A single membrane-embedded negative charge is critical for recognizing positively charged drugs by the *Escherichia coli* multidrug resistance protein MdfA

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The nature of the broad substrate specificity phenomenon, as manifested by multidrug resistance proteins, is not yet understood. In the *Escherichia coli* multidrug transporter, MdfA, the hydrophobicity profile and PhoA fusion analysis have so far identified only one membrane-embedded charged amino acid residue (E26). In order to determine whether this negatively charged residue may play a role in multidrug recognition, we evaluated the expression and function of MdfA constructs mutated at this position. Replacing E26 with the positively charged residue lysine abolished the multidrug resistance activity against positively charged drugs, but retained chloramphenicol efflux and resistance. In contrast, when the negative charge was preserved in a mutant with aspartate instead of E26, chloramphenicol recognition and transport were drastically inhibited; however, the mutant exhibited almost wild-type multidrug resistance activity against lipophilic cations. These results suggest that although the negative charge at position 26 is not essential for active transport, it dictates the multidrug resistance character of MdfA. We show that such a negative charge is also found in other drug resistance transporters, and its possible significance regarding multidrug resistance is discussed.

*Keywords:* Escherichia coli/MdfA/multidrug resistance/negative charge/transport

Introduction

The simultaneous emergence of resistance in eukaryotic and prokaryotic cells to many chemically unrelated drugs is termed multidrug resistance (Mdr). Transporters (Mdr proteins) that remove the drugs from their target, usually from the cell cytoplasm to the external medium, cause one major form of Mdr. The best-characterized Mdr system is the mammalian P-glycoprotein found in drug-resistant tumors and selected cell lines (Gottesman *et al.*, 1996). In addition, multidrug efflux systems are also widely distributed among prokaryotic microorganisms including pathogenic bacteria (Levy, 1992; Paulsen and Skurray, 1993; Lewis, 1994; Nikaido, 1994; Paulsen *et al.*, 1996a). Moreover, although Gram-negative bacteria are generally more resistant to various drugs than Gram-positive bacteria because of their impermeable outer membrane, they also have Mdr transporters. The Gram-negative transporters belong to at least three different families of transport proteins: the major facilitator superfamily (MFS) (Marger and Saier, 1993), the resistance–nodulation–division (RND) family (Saier *et al.*, 1994; Okusu *et al.*, 1996) and the SMR family of small translocases (Paulsen *et al.*, 1996b). The transporters of the MFS, RND and SMR groups are usually driven by the transmembrane proton electrochemical gradient, as shown with intact cells using ionophores, and with unc mutants (Grinius *et al.*, 1992; Littlejohn *et al.*, 1992; Li *et al.*, 1994; Bolhuis *et al.*, 1996; Edgar and Bibi, 1997). Gram-positive bacteria also contain, in addition to MFS and SMR proteins, Mdr proteins of the ATP-binding cassette (ABC) or traffic ATPase superfamily (van Veen *et al.*, 1996). In general, similarly to the P-glycoprotein-mediated Mdr in mammalian systems, many of the prokaryotic Mdr transporters are also able to extrude a variety of unrelated lipophilic compounds, many of which tend to be positively charged under physiological conditions. However, many bacterial Mdr proteins also interact with neutral drugs, some of which are relatively hydrophilic, and some transporters probably export lipophilic anionic drugs (Lewis *et al.*, 1994). Therefore, in addition to their potential clinical importance, the multispecific Mdr proteins pose intriguing questions regarding their transport mechanism.

Despite intensive attempts to understand the basics of the transport-related Mdr, still no conclusive descriptions are available for a few major aspects. (i) Concerning the mechanism of action of Mdr proteins, it is unknown how the driving force is coupled stoichiometrically to the export process, especially when a single Mdr protein simultaneously recognizes neutral substrates, compounds bearing a single positive charge or even divalent organic cations. (ii) The normal functions of the bacterial Mdr proteins and their physiological substrates are still unclear in many instances (Neyfakh, 1997). (iii) With respect to the drug-binding and/or translocation site(s) of Mdr proteins, it is not understood how a single transport protein can handle such an extremely broad spectrum of chemically unrelated species. Although the substrate recognition spectrum of the mammalian P-glycoprotein is probably limited to lipophilic compounds, most of which are protonated at physiological pH, some bacterial Mdr proteins recognize antibiotics that are uncharged or zwitter-ionic in physiological solutions, in addition to the cationic drugs (Bibi *et al.*, 1999). Here we have focused mainly on the substrate specificity determinants of the *Escherichia coli* Mdr protein. MdfA (also termed Cmr, Nilsen *et al.*, 1996) and Mine (also termed unci *et al.*, 1996), which represents multidrug transporters that recognize charged and uncharged substrates such as ethidium and chloramphenicol, respectively (Edgar and Bibi, 1997; Mine *et al.*, 1998).

