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Mutations Affecting Substrate Specificity of the *Bacillus subtilis* Multidrug Transporter Bmr

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The *Bacillus subtilis* multidrug transporter Bmr, a member of the major facilitator superfamily of transporters, causes the efflux of a number of structurally unrelated toxic compounds from cells. We have shown previously that the activity of Bmr can be inhibited by the plant alkaloid reserpine. Here we demonstrate that various substitutions of residues Phe^{143} and Phe^{306} of Bmr not only reduce its sensitivity to reserpine inhibition but also significantly change its substrate specificity. Cross-resistance profiles of bacteria expressing mutant forms of the transporter differ from each other and from the cross-resistance profile of cells expressing wild-type Bmr. This result strongly suggests that Bmr interacts with its transported drugs directly, with residues Phe^{143} and Phe^{306} likely to be involved in substrate recognition.

The *Bacillus subtilis* membrane protein Bmr belongs to the superfamily of major facilitator proteins and is one of the first-discovered bacterial multidrug-efflux transporters (12). These transporters actively export structurally diverse organic molecules out of cells, thus making bacteria simultaneously resistant to many toxic compounds (reviewed in references 10, 14, 15, and 17). Bmr, for instance, causes resistance to such structurally diverse toxins as ethidium, rhodamine, tetraphenylphosphonium (TPP), acridine dyes, fluoroquinolone antibiotics, doxorubicin, chloramphenicol, and puromycin (3, 12). In addition, Bmr interacts with the plant alkaloid reserpine, which exerts a strong inhibitory effect on the Bmr-mediated efflux of drugs (12).

The ability of a single protein to recognize so many structurally different molecules seemingly contradicts the accepted views on the molecular mechanism of enzyme-substrate interaction. Theoretically, this problem can be avoided by assuming that Bmr does not interact directly with the effluxed compounds. Instead, it may create such conditions that all compounds sharing certain physical characteristics leave the cell. For example, according to one of the hypotheses (18) intended to explain the broad substrate specificity of the mammalian multidrug transporter, P-glycoprotein (reviewed in references 7 and 18), Bmr may reduce the transmembrane electric potential, thus leading to an outward diffusion of positively charged drugs. All of the drugs effluxed by Bmr, with the exception of quinolone antibiotics, are indeed cationic; quinolones potentially may also acquire a positive charge by chelating divalent cations (19).

The question of whether Bmr interacts with the effluxed compounds directly or causes their efflux by some indirect mechanism can potentially be resolved by mutational analysis. Indeed, if the drugs are recognized directly by Bmr, then one would expect to obtain mutations in the Bmr molecule influencing the recognition process: the efflux of different drugs could be differentially affected by such mutations. If, however, Bmr causes the efflux of drugs in an indirect way, then the transport of all drugs should be affected by Bmr mutations in similar manners.

We have previously shown that substitution of leucine for Bmr residue V286 dramatically reduces the sensitivity of this protein to reserpine inhibition and the binding of reserpine to Bmr-containing membrane preparations of *B. subtilis* (1). Although strongly suggesting that reserpine interacts with Bmr directly, the results obtained with this mutation did not allow any conclusions to be drawn regarding the interaction of Bmr with its drug substrates because it had no effect on the efflux of any tested drug.

In this report, we describe several additional mutations in the Bmr molecule that were obtained by selecting *B. subtilis* for the loss of reserpine sensitivity of the Bmr transporter. Unlike the previously described mutations of V286 (1), the new mutations have distinct effects on Bmr-mediated resistance to specific drugs, strongly suggesting that Bmr interacts directly not only with reserpine but also with its diverse substrates.

