Determinants of Substrate Recognition by the *Escherichia coli* Multidrug Transporter MdfA Identified on Both Sides of the Membrane*

**Julia Adler and Eitan Bibi‡**

*From the Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel*

The *Escherichia coli* multidrug transporter MdfA contains a membrane-embedded charged residue (Glu-26) that was shown to play an important role in substrate recognition. To identify additional determinants of multidrug recognition we isolated 58 intragenic second-site mutations that restored the function of inactive MdfA E26X mutants. In addition, two single-site mutations that enhanced the activity of wild-type MdfA were identified. Most of the mutations were found in two regions, the cytoplasmic half of transmembrane segments (TMs) 4, 5, and 6 (cluster 1) and the periplasmic half of TM 1 and 2 (cluster 2). The identified residues were mutated to cysteines in the background of a functional cysteine-less MdfA, and substrate protection against alkylation was analyzed. The results support the suggestion that the two clusters are involved in substrate recognition. Using inverted membrane vesicles we observed that a proton electrochemical gradient ($\Delta \mu_H^+$, inside positive and acidic) enhanced the substrate-protective effect in the cytoplasmic region, whereas it largely reduced this effect in the periplasmic side of MdfA. Therefore, we propose that substrates interact with two sites in MdfA, one in the cytoplasmic leaflet of the membrane and the other in the periplasmic leaflet. Theoretically, these domains could constitute a large part of the multidrug pathway through MdfA.

*This research was supported in part by a grant from the Y. Leon Benoziyo Institute for Molecular Medicine and the M. D. Moross Institute for Cancer Research at the Weizmann Institute of Science, Rehovot, Israel. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 972-8-9343464; Fax: 972-8-9344118; E-mail: e.bibi@weizmann.ac.il.

The abbreviations used are: MDR, multidrug resistance; $\Delta \mu_H^+$, proton electrochemical gradient; TM, transmembrane; EtBr, ethidium bromide; TPP, tetraphenylphosphonium; NEM, N-ethylmaleimide; DTT, dithiothreitol; HRP, horseradish peroxidase.

Received for publication, December 9, 2003
Published, JBC Papers in Press, December 19, 2003, DOI 10.1074/jbc.M313422200

The simultaneous emergence of resistance in cells to many chemically unrelated drugs is termed multidrug resistance (MDR).* MDR transporters that remove the drugs from the cell cytoplasm or cytoplasmic membrane to the external medium cause one major form of multidrug resistance. These efflux systems are widely distributed among prokaryotic microorganisms including pathogenic bacteria (1–4). MDR transporters are usually able to extrude a variety of lipophilic compounds, many of which are positively charged under physiological conditions; others are neutral or zwitterionic, and some substrates are relatively hydrophilic. Although interesting features of multidrug recognition have been recently revealed by structural analysis of a multidrug-binding protein (5) and an MDR transporter (6, 7) crystallized at certain conformations, it is not yet fully understood how dissimilar drugs interact with MDR transporters at various stages of the transport cycle. Here we addressed this question in vivo and in vitro and searched for substrate recognition determinants in the MDR transporter MdfA.

MdfA is an *Escherichia coli* MDR transporter (8, 9). It is a 410-amino acid-long membrane protein of the major facilitator superfamily of secondary transporters (10). Recently, MdfA orthologues were identified in several pathogenic bacteria: *Shigella flexneri* (99% identity) (11), *Salmonella enterica* serovar Typhi (90% identity) (12), and *Yersinia pestis* (73% identity) (13). Cells expressing MdfA from a multicopy plasmid exhibit multidrug resistance to a diverse group of toxic compounds and also export other substrates including (i) lipophilic cations such as ethidium bromide (EtBr), tetraphenylphosphonium (TPP$^+$), and benzalkonium; (ii) zwitterionic drugs such as ciprofloxacin, and (iii) neutral compounds such as chloramphenicol, thiamphenicol, and the sugar isopropyl-1-thio-D-galactopyranoside (8, 14, 15). A recent study demonstrates that MdfA is able to bind chloramphenicol and TPP$^+$ simultaneously and that the two binding sites are closely related (16). Transport experiments show that MdfA is driven by the proton electrochemical gradient and functions as a drug/proton antiporter (17, 15). As predicted from the hydropathy plot of the protein and gene fusion analysis, the putative 12 transmembrane domains (TMs) of the protein contain at least one membrane-embedded charged amino acid residue, Glu-26, in the middle of the TM1 (18, 19) (see Fig. 1). The localization of this acidic residue within the membrane and its high conservation among other drug transporters strongly suggested its functional relevance. Indeed, as demonstrated by mutational analysis, Glu-26 constitutes an important part of the drug binding domain in MdfA; the negative charge at position 26 is important for recognition and transport of a variety of positively charged substrates but not of neutral compounds (18, 20).

