

## A Two-Component Multidrug Efflux Pump, EbrAB, in *Bacillus subtilis*

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**Genes (*ebrAB*) responsible for ethidium resistance were cloned from chromosomal DNA of *Bacillus subtilis* ATCC 9372. The recombinant plasmid produced elevated resistance against ethidium bromide, acriflavine, pyronine Y, and safranin O not only in *Escherichia coli* but also in *B. subtilis*. It also caused an elevated energy-dependent efflux of ethidium in *E. coli*. EbrA and EbrB showed high sequence similarity with members of the small multidrug resistance (SMR) family of multidrug efflux pumps. Neither *ebrA* nor *ebrB* was sufficient for resistance, but introduction of the two genes carried on different plasmids conferred drug resistance. Thus, both EbrA and EbrB appear to be necessary for activity of the multidrug efflux pump. In known members of the SMR family, only one gene produces drug efflux. Thus, EbrAB is a novel SMR family multidrug efflux pump with two components.**

Drug efflux from cells is one of the major mechanisms of drug resistance. Multidrug efflux pumps are widely distributed in membranes ranging from bacterial cells to animal cells and remove toxic substances from the cytoplasm or membranes in an energy-dependent manner. The multidrug efflux pumps are responsible for multidrug resistance in bacterial cells and in cancer cells. Thus, the presence of multidrug efflux pumps is a serious problem in the treatment of infectious diseases and cancer. Several major groups of multidrug extrusion systems are known in microorganisms (2, 11, 17). One such group is the small multidrug resistance (SMR) family, and members of this family have been found in many microorganisms (19). Transporters of the SMR family are rather small and usually possess four transmembrane domains in one polypeptide. The SMR family includes more than 40 proteins in eubacteria, and a few of them have been studied in detail (19, 24). These include Smr (*Staphylococcus aureus*) (6) and EmrE (*Escherichia coli*) (25). It is very likely that the SMR family drug transporters are drug/H<sup>+</sup> antiporters. During the course of our studies of multidrug transporters in bacterial cells, we found a unique system in *Bacillus subtilis* ATCC 9372: a two-component drug transporter that belongs to the SMR family.

### MATERIALS AND METHODS

**Bacteria and growth.** *B. subtilis* ATCC 9372 was used as a donor of chromosomal DNA. *E. coli* KAM3 (15), a derivative of K-12 that lacks a restriction system and AcrAB (13), was used as the cloning host and for drug susceptibility testing. *B. subtilis* ISW1214 (*hsrM leuA8 metB5*; Tet<sup>r</sup>) was purchased from TaKaRa Co. and used for the drug susceptibility test. *B. subtilis* and *E. coli* cells were grown in Luria-Bertani (LB) broth (10) under aerobic conditions at 37°C. Where indicated, drugs were added to the medium.

**Drug susceptibility test.** The MICs of drugs were determined in Mueller-Hinton broth (Difco) containing various drugs at various concentrations as indicated. Cells in the test medium (10<sup>5</sup> cells/ml) were incubated at 37°C for 24 h, and thereafter the growth was judged.

**Gene manipulation.** The gene responsible for ethidium resistance was cloned from *B. subtilis* ATCC 9372 cells as follows. Chromosomal DNA was prepared

from *B. subtilis* ATCC 9372 by the method of Lovett and Keggins (12). The DNA was partially digested with *Sau3AI*, and fragments with 4 to 10 kbp were separated by sucrose density gradient centrifugation. The DNA fragments were ligated into pUC19, which had been digested with *Bam*HI, and dephosphorylated with bacterial alkaline phosphatase. Competent cells of *E. coli* KAM3 prepared by the method of Hanahan (8) were transformed with the ligated recombinant plasmids and were spread on LB agar plates containing 9 µg of ethidium bromide per ml and 60 µg of ampicillin per ml. Plasmids contained in the transformants were isolated, reintroduced into KAM3 cells, and spread on the same type of plates again. One of the resulting hybrid plasmids were designated pBET5. The DNA insert in the pBET5 was about 3.5 kbp. Two *Hinc*II sites were present in pBET5, one in the multicloning site derived from pUC19 and another one in the insert. The pBET5 was digested with *Hinc*II and self ligated. The resulting recombinant plasmid pBET52 carries *ebrAB*. Two *Hind*III sites were present in pBET5, one site in the *ebrB* gene and another in the multicloning site derived from pUC19. The pBET5 was digested with *Hind*III and self ligated. The resulting pBET53 carries *ebrA* but not *ebrB*. pBET52 was cleaved with *Cla*I, digested with mung bean nuclease, which resulted in blunt ends, and self ligated, to produce pBET51. Thus, the *ebrA* gene was inactivated by removing two nucleotides from the *Cla*I site while leaving the *ebrB* gene intact. The DNA insert in the pBET53 was subcloned into pSTV29, a derivative of pACYC184, and pTS93 was obtained. pHAB was constructed as follows. The DNA insert in the pBET52 was cut out and ligated to a *B. subtilis* shuttle vector, pHY300PLK (Amp<sup>r</sup> Tet<sup>r</sup>) (purchased from TaKaRa Co.). The plasmids used in this study, their vectors, and *B. subtilis* genes carried are listed in Table 1.