MATERIALS AND METHODS

Bacterial strain. *B. subtilis* BD170-VB was created from a derivative of strain BD170 (*thr-5 trpC2*) in which both the gene encoding the transcriptional regulator of *bmr*, BmrR (2), and the gene for the second *B. subtilis* multidrug transporter, Blt (3), were disrupted by the chloramphenicol (*cat*) and erythromycin (*erm*) resistance determinants, respectively (see references 2 and 3 for details). This strain was further genetically modified by replacing the promoter of the chromosomal *bmr* gene with a variant of the strong constitutive promoter P_{vegII} (9). A PCR product was generated in which the P_{vegII} promoter was placed between a 580-bp-long DNA fragment located immediately upstream of the *bmr* promoter in the chromosome and a 471-bp-long DNA fragment located immediately downstream of the *bmr* promoter. This PCR fragment was used for transforming BD170/*bmrR::cat blt::erm* cells, with subsequent selection for resistance to 10 μg of ethidium bromide per ml. Transformation of *B. subtilis* was conducted by using the method described in reference 4. Sequencing of the PCR-amplified *bmr* promoter region in the transformants obtained confirmed that P_{vegII} had been integrated into the chromosome, although two nucleotide substitutions were detected in the P_{vegII} spacer region. In spite of these substitutions, which were likely due to the PCR errors that occurred when the promoter had been constructed, this promoter was effective enough to direct efficient transcription of the *bmr* gene and thus provide strong resistance to ethidium (MIC equals 40 μg of ethidium bromide per ml, compared to 1 μg/ml for BD170/*bmrR::cat blt::erm* cells). The sequence of this variant of P_{vegII} is TTGACAACGGCCTATTAACGTTGATATAAT (the −35 and −10 promoter consensus elements are underlined). The resulting strain, designated BD170-VB, was used in this work for generating mutations in the *bmr* gene.

Random mutagenesis of BD170-VB cells and selection for the loss of reserpine sensitivity. BD170-VB cells were grown in 50 ml of Luria-Bertani (LB) medium

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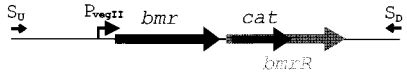

FIG. 1. Organization of the *bmr* locus in the BD170-VB strain of *B. subtilis*. In this strain, the strong constitutive promoter P_{vegII} was inserted upstream of *bmr* in place of its own promoter. The *bmrR* gene, encoding the transcriptional regulator of *bmr*, was disrupted by *cat*. In addition, the distantly located *blt* gene, encoding the second multidrug transporter of *B. subtilis*, was disrupted by *erm*. Primers S_U and S_D were used to amplify the entire *bmr* locus by PCR.

to an optical density at 600 nm (OD_{600}) of 0.5, washed by centrifugation in 1 M Tris-HCl (pH 7.5), and resuspended in 1 ml of the same buffer. Ethyl methanesulfonate (Sigma) was added to the cell suspension to a final concentration of 40 mM, and after 3 min of incubation at room temperature with vigorous shaking, the suspension was diluted with 49 ml of LB medium. Cells were harvested by centrifugation, resuspended in 50 ml of LB medium, and incubated in a 37°C shaker for 2 h before plating on selective plates. In an alternative mutagenesis procedure, 10^9 logarithmic-phase BD170-VB cells were spread on an LB agar plate and irradiated by a 30-W bactericidal UV lamp (1-m distance, 30 s) so that the plating efficiency of the bacteria was reduced by approximately 1 order of magnitude. Irradiated cells were resuspended in LB medium and incubated for 2 h in a 37°C shaker before plating on selective plates. Selective LB agar plates contained 1 μg of reserpine (Sigma) per ml and either ethidium bromide (10 μg/ml; Sigma), rhodamine 6G (1 μg/ml; Sigma), or TPP (75 μg/ml; Aldrich).

Sequencing of the *bmr* gene of the mutants. The entire *bmr* gene was amplified by PCR, using the chromosomal DNA isolated from the selected colonies as a template. Sequencing of the obtained PCR products was performed using a PCR-based *fmol* sequencing kit (Promega).