In this study we combined a genetic approach with species-specific sulfhydryl labeling to identify additional residues that may constitute parts of the drug binding pocket(s) of MdfA. Initially, we utilized several inactive E26X mutants to isolate second-site mutations that restore the chloramphenicol transport activity of these mutants. In addition, using the wild-type *mdfA* as a template, we selected mutants that exhibit higher chloramphenicol resistance activities. To provide further information about the involvement of the identified residues in drug recognition, we applied *N*-ethylmaleimide (NEM) labeling of single-cysteine (Cys) replacements at these positions and studied the effect of different substrates on the labeling. The results revealed regions that may participate in the formation of the multidrug pathway in MdfA.
**Experimental Procedures**

**Materials**—N-(ethy1-l-lysyl) Ethylmaleimide (34.2 mM/mmol) was purchased from PerkinElmer Life Sciences. 

**Plasmids**—pT7-5/mdfA-His6 was expressed in E. coli UTL2 (kan) containing defective MdfA. Multidrug Recognition by MdfA.

**Strains**—E. coli strain UTL2 was used for membrane vesicle preparations and E. coli strain UTL12 was used as the background strain.

**Cell Disruption**—Leaky cells were disrupted by either high-pressure homogenization or sonication.

**Protein Electrophoresis**—Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes.

**Western Blotting**—Membranes were probed by ECL.

**Drug Resistance Assays**—Drug resistance was assayed in solid or liquid LB medium. When tested on solid media, drug resistance assays were performed as described.

**Efflux of Ethidium Bromide**—EthBr efflux assays were conducted as described with modifications. Overnight cultures of E. coli UTL2/mdfA::kan cells harboring plasmid pT7-5 (vector alone) or the indicated pT7-5/mdfA-His6 plasmids were diluted to 0.04 A_600 unit, grown at 37 °C in LB supplemented with ampicillin (200 μg/ml) and kanamycin (30 μg/ml), and 1.0 A_600 unit, and kept on ice. Aliquots of cells (0.3 A_600 units) were pelleted, resuspended in 2 ml of KP, buffer (K/HPO_4, KHPO_4, 50 mM, pH 7.0), and loaded with EthBr (5 μM) at 37 °C for 5 min in the presence of carbonyl cyanide p-chlorophenylhydrazone (100 μM). Loaded cells were then centrifuged, resuspended in the same buffer containing only EthBr (5 μM), and subjected to fluorescence measurements. After 1 min in the fluorescence microplate, 10 mM DTT was added (final concentration 0.4%), and the EthBr efflux was monitored continuously by measuring the fluorescence excitation and emission wavelengths of 545 and 610 nm, respectively.

**Recombinant DNA Techniques**—To generate cysteine (Cys)-less MdfA, all the native Cys residues were simultaneously mutagenized (C21S, C96A, C120A, and C198S) in pT7-5/mdfA-His6. A series of single Cys replacements were then generated at sites identified in the genetic screens.

**Materials—**N-ethylmaleimide (34.2 mM/mmol) was purchased from PerkinElmer Life Sciences. N-terminal nitrocellulose acid-agarose was purchased from New England Biolabs. India HisProbe-HRP (Fierce) was used for MdfA-His6 detection by Western blotting. Oligonucleotides were synthesized by the Scientific Services unit at the Wadsworth Center Institute of Science. Prescreened protein molecular weight markers were obtained from New England Biolabs, and DNA molecular weight markers were from Fermentas. All other materials were reagent grade and obtained from commercial sources.

**Bacterial Strains—**E. coli HB101 (rpsL20, tnlA13, ara-14, proA2, lacY1, galK2, rpsL20 (35°), xyl-5, mtl-1, supE44, l F) was used for the propagation and preparation of various plasmid constructs. E. coli UTL2/mdfA::kan or the leaky strain UTL2/mdfA::kan (18) were used in drug resistance and transport experiments.