MdfA is a 410 amino acid residue MFS-related membrane protein. Cells expressing MdfA from a multi-
Drug recognition by the Mdr transporter, MdfA

The physiological role of the bacterial Mdr proteins and Mdr proteins in general is frequently questioned, and was discussed in detail in a recent review by Neyfakh (1997). One possible way to determine their role is by studying mdr knockout strains. However, in a limited number of cases where knockout mutants have been generated in bacteria, the deleted strains that became somewhat more sensitive to drugs were otherwise indistinguishable from their wild-type counterpart (Ma et al., 1993, 1994). To obtain cells disrupted in mdfA, the chromosomal mdfA gene in a number of E.coli strains (see Materials and methods) was replaced with a disrupted version of the gene containing an antibiotic marker (the kanamycin resistance gene) inserted inside a truncated mdfA (Figure 2A). In general, we followed the homologous recombination protocols described by Pinner et al. (1993), using E.coli JC7623. The disrupted gene was then transferred from JC7623 to other E.coli strains by P1 transduction. The chosen strain for these studies was UTL2 because it is significantly more sensitive to various drugs (Béjà and Bibi, 1996). Recently we have mapped the mutation responsible for UTL2 drug sensitivity to galU (unpublished data), thus supporting our previous interpretation that UTL2 may have an impaired outer membrane (Nikaido and Vaara, 1987). PCR experiments (data not shown) and Southern blot analysis (Figure 2B) were used to confirm the disruption of mdfA in UTL2. Knockout of the mdfA gene led to a decrease in the EtBr efflux activity (Figure 2C) and this reduced activity was translated into appreciable EtBr sensitivity (Figure 2D). This EtBr sensitivity is the only apparent phenotype in the mdfA::kan strains, which otherwise grow and exhibit cellular and colony morphologies, as well as chloramphenicol resistance, indistinguishable from the parent strains (data not shown). Because of the effect of the mdfA disruption on drug resistance, the knockout strains were used for studying the function of MdfA and MdfA mutants expressed from plasmids.

Another approach to studying the physiological role of Mdr proteins is to identify the mechanism by which their expression is regulated. With mdfA, the situation seems to be more complex, as described by Nilsen et al. (1996). So far, we have not identified inducers of MdfA expression, and it seems to be expressed constitutively. Interestingly, we failed to obtain better expression of MdfA by placing the structural gene under control of the lac promoter. This may be due to the recently identified isopropyl-β-D-thiogalactopyranoside (IPTG) export activity of MdfA (Bohn and Bouloc, 1998). The greatest expression level was achieved with the wild-type 231 bp 5' region of mdfA. Therefore, throughout this study, we used a configuration in which mdfA or its mutants are cloned in plasmid pT7-5 with the 231 bp upstream region. However, the constitutive expression level of MdfA from plasmid pT7-5 was not sufficiently high for detection by Coomassie or silver staining, as usually apparent also in the case of other transporters in E.coli. In order to visualize the protein, we utilized a biotin acceptor domain from the oxaloacetate decarboxylase of Klebsiella pneumoniae (Cronan, 1990).

Results

The mdfA gene: knockout, expression, and detection of the gene product

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Fig. 2. Disruption of the chromosomal mdfA gene. (A) Organization of the chromosomal mdfA gene and its disrupted form, harboring a small deletion (Md/u–Md/u) and an insertion of the kanamycin resistance gene kan inside the BstBI site of mdfA. The probe used for Southern blot experiments encompasses the indicated region in both cases. (B) Southern blot analysis of the chromosomal DNA preparations from wild-type (UTL2) and mutated (UTL2/mdfA::kan) cells. Plasmid DNAs harboring wild-type (pT7-5/mdfA) or disrupted (pT7-5/mdfA::kan) mdfA were used as controls. (C) EtBr efflux by UTL2 and UTL2/mdfA::kan cells. Efflux from cells loaded with EtBr was monitored continuously by following the fluorescence as described in Materials and methods. (D) Resistance of UTL2 and UTL2/mdfA::kan to EtBr. Cells were grown in LB plates in the presence of given concentrations of various drugs. The minimal inhibitory EtBr concentration for each strain is shown. The results represent four independent experiments, and standard deviations are indicated as ±SD bars.

fused to the C-terminus of MdfA (mdfA-bio) (Figure 3A). The biotin acceptor domain is biotinylated in vivo in E.coli, suggesting that the C-terminus of MdfA is oriented towards the cytoplasm. The biotinylated tag enables specific detection of the MdfA fusion protein (Figure 3B) in sub-nanogram quantities (data not shown) by using streptavidin–horseradish peroxidase (HRP). The fusion protein is fully active as shown by chloramphenicol and EtBr resistance experiments (Figure 3C), and it was used throughout the following studies.