Transfer of *bmr* mutations into nonmutagenized bacteria. Using DNA from the selected clones as templates, 5-kb PCR products were generated by using the Expand Long Template PCR system (Boehringer Mannheim). These products contained a 1.1-kb fragment of DNA located upstream of the *bmr* gene, the mutated *bmr* gene controlled by the variant of P_{vegII} promoter, the *cat* gene integrated into the site located 180 bp downstream of the termination codon of the *bmr* gene and used to disrupt the *bmrR* gene, and a 1.2-kb fragment of DNA located downstream of the *cat* integration site (Fig. 1). These PCR products were used for transforming the BD170/*blt::erm* strain with selection for resistance to either ethidium (for V286L, F306L, and F306S variants) or TPP (for F143I and F143L variants) and for chloramphenicol resistance. Theoretically, the only bacterial clones that could survive this selection were the clones in which two separate acts of homologous recombination between the PCR product and the chromosomal DNA occurred in the distal parts of the PCR fragment. Upon recombination, the P_{vegII} promoter upstream of *bmr* and the *cat* gene downstream of *bmr*, and therefore the entire *bmr* gene carrying a mutation, were transferred into the chromosome. The transfer of the mutation was then confirmed by direct sequencing.

Site-directed mutagenesis of *bmr*. Two complementary mutagenic primers were used to obtain each mutant variant of *bmr*. These primers were used in conjunction with the standard upstream and downstream primers (S_U and S_D in Fig. 1) oriented toward each other and separated by 5 kb, with the *bmr* gene located approximately midway between them. Using DNA from BD170-VB cells as a template, we synthesized two overlapping PCR products: one defined by the upstream standard primer and the reverse mutagenesis primer and the other defined by the direct mutagenesis primer and the downstream standard primer. Subsequently, the mixture of these two PCR products was used as a template to generate a 5-kb PCR product between the two standard primers. All PCRs were conducted with the Expand Long Template PCR system (Boehringer Mannheim). The resulting PCR product contained the desired mutation in the *bmr* gene, the P_{vegII} promoter upstream of *bmr*, and *cat* downstream of *bmr*. This product was used to transform BD170/*blt::erm* cells, with simultaneous selection for resistance to ethidium (in the case of F306 mutations) or TPP (in the case of F143 mutations), which ensured integration of the P_{vegII} promoter, and for chloramphenicol resistance, which ensured integration of the *cat* gene. The integration of the desired mutation, which is located between these two reference points, into the chromosomes of selected clones was then confirmed by sequencing their *bmr* genes as described above.

Determination of bacterial sensitivity to drugs. Cells at a logarithmic stage of growth (OD_{600} of 0.4 to 0.8) were diluted to an OD_{600} of 0.005 in LB medium. Drugs diluted 1:1.5 in 8 to 10 serial dilutions were added to 1-ml aliquots of cell suspension. After incubation in a 37°C shaker for 3 to 4 h, at which point the control culture without added drugs reached an OD_{600} of approximately 1.0, the ODs of all cultures were measured. The IC_{50}s (50% inhibitory concentrations) were determined from the obtained OD_{600}-drug concentration graphs.

Measurement of the efflux of ethidium. Efflux of ethidium was measured fluorimetrically as described previously (12, 13). In brief, cells were loaded with ethidium (5 μg/ml) in the presence of 20 μg of reserpine per ml for 20 min in Spizizen salt medium supplemented with 0.5% glucose, pelleted in a microcentrifuge, and resuspended to an OD_{600} of 0.2 in the same medium containing different concentrations of reserpine. Release of ethidium from cells was monitored by the decrease in the fluorescence of cell suspension at a λ_{ex} of 530 nm and a λ_{fluor} of 600 nm.