**Second-site Genetic Screens—**As templates we used defective MdfA E2X mutants tagged with six histidines, encoded from plasmid pT7-5/mdfA-His6. To avoid possible recombination events, we used an E. coli UTL2/mdfA::kan plasmid containing a chromosomal mdfA gene (UTL2/mdfA::kan) (18). Cells transformed with any of the inactive E2X mutants were plated on LB agar containing lethal concentrations of chloramphenicol (10 μg/ml) in addition to ampicillin (200 μg/ml) and kanamycin (30 μg/ml) for selection of the plasmid and the strain, respectively. After 24–36 h, single colonies developed on chloramphenicol plates due to spontaneous mutations. To discriminate between mutations within mdfA versus chromosomal changes, plasmid DNA was purified from resistant clones, and the phenotype was confirmed by retransformation. Plasmid DNA was isolated from resistant colonies, and the entire mdfA gene (including the 231-bp 5’-untranslated region) was sequenced. In addition, to ensure that the acquired phenotype was not associated with another mutation elsewhere in the plasmid, the mdfA gene was transferred to a new plasmid.

**Screening of Mutations with Elevated Chloramphenicol Resistance—**To select for mutants with increased resistance to chloramphenicol, E. coli UTL2/mdfA::kan cells harboring wild-type MdfA (pT7-5/mdfA-His6) were plated on LB agar containing ampicillin (200 μg/ml) and kanamycin (30 μg/ml) for selection of the plasmid and the strain, respectively. Chloramphenicol was added in a concentration that inhibits growth of the test drug. TPP was used for MdfA-His6 detection by Western blotting. Oligonucleotides were synthesized by the Scientific Services unit at the Wadsworth Center Institute of Science. Prescreened protein molecular weight markers were obtained from New England Biolabs, and DNA molecular weight markers were from Fermentas. All other materials were reagent grade and obtained from commercial sources.
Multidrug Recognition by MdfA

Isolation of Second-site Mutations That Restore the Function of Inactive E26X Mutants—In an attempt to identify residues involved in multidrug recognition by MdfA we screened for second-site mutations that suppress the inhibitory effect of mutations at position 26. As templates we used several E26X mutants (X = Ala, Asp, Asn, or Leu) that exhibit almost no chloramphenicol resistance activity of the original inactive E26X mutants (Table I).

Remarkably, as much as half of the second-site clones possessed mutations within the cytoplasmic half of TMs 4, 5, and 6 (cluster 1 in Fig. 1, Table I). Several sites were identified once (one replacement), including V125A, A128T, S133F, A147T, A150V, and three of the sites were mutated twice: Y127N or Y127H, G187S or G187A, and A191V or A191T. Another cluster of second-site mutations was found in the periplasmic side of TMs 1 and 2 (cluster 2 in Fig. 1 and Table I). Here, the most abundant second-site mutation was V23A, which restored the function of all the inactive E26X mutants. Other recurrent second-site mutations occurred at position 21, where the inactive mutants E26A, E26D, and E26L were restored by introduction of aromatic residues (Tyr, Phe, or Trp) instead of Cys-21. The other second-site mutations isolated in the periplasmic cluster 2 were G39V, V54L, and T56A. In addition to clusters 1 and 2, second-site mutations were found also in TM 7 (V231G), TM 8 (L268S) and within the cytoplasmic loop connecting TMs 10 and 11 (V335M). Western blot analysis (Fig. 2) revealed that all the second-site mutants were expressed in the membrane at levels similar to those of wild-type MdfA and the original E26X mutants (Fig. 2) (20).

Isolation of Mutants with Enhanced Chloramphenicol Resistance Activity—As an alternative approach to identify residues that might contribute to drug recognition, we utilized cells expressing wild-type MdfA and selected for spontaneous mutants that enabled growth on high chloramphenicol concentrations. Two MdfA mutants were isolated, and sequencing the entire mdfA gene identified a single mutation in each of them. One mutant harbors a M146T mutation at the putative cytoplasmic interface of TM5, and the second mutant has a S204P mutation within the putative cytoplasmic loop between TMs 6 and 7 (Table I). Strikingly, both mutations are confined to cluster 1, which was identified in the course of the second-site mutation analysis (Fig. 1). As shown by Western blot analysis (Fig. 2), the mutations did not appreciably change the expression level of MdfA in the membrane, but as expected, the mutants were more effective than wild-type MdfA in reducing intracellular chloramphenicol concentration as a result of active transport (data not shown).

