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bfr1$^+$, a Novel Gene of *Schizosaccharomyces pombe* Which Confers Brefeldin A Resistance, Is Structurally Related to the ATP-Binding Cassette Superfamily

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We have isolated a *Schizosaccharomyces pombe* gene, *bfr1*$^+$, which on a multicopy plasmid vector, pDB248′, confers resistance to brefeldin A (BFA), an inhibitor of intracellular protein transport. This gene encodes a novel protein of 1,531 amino acids with an intramolecular duplicated structure, each half containing a single ATP-binding consensus sequence and a set of six transmembrane sequences. This structural characteristic of *bfr1*$^+$ protein resembles that of mammalian P-glycoprotein, which, by exporting a variety of anticancer drugs, has been shown to be responsible for multidrug resistance in tumor cells. Consistent with this is that *S. pombe* cells harboring *bfr1*$^+$ on pDB248′ are resistant to actinomycin D, cerulenin, and cytochalasin B, as well as to BFA. The relative positions of the ATP-binding sequences and the clusters of transmembrane sequences within the *bfr1*$^+$ protein are, however, transposed in comparison with those in P-glycoprotein; the *bfr1*$^+$ protein has N-terminal ATP-binding sequence followed by transmembrane segments in each half of the molecule. The *bfr1*$^+$ protein exhibited significant homology in primary and secondary structures with two recently identified multidrug resistance gene products of *Saccharomyces cerevisiae*, Snq2 and Sts1/Pdr5/Ydr1. The *bfr1*$^+$ gene is not essential for cell growth or mating, but a Δ*bfr1* mutant exhibited hypersensitivity to BFA. We propose that the *bfr1*$^+$ protein is another member of the ATP-binding cassette superfamily and serves as an efflux pump of various antibiotics.

Living organisms can export and import a variety of molecules across the plasma membrane. For this purpose, they have evolved specialized membrane-associated transport systems, one of which is mediated by transport proteins belonging to the ATP-binding cassette (ABC) superfamily (1, 13). The ABC superfamily consists of transporters which typically have four functional domains, two of which are hydrophobic and have several membrane-spanning sequences while the other two domains are hydrophilic and have highly conserved ATP-binding sequences that couple ATP hydrolysis to transport processes. In procaryotes the ATP-binding and transmembrane domains are frequently expressed as separate polypeptides, while in eucaryotic ABC transporters they are fused to larger proteins. A well-known example of the latter is multidrug-resistance-conferring P-glycoprotein, which, by exporting a variety of anticancer drugs from the cells, is involved in pleiotropic drug resistance in tumor cells (10). P-glycoprotein has an intramolecular dimer-like structure, each half of the molecule containing a cluster of six transmembrane sequences and a single ATP-binding sequence, the former followed by the latter. In contrast, *white* and *brown* proteins of *Drosophila melanogaster*, two of the members of the ABC superfamily which have been suggested to be involved in import of eye pigments into the cells, have a transposed structure; they have N-terminal ATP-binding sequences and C-terminal transmembrane segments, though they do not have an intramolecularly duplicated structure (9, 23).

Brefeldin A (BFA) is a fungal metabolite which inhibits intracellular protein transport between the endoplasmic reticulum and the Golgi apparatus in animal cells and in yeasts (11, 29). Interestingly, electron microscopical observation revealed that BFA caused disappearance of the Golgi apparatus in animal cells and induced aberrant membrane structures in *Candida albicans* (2, 19). Recently it has been reported that BFA inhibits exchange of GDP for GTP bound to ADP ribosylation factor (ARF) on the Golgi membranes, resulting in perturbation of the budding process of transport vesicles (8, 12). However, the BFA-sensitive cellular component(s), which is thought to be involved in the GDP-GTP exchange reaction of ARF on the Golgi membrane, is yet to be identified. To elucidate the molecular mechanism of action of BFA, we have isolated genes which on a multicopy plasmid, pDB248′, conferred on *Schizosaccharomyces pombe* cells resistance to BFA. A similar strategy was undertaken in a search for the target molecule of antibiotics such as tunicamycin and compactin in *Saccharomyces cerevisiae* (24). Analysis of one of the BFA-resistant transformants obtained revealed that the cloned gene encoded a novel protein that might function as a membrane-associated transporter belonging to the ABC superfamily.

MATERIALS AND METHODS

Materials. BFA was isolated and purified from a culture broth of *Penicillium brefeldianum* and dissolved in methanol at 100-fold concentrations of the final concentrations. All other reagents used were analytical grade. *S. pombe* was grown in minimal SD medium containing 2% glucose and 0.67% yeast nitrogen base without amino acids (Difco) or YEPD medium containing 1% yeast extract, 2% Polypepton (Wako Pure Chemical Industries, Osaka, Japan), and 2% glucose.