RESULTS

Selection of mutant Bmr variants with reduced sensitivity to reserpine. In our earlier work (1), we identified residue V286 of Bmr as being critical for the binding of reserpine to the Bmr transporter. At that time, we failed to identify any other Bmr residues involved in the interaction with reserpine. The reason for this was technical: to obtain Bmr mutants, we mutagenized a *B. subtilis* strain in which Bmr was expressed from a multicopy plasmid. As a result, in every cell, mutated Bmr molecules were diluted by wild-type copies of Bmr. The selection protocol relied on plasmid segregation and therefore was relatively inefficient.

In this study, we used a different approach: the strain to be mutagenized, BD170-VB, contained a single copy of the chromosomal *bmr* gene so that mutations in this gene were manifested in all the cellular Bmr molecules. This gene was overexpressed by replacing its weak promoter (2) with a strong constitutive promoter, P_{vegII} (9). To avoid possible interference from the transcriptional regulator of *bmr*, BmrR (2), the gene encoding BmrR, located immediately downstream of *bmr*, was disrupted by the chloramphenicol resistance determinant. In addition, the gene encoding the second *B. subtilis* multidrug transporter, Blt (3), was disrupted by the erythromycin resistance determinant. As a result of these genetic manipulations, the constitutive overexpression of a single copy of the *bmr* gene was solely responsible for the resistance of BD170-VB cells to a number of toxic compounds.

BD170-VB cells were mutagenized by treatment with ethyl methanesulfonate and plated on LB agar containing a Bmr substrate (either ethidium bromide [10 μg/ml], rhodamine 6G [1 μg/ml], or TPP [75 μg/ml]), and the Bmr inhibitor reserpine at 1 μg/ml. Only the cells whose resistance to the Bmr substrate could no longer be inhibited by reserpine should theoretically have survived on such plates. The *bmr* gene was amplified from the obtained colonies by PCR and sequenced.

Fifty-nine colonies selected on ethidium-reserpine plates were analyzed. In five of them, the *bmr* gene was sequenced fully, and only one mutation was detected in each of the clones. Each of these mutations was in the codon encoding residue F306 of Bmr and converted phenylalanine into either leucine (F306L; in three clones), or serine (F306S; in two clones). The *bmr* genes amplified from the other 54 selected colonies were sequenced only in the region surrounding the F306 codon, revealing a single mutation in this region in each of them. These mutations were of three types: the previously described substitution V286L (1) in two of the selected clones (codon GTA was converted into either TTA or CTA, both encoding leucine) and two substitutions of the F306 residue of Bmr: F306L (TTT→CTT, TTA, or TTG; 28 clones) and F306S (TTT→TCT; 24 clones).

Each of the six sequenced mutants selected on rhodamine-reserpine plates contained the F306L mutation. Surprisingly, of 15 sequenced mutants selected on TPP-reserpine plates, only one contained the F306S mutation, while the remainder contained mutations of residue F143 of Bmr: F143I (TTT→ATT; nine clones) or F143L (TTT→CTT or TTA; five clones).

To verify that the detected mutations in the *bmr* gene are responsible for the ability of the BD170-VB cells to display reserpine-insensitive drug resistance, we transferred each of the obtained mutant variants of the *bmr* gene (F143I, F143L, V286L, F306L, and F306S) into nonmutagenized bacteria (see Materials and Methods). All of the obtained transformants

grew not only on plates with a drug (ethidium or TPP) but also on plates with a drug plus reserpine, thus indicating that the detected mutations in the *bmr* gene were indeed responsible for the inability of reserpine to reverse the Bmr-mediated drug resistance.

Since the selected mutations affected only three residues (F143, V286, and F306), it was important to determine whether this specificity was due to the specificity of the mutagenizing procedure. To test for this, BD170-VB cells were mutagenized by a different procedure (UV irradiation) and plated on the ethidium-reserpine plates. Of the 16 sequenced clones, the F306L mutation was detected in 9 and the F306S mutation was detected in 7, thus demonstrating that the same mutations are selected regardless of the nature of the mutagen.

