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NOTES

A Broad-Specificity Multidrug Efflux Pump Requiring a Pair of Homologous SMR-Type Proteins

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The *Bacillus subtilis* genome encodes seven homologues of the small multidrug resistance (SMR) family of drug efflux pumps. Six of these homologues are paired in three distinct operons, and coexpression in *Escherichia coli* of one such operon, *ykkCD*, but not expression of either *ykkC* or *ykkD* alone, gives rise to a broad specificity, multidrug-resistant phenotype including resistance to cationic, anionic, and neutral drugs.

Five currently recognized ubiquitous families of transport proteins are known to include members that are capable of functioning in multidrug resistance (MDR) (1, 8; D. L. Jack and M. H. Saier, Jr., unpublished observations). Of these, the small multidrug resistance (SMR) family is unusual in that it consists of proteins with only 100 to 120 aminoacyl residues and four transmembrane α-helical spanners (see references 5 and 7 for reviews). Although the subunit stoichiometry has not been defined for any member of this family, previously characterized SMR-type drug efflux pumps are thought to exist in the membrane as homo-oligomers (9, 11). Some SMR family members have not been shown to exhibit an MDR phenotype in spite of extensive effort in this direction (5). Those that have been shown to export drugs from the bacterial cell are specific for cationic drugs and are believed to translocate their substrates via a fairly hydrophobic transmembrane pathway (4).

Analysis of the *Bacillus subtilis* genome has revealed that this gram-positive bacterium encodes seven SMR homologues (6) (Table 1). The *Escherichia coli* genome encodes four such homologues (plus a plasmid-encoded homologue), and distant SMR homologues have been detected in a variety of other bacteria as well as in archaea and eukaryotes (D. L. Jack and M. H. Saier, Jr., unpublished observations). Surprisingly, six of the *B. subtilis* homologues and two of the *E. coli* homologues are encoded from gene pairs in four distinct operons. These gene pairs are *ebrA* and *ebrB*, *yvdR* and *yvdS*, and *ykkC* and *ykkD* in *B. subtilis* as well as b1599 and b1600 in *E. coli* (Table 1).

One member of each *B. subtilis* protein pair is short (105 to 106 aminoacyl residues), while the other is longer (111 to 117 residues) (Table 1). This difference proved to be due to a partially conserved C-terminal hydrophilic extension present in the latter proteins but lacking in the former proteins. The short SMR family homologues could also be distinguished from the longer homologues of each of these protein pairs on the basis of topological features revealed by hydropathy plots (data not shown). Similar features are observed for the *E. coli* b1599-b1600 pair, and possibly also for the *E. coli* SugE-Ebr pair (Table 1). Interestingly, *sugE* and *ebr* of *E. coli* are chromosomally and plasmid encoded, respectively. These differences between the two members of each protein pair may provide the molecular basis for a requirement for the functional heterodimeric structure proposed here.

We initially cloned each of the seven *B. subtilis* genes and expressed them individually in *E. coli* strain DH5α. A drug resistance phenotype was not observed for any of them. We therefore initiated studies to determine if both genes in any one operon needed to be simultaneously expressed in order to observe an MDR phenotype. Results of the experiments with the *ykkCD* gene pair are reported below.

The *B. subtilis* genes *ykkC* and *ykkD* and the gene pair *ykkCD* were cloned into the expression vector pBAD24 (2). The procedure was as follows. (i) The targeted gene (or genes) was were amplified by PCR with *Pyrococcus woesei* (*Pwo*) polymerase. For *ykkC*, the primers (5′ to 3′) were CATGCCATG GAATGGGGATTGGTCGTG (sense) and AAACTGCAGT TATGCCTCGCCTCCTTTTTCC (antisense); for *ykkD*, the primers were CATGCCATGGTGCACTGGATCAGTTTAT TGTG (sense) and ACGCGTCGACACCAACTGCTGAGC (antisense); for *ykkCD*, the *ykkC* sense and *ykkD* antisense primers were used. (ii) The DNA was digested with the *Nco*I and *Sal*I (*ykkD*; *ykkCD*) or *Nco*I and *Pst*I (*ykkC*) restriction enzymes with restriction sites flanking the target gene created during the PCR. (iii) The included genes were then cloned into the pBAD24 polylinker region. (iv) The pBAD24 ligation mixture was heat shocked or electroporated into *E. coli* DH5α. (v) Finally, transformants were selected on the basis of ampicillin (50 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% arabinose.