Strains, plasmids, and media. *S. pombe* haploid strains JY333 (h$^-$ *ade6-M216 leu1*) and JY741 (h$^-$ *ade6-M216 leu1 ura4-D18*) were used in the experiments. For disruption of *bfr1*$^+$, diploid Ura$^-$ strain JY765 (h$^-$/h$^+$ *ade6-M216*/*ade6-M210 leu1*/*leu1 ura4-D18*/*ura4-D18*) was used. *Escherichia coli* JM109 (F′[*traD36 lacI*^{q} Δ*lacZ*M15 *proAB*$^+$] *recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ[*lac-*

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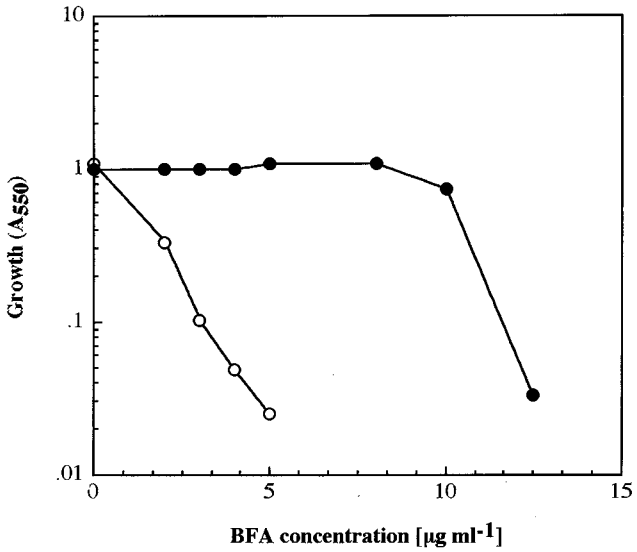

FIG. 1. BFA sensitivity of *S. pombe* JY333 carrying vector pDB248′ (open circles) and pBF9-1 (closed circles). Cells were incubated in liquid SD media containing 0.006% SDS plus various concentrations of BFA, and the growth (A_{550}) was monitored as described in Materials and Methods.

proAB]) and HB101 (*supE44 hsdS20*[r_B^- m_B^-] *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*) were used for cloning and sequencing. A genomic library of *S. pombe* constructed on *S. pombe-E. coli* shuttle vector pDB248′ was a generous gift from M. Yamamoto (The University of Tokyo, Tokyo, Japan) and was used for isolation of genes which confer BFA resistance.

Genetic methods. Transformation of *S. pombe* was done by the lithium acetate method developed by Ito et al. (15). Standard molecular cloning methods were used as described elsewhere (25). The nucleotide sequence of the *bfr1*$^+$ gene was determined by the dideoxy method of Sanger et al. (26) by using a DNA sequencer (model ABI370I; Applied Biosystems).

Measurement of drug sensitivity. *S. pombe* cells carrying the plasmids were grown either on SD agar plates or in liquid SD media containing various concentrations of each antibiotic and 0.006% sodium dodecyl sulfate (SDS). Growth in liquid media (A_{550}) was monitored and recorded every 90 min by using a bioscanner (Ohtake Kikai Incorp., Tokyo, Japan). Since the lag period was considerably long and the growth of cells was very slow in the presence of SDS, cells were grown for 2 to 3 days and growth of cells was evaluated by dividing the absorbance at the end of the exponential phase by the length of the exponential growing phase.

Construction of *bfr1* null mutant. The *Sac*I-*Nsi*I fragment of pMA182, which carries the whole insert sequence of pBF9-1 on pUC118, was replaced with the *Sac*I-*Pst*I fragment containing the *S. pombe ura4*$^+$ gene. The resultant plasmid was linearized and introduced into Ura$^-$ diploid JY765.

RESULTS

Isolation of *bfr1*$^+$ gene. To isolate genes which confer BFA resistance in a multicopy state, *S. pombe* JY333 was transformed with the genomic library of JY333 constructed on multicopy plasmid vector pDB248′ and directly plated onto SD plates containing 0.006% SDS and 5 μg of BFA ml^{-1}. Growth of *S. pombe* cells or cells transformed with pDB248′ was severely inhibited at this concentration of BFA, since addition of SDS increased sensitivity to BFA more than 10-fold. Colonies growing faster were selected, and plasmids were recovered from them, propagated in *E. coli*, and reintroduced into JY333 to check if the plasmids were reproducibly capable of conferring BFA resistance. BFA resistance was confirmed by using liquid SD medium containing SDS and various concentrations of BFA. More than 10^5 Leu$^+$ transformants were examined, and 10 transformants were selected in this manner as candidates. One of the plasmids, named pBF9-1, was chosen for further analysis because it conferred a higher degree of BFA resistance than any other plasmid did. In the presence of SDS, addition of 5 μg of BFA ml^{-1} almost completely inhibited growth of *S. pombe* cells harboring only the vector plasmid, while the MIC of BFA at which growth of cells transformed with pBF9-1 was inhibited increased to 12.5 μg ml^{-1} (Fig. 1).

The physical map of the 6-kb insert of pBF9-1 was determined as shown in Fig. 2. Preliminary subcloning analysis demonstrated that a substantial portion of the insert was necessary for BFA resistance, since partial deletion of fragments from the insert sequence resulted in loss of BFA resistance; cells containing neither pBF9-1-D1 nor pBF9-1-D2 were resistant to BFA (Fig. 2). Therefore, we determined the whole nucle-