Site-directed mutagenesis of residues F143 and F306 of Bmr. Single nucleotide substitutions within TTT triplets encoding residues F143 and F306 of Bmr could potentially lead to six possible substitutions at the protein level: amino acid C, I, L, S, V, or Y instead of F. Of these, we obtained, by selection, only two in each case: either F143I and F143L or F306L and F306S. To determine the properties of the Bmr transporter having other substitutions of F143 and F306, we generated *B. subtilis* strains, each isogenic to the BD170-VB strain but with one of the following substitutions in the Bmr transporter: F143V, F306C, F306I, F306V, and F306Y. This was accomplished by using PCR-based site-directed mutagenesis as described in Materials and Methods.

Obtained mutations exert complex effects on the transporter properties. To characterize the mutant Bmr variants, mutated derivatives of the BD170-VB cells were grown, in either the presence or absence of reserpine, with different concentrations of a Bmr-effluxed drug. ODs of the cultures were determined at the time when the control culture, which had no drugs added, reached an OD_{600} of approximately 1 (approximately 3 to 4 h of growth). Figure 2 demonstrates that mutations obtained for two Bmr mutants led to complex changes in the properties of the Bmr transporter. Not only reserpine sensitivity but also the substrate specificity of Bmr was affected.

Figure 2A shows that cells selected on a TPP-reserpine plate and expressing F143I Bmr are inhibited at similar concentrations of TPP as are the cells expressing wild-type Bmr. In the presence of reserpine, however, cells of these two types behaved differently: while reserpine dramatically reduced the TPP resistance of the cells expressing wild-type Bmr, it had little effect on the resistance of the mutant cells. When the same experiment was performed with ethidium instead of TPP, it was found that the mutant transporter, in contrast to the wild-type, conferred a markedly reduced resistance to this drug (Fig. 2B).

Similar results were obtained with the F306L variant of Bmr selected on ethidium-reserpine plate. Here, the level of ethidium resistance provided by the mutant transporter was similar to that provided by the wild-type transporter. Again, reserpine at 1 μg/ml had little effect on the ethidium resistance of the mutant cells (Fig. 2C). At the same time, the F306L transporter showed very little resistance to norfloxacin (Fig. 2D).

Cross-resistance profiles provided by mutant Bmr variants. To characterize systematically the changes in the cross-resistance profiles of cells carrying different mutant forms of Bmr, we determined the IC_{50}s of five Bmr substrates, TPP, ethidium, norfloxacin, acriflavine, and rhodamine 6G, for BD170-VB cells and of all its mutant derivatives (Fig. 3). Since each IC_{50} in Fig. 3 was determined from growth inhibition curves containing multiple experimental points, the experimental errors in their determination were negligibly small. Practically, however, the IC_{50}s determined in independent experiments for the same drug-strain combination were found to vary, although this variation never exceeded 25%.

