Functional Characterization of the Heterooligomeric EbrAB Multidrug Efflux Transporter of *Bacillus subtilis*†

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ABSTRACT: The *Bacillus subtilis* genome contains two tandem genes, *ebrA* and *ebrB*, which encode two homologues of the SMR family of multidrug efflux transporters. The sequences of EbrA and EbrB are highly similar to each other and to that of EmrE, the prototypical SMR transporter of *Escherichia coli*. Drug resistance profiling and drug binding experiments showed that the presence of both EbrA and EbrB is required for proper transport function. EbrA and EbrB directly interact and combine to form a functional transporter. They likely form a heterodimer in analogy to the EmrE homodimer. Mutagenesis experiments indicate that the conserved membrane-embedded glutamates in the first transmembrane helices of both EbrA and EbrB are required for multidrug efflux activity. However, the two glutamates are nonequivalent since EbrA E15 is required for substrate binding while EbrB E14 is not. Our studies support a model in which functional residues in EbrAB are relegated to at least two sets that participate in distinct steps of the active drug transport process.

One important mechanism of multidrug resistance involves drug efflux mediated by integral membrane MDR[1] transporters (*1*). These transporters are found in all organisms from bacteria to humans, and they extrude various cytotoxic compounds from cells using ATP or monovalent cation gradients as energy sources. One group of these MDR transporters, the small multidrug resistance (SMR) family (*2*, *3*) within the drug/metabolite transporter (DMT) superfamily (*4*), includes proteins that are the smallest of such transporters. The polypeptide chains of these efflux pumps, found in the inner membranes of Gram-negative bacteria, are only ∼110 amino acyl residues in length, contain four transmembrane (TM) helices, and have a molecular mass of ∼12 kDa. SMR pumps use proton gradients as the energy source to pump out various cationic hydrophobic compounds such as ethidium bromide, methyl viologen, acriflavine, tetracycline, and tetraphenylphosphonium (TPP), as well as various antiseptics and intercalating dyes (*2*, *3*).

The best-characterized SMR transporter is the EmrE protein of *Escherichia coli* (*5*). EmrE was originally identified on the basis of its ability to confer resistance to ethidium (*6*). Biochemical and genetic studies have shown that a membrane-embedded glutamate residue (E14), located in the middle of the first TM helix, is indispensable for proper drug efflux activity (*5*). Nonconservative mutations of E14 completely abrogate drug binding, and even a relatively conservative mutation to aspartate severely inhibits drug transport (*7*, *8*). Consistent with its central role in drug efflux, E14 is absolutely conserved in all SMR family members (for example, see Figure 1).

Studies also show that the basic functional unit of EmrE is an oligomer, which may be expected for an integral membrane protein of its small size. The exact oligomeric state of the basic functional unit of EmrE is still in dispute: it has been described to function as a dimer [based on electron microscopy and biochemical studies (*9*−*12*)], a trimer [based on substrate binding studies (*7*, *13*)], and a tetramer [based on subunit complementation experiments (*14*)]. In light of recent analyses, which indicate that the basic structural unit of EmrE is an asymmetric dimer of inverted monomers (*12*, *15*, *16*), it appears that the functional unit of EmrE is dimeric.

The conclusion that SMR pumps function as oligomers is strongly supported by the existence of tandem SMR genes, which include the *ebrA* and *ebrB* genes of *Bacillus subtilis*. These two genes are organized as an operon in the *B. subtilis* genome, and their expression is controlled by a single promoter (*2*). The EbrA and EbrB proteins are significantly homologous to each other and to EmrE (Figure 1). However,

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[1] Abbreviations: BDTA, benzyldimethyltetradecylammonium chloride; CTAB, hexadecyltrimethylammonium bromide; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DDM, *n*-dodecyl β-D-maltoside; EPR, electron paramagnetic resonance; IPTG, isopropyl β-D-thiogalactoside; MBP, 3-(*N*-maleimidopropinyl)biocystin; MDR, multidrug resistance; SMR, small multidrug resistance; TM, transmembrane helix; TPA, tetraphenylarsonium; TPP, tetraphenylphosphonium.

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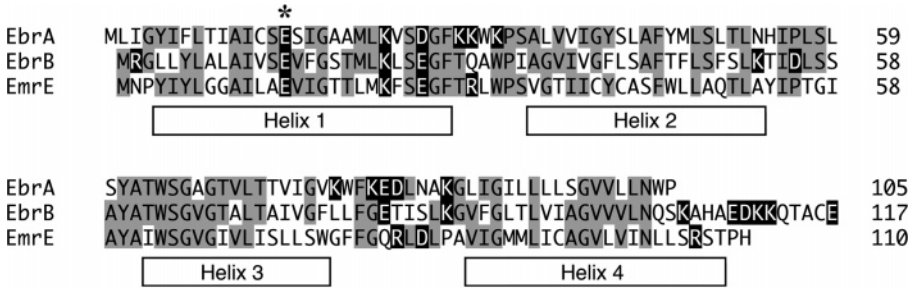