**Localization of Glu26 to the N-terminal transmembrane segment by phoA fusions**

Previously, we proposed that MdfA belongs to the 12 TMs subclass of the MFS (Edgar and Bibi, 1997). The detailed hydropathy plot of the hydrophobic region flanking glutamate at position 26 (E26) suggests that it is located inside TM1 of MdfA (Figure 4A). Interestingly, according to the secondary structure prediction, E26 is the only charged residue inside putative TMs of MdfA. We used the phoA gene fusion approach to test the proposal that E26 is inside the putative N-terminal TM and to determine the orientation of this segment with respect to the plane of the membrane. Briefly, the phoA gene encodes the mature form of alkaline phosphatase (Manoil and Beckwith, 1985), a periplasmic reporter that requires export to the periplasm to be enzymatically active. It acts as a sensor for periplasmic location of the protein sequence to which it is attached (Calamia and Manoil, 1992). All the phoA fusions were directed to chosen sites flanking the putative TM1s (hybrids A15, in; I31, V43 and W53, out) and -2s (W53, out; P83, in) of MdfA (Figure 4B). The expression of the resulting hybrids was investigated by labeling with [35S]methionine and immunoprecipitation by anti-alkaline phosphatase antibodies (Figure 4C), and their alkaline phosphatase activities were analyzed (Figure 4D). As shown, the size of the hybrid proteins or the location of the reporters with respect to the plan of the membrane did not affect the level of expression and, except for hybrid P83, the constructs are expressed at similar levels. Using densitometry, the densities of the bands representing the chimeric proteins were quantitated. In addition, the number of methionine residues was taken into account because [35S]methionine was used for the labeling. The level of expression (band intensity) divided by the number of methionines was then used to normalize the alkaline phosphatase activity, as presented in Figure 4D. Fusions I31, V43 and W53 exhibited high alkaline phosphatase activity (23.5, 19.7 and 25.3 U, respectively) and, therefore, we suggest that they are located on the periplasmic side of TM1. Hybrids A15 and P83 exhibited lower activities (0.1 and 9.2 U, respectively), consistent with their cytoplasmic disposition as in the secondary structure model of MdfA. In conclusion, these results support the proposal that E26 is located inside TM1 and that the topology of TM2 is N-out C-in, as predicted.

**The negative charge at position 26 is essential for resistance to and transport of lipophilic cations**

Being the only charged residue inside putative TMs of MdfA, E26 may play an important mechanistic role in drug transport. As mentioned in the Introduction, sequence alignment of the first putative TMs from different Mdr
proteins revealed that some of them contain a negatively charged residue at approximately the same location as E26 in MdfA (Figure 1). In order to evaluate the importance of this conserved charge, we replaced it by the smaller residue aspartate (mutant E26D) that preserves the negative charge, by the positively charged residue lysine (mutant E26K) or by the uncharged residue alanine (mutant E26A). The three mutants were fused to the biotin acceptor domain as previously described, and Western blot analysis demonstrated that all of the mutants were expressed as well as wild-type MdfA (Figure 5). We used two groups of drugs to characterize the function of the mutants: the positively charged MdfA substrates EtBr, benzalkonium and doxorubicin, and the dissimilar compound chloramphenicol, which is neutral in aqueous solution at physiological pH. In order to illustrate the differences between the drugs, their planar chemical structures are shown in Figure 6. Unlike chloramphenicol, EtBr, benzalkonium and doxorubicin are positively charged. Drug resistance assays on Luria Bertani (LB) plates containing the indicated drugs showed that cells expressing mutant E26D exhibit resistance to EtBr and benzalkonium, but not to chloramphenicol, whereas cells expressing mutant E26K grew on plates with chloramphenicol but not with the positively charged drugs (Figure 7A). Similar results were obtained in quantitative growth experiments (Figure 7B). Mutant E26D confers high resistance only to the cationic drugs EtBr and benzalkonium; mutant E26K confers only chloramphenicol resistance, whereas mutant E26A exhibits only marginal chloramphenicol resistance activity. In order to determine whether the mutations in position 26 also influence the inhibitory effect of the plant alkaloid reserpine on MdfA, we used *E. coli* UTL2mdfA::kan, tolC. The UTL2mdfA::kan, tolC strain was used only with reserpine and doxorubicin (see below, Figure 8) because our test strain UTL2mdfA::kan exhibits only very low reserpine and doxorubicin permeability (data not shown). The tolC mutation causes pleiotropic efflux problems and renders cells hypersensitive to various drugs (Fralick, 1996). To test the effect of reserpine, growth experiments were conducted in the presence of increasing concentrations of EtBr (mutant E26D) or chloramphenicol (mutant E26K), with or without reserpine (Figure 7C). Interestingly, reserpine abolished drug resistance in both cases, regardless of the mutation or the test drug. Notably, *E. coli* UTL2mdfA::kan, tolC cells are in general significantly more sensitive to the lipophilic cation EtBr than UTL2mdfA::kan, as expected (compare the left panels of Figure 7C and B, respectively).

Drug resistance assays provided indirect information about the activity of the mutants. In order to confirm these observations, the activity of the mutants was tested directly by transport and efflux assays. The experiments were conducted with the outer membrane permeability mutants, *E. coli* UTL2mdfA::kan and *E. coli* UTL2mdfA::kan, tolC, as indicated, harboring plasmid pT7-5/mdfA-bio, or each of the mutants, or pT7-5 with no insert as control. Transport of [dichloroacetyl-1-14C]chloramphenicol was assayed as described previously (Edgar and Bibi, 1997), by rapid filtration (Figure 8A). As shown, cells harboring mutant E26A or E26D behaved like cells harboring the vector alone and transported dichloroacetyl-1-14C chloramphenicol relatively quickly (within \(< 1\) min) to a steady-state level of accumulation that is at least 2-fold higher.

