

# Two-component bacterial multidrug transporter, EbrAB: Mutations making each component solely functional

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## Abstract

EbrAB in *Bacillus subtilis* belongs to a novel small multidrug resistance (SMR) family of multidrug efflux pumps. EmrE in *Escherichia coli*, a representative of SMR, functions as a homo-oligomer in the membrane. On the other hand, EbrAB requires a hetero-oligomeric configuration consisting of two polypeptides, EbrA and EbrB. Although both polypeptides have a high sequence similarity, expression of either single polypeptide does not confer the multidrug-resistance. We performed mutation studies on EbrA and B to determine why EbrAB requires the hetero-oligomerization. Mutants of EbrA and B lacking both the hydrophilic loops and the C-terminus regions conferred the multidrug-resistance solely by each protein. This suggests that the hydrophilic loops and the C-terminus regions constrain them to their respective conformations upon the formation of the functional hetero-oligomer.

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## 1. Introduction

The small multidrug resistance (SMR) family consists of small membrane proteins that extrude various toxicants from cells by utilizing the proton gradient across the membrane [1]. SMR proteins are widespread in bacteria and archaeobacteria including pathogenic organisms and have several unique characteristics [2]: they are only 100–120 amino acids long, forming four tentative transmembrane  $\alpha$ -helices, and have very high hydrophobicity, making them soluble in an organic solvent [1,3]. Many aspects of SMR proteins have been clarified so far through studies on EmrE, the SMR representative, in *E. coli*. It has been shown that EmrE functions in an oligomeric state [4–8]. This is consistent with the results of electron and X-ray crystallographic studies [9–12]. Although structures proposed by these studies are different, both reveal the dimer as a repetitive unit in the crystal, and of interest, the dimer shares a unique feature that EmrEs form a homo-dimer with an asymmetric structure.

EbrAB and YkkCD in *B. subtilis* belong to a novel SMR family [13,14]. These proteins are encoded from a gene pair (*ebrA* and *B* or *ykkC* and *D*) in distinct operons, and expression of a sole member cannot confer the multidrug-resistance. Both members in the operon (EbrAB or YkkCD) are necessary for the multidrug efflux pump. This implies that EbrA and B, or YkkC and D, function as hetero-dimers. Here, a question arises: why does EbrAB require the hetero-oligomerization although EmrE does not? There must be factors that determine the respective roles and/or conformations of EbrA and B (or YkkC and D). To clarify these factors, we compared the amino acid sequences of the monomers of SMR pairs (EbrA and B types) and other SMR homologues (EmrE type) and then focused on the hydrophobicity differences in the loop and C-terminus regions between these three types.

Three operons of *B. subtilis* and one operon of *E. coli* encode the respective pairs of component proteins constituting the SMR family [14]. In these SMR pairs, one member is commonly shorter (105–109 residues), while the other is longer (109–121 residues) due to the hydrophilic C-terminus extension. Moreover, the shorter member has hydrophilic regions in tentative loops, while the longer member and other SMR homologues do

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not. We thus focus on these hydrophilic regions and examine the roles of their hydrophobicities in the efflux function by using a mutation approach. The resulting EbrA and B mutants are functional solely by the expression of each single member.

## 2. Materials and methods

### 2.1. Construction of expression plasmids

At first, we constructed three plasmids for the respective and simultaneous expressions of EbrA and B. The *B. subtilis* genes *ebrA*, and *ebrB* and the gene pair *ebrAB* were amplified by polymerase chain reaction (PCR) from *B. subtilis* ATCC 6051. The primers were designed based on the sequences in the GenBank database (accession number, NC\_000964). The *ebrA* sequence in the database includes the *NdeI* site near the start codon, so the *ebrA* sense primer was designed to substitute the *NdeI* site with a synonymous sequence. By PCR, additional *NdeI* and *SalI* sites were introduced at the ends of the target genes. The primers used were as follows: for *ebrA*, 5'-TTATGAATTCATATGTTGATAGGATATA-TATTCCTCACGATTGCCATTGTTCCGAATCGATAGGAG-3' (sense) and 5'-TCTGGTCGACTTACGGCCAATTAAGTAACAC-3' (antisense); for *ebrB*,

5'-TTATCATATGAGAGGATTGCTTTATTTG-3' (sense) and 5'-TCTGGTCGACTCACTCACAGGCCGTCTG-3' (antisense); and for *ebrAB*, the *ebrA* sense and *ebrB* antisense primers. The PCR products were restricted, and the resultant DNA fragments were ligated to the *NdeI* and *SalI* sites of pFLAG-CTC (Sigma). The DNA sequences were determined using a standard procedure (377 DNA sequencer, Applied Biosystems). Except for the primer regions, the obtained sequences were the same as those in the GenBank database. The plasmids constructed for the respective expressions of EbrA and B were used as templates for the following mutations.