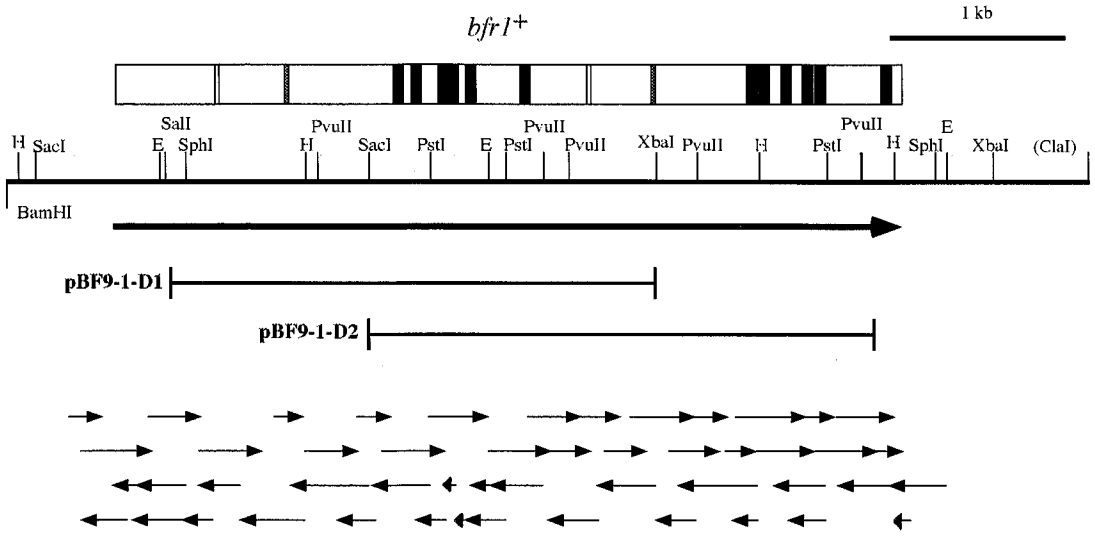

FIG. 2. Restriction map, subcloned fragments, and DNA sequencing strategy for the insert of pBF9-1. The insert sequence of pBF9-1 is shown by the bar labeled with restriction enzyme sites, and the thick arrow indicates the location and direction of the ORF encoding the *bfr1*$^+$ gene product. The predicted secondary structure of the *bfr1*$^+$ gene product is shown by the striped box; Walker A and B motifs are indicated by open and hatched boxes, respectively, while transmembrane sequences are indicated by solid black boxes. The subcloned fragments carried by pBF9-1-D1 and -D2 are shown below. Thin arrows indicate the direction of DNA sequencing. Abbreviations: E, *Eco*RI; H, *Hin*dIII.

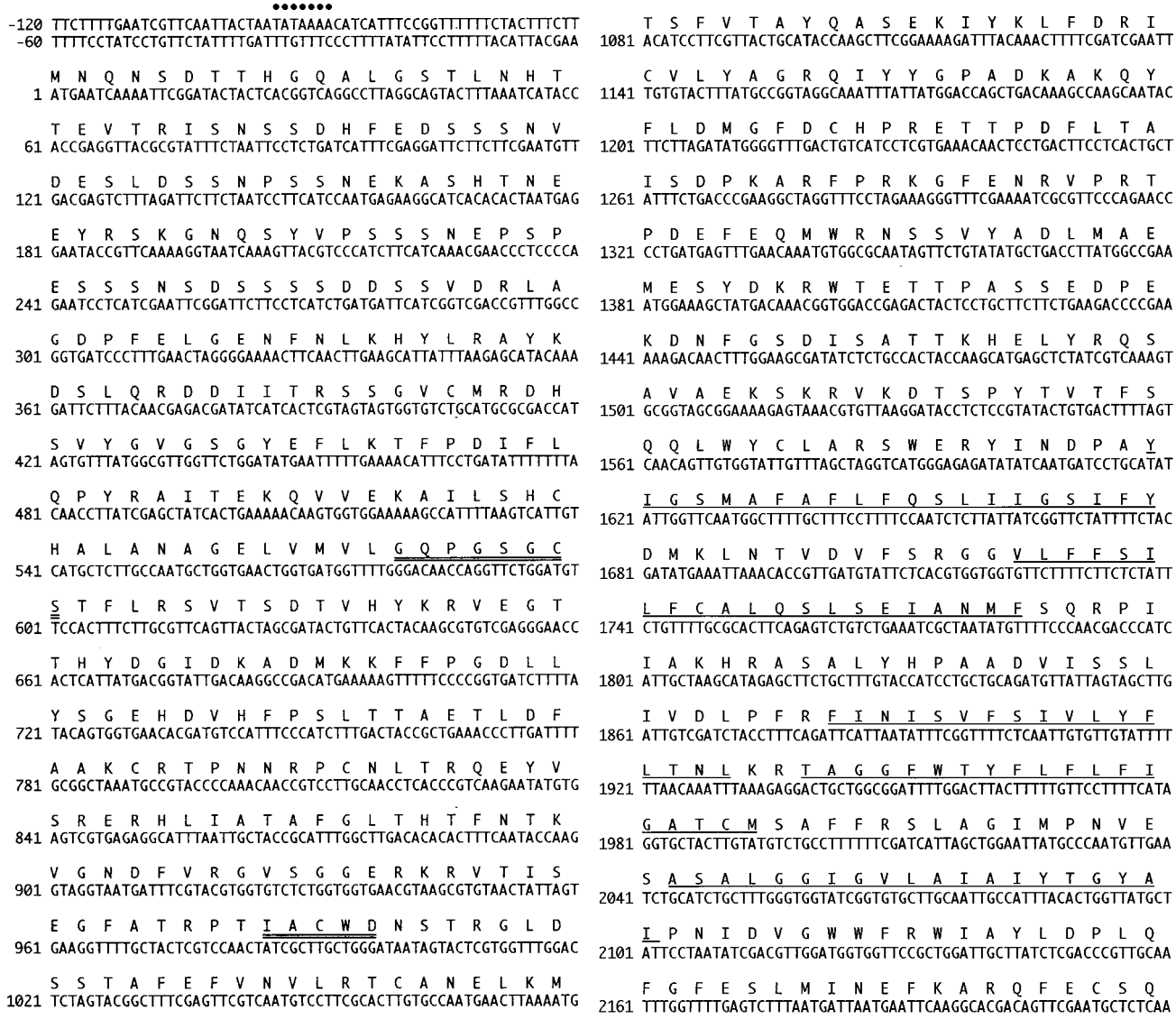

FIG. 3. Nucleotide sequence of *bfr1*$^+$ and deduced amino acid sequence of *bfr1*$^+$ protein. Nucleotide +1 is the A of the ATG translational start codon. The consensus ATP-binding sequences (Walker A and B motifs) reported by Walker et al. (30) are doubly underlined, and hydrophobic transmembrane segments are underlined. The putative TATA sequence within the 5′-upstream region is marked by small solid circles. The asterisk indicates the stop codon.