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**Fig. 3.** Tagging of MdfA with a biotin acceptor domain. (A) Organization of the tagged *mdfA* gene (*mdfA-bio*) and the factor Xa site-coding sequence inserted between *mdfA* and the biotin acceptor coding region. 5’ UTR corresponds to the wild-type 231 bp segment upstream of the *mdfA* start codon. (B) Western blot analysis of the tagged MdfA protein. *Escherichia coli* UTL2 expressing MdfA or MdfA-Bio were grown as described in Materials and methods. Membrane fractions were analyzed by SDS–PAGE followed by Western blotting using HRP-conjugated streptavidin. Every lane contains 1 μg of *E.coli* membrane proteins from cells carrying the respective plasmids. (C) Cytotoxic effect of chloramphenicol and EtBr. Relative growth (calculated from the cell density, measured by absorption at 600 nm) of the drug-sensitive UTL2 *mdfA::kan* cells transformed with the vector (pT7-5) or with plasmids harboring *mdfA* or *mdfA-bio* in medium containing increasing concentrations of chloramphenicol (left panel) or EtBr (right panel). The results represent four independent experiments, and standard deviations are indicated as ±SD bars.
Fig. 4. Characterization of MdfA–PhoA hybrids. (A) The average local hydrophobicity (using a window of five residues) at each residue throughout the N-terminal sequence of MdfA, calculated by the method of Kyte and Doolittle (1982) and plotted on the vertical axis versus the residue number on the horizontal axis. Higher values represent greater hydrophobicity. The figure is adapted from the output of the program DNA Strider. Filled circles indicate the MdfA–PhoA fusion joints. (B) Secondary structure model of TM1 and TM2 of MdfA. The TM segments were chosen based on the hydropathy profile shown in (A). Residues at the MdfA–PhoA fusion joints are circled, and the conserved negatively charged glutamate is emphasized. (C) Immunoprecipitation of [35S]methionine-labeled E. coli T184 cells expressing various MdfA–PhoA hybrids. Solubilized membrane fractions from E. coli T184 harboring given mdfA–phoA constructs were labeled with [35S]methionine, immunoprecipitated with anti-alkaline phosphatase antibodies and subjected to SDS–PAGE and autoradiography. Dried gels were exposed to film for 4 days. (D) Normalized alkaline phosphatase activity in cells expressing the various hybrids was measured and calculated as described previously (Bibi and Béja, 1994).

Fig. 5. Expression of the biotinylated wild-type MdfA and its mutants. Escherichia coli UTL2mdfA: kan harboring the vector alone, mdfA-bio or the biotinylated mutants E26A, E26D or E26K were grown as described in Materials and methods. Membrane fractions were analyzed by SDS–PAGE followed by Western blotting using HRP-conjugated streptavidin. Every lane contains 100 ng of E. coli membrane proteins prepared from cells carrying the respective plasmids.

than that observed in cells expressing functional wild-type MdfA or mutant E26K. Efflux of EtBr and doxorubicin was measured by fluorescence spectroscopy. The various transformants were loaded with EtBr or with doxorubicin, and their efflux was initiated by adding glucose. The efflux of EtBr was measured continuously by monitoring the decrease in fluorescence. Similarly, the doxorubicin efflux was monitored continuously but, unlike EtBr, the doxorubicin efflux is characterized by an increase in fluorescence. As shown in Figure 8B, cells expressing MdfA or mutant E26D rapidly expel the respective drugs, EtBr and doxorubicin, relative to control cells or cells expressing E26K or E26A. The slow EtBr efflux observed with control cells is probably due to another efflux protein(s) (Ma et al., 1993).

It was shown previously that chloramphenicol efficiently inhibits the EtBr efflux by MdfA (Edgar and Bibi, 1997; Mine et al., 1998), suggesting that both drugs compete for a similar transport pathway and may also share their MdfA-binding site. This observation provided us with a tool for testing whether or not the binding of chloramphenicol to the E26D mutant is preserved, despite the fact that this mutant is unable to transport chloramphenicol. In other words, the transport of chloramphenicol by the mutant (E26D) can also be inhibited if chloramphenicol binding is drastically increased and becomes relatively irreversible, but then the transport of other drugs must also be blocked by chloramphenicol if they use the same pathway. The influence of various concentrations of chloramphenicol on EtBr uptake was examined with cells expressing MdfA or mutant E26D (Figure 8D). The results clearly demonstrated that unlike with the wild-type protein, EtBr uptake by cells expressing mutant E26D is only slightly affected by high chloramphenicol concentration (40 times the concentration of EtBr). This suggests that the affinity of MdfA for chloramphenicol has decreased significantly due to the E26D mutation.

Discussion

The ability of many Mdr proteins to confer resistance against an extremely broad range of toxic agents has led to many mechanistic hypotheses. The most prevailing hypothesis favors a direct mechanism by which Mdr proteins are capable of recognizing a variety of
compounds and actively exporting them across the membrane. One of the experimental approaches that were taken to evaluate the active transport hypothesis was to examine the outcome of specific mutations. Some of the amino acid substitutions abolished transport completely, as shown in a number of Mdr systems, but this could reflect the possibility that such residues play a critical role in energy utilization and coupling or that their replacement may have caused detrimental structural modifications. Other mutations clearly alter the drug specificity pattern by exerting a differential effect on the transport of various drugs, thus providing strong support for direct interaction between Mdr proteins and drugs.