A twofold dilution series of the drugs listed in Table 2 was analyzed. Drug assay plates were prepared with Luria-Bertani (LB) agar, 50 μg of ampicillin per ml, 0.2% arabinose, and a twofold series of drug concentrations (1, 3) (Table 2). *E. coli* strain DH5α bearing the pBAD24 vector or bearing this plasmid with the gene(s) *ykkC*, *ykkD*, or *ykkCD* was grown overnight in LB broth with 50 μg of ampicillin per ml at 37°C with shaking (250 rpm). Subcultures were grown to an A_{600} of 0.06

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TABLE 1. SMR family members identified in *B. subtilis* and *E. coli*[a]

Abbreviation	Description in database	Organism	No. of residues	Database and accession no.	NCBI (gi) no.
YkkC Bsu	Hypothetical protein	*B. subtilis*	112	spP49856	2632029
YkkD Bsu	Hypothetical protein	*B. subtilis*	105	gbAJ002571	2632030
EbrA Bsu	MDR protein	*B. subtilis*	105	gbZ99113	2634114
EbrB Bsu	MDR protein	*B. subtilis*	117	gbZ99113	2634113
YvdR Bsu	Hypothetical protein	*B. subtilis*	106	gbZ94043	1945677
YvdS Bsu	Hypothetical protein	*B. subtilis*	114	gbZ94043	1945678
YvaE Bsu	Similar to multidrug-efflux transporter	*B. subtilis*	119	gbZ99121	2635870
Ebr Eco	Putative ethidium bromide resistance protein; plasmid encoded	*E. coli*	115	spP14502	119115
SugE Eco	SugE protein	*E. coli*	105	spP30743	3915875
b1599 Eco	Putative ethidium bromide resistance protein	*E. coli*	109	gbD90802	1742633
b1600 Eco	Putative ethidium bromide resistance protein	*E. coli*	121	gbD90802	1742634
EmrE Eco	Ethidium bromide-methyl viologen resistance protein	*E. coli*	110	spP23895	127565

[a] This table lists SMR family homologues identified in the completely sequenced genomes of *B. subtilis* and *E. coli*. Homologues in archaea and eukaryotes, as well as numerous bacteria, have also been identified (D. L. Jack and M. H. Saier, Jr., unpublished results).

optical density unit in LB broth with 50 μg of ampicillin per ml and 0.2% arabinose at 37°C with shaking. These cultures were diluted 10^{-1}, 10^{-2}, and 10^{-3} 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 after 12, 18, and 24 h of growth. The results presented in Table 2 are those performed with 0.2% arabinose present in the plates.

When both the *ykkC* and *ykkD* genes were expressed together in *E. coli* strain DH5α, a broad-spectrum MDR phenotype was observed (Table 2). We observed resistance to a broader range of toxic compounds than was observed for any previously studied SMR pump (5, 7). These compounds included representative cationic dyes and neutral and anionic antimicrobials (Table 2). The effects were at least 1 order of magnitude greater than the additive effect of the two individual genes, which were essentially inactive when present alone (Table 2). It seems unlikely that the effects of YkkC and YkkD are due to the activation of an endogenous *E. coli* MDR pump, since each protein when synthesized alone had no effect. Coexpression of the *ykkC* and *ykkD* genes led to greater than 20-times-higher MICs of many of the compounds tested and as great as 100-times-higher MICs of pyronin Y and phosphonomycin. Transport studies (not shown) revealed that expression of the *ykkCD* operon greatly inhibited ethidium bromide accumulation, and this effect was abolished by the addition of carbonylcyanide *m*-chlorophenylhydrazone (20 μM).

TABLE 2. MICs for *E. coli* DH5α bearing the pBAD24 vector with various inserts[a]

Compound	MIC (μg/ml) with insert			
	None	*YkkC*	*YkkD*	*YkkC-YkkD*
Cationic dyes				
Ethidium bromide	50	50	50	2,000
Proflavine	20	20	20	500
Tetraphenylarsonium chloride	200	200	200	1,000
Crystal violet	2	2	2	50
Pyronin Y	5	5	5	500
Methyl viologen	50	50	50	1,000
Cetylpyridinium chloride	50	50	50	500
Neutral antimicrobial				
Chloramphenicol	2	2	2	10
Other antimicrobials				
Streptomycin	2	2	2	100
Tetracycline	0.5	0.5	0.5	2
Anionic antimicrobial				
Phosphonomycin	0.1	0.1	0.1	10

[a] The proteins produced by expression in pBAD24 (all SMR genes are from *Bacillus subtilis*) are as follows: none (no insert); YkkC (*ykkC*, gi 2632029); YkkD (*ykkD*, gi 2632030); and YkkC/YkkD (both the *ykkC* and *ykkD* genes are expressed under the control of the single P_{BAD} promoter).

This report provides the first demonstration that a naturally occurring, proton motive force-dependent, secondary carrier consists of a hetero-oligomer of two or more dissimilar but homologous subunits. A heterodimer is proposed to be the actual structure. The YkkCD permease contrasts with other characterized members of the SMR family which are believed to be homo-oligomeric (7, 10). The results reported lead to a number of interesting questions. (i) Can each subunit function with just one subunit partner, or can it pair with multiple partners? (ii) Does just one of these subunits comprise the channel and determine the substrate specificity of the permease, or do both subunits participate in channel formation and substrate recognition? (iii) If multiple partners when paired are active, do the different possible combinations give rise to novel substrate specificities, or do these specificities merely reflect the specificities of the constituent subunits? (iv) Are the Sug proteins (5, 7), which have never been shown to exhibit a transport function, active only as hetero-oligomers? (v) What are the molecular determinants that allow an SMR polypeptide chain to function as a homo- or hetero-oligomer? These and other questions concerning the functionality of SMR superfamily members are currently under study in our laboratory.

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