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# A Drug Efflux Pump for Cationic Drugs including Disinfectants in *Bacillus subtilis*

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The *Bacillus subtilis* YvaE protein, the small multidrug resistance (SMR) family (TC #2.A.7.1), is shown to catalyze efflux of multiple cationic drugs including many disinfectants, when it was cloned and expressed in *Escherichia coli*. When the *yvaD* gene was coexpressed with *yvaE* gene, the *yvaD* protein, encoded within a single operon with the *yvaE* gene, is shown to counteract the action of YvaE. By ethidium efflux analysis, the cells harvoring a vector with *yvaE* gene showed a rapid ethidium efflux, compared with the control cells. These results clearly suggest that YvaE mediates drug export from the cell cytoplasm.

**Key words:** Efflux pump, cationic drugs, *Bacillus subtilis*

Multidrug resistance efflux pumps represent major problems for microbial infections in man and other animals, yet many such systems encoded within microbial genomes are not characterized functionally [6]. Both multidrug and drug-specific efflux systems are responsible for clinically significant resistance to chemotherapeutic agents in pathogenic bacteria, fungi, parasites and in human cancer cells [10, 12]. Approximately 1% of all genes present within the genome of most bacteria encode such putative systems [13, 14]. However, little is known regarding the details of their genetic regulation or their mechanisms of action. Of the 250 currently recognized families, six families (or superfamilies) of transport systems (TC system) are currently known to include members that pump multiple drugs out of living cells [15]. There are five universal pump families [MFS, TC #2.A.1; RND, TC #2.A.6; DMT, TC #2.A.7; MATE, TC #2.A.66; and ABC, TC #3.A.1] and one eukaryotic-specific pump family [MET; TC #2.A.74]. One family within the DMT superfamily, the Small Multidrug Resistance (SMR) family (TC #2.A.7.1), consists of small hydrophobic proteins of between 103 and 121 amino acyl residues with four established or putative transmembrane a-helical spanners. These proteins can exist in the membrane as homooligomers 18 or putative heterooligomers [5, 7]. They primarily expel cationic drugs using a drug: $H^+$ antiport mechanism [12, 17]. The drug is believed to pass through a hydrophobic pore in the interior of the protein [8]. A membrane embedded glutamate at the presumed active site is essential for high affinity binding of cationic drugs [9, 19]. Jack *et al* have previously reported the properties of a heterologous drug efflux pumps, YkkCD of *Bacillus subtilis* that compose functional heterodimeric structure. These two subunits are encoded by two genes within a single operon [5]. *B. subtilis* has seven established paralogues of the SMR family while *Escherichia coli* has four. All of these eight *B. subtilis* genes were recently cloned into expression vectors. However, two pairs of the cloned *B. subtilis* homologues (EbrAB and YkkCD) conferred increased resistance to cationic dyes and antiseptics, respectively, when both members of the pair were simultaneously produced in *E. coli* [5, 7]. Possibly the two proteins of each pair form a heterooligomer, or one functions as a chaperone protein to allow proper folding and insertion of the other.

Examination of the fully sequenced genome of *B. subtilis* reveals these additional operons, each encording two SMR family homologues. In this report, this YvaDE pair was characterized, showing unexpectedly that while the expression of YvaE alone in *E. coli* catalyzed efflux of cationic drugs and dyes including quarternary ammonium compounds (QAC), YvaD, when expressed together with YvaE, counteracts the activity of the former protein.

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## Materials and Methods

### Gene cloning and expression

The *B. subtilis* genes *yvaE* and *yvaD* and the gene pair *yvaED* were cloned into the expression vector pBAD24 [3]. The procedure was as follows. The targeted gene (or genes) was (were) amplified by PCR with *Taq* polymerase using *B. subtilis* 168 chromosomal DNA as a template. For *yvaE*, the primers (5' to 3') were CAGAATTCCACATGAATTGGG-TGTTTC (sense) and CACTGCAGCTTCATATATCTCA-TTTGTACTGC (antisense); for *yvaD*, the primers were CAGAATTCCAAATGAGATATATGAAGCTG (sense) and CAGTCGACCTGTCAGGACTGTTTGTCC (antisense); for *yvaED*, the *yvaE* sense and *yvaD* antisense primers were used. The DNA was digested with the *Eco*RI and *Pst*I (*yvaE*) or *Eco*RI and *Sal*I (*yvaE*; *yvaED*) restriction enzymes with restriction sites flanking the target gene copied by the PCR. The included genes were then cloned into the pBAD24 polylinker region. The pBAD24 ligation mixture was introduced into competent *Escherichia coli* DH5a (F-ϕ80d*lacZΔM15*D (*lacZYA-argF*)U169 *deoR recA*1 *endA1 hsdR17(rk-, mk+) gal-phoA supE44 λ thi-1 gyrA96 relA1).* Finally, transformants were selected on the basis of ampicillin (60 μg/ml) resistance. Recombinant plasmids were checked by restriction enzyme digestion and direct sequencing. Expression of the cloned gene (s) was induced by the addition of 0.2% L-arabinose.