Here we have focused on the drug recognition properties of the E. coli Mdr protein, MdfA, by analyzing the consequences of substitutions at position 26, which attracted our attention for the following reasons. First, the native residue at position 26 is glutamate, and it seems to be the only charged residue inside the putative TM segments of the protein. We have shown by PhoA fusion analyses that it is most likely located in the middle of TM1. Second, as shown in Figure 1, a similar negative charge is found inside the putative TM1 of other drug transport proteins, and sometimes it has been proved essential for drug transport and resistance (see later). Briefly, we have shown that mutations at position 26 have a drastic effect on the substrate recognition profile of MdfA in general, suggesting that MdfA causes Mdr by directly interacting with and transporting the drugs. Moreover, the most important outcome of this set of experiments is showing that the negative charge at position 26 is essential only for the transport of lipophilic cations, an observation that may have a few tentative explanations. First, it is possible that the acidic residue plays an important role in active transport and coupling. This seems unlikely because mutant E26K transports chloramphenicol quite efficiently. However, if MdfA is assumed to use different energy-coupling mechanisms for different drugs, then this explanation becomes theoretically feasible. For example, this could be true if MdfA was a proton–drug antiporter only with lipophilic cations, but not with chloramphenicol. Then, if E26 plays a role in proton translocation, its replacement with a positively charged residue would affect only the exchange of the cationic drugs with protons, but not the export of chloramphenicol. To the best of our knowledge, such a mechanism has never been described previously for secondary trans-
Fig. 8. Transport activity of MdfA and mutants E26D, E26K and E26A. (A) Uptake of 1.3 μM [dichloroacetyl-1-14C]chloramphenicol was assayed by rapid filtration with E.coli UTL2 mdfA::kan. (B) EtBr (left panel) or doxorubicin (right panel) efflux were monitored continuously by following the fluorescence as described in Materials and methods. Exponential cultures of E.coli UTL2 mdfA::kan (left panel) or UTL2 mdfA::kan tolC (right panel) were collected and washed in M9 minimal medium (for EtBr efflux) or KPi and MgSO4 (for doxorubicin efflux). To enable maximal accumulation of the fluorophore, the cells (OD600 = 0.1 for EtBr, OD600 = 20 for doxorubicin) were incubated with EtBr (2.5 μM) or doxorubicin (11.5 μM) and the proton ionophore, CCCP (40 μM), as described previously (Edgar and Bibi, 1997). Cells were then centrifuged, resuspended in the same medium containing only EtBr (2.5 μM) or doxorubicin (11.5 μM) as indicated, and subjected to fluorescence measurements. After ~0.5 min in the fluorimeter, glucose was added (final concentration 0.4%) to energize the cells, and the efflux was monitored continuously. (C) Analysis of the inhibitory effect of chloramphenicol on EtBr transport by MdfA and mutant E26D. E.coli UTL2 mdfA::kan expressing MdfA or mutant E26D were grown and washed as in (A). Cells (OD600 = 0.5) were resuspended in M9 containing potassium lactate (final concentration 0.4%) and placed in the fluorimeter. EtBr (10 μM) was then added and its accumulation under conditions of active MdfA was monitored continuously. Ten seconds after EtBr accumulation has reached an equilibrium, various amounts of chloramphenicol (CAM) were added (indicated as 5, 10 or 40 times the concentration of EtBr). After 6 min, the proton ionophore CCCP (40 μM) was added to allow EtBr accumulation under de-energized conditions.

Porters and, in addition, there are indications (Mine et al., 1998) that MdfA also exchanges chloramphenicol for protons. Another explanation could be that mutations at position 26 cause conformational alterations that indirectly influence the drug-binding domain located elsewhere in MdfA. We do not favor this possibility because of the findings that all the positively charged drugs that were tested were affected similarly by the mutations, regardless of their size or chemical structure. Finally, the most feasible hypothesis, which also serves as our current working model, is that E26 forms part of the drug recognition domain and that the negative charge may interact directly with the positive charge of the lipophilic cations. Generally, we think that the results presented here strongly support this proposition, mainly because each of the three mutations leads to identical consequences with respect to the interaction of MdfA with every one of the positively charged, but structurally dissimilar drugs tested. Chloramphenicol recognition, however, is not sensitive to the charge but possibly to the size or shape of the side chain of the amino acid residue at position 26. Furthermore, these results and the observation that chloramphenicol competes with EtBr raise the possibility that chloramphenicol, EtBr, benzalkonium and doxorubicin share, at least to some extent, a common binding site in MdfA. If this is true, then the drug-binding site in MdfA should be either sufficiently large or very flexible to accommodate the different substrates. Interestingly, the close homolog of MdfA, the putative E.coli open reading frame YjiO, contains aspartate instead of glutamate at the same site.
YjiO confers low levels of resistance against the lipophilic cation tetraphenylphosphonium (Edgar and Bibi, 1997), but cells expressing YjiO do not exhibit chloramphenicol resistance, similarly to mutant E26D of MdfA.