Construction and Characterization of a Cys-less MdfA—The fact that many of the mutations found in the genetic screens were clustered and isolated more than once suggests that the mutated sites might be located in the drug binding pocket of MdfA. To study this possibility further, we implemented a cysteine modification approach. Site-directed sulphydryl modification of single-Cys mutants with [14C]NEM has been useful in studying structural and functional aspects of membrane proteins (24). The extent of alkylation with NEM is a measure of the reactivity and/or accessibility of a given Cys residue to this small, relatively hydrophobic, membrane-permeable, thiol-specific reagent. Changes in the labeling of a given Cys residue upon substrate binding indicate an alteration in the local environment around the Cys residue. Hence, in situ labeling is valuable for testing whether a specific region in the protein is involved in interaction with a substrate (24). As a prerequisite for site-directed sulphydryl modification, a functional cysteine-less protein must be constructed. MdfA contains four native cysteines at positions 21, 96, 120, and 139 (Fig. 1). These cysteine residues were simultaneously replaced (C21S, C96A, C120A, and C139S) by site-directed mutagenesis to generate a Cys-less MdfA. Western blot analysis of membrane fractions revealed normal steady-state expression of the mutant (Fig. 3A). Moreover, transport experiments demonstrated Cys-less MdfA-mediated efficient efflux of EtBr (Fig. 3B). In

| Table I | Summary of the selected mutations |
|---------|----------------------------------|
| Drug resistance was measured as described under “Experimental Procedures.” |

| Clone | Defective MdfA mutant (E26X) | Second-site mutation | Chloramphenicol resistance μg/ml |
|-------|-------------------------------|---------------------|---------------------------------|
| Vector | Ala | Leu | Asp | Asn | Wild-type | Ala | Asp | Leu | Asp | Promoter |
| 1 | Ala | C21Y | <2 |
| 2 | Ala | C21Y | 10 |
| 3 | Ala | C21Y | 10 |
| 4 | Ala | C21Y | 10 |
| 5 | Ala | C21F | 10 |
| 6 | Ala | C21F | 10 |
| 7 | Ala | V23A | 10 |
| 8 | Ala | V23A | 10 |
| 9 | Ala | V23A | 10 |
| 10 | Ala | V23A | 10 |
| 11 | Ala | V23A | 10 |
| 12 | Ala | V23A | 10 |
| 13 | Ala | V23A | 10 |
| 14 | Ala | V23A | 10 |
| 15 | Ala | V23A | 10 |
| 16 | Ala | V23A | 10 |
| 17 | Ala | V23A | 10 |
| 18 | Ala | V23A | 10 |
| 19 | Ala | V23A | 10 |
| 20 | Ala | V23A | 10 |
| 21 | Ala | V23A | 10 |
| 22 | Ala | V23A | 10 |
| 23 | Ala | V23A | 10 |
| 24 | Ala | V23A | 10 |
| 25 | Ala | V23A | 10 |
| 26 | Ala | V23A | 10 |
| 27 | Ala | V23A | 10 |
| 28 | Ala | V23A | 10 |
| 29 | Ala | V23A | 10 |
| 30 | Ala | V23A | 10 |
| 31 | Ala | V23A | 10 |
| 32 | Ala | V23A | 10 |

0.5 ml of buffer C (Buffer B supplemented by 40 mM imidazole). The protein was eluted with 50 μl of buffer D (50 mM KF, pH 7.5, supplemented by 350 mM imidazole) and incubated with sample buffer (1% SDS, 25 mM Tris, pH 8, 8% glycerol, 0.01% bromphenol blue, and 25 mM DTT) for 1 h at 37 °C. The samples were subjected to SDS-PAGE (12.5%), stained with Coomassie Brilliant Blue, and treated with Amplify fluorographic reagent. Dried gels were exposed to x-ray film for 1–14 days at −80 °C.

RESULTS

0.5 ml of buffer C (Buffer B supplemented by 40 mM imidazole). The protein was eluted with 50 μl of buffer D (50 mM KF, pH 7.5, supplemented by 350 mM imidazole) and incubated with sample buffer (1% SDS, 25 mM Tris, pH 8, 8% glycerol, 0.01% bromphenol blue, and 25 mM DTT) for 1 h at 37 °C. The samples were subjected to SDS-PAGE (12.5%), stained with Coomassie Brilliant Blue, and treated with Amplify fluorographic reagent. Dried gels were exposed to x-ray film for 1–14 days at −80 °C.
addition Cys-less MdfA was able to confer resistance to multiple drugs, such as chloramphenicol, EtBr, TPP¹¹⁰/¹¹⁰¹, ciprofloxacin, erythromycin, and tetracycline (Fig. 4B) and also thiamphenicol and benzalkonium, (data not shown). Thus, because the Cys-less transporter retained multidrug transport activity, it was used as a template for site-directed sulfhydryl modification of newly inserted single-Cys residues.