The mutations of EbrA on loop 1–2 or 3–4 were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and sets of two overlapping primers containing the desired mutations. These mutations represent the residue replacement in the loop regions: for loop 1–2, KKWK at position 29–32 was replaced with TQAW; for loop 3–4, KWFKEK at position 78–83 was replaced with LLFGET. For mutation in both loops, the same procedure for loop 3–4 mutation was repeated relative to the plasmid of EbrA mutated in loop 1–2. The C-terminus mutants of EbrA, EbrB, and EbrA mutated in both loops 1–2 and 3–4 were obtained by PCR and followed by insertion of the PCR products into the pFLAG-CTC plasmid, where the antisense primers for PCR contain the desired mutations for the C-terminus regions. These mutations represent that for EbrB, the truncation after the histidine residue at position 107, and that for EbrA and the EbrA mutant in the loop regions, the

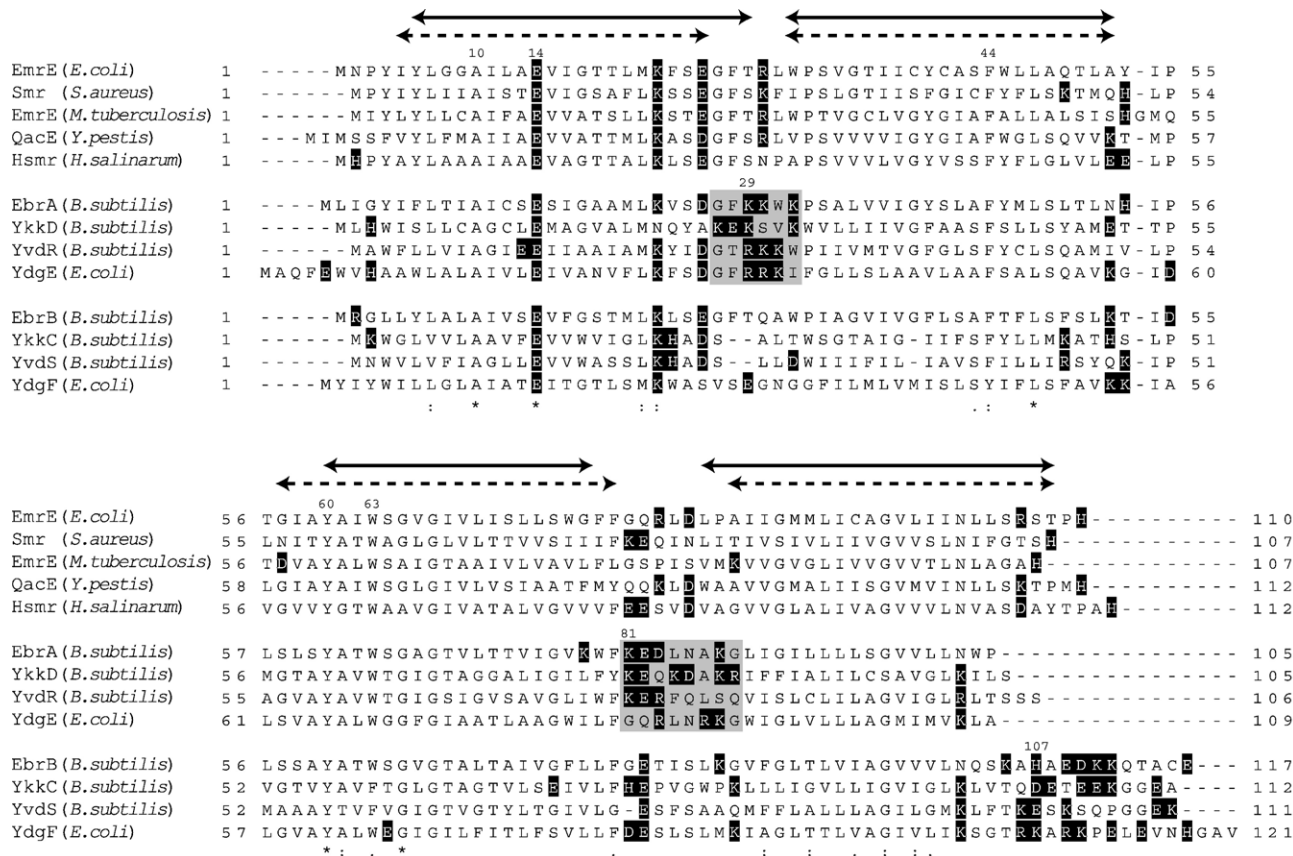


Fig. 1. Multiple alignments of amino acid sequences of SMR homologues. These homologues are classified into three groups: top, homologues encoded solely in the respective operons; middle and bottom, short and long components encoded in one operon as gene pairs. Solid and broken line arrows indicate the  $\alpha$ -helical regions of the asymmetric dimer in the X-ray crystal structure of EmrE (PDB entry 2F2M) [12]. The differences in the regions indicated by solid and broken lines originate from the asymmetric orientation of the monomer in the dimeric structure. The numbers above each group represent the amino acid numbers of EmrE of *E. coli*, EbrA, and EbrB of *B. subtilis*, and acidic and basic amino acid residues are marked in black. For the middle group, the loop regions having closely packed charged residues are marked by gray boxes. Strictly