### Assay of drug resistance

Drug assay plates were prepared with M9 minimal agar, using glycerol instead of glucose as a carbon source, 0.2% L-arabinose as an inducer, and a twofold series of drug concentrations [5]. *E. coli* strain DH5a bearing the pBAD24 vector or bearing this plasmid with the gene(s) *yvaE*, *yvaD*, or *yvaED* was grown overnight in Luria-Bertani (LB) broth with 60 μg/ml of ampicillin per ml at 37°C with shaking (220 rpm). Subcultures were grown to an $A_{600}$ of 0.6 optical density units in LB broth with 60 μg/ml of ampicillin per ml and 0.2% L-arabinose at 37°C with shaking. These cultures were diluted 100, $10^{-1}$ and $10^{-2}$ in LB broth, and 5 μl samples of each transformant at each dilution were plated on the above mentioned assay plates. The plates were incubated overnight at 37°C, and drug resistance was scored and minimal inhibitory concentration (MIC) was measured after 12, 18, and 24 h of growth.

### Ethidium efflux experiments

Overnight cultured cells grown in LB broth containing 60 μg of ampicillin per ml at 37°C were inoculated into the same medium with 0.2% L-arabinose. The culture was incubated at 37°C with shaking. The cells were harvested at an $A_{600}$ of 0.6, washed twice with 0.1 M sodium phosphate buffer (pH 7.0), and resuspended in the same buffer to an $A_{600}$ of about 0.5. Ethidium bromide and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) were added to the cell suspension to 15 μM and 40 μM, respectively. The cell suspensions were incubated at 37°C for 30 min to load the cells with dye. The cells were then harvested and washed twice with the same buffer supplemented with ethidium bromide to a final concentration of 7.5 μM. The ethidium-loaded cells were then resuspended in the same wash buffer supplemented with ethidium bromide to give a cell suspension of 0.5 OD units at $A_{600}$. Glucose was added to the cell suspension at a final concentration of 11 mM to energize the cells. Transport experiment were performed by using a Perkin-Elmer fluorescence spectrophotometer MPF-4. The fluorescence of the assay mixture was measured at 37°C with mixing [1]. Excitation and emission wavelengths were 530 and 600 nm with a 10 nm bandwidth.

### Analysis of Multiple alignment

The PSI-BLAST data base search method was used to identify homologous proteins. Multiple alignments were generated using the Clustal X program [16], and hydropathy and putative transmembrane spanning (TMS) analyses were conducted using the TMpred program [4].

## Results and Discussion

### Multiple alignment of Qac protein sequences

In recent report, Masaoka *et al.* [7] provided evidence that two *B. subtilis* gene products of the SMR family, EbrA and EbrB, are required for energy-dependent drug efflux. In an independent study, Jack *et al.* [5] suggested that the same is true for two other *B. subtilis* SMR family homologues, YkkC and YkkD. The *ykkCD* genes comprise an operon as do the *ebrAB* genes, and a third operon, *yvdSR*, encodes still a third pair of SMR family homologues in *B. subtilis* [5]. Finally, a fourth gene pair, *yvaED*, encodes one established SMR family member, *yvaE*, as well as *yvaD*, which shows characteristics of an SMR protein although there is insufficient sequence

**Fig. 1. Well conserved portions of the complete multiple alignment of functionally characterized quaternary ammonium compounds resistant proteins.** Bsu: *Bacillus subtilis*, Sau: *Staphylococcus aureus,* Pae: *Pseudomonas aeruginosa*, Kpn: *Klebsiella pneumoniae*, Eae: *Enterobacter aerogenes*, Sta.sp.: *Staphylococcus* sp., Ssa: *Staphylococcus saprophyticus*. NCBI(gi) number: QacC (1848269), QacE2 (4210828), QacE (477357), QacF (4539649), QacG (3647360), QacH (3647363).
*, identity shared by all proteins; :, a near similarity; ., a distant similarity.