otide sequence of the insert of pBF9-1, which predicted an open reading frame (ORF) consisting of 1,531 amino acids (Fig. 2 and 3). Since this ORF covered both the *Sac*I and *Xba*I sites used to construct pBF9-1-D1 and -D2 and no other ORF long enough to be considered to encode a protein was found, we assumed that this ORF encoded a protein whose overproduction could bring about BFA resistance. This assumption was reinforced by the fact that 95 bp upstream from the first ATG, a putative TATA sequence was located (Fig. 3, positions −95 to −89; TATAAAA), and no other start codon was found within this 5′-flanking region. Furthermore, the presence of a purine residue (G) 3 nucleotides upstream from the initiation codon is in good agreement with the observation made by Kozak (16).

Structural analysis of *bfr1*$^+$ gene. A search for sequences with homology to the deduced amino acid sequence demonstrated that the ORF encoded a novel protein; therefore, we named the cloned gene *bfr1*$^+$ for brefeldin A resistance. Sequence analysis revealed that the *bfr1*$^+$ gene product had two putative ATP-binding sequences located near the N-terminal and central regions of the molecule and, as indicated by hydropathy profile analysis, two hydrophobic regions (Fig. 2 and 3; also data not shown) (18). The first putative ATP-binding sequence (GQPGSGCS) did not completely match the consensus Walker A motif (GXXXXGK[S/T]), where the lysine residue highly conserved in other ATP-binding proteins was replaced with cysteine in the *bfr1*$^+$ protein (27, 30). Each ATP-binding sequence was followed by a hydrophobic region, forming a tandemly repeated structure consisting of an N-terminal ATP-binding sequence plus C-terminal transmembrane segments (Fig. 2). Indeed, a low level of homology was found between the N-terminal and C-terminal halves of the *bfr1*$^+$ protein (data not shown). One hydrophobic region contained 6 putative transmembrane sequences, resulting in 12 transmembrane segments in the molecule (Fig. 3). Since the typical N-terminal signal-sequence-like motif was not found,