Paulsen et al. (1996c) described a very similar but distinct charge-related phenomenon with the staphylococcal Mdr protein, QacA. They observed that the negative charge at position 323 within putative TM10 of the QacA protein plays a critical role in conveying resistance to divalent cations. Similarly, the close homolog QacB can also be converted into a divalent cation transporter, provided that a negatively charged residue replaces its native neutral residue at position 323. Unlike in MdfA, however, no change occurred in the substrate recognition profile other than the change observed with divalent cations. QacA belongs to the 14 TMs MFS-related transporters, and it was proposed that in addition to Asp323, it also has an aspartate residue at position 34, located inside TM1 (Figure 1), like E26 in MdfA. It would be interesting to evaluate the role of this negative charge as well, because it may form, together with Asp323, a recognition site for divalent organic cations.

As shown, proteins of the SMR family of small Mdr transporters also have conserved glutamic acids in the middle of their TM1 (Figure 1). This is also the only charged residue inside membrane segments of this group of proteins. Moreover, site-directed mutagenesis studies have demonstrated that this glutamate is essential for transport activity (see later). In the E.coli transporter, EmE, E14 was replaced with cysteine and the function of the mutant was tested in intact cells as the ability to confer resistance to methyl viologen, ethidium and acriflavin. The mutant exhibited no Mdr activity against these drugs, all of which are considered to be positively charged under physiological conditions. Recently it has been proposed that the EmE mutant E14C is incapable of driving ΔpH-driven transport as well as downhill efflux and exchange (Schuldiner et al., 1997). From their experiments and from the observation that even a conservative substitution (E13D) in the staphylococcal protein Smr effectively abolished its activity (Grinius and Goldberg, 1994), Schuldiner et al. (1997) proposed that this glutamate may be involved in proton translocation. This situation is different from what we observed with MdfA, where the negative charge (represented by E26 in the wild-type protein) is not essential for the transport activity, since a mutant E16K is functional with chloramphenicol as a substrate.

The results presented here offer a tentative mechanism by which charge–charge interaction may play a role in the recognition and export of lipophilic cations by Mdr proteins. This has never been proposed in Mdr systems other than those described here. For example, in the widely accepted secondary structure model of the ABC-related human Mdr1 protein, there are no aspartate, glutamate, arginine or lysine residues inside predicted TMs (Loo and Clarke, 1995). Many point mutations inside these predicted TM segments were shown to affect drug specificity, but not in a charge-related fashion (reviewed in Gottesman et al., 1995). However, in some of the alternative topological arrangements (cf. Bibi and Béja, 1994; Béja and Bibi, 1995; reviewed in Levy, 1996), charged residues have been placed experimentally inside TM segments of mouse Mdr1, but their role in drug transport is not known.

We plan to test further the proposal that the recognition of a subset of drugs by the Mdr protein MdfA is mediated by opposite charge–charge interactions between the protein and the drug. Screening for active second site revertants with the MdfA mutant harboring lysine or alanine at position 26 as a template may reveal other important residues in the drug-binding pocket of the protein. Finally, studying the energetics and its stoichiometrical usage in the MdfA-mediated transport process with uncharged substrate (e.g. chloramphenicol) and with any of the lipophilic cations is expected to shed light on the mechanism of action of this and similar Mdr proteins.

**Materials and methods**

**Materials**

[Dichloroacetyl-1-14C]chloramphenicol (55.0 mCi/mmol) and [35S]methionine (1000 Ci/mmol) were purchased from Amersham Corp. Carboxyl cyanide m-chlorophenylhydrazone (CCCP), EtBr, chloramphenicol, kanamycin, ampicillin, phenylmethylsulfonyl fluoride (PMSF), doxorubicin, p-nitrophenyl phosphate and protein A (cell suspension of *Staphylococcus aureus* Cowman strain) were all purchased from Sigma. Benzalkonium chloride (17.5% solution) was from Calbiochem. Purification and modifying enzymes were obtained from New England Biolabs. Oligodeoxynucleotides were synthesized by the scientific services unit at the Weizmann Institute of Science. Polyclonal antibodies to alkaline phosphatase were from 5 Prime-3 Prime Inc. Pre-stained protein molecular weight markers were obtained from BioLabs, and DNA molecular weight markers from Fermentas. GenesClean glassmilk DNA purification kits were obtained from Bio 101, and Wizard phosphor prep kits from Promega. Southern blots were carried out using Supersignal™ nucleic acid with HRP-conjugated streptavidin obtained from Pierce. Biotinylated DNA probes were prepared by the NEBlot phototope kit (New England Biolabs). All other materials were reagent grade and obtained from commercial sources.