The nucleotide sequence was determined by the dideoxy chain termination method (20) using a DNA sequencer (ALF express; Pharmacia Biotech). Sequence data were analyzed with GENETYX sequence analysis software (Software Development Co.). The SwissProt and GenBank databases were screened for sequence similarities.

**Ethidium efflux assay in cells.** *E. coli* KAM3 cells harboring each hybrid plasmid were grown in LB broth (10) under aerobic conditions at 37°C. The cells were harvested at the late exponential phase of growth, washed twice with a minimal medium (23), and suspended in the same medium to an optical density of about 0.2 at 650 nm. Carbonylcyamide *m*-chlorophenylhydrazone (C CCP) and ethidium bromide were added to the cell suspension to 40 µM and 2.5 µM, respectively. The cell suspension was shaken for 1 h at 37°C to deplete the energy of cells and to load the cells with ethidium. Thereafter, cells were harvested and washed twice with the minimal medium supplemented with ethidium bromide (2.5 µM, final concentration) and resuspended in the same medium to an optical density of about 0.1 at 650 nm. The cell suspension was preincubated at 37°C for 5 min, and the assay was initiated. The fluorescence of the assay mixture was measured with excitation and emission wavelengths of 500 nm and 580 nm (1), respectively, using the Hitachi fluorescence spectrophotometer F-2000.

**Other.** Chemicals and enzymes used in this study were from commercial sources.

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession no. AB029306.

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TABLE 1. Plasmids used in this study

| Plasmid | Vector                            | <i>B. subtilis</i> genes carried |
|---------|-----------------------------------|----------------------------------|
| pBET5   | pUC19                             | <i>ebrA ebrB</i>                 |
| pBET51  | pUC19                             | <i>ebrB</i>                      |
| pBET52  | pUC19                             | <i>ebrA ebrB</i>                 |
| pBET53  | pUC19                             | <i>ebrA</i>                      |
| pTS93   | pSTV29 (a derivative of pACYC184) | <i>ebrA</i>                      |
| pHAB    | pHY300PLK (a shuttle vector)      | <i>ebrA ebrB</i>                 |

## RESULTS

**Cloning of ethidium resistance genes.** *E. coli* mutant KAM3 lacks both the restriction system (*hsd* mutant) and the principal multidrug efflux pump AcrAB and is suitable for cloning of multidrug resistance gene(s) from other organisms (15). In fact, we have cloned multidrug efflux genes from *Vibrio parahaemolyticus* (15), *Pseudomonas aeruginosa* (14), and others (unpublished results) with KAM3 as host. We also tried to clone a multidrug resistance gene(s) from the chromosome of gram-positive bacterium *B. subtilis* with KAM3 as host. Ethidium resistance is a useful marker for cloning a drug efflux gene(s), because cells have to extrude ethidium to escape its toxicity. We obtained two recombinant plasmids by using ethidium as drug for the selection. Restriction analysis revealed that these two plasmids carried the same DNA region. We further analyzed one of the plasmids, pBET5. Table 2 shows MICs of several drugs for KAM3/pUC19 (control) and KAM3/pBET5. When compared to the control, KAM3/pBET5 cells required a MIC that was eight times higher with ethidium bromide and four times higher with acriflavine, pyronine Y, safranin O, and tetraphenylphosphonium (TPP) chloride. No significant change in the MIC was observed with other drugs tested (Table 2).