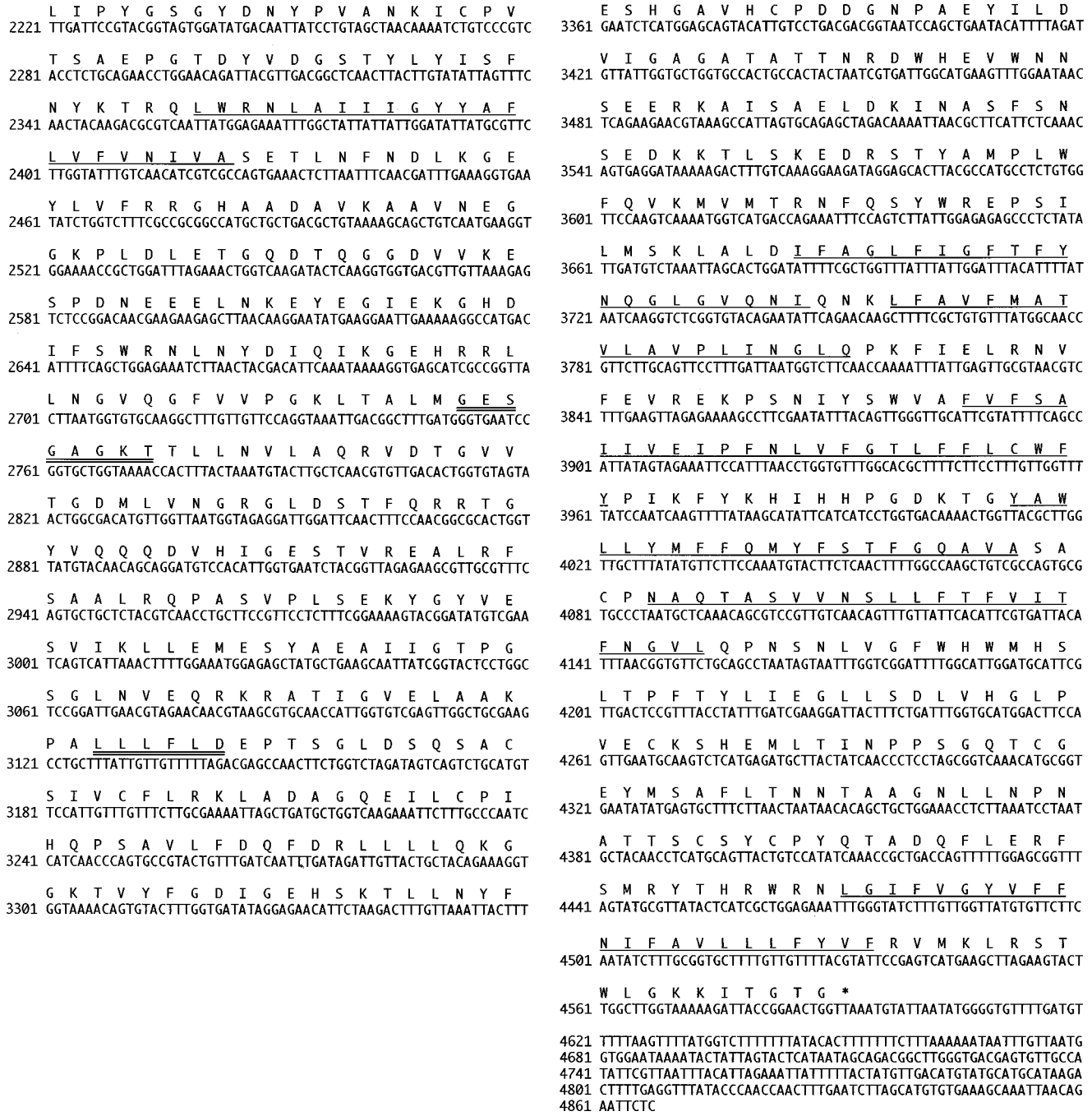

FIG. 3—*Continued.*

we speculate that both N and C termini of the *bfr1*$^+$ protein face the cytoplasm. These structural features of the *bfr1*$^+$ protein were remarkably similar to those of the mammalian P-glycoprotein that was responsible for pleiotropic drug resistance in tumor cells, except that the relative locations of ATP-binding and transmembrane sequences were in reverse order between the P-glycoprotein and the *bfr1*$^+$ protein; P-glycoprotein has N-terminal transmembrane regions fused to C-terminal ATP-binding sequences (Fig. 4).

The *bfr1*$^+$ protein exhibited limited but distinct similarity to proteins belonging to the ABC superfamily. The highest level of homology was observed with the Sts1/Pdr5/Ydr1 and Snq2 proteins of *S. cerevisiae* (43 and 40%, respectively) (Fig. 4) (4, 6, 14, 28). Both of the *S. cerevisiae* genes were first identified as multidrug resistance genes. Their proteins share structural features with the *bfr1*$^+$ protein; they have an intramolecularly duplicated structure consisting of N-terminal ATP-binding plus C-terminal transmembrane regions. The degree of similarity of the *bfr1*$^+$ protein with these two gene products was considerably high around the Walker A, the ABC signature, and the Walker B sequences (Fig. 4). A relatively low level of homology was also observed with the *white* and *brown* proteins

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                          Walker A                                    ABC-Signature          Walker B
bfr1*(N) 185-NAGELVMVLGQPGSGCSTFLRS     292-GLTHTFNTKVGNDFVRGVSGGERKRVTISEGFATRPTIACWDNSTRGLDSS
                 * ***  *** ***** * * *          ** ** ******* *********** * *          **** ******
PDR5(N)  184-NPGELLVVLGRPGSGCTTLLKS     292-GLSHTRNTKVGNDIVRGVSGGERKRVSIAEVSICGSKFQCWDNATRGLDSA
SNQ2(N)  184-EAGEMILVLGRPGAGCSSFLKV     292-GLRHTYNTKVGNDFVRGVSGGERKRVSIAEALAAKGSIYCWDNATRGLDAS
Brown     57-KTGDLIAILGGSGAGKTTLLAA     155-GLRDAAHTR-----IQQLSGGERKRLSLAEELITDPIFLFCDEPTTGLDSF

                          Walker A                                    ABC-Signature          Walker B
bfr1*(C) 909-VPGKLTALMGESGAGKTTLLNV     1006-LEMESYAEAIIGTPG--SGLNVEQRKRATIGVELAAKPALLLFLDEPTSGLDSQ
                 ** ******  *********          **** ** *  *  *   ********* ******  *** ** ************
PDR5(C)  896-KPGTLTALMGASGAGKTTLLDC     993-LEMEKYADAVVGVAG--EGLNVEQRKRLTIGVELTAKPKLLVFLDEPTSGLDSQ
SNQ2(C)  880-IPGTMTALMGESGAGKTTLLNT     976-LGMEEYAEALVGEVG--CGLNVEQRKKLSIGVELVAKPDLLLFLDEPTSGLDSQ
White    130-YPGELLAVMGSSGAGKTTLLNA     231-LSLSKCQHTIIGVPGRVKGLSGGERKRLAFASEALTDPPLLIC-DEPTSGLDSF
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FIG. 4. Alignment of Walker A, ABC signature, and Walker B sequences of the *bfr1*$^+$ protein and other members of the ABC superfamily. The N-terminal (N) and the C-terminal (C) ATP-binding domains of the *bfr1*$^+$ protein are aligned with the Sts1/Pdr5/Ydr1, Snq2, *white*, and *brown* proteins. Amino acid numbers are indicated at the beginning of each sequence. Asterisks indicate amino acid residues identical between the *bfr1*$^+$ protein and the Sts1/Pdr5/Ydr1 protein.

of *D. melanogaster*, which are known to be involved in uptake of the precursor of eye pigment from the bloodstream (9, 23).

Multidrug resistance phenotype conferred by *bfr1*$^+$ gene. The structural similarity between the *bfr1*$^+$ protein and P-glycoprotein, together with the fact that the *bfr1*$^+$ protein exhibited significant homology to multidrug resistance gene products of *S. cerevisiae*, led us to investigate whether the *bfr1*$^+$ gene on a multicopy vector could confer resistance to drugs other than BFA. As shown in Table 1, the cells carrying the plasmid pBF9-1 exhibited increased resistance to actinomycin D, cerulenin, and cytochalasin B, while sensitivity to oligomycin and cycloheximide was unaffected. Since the former three drugs and BFA differed from each other in their chemical nature, size, and cellular target and since P-glycoprotein and the two *S. cerevisiae* gene products are thought to transport chemically unrelated drugs, we suggest that the *bfr1*$^+$ protein functions as an ATP-dependent membrane transporter which pumps a variety of substrates out of the cells.