Construction of Single-cysteine Mutants at Positions Identified by the Genetic Screens—All the amino acid residues that were identified in the screens as putative substrate recognition sites were individually replaced by cysteines in a Cys-less version of MdfA. All the single-Cys mutants were detected in the cytoplasmic membrane at levels comparable with that of Cys-less MdfA or the wild-type protein, as judged from Western blotting analysis (Fig. 4A). The activity of the mutants was examined by drug resistance assays, and most of the mutants exhibited considerable drug resistance activity toward multiple drugs (Fig. 4B). Interestingly, however, many of the Cys replacements affected the substrate recognition profile of MdfA, and the activity of three mutants (E26C, A147C, and L268C) was dramatically changed. These observations suggest that many of the identified sites might indeed be involved in multidrug recognition.

[^14]C]NEM Labeling of Single-Cys Mutants of MdfA—To examine the susceptibility of the single-Cys mutants to NEM modification, inside-out membrane vesicles containing overexpressed His₆-tagged versions of the mutants were incubated with[^14]C]NEM. The membranes were then solubilized, and the MdfA constructs were purified by Ni²⁺ nitritotriacetic acid-agarose and analyzed by SDS-PAGE and autoradiography. The amount of MdfA in each sample was estimated by staining the gel with Coomassie Brilliant Blue. In control experiments we observed that Cys-less MdfA (negative control) was not labeled with NEM, even after prolonged exposure, whereas wild-type MdfA (positive control) was readily labeled (data not shown). As shown in Fig. 5, there was no significant difference in the amounts of the purified mutants (lower panel), and all of the mutants were modified by[^14]C]NEM (upper panel). However, there were clear differences in the amounts of the radiolabeled products, indicating different levels of reactivity with NEM. These differences might be due to changes within the local environment of the various Cys side chains, because water exposed Cys side chains are usually highly reactive, whereas those tightly packed in a hydrophobic environment are usually less reactive or inaccessible to the sulfhydryl reagent (25–29).

The single-Cys mutants V54C, T56C, Y127C, A128C, A147C, A191C, S204C, and V335C exhibited high reactivity with NEM, whereas the mutants G39C, V125C, S133C, M146C, and
A150C were also efficiently labeled, but to a lesser extent. This pattern supports the topological model of MdfA (18, 19), since the moderately or highly reactive mutants are found within the putative loops or at the edges of transmembrane domains.

Another group of mutants, including Cys-21 (a single native cysteine in otherwise Cys-less MdfA), V23C, E26C, G187C, V231C, and L268C were much less reactive with NEM. Except for G187C, which is thought to be at the cytoplasmic edge of TM 6, all of these mutated residues are located within the putative membrane domains of MdfA.

Effect of the substrates on [14C]NEM Labeling of Single-cysteine Mutants of MdfA—Because all the putative substrate recognition sites were amenable to modification by NEM to various extents when substituted for Cys residues, we tested whether substrates can modify their reactivity with the sulfhydryl reagent. Inverted membrane vesicles harboring the different single-Cys mutants were incubated with a given test substrate for 25 min at 22 °C before adding the radiolabeled NEM. The labeling of each mutant was examined after different incubation times and at different temperatures to identify appropriate conditions for detection of the substrate effects. The effect of three MdfA substrates on the labeling was studied; they are chloramphenicol (which was used in the genetic screen), TPP(H11001, and EtBr. With mutant E26C we also tested the effects of tetracycline, benzalkonium, and erythromycin.

Initially, the sites in cluster 1 (Fig. 1) were examined; the results are shown in Fig. 6. As shown, the labeling of two mutants (A128C and A147C) was inhibited by all the substrates. With A128C the inhibitory effects were observed under all conditions (time and temperature), whereas with MdfA A147C, the protective effects of the substrates were observed only on ice, indicating that the substrates may interact more tightly at position 128. Interestingly, despite being close to the protected residue Cys-128, the NEM modification of the adjacent Cys replacements at positions 125, 127, and 133 was not affected by any of the test substrates. Similarly, the labeling of Cys residues at position 146 or 150 was not affected by the substrates despite their proximity to the protected site at po-
sition 147. Finally, whereas the labeling of mutant G187C was stimulated by TPP\(^+\)/H11001, suggesting a conformation change, the modification of mutants A191C and S204C remained almost unaffected. These results suggest that among the residues identified in cluster 1, those at positions 128 and 147 might constitute an important part of the multidrug recognition pocket of MdfA.