**Sequence and characteristics of the gene products.** Partial sequencing of the DNA insert in the pBET5 plasmid revealed that it carried the putative *ebrAB* genes, which were suggested from the genome sequence of *B. subtilis* 168 (9). Since we cloned the genes from *B. subtilis* ATCC 9372, we determined the sequence of the whole *ebrAB* region. We found that there were some differences in the sequence of the *ebrAB* region between strain ATCC 9372 and strain 168 (91% identity). The putative *ebrAB* genes were preceded by a possible promoter-like sequence and followed by a possible terminator-like sequence. The *ebrAB* region was located just downstream from the *lac* promoter of pUC19 but in the opposite orientation in the plasmid pBET5. Cells harboring pBET5 showed elevated drug resistance, as described above. Therefore, we conclude

TABLE 2. Changes in drug susceptibility in *E. coli* transformant

| Drug             | MIC ( $\mu\text{g/ml}$ ) for: |            |
|------------------|-------------------------------|------------|
|                  | KAM3/pUC19                    | KAM3/pBET5 |
| Ethidium bromide | 1                             | 8          |
| Acriflavine      | 4                             | 16         |
| Pyronine Y       | 1                             | 4          |
| Safranin O       | 4                             | 16         |
| TPP Cl           | 8                             | 32         |
| Rhodamine 6G     | 2                             | 2          |
| Erythromycin     | 1                             | 1          |
| Chloramphenicol  | 1                             | 1          |
| Tetracycline     | 0.5                           | 0.5        |
| Kanamycin        | 1                             | 1          |
| CCCP             | 8                             | 8          |

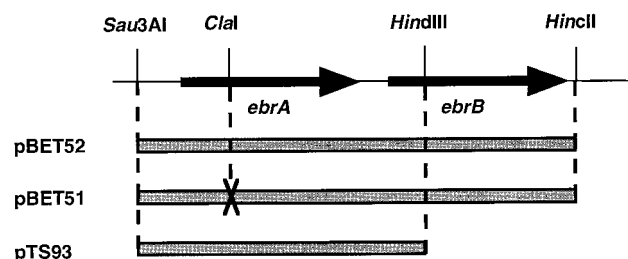


FIG. 1. Construction of plasmids carrying *ebrA* and/or *ebrB*. The plasmid pBET52 carries normal *ebrA* and *ebrB*. Plasmid pBET51 carries intact *ebrB* and defective *ebrA* in which two nucleotides were removed from the *ClaI* site. Plasmid pTS93 carries intact *ebrA*. Both pBET52 and pBET51 are derivatives of pUC19, and pTS93 is a derivative of pACYC184.

that the original promoter of the *ebrAB* genes derived from *B. subtilis* is functional in the *E. coli* cells. There were no terminator-like sequence or promoter-like sequence between the two putative genes, *ebrA* and *ebrB*. Thus, it is likely that *ebrA* and *ebrB* are members of one operon. Both *ebrA* and *ebrB* were preceded by Shine-Dalgarno sequences (22) at a proper distance.

The deduced EbrA and EbrB proteins consist of 105 and 118 amino acid residues, respectively, similar to the size of EbrA and EbrB in strain 168 (9). The identity in the amino acid sequence of the EbrA (and EbrB) between strain ATCC 9372 and strain 168 was 94% and similarity was 99%. Strikingly, the deduced amino acid sequence of the EbrA was highly similar (80%) to that of EbrB. A homology search in the SwissProt database revealed that the EbrA and EbrB have sequence similarity (80 to 85%) with EmrE and Ebr of *E. coli* and Smr of *S. aureus*. Thus, EbrA and EbrB are both members of the SMR family of drug transporters (19). In addition, significant sequence similarities were detected between EbrAB and SugEs of *E. coli* (5), *Proteus vulgaris* (3), and *Citrobacter freundii* (19). Hydrophathy plots for EbrA and EbrB as calculated by the method of Eisenberg et al. (4) showed four hydrophobic, presumably transmembrane, regions as expected for the SMR family (6, 18, 24). EbrB possesses a hydrophilic region at the carboxyl terminus.