It was previously reported that overexpression of the *pmd1*$^+$ gene of *S. pombe*, whose protein is a structural and functional homolog of P-glycoprotein, resulted in resistance to several antibiotics, such as leptomycin B, cycloheximide, and valinomycin (22). Since the *bfr1*$^+$ gene on the multicopy vector also confers resistance to several antibiotics and we observed that the *bfr1*$^+$ protein structurally belongs to the ABC superfamily, we examined the drug resistance phenotypes induced by the *pmd1*$^+$ and *bfr1*$^+$ genes. As shown in Fig. 5, *pmd1*$^+$ overexpression had no effect on BFA resistance, while overexpression of the *bfr1*$^+$ gene conferred BFA resistance. Furthermore, the *pmd1* null mutant did not exhibit hypersensitivity toward BFA, a phenotype different from that of the Δ*bfr1* mutant (see below). Thus, the *bfr1*$^+$ gene product seems to have a substrate specificity and a function different from those of the *pmd1*$^+$ gene product.

TABLE 1. *S. pombe* cells harboring *bfr1*$^+$ gene on a multicopy vector exhibit resistance to various drugs

Drug	MIC (μg ml^{-1}) for cells carrying:	
	pDB248′	pBF9-1
Brefeldin A	6.0	20.0
Cycloheximide	10.0	10.0
Actinomycin D	1.5	6.0
Cytochalasin B	6.0	20.0
Oligomycin	0.5	0.75
Cerulenin	0.05	0.15

***bfr1*$^+$ gene disruption.** To test if the *bfr1*$^+$ gene is required for cell viability, a disruption mutant of the *bfr1*$^+$ gene was constructed (Fig. 6a). A *Sal*I-*Nsi*I fragment covering 90% of the *bfr1*$^+$ coding region was replaced with a *Sal*I-*Pst*I fragment containing the *ura4*$^+$ gene, and the resultant linear fragment carrying the *bfr1*::*ura4*$^+$ allele was introduced into diploid Ura$^-$ strain JY765 to generate a one-step gene replacement by homologous recombination. Stable Ura$^+$ transformants were selected, and disruption of one chromosomal *bfr1*$^+$ gene in diploid cells was confirmed by Southern blot analysis with a 1.9-kb *Sac*I fragment used as a probe (Fig. 6a). The probe hybridized with a 4.5-kb *Xba*I fragment from the parental allele and a 3.7-kb fragment from the *bfr1*$^+$-deleted allele, indicating that one chromosomal *bfr1*$^+$ gene is disrupted (Fig. 6b, lane 1). Sporulation of the heterozygous diploid *bfr1*$^+$/*bfr1*::*ura4*$^+$ strain yielded four viable spores, two Ura$^+$ and two Ura$^-$, indicating that the *bfr1*$^+$ gene is not essential for cell growth.

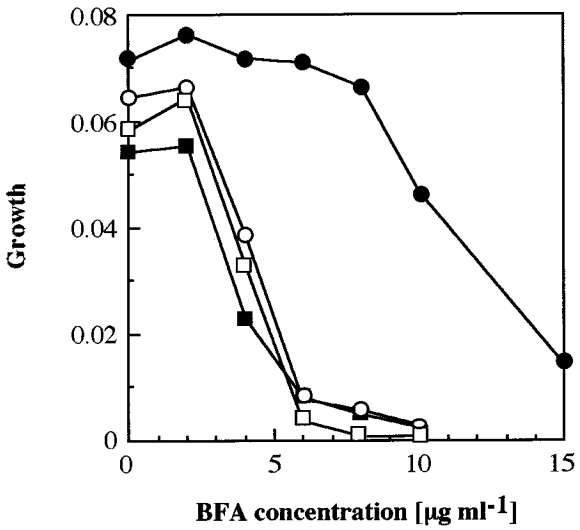

FIG. 5. Effect of overexpression of *pmd1*$^+$ gene and *bfr1*$^+$ gene on BFA resistance. JY333 cells harboring multicopy plasmid pDB248′ (open circles), the *bfr1*$^+$ or *pmd1*$^+$ gene on pDB248′ (closed circles and closed squares, respectively), or the *pmd1*$^+$ null mutant (open squares) were tested for BFA resistance. Growth of cells in liquid media was measured as described in Materials and Methods.