conserved residues are marked with an asterisk (\*). Conserved and semiconserved substitutions are represented by (:) and (.), respectively. Sequences were obtained from the National Center for Biotechnology Information Protein database, accession numbers: NP\_415075, EmrE of *E. coli*; NP\_863640, Smr of *S. aureus*; NP\_337671, EmrE of *M. tuberculosis*; NP\_405870, QacE of *Y. pestis*; NP\_389612, EbrA of *B. subtilis*; NP\_391330, YvdR of *B. subtilis*; NP\_416116, YdgE of *E. coli*; NP\_389611, EbrB of *B. subtilis*; NP\_389192, YkkC of *B. subtilis*; NP\_391329, YvdS of *B. subtilis*; NP\_416117, YdgF of *E. coli*. Sequence for Hsmr of *H. salinarum* was obtained from HaloLex (*H. salinarum* database <http://www.halolex.mpg.de>) code OE3652F. Alignment was performed using ClustalW at <http://clustalw.genome.jp/>.

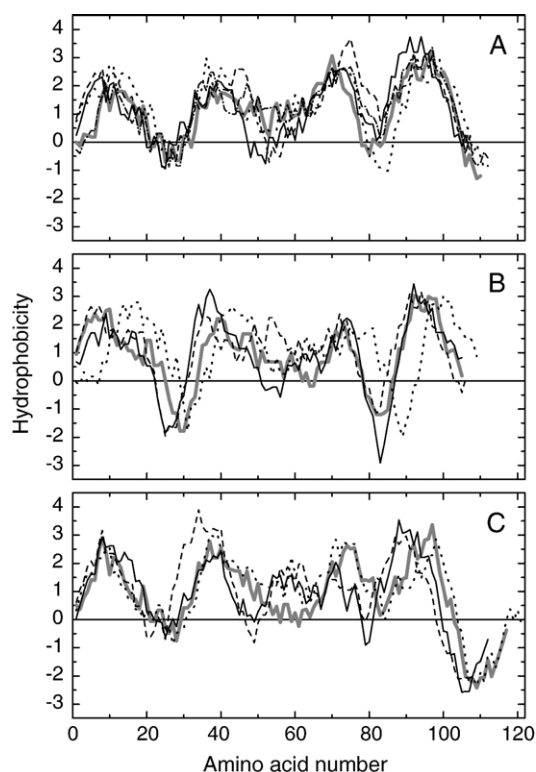


Fig. 2. Hydrophobicity plots of SMR homologues. Panels of A, B, and C correspond to three groups in Fig. 1, respectively. A: ■, EmrE (*E. coli*); □, QacC (*S. aureus*); - - -, EmrE (*M. tuberculosis*); ···, QacE (*Y. pestis*); -·-·, Hsmr (*H. salinarum*); B: ■, EbrA (*B. subtilis*); □, YkkD (*B. subtilis*); - - -, YvdR (*B. subtilis*); ···, YdgE (*E. coli*); C: ■, EbrB (*B. subtilis*); □, YkkC (*B. subtilis*); - - -, YvdS (*B. subtilis*); ···, YdgF (*E. coli*). The top group has high hydrophobicity over the entire proteins, while the middle and bottom groups have hydrophilic regions in loops 1–2 and 3–4 for the middle group and in the C-terminus regions for the bottom group. Hydrophobicities were calculated using the Kyte and Doolittle scale [21] by the window size of 9. To calculate hydrophobicities of the N- and C-terminus regions, we assumed that four amino acid residues having a hydrophobicity index of 0 connect with the protein terminuses.