**Both EbrA and EbrB are needed for drug resistance.** The known drug efflux pumps of the SMR family consist of one component (19). We tested whether both EbrA and EbrB are necessary for drug resistance. We constructed a plasmid carrying the normal *ebrAB* genes (pBET52), a plasmid carrying the normal *ebrA* gene alone (pTS93), and a plasmid carrying a defective *ebrA* gene and a normal *ebrB* gene (pBET51) (Fig. 1). *E. coli* KAM3 cells harboring pBET52 had an elevated ethidium bromide MIC (16  $\mu\text{g/ml}$ ) compared with cells harboring pUC19 (2  $\mu\text{g/ml}$ ). Cells harboring either pBET51 or pTS93 showed no change in resistance compared with cells harboring pUC19. Therefore, it seems that neither *ebrA* nor *ebrB* is sufficient for ethidium bromide resistance. However, cells harboring both pTS93 and pBET51 showed the same level of resistance (MIC of ethidium bromide, 16  $\mu\text{g/ml}$ ) as compared to cells harboring pBET52. Based on these results, we conclude that *ebrB* gene in pBET51 is functional, and therefore there is no polar effect in pBET51 in which an upstream *ebrA* gene is defective. Similar resistance patterns were obtained with cells harboring both pTS93 and pBET51 when acriflavine, pyronine Y, safranin O, or TPP Cl was used instead of ethidium bromide (data not shown). Thus, we conclude that both *ebrA* and *ebrB* are necessary for drug resistance. We also conclude that the

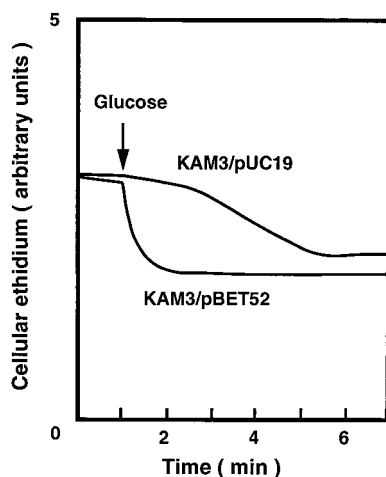


FIG. 2. Accumulation of ethidium in cells. Energy-starved cells of *E. coli* KAM3/pUC19 and KAM3/pBET52 were loaded with ethidium bromide. Cellular ethidium was monitored continuously by measuring the fluorescence of ethidium at the excitation and emission wavelengths of 500 nm and 580 nm, respectively. After 1 min (arrow), glucose was added to the cell suspension at a final concentration of 20 mM to energize the cells.

EbrAB system is a new type of SMR family member, a drug efflux pump consisting of two small components.

**Ethidium efflux activity.** As mentioned above, sequence similarity with other SMR family multidrug efflux proteins suggested that the putative EbrAB is a multidrug efflux pump. We tested this possibility by measuring ethidium efflux. Energy-starved cells were first loaded with ethidium. Thereafter, glucose was added to energize the cells. As shown in Fig. 2, rapid ethidium efflux was observed with KAM3/pBET52 cells just after the addition of glucose. On the other hand, only a slow efflux of ethidium was observed with cells of KAM3/pUC19. Addition of an H<sup>+</sup> conductor, CCCP, greatly reduced the ethidium efflux elicited by glucose (data not shown). Thus, the driving force for the ethidium efflux is likely an electrochemical potential of H<sup>+</sup>. Addition of Na<sup>+</sup> or Li<sup>+</sup> did not produce any significant effect on ethidium efflux (data not shown).

We observed similar glucose-induced ethidium efflux with cells of KAM3/pBET51 and KAM3/pTS93, but not with *E. coli* KAM3/pBET51, KAM3/pTS93, and KAM3/pUC19 (data not shown). Thus, ethidium resistance and ethidium efflux activity in cells corresponded well. These results also support the idea that products of both *ebrA* and *ebrB* genes are involved in drug efflux.