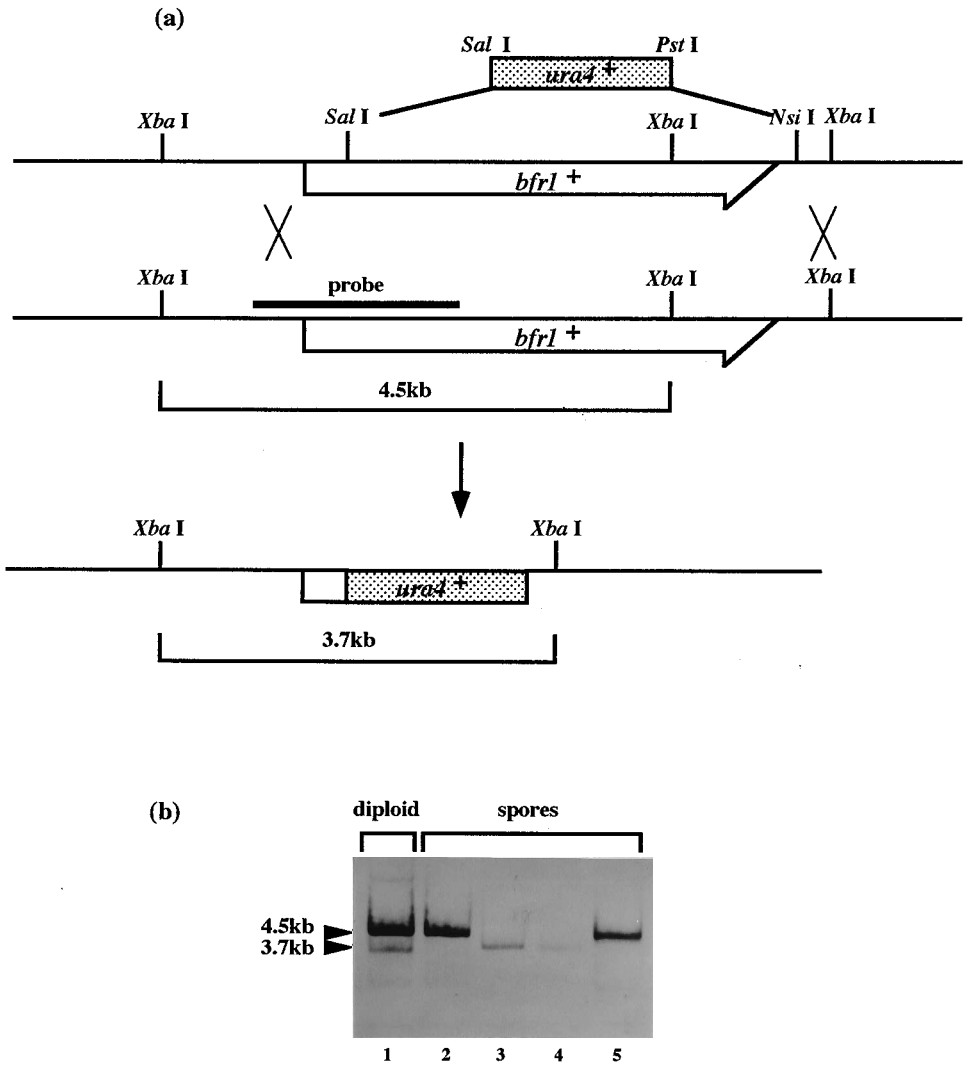

FIG. 6. Partial restriction map of *bfr1*$^+$ gene and the construct used for disruption of the *bfr1*$^+$ gene (a) and result of Southern blot analysis (b). (a) A *Sal*I-*Nsi*I fragment containing almost the entire coding region of the *bfr1*$^+$ gene (open half-arrow) was replaced with a *Sal*I-*Pst*I fragment containing the *ura4*$^+$ gene (dotted box), and the linearized DNA fragment was used to transform a Ura$^-$ diploid strain to generate a chromosomally disrupted allele of *bfr1*$^+$. (b) Genomic DNAs from *bfr1*$^+$/*bfr1*::*ura4*$^+$ heterozygotes (lane 1) and two Ura$^+$ (lanes 2 and 5) and two Ura$^-$ (lanes 3 and 4) spores derived from them were digested with *Xba*I and were subjected to Southern blot analysis with 1.9-kb *Sac*I fragments as a probe (thick line in panel a).

Chromosomal deletion of the *bfr1*$^+$ allele in two Ura$^+$ haploids was further confirmed by Southern blots of genomic DNA (Fig. 6b, lanes 3 and 4). Deletion of the *bfr1*$^+$ gene in either the h$^+$ or the h$^-$ strain had no effect on mating, suggesting that unlike the *STE6* gene of *S. cerevisiae*, the *bfr1*$^+$ gene is not involved in export of mating pheromone. However, the Δ*bfr1* mutant exhibited hypersensitivity to BFA; while the isogenic parental strain JY741 was resistant up to 6 μg of BFA ml^{-1}, growth of the Δ*bfr1* mutant was severely inhibited at 2 μg of BFA ml^{-1} (Fig. 7). Thus, the *bfr1*$^+$ gene is required for a moderate level of BFA resistance in parental cells, probably acting to export the drug out of the cells.

DISCUSSION

Several examples of ABC transporters that mediate resistance to drugs or antibiotics by pumping them out of the cell have been identified. In tumor cells, overexpression of the 170-kDa P-glycoprotein is associated with resistance to various chemotherapeutic drugs, a phenomenon known as multidrug resistance (10). Proteins with a structural organization similar to that of mammalian P-glycoprotein also have been found in yeasts. In *S. cerevisiae*, the *STE6* gene product involved in *sec*-independent export of **a**-factor was found to be a membrane-associated, ATP-dependent efflux pump with characteristic tandemly duplicated structures (17, 20). Overexpression of the *STE6* gene did not, however, result in resistance to various antibiotics. On the other hand, the *pmd1*$^+$ gene of *S. pombe* was identified as a structural and functional homolog of P-glycoprotein, since this gene on a multicopy plasmid conferred resistance to various growth inhibitors, such as leptomycin B, cycloheximide, and valinomycin (22).