**Bacterial strains and plasmids**

*Escherichia coli* HB101 [hsdS20 (r−B, m−B), recA13, ara−14, proA2, lacY1, galK2, rpsL20 (Sm̅), syl−1, supE44, λ−/F−] was used for propagation and preparation of various plasmid constructs. The E.coli strain JC7623 (Kushner et al., 1971) was used for knockout of mdfA by homologous recombination of linear DNA fragments. The outer membrane permeability mutant *E.coli* UTL2 (Béja and Bibi, 1996) was disrupted in mdfA in this study (see Results). The resulting strain, *E.coli* UTL2mdA::kan, was used in drug resistance and transport experiments. The *E.coli* UTL2mdA::kan strain was deleted further of the tolC gene encoding the outer membrane protein TolC. The construction of the tolC knockout was accomplished by P1 transduction using the donor strain CS1562tolC::Tn10. The tolC mutant UTL2mdA::kan,tolC is supersensitive to various drugs (data not shown) and it was used in doxorubicin efflux experiments and in studies of the inhibitory effect of reserpine on MdfA. Plasmid pBT7-5mdA (Edgar and Bibi, 1997) was used in these studies in order to allow expression of mdfA from its native 231 bp 5’ untranslated region (see below). Plasmid pTCT175 containing the gene encoding the biotin acceptor domain from *Klebsiella pneumoniae* was obtained from H.R.Kaback, UCLA. Plasmid pCV3 is a derivative of pACYC184 and will be described elsewhere.

**Growth conditions and drug resistance assays**

Cultures were grown at 37°C in LB medium supplemented with ampicillin (100 μg/ml), kanamycin (30 μg/ml) and tetracycline (12.5 μg/ml) when necessary. Resistance of cells harboring the indicated plasmids was assayed in both solid and liquid media. When tested on solid media, *E.coli* UTL2mdA::kan was transformed with the indicated plasmid and plated over LB plates (1.5%) supplemented with ampicillin (100 μg/ml) and various concentrations of the test compound (chloramphenicol, benzalkonium or EtBr). The ability of the transformants to form single colonies was recorded after 36 h at 37°C. When tested in liquid media, overnight cultures were diluted into fresh LB containing ampicillin (100 μg/ml) and grown up to optical density at 600 nm (OD600) of 0.6 units. Cells were then diluted again and aliquoted (50 μl) into...
96-well microplates containing 50 μl of increasing concentrations of the test drug (chloramphenicol, EtBr or benzalkonium). At the beginning of a typical experiment, the cell density in the wells was measured in a microplate autoreader (model EL309, Bio-Tek, Burlington, VT) to 0.03 OD₄₀₀ units. Plates were incubated at 37°C with a cell density monitored by following the absorption at 600 nm after 6–8 h.

**Disruption of the chromosomal mdfA gene**
In order to disrupt the chromosomal mdfA gene, a plasmid containing disrupted mdfA was constructed. First, an MluI fragment was deleted from pT7-5/mdfA. In addition, the kanamycin resistance gene from plasmid pC73 was excised by digestion with AvaI, treated with Klenow and inserted into the Klenow-treated BioBlI site of the deleted version of mdfA, thus constructing plasmid pT7-5/mdfA::kan. This plasmid was used for replacement of the chromosomal mdfA gene by homologous recombination experiments as described (Pinner et al., 1993). Briefly, a linear DNA fragment containing the disrupted gene was released from plasmid pT7-5/mdfA::kan by digestion with PstI and PvuII and used to transform E. coli strain JC7623 by electroporation. The transformants were plated on LB plates containing kanamycin. Single colonies were tested by PCR and by Southern blot (see below), and positive clones harboring the disrupted gene were used as donors for P1 transduction experiments. The deletion/insertion mutation was transferred by P1vir phage as described (Miller, 1972), into UT2L and UT2L20/c. In order to verify the disrupted genotype, the kanamycin-resistant transductants were tested by Southern blot as follows. Chromosomal DNA was prepared as described (Sambrook et al., 1989) and ~10 μg of DNA was digested by PstI and HindIII and resolved on 1% agarose gels. After blotting, the filters were hybridized with a biotinylated 400 bp MfdI-NruI fragment probe made from pT7-5/mdfA::kan. The detection was carried out with a 0.25 ng/ml HRH-conjugated streptavidin.

**Construction of mdfA–phoA fusions**
Using synthetic deoxyoligonucleotides (Table I) and PCR, the given fusions between mdfA and the alkaline phosphatase gene, phoA, were constructed. Each fusion, to codons A15, I31, V43, W53 and P83, was made by PCR amplification of a specific 5′ portion of the mdfA gene using pT7-5/mdfA as a template. All the PCR fragments were designed to contain a Nhel site at the 3′ end immediately following the junction codon, and an EcoRI site in the 5′ end. The vector (pT7-5/mdr1-phoA, Bibi and Béja, 1994) and each of the mdfA PCR fragments were digested with the two restriction enzymes, EcoRI and Nhel, and ligated to form the various mdfA-phoA constructs. Plasmids prepared from positive transformants were analyzed by restriction analysis and by sequencing of the PCR-amplified region through the Nhel junction.