**EbrAB is functional in *B. subtilis*.** We tested whether the putative EbrAB pump is functional in *B. subtilis*. A plasmid, pHAB, carrying the *ebrAB* genes was introduced into *B. subtilis* ISW1214, and changes in drug resistance were tested. As shown in Table 3, a several-fold increase in MICs was observed with ethidium bromide, acriflavine, pyronine Y, and safranin O but not with other antimicrobial drugs tested in ISW1214/pHAB compared with ISW1214. Thus, the EbrAB system is functional in *B. subtilis* cells. A slightly smaller increase in MIC was observed with *B. subtilis* ISW1214/pHAB cells (Table 3) than with *E. coli* KAM3/pBET5 cells (Table 2). This may be partly due to the fact that *B. subtilis* ISW1214 has a wild-type phenotype regarding drug sensitivity; perhaps it possesses intrinsic drug efflux pumps, such as Bmr (16) and EbrAB, while *E. coli* KAM3 is deleted in the major drug efflux pump AcrAB. Another factor(s) which may affect the MIC would be the copy number of the plasmids in cells and/or efficiency of the gene expression.

## DISCUSSION

The presence of putative *ebrAB* genes in the chromosomal DNA of *B. subtilis* has been revealed by whole-genome sequencing (9). Judging from the similarity of the deduced amino acid sequences between EbrA or EbrB and members of SMR family of multidrug efflux proteins, it seemed that the EbrAB is responsible for ethidium bromide resistance. We cloned the *ebrAB* genes from *B. subtilis* ATCC 9372 and expressed them in *E. coli* cells. In fact, our data suggest that the EbrAB is a multidrug efflux pump and is involved in multidrug resistance against cationic lipophilic dyes such as ethidium bromide, acriflavine, pyronine Y, and safranin O. Three signature sequences that are specific to the SMR family members (19) were all present in deduced amino acid sequences of EbrA and EbrB (data not shown). Thus, we believe that the EbrAB is a member of the SMR family.

Each member of the SMR family so far reported is encoded by a single gene. Smr of *S. aureus* (6) and EmrE of *E. coli* (25) have been purified to homogeneity and reconstituted into liposomes. The reconstituted liposomes then showed drug efflux activity, indicating that a single polypeptide is sufficient for function although it may work as homo-oligomer (26). Interestingly, however, we found two consecutive genes in the *ebr* region of *B. subtilis*. It seemed possible that either of the products from the two genes (*ebrA* and *ebrB*) could function as a drug efflux pump. However, introduction of either gene alone into cells did not confer resistance. On the other hand, concomitant introduction of the two genes made cells resistant. Thus, both EbrA and EbrB are necessary for drug efflux activity. Sasatsu and coworkers reported that there were two consecutive *ebr* genes in plasmid pTZ22, a transferable plasmid of *S. aureus* (21). Deletion of one of the genes only lowered the resistance level to ethidium (21). One copy of the *ebr* gene was thus enough for the drug efflux in their case. Thus, our case is the first example of a drug efflux pump of the SMR family that seems to be composed of two very similar, but different, components.

The EbrAB system was functional in both *E. coli* and *B. subtilis*. It is very likely that the promoter of the *ebrAB* operon and products of the operon from a gram-positive bacterium, *B. subtilis*, are functional in a gram-negative bacterium, *E. coli*.

Two more sets of putative genes similar to the *ebrAB* genes are present in the whole genome of *B. subtilis* 168, *yvdR* and *yvdS* and *ykkD* and *ykkC* (9). The deduced products of these putative genes showed roughly 50% sequence similarity with EbrA and EbrB. It would be interesting to test whether they encode drug efflux pumps.

Grinius and Goldberg reported that Glu-13 of the Smr, a unique acidic residue located in the hydrophobic domain, is directly involved in the drug/H<sup>+</sup> antiporter (7). They also re-

TABLE 3. Changes in drug susceptibility in *B. subtilis* transformant

| Drug             | MIC ( $\mu$ g/ml) for: |              |
|------------------|------------------------|--------------|
|                  | ISW1214                | ISW1214/pHAB |
| Ethidium bromide | 4                      | 16           |
| Acriflavine      | 2                      | 4            |
| Pyronine Y       | 4                      | 8            |
| Safranin O       | 8                      | 16           |
| Rhodamine 6G     | 1                      | 1            |
| Erythromycin     | 0.25                   | 0.25         |
| Chloramphenicol  | 0.5                    | 0.5          |
| Ofloxacin        | 0.06                   | 0.06         |

ported that Glu-24 of the Smr is involved in determining the specificity of drug resistance. These two Glu residues are conserved in both EbrA and EbrB. Paulsen and coworkers reported that Tyr-59 and Trp-62 play an essential role in drug resistance in QacC (Smr) (18). These two residues are also conserved in EbrA and EbrB.

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