FIGURE 1: Primary sequence alignment of EbrA, EbrB, and EmrE. The alignment was created using ClustalW, including 20 other SMR homologues. For clarity, only EbrAB and EmrE are shown as representative heterooligomeric and homooligomeric SMR transporters, respectively. Charged residues are shaded in black. Hydrophobic and polar residues that are identical in at least two proteins are shaded in gray. The conserved glutamates in the middle of the first transmembrane helices are highlighted with an asterisk. The secondary structure is derived from the X-ray crystal structure of unbound EmrE (*15*).

unlike EmrE, the efflux pump is functional only when both of its components, EbrA and EbrB, are present (*17*). EbrA and EbrB have been proposed to combine to form a heterooligomeric efflux pump. To test this hypothesis, we investigated the multidrug transport activity and performed a preliminary characterization of oligomerization of EbrA and EbrB. We also tested the relative importance of the two conserved glutamates in EbrAB (E15 in EbrA and E14 in EbrB) in drug binding and recognition, as well as the overall transport process.

MATERIALS AND METHODS

Bacterial Strains and Growth. *E. coli* DH5α, TG1Δ*acrAB* (kindly provided by A. Yamaguchi of Osaka University, Osaka, Japan), and BL21(DE3) and *B. subtilis* BAL218 (*18*) and M168 (*19*) were grown in Luria-Bertani (LB) medium at 37 °C. When indicated, induction agents (10 mM L-arabinose and/or 1 mM IPTG), antibiotics (ampicillin and kanamycin at 100 and 25 μg/mL, respectively, for *E. coli*; kanamycin at 10 μg/mL for *B. subtilis*), and substrate drugs were included in the medium.

Gene Cloning and Mutagenesis. The *ebrAB* structural genes were PCR-cloned from the chromosome of wild-type *B. subtilis* strain BAL218 using primers (*ebrAB*-F and *ebrAB*-R) described in Table S1 of the Supporting Information. The 5′ end of the forward primer contained a typical ribosome-binding site (AGGAGG). The PCR products were purified using a PCR purification kit (Qiagen), digested with *Sac*I and *Sal*I, and ligated into the same sites of pBluescript SK. The EbrA E15A/E15D and EbrB E14A/E14D mutants were obtained using the Quickchange site-directed mutagenesis kit (Stratagene). All constructs were verified by sequencing the insert and flanking vector regions (SeqWright, Fisher Scientific).

E. coli protein expression vectors were created by subcloning the *ebrAB* genes into pBAD28, which harbors the *araBAD* promoter (*20*), or pET28a, which harbors the T7 promoter (Novagen). Genes were subcloned either separately or in tandem to allow individual expression of each protein or coexpression of the two polypeptides. Proteins expressed from the pET28a construct contained an N-terminal hexahistidine tag. For protein expression in *B. subtilis*, wild-type and mutant *ebrAB* were subcloned into the *E. coli*−*B. subtilis* shuttle plasmid pDG148-Stu as described by Joseph et al. (*21*).

Drug Resistance Assays. Assays performed with *E. coli* involved transformation of the DH5α, TG1Δ*acrAB*, and BL21(DE3) strains with the appropriate plasmid or plasmid pairs; 10^4 cells were inoculated into the wells of 96-well microtiter plates containing 120 μL of LB medium per well, the appropriate antibiotics and inducers, and serial dilutions of each drug being tested. Plates were incubated at 37 °C for 24 h, and the minimal inhibitory concentration (MIC) was determined for each drug by examining the medium turbidity. Assays with *B. subtilis* were performed in the same manner using strain M168.

Pull-Down Assay. To determine if EbrA and EbrB interact directly, N-terminally hexahistidine-tagged EbrA (His-EbrA) and untagged EbrB were coexpressed in *E. coli* BL21(DE3) cells. Total membranes were prepared from cells derived from 1 L of culture using a microfluidizer (Microfluidics, Inc.) and ultracentrifugation and then solubilized in 10 mL of MonoA buffer [20 mM Tris (pH 8), 100 mM NaCl, and 5 mM β-mercaptoethanol] containing 2% (w/v) *n*-dodecyl β-D-maltoside (DDM) for 2 h. The insoluble material was removed by centrifugation, and the supernatant was applied to a gravity-flow column containing 0.2 mL of Ni-NTA resin (Qiagen). The column was washed with 10 mL of MonoA containing 0.1% DDM, followed by 10 mL of MonoA-DDM with 500 mM NaCl, and 10 mL of MonoA-DDM with 60 mM imidazole. Bound fractions were collected by elution with MonoA-DDM containing 300 mM imidazole (3 × 0.1 mL) and visualized by SDS−PAGE with Coomassie staining. Protein identities were confirmed by sequencing the N-termini of excised bands using the Edman method (University of Utah Oligo/Peptide Facility, University of Utah, Salt Lake City, UT).

[^{3}H]TPP Binding Assays. The various EbrAB constructs were expressed in *E. coli* TG1Δ*acrAB*. Cells were collected by centrifugation, washed once with M63 minimal medium (*22*), and resuspended in the same medium containing DNase I. The cells were lysed via two passes through a French pressure cell, and cellular debris was removed by centrifugation at 10000*g* for 10 min. Membranes were collected by centrifugation at 100000*g* for 2 h, resuspended in M63, and stored at −80 °C prior to use. Protein concentrations were determined using a colorimetric assay (Bio-Rad) with bovine serum albumin as the standard.

