland, V., G. Plunkett III, H. J. Sofia, D. L. Daniels, and F. R. Blattner.** 1995. Analysis of the *Escherichia coli* genome VI: DNA sequence of the region from 92.8 through 100 minutes. Nucleic Acids Res. **23:**2105–2119.
8. **Dinh, T., I. T. Paulsen, and M. H. Saier, Jr.** 1994. A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria. J. Bacteriol. **176:**3825–3831.
9. **Endicott, J. A., and V. Ling.** 1989. The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu. Rev. Biochem. **58:**137–181.
10. **Gottesman, M. M., and I. Pastan.** 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu. Rev. Biochem. **62:**385–427.
11. **Griffith, J. K., M. E. Baker, D. A. Rouch, M. G. P. Page, R. A. Skurray, I. T. Paulsen, K. F. Chater, S. A. Baldwin, and P. J. F. Henderson.** 1992. Membrane transport proteins: implications of sequence comparisons. Curr. Opin. Cell Biol. **4:**684–695.
12. **Grinius, L. L., and E. B. Goldberg.** 1994. Bacterial multidrug resistance is due to a single membrane protein which functions as a drug pump. J. Biol. Chem. **269:**29998–30004.
13. **Gros, P., J. Croop, and D. E. Housman.** 1986. Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. Cell **47:**371–380.
14. **Henderson, P. J. F.** 1991. Sugar transport proteins. Curr. Opin. Struct. Biol. **1:**590–601.
15. **Higgins, C. F.** 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. **8:**67–113.
16. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
17. **Levy, S. B.** 1992. Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. **36:**695–703.
18. **Lewis, K.** 1994. Multidrug resistance pumps in bacteria: variations on a theme. Trends Biochem. Sci. **19:**119–123.
19. **Lewis, K., V. Naroditskaya, A. Ferrante, and I. Fokina.** 1994. Bacterial resistance to uncouplers. J. Bioenerg. Biomembr. **20:**639–646.
20. **Lomovskaya, O., and K. Lewis.** 1992. Emr, an Escherichia coli locus for multidrug resistance. Proc. Natl. Acad. Sci. USA **89:**8938–8942.
21. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1993. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. J. Bacteriol. **175:**6299–6313.
22. **Marger, M. D., and M. H. Saier, Jr.** 1993. A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. Trends Biochem. Sci. **18:**13–20.
23. **McMurry, L. M., A. M. George, and S. B. Levy.** 1994. Active efflux of chloramphenicol in susceptible *Escherichia coli* strains and in multiple-antibiotic-resistant (Mar) mutants. Antimicrob. Agents Chemother. **38:**542–546.
24. **Naroditskaya, V., M. J. Schlosser, N. Y. Fang, and K. Lewis.** 1993. An *E. coli* gene *emrD* is involved in adaptation to low energy shock. Biochem. Biophys. Res. Commun. **196:**803–809.
25. **Neyfakh, A. A.** 1992. The multidrug efflux transporter of *Bacillus subtilis* is a structural and functional homolog of the *Staphylococcus* NorA protein. Antimicrob. Agents Chemother. **36:**484–485.

25a. **Neyfakh, A. A.** Personal communication.

26. **Neyfakh, A. A., V. E. Bidnenko, and L. B. Chen.** 1991. Efflux-mediated multidrug resistance in *Bacillus subtilis*: similarities and dissimilarities with the mammalian system. Proc. Natl. Acad. Sci. USA **88:**4781–4785.
27. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264:**382–388.
28. **Okusu, H., D. Ma, and H. Nikaido.** 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. **178:**306–308.
29. **Paulsen, I. T., and R. A. Skurray.** 1993. Topology, structure and evolution of two families of proteins involved in antibiotic and antiseptic resistance in eukaryotes and prokaryotes—an analysis. Gene **124:**1–11.
30. **Paulsen, I. T., M. H. Brown, T. G. Littlejohn, B. A. Mitchell, and R. A. Skurray.** 1996. Multidrug resistance proteins QacA and QacB from *Staphylococcus aureus*: membrane topology and identification of residues involved in substrate specificity. Proc. Natl. Acad. Sci. USA **93:**3630–3635.
31. **Paulsen, I. T., R. A. Skurray, R. Tam, M. H. Saier, Jr., R. J. Turner, J. H. Weiner, E. B. Goldberg, and L. L. Grinius.** 1996. The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs. Mol. Microbiol. **19:**1167–1175.
32. **Saier, M. H., Jr., R. Tam, A. Reizer, and J. Reizer.** 1994. Two novel families of bacterial membrane proteins concerted with nodulation cell division and transport. Mol. Microbiol. **11:**841–847.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
35. **Tabor, S., and C. C. Richardson.** 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA **84:**4767–4771.
36. **von Heijne, G.** 1989. Control of topology and mode of assembly of a polytopic membrane protein by positively charged residues. Nature **341:**456–458.
37. **Wandersman, C.** 1996. Secretion across the bacterial outer membrane, p. 955–967. *In* F. C. Neidhart, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D.C.
38. **Yerushalmi, H., M. Lebendiker, and S. Schuldiner.** 1995. EmrE, an *Escherichia coli* 12-kDa multidrug transporter, exchanges toxic cations and H+ and is soluble in organic solvents. J. Biol. Chem. **270:**6856–6863.