similarity to established homology. A multiple alignment of the YvaE protein sequence with other quarternary ammonium compound resistance proteins (Qac proteins) found in other bacteria is presented in Fig. 1. The proteins exhibits an strong sequece homology, specially showing the well-conserved sequences of the presumed active sites for binding of substrates. As a result, the common structural and sequence features suggest a distant relationship between these proteins. Grinius and Goldberg reported that Glu-13 of SMR, a unique acidic residue located in the hydrophobic domain, is directly involved in the drug/$H^+$ antiporter [2]. This Glu residue is conserved in the sequence of YvaE. Paulsen *et al.* reported that Tyr-59 and Trp-62 play an essential role in drug resistance in QacC (Smr) of *Staphylococcus aureus* [11]. These two residues are also found to be conserved in the sequence of YvaE.

### Drug and dye resistance of YvaE protein

Table 1 summarized the drug and dye resistance phenotypes of parental wild type *E. coli* DH5a cells bearing the control plasmid, pBAD24, the experimental plasmid with the *yvaE* gene and *yvaDE* operon inserted behind the pBAD24 promoter. Overexpression of the *yvaE* gene alone gives rise to a broad specificity resistance phenotype when a wide variety of cationic drugs and dyes was assayed. (Table 1) When various quarternary ammonium compounds including cetyl compounds were used to assay, overexpression of *yvaE* gene in *E. coli* increased 3-6 times in resistance compared with cells harboring pBAD24. Similar resistance patterns were obtained when pyronineY and crystal violet were used. However, benzalkonium chloride and tetraphenylarsornium chloride relatively gave a lower increase by 1.5-2 times. When ethidium bromide was used for a substrate, th level of resistance was elevated greatly to 500 μg/ml of MIC, compared with the control (50 μg/ml of MIC).

When *yvaD* gene was cloned and overexpressed in *E. coli*, the cells were not able to grow, because the overexpressed YvaD protein might give a toxicity to the cells. So *yvaE-D* genes were clone together in the same vector and coexpressed. As a results, suprisingly, coexpression of *yvaD* totally reversed the drug resistance phenotype caused by *yvaE* expression when some cationic drugs and dyes were used, but had no effect or less effect exceptionally only when ethidium bromide were assayed. Masaoka *et al.* [7] reported that EbrAB, a SMR family multidrug efflux pump in *B. subtilis* showed to be a heterologous two-components drug transporter. Thus, both EbrA and EbrB appear to be necessary for activity of the multidrug efflux pump. Another heterologous drug efflux pump, YkkCD

**Table 1. MICs for *E. coli* DH5α bearing the pBAD24 vector with various inserts**

| Compounds | MIC ( μg/ml ) with insert[a] | | |
|---|---|---|---|
| | None | YvaE | YvaED |
| **Quaternary Ammonium Compounds** | | | |
| Benzyldimethyl tetradecylammonium chloride ($C_{23}H_{42}NCl$) | 20 | 120 | 100 |
| Benzalkonium chloride ($C_{12}H_{25}N(CH_3)_2$-$C_7H_7Cl$) | 25 | 50 | 50 |
| Hexadecyltrimethylammonium bromide (cetrimide) ($C_{19}H_{42}NBr$) | 50 | 150 | 20 |
| Cetylpyridinium chloride ($C_{21}H_{38}NCl$) | 10 | 100 | 80 |
| Cetylpyridinium bromide ($C_{21}H_{38}NBr$) | 40 | 120 | 16 |
| Cetyldimethylethyl ammonium bromide | 40 | 120 | 8 |
| **Lipophilic Cations** | | | |
| Tetraphenylarsonium chloride ($C_{24}H_{20}AsCl$) | 500 | 800 | 600 |
| **Pyronins** | | | |
| PyronineY | 8 | 32 | 24 |
| **Triphenylmethanes** | | | |
| Crystal violet | 2 | 12 | 10 |
| **Phenathridines** | | | |
| Ethidium bromide | 50 | 500 | 400 |

[a]All strains showed the same level of resistance against cloramphenicol, erythromycin, kanamycin, tetracycline and streptomycin, showing that YvaE and YvaED do not transport these antibiotics.

system of *B. subtilis* also was recently reported [5]. In this result as shown in Table 1, for the activity of YvaE efflux pump, one of pair protein, YvaD was apparently not required, but a single component, only YvaE appears to be sufficient in efflux function, differently from both previous reports. However, the actual role of YvaD for the YvaE-D efflux system was not yet known. In addition, this result is the first report for SMR-type multidrug transporter in *B. subtilis* with a wider specificity to drugs including various quarternary ammonium compounds. Further molecular biochemical study may lead to an explanation for these interests.