To measure TPP binding activity, 180 μL of membrane vesicles (containing ∼0.5 mg of total protein) was incubated with 20 μL of 1 μM [^{3}H]TPP (30 Ci/mmol) (Amersham Life Sciences) in M63 (pH 7.5) at 4 °C for 30 min. The assay mixture was then centrifuged at 250000*g* for 30 min. Membranes were washed once with M63 and then resus-

Table 1: Recombinant Expression of EbrAB Confers Multidrug Resistance in *E. coli*[a]

drug[b]	vector control	EbrA	EbrB	EbrAB	EbrA^{E15A}B	EbrAB^{E14A}	EbrA^{E15A}B^{E14A}
(A) DH5α							
ethidium bromide	50	50	50	500	50	50	50
acriflavine	25	25	25	140	25	25	25
crystal violet	4	4	4	10	4	4	4
cetalkonium chloride	3	3	3	8	3	3	3
benzalkonium chloride	8	8	8	14	8	8	8
CTAB	4	4	4	7	4	4	4
cetylpyridinium chloride	2	2	2	3	2	2	2
pyronin Y	4	4	4	>120	4	4	4
TPA	200	200	200	1500	200	200	200
TPP	400	400	400	1500	400	400	400
(B) TG1Δ*acrAB*							
ethidium bromide	8	ND[c]	ND[c]	>100	8	8	8
acriflavine	8	ND[c]	ND[c]	40	8	8	8
crystal violet	0.5	ND[c]	ND[c]	1	0.5	0.5	0.5
pyronin Y	0.5	ND[c]	ND[c]	18	0.5	0.5	0.5
TPA	10	ND[c]	ND[c]	50	10	10	10
TPP	10	ND[c]	ND[c]	>80	10	10	10

[a] MICs (micrograms per milliliter) for *E. coli* DH5α (A) and TG1Δ*acrAB* (B) Expressing the Indicated Recombinant EbrAB Constructs. [b] All DH5α strains exhibited the same level of resistance to methyl viologen, and all TG1Δ*acrAB* strains exhibited the same level of resistance to methyl viologen, cetalkonium chloride, CTAB, and cetylpyridinium chloride. Values represent the averages of triplicate determinations. [c] Not determined.

pended in 0.5 mL of the same buffer containing 300 mM imidazole. Radioactivity was measured by liquid scintillation counting. Membranes from cells containing the vector alone were used as a control. Competition binding assays were performed similarly, except that a 20- or 200-fold molar excess of nonradioactive drug was added.

To test the effect of pH on TPP binding, assay solutions were buffered with 50 mM MES (for pH 5.5−7.5) or 50 mM Tris-HCl (for pH 7.5−10). To test the effect of DCCD on binding, DCCD was added to the binding buffer (M63 at pH 7.5) at final concentrations of 0−1 mM.

Ethidium Efflux Assays. Transformed *E. coli* TG1Δ*acrAB* cells were cultured in LB containing the appropriate antibiotics. Cells were harvested in the late exponential phase of growth, washed twice with M63 minimal medium at pH 7.5, and resuspended in the same medium to an optical density (OD) of 0.6 at 600 nm. The cell suspension (25 mL) was incubated with 5 μM ethidium bromide while being shaken at 37 °C for 2 h, after which 25 mM glucose was added. At various time intervals, 1 mL aliquots were removed and immediately centrifuged at 14 000 rpm for 2 min, and 0.5 mL of the supernatant was mixed with 1 mL of M63 containing 0.1 mM fragmented calf thymus DNA prepared as described by Bolhuis et al. (*23*). The fluorescence of the assay mixture was measured using excitation and emission wavelengths of 500 and 580 nm, respectively.

In a second method of ethidium efflux measurement, the cell suspension was incubated with 5 μM ethidium bromide at 37 °C for 1 h. Ethidium efflux was again initiated by adding 25 mM glucose. The fluorescence in the cell suspension was monitored at 10 s time intervals.

RESULTS

B. subtilis EbrA and EbrB Combine To Form a Hetero-oligomeric SMR Efflux Transporter. Tsuchiya and co-workers first described the multidrug resistance activity of *B. subtilis* EbrAB (*17*). Similarly, we found that recombinant expression of both proteins in *E. coli* conferred resistance to a variety of cytotoxic drugs. Both polypeptides proved to be required for activity as expression of either *ebrA* or *ebrB* alone did not confer a drug resistant phenotype, although coexpression of *ebrA* and *ebrB* from a bicistronic plasmid construct (pBAD28-*ebrAB*) in *E. coli* DH5α cells conferred an at least 10-fold enhanced resistance to ethidium bromide and pyronin Y compared to control cells (Table 1A). We also observed a 2−8-fold increase in resistance to TPP, TPA, crystal violet, and CTAB. Similar results were obtained with a second *E. coli* strain, TG1Δ*acrAB*, from which the major drug efflux transporter, encoded by the *acrAB* genes, has been deleted. Indeed, we found that although the overall levels of drug resistance were lower for TG1Δ*acrAB* than for DH5α, the enhancement was significantly greater for certain drugs, e.g., 8-fold versus 4-fold for TPP (Table 1B; see also Figure 5B below for relative expression levels). Similar resistance profiles were observed when EbrA and EbrB were coexpressed from two separate plasmids (data not shown).