Next, we tested the effects of the substrates on NEM labeling of Cys residues in cluster 2 (Fig. 1); the results are shown in Fig. 7. Remarkably, we observed that TPP\(^+\)/H11001 always induced various degrees of stimulation of labeling, suggesting a major conformational rearrangement in this region of the protein upon interaction with TPP\(^+\). A similar stimulatory effect was observed with benzalkonium on the NEM labeling of mutant E26C. As expected, none of the substrates inhibited the labeling of mutant E26C, which exhibits only marginal reactivity and probably poorly interacts with the substrates (20). In four cases (single Cys-21, G39C, V54C, and T56C), inhibition of NEM labeling was observed in the presence of chloramphenicol and EtBr, in support of the notion that these sites may directly contribute to substrate recognition by MdfA. Finally, none of the test substrates exerted significant effects on NEM-labeling of mutant V23C.

The remaining three mutants, which were not confined to any of the clusters (V231C, L268C, and V335C) (Fig. 1), were also examined by the substrate protection assay (Fig. 8). As shown, the labeling of mutants V231C and L268C was somewhat stimulated by TPP\(^+\), suggesting a conformational response. In contrast, the labeling of mutant V335C was markedly inhibited by chloramphenicol and TPP\(^+\), indicating the possible direct involvement of this site in multidrug recognition.

**Effect of Δ\(\mu_{H^+}\) on Substrate Protection against NEM Labeling**—To study the effect of Δ\(\mu_{H^+}\) on the interaction of MdfA with substrates, we used inverted membrane vesicles expressing two single-Cys mutants, A128C (cluster 1) and V54C (cluster 2). Both cysteines exhibited substrate protection against NEM modification (Figs. 6 and 7). Inverted vesicles prepared from cells expressing either of these mutants generated a stable electrochemical gradient (inside positive and acid) in the presence of ascorbate and phenazine methosulfate (30), as demonstrated by fluorescence spectroscopy using \(\Delta\psi\)- and \(\Delta\mathrm{pH}\)-sensitive dyes (data not shown). Under these conditions chloramphenicol is actively transported into the vesicles (15). The vesicles were preincubated with or without chloramphenicol, energized to generate Δ\(\mu_{H^+}\), and then labeled by \([^{14}\mathrm{C}]\)NEM. As shown in Fig. 9, chloramphenicol protected both MdfA A128C and V54C against NEM modification. Interestingly however, when the reactions were carried out in the presence of Δ\(\mu_{H^+}\), reproducible differential effects were observed. Δ\(\mu_{H^+}\) enhanced the chloramphenicol protection effect with A128C, whereas with V54C, the protection was largely reduced.

**FIG. 6.** Effect of substrates on \([^{14}\mathrm{C}]\)NEM labeling of single cysteine mutants of MdfA in cluster 1. Membranes (3 mg of protein) from *E. coli* UTL2mdfA::kan expressing the indicated single-Cys mutants were incubated with 0.2 mM \([^{14}\mathrm{C}]\)NEM (34 mCi/mmol) on ice or at 22 °C. When indicated, 5 mM of chloramphenicol (Chl), tetraphenylphosphonium (TPP\(^+\)), or ethidium bromide (EtBr) was present before and during the reaction with NEM. The labeled material was analyzed as described in the legend of Fig. 5.
Among these mutants we isolated three single-Cys mutants of MdfA in cluster 2. See legend of Fig. 6. The E26C mutant was also tested with tetracycline (Tet, 0.625 mM), erythromycin (Ery, 1 mM), and benzalkonium Cl (BzCl, 5 mM).

We speculate that when energized, MdfA binds substrates with a higher affinity to cluster 1 in the inner leaflet of the membrane and releases them from cluster 2 at the end of the transport cycle.

**DISCUSSION**

Recent studies demonstrated that the membrane-embedded, negatively charged residue Glu-26 is involved in multidrug recognition by MdfA (18, 20). Here, we combined a genetic approach with site-specific sulfhydryl labeling to identify additional residues that may contribute to the drug binding pocket of MdfA. Initially, we used several inactive E26X mutants to isolate intragenic second-site mutations that restore the function of these mutants. Using chloramphenicol for the selection, we identified a series of mutants that regained high chloramphenicol resistance activity. Among these mutants we found two independent clones, both of which had acquired an identical single A → G base change at position -14 relative to the transcription start site of mdfA (31). The parental mutants (E26N and E26L) exhibit some marginal chloramphenicol resistance activity. From these mutations are located in cluster 1. These results further support the proposed putative role of cluster 1 in multidrug resistance.