**Ethiduim efflux activity**

As mentioned above, sequence similarity with other quarternary ammonium compounds resistance proteins suggested that the YvaE is a multidrug efflux pump. So a possibility was tested by measuring ethidium efflux. Energy-starved cells were first loaded with ethidium, thereafter, glucose was added to energize the cells. The transport assay with ethidium bromide is shown in Fig. 2. Cells expressing the *yvaE* gene showed greatly increased rates of drug efflux when cells were exposed to glucose at 4 minute after starting the reaction and the addition of CCCP reversed this effect at 12 minute. which showed a rapid ethidium efflux. On the other hand, only a slow efflux of ethidium was observed with the cells harvoring vector only, pBAD24. Addition of an $H^+$ conductor, CCCP, greatly reduced the ethidium efflux elicited by glucose. Thus, the resistance phenotype is mediated by an elevated energy-dependent efflux pump and the driving force for

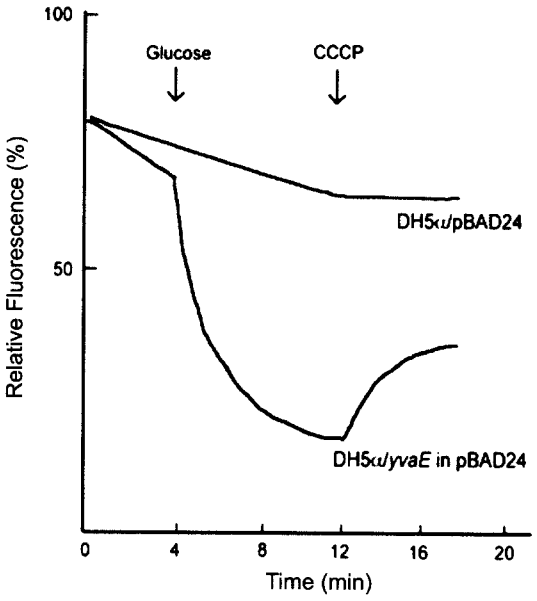


**Fig. 2. Efflux of ethidium into *E.coli* cells.** Energy-starved cells of *E. coli* DH5α/pBAD24 and *E. coli* DH5α/pBAD24 containing *yvaE* gene were loaded with ethidium bromide. After 4 min (arrow), glucose was added to the cell suspension at a final concentration of 11 mM to energize the cells.

ethidium efflux is likely an electrochemical potential of $H^+$. Similar results for the ethidium efflux experiment were obtained in EbrAB system of *B. subtilis* [7] and QacC system of *S. aureus* [11]. Conclusively, these results clearly suggest that YvaE mediates drug export from the cell cytoplasm and confers a multidrug resistance phenotype to the cells. This information obtained may lead to the investigation of mechanistic features of SMR-type efflux pumps and pmf-driven secondary carrier in general.

## 국문초록

### 양이온약제내성을 유도하는 *Bacillus subtilis*의 Drug Efflux Pump

**정용준**

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Small multidrug resistance(SMR) family(TC #2.A.7.1)에 속하는 막단백질 중 하나를 coding하는 *Bacillus subtilis*의 yvaE 유전자의 발현을 유도한 결과, 발현된 YvaE 단백질에 의해 대장균세포내에서 살균제를 포함한 다양한 양이온 약제에 대한 efflux활성을 촉매하는 것으로 확인되었다. 같은 operon내에 인접한 yvaD유전자의 동시발현을 유도한 결과, 이러한 efflux활성은 억제가 되는 것으로 나타났다. Ethidium bromide를 기질로 하여 fluorimeter를 이용한 efflux transport실험결과, YvaE 단백질이 발현된 대장균세포의 경우 vector만을 함유한 대조세포에 비해 현저히 빠른 efflux활성을 보여주었다. 따라서 YvaE 막단백질은 multidrug export를 촉매하는 SMR-type efflux pump임을 확인하였다.

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