Table 2: Recombinant Expression of EbrAB Confers Multidrug Resistance in *B. subtilis*[a]

drug	vector control	EbrAB	EbrA^{E15A}B	EbrAB^{E14A}
ethidium bromide	4	>18	4	4
acriflavine	2	>11	2	2
pyronin Y	3	>20	4	3
TPA	10	50	10	10
TPP	10	50	10	10

[a] MICs (micrograms per milliliter) for *B. subtilis* M168 expressing recombinant EbrAB constructs. The experiments were conducted as described in the footnote of Table 1.

We also measured drug resistance associated with the EbrAB constructs in *B. subtilis*. Plasmid pDG148-Stu, harboring *ebrAB*, was introduced into *B. subtilis* M168, and drug resistance profiles were monitored. As shown in Table 2, expression of wild-type EbrAB conferred a 3−12-fold higher resistance to ethidium, acriflavine, pyronin Y, TPA, and TPP. These results indicate that the EbrAB drug efflux pump is functional in both *E. coli* and *B. subtilis* and that both EbrA and EbrB are required for the drug resistant phenotype.

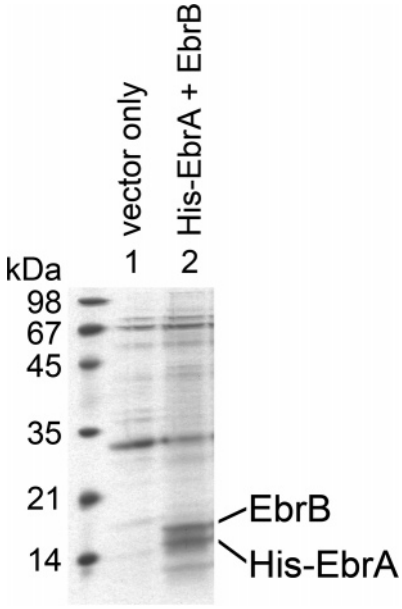

FIGURE 2: Untagged EbrB copurifies with His-tagged EbrA in a "pull down" experiment. Membranes from cells expressing both His-EbrA and untagged EbrB and cells harboring vector alone were extracted with the detergent DDM and applied to a nickel column. Shown is a Coomassie-stained gel of the bound fractions. The identities of the two indicated bands were verified by N-terminal sequencing to be His-EbrA and EbrB.

EbrA and EbrB Interact Directly. The data given above strongly suggest that the EbrA and EbrB polypeptides combine to form a heterooligomeric multidrug transporter. We therefore tested for a direct interaction between the two proteins, using a simple "pull-down" binding assay. For this experiment, EbrA was expressed with an N-terminal His tag while EbrB remained untagged. The two proteins were coexpressed in *E. coli* BL21(DE3) cells. Importantly, we noted that addition of the His tag did not have a significant deleterious effect on drug resistance in these cells (data not shown). Total membranes were prepared, then solubilized in the detergent *n*-dodecyl β-D-maltoside (DDM), and applied to a nickel column. The resin was washed, and the bound fractions were collected and analyzed using SDS−PAGE. As a negative control, and to identify nonspecific binders, a membrane sample prepared from cells carrying the vector alone was analyzed similarly. Results are shown in Figure 2. We found that two small proteins remained associated with the nickel resin when DDM-solubilized membranes containing both His-EbrA and untagged EbrB were applied to the column (lane 2). These bands are absent in the control sample (compare lane 1 with lane 2). N-Terminal Edman analysis showed that the lower band corresponded to His-EbrA (NH_2-GHHHH), while the upper band corresponded to untagged EbrB (NH_2-MRGLL). These results demonstrate a direct interaction between EbrA and EbrB. In addition, the band intensities indicate that the two constituent proteins are present at equimolar amounts in the EbrAB complex.

Biochemical Assay of EbrAB Multidrug Binding Activity. To examine the multidrug binding function of EbrAB in vitro, we performed drug binding experiments using radioactive [^{3}H]TPP as a substrate. Membranes were prepared from *E. coli* cells producing EbrA and EbrB. The membranes were incubated with 100 nM [^{3}H]TPP for 30 min and washed, and the amounts of bound TPP were measured. To assess