addition of three residues of KAH to their C-terminuses. The plasmid of EbrA mutated in the loops was used as a PCR template for its C-terminus mutation. The mutations introduced into the plasmids were confirmed by DNA sequencing to ensure that no other mutations occurred.

## 2.2. Ethidium efflux assay in *E. coli* cells

Ethidium is a representative substrate of SMR proteins. We measured the ethidium efflux activities of cells harboring various types of plasmids using the method of Masaoka et al. [13] with a few modifications. The pFLAG-CTC plasmid with no insert was used for a negative control. The expression host was *E. coli* strain AS1, which lacks AcrA, a subunit of a major multidrug efflux transporter. Cells were grown at 37 °C in LB broth [15] supplemented with 100 µg/ml of ampicillin. The precultures were prepared by growing the cells until the optical density at 660 nm was between 0.5 and 0.7 and were then stored at 4 °C. The next day, the main cultures, supplemented with 10 µM of IPTG, were inoculated with 0.5% preculture. The IPTG concentration we used (10 µM) led to strong efflux activity in the cell harboring the EbrAB plasmid without significant hindering of the growth. After 6-h incubation, the optical density at 660 nm was 0.4 to 0.9. The cells were then harvested by centrifugation.

In the following procedure, the minimum medium derived from M9 [15] was used as a basal medium, which lacked glucose and was supplemented with 0.1 mM of CaCl<sub>2</sub>, 0.2 mM of MgSO<sub>4</sub> and 40 µg/ml of chloramphenicol. The cell pellets were washed twice with the basal medium supplemented with 40 µM of

carbonylcyanide *m*-chlorophenylhydrazone (medium A). They were then resuspended in the medium A supplemented with 5 µM of ethidium bromide (medium B) at the optical density of 0.5 at 660 nm. The cell suspensions were shaken at 37 °C to deplete the energy of the cells and to load them with ethidium. After 1 h, they were harvested by centrifugation and washed twice with the basal medium supplemented with 5 µM of ethidium bromide (medium C). Finally, the cells were resuspended in the medium C at the optical density of 0.25 at 660 nm. The time-dependent decrease in the ethidium remaining in the cells was measured with a Hitachi F-2000 fluorometer at 37 °C. After 5-min preincubation, glucose was added (final concentration of 0.5% W/V) to the suspension to energize the cells for the ethidium efflux. The excitation and emission wavelengths were 545 and 610 nm, respectively.

## 2.3. Drug susceptibility test

Drug assay plates were prepared with LB agar containing 50 µg/ml of ampicillin, 10 µM IPTG, and various concentrations of drugs. *E. coli* AS1 cells harboring various plasmids were grown in the above LB medium lacking drugs at 37 °C until the optical density at 660 nm was between 0.2 and 0.4. These cultures were diluted in the same medium at the optical density of 0.005 at 660 nm, and 5 µl of the samples were plated on the drug assay plates. These plates were incubated at 37 °C for 11 h, and thereafter the growth was evaluated.

## 2.4. SDS-PAGE analysis of *E. coli* membrane fractions

Cells grown using the same procedure for ethidium efflux assay were harvested and washed twice with a buffer solution containing 400 mM of NaCl and 50 mM of sodium phosphate (pH 7.0). The cells were resuspended in the same buffer and disrupted by sonication. The membrane fraction was collected by ultracentrifugation at 106,000×g for 1.5 h at 4 °C and analyzed by tricine-SDS-PAGE with 4% acrylamide stacking and 16.5% acrylamide separating gels.

## 3. Results and discussion

Four pairs of SMR homologues encoded in distinct operons are reported in *B. subtilis* (EbrAB, YkkCD, and YvdRS) and *E. coli* (YdgEF) [14]. Because the pair consists of two components, we call the short and long members of these SMR pairs EbrA and EbrB homologues, respectively. In addition, the SMR proteins solely encoded in the respective operon are called EmrE homologues.