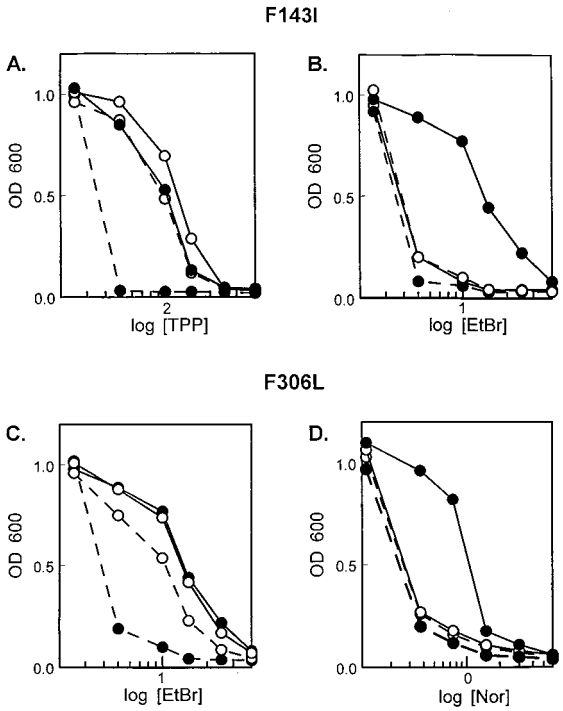

FIG. 2. Inhibition of growth of BD170-VB cells expressing wild-type Bmr (filled circles) or the F143I (open circles in panels A and B) or F306L (open circles in panels C and D) variant by TPP, ethidium bromide (EtBr), or norfloxacin (Nor). All the drug concentrations are given in micrograms per milliliter. Cells were cultivated with drugs in the absence (solid lines) or presence (dashed lines) of 1 μg of reserpine per ml. The mutant Bmr variants demonstrate reduced sensitivity to reserpine inhibition (A and C) and reduced the ability to protect cells from certain drugs: ethidium bromide for the F143I variant (B) and norfloxacin for the F306L variant (D).

With this level of variation in mind, the following conclusions can be made. Mutation V286L, in full accordance with what we reported earlier (1), did not lead to any significant changes in the ability of Bmr to confer drug resistance. In contrast, all analyzed mutations in residue F143 caused a dramatic reduction in the levels of norfloxacin, ethidium, and acriflavine resistances without affecting resistance to TPP or rhodamine. Mutations of residue F306 dramatically reduced the level of norfloxacin resistance. Resistance to acriflavine was either unaffected or reduced, depending on the nature of the residue substituting for F306. Resistances to ethidium, rhodamine, and TPP were only marginally affected by F306 mutations, with the notable exception of the F306Y substitution, which caused significant reduction of both TPP and rhodamine resistances.

Reserpine sensitivity of the mutant Bmr variants. To analyze systematically the effects of different mutations on the reserpine sensitivity of Bmr, we made use of the fact that almost none of the analyzed mutations alter the rhodamine resistance of BD170-VB cells. In these experiments, cells were grown in the presence of a standard concentration of rhodamine (0.5 μg/ml), and IC_{50} of reserpine (as defined in the legend to Fig. 4) was determined for each strain. Like the IC_{50}s for drugs, the reserpine IC_{50} varied in independent experiments, but these variations did not exceed 20%. Figure 4 shows that all mutations selected after random mutagenesis increase

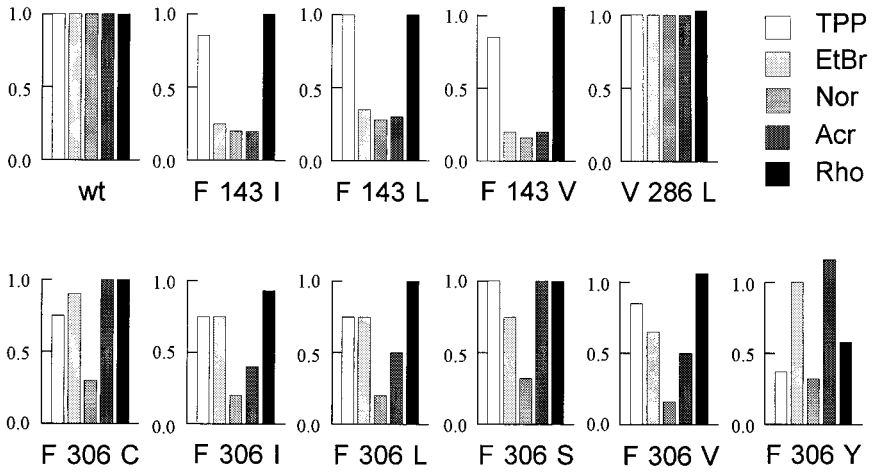

FIG. 3. Relative IC_{50}s of five drugs for BD170-VB cells expressing wild-type (wt) Bmr and mutant variants. The IC_{50} of a drug for wild-type BD170-VB cells is assumed to be 1. The actual IC_{50}s for these cells were as follows: 115 μg/ml for TPP; 15 μg/ml for ethidium bromide (EtBr); 1.25 μg/ml for norfloxacin (Nor); 30 μg/ml for acriflavine (Acr); and 0.8 μg/ml for rhodamine 6G (Rho). Typical results are shown. The experiment-to-experiment variations did not exceed 25%.