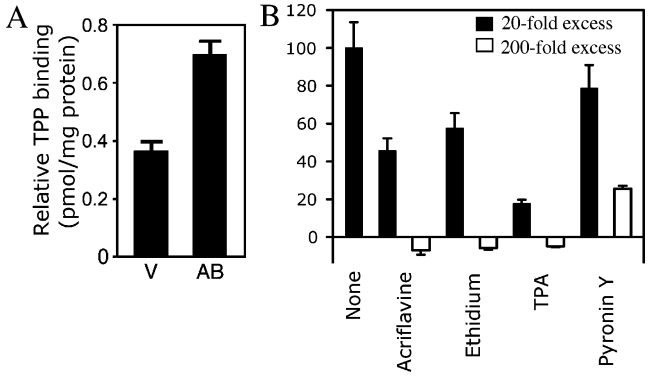

FIGURE 3: Drug binding activity of the EbrAB transporter in vitro. (A) V stands for vector and AB for the vector expressing the *ebrAB* genes. Membranes prepared from *E. coli* cells either producing or not producing EbrA and EbrB were incubated with 100 nM [^{3}H]-TPP for 30 min (specific activity of 30 mCi/μmol). After the membranes had been washed, the amount of bound [^{3}H]TPP was measured using a scintillation counter. Values represent the averages of triplicate measurements. (B) Competition binding experiments for binding of [^{3}H]TPP to EbrAB using the indicated aromatic cationic compounds. The concentrations of the inhibitor were either 20- or 200-fold greater than that of the [^{3}H]TPP as indicated. The 100% value refers to 0.62 pmol/mg of protein. These experiments were repeated several times with nearly identical results.

nonspecific binding, measurements were also performed on a membrane sample prepared from cells carrying the vector alone. As shown in Figure 3A, membranes containing EbrA and EbrB reproducibly showed a significantly increased level of [^{3}H]TPP binding over the vector control. This indicates that the EbrAB heterooligomer harbors drug binding activity, as expected for a drug transporter. However, it should be noted that our measurements should not be equated to binding per se since equilibrium binding has not been assessed. Thus, the mutation might decrease the association rate and/or increase the dissociation rate rather than blocking binding altogether. It is also important to note that binding of TPP to wild-type EbrAB probably reflects only a small fraction of the total protein that is present.

Competition binding experiments were then performed by adding an excess of other cationic hydrophobic compounds to the binding reaction mixture. These assays showed that acriflavine, ethidium bromide, pyronin Y, and TPA compete with TPP for EbrAB binding with varying efficiencies (Figure 3B). This confirms that EbrAB drug binding activity is polyspecific. Curiously, we found that there is no positive correlation between the extent of TPP binding inhibition and drug resistance profiles for these drugs. For example, pyronin Y and acriflavine inhibited TPP binding by ~20 and ~50%, respectively, when introduced at a 20-fold molar excess (Figure 3B). In the drug resistance assays, EbrAB conferred >30-fold resistance to pyronin Y and only ~5-fold resistance to ethidium (Table 1). These data suggest that transport efficiency is not directly related to substrate binding affinity and is likely influenced by other factors.

Conserved Glutamates in both EbrA and EbrB Are Required for Multidrug Resistance. Previous studies have shown that a membrane-embedded glutamate in TM helix 1 of *E. coli* EmrE is required for drug efflux activity (*7*, *8*). This glutamate is present in both EbrA and EbrB (Figure 1) and is absolutely conserved in all SMR family members (*7*, *8*). In analogy to EmrE, one or both glutamates in EbrAB would be expected to be required for drug binding and

Table 3: Effect of EbrA E15D and EbrB E14D Mutations on EbrAB Multidrug Resistance[a]

drug	vector control	EbrAB	EbrA^{E15D}B	EbrAB^{E14D}
ethidium bromide	50	500	85	100
acriflavine	25	140	30	35
crystal violet	4	8	5	5
benzalkonium chloride	8	13	10	8
BDTA	3	8	6	4
CTAB	2	4	3	3
cetylpyridinium chloride	3	5	3.5	3.5
pyronin Y	3	>120	8	10
TPA	200	1000	650	650
TPP	350	1500	450	500

[a] MICs (micrograms per milliliter) for *E. coli* DH5α expressing recombinant EbrAB constructs. The experiments were conducted in triplicate and the results were averaged.

transport. To test this hypothesis, we substituted the corresponding glutamates (E15 in EbrA and E14 in EbrB) with alanine, either singly or doubly, and tested cells harboring these mutants for drug resistance. Results showed that expression of either single mutants (EbrA^{E15A}B or EbrAB^{E14A}) or the double mutant (EbrA^{E15A}B^{E14A}) in *E. coli* (Table 1) or *B. subtilis* (Table 2) did not result in enhanced resistance to the drugs that were tested. Similar results were obtained when EbrA^{E15A} and EbrB or EbrA and EbrB^{E14A} were coexpressed from two separate plasmids. However, coexpression of EbrA^{E15A}B and EbrA or EbrAB^{E14A} and EbrB led to elevated drug resistance similar to that of EbrAB (data not shown). These results indicate that both glutamates are required for a functional drug resistance phenotype.

We also tested EbrAB constructs containing aspartate substitutions for the membrane-embedded glutamates, which are generally considered conservative mutations (EbrA E15D and EbrB E14D). As shown in Table 3, cells harboring these mutants had dramatically impaired drug resistance profiles, although we reproducibly observed some residual resistance, notably for ethidium bromide, TPP, and TPA. This is consistent with studies of EmrE, which showed that transporters with aspartate substitutions for the membrane-embedded glutamates in the dimer bound drug with near-wild type affinity but had impaired transport function (*7*, *8*). In addition, these experiments show that the presence of a negative charge in the middle of the first TM helices of EbrAB is insufficient for proper drug efflux function. This suggests that the glutamate side chains also fulfill structural or positional constraints in addition to the electrostatic requirement for the transport mechanism.