Fig. 1 shows the amino acid sequence alignments of these homologues. The arrows indicate the tentative  $\alpha$ -helical regions from the X-ray crystal structure of EmrE [12]. The acidic and basic amino acid residues are marked in black. Five sequences in the top group are those of EmrE and its homologues from human pathogens and archaeal bacteria. The middle and bottom groups are the EbrA and EbrB homologues, respectively. The most distinct difference among these groups is seen in the C-terminus region. The EbrB homologues have many charged residues at their prolonged C-terminus, while the EmrE homologues have only one or two charged residues in this region. The EbrA homologues terminate in the middle of the last  $\alpha$ -helical domain of EmrE, so they lack such a hydrophilic C-terminal region. Except for this region, the charged residues are similarly distributed in the EmrE and EbrB homologues. The loop regions of the EbrA homologues differ from those of the other homologues. In the regions of loops 1–2 and 3–4, which are loops connecting helices 1 and 2 and helices 3 and 4, the EbrA homologues have closely packed charged residues.

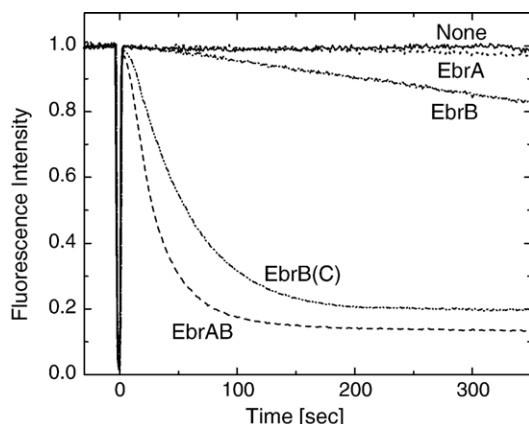


Fig. 3. Time-dependent changes in intracellular ethidium concentrations. *E. coli* strain AS1 cells harboring various expression plasmids were energy-starved and loaded with ethidium. Ethidium remaining in the cells was monitored continuously by measuring the fluorescence of ethidium. At time 0, glucose (final concentration of 0.5% W/V) was added to the suspension to energize the cells. Large downward deflections at time 0 are artifacts due to the glucose addition. Expression plasmids harbored in particular cells are denoted in the figure, where “None” means pFLAG-CTC having no insert, and EbrB(C) represents the EbrB mutant lacking the region from Ala108 to its C-terminus. It is noteworthy that EbrB(C) confers high efflux activity on the cells.

To evaluate the hydrophobicity differences in the loop and C-terminus regions of these three groups, we created their hydrophobicity plots. As shown in Fig. 2A, the EmrE homologues have high hydrophobicity over the entire protein, and the four transmembrane regions are, to some extent, clearly indicated. The EbrB homologues (Fig. 2C) have similar hydrophobicity profiles, but there are distinct differences from the EmrE homologues in the C-terminus regions. The EbrB homologues have a prolonged hydrophilic C-terminus, while the EbrA homologues (Fig. 2B) lack the hydrophilic C-terminus and have two hydrophilic regions in loops 1–2 and 3–4.

Mutation studies on EmrE of *E. coli* have identified essential amino acid residues: the representatives are Ala 10, Glu 14, Phe 44, Tyr 60, and Trp 63 [6,16–20]. These residues are essential for substrate binding, coupling between proton and substrate flux, and stable expression of the protein itself. Most of these residues are conserved in the three groups shown in Fig. 1. Although an exception is seen in Tyr of YdgF corresponding to Phe 44 of EmrE, the other EbrB homologues fully conserve the Phe residue. Another exception is seen in Phe of YkkC and YvdS corresponding to Trp 63 of EmrE. However, two other EbrB homologues still conserve the Trp residues. These facts contradict the idea that EbrA and B homologues complementarily provide amino acid residues indispensable for the multidrug efflux function. Thus, the reason that the efflux function requires the complex formation of EbrA and B homologues may originate from the asymmetrical structure in the hetero-dimer. Upon the formation of the functional complex, in other words, the respective structure of EbrA and B homologues may be complementary. The differences in hydrophobicities of the loops and the C-terminus regions between the EbrA and B homologues may determine their respective structures. Based on this idea, we performed the following mutation study of EbrA and B.