**Construction of a hybrid of MdfA fused to the biotin acceptor domain**
The gene encoding a biotin acceptor domain from the oxaolactate decarboxylase of *K. pneumoniae* (Cronan, 1990; Consler et al., 1993) was excised from plasmid pTC175 using AsclI. After treatment with Klenow, it was inserted in-frame with the mdfA gene into plasmid pT7-5/mdfA-phoA which was digested with Nhel and HindIII to release the phoA gene and treated with Klenow. The resulting plasmid, pT7-5/mdfA-bio, encodes a hybrid in which the biotin acceptor domain is fused to the C-terminus of MdfA.

**Site-directed mutagenesis**
Mutants E26A, E26D and E26K were constructed by introducing mutations into plasmid pT7-5/mdfA-bio using oligonucleotide-directed (Table I), site-specific mutagenesis via two-step PCR experiments (Ho et al., 1989). The final PCR products were digested with SphI and BsrBI and ligated to the SphI–BsrBI 3.6 kb fragment of plasmid pT7-5/mdfA-bio to produce plasmids pT7-5/mdfA-bio E26A, pT7-5/mdfA-bio E26D and pT7-5/mdfA-bio E26K. The mutations were verified by sequencing the length of the PCR-generated segments through the ligation junctions.

**Western blotting**
Overnight cultures of cells constitutively expressing biotinylated MdfA constructs were diluted 1:50 in LB supplemented with ampicillin (100 μg/ml) and grown to an OD₆₀₀ of 0.8. Cultures were harvested and membranes were prepared as described previously (Bibi et al., 1993). Membrane fractions were then subjected to SDS–PAGE using 10% polyacrylamide in the running gel. Proteins were electroblotted to nitrocellulose membranes and probed as described by Consler et al. (1993) with streptavidin–HRP.

**Alkaline phosphatase assays**
Overnight cultures of E. coli UT2L2 cells harboring the mdfA–phoA gene fusions or the vector as a negative control were diluted 1:50 into 2 ml of LB containing 100 μg/ml ampicillin and grown for 3 h at 37°C. Then 1 ml of each culture was centrifuged and assayed for alkaline phosphatase activity in the presence of 1 mM lodoacetamide as described previously (Bibi and Béja, 1994), by measuring the rate of hydrolysis of p-nitrophenyl phosphate by permeabilized cells. Alkaline phosphatase activity was calculated as suggested by Brickman and Beckwith (1975).

**[35S]Methionine labeling and immunoprecipitation**
*Escherichia coli* cells expressing the MdfA–PhoA hybrids or harboring pT7-5 for control were grown overnight at 37°C in LB media containing 100 μg/ml ampicillin and grown for 3 h at 37°C. Then 1 ml of each culture was centrifuged and assayed for alkaline phosphatase activity using the presence of 1 mM lodoacetamide as described previously (Bibi and Béja, 1994), by measuring the rate of hydrolysis of p-nitrophenyl phosphate by permeabilized cells. Alkaline phosphatase activity was calculated as suggested by Brickman and Beckwith (1975).

**Transport assays**
 Cultures were harvested and washed twice with 100 mM KP buffer pH 7.5 (with 5 mM MgSO₄ for doxorubicin efflux assays). For chloramphenicol transport, cells were resuspended in the same buffer to an OD₆₀₀ of 20 units (~2 mg protein/ml). For EtBr or doxorubicin efflux, cells were suspended in minimal medium to an OD₆₀₀ of 0.1 or

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**Table I. Synthetic deoxyoligonucleotides used for constructing mdfA-phoA fusions and PCR-directed mutagenesis**

<table>
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<tr>
<th>Sequence</th>
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<th>Orientation</th>
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<tbody>
<tr>
<td>Primers for phoA fusions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5′-AATTGCTAGCCGGACAGTAAAGCG</td>
<td>A15</td>
<td>antisense</td>
</tr>
<tr>
<td>5′-AATTGCTAGCGATAGGTTGAAATTCGTAAGC</td>
<td>I31</td>
<td>antisense</td>
</tr>
<tr>
<td>5′-AATTGCTAGACGGCCGCAATCAGCGG</td>
<td>V43</td>
<td>sense</td>
</tr>
<tr>
<td>5′-AATTGCTAGCCCAATCCTAGCCGCTTG</td>
<td>W53</td>
<td>antisense</td>
</tr>
<tr>
<td>5′-AATTGCTAGAGGCGGACACATACG</td>
<td>P83</td>
<td>antisense</td>
</tr>
<tr>
<td>5′-GGAGCTGCATGTGTCAGAGG</td>
<td>SOv3</td>
<td>sense</td>
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<tr>
<td>Primers for site-directed mutagenesis</td>
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<tr>
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Drug recognition by the Mdr transporter, MdfA

20 units, respectively. Transport of [dichloroacetyl-1-14C]chloramphenicol (1.3 μM, 55 mCi/mmol) by rapid filtration and EtBr efflux from UTL2mdfA::kan cells harboring plasmid vector or expressing MdfA constructs were monitored as described (Edgar and Bibi, 1997). Uptake of EtBr and inhibition by chloramphenicol were measured as described previously (Mine et al., 1998). The E.coli UTL2mdfA::kan::toLC strain was used for measuring efflux of doxorubicin essentially as described for daunomycin (Bolhuis et al., 1994), using excitation light at 478 nm and emission at 591 nm.

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References


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