All the other mutations were found within the coding sequence of mdfA. Among these mutants we isolated three “first-site” mutations, A26T, L26V, and A26V, which led to a strong chloramphenicol resistance phenotype. These and other Glu-26 mutants were characterized in detail elsewhere (20). The analysis of the other second-site mutations at 16 different positions revealed that most of them were clustered in two regions of MdfA (Fig. 1); one cluster (cluster 1, TMs 4–6) is within the cytoplasmic side of the membrane, and the other (cluster 2, TMs 1 and 2) is within the periplasmic side, suggesting that collectively they might constitute part of the multidrug pathway of MdfA. To identify additional putative multidrug recognition determinants we also used an alternative screen in which mutations that led to increased chloramphenicol resistance activity were isolated. Two single mutants were identified and characterized, M146T and S204P, and remarkably, both of these mutations are located in cluster 1. These results further support the proposed putative role of cluster 1 in multidrug recognition.

The new 18 single-Cys mutants of MdfA provided us with a biochemical tool for further studying the involvement of these sites in substrate recognition. Because all the single-Cys residues were accessible to the sulfhydryl reagent NEM to various degrees, we tested the ability of several substrates to protect against NEM modification. As shown in this work, several types of substrate effects were observed. (i) Certain sites were protected by all three test substrates, (ii) several sites were protected by one or two of the substrates, (iii) some sites were not protected by any of the test substrates, and (iv) the NEM modification of many sites was stimulated by TPP " and not by the other substrates. The substrate-protective effect...
could be interpreted as conformational or as a result of the direct competition between the substrate and NEM. With this notion in mind, we considered sites that were appreciably protected by two of the substrates or only one might contribute differentially to the multidrug recognition pocket (21, 39, 54, 56, 191, and 335), whereas sites that were not protected by the substrates probably do not contribute to drug recognition directly, or if they do, the Cys mutation at these positions may have altered their drug recognition capabilities. The fact that certain residues interact with structurally diverse substrates by MdfA is consistent with similar studies carried out on the MDR residues interact with structurally diverse substrates by MdfA altered their drug recognition capabilities. The fact that certain

vesicles harboring the single-Cys mutant V54C (located in cluster 2), the protective effect of chloramphenicol against labeling with NEM was largely reduced. One possible interpretation of the results is that during transport, $\Delta \mu_{\text{H}}$ increases the substrate affinity to cluster 1, whereas at the same time it decreases the substrate affinity to cluster 2, to facilitate release of the substrate into the periplasm. Therefore, we suggest that the differential energy-dependent effects that take place in clusters 1 and 2 of MdfA support their classification as sequential substrate-interacting domains along the multidrug pathway in MdfA.

Acknowledgments—We thank Lior Izhav for help during the initial stages of this study, Dr. Elena Bochkareva for helpful suggestions, and Oded Levinson for comments on the manuscript.