The distinct difference in hydrophobicity between the EmrE and EbrB homologues lies in the prolonged hydrophilic C-terminus regions of the EbrB homologues. Deletion of these regions may make EbrB homologues solely functional. Thus, we constructed the C-terminus-truncated EbrB and examined its efflux activity for ethidium, a representative substrate for SMR proteins. Four expression vectors were constructed for the simultaneous expression of EbrA and B and the sole expression of EbrA or EbrB or the EbrB mutant lacking the region from Ala at position 108 to the C-terminus.

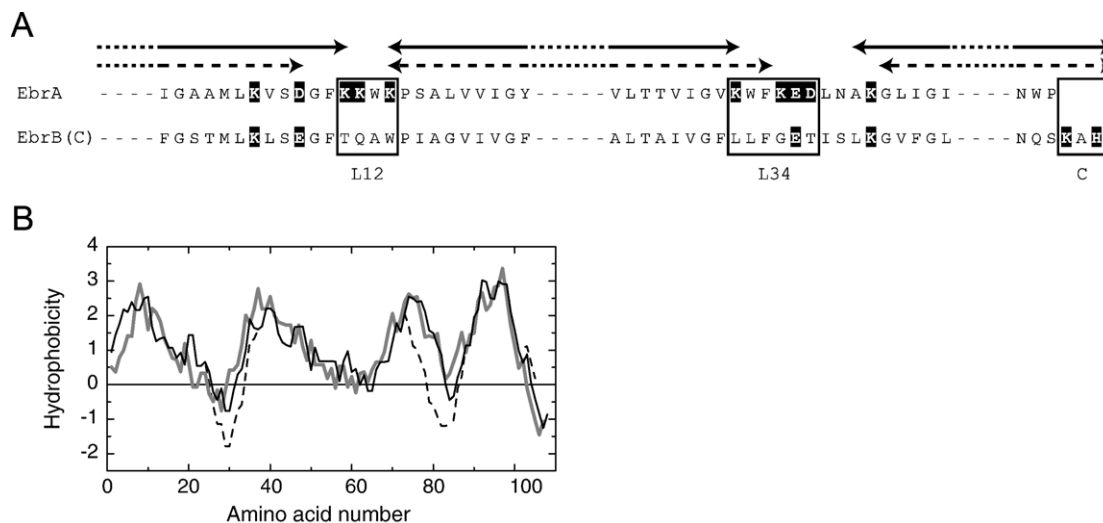


Fig. 4. Mutated regions and hydrophobicity plots of EbrA mutants. (A) Comparison of amino acid sequences between EbrA and EbrB(C). Target regions for mutations are marked with boxes. The sequences of EbrA within the boxes were replaced by the corresponding sequences of EbrB(C). B: Hydrophobicity plots of EbrA (---), EbrB(C) (—), and EbrA mutant (Δ) assuming identical sequences in its three regions, loops 1–2 and 3–4 and the C-terminus, as EbrB(C). Due to mutations in all three regions, the hydrophobicity of EbrA closely resembles that of EbrB(C). Hydrophobicities were calculated by the same method for Fig. 2.



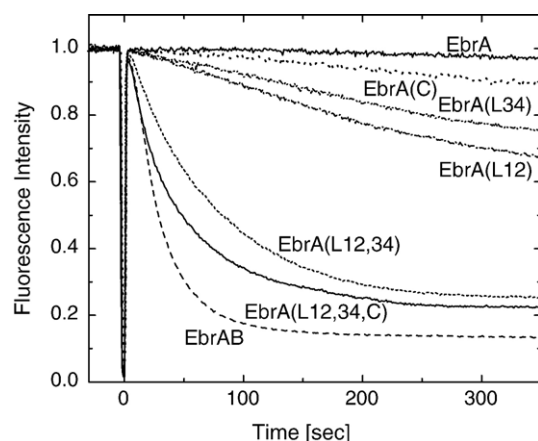


Fig. 5. Time-dependent changes in intracellular ethidium concentrations. *E. coli* cells harboring expression plasmids of EbrA mutants are used. Measurements were performed the same as in Fig. 3. In the respective EbrA mutants, the regions identical with those of EbrB(C) are the C-terminus for EbrA(C), loop 1–2 for EbrA(L12), loop 3–4 for EbrA(L34), loops 1–2 and 3–4 for EbrA(L12, 34), and loops 1–2 and 3–4 and the C-terminus for EbrA(L12,34,C). Efflux activities of the cells were not increased significantly by mutations in single regions, i.e., the C-terminus, loop 1–2 or loop 3–4. However, it is noteworthy that EbrA becomes able to confer high efflux activities on the cells by the mutations in the two loops or all three regions (EbrA(L12,34) and EbrA(L12,34,C)).