the IC_{50} of reserpine by 1 order of magnitude or more. Mutations of residues F143 and F306 obtained artificially also led to a decrease in the reserpine sensitivity of the transporter, although the magnitude of this effect was significantly smaller.

For the substitution F306Y, which reduces the level of rhodamine resistance, a similar experiment was performed with ethidium instead of rhodamine. Here again, the artificially obtained F306Y substitution led to a smaller decrease of the reserpine sensitivity than did the selected substitution V286L. These results explain why only a limited set of possible substitutions was selected on the drug-reserpine plates.

The decrease of the sensitivity of the mutant Bmr transporters to reserpine was also demonstrated in more direct experiments. Here, the kinetics of ethidium efflux from the Bmr-expressing *B. subtilis* was determined fluorimetrically in the presence of different concentrations of reserpine. As demonstrated in Fig. 5, cells expressing either wild-type Bmr or the F306L variant showed rapid efflux of ethidium. However, while the efflux from cells expressing the wild-type transporter could be inhibited by low concentrations of reserpine (maximum inhibition could be achieved at 1 μg/ml), efflux from the cells expressing the F306L variant required more than 8 μg of reserpine per ml to be fully inhibited. Similar results were obtained with the V286L and F306S Bmr variants (not shown).

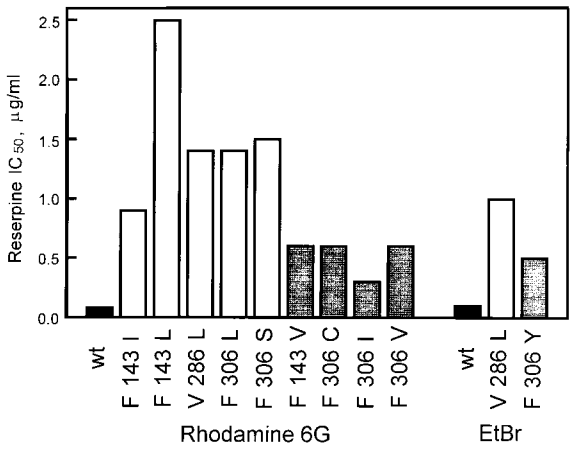

FIG. 4. Concentrations of reserpine required to inhibit by 50% the Bmr-mediated resistance to rhodamine 6G or ethidium bromide (EtBr) for the wild-type (wt) Bmr and mutant variants. BD170-VB cells expressing different variants of Bmr were grown in the presence of serial dilutions of reserpine and a Bmr-effluxed drug, either rhodamine 6G at 0.5 μg/ml or ethidium bromide at 10 μg/ml. The IC_{50} is defined as the concentration of reserpine promoting the drug-induced inhibition of growth by 50% compared to the control culture without reserpine. Note that all the Bmr mutants obtained by selection (open bars) lose reserpine sensitivity to a greater extent than the Bmr variants with artificial substitutions of the same residues (shaded bars). Typical results are presented. The experiment-to-experiment variations did not exceed 20%.