We report here that the *bfr1*$^+$ protein of *S. pombe* is another member of the ABC superfamily with a P-glycoprotein-like internally duplicated structure. The *bfr1*$^+$ gene on a multicopy plasmid could confer resistance to various antibiotics with unrelated structures, sizes, and molecular targets, suggesting that the *bfr1*$^+$ protein is not only structurally but also functionally

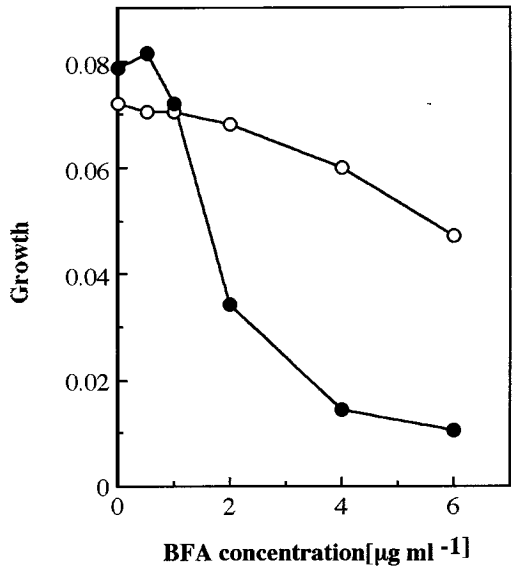

FIG. 7. BFA sensitivity of Δ*bfr1* mutant and isogenic parental strain JY741. JY741 (open circles) and the Δ*bfr1* mutant (closed circles) were tested for BFA sensitivity in the presence of 0.006% SDS. Growth of cells in liquid media was measured as described in Materials and Methods.

homologous to P-glycoprotein and the *pmd1*$^+$ gene product. However, the functional properties of the *bfr1*$^+$ protein may be different from those of the *pmd1*$^+$ protein, since *S. pombe* cells harboring the *pmd1*$^+$ gene on a multicopy plasmid did not exhibit resistance to BFA and chromosomal disruption of *pmd1*$^+$ did not result in increased BFA sensitivity (Fig. 5), while it caused a severalfold increase in sensitivity to leptomycin B.

The unique structural feature of the *bfr1*$^+$ protein is that it is intramolecularly tandemly duplicated with the N-terminal ATP-binding region fused to the C-terminal hydrophobic region (Fig. 2). In P-glycoprotein the relative locations of these two regions are transposed, and this is also the case with HlyB protein of *E. coli*, which, together with HlyD protein, forms a transmembrane complex and is responsible for export of hemolysin (7). Structural organization similar to that of the *bfr1*$^+$ protein is found in *white* and *brown* proteins of *D. melanogaster*, two of the members of the ABC superfamily, although they are not internally duplicated. These two proteins are thought to be involved in import of the pteridine precursor of eye pigment across the plasma membrane. It is unlikely, however, that the *bfr1*$^+$ protein confers resistance to BFA and to various drugs by involvement with the import process. Rather, it is reasonable to assume that the *bfr1*$^+$ protein functions as a P-glycoprotein-like efflux pump and that there is little correlation between the structure of the transporter and the direction of movement of transported molecules.

The products of two genes of *S. cerevisiae*, *SNQ2* and *STS1*/*PDR5*/*YDR1*, have been reported as novel members of the ABC superfamily (4, 6, 14, 28). Predicted secondary structures of these gene products, together with the relative locations of functional domains, were quite similar to those of the *bfr1*$^+$ protein; the gene products have internally duplicated structures consisting of N-terminal ATP-binding sequences and the C-terminal six transmembrane helices (Fig. 2).

The first and the second glycine residues in the Walker A motif (GXXXXGK[S/T]) (3, 5, 30), completely conserved in ATP-binding proteins, had been shown to be essential for the function of P-glycoprotein and Ste6 protein. The *bfr1*$^+$ protein had these conserved glycine residues within both of the putative ATP-binding sequences. The lysine residue highly conserved in other members of the ABC transporter family, however, was replaced with cysteine in the first ATP-binding sequence of the *bfr1*$^+$ protein. Substitution of this lysine with arginine resulted in loss of function of the Ste6 protein and P-glycoprotein, while substitution with alanine partially disturbed its function (3). Since in none of the experiments was the lysine residue substituted with cysteine, it is not clear whether the first ATP-binding sequence is functional. Interestingly, in the first Walker A motifs of Snq2 and Sts1/Pdr5/Ydr1 proteins, the consensus lysine is replaced with cysteine, as observed with the *bfr1*$^+$ protein (Fig. 4). Because both of the two functional ATP-binding sequences in one molecule are required for proper transport function of the Ste6 protein and P-glycoprotein (5), it is likely that the first Walker A motif in the *bfr1*$^+$ protein is functional.

The natural ligand transported by the *bfr1*$^+$ protein is unknown, and so is the ligand transported by the *pmd1*$^+$ protein. It was recently demonstrated that deletion of the *pmd1*$^+$ gene in either the h^+ or the h^- strain had no effect on the mating efficiency of *S. pombe* (21). In addition, overexpression of the *STE6* gene did not result in multidrug resistance in *S. cerevisiae*. Therefore, despite the structural similarity between the *pmd1*$^+$ protein and Ste6 protein, it seems unlikely that they have similar functions. Similarly, mating deficiency was not observed in the null mutant of the *bfr1*$^+$ gene, indicating that the *bfr1*$^+$ protein also has a function distinct from that of the Ste6 protein. Since the *bfr1*$^+$ protein seems to have structural and functional characteristics similar to but substrate specificity different from those of the *pmd1*$^+$ protein, it can be speculated that the two proteins play complementary roles in *S. pombe* cells, exporting different toxic substances out of cells. Therefore, it may be of interest to examine whether double deletion of the *bfr1*$^+$ and *pmd1*$^+$ genes results in much-increased drug sensitivity or lethality, although neither of the genes is essential for cell growth.

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