REFERENCES
1. Nikaido, H. (1998) Curr. Opin. Microbiol. 1, 516–523
2. Poole, K. (2001) Curr. Opin. Microbiol. 4, 500–508
3. Levy, S. B. (2002) J. Appl. Microbiol. 92, suppl. 65–71
4. Zegerskaya, H. I. (2002) Int. J. Med. Microbiol. 292, 95–105
5. Schumacher, M. A., Miller, M. C., Grkovic, S., Brown, M. H., Skurray, R. A., and Brennan, R. G. (2001) Science 294, 2158–2163
6. Murakami, S., Nakashima, E., Yamashita, E., and Yamaguchi, A. (2002) Nature 419, 587–593
7. Yu, E. W., McDermott, G., Zegerskaya, H. I., Nikaido, H., and Koshland, D. E., Jr. (2003) Science 300, 976–980
8. Edgar, R., and Bibi, E. (1997) J. Bacteriol. 179, 2274–2280
9. Bibi, E., Adler, J., Lewinson, O., and Edgar, R. (2001) J. Mol. Microbiol. Biotechnol. 3, 171–177
10. Saer, M. H., Jr., and Paulsen, I. T. (2001) Semin. Cell Dev. Biol. 12, 205–213
11. Jin, Q., Yuan, Z., Xu, J., Wang, Y., Shen, Y., Lu, W., Wang, J., Liu, H., Yang, J., Yang, P., Zhang, X., Zhang, J., Yang, G., Wu, Q., Dang, J., Sun, L., Xu, Y., Zhao, A., Gao, Y., Zhu, J., Kan, B., Ding, R., Chen, S., Cheng, H., Yao, Z., He, B., Chen, R., Ma, D., Qiang, B., Wen, Y., Hou, Y., and Yu, J. (2002) Nucleic Acids Res. 30, 4432–4441
12. Parkhill, J., Dougan, G., James, K. D., Thomson, N. R., Pickard, D., Wain, J., Churcher, C., Mungall, K. L., Bentley, S. D., Holden, M. T., Sebaihia, M., Barker, S., Basham, D., Brooks, K., Cerezo-Tarraga, A. M., Chillingworth, T., Cronin, A., Davies, P., Davies, R. M., Dow, L., White, K., Wild, P., Farrar, J., Fellwell, T., Hamlin, N., Haque, A., Hien, T. T., Holroyd, S., Jagels, K., Krog, A., Larsen, T. S., Leather, S., Moule, S., O’Gara, P., Parry, C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S., Bibi, E., and Barrett, B. G. (2001) Nature 413, 848–852
13. Parkhill, J., Wren, B. W., Thomas, N. R., Tithall, R. W., Holden, M. T., Prestine, M. B., Sebaihia, M., James, K. D., Churcher, C., Mungall, K. L., Baker, S., Basham, D., Bentley, S. D., Brooks, K., Cerezo-Tarraga, A. M., Chillingworth, T., Cronin, A., Davies, R. M., Davies, P., Dougan, G., Fellwell, T., Hamlin, N., Holroyd, S., Jagels, K., Krog, A., Larsen, T. S., Leather, S., Moule, S., O’Gara, P., Parry, C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S., and Barrett, B. G. (2001) Nature 413, 523–527
14. Rauen, C., and Boule, P. (2000) J. Bacteriol. 180, 6072–6075
15. Levinson, O., Adler, J., Poelarends, G. J., Mazurkiewicz, P., Dreissen, A. J., and Bibi, E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1667–1672
16. Levinson, O., and Bibi, E. (2004) Biochemistry 43, 518–525
17. Mine, T., Morita, Y., Katoaka, M., Mizusawa, T., and Tsuchiya, T. (1998) J. Biochem. (Tokyo) 124, 187–193
18. Edgar, R., and Bibi, E. (1999) EMBO J. 18, 822–832
19. Adler, J., and Bibi, E. (2002) J. Bacteriol. 184, 3313–3320
20. Adler, J., and Bibi, E. (2004) Biochemistry 43, 518–525
21. Bibi, E., Gros, P., and Kacabak, H. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9209–9213
22. Yerushalmi, H., and Schuldiner, S. (2000) J. Biol. Chem. 275, 5264–5269
23. Ho, S. N., Hunt, D. H., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 51–55
24. Frillings, S., Sainin Toth, M., Wu, J., and Kacabak, H. R. (1998) FASEB J. 12, 1281–1299
25. Mochi, S. S., Granot, D., Lebedniker, M., and Schuldiner, S. (1999) J. Biol. Chem. 274, 19480–19486
26. Venkatesan, P., Liu, Z., Hu, Y., and Kacabak, H. R. (2000) Biochemistry 39, 10649–10655
27. Venkatesan, P., Hu, Y., and Kacabak, H. R. (2000) Biochemistry 39, 10656–10661
28. Kiew, I., Zen, K. C., Hu, Y., and Kacabak, H. R. (2001) Biochemistry 40, 9489–9499
29. Tamura, N., Konishi, I., Iwaki, S., Kimura-Someya, T., Nada, S., and Yamaguchi, A. (2000) J. Biol. Chem. 275, 20330–20339
30. Kaback, H. R., and Patel, H. (1978) Biochemistry 17, 1640–1646
31. Nilsen, I. W., Bakke, I., Vater, A., Olvik, O., and El-Gewely, M. F. (1996) J. Bacteriol. 178, 3188–3193
32. Lou, T. W., and Clarke, D. M. (2000) J. Biol. Chem. 275, 39272–39278
33. Lou, T. W., and Clarke, D. M. (2001) J. Biol. Chem. 276, 14892–14897
34. Lou, T. W., and Clarke, D. M. (2002) J. Biol. Chem. 277, 44332–44338
35. van Iwaarden, P. R., Dreissen, A. J., and Konings, W. N. (1992) Biochim. Biophys. Acta 1113, 161–170
36. Bolhuis, H., van Veen, H. W., Molenaa, D., Poolman, B., Dreissen, A. J., and Konings, W. N. (1996) EMBO J. 15, 4239–4245
Multidrug Recognition by MdfA