Fig. 3 shows time-dependent changes in ethidium remaining in *E. coli* cells harboring these expression vectors. The decrease in the fluorescence intensity corresponds to the ethidium efflux from the cells. The cells harboring the EbrA plasmid did not show efflux activity. These harboring the EbrB plasmid showed a slight activity, but it was very small compared with that of cells expressing the complete pair of EbrA and B (EbrAB). It is noteworthy that the cells expressing the C-terminus truncated EbrB (EbrB(C)) shows high efflux activity. The mutation supplied a sole protein with the efflux activity.

We also constructed five EbrA mutants. The target regions for the mutations are shown in Fig. 4A. We constructed EbrA mutants having loop and C-terminus regions identical with

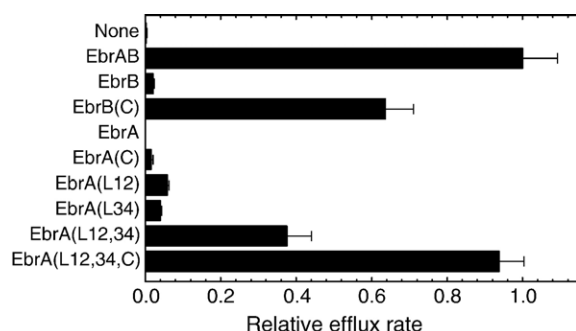


Fig. 6. Ethidium efflux rate by cells harboring various expression plasmids. Initial slopes of efflux curves shown in Figs. 3 and 5 were taken as indexes for efflux activities of the cells, and relative efflux rates are plotted. Each bar represents the mean  $\pm$  S.D. for between three and six measurements. With only the C-terminus truncation, EbrB was able to confer high efflux activity on the cells. For EbrA, on the other hand, mutations in both loops 1–2 and 3–4 conferred high efflux activity. Improvement in activity came from additional mutation in the C-terminus region.

Table 1

MICs for *E. coli* AS1 cells harboring various expression plasmids

Plasmid for	MIC ( $\mu$ M)		
	EtBr <sup>a</sup>	TPPCL <sup>b</sup>	Acridavine
None <sup>c</sup>	10	10	5
EbrA	10	10	5
EbrB	10	20	5
EbrAB	200	500	100
EbrB(C) <sup>d</sup>	50	200	10
EbrA(L12,34,C) <sup>e</sup>	50	100	10

<sup>a</sup> Ethidium bromide.

<sup>b</sup> Tetraphenylphosphonium chloride.

<sup>c</sup> pFLAG-CTC plasmid having no insert.

<sup>d</sup> EbrB mutant lacking the region from Ala108 to its C-terminus.

<sup>e</sup> EbrA mutant having sequences on loops 1–2 and 3–4 and the C-terminus regions identical with those of EbrB(C).

those of EbrB(C). Fig. 4B shows the hydrophobicities for EbrA, EbrB(C), and the EbrA mutant in three regions of loops 1–2 and 3–4 and the C-terminus. With mutation in all three regions, the hydrophobicity is close to that of EbrB(C) over the entire protein.

The ethidium efflux activities of the cells harboring EbrA mutant plasmids are shown in Fig. 5. The mutations in single regions, i.e., the C-terminus (EbrA(C)), loop 3–4 (EbrA(L34)), and loop 1–2 (EbrA(L12)), did not confer strong efflux activity on the cells. However, simultaneous mutations in two loops or all three regions (EbrA(L12,34) and EbrA(L12,34,C)) remarkably increased the efflux activity of the cells.

In Fig. 6, the relative efflux activities of cells harboring various plasmids are shown that were deduced from the initial slopes of the curves in Figs. 3 and 5. For EbrB, a single mutation of the C-terminus truncation dramatically increases the efflux activity. For EbrA, mutations at a single site were not adequate. Mutations in loops 1–2 and 3–4 were necessary to confer high efflux activity on the cells. Additional mutation at the C-terminus further improved the activity.