DISCUSSION

The original goal of this work was to identify residues of the Bmr transporter involved in reserpine recognition. In previous work (1), residue V286 was identified as being important for the reserpine sensitivity of Bmr and for the binding of reserpine to Bmr. We demonstrate here that at least two more residues, F143 and F306, are critical for the sensitivity of the transporter to reserpine inhibition. These residues are located

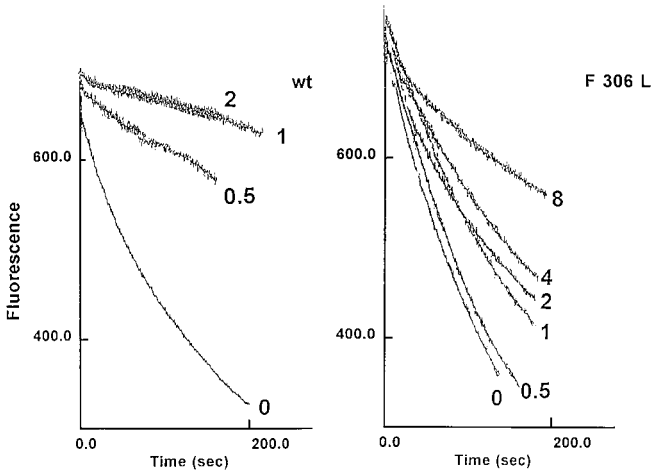

FIG. 5. Reserpine sensitivity of ethidium efflux from BD170-VB cells expressing wild-type (wt) Bmr or the F306L variant. Cells loaded with ethidium were incubated in the presence of the indicated concentrations (micrograms per milliliter) of reserpine. The fluorescence of the cell suspension is proportional to the amount of ethidium remaining inside the cells.

in different regions of the Bmr transporter: the putative 5th (F143), 9th (V286) and 10th (F306) transmembrane domains, thus suggesting that the reserpine-binding site of Bmr may represent a spatial structure formed by interacting transmembrane domains.

The major result of our work, however, is the finding that mutations of F143 and F306 strongly affect not only reserpine sensitivity but also the chemical specificity of drug transport by Bmr. It appears that the best explanation for our results, particularly the data presented in Fig. 3, is that Bmr recognizes transported drugs directly. Indeed, the fact that mutations of F143 and F306 affect the IC_{50}s of some drugs but not others strongly argues against any hypothesis postulating that Bmr causes drug efflux in an indirect way, such as by changing the properties of the bacterial membrane. In this respect, the most convincing observation is the finding that different mutations may have diametrically opposite effects on the transport of specific drugs (compare, for example, the data in Fig. 3 obtained with mutations F306Y and F306V).

The results presented in this report are not the first to demonstrate that mutations of a multidrug transporter may affect its substrate specificity. Previously, individual mutations affecting transport of specific drugs have been described for the mammalian multidrug transporter P-glycoprotein (5, 6, 8, 11). More recently, it has been demonstrated that the difference in substrate specificity between the two highly homologous plasmid-encoded multidrug transporters of *Staphylococcus aureus*, QacA and QacB, is due to a difference in a single amino acid residue (16). Our results, however, constitute probably the most comprehensive set of data on the effects of various substitutions of specific residues on the substrate specificity of a multidrug transporter.

These data provide the basis for the preliminary conclusion that each of the identified residues is critical for the recognition of only a limited subset of drugs. Residue V286 appears to be important only for the recognition of reserpine and not recognition of any of the transported drugs. Three analyzed mutations of F143, in addition to affecting reserpine recognition, diminish greatly the transport of norfloxacin, acriflavine, and ethidium, but none of them exerts a significant effect on the transport of TPP or rhodamine. Similarly, none of the six analyzed mutations of F306 has a strong effect on the transport of ethidium. These results may be explained by the assumption that different drugs interact with different domains of a substrate recognition site of the Bmr transporter, with residues F143, V286, and F306 being involved, either directly or through allosteric interactions, in the formation of only some of these domains. The enigmatic phenomenon of multidrug transport may, therefore, be explained not by some novel type of molecular recognition but merely by the large size and/or chemical complexity of the substrate recognition site of a transporter. Further mutational analysis of Bmr may test the validity of this hypothesis and, more generally, lead to at least a conceptual understanding of the presently obscure process of multidrug recognition.

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