Both EbrA E15 and EbrB E14 Are Required for Drug Efflux, but Only EbrB E14 Appears To Be Required for TPP Binding. The deleterious effects of substitutions of the two conserved glutamates in EbrA and EbrB could be due to impaired drug binding or disruption of the subsequent transport process. We tested whether the two glutamates are important for drug transport, using ethidium efflux measurements in intact cells. Cells expressing EbrAB constructs were energy-starved by incubation in a culture medium lacking a carbon source and loaded with ethidium. After 2 h, glucose was added to provide energy. Ethidium efflux was then monitored by measuring the change in the concentration of extracellular ethidium. As shown in Figure 4, cells expressing EbrAB exhibited rapid ethidium efflux, with a substantial

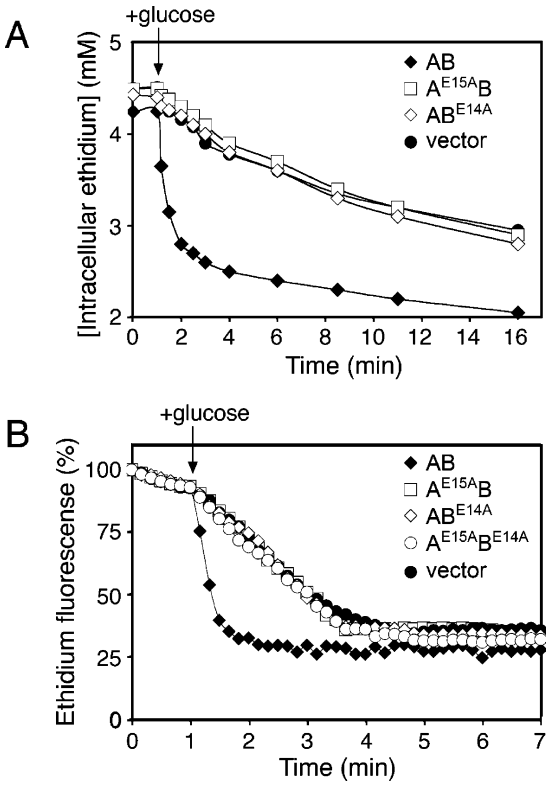

FIGURE 4: Conserved membrane-embedded glutamates in EbrA and EbrB are both required for ethidium efflux. Energy-starved *E. coli* cells expressing wild-type or mutant *ebrAB* constructs were loaded with ethidium bromide. At the indicated times (denoted with the arrows), glucose was added to energize the cells. (A) Aliquots were removed at the indicated time points and assayed for ethidium fluorescence at excitation and emission wavelengths of 500 and 580 nm, respectively. The intracellular ethidium concentration was calculated from the difference between the concentrations of total and external ethidium (*23*). (B) Intracellular ethidium fluorescence was directly monitored at 10 s intervals. Intensities after addition of ethidium and before addition of glucose were normalized to 100%: wild-type EbrAB (◆), EbrA^{E15A}B (□), EbrAB^{E14A} (◇), EbrA^{E15A}B^{E14A} (○), and vector control (●).

amount of the drug being extruded from the cells within 1 min [Figure 4A (◆)]. Conversely, cells expressing the mutant constructs (EbrA^{E15A}B or EbrAB^{E14A}) exhibited little efflux (□ and ◇, respectively), similar to control cells harboring the vector alone (●). When ethidium efflux was examined using a second method that measures the decrease in the total fluorescence, similar results were observed with rapid efflux for EbrAB cells but slow efflux for EbrA^{E15A}B or EbrAB^{E14A} cells (Figure 4B). As expected from these results, the EbrA^{E15A}B^{E14A} double mutant also failed to facilitate efficient ethidium efflux [Figure 4B (○)]. Thus, there is an excellent correlation between the drug resistance and ethidium transport, confirming the conclusion that both glutamate residues of TM helices 1 of EbrA and EbrB are essential for drug transport activity.

We next investigated whether both glutamates are important for drug binding, using the [^{3}H]TPP binding assay. As shown in Figure 5A, EbrA E15 is important for drug binding, because mutation of this residue reduced the level of binding to background levels (EbrA^{E15A}B). Surprisingly, EbrB E14 is dispensable for drug binding since membranes containing EbrAB^{E14A} bound TPP at levels similar to that of the wild type. As expected, the double mutant EbrA^{E15A}B^{E14A} failed to bind TPP. These results indicate a crucial role for EbrA

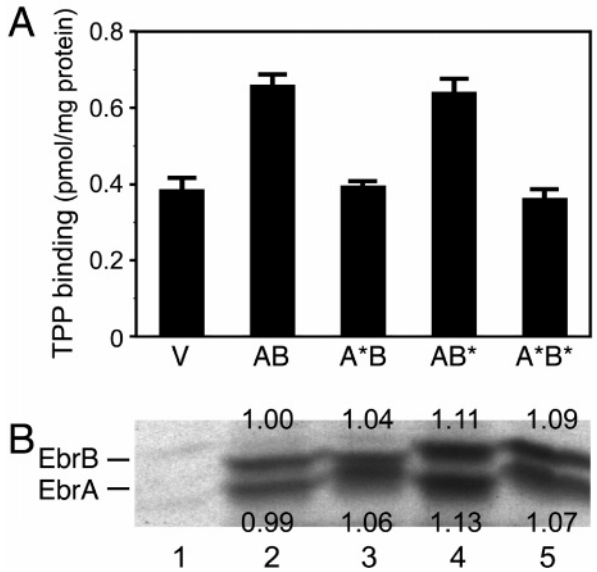

FIGURE 5: EbrA E15, but not EbrB E14, is required for TPP binding. (A) Membranes prepared from *E. coli* TG1Δ*acrAB* cells producing wild-type or mutant EbrAB proteins were incubated with 100 nM [^{3}H]TPP for 30 min. After the membranes had been washed, the amount of bound [^{3}H]TPP was measured using a scintillation counter. Values represent the averages of triplicate measurements. (B) Coomassie-stained gel showing relative expression levels of the different EbrAB constructs: lane 1, vector alone; lane 2, wild-type EbrAB; lane 3, EbrA^{E15A}B; lane 4, EbrAB^{E14A}; and lane 5, EbrA^{E15A}B^{E14A}. The intensities of bands were determined by densitometry with the EbrB band being 1.

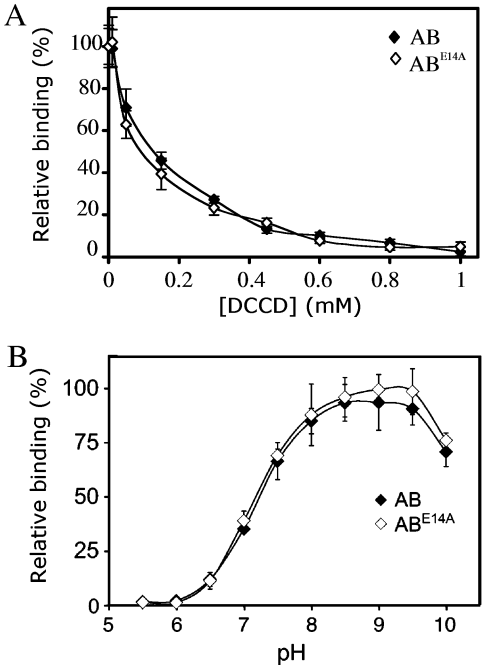

FIGURE 6: Effects of DCCD and pH on binding of TPP by the EbrAB transporter. (A) Effect of DCCD on TPP binding. Binding reactions at pH 7.5 were conducted in the presence of increasing concentrations of DCCD (from 0 to 1 mM). Specifically bound [^{3}H]TPP was calculated by subtracting the signal from that of a control sample containing membranes from cells harboring the vector alone. (B) Effect of pH on the TPP binding activity of EbrAB. Binding assays were performed in the pH range of 5.5−9.0. For both panels, error bars represent standard deviations following triplicate determinations: wild-type EbrAB (◆) and EbrAB^{E14A} (◇).

E15 in drug recognition. Importantly, the observed differences in binding affinity are not due to differences in expression levels, because the wild-type and mutant EbrAB constructs were expressed in similar amounts (Figure 5B). Note that the EbrA and EbrB mutants, identified by N-terminal sequence analysis (not His-tagged), have mobilities slightly different from those of the wild-type proteins. This is because the removal of a single glutamate significantly alters the overall charge in these small proteins. In summary, these experiments demonstrate that although the two EbrAB glutamates are both required for multidrug efflux activity, only EbrA E15 is required for drug binding. Thus, the two glutamates are not equivalent, and as a corollary, the two constituent proteins are likely to perform different functions during drug efflux.

TPP Binding Is Inhibited by Chemical Modification of the EbrA E15 Side Chain. One interpretation of the results given above is that the negatively charged EbrA E15 side chain participates in an electrostatic interaction with the TPP molecule. This is in line with its membrane-embedded location and the positively charged nature of EbrAB substrates. Modification of the side chain would be expected to disrupt this interaction. We therefore tested the effect of *N*,*N*′-dicyclohexylcarbodiimide (DCCD) on substrate binding. DCCD has been shown to specifically react with activated carboxylate groups embedded in hydrophobic environments (*24*), with the effect of neutralizing the negative charge and introducing steric constraints. Membranes from cells expressing EbrAB or EbrAB^{E14A} were incubated with [^{3}H]TPP and various concentrations of DCCD. As shown in Figure 6A, DCCD effectively blocked TPP binding in this assay, with higher concentrations of DCCD leading to lower levels of binding. In a separate experiment, the EbrAB-containing membranes were treated with DCCD prior to the binding assay. As shown in Figure S1 of the Supporting Information, DCCD pretreatment further inhibited the binding of TPP to EbrAB, but this difference was only slight. These results confirm the conclusion that EbrA E15 is required for binding positively charged drug substrates.

Binding of TPP to EbrAB Is Dependent on the Protonation State of EbrA E15. The central role for EbrA E15 also suggests that EbrAB should display pH-dependent drug binding. Indeed, this is the case; as shown in Figure 6B, EbrAB bound TPP only at pH >6.5. The extent of TPP binding increased with an increase in pH and reached a maximum near pH 8. The EbrAB^{E14A} mutant also displayed a pH binding profile that is essentially the same as that of the wild-type transporter. The pH dependence of EbrAB TPP binding activity is therefore likely a reflection of the protonation−deprotonation states of EbrA E15 and not EbrB E14. Moreover, these results indicate that protonation of EbrA E15 inhibits drug binding, consistent with the idea that the negatively charged EbrA E15 residue participates in an electrostatic contact with the positively charged TPP substrate. This is in line with biochemical studies of EmrE, which showed that the membrane-embedded glutamyl residue in the first transmembrane helix directly interacts with the bound drug (*7*, *8*).

DISCUSSION

The *ebrA* and *ebrB* genes of *B. subtilis* encode two small proteins that are members of the SMR family of multidrug

efflux transporters (*2*, *4*, *17*). In contrast to the *E. coli* EmrE protein, EbrA and EbrB confer multidrug resistance to bacterial cells only when coexpressed (ref *17* and this study). These results indicate that the two proteins combine to form a functional MDR transporter. In support of this hypothesis, we found that EbrA and EbrB form a purifiable heterooligomer. The oligomerization property of EbrAB is consistent with biochemical studies of EmrE that show this protein to function as a homooligomer (*9*, *13*, *14*). In particular, EmrE has been shown to be a dimer when solubilized in detergent and binds the drug with a 2:1 protein:drug stoichiometry (*9*, *11*). Recent structural studies of EmrE have also shown that the primary structural unit of this protein is an asymmetric homodimer (*12*, *15*). Taken together, these studies indicate that SMR transporters are composed of dimeric units, which can be assembled from different subunits as in the case of EbrAB, or identical subunits as in EmrE.

Interestingly, the structural analyses of the EmrE dimer suggested that the two subunits are antiparallel and are therefore inserted in opposite orientations in the cell membrane (*12*, *15*). This is consistent with the differing distributions of charged residues in EbrA and EbrB (Figure 1). In EbrA, six of seven lysines are located in the first and third extramembrane loops. In contrast, although EbrB loops do not exhibit a marked charge bias, both the N- and C-termini contain positively charged residues. According to the "positive-inside rule" (*25*), EbrA should have an N_{out}/C_{out} and EbrB an N_{in}/C_{in} conformation in the cell membrane. Another pair of SMR homologues, the YdgE and YdgF proteins of *E. coli*, have been experimentally determined to have opposite orientations in the membrane (*26*). Like EbrAB, YdgE and YdgF catalyze drug efflux only when coexpressed (*27*, *28*). Thus, the unifying structural model for SMR transporters is likely to be an antiparallel dimer.

Like all members of the SMR family, EbrA and EbrB each contain an absolutely conserved glutamate (E15 in EbrA and E14 in EbrB), located in the middle of TM helix 1. We found that as in the case of EmrE, these glutamates are required for proper function, since cells harboring substitutions for either of these glutamates have impaired drug resistance. Both glutamates are required for drug transport as demonstrated by ethidium efflux measurements. However, our drug binding studies demonstrated that while EbrA E15 is required for substrate binding, EbrB E14 is dispensable. An important conclusion from this study is that the two glutamates perform different functions in the drug transport mechanism, and by extension, the EbrA and EbrB subunits in the putative EbrAB heterodimer are not equivalent. This is consistent with the asymmetric nature of the EmrE homodimer. Moreover, EbrB contains a highly charged stretch of amino acids (of unknown function) at its C-terminus that is absent in EbrA (Figure 1). This is a common feature among paired SMR homologues (*27*).

Nonequivalence of the SMR subunits is presaged by studies of the major facilitator superfamily of transporters (*29*). The prototypical MFS transporter, LacY, transports lactose and protons in a symport mechanism (*29*, *30*). LacY is composed of two internal repeat units that have the same tertiary structure. The two repeat units are nonequivalent because one subunit confers most of the energetic determinants for substrate recognition and specificity while the other subunit provides the bulk of the proton translocation pathway (*30*). Our results raise the intriguing possibility that a similar functional delineation of subunits may exist in SMR transporters, although this requires further experimental confirmation.

CONCLUSIONS

In summary, we showed that the *B. subtilis* EbrA and EbrB proteins associate into a heterooligomeric SMR transporter. Although the two proteins cooperate in drug transport, EbrA and EbrB are likely to play different mechanistic roles in the process. Further studies are now required to clarify these roles. Because the two components can be manipulated independently, the EbrAB transporter is an ideal model system for understanding the mechanistic implications of structural and functional nonequivalence of the two subunits in SMR transporters.

SUPPORTING INFORMATION AVAILABLE

Effect of DCCD pretreatment on binding of TPP to EbrAB (Figure S1) and primers used in this study (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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