We also tested the abilities for cellular resistance to toxic compounds by the cells harboring various plasmids. Table 1 shows the MICs of three representative drugs. Mutant cells of EbrB(C) and EbrA(L12,34,C) led to clear increases in the MICs compared with the control cell (“None”) and those expressing EbrA and EbrB alone. However, the abilities of these mutants did not attain the level expected from the ethidium efflux assay.

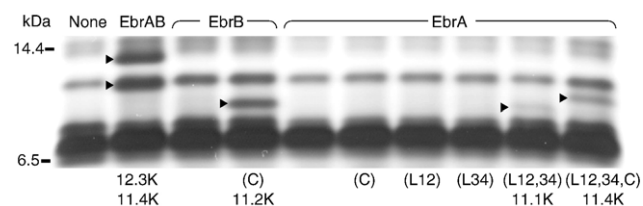


Fig. 7. SDS-PAGE analysis of EbrAB and the mutants. Protein bands were visualized by silver staining. Bands were detected for EbrAB, EbrB(C), EbrA(L12,34), EbrA(L12,34,C). Their molecular weights calculated from amino acid sequences are shown at the bottom. The bands similar to the EbrA band, the lower band of “EbrAB” lane, appear in all samples including “None”. These bands, which do not originate in the expression plasmids, seem to have a slightly lower mobility than the EbrA band.

As shown in Fig. 6, the relative activities for ethidium efflux of EbrB(C) and EbrA(L12,34,C) were 0.67 and 0.94, respectively. On the other hand, the MICs for ethidium were 200  $\mu$ M for EbrAB and 50  $\mu$ M for both EbrB(C) and EbrA(L12,34,C). Thus, the mutations enable the cells to extrude toxic drugs by expression of a single component of EbrA or EbrB, but these mutants may still be inferior to the native EbrAB especially in the ability to confer drug resistance.

Jack et al. reported that YkkCD, another EbrAB-type SMR of *B. subtilis*, confers resistance to a broader range of drugs than the homo-oligomeric SMR [14]. Thus, the hetero-oligomerization may bring about a certain advantage for the drug resistance phenotype. The elucidations of the functional and mechanical differences between homo- and hetero-oligomeric SMRs should be an attractive subject for a future study.

In the present work, we utilized the efflux rate of ethidium from the cells or MIC as the indicator for the protein activity of substrate efflux function. However, these parameters also depend on the amount of protein expressed in the cell. The CBB staining of the SDS-PAGE gels of the membrane fractions did not clarify the bands corresponding to the target proteins. However, we found that those bands could be enhanced by silver staining. Fig. 7 shows images of the silver-stained SDS-PAGE gel. Two bands, one corresponding to EbrA and one to EbrB, were observed for a sample that originated from cells harboring the EbrAB plasmid. Bands for EbrB(C) and EbrA(L12,34,C) were also observed for samples from their corresponding cells. Moreover, a weak band of EbrA(L12,34) was also observed. For other samples, however, we could not detect distinct bands. Thus, the expressions of EbrAB and those mutants were detected only in the cells having strong activities for the ethidium efflux. Why does the simultaneous expression of EbrA and B result in the stable expression of both proteins while the expressions of the single components do not? The formation of the EbrAB complex is considered to be indispensable for their stable expression. The formation of this complex appears to be essential not only for their function but also for assuming a stable structure within the membrane. Mutations of EbrA or EbrB that confer the efflux activities by the single components may induce their stable expressions, possibly due to the oligomerization of the homo-component.

The mutations conferring the activities by only one component were only the C-terminus truncation for EbrB and the modifications of the hydrophobicities at two or three regions for EbrA. These results suggest that native EbrA and B are fully equipped with the amino acid residues essential for drug efflux function. In other words, EbrA and B do not seem to provide complementarily the essential residues for the formation of the functional EbrAB. Their respective conformations may be complementary for the functional EbrAB complex. As shown by the crystal structures of EmrE [9–12], it assumes asymmetric structures in the homo-dimer. Moreover, Pornillos et al. recently reported that the two subunits in the dimer assume antiparallel configurations. This asymmetric structure may originate from the high hydrophobicities on the entire EmrE molecule. For

EbrA and B, on the other hand, the presence of their hydrophilic regions may restrict them to their respective conformations. Mutations that remove their hydrophilic regions probably enable them to solely assume antiparallel configurations